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Assessment of heat-sensitive thiophosphate protecting groups in the development of thermolytic DNA oligonucleotide prodrugs

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ABSTRACT

Heat-sensitive thiophosphate protecting groups derived from the alcohol **4** or **10** have provided insights in the design of DNA oligonucleotide prodrugs. Indeed, functional groups stemming from the alcohol **9**, **15**, **16** or **22** may be applicable to thiophosphate protection of immunostimulatory CpG DNA motifs, whereas those originating from the alcohol **3**, **5**, **12**, **13**, **18**, **20** or **22** offer adequate protection of terminal phosphodiester functions against ubiquitous exonucleases that may be found in biological environments. Functional groups derived from the alcohol **9**, **15**, **16**, **19** or **23** are suitable for the protection of phosphodiester functions flanking the CpG motifs of immunomodulatory DNA sequences.

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1. Introduction

The development of oligonucleotide prodrugs has attracted considerable attention in recent years in an effort to facilitate cellular uptake of DNA sequences and provide these biomolecules with increased resistance to extracellular and intracellular nucleases. An approach to achieve these objectives is to protect the negatively charged phosphodiester groups of DNA sequences as, for example, acylthioethyl,[1] acyloxymethyl,[2] and 4-acyloxybenzyl[3] esters or as derivatives of bis(hydroxymethyl)-1,3-dicarbonyl compounds.[4] The phosphotriester functions of these DNA oligonucleotide prodrugs are expected, upon cellular entry, to be converted to their bioactive phosphodiester state by intracellular esterases. From this perspective, we had described the preparation of DNA oligonucleotide prodrugs, which did not require the assistance of intracellular enzymes for prodrug-to-drug conversion. Indeed, oligonucleoside phosphorothioates with 2-(*N*-formyl-*N*-methylamino)ethyl groups for thiophosphate protection[5] exhibited the characteristics of oligonucleotide prodrugs in that of being uncharged and resilient to the hydrolytic activity of extracellular and intracellular nucleases. A distinctive feature of this class of modified oligonucleotides is that only an aqueous environment at a nominal temperature of 37 °C is necessary to thermolytically convert oligonucleoside 2-(*N*-formyl-*N*-methylamino)ethyl phosphorothioate triesters to bioactive oligonucleoside phosphorothioate diesters. When applied in the context of single-stranded DNA oligonucleoside phosphorothioates containing unmethylated CpG motifs (CpG ODNs),[6] a thermolytic oligonucleotide prodrug produced an immunostimulatory response in mice similar to that generated from a conventional CpG ODN.[5b,6] However, the induction of the immunostimulatory events was delayed commensurately with the thermolytic conversion half time of 2-(*N*-formyl-*N*-methylamino)ethyl thiophosphate triesters to the biologically active diesters.[5b] Interestingly, the co-administration of a CpG ODN prodrug with a conventional CpG ODN in an animal model resulted in a significantly prolonged therapeutic protection of the animal against specific viral infections.[5b] These observations prompted us to design new thermolytic groups that would induce a persistent immunoprotection against infectious diseases in animals over extended periods of time. This objective can hopefully be achieved through the selection of thermosensitive phosphate/thiophosphate protecting groups exhibiting different thermolytic deprotection rates within the immunostimulatory DNA sequence. Typically, the protection of one or preferably two terminal phosphate/thiophosphate diesters with thermostable groups would safeguard the DNA sequence against extracellular and intracellular exonucleases. Conversely, the phosphate/thiophosphate diesters flanking the CpG motif in both directions would preferably be protected with groups exhibiting sufficient thermostability at 37 °C to facilitate early cellular uptake of the oligonucleotide prodrug while allowing some thermal phosphate/thiophosphate deprotection to enhance its aqueous solubility. Given the criticality of the CpG motif for inducing immunostimulatory events, the protection of its phosphate/thiophosphate functions with groups displaying variable thermolytic deprotection kinetics is indicated for generating a population of CpG ODN sequences, which when co-administered in an animal model would trigger a strong and sustained immunoprotection of the host against infectious diseases.

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We report herein the results of our search for thermolytic groups that can be used for thiophosphate protection of potential immunotherapeutic oligonucleotide prodrugs. Simple DNA prodrug models, such as thymidylyl-(3′→5′)-thymidine thiophosphate (TpsT) with various thermosensitive thiophosphate protecting groups were selected for this investigation. Deprotection kinetic data of each thermolytic thiophosphate protecting group are provided to enable the rational design of lipophilic CpG ODN prodrugs with the potential of developing adequate aqueous solubility properties.

2. Results and discussion

The dinucleoside phosphorothioate TpsT is preferred over the native dinucleoside phosphate diester TpT for comparing the deprotection rates of thermolytic protecting groups only to ensure that no S-alkylation of the internucleoside thiophosphate linkage occurred during the heat-assisted deprotection reaction. S-Alkylation of the internucleoside linkage by deprotection side products would result in its extensive hydrolytic desulfurization. Consequently, S-alkylation and desulfurization of phosphorothioate diester groups must be avoided, as these functional groups play an important role in safeguarding the immunostimulatory properties of type K[5b,6] and type D[6,7] CpG ODNs against the nucleolytic activities of extracellular and intracellular nucleases.

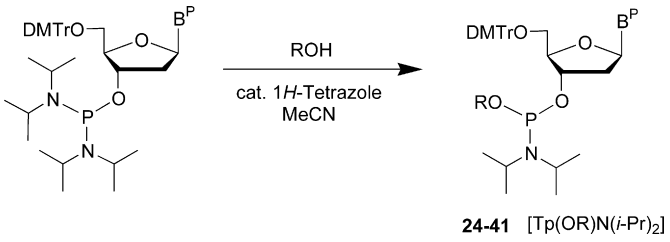
The structural design of new thermolytic thiophosphate protecting groups is based on selected commercial and synthetic alcohols (Table 1), a number of which have already shown to exhibit thermolabile properties as phosphate/thiophosphate esters.[5,8] These alcohols were allowed to react with an equimolar amount of 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-bis(*N*,*N*-diisopropylamino)phosphinyl-2′-deoxythymidine and catalytic amount of 1*H*-tetrazole in anhydrous MeCN to generate the corresponding phosphoramidite derivatives (Scheme 1). The phosphoramidites **24**–**39** were activated with 1*H*-tetrazole and reacted with 2′-deoxythymidine that is covalently attached to long chain alkylamine controlled-pore glass through a 3′-*O*-hemisuccinate linker (TsuccCPG). The resulting dinucleoside phosphite triesters were then oxidized to the corresponding dinucleoside phosphorothioate derivatives by treatment with 3*H*-1,2-benzodithiol-3-one-1,1-dioxide, 5′-deprotected under acidic conditions and released from CPG on exposure to pressurized $MeNH_2$ gas (Scheme 2). The dinucleoside phosphorothioate triesters (**42**–**57**) were purified by reversed-phase (RP) HPLC prior to removal of the thiophosphate protecting groups under thermolytic conditions [phosphate-buffered saline (PBS), pH 7.4, 37 °C or 90 °C].

Our earlier work on the use of the 2-(*N*-formyl-*N*-methyl-amino)-ethyl (FMA) group for thiophosphate protection[5] led to the preparation of thermosensitive oligonucleotide prodrugs.[5b] Removal of the FMA group proceeded smoothly in PBS (pH 7.4) with a half time of 23 min at 90 °C or 72 h at 37 °C (Table 2).

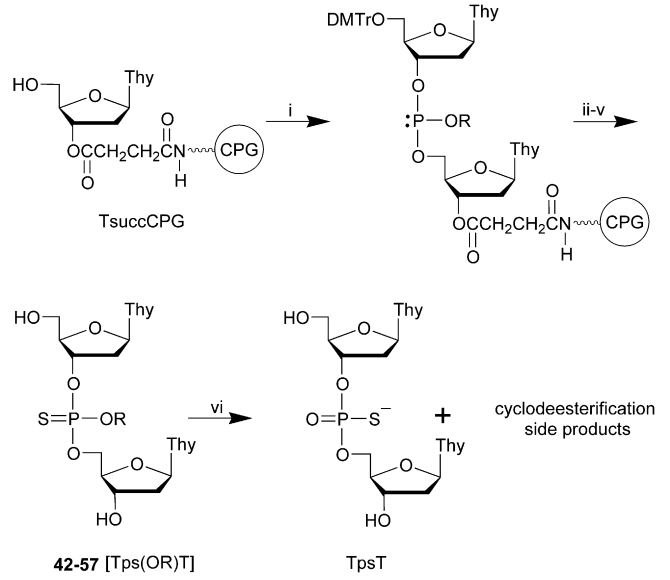
Table 1
Alcohols used in the synthesis of phosphoramidites **24**–**41**

Alcohol	
3	$HC(O)N(CH_3)CH_2CH_2CH_2OH$
4	$HC(O)N(CH_3)CH_2CH_2OH$
5	$HC(O)NHCH_2CH_2OH$
8	$HC(S)NHCH_2CH_2OH$
9	$CH_3C(O)NHCH_2CH_2OH$[a]
10	$CH_3SCH_2CH_2CH_2CH_2OH$[a]
11	$CH_3SCH_2CH_2OH$[a]
12	$CH_3OCH_2CH_2OH$[a]
13	$CH_3SCH_2CH_2CH_2CH_2CH_2CH_2OH$
15	$CH_3SCH_2CH_2CH_2CH_2CH_2OH$
16	$(CH_3)_2CHSCH_2CH_2CH_2CH_2CH_2CH_2OH$
18	$CF_3SCH_2CH_2CH_2CH_2OH$
19	$CH_3OCH_2SCH_2CH_2CH_2CH_2OH$
20	$CH_3SCH_2CH_2OCH_2CH_2OH$
22	$CH_3SCH_2OCH_2CH_2CH_2OH$
23	$CH_3SCH_2OCH_2CH_2OH$

[a] Commercially available.



Scheme 1. Preparation of the phosphoramites **24**–**41** from deoxyribonucleoside phosphoramidites. Abbreviations: DMTr, 4,4′-dimethoxytrityl; B^P, thymin-1-yl (**24**–**39**), N^4-benzoylcytosin-1-yl (**40**) or N^2-isobutyrylguanin-9-yl (**41**); ROH, **3**–**5**, **8**–**13**, **15**, **16**, **18**–**20**, **22**, **23**.



Scheme 2. Solid-phase synthesis of the dinucleoside phosphorothioate triesters **42**–**57** and their thermolytic conversion to TpsT. Conditions: (i) **24**–**39**, 1*H*-tetrazole, MeCN; (ii) 0.05 M 3*H*-1,2-benzodithiol-3-one-1,1-dioxide, MeCN; (iii) 3% TCA, CH_2Cl_2; (iv) $MeNH_2$ gas (~2.5 bar); (v) RP-HPLC purification; (vi) PBS, pH 7.4, 37 °C or 90 °C. Abbreviations: Thy, thymin-1-yl; CPG, long chain alkylamine controlled-pore glass; R, thermolytic thiophosphate protecting group derived from the alcohols listed in Table 1; TCA, trichloroacetic acid; PBS, phosphate-buffered saline.

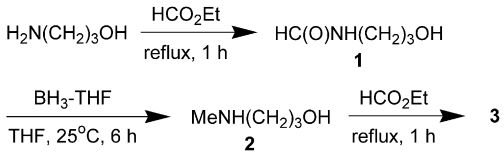
In order to develop thermosensitive thiophosphate protecting groups with slower deprotection rates than that of the FMA group, the preparation of an FMA homolog, such as the 3-(*N*-formyl-*N*-methylamino)prop-1-yl group was carried out. Actually, the synthesis of the alcohol **3** (Table 2) was accomplished from the reaction of 3-aminopropan-1-ol with ethyl formate to give the formamido alcohol **1** in an isolated yield of 67% (Scheme 3). Reduction of **1** by treatment with the borane–THF complex in THF produced the aminoalcohol **2**, which was allowed to react with ethyl formate to afford 3-(*N*-formyl-*N*-methylamino)propan-1-ol (**3**). The incorporation of **3** into the dinucleoside phosphorothioate triester **42** (Table 2) was effected under the general conditions outlined in Schemes 1 and 2.

The thermolytic deprotection of **42** to TpsT was performed under essentially physiological conditions (Scheme 2). The deprotection half time of the 3-(*N*-formyl-*N*-methyl-amino)prop-1-yl group was determined by monitoring the formation of TpsT by RP-HPLC and was found to be 36 h at 90 °C (Table 2). When compared to the deprotection half time of the FMA group under the same conditions (23 min) it was anticipated that the thermal deprotection of **42** would to be too slow at 37 °C for adequate prodrug-to-

Table 2
Thermolytic deprotection rates of the dinucleoside phosphorothioate triesters **42**–**57** to TpsT

ROH →	Tp(OR)N(*i*-Pr)$_2$ →	Tps(OR)T →	TpsT	
			$t_{1/2}$ (90 °C)	$t_{1/2}$ (37 °C)
3	**24**	**42**	36 h	ND[a]
4	**25**[b]	**43**[b]	23 min[b]	72 h[b]
5	**26**	**44**	6 h	ND
8	**27**	**45**	ND[c]	ND[c]
9	**28**	**46**	15 min	38 h
10	**29**	**47**	ND	15 min
11	**30**	**48**	ND	<3 min
12	**31**	**49**	ND[d]	ND
13	**32**	**50**	13 h	ND
15	**33**	**51**	30 min	33 h
16	**34**	**52**	34 min	35 h
18	**35**	**53**	17 h	ND
19	**36**	**54**	9 min	6.5 h
20	**37**	**55**	5 h	ND
22	**38**	**56**	2.5 h	375 h
23	**39**	**57**	12 min	22 h

[a] Not determined.
[b] See Ref. 5a.
[c] Not determined. The formation of TpsT was accompanied with internucleoside bond cleavage, thereby preventing the use of **8** as a thermolytic DNA prodrug thiophosphate protecting group.
[d] Not determined as **49** is essentially thermostable. Only traces of TpsT (~2%) are formed after 10 h at 90 °C.

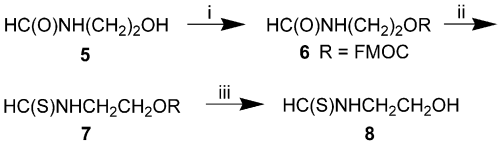


Scheme 3. Preparation of the amido alcohol **3** from 3-aminopropan-1-ol.

drug conversion. However, the 3-(*N*-formyl-*N*-methylamino)-prop-1-yl group may serve as a thermostable thiophosphate protecting group for the 5′- and/or 3′-terminus of DNA prodrug sequences and provide resilience against ubiquitous extracellular and intracellular exonucleases.

The 2-(*N*-formylamino)ethyl group, as an *N*-unmethylated homolog of the FMA group, was expected to have a slower thermolytic thiophosphate deprotection rate than that of the FMA group on the basis of a lesser electronic density on the amide function. The preparation of the amido alcohol **5** was accomplished by the reaction of ethanolamine with ethyl formate under conditions nearly identical to those employed in the synthesis of the amido alcohol **1** (Scheme 3). The amido alcohol **5** was incorporated into the dinucleoside phosphorothioate triester **44** as shown in Table 2 under the conditions provided in Scheme 2. The thermolytic cleavage of the 2-(*N*-formylamino)ethyl group from **44** occurred at 90 °C with a half time of 6 h (Table 2). This deprotection rate is considerably slower (~15-fold) than that of the FMA group under similar conditions. It is therefore probable that the 2-(*N*-formylamino)ethyl group would be too thermostable at 37 °C for thiophosphate protection of the CpG motif of CpG ODN prodrugs. These results prompted us to evaluate the 2-(*N*-thioformyl-amino)ethyl group as a potentially faster-deprotecting thiophosphate protecting group by virtue of the inherent nucleophilicity of the thiocarbonyl function in the context of a thermolytic cyclodeesterification process. The synthesis of the thioformamido alcohol **8** was undertaken following the sequence of reactions shown in Scheme 4.

The reaction of 9-fluorenylmethoxycarbonyl chloride (Fmoc-Cl) with 2-(*N*-formylamino)ethan-1-ol in pyridine at 5 °C afforded the carbonate **6**, which was isolated in a yield of 66% after chromatography on silica gel. Treatment of **6** with the Lawesson reagent[9] in THF for 10 min at 25 °C gave the 2-(*N*-thioformylamino)ethyl carbonate **7**, which was isolated chromatographically pure in a yield of



Scheme 4. Synthesis of the thioformamido alcohol **8** from 2-(*N*-formylamino)ethan-1-ol. Conditions: (i) FMOC–Cl, pyridine, 2 h, 5 °C; (ii) Lawesson reagent, THF, 10 min, 25 °C; (iii) 20% piperidine in DMF, 30 min, 25 °C. Abbreviations: FMOC, 9-fluorenylmethoxycarbonyl.

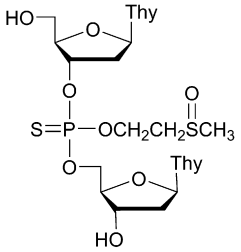
79%. Removal of the Fmoc group from **7** was effected cleanly and rapidly in a solution of 20% piperidine in DMF.

The reaction was complete within 30 min at ambient temperature. Following work-up and purification by chromatography on silica gel, pure 2-(*N*-thioformyl-amino)ethan-1-ol (**8**) was isolated in a yield of 84%. Incorporation of **8** into the dinucleoside phosphorothioate triester **45** was accomplished by the reaction of **27** (Table 2) with TsuccCPG under the general conditions described in Scheme 2. The thermolytic deprotection of **45** to TpsT did not proceed cleanly; the presence of deoxythymidine and other side products was detected by RP-HPLC. These side products were indicative of the thiocarbonyl group attacking, intramolecularly, the phosphorus atom of the thiophosphotriester function to ultimately result in the splitting of the internucleoside linkage. This observation is reminiscent of the findings reported by Mikhailov and Smrt[10] on the sensitivity of a dinucleoside hydroxyethyl phosphate triester to basic conditions. It was postulated that cleavage of the internucleoside linkage occurred under these conditions as a consequence of an intramolecular nucleophilic attack of the phosphotriester function by the hydroxyethyl group.

The commercially available *N*-acetylethanolamine (**9**) was then investigated as a potential thermolytic group in the design of CpG ODN prodrugs. The amido alcohol was employed in the synthesis of the deoxyribonucleoside phosphoramidite **28** (Table 2), which led to the dinucleoside phosphorothioate triester **46** (Scheme 2). Thermolytic deprotection of the thiophosphate protecting group from **46** proceeded smoothly at 90 °C with a half time of 15 min or 38 h at 37 °C. Although the removal of the 2-(*N*-acetylamino)ethyl group took place at a rate that is faster (approx. two-fold) than that of the FMA group at 37 °C, this heat-sensitive thiophosphate protecting group may be of value in the protection of the CpG motif of CpG ODN prodrugs. Moreover, when concurrently protecting the thiolated phosphodiester functions flanking the CpG motif, the 2-(*N*-acetylamino)ethyl groups may facilitate the cellular uptake of these modified oligonucleotides while gradually increasing their aqueous solubility as a result of a relatively faster thermal loss of thiophosphate protection.

Another class of thermolytic phosphate/thiophosphate protecting groups, derived from 4-(methylthio)butan-1-ol (**10**) and 2-(methylthio)ethanol (**11**), was evaluated in the development of DNA oligonucleotide prodrugs. Previous studies had shown that the 4-(methylthio)but-1-yl phosphate/thiophosphate protecting group was completely removed from DNA sequences when heated for 30 min at 55 °C in PBS (pH 7.2).[11] Consistent with these studies, the thermolytic cleavage of the 4-(methylthio)but-1-yl group from the dinucleoside thiophosphate triester **47**, which was prepared from the phosphitylation of TsuccCPG by **29** (Table 2) under standard conditions (Scheme 2), gave TpsT with a half time of 15 min at 37 °C. The dinucleoside thiophosphate triester **48** was similarly synthesized using phosphoramidite **30** (Table 2). The thermolytic deprotection of the 2-(methylthio)ethyl group from **48** occurred with a half time of less than 3 min at 37 °C. On the basis of such rapid deprotection rates, the 4-(methylthio)but-1-yl and 2-(methylthio)ethyl groups are unsuitable for thiophosphate protection in the development of DNA oligonucleotide prodrugs.

In order to decrease the deprotection rate of the 2-(methylthio)ethyl thiophosphate protecting group, **48** was treated, prior to removal of the 5′-DMTr group and release from the CPG support, with 5 M *tert*-butyl hydroperoxide in decane to oxidize the 2-(methylthio)ethyl function to its sulfoxide derivative. Subsequent to 5′-deblocking and release of the dinucleoside thiophosphate triester **58** from the CPG support, the 2-(methylsulfinyl)ethyl group was thermolytically removed from the RP-HPLC-purified dinucleotide with a half time of 146 min at 90 °C.



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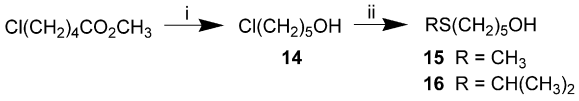
Another way to decrease the deprotection rate of the 2-(methylthio)ethyl group from **48** is to replace the group with a 2-methoxyethyl group for thiophosphate protection (**12**→**31**→**49**, Table 2). This change resulted in a strikingly slow thiophosphate deprotection under thermolytic conditions. After heating **49** for 10 h at 90 °C, TpsT was formed to the extent of ~2% on the basis of RP-HPLC analysis of the deprotection reaction. Thus, selection of the 2-(methylsulfinyl)ethyl or 2-methoxyethyl group for the protection of terminal thiophosphate groups at the 5′- and/or 3′-terminus of DNA prodrug sequences would be an excellent choice for preventing extracellular and intracellular nucleolytic hydrolysis of these DNA sequences.

The deprotection rates of thermolytic thiophosphate protecting groups derived from 4-(methylthio)butan-1-ol and 2-(methylthio)ethanol may likely be controlled by increasing the size of the cyclic transition state operating in the well-studied cyclodeesterification process.[5a,8,11,20,21,32] In order to evaluate this hypothesis, the synthesis of 6-(methylthio)hexan-1-ol (**13**) was carried out by reacting 6-chlorohexan-1-ol with an aqueous solution of sodium methylmercaptide for 8 h at 25 °C. After extractive work-up, crude **13** was purified by chromatography on silica gel to give pure 6-(methylthio)hexan-1-ol in a yield of 97%. This alcohol led to the phosphoramidite **32**, which in turn was used in the synthesis of the dinucleoside phosphorothioate triester **50**. Thermolytic removal of the 6-(methylthio)hex-1-yl group from **50** was slow; TpsT was generated with a half time of 13 h at 90 °C (Table 2). With the exception of terminal 5′- and/or 3′-phosphodiester protection, the 6-(methylthio)hex-1-yl group is anticipated to be too thermostable at 37 °C for thiophosphate protection of thermolytic CpG ODN prodrugs.

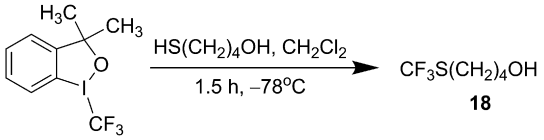
The 5-(methylthio)pent-1-yl group was considered next, as a compromise between the 4-(methythio)but-1-yl and the 6-(methylthio)hex-1-yl groups, in terms of rates of thermolytic thiophosphate triester deprotection. The synthesis of 5-(methylthio)pentan-1-ol (**15**) was completed in two steps from methyl 5-chlorovalerate (Scheme 5). Specifically, the reduction of methyl 5-chlorovalerate by treatment with $LiAlH_4$ in diethyl ether gave, after silica gel chromatography, 5-chloropentan-1-ol[12] (**14**) in a yield of 58%. The reaction of **14** with an aqueous solution of sodium methylmercaptide for 8 h at 25 °C produced 5-(methylthio)pentan-1-ol[13] in a yield of 61% after extractive work-up and purification by chromatography on silica gel. The alcohol **15** was converted to the deoxyribonucleoside phosphoramidite **33**, which led to the dinucleoside phosphorothioate triester **51** (Schemes 1 and 2). The 5-(methylthio)pent-1-yl group was thermolytically removed from **51** under the conditions described in Scheme 2 to generate TpsT with a half time of 30 min at 90 °C or 33 h at 37 °C.



Scheme 5. Synthesis of 5-(methylthio)pentan-1-ol (**15**) and 5-(isopropylthio)pentan-1-ol (**16**) from methyl 5-chlorovalerate. Conditions: (i) $LiAlH_4$, Et_2O, 1 h, 25 °C; (ii) CH_3SNa, H_2O, 8 h, 25 °C or $(CH_3)_2CHSNa$,[14] EtOH, 1.5 h, reflux.

The alcohol **16** was selected for assessing the effect of steric hindrance around the sulfur atom on the thermolytic deprotection rate of **52** (Table 2). The steric bulk of the 5-isopropylthio function had only a marginal effect on the thiophosphate deprotection of **52** relative to that of **51**. TpsT was generated from **52** with a half time of 35 min at 90 °C or 35 h at 37 °C. Such a deprotection rate is only modestly slower than that determined for the 5-(methythio)pent-1-yl group under similar conditions (Table 2). Although the 5-(methythio)pent-1-yl and 5-(isopropylthio)pent-1-yl groups can be used for thiophosphate protection of the CpG motif and/or its flanking sequences in the development of immunotherapeutic CpG ODN prodrugs, the search for thermolytic groups that would impart moderately slower deprotection kinetics than that of the FMA group was further investigated.

The placement of an electron-withdrawing group near the sulfur atom of the 4-(methylthio)but-1-yl group, on either side, is likely to decrease its nucleophilicity and should yield thiophosphate protecting groups exhibiting relatively slower deprotection rates. This hypothesis was tested by replacing the methyl group, directly linked to the sulfur atom of the 4-(methylthio)but-1-yl group, with a trifluoro-methyl group. The preparation of 4-(trifluoromethyl-thio)butan-1-ol (**18**) was carried out through the reaction of 4-mercaptobutan-1-ol with the 1,2-benziodoxole **17** (Scheme 6) in CH_2Cl_2 at −78 °C. By the way, **17** was synthesized according to the method of Kieltsch et al.[15] The dinucleoside phosphorothioate triester **53** was routinely prepared following the sequence of reactions shown in Table 2 under the conditions given in Scheme 2.



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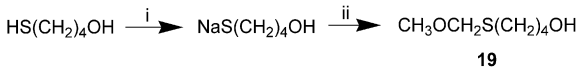
Scheme 6. Preparation of 4-(trifluoromethylthio)butan-1-ol (**18**) from 4-mercaptobutanol and 1-trifluoromethyl-1,3-dihydro-3,3-dimethyl-1,2-benziodoxole (**17**).

Thermolytic cleavage of the 4-(trifluoromethylthio)but-1-yl group from **53** under standard conditions (Scheme 2) led to the formation of TpsT with a half time of 17 h at 90 °C. The strong electron-withdrawing properties of the trifluoro-methyl group drastically decreased the deprotection rates of the thiophosphate protecting group. Consequently, the 4-(trifluoromethylthio)but-1-yl group can only be used for the protection of terminal 5′- and/or 3′-thiophosphodiester functions of DNA prodrugs.

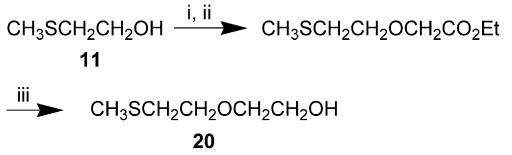
The search for weaker electron-withdrawing groups neighboring the sulfur atom of the 4-(methylthio)but-1-yl group was then undertaken. The selection of the 4-(methoxymethylthio)but-1-yl group for thiophosphate protection seemed appropriate. The required alcohol, 4-(methoxymethylthio)butan-1-ol (**19**), was synthesized from the reaction of chloromethyl methyl ether with an alkaline ethanolic solution of 4-mercaptobutan-1-ol under the conditions described in Scheme 7.[16] After work-up and purification by chromatography on silica gel, pure **19** was isolated in a yield of 29%.



Scheme 7. Synthesis of 4-(methoxymethylthio)butan-1-ol (**19**) from 4-mercaptobutan-1-ol. Conditions: (i) NaOH, EtOH, 40 °C, 15 min; (ii) CH_3OCH_2Cl, 5–10 °C, 30 min, then 16 h, 25 °C.

The dinucleoside thiophosphate triester **54** (Table 2) was then synthesized from the phosphoramidite **36** under the conditions given in Scheme 2. The thermolytic removal of the 4-(methoxymethylthio)but-1-yl group from **54** occurred with a half time of 9 min at 90 °C or 6.5 h at 37 °C. Such a deprotection rate is too rapid at 37 °C for thiophosphate protection of the CpG motif of immunomodulatory DNA oligonucleotide prodrugs, but may be suitable for the protection of its flanking thiophosphodiester functions.

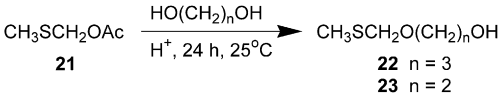
The insertion of an oxygen atom in the carbon chain of the 4-(methylthio)but-1-yl group is expected to slow down the thermolytic deprotection of the thiophosphate protecting group for the following reasons: (i) the inductive effect of the oxygen atom should reduce the nucleophilicity of the methylthio function participating in the cyclodeesterification process; and (ii) the transition state of the cyclodeesterification reaction is expanded from a five- to a six-membered transition state. In this context, the thermolytic properties of the 2-(2-methylthioethoxy)ethyl group for thiophosphate protection have been investigated. The synthesis of 2-(2-methylthioethoxy)ethanol[17] (**20**) was carried out by reacting the sodium salt of 2-(methylthio)ethanol with ethyl bromoacetate in 1,2-dimethoxyethane to give ethyl 2-(2-methylthioethoxy)acetate. Reduction of the ester was effected by treatment with $LiAlH_4$ in Et_2O to provide, after work-up and vacuum distillation, the pure alcohol **20** in a yield of 36% (Scheme 8).



Scheme 8. Preparation of 2-(2-methylthioethoxy)ethanol (**20**) from 2-(methylthio)ethanol. Conditions: (i) NaH, dry 1,2-dimethoxyethane, 2 h, 25 °C; (ii) $BrCH_2CO_2Et$, 10 h, reflux; (iii) $LiAlH_4$, Et_2O, 30 min, 25 °C.

The alcohol **20** was incorporated into the dinucleoside phosphorothioate triester **55** via its phosphoramidite derivative **37** (Table 2). Removal of the 2-(2-methylthioethoxy)ethyl group from **55** under thermolytic conditions (Scheme 2) occurred with a half time of 5 h at 90 °C. Similar to the 2-(*N*-formylamino)ethyl thiophosphate protecting group, the 2-(2-methylthioethoxy)ethyl group is inadequate for thiophosphate protection of the CpG motif of DNA prodrugs because its thermolytic deprotection rate would be too slow at 37 °C for practical prodrug-to-drug conversion. This group may nonetheless be used for the protection of terminal 5′- and/or 3′-thiophosphate functions of these DNA sequences.

It is often difficult to predict the effect of simplistic structural modifications on the deprotection kinetics of thermosensitive thiophosphate protecting groups. For example, the 3-(methylthiomethoxy)prop-1-yl group, which is an isostere of the 2-(2-methylthioethoxy)ethyl group for thiophosphate protection, was expected to be slower-deprotecting than the 2-(2-methylthioethoxy)ethyl group given the stronger inductive effect generated by the closer proximity of the oxygen atom to the nucleophilic methylthio function. Surprisingly, the thermolytic deprotection of the dinucleoside phosphorothioate triester **56** took place with a half time of 2.5 h at 90 °C or 375 h at 37 °C. The two-fold faster thiophosphate deprotection rate of **56** relative to that of **55** is unclear and requires further investigation. The dinucleotide **56** was obtained from the reaction of phosphoramidite **38** with TsuccCPG under the conditions reported in Scheme 2. The reaction of methylthiomethyl acetate (**21**)[18] with 1,3-propanediol under catalytic acidic conditions (Scheme 9) gave 3-(methylthiomethoxy)-propan-1-ol (**22**, Tables 1 and 2).



Scheme 9. Synthesis of 3-(methylthiomethoxy)propan-1-ol (**22**) and 2-(methylthiomethoxy)ethanol (**23**) from methythiomethyl acetate (**21**).

The 3-(methylthiomethoxy)prop-1-yl group may, in a worst case study in terms of slow thermolytic deprotection rates, be applied to thiophosphate protection of the CpG motif of CpG ODN prodrugs to assess the biological significance of sustained immunostimulation in animal models. Alternatively, this protecting group may be applied to the protection of terminal 5′- and/or 3′-thiophosphate functions of DNA prodrugs as a means to extend the half time of their circulation in vivo.

In order to increase the thiophosphate deprotection rate of **56** at 37 °C, the use of the 2-(methylthiomethoxy)ethyl group for thiophosphate protection was considered. The alcohol **23** was obtained under conditions similar to those used for the synthesis of **22** (Scheme 9).[18] The dinucleoside phosphorothioate triester **57** was prepared from **23** in accordance with the sequence of reactions shown in Table 2 under standard conditions (Scheme 2). The thermolytic deprotection of the 2-(methylthiomethoxy)ethyl group from **57** went smoothly to generate TpsT with a half time of 12 min at 90 °C or 22 h at 37 °C. In a worst case study in terms of fast thermolytic deprotection rates, this group may be suitable for thiophosphate protection of the CpG motif of CpG ODN prodrugs and/or that of its flanking DNA sequences.

It is worth mentioning that phosphate diester can similarly be protected by heat-sensitive groups derived from the alcohols listed in Tables 1 and 2. As shown in Table 3, the thermolytic deprotection half times of exemplary thymidylyl-(3′→5′)-thymidine phosphate triesters [Tp(OR)T] to TpT are approximately 10% longer than those of the corresponding thiophosphate triester analogs.

Table 3
Thermolytic deprotection rates of selected dinucleoside phosphate triesters (**62**–**64**)

Tp(OR)T	→	TpT	
		$t_{1/2}$ (90 °C)	$t_{1/2}$ (37 °C)
62 R=$(CH_2)_2S(O)CH_3$		162 min[a]	93 d
63 R=$(CH_2)_4SCH_3$		ND[b]	17 min[c]
64 R=$(CH_2)_5SCH(CH_3)_2$		36 min[d]	38 h[d]

[a] The thermolytic conversion of **58** to TpsT proceeded with a $t_{1/2}$ of 146 min at 90 °C.
[b] Not determined.
[c] The thermolytic conversion of **47** to TpsT proceeded with a $t_{1/2}$ of 15 min at 37 °C.
[d] The thermolytic conversion of **52** to TpsT proceeded with a $t_{1/2}$ of 34 min at 90 °C or 35 h at 37 °C.

This work has led to the identification of a number of thermolytic groups for thiophosphate protection in the design and development of thermosensitive DNA oligonucleotide prodrugs. Most of the thermolytic groups investigated are relatively lipophilic and while the lipophilicity of DNA prodrugs facilitates their cellular uptake,[1d] it negatively affects their solubility in aqueous media. Thus, thermolytic thiophosphate protecting groups with relatively short deprotection kinetics are likely to enhance early cellular uptake of lipophilic DNA oligonucleotide prodrugs by generating, in a time-dependent manner, negatively charged phosphorothioate diester functions, which should progressively increase the solubility of the prodrugs in aqueous media.

In this regard, the thermosensitive 4-(methoxymethylthio)-but-1-yl and 2-(methylthiomethoxy)ethyl groups for thiophosphate

protection are excellent candidates for time-dependent negative charge generation on lipophilic DNA oligonucleotide prodrugs, given their respective deprotection half times of 6.5 h and 22 h at 37 °C.

The synthesis of an oligonucleotide model was carried out to further assess the validity of our approach to the design of heat-sensitive DNA oligonucleotide prodrugs. The DNA sequence **59** [5′-d($C_{PS(MOE)}C_{PS(MOE)}T_{PS(FMA)}T_{PS(FMA)}T_{PS(FMA)}T_{PS(FMA)}C_{PS(MOE)}G_{PS(FMA)}T_{PS(FMA)}T_{PS(FMA)}T_{PS(FMA)}T_{PS(FMA)}C_{PS(MOE)}C_{PS(MOE)}T$)] was selected for a simplistic and economical demonstration of our strategy. The acronyms PS(FMA) and PS(MOE) identify the thermolytic 2-(*N*-formyl-*N*-methylamino)ethyl[5] and thermostable 2-methoxyethyl phosphorothioate triester functions, respectively. The MOE group was chosen for the protection of the CpG motif to mimic any thermolytic group exhibiting thiophosphate deprotection rates slower than that of the FMA group. As mentioned above, the MOE group by virtue of its thermostability, size, and relatively moderate lipophilicity is ideal for the protection of both 5′-and 3′-terminal thiophosphate functions of the DNA sequence against potential exonucleases that may be found in biological media. The thermolytic FMA group was selected to protect the thiophosphate functions flanking the CpG motif on either side to demonstrate the time-dependent generation of charged phosphorothioate diesters, which should impart a progressive solubility of the DNA sequence and facilitate its cellular uptake. Thus, the deoxyribonucleoside phosphoramidites **25**, **40** (R=MOE), and **41** (R=FMA) were employed in the solid-phase synthesis of the thermolytic DNA oligonucleotide **59** under the conditions described in the Experimental procedures section. Following synthesis, the DNA sequence was exposed to pressurized ammonia gas[19] to *N*-deacylate the nucleobases and release the oligonucleoside phosphorothioate triester from the solid support. The 5′-dimethoxytritylated DNA sequence was purified by RP-HPLC, treated with 80% AcOH to remove the 5′-DMTr group, and re-purified by RP-HPLC employing the chromatographic conditions described in the Experimental procedures section. The identity of **59** was confirmed by MALDI-TOF MS. The DNA sequence was then heated in PBS (pH 7.4) at 90 °C for 23 min. Under these conditions, half the number of FMA thiophosphate protecting groups were thermolytically cleaved, as indicated by RP-HPLC analysis of the deprotection reaction. The RP-HPLC profile of **59** prior to heating is shown in Figure 1A and consists of a major broad peak with a retention time of ~35 min. The shape and retention time of this peak have changed significantly after heat treatment (Fig. 1B). Consistent with the increased number of phosphodiester functions that have been generated from the thermal cleavage of the FMA thiophosphate protecting groups, a shorter retention time (~32 min) and a sharper peak representation of the P-diastereomeric population were apparent. Extension of the heat treatment to 3 h at 90 °C resulted in the complete thermolytic removal of the FMA groups while leaving the MOE groups intact.

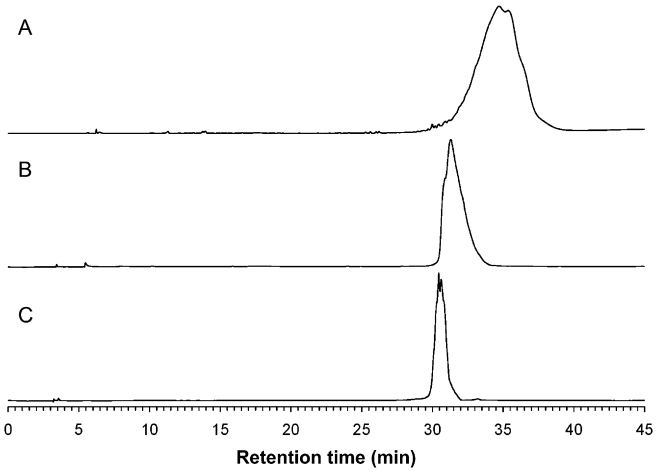


Figure 1. RP-HPLC analysis of the conversion of the DNA sequence **59** to **60** under thermolytic conditions. (A): chromatographic profile of the RP-HPLC-purified DNA sequence **59** prior to thermolysis. (B): chromatogram of **59** after being heated for 23 min at 90 °C in PBS (pH 7.4). (C): chromatogram of **60**, which is that of **59** after being heated for 3 h at 90 °C in PBS (pH 7.4).

Figure 1C shows the RP-HPLC profile of 5′-d($C_{PS(MOE)}C_{PS(MOE)}T_{PS}T_{PS}T_{PS}T_{PS}C_{PS(MOE)}G_{PS}T_{PS}T_{PS}T_{PS}T_{PS}C_{PS(MOE)}C_{PS(MOE)}T$) (**60**) consisting of significantly sharper peaks with retention times of ~31 min. The acronym PS stands for a phosphorothioate diester function. The identity of the FMA-free DNA sequence **60** was corroborated by ESI-MS and by the solid-phase synthesis of the control DNA sequence **61** [5′-d($C_{PS(MOE)}C_{PS(MOE)}T_{PS(CE)}T_{PS(CE)}T_{PS(CE)}T_{PS(CE)}C_{PS(MOE)}G_{PS(CE)}T_{PS(CE)}T_{PS(CE)}T_{PS(CE)}T_{PS(CE)}C_{PS(MOE)}C_{PS(MOE)}T$)] differing from **59** only by the replacement of the FMA groups with 2-cyanoethyl (CE) groups for thiophosphate protection. Post-synthesis deprotection of this DNA sequence was effected by treatment with pressurized ammonia gas[19] to *N*-deacylate the nucleobases, remove the 2-cyanoethyl thiophosphate protecting groups, and release the thioated oligonucleotide **60** from the solid support. The electrophoretic mobility of the DNA sequence **60** obtained from either **59** or **61** was compared by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions. A photograph of the stained gel is shown in Figure 2.

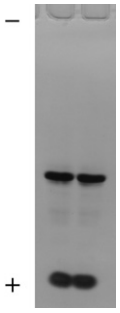


Figure 2. PAGE analysis of the DNA sequence **60** under denaturing conditions. Electrophoretic conditions are described in the Experimental procedures section. Left lane: RP-HPLC-purified **60** that was obtained from **61**, which was deprotected by treatment with pressurized ammonia gas (~10 bar) for 12 h at 25 °C. Right lane: RP-HPLC-purified **60** that was obtained from **59**, which was deprotected by treatment with pressurized ammonia gas (~10 bar) for 12 h at 25 °C followed by heating in PBS (pH 7.4) for 3 h at 90 °C. Oligonucleotides are visualized as blue bands upon staining the gel with Stains-all. Bromophenol blue is used as a marker and shows as a large band, in each lane, at the bottom of the gel.

The thermolytic cleavage of the FMA groups from the DNA sequence **59** and the base-assisted removal of the 2-cyanoethyl groups from the control DNA sequence **61** resulted in the production of a DNA sequence exhibiting an electrophoretic mobility identical to that of **60** and further corroborated the identity of the thermolytic DNA sequence **59**.

The lipophilicity of oligonucleotide prodrugs is an important parameter for cellular uptake, and yet it is very challenging to design and synthesize lipophilic DNA sequences with adequate solubility properties in aqueous media. The DNA oligomer **59** serves as an acceptable model to demonstrate the paradox of lipophilicity versus aqueous solubility. The oligomer was dissolved in a minimal amount of MeCN and a fraction of the solution was added to pre-heated PBS (pH 7.4) at 90 °C in a thermo-controlled quartz cuvette. The UV absorbance of the oligomer was monitored at 268 nm at selected time points over a period of 3 h to ensure complete thermal cleavage of the FMA thiophosphate protecting groups. Figure 3 shows that the solubility of **59** at the time of addition (t=0) was only 16% that of the DNA sequence **60** under these conditions. The solubility profile of **59**/**60** shown in Figure 3 underscores the

importance of time-dependent generation of negatively charged phosphorothioate diester functions as a viable approach to enhance the aqueous solubility of lipophilic DNA oligonucleotide prodrugs.

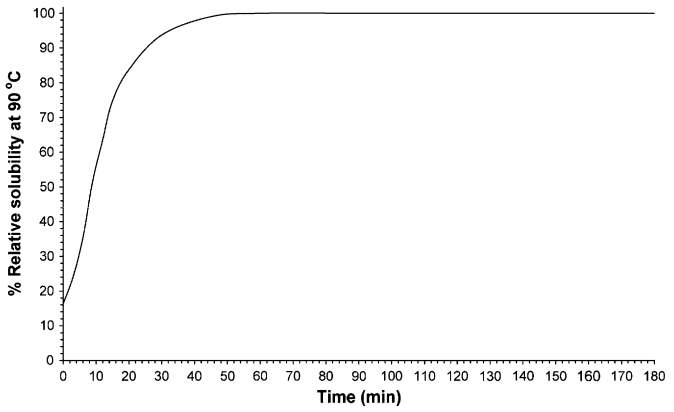


Figure 3. Effect of the thermal deprotection rate of FMA thiophosphate protecting groups on the relative solubility of the DNA sequence **59**. 100% relative solubility is defined as the maximum absorbance (A_{max}) of the DNA sequence at 268 nm and 90 °C. Thus, % relative solubility=(A/A_{max})100.

In summary, a systematic investigation of heat-sensitive thiophosphate protecting groups, derived from the previously studied FMA[5] and 4-(methylthio)but-1-yl[11] groups, has identified additional groups for the protection of immunomodulatory DNA oligonucleotide prodrugs. These groups reveal faster and slower thermolytic thiophosphate deprotection rates than that of the FMA and 4-(methylthio)but-1-yl groups. Typically, thiophosphate protecting groups with deprotection kinetics slower than that of the FMA group are derived from alcohols **3**, **5**, **12**, **13**, **18**, **20** or **22** were found essentially thermostable. These groups, in addition to the 2-(methylsulfinyl)ethyl group, may serve as protecting groups for the terminal phosphodiester functions of DNA prodrug sequences and provide resistance against the extracellular and intracellular exonucleases found in biological systems.

Thermosensitive thiophosphate protecting groups derived from alcohols **9**, **15**, **16**, **19** or **23** were found to deprotect faster than the FMA group at 37 °C. These groups are more appropriate for the protection of the thiophosphates flanking the CpG motif of DNA prodrugs given that they are likely to fulfill the dual function of providing both lipophilicity and time-dependent hydrophilicity through the generation of negatively charged diester functions. The relationship between lipophilicity and hydrophilicity is undoubtedly an important parameter for optimal cellular uptake of DNA oligonucleotide prodrugs.

The thermolabile thiophosphate protecting groups derived from alcohols **9**, **15** or **16** can also be used for the protection of CpG motifs in cases where the induction of early immunostimulatory events in animal models is necessary. Conversely, the 3-(methylthiomethoxy)-prop-1-yl group should be useful in evaluating the effect of sustained immunostimulation in animal models given its relatively slow thiophosphate deprotection rate at 37 °C ($t_{1/2}$=375 h). In the context of further assessing the biological consequences of sustained immunostimulation in animal models, we had hoped to identify thiophosphate protecting groups for the CpG motif of DNA prodrugs that would have had displayed thermolytic deprotection half times in the range of 100–200 h at 37 °C. Investigations regarding the development of phosphate/thiophosphate protecting groups derived from hydroxyalkyl thiophosphoramidate intermediates are currently ongoing in our laboratory. Incidentally, we have recently reported the use of a 3-hydroxypropyl thiophosphoramidate function in the thermosensitive release of covalently linked DNA sequences from controlled-pore glass under essentially neutral conditions.[20] The results of our studies on the use of this class of thermolytic protecting groups for the protection of CpG motifs in the development of DNA prodrugs will be reported in due course.

Our findings also address the paradox of oligonucleotide lipophilicity versus aqueous solubility in that thiophosphate protecting groups with thermolytic deprotection half times, in the particular range of 6–40 h at 37 °C (Table 2) are likely to increase the solubility of lipophilic DNA oligonucleotide prodrugs through time-dependent generation of negatively charged phosphodiester functions (Fig. 3). We recently demonstrated that the aqueous solubility of an FMA thiophosphate-protected DNA oligonucleotide prodrug (15-*mer*) increased by 25% or 49% as a consequence of the incorporation of one thiophosphate or phosphate monoester group, respectively, into the DNA sequence.[21] An investigation, beyond the scope of the present study, on the correlation of thermolytic time-dependent generation of negatively charged phosphodiester groups with the uptake of DNA oligonucleotide prodrugs in human cell culture is underway in the laboratory. The details of this investigation will be reported elsewhere.

Thermosensitive groups have also been applied to the protection of the 5′-hydroxyl[22] and exocyclic amino[23] functions of deoxyribonucleosides, thereby supporting the potential application of these groups for the protection of alcohols and amine functions in various areas of synthetic organic and medicinal chemistry. Particularly noteworthy is the incorporation of thermosensitive phosphate protecting groups into DNA oligonucleotide primers[24] to prevent the extension of these primers at the initial set up stages of the polymerase chain reaction. A thermal activation step induced the cleavage of the phosphate protecting groups and generated the corresponding unmodified DNA primers, which then supported clean amplification of the desired DNA target sequences. Such a useful application underscores the significance of thermolytic groups in the development of DNA prodrugs and DNA diagnostics.

3. Experimental procedures

3.1. Materials and methods

Common chemicals and solvents were purchased from commercial sources and used without further purification. Preparative chromatographic purifications were performed on columns packed with silica gel 60 (230–400 mesh), whereas analytical thin-layer chromatography (TLC) analyses were conducted on 2.5×7.5 cm glass plates coated with a 0.25 mm thick layer of silica gel 60 F_{254}.

NMR spectra were recorded at 7.05 T (300 MHz for ^{1}H) at 25 °C. ^{1}H and proton-decoupled ^{31}P NMR spectra were run in deuterated solvents as indicated. Tetramethylsilane (TMS) was used as an internal reference for ^{1}H NMR spectra, and 85% phosphoric acid in deuterium oxide served as an external reference for ^{31}P NMR spectra. Proton-decoupled ^{13}C NMR spectra were recorded in DMSO-d_6. Chemical shifts δ are reported in parts per million (ppm).

MALDI-TOF mass spectrometric analyses of oligonucleotides were performed operating in delayed extraction reflector mode, using 2′,4′,6′-trihydroxyacetophenone as a matrix and ammonium citrate as a cation exchanger. Mass spectrometric analyses of oligonucleotides were also carried out using an LC–MS/MS instrument equipped with an electrospray ionization source. Measurements were made in the negative ion mode. Accurate mass measurements of alcohol derivatives and deoxyribonucleoside phosphoramidites were obtained using a time-of-flight (TOF) mass spectrometer with electrospray ionization in the positive ion mode.

*3.1.1. 3-(N-Formylamino)propan-1-ol (**1**).* 3-Aminopropan-1-ol (26 mL, 0.34 mol) was placed in a flask connected to a condenser and was cooled to ~5 °C in an ice-water bath. Ethyl formate

(41 mL, 0.51 mol) was added, portionwise through the condenser, to the stirred aminoalcohol over a period of 5 min. The solution was removed from the ice-bath and was heated to a reflux, which was maintained for 1 h. The solution was then distilled at atmospheric pressure to remove the excess of ethyl formate. Residual ethyl formate was eliminated under high vacuum leaving the desired product **1**[25] as a colorless oil (23.6 g, 230 mmol, 67%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.07 (s, 1H), 4.60 (s, 1H), 3.65 (s, 1H), 3.50 (d, J=6.0 Hz, 2H), 3.22 (d, J=6.0 Hz, 2H), 1.54 (t, J=6.0 Hz, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 161.4, 58.5, 34.5, 32.3.

*3.1.2. 3-(Methylamino)propan-1-ol (**2**).* To a cold (0 °C) stirred solution of 3-(*N*-formyl-amino)propan-1-ol (**1**, 1.72 g, 17.0 mmol) in THF (50 mL) was added 1 M borane/THF complex in THF (50 mL, 50 mmol), dropwise, over a period of 1 h. The solution was stirred for an additional 5 h at ~25 °C and was then cooled to 0 °C. NaOH (3 M, 15 mL) was added to the solution, which was allowed to stir for 12 h at ~25 °C. The aqueous phase was saturated with K_2CO_3 and the organic phase was collected. The aqueous phase was extracted with Et_2O (3×50 mL); the combined organic extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The crude aminoalcohol **2**[25a] (1.33 g, 15.0 mmol) was sufficiently pure to be used without further purification in the synthesis of 3-(*N*-formyl-*N*-methylamino)propan-1-ol (**3**).

*3.1.3. 3-(N-Formyl-N-methylamino)propan-1-ol (**3**).* 3-(Methylamino)propan-1-ol (**2**, 1.33 g, 15.0 mmol) was placed in a flask connected to a condenser and was cooled to 0 °C. Ethyl formate (1.66 g, 22.5 mmol) was added, portionwise through the condenser, to the cold aminoalcohol over a period of 5 min. The solution was removed from the cold bath and was heated to a reflux, which was continued for 1 h. The excess ethyl formate was distilled off at atmospheric pressure leaving the amido alcohol **3**,[25b] which was used without further purification in the synthesis of the deoxyribonucleoside phosphoramidite **24**.

*3.1.4. 2-(N-Formylamino)ethan-1-ol (**5**).* Freshly distilled ethanolamine (2.00 g, 32.7 mmol) was placed in a flask connected to a condenser and was cooled to ~5 °C by immersion in an ice-water bath. Ethyl formate (3.64 g, 49.1 mmol) was added in portions of 1 mL, through the condenser, to the stirred ethanolamine over a period of 5 min. The solution was removed from the ice-bath and brought to a reflux, which was continued for 1 h. The excess ethyl formate was removed by distillation at atmospheric pressure and the crude product was purified by chromatography on silica gel using a gradient of MeOH (0→5%) in CH_2Cl_2 to give **5**[26] (2.02 g, 22.7 mmol) in a yield of 69%. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.09 (br s, 1H), 4.84 (t, J=5.5 Hz, 1H), 3.51 (m, 2H), 3.25 (m, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 161.7, 60.0, 40.3.

*3.1.5. 9-Fluorenylmethyl 2-(N-formylamino)ethyl carbonate (**6**).* A solution of 2-(*N*-formylamino)ethan-1-ol (**5**, 1.00 g, 11.2 mmol) in 60 mL of pyridine was cooled to ~5 °C in an ice-water bath. 9-Fluorenylmethoxycarbonyl chloride (3.49 g, 13.5 mmol) was added to the solution, which was left stirring for 2 h at ~5 °C. The reaction was quenched by adding ethanediol (2 mL); after 10 min, the reaction mixture was evaporated to an oil under reduced pressure. The crude material was dissolved in CH_2Cl_2 (100 mL) and was washed with a saturated aqueous solution of $NaHCO_3$ (50 mL). The aqueous phase was collected and was extracted with CH_2Cl_2 (2×40 mL); the organic extracts were pooled together, dried over sodium sulfate, and filtered. The filtrate was evaporated under low pressure and the material left was purified by chromatography on silica gel using a gradient of MeOH (0→2%) in CH_2Cl_2. Pure **6** (2.3 g, 7.4 mmol) was isolated in a yield of 66%. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.15 (br s, 1H), 8.00 (d, J=7.4 Hz, 2H), 7.76 (d, J=7.4 Hz, 2H), 7.53 (t, J=7.4 Hz, 2H), 7.45 (t, J=7.4 Hz, 2H), 4.67 (d, J=6 Hz, 2H), 4.41 (t, J=6 Hz, 1H), 4.18 (t, J=5.2 Hz, 2H), 3.44 (t, J=5.2 Hz, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 161.3, 154.3, 143.2, 140.7, 127.6, 127.1, 124.8, 120.1, 68.5, 66.1, 46.2, 36.1. $^+$ESI-TOF MS: calcd for $C_{18}H_{17}NO_4$ $(M+H)^+$ 312.1236, found 312.1228.

*3.1.6. 9-Fluorenylmethyl 2-(N-thioformylamino)ethyl carbonate (**7**).* 9-Fluorenylmethyl 2-(*N*-formylamino)ethyl carbonate (**6**, 2.3 g, 7.4 mmol) and Lawesson reagent (1.49 g, 3.69 mmol) were dissolved in THF (75 mL). The solution was stirred at ~25 °C for 10 min. Silica gel (10 g) was added to the reaction mixture and the resulting suspension was evaporated under reduced pressure. The material left was loaded on the top of a column packed with silica gel (120 g). The product **7** eluted from the column using CH_2Cl_2 as the eluent and was isolated in a yield of 79% (1.93 g, 5.89 mmol). ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.50 (br s, 1H), 9.39 (s, 1H), 7.98 (d, J=7.2 Hz, 2H), 7.75 (d, J=7.2 Hz, 2H), 7.52 (t, J=7.2 Hz, 2H), 7.44 (t, J=7.2 Hz, 2H), 4.66 (d, J=6.0 Hz, 2H), 4.40 (t, J=6.0 Hz, 1H), 4.35 (t, J=5.0 Hz, 2H), 3.88 (q, J=5.0 Hz, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 189.3, 154.2, 143.2, 140.7, 127.7, 127.1, 124.8, 120.0, 68.6, 64.6, 46.2, 41.4. $^+$ESI-TOF MS: calcd for $C_{18}H_{17}NO_3S$ $(M+H)^+$ 328.1007, found 328.1005.

*3.1.7. 2-(N-Thioformylamino)ethan-1-ol (**8**).* 9-Fluorenylmethyl 2-(*N*-thioformylamino)ethyl carbonate (**7**, 1.93 g, 5.89 mmol) was dissolved in a solution of 20% piperidine in DMF. The resulting solution was stirred at ~25 °C for 30 min and was then poured into water (75 mL). The aqueous solution was extracted with CH_2Cl_2 (2×50 mL). The organic extracts were dried over sodium sulfate and filtered. The filtrate was evaporated under low pressure and the material left was purified by chromatography on silica gel using a gradient of MeOH (0→2%) in CH_2Cl_2. Pure **8**[27] (523 mg, 4.98 mmol) was isolated in a yield of 84%. ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.27 (br s, 1H), 9.27 (s, 1H), 4.90 (br s, 1H), 3.49 (t, J=5.2 Hz, 2H), 3.36 (t, J=5.2 Hz, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 188.0, 57.6, 45.1.

*3.1.8. 6-(Methylthio)hexan-1-ol (**13**).* To a stirred aqueous solution (25 mL) of sodium methylmercaptide (3.00 g, 42.8 mmol) was added 6-chlorohexanol (5.08 g, 37.2 mmol). The solution was left stirring at ~25 °C for 8 h and was then extracted with chloroform (25 mL). The organic phase was collected and dried over anhydrous K_2CO_3. Following filtration, the filtrate was evaporated under reduced pressure and the crude product was purified by chromatography on silica gel using CH_2Cl_2/MeOH (95:5 v/v) as the eluent. Pure **13**[28] was isolated as a light yellow oil (5.34 g, 36.0 mmol, 97%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 4.41 (t, J=5.1 Hz, 1H,), 3.48 (dt, J=5.1, 5.8 Hz, 2H), 2.54 (t, J=7.3 Hz, 2H), 2.12 (s, 3H), 1.7–1.2 (m, 8H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 60.6, 33.3, 32.4, 28.7, 28.1, 25.1, 14.6.

*3.1.9. 5-Chloropentan-1-ol (**14**).* To a stirred solution of methyl 5-chlorovalerate (1.80 g, 11.9 mmol) in 40 mL of Et_2O was added, dropwise, a suspension of $LiAlH_4$ (0.49 g, 13 mmol) in THF (13 mL). The suspension was stirred for 1 h at 25 °C, at which point, the excess $LiAlH_4$ was quenched by addition of ice-cold diluted sulfuric acid. The white solid that was formed was removed by filtration. The filtrate was extracted with Et_2O (3×30 mL). The etheral extracts were collected, dried over anhydrous Na_2SO_4, and filtered. The filtrate was evaporated under low pressure and the crude product was purified by silica gel chromatography using hexane/EtOAc (9:1 v/v) as the eluent. The pure alcohol **14**[12] (850 mg, 6.93 mmol, 58%) was isolated as a colorless oil. ^{1}H NMR (300 MHz, DMSO-d_6): δ 4.37 (t, J=5.2 Hz, 1H), 3.61 (t, J=6.6 Hz, 2H), 3.39 (m, 2H), 1.71 (t, J=6.6 Hz, 2H), 1.43 (m, 4H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 60.5, 45.3, 31.9, 31.6, 22.9.

*3.1.10. 5-(Methylthio)pentan-1-ol (**15**).* 5-Chloropentanol (**14**, 836 mg, 6.82 mmol) was added to a stirred aqueous solution (5 mL) of sodium

methylmercaptide (550 mg, 7.84 mmol). The solution was further stirred at 25 °C for 8 h and was then extracted with chloroform (25 mL). The organic phase was collected and dried over anhydrous K_2CO_3. Following filtration, the filtrate was evaporated under reduced pressure and the crude product was purified by chromatography on silica gel using CH_2Cl_2/MeOH (95:5 v/v) as the eluent. The pure alcohol **15**[13] was isolated as a colorless oil (560 mg, 4.17 mmol, 61%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 4.44 (t, J=5.1 Hz, 1H), 3.48 (m, 2H), 2.54 (t, J=7.3 Hz, 2H), 2.12 (s, 3H), 1.7–1.3 (m, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 60.6, 33.3, 32.1, 28.5, 24.7, 14.7.

*3.1.11. 5-(Isopropylthio)pentan-1-ol (**16**).* 2-Propanethiol (326 mg, 4.28 mmol) was added to a solution of sodium ethoxide (277 mg, 4.08 mmol) in ethanol (4 mL). The stirred solution was brought to a reflux while 5-chloropentanol (**14**, 500 mg, 4.08 mmol) was added over a period of 1 h. The suspension was further stirred at reflux temperature for 30 min and was then concentrated to 50% of its original volume under reduced pressure. The suspension was filtered and the filtrate was evaporated under low pressure to give the crude material, which was purified by chromatography on silica gel using CH_2Cl_2 as eluent. Pure **16**[29] was isolated as a colorless oil (595 mg, 3.67 mmol, 90%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 4.44 (t, J=5.1 Hz, 1H), 3.47 (m, 2H), 2.99 (sept, J=7.0 Hz, 1H), 2.58 (t, J=7.1 Hz, 2H), 1.7–1.4 (m, 6H), 1.29 (d, J=7.0 Hz, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 60.5, 33.9, 32.0, 29.6, 29.2, 24.9, 23.2.

*3.1.12. 4-(Trifluoromethylthio)butan-1-ol (**18**).* A solution of **17**[15] (1.5 g, 4.5 mmol) in CH_2Cl_2 (1 mL) was added dropwise over 5 min to a cold (−78 °C) solution of 4-mercaptobutan-1-ol (439 mg, 4.13 mmol) in CH_2Cl_2 (3 mL). The reaction mixture was stirred at −78 °C for 1.5 h and then allowed to warm up to 25 °C. The solvent was evaporated under reduced pressure and the material left was purified by chromatography on silica gel using a gradient of MeOH (0→10%) in CH_2Cl_2 as the eluent. The pure product **18** was isolated as a colorless oil (520 mg, 2.99 mmol, 72%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 4.20 (br s, 1H), 3.41 (t, J=6.3 Hz, 2H), 3.00 (t, J=7.4 Hz, 2H), 1.69 (m, 2H), 1.5 (m, 2H). ^{13}C NMR (75 MHz, C_6D_6): δ: 131.3 (q, $^1J_{C-F}$=306 Hz), 59.9, 31.0, 29.4, 26.0. $^+$ESI-TOF MS (FMOC derivative): calcd for $C_{20}H_{19}F_3O_3S$ $(M+H)^+$ 397.1085, found 397.1122.

*3.1.13. 4-(Methoxymethylthio)butan-1-ol (**19**).* 4-Mercaptobutan-1-ol (**10**, 5.00 g, 47.1 mmol) and NaOH (1.58 g, 45.7 mmol) were mixed in anhydrous ethanol (18 mL). The reaction mixture was stirred at 40 °C until a clear solution was obtained (15 min). The solution was cooled to ~5 °C in an ice-bath and chloromethyl methyl ether (3.3 mL, 44 mmol) was added slowly over a period of 30 min while maintaining the reaction temperature below 10 °C. The ice-bath was removed and the reaction mixture was stirred overnight at ~25 °C. The salt that was generated during the course of the reaction was removed by filtration and washed with ethanol (2×15 mL). The ethanolic filtrates were pooled together and evaporated under reduced pressure. The crude product was purified by chromatography on silica gel using a gradient of MeOH (0→20%) in CH_2Cl_2 as the eluent. The pure product **19** was isolated as an oil (2.1 g, 14 mmol, 29%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 4.63 (s, 2H), 4.42 (t, J=5.2 Hz, 1H), 3.38 (dt, J=6.3, 5.2 Hz, 2H), 3.23 (s, 3H), 2.54 (t, J=7.1 Hz, 2H), 1.57 (m, 2H), 1.5 (m, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 74.7, 60.2, 54.9, 31.6, 30.2, 26.1. $^+$ESI-TOF MS (DMTr derivative): calcd for $C_{27}H_{32}O_4S$ $(M+H)^+$ 453.2100, found 453.2083.

*3.1.14. 2-(2-Methylthioethoxy)ethanol (**20**).* To a solution of 2-(methylthio)ethanol (**11**, 4.00 g, 43.0 mmol) in dry 1,2-dimethoxyethane (200 mL) was added sodium hydride (1.04 g, 43.0 mmol). The suspension was stirred for 2 h at ~25 °C when ethyl bromoacetate (7.25 g, 43.0 mmol) was added. The suspension was brought to reflux and was left stirring for 10 h at reflux temperature. The reaction mixture was allowed to cool to ~25 °C and was then filtered to remove the sodium salt. The filtrate was concentrated to ~10 mL and added to a suspension of $LiAlH_4$ (1.65 g, 430 mmol) in dry Et_2O (100 mL); the suspension was stirred for 30 min at ~25 °C and was then treated with a 15% aqueous solution of KOH (10 mL). The reaction mixture was diluted with water (50 mL), filtered, and extracted with Et_2O (3×50 mL). The organic extracts were collected and dried over anhydrous Na_2SO_4. Following filtration, the volatiles were evaporated under low pressure. Vacuum distillation of the crude material afforded pure **20**[17] (2.1 g, 15 mmol, 36%), bp 84 °C at 3 mmHg. ^{1}H NMR (300 MHz, DMSO-d_6): δ 4.66 (t, J=5.9 Hz, 1H), 3.65 (t, J=6.6 Hz, 2H), 3.58 (t, J=5.1 Hz, 2H), 3.51 (t, J=5.1 Hz, 2H), 2.71 (t, J=6.6 Hz, 2H), 2.17 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 72.0, 69.8, 60.2, 32.6, 15.1.

*3.1.15. Methylthiomethyl acetate (**21**).* To a stirred solution of DMSO (10 mL, 0.14 mol) in benzene (20 mL) was added acetic anhydride (13.2 mL, 140 mmol). The solution was refluxed for 5 h and AcOH was distilled off under atmospheric pressure. The crude product was purified by distillation under reduced pressure (bp 40 °C at 0.5 mmHg) to give **21**[18] (14.5 g, 120 mmol, 86%) as a colorless liquid. ^{1}H NMR (300 MHz, DMSO-d_6): δ 5.13 (s, 2H), 2.19 (s, 3H), 2.05 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 170.0, 67.4, 20.6, 14.5.

*3.1.16. 3-(Methylthiomethoxy)propan-1-ol (**22**).* Methylthiomethyl acetate (**21**, 978 mg, 8.14 mmol) and 1,3-propanediol (1.23 g, 16.1 mmol) were stirred together at ~25 °C in the presence of a catalytic amount of acid (Amberlyst H^+ form, 20 mg) over a period of 24 h. The catalyst was removed by filtration and a solution (4 mL) of NaOH (33 mmol) in MeOH/H_2O (3:1 v/v) was added to the filtrate. The resulting solution was stirred for 2 h at ~25 °C and neutralized to pH 7 upon addition of aqueous 5% HCl. The volume of the solution was reduced by 50% under low pressure. Water (10 mL) was added to the solution, which was extracted with CH_2Cl_2 (2×15 mL). The organic extracts were collected, dried over $MgSO_4$, filtered, and evaporated under reduced pressure to afford **22** (297 mg, 2.18 mmol, 27%) as a light yellow oil. ^{1}H NMR (300 MHz, DMSO-d_6): δ 4.60 (s, 2H), 4.58 (m, 1H), 3.52 (t, J=6.6 Hz, 2H), 3.44 (t, J=6.6 Hz, 2H), 2.07 (s, 3H), 1.66 (t, J=6.6 Hz, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 74.4, 64.6, 57.8, 32.3, 13.3. $^+$ESI-TOF MS (FMOC derivative): calcd for $C_{20}H_{22}O_4S$ $(M+H)^+$ 359.1317, found 359.1281.

*3.1.17. 2-(Methylthiomethoxy)ethanol (**23**).* Methylthiomethyl acetate (**21**, 2.00 g, 16.6 mmol), ethylene glycol (2.06 g, 33.3 mmol), and an acidic catalyst (Amberlyst H^+ form, 50 mg) were stirred together at ~25 °C over a period of 24 h. The catalyst was removed by filtration and a solution (8 mL) of NaOH (66 mmol) in MeOH/H_2O (3:1 v/v) was added to the filtrate. The resulting solution was stirred for 2 h at ~25 °C and was then neutralized by addition of aqueous 5% HCl. The volume of the solution was reduced by 50% under reduced pressure. Water (10 mL) was added and the solution was extracted with CH_2Cl_2 (2×15 mL). The organic phase was collected and dried over anhydrous $MgSO_4$, filtered, and evaporated under low pressure to give **23**[18] (870 mg, 7.12 mmol, 43%) as a light yellow oil. ^{1}H NMR (300 MHz, DMSO-d_6): δ 4.73 (s, 2H), 3.60 (m, 4H), 2.17 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 74.7, 69.4, 60.1, 13.3.

3.2. General procedure for the preparation of deoxyribonucleoside phosphoramidites 24–41

1*H*-Tetrazole (28 mg, 0.40 mmol) was added, under an inert atmosphere, to a solution of any of the alcohols listed in Table 1 (1 mmol) and appropriately protected deoxyribonucleoside 3′-*O*-phosphordiamidite (1 mmol) in anhydrous MeCN (10 mL). The solution was stirred at ~25 °C for 2 h and Et_3N (1 mL) was then added. The reaction mixture was concentrated to a syrup under

reduced pressure and the crude phosphoramidite was purified using a 2.5×20 cm Flex chromatography column packed with silica gel (25 g), which was equilibrated in C_6H_6/Et_3N (9:1 v/v). The equilibration solvent was 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) and the resulting solution was cooled to −78 °C in a dry ice-acetone bath. The frozen solution was lyophilized under high vacuum to give the phosphoramidite as a white powder. Phosphoramidites **24**–**41** were isolated in yields ranging from 70% to 85%.

*3.2.1. 5′-O-(4,4′-Dimethoxytrityl)-3′-O-[3-(N-formyl-N-methylamino)propoxy]-(N,N-diisopropylamino)phosphinyl-2′-deoxythymidine (**24**).* ^{31}P NMR (121 MHz, C_6D_6): δ 147.5, 147.4, 147.0, 146.9. $^+$ESI-TOF MS: calcd for $C_{42}H_{55}N_4O_9P$ $(M+H)^+$ 791.3785, found 791.3651.

*3.2.2. 5′-O-(4,4′-Dimethoxytrityl)-3′-O-[2-(N-formyl-N-methylamino)ethoxy]-(N,N-diisopropylamino)phosphinyl-2′-deoxythymidine (**25**).* ^{31}P NMR (121 MHz, C_6D_6): δ 145.3, 145.2, 145.0, 144.8. FAB-HRMS: calcd for $C_{41}H_{53}N_4O_9P$ $(M+Cs)^+$ 909.2604, found 909.2544.[5]

*3.2.3. 5′-O-(4,4′-Dimethoxytrityl)-3′-O-[2-(N-formylamino)ethoxy]-(N,N-diisopropylamino)phosphinyl-2′-deoxythymidine (**26**).* ^{31}P NMR (121 MHz, C_6D_6): δ 149.2, 148.6. $^+$ESI-TOF MS: calcd for $C_{40}H_{51}N_4O_9P$ $(M+H)^+$ 763.3472, found 763.4574.

*3.2.4. 5′-O-(4,4′-Dimethoxytrityl)-3′-O-[2-(N-thioformylamino)ethoxy]-(N,N-diisopropylamino)phosphinyl-2′-deoxythymidine (**27**).* ^{31}P NMR (121 MHz, C_6D_6): δ 147.7, 147.5. $^+$ESI-TOF MS: calcd for $C_{40}H_{51}N_4O_8PS$ $(M+H)^+$ 779.3243, found 779.3579.

*3.2.5. 5′-O-(4,4′-Dimethoxytrityl)-3′-O-[2-(N-acetylamino)ethoxy]-(N,N-diisopropylamino)phosphinyl-2′-deoxythymidine (**28**).* ^{31}P NMR (121 MHz, C_6D_6): δ 147.0, 146.8. $^+$ESI-TOF MS: calcd for $C_{41}H_{53}N_4O_9P$ $(M+H)^+$ 777.3628, found 777.3555.

*3.2.6. 5′-O-(4,4′-Dimethoxytrityl)-3′-O-(4-methylthiobutoxy)-(N,N-diisopropylamino)phosphinyl-2′-deoxythymidine (**29**).* ^{31}P NMR (121 MHz, C_6D_6): δ 149.4, 148.7. FAB-HRMS: calcd for $C_{42}H_{56}N_3O_8PS$ $(M+Cs)^+$ 926.2580, found 926.2537.[11]

*3.2.7. 5′-O-(4,4′-Dimethoxytrityl)-3′-O-(2-methylthioethoxy)-(N,N-diisopropylamino)phosphinyl-2′-deoxythymidine (**30**).* ^{31}P NMR (121 MHz, C_6D_6): δ 149.1, 148.8. FAB-HRMS: calcd for $C_{40}H_{52}N_3O_8PS$ $(M+Cs)^+$ 898.2267, found 898.2256.[11]

*3.2.8. 5′-O-(4,4′-Dimethoxytrityl)-3′-O-(2-methoxyethoxy)-(N,N–diisopropylamino)phosphinyl-2′-deoxythymidine (**31**).* ^{31}P NMR (121 MHz, C_6D_6): δ 150.5, 150.1. $^+$ESI-TOF MS: calcd for $C_{40}H_{52}N_3O_9P$ $(M+H)^+$ 750.3519, found 750.3496.

*3.2.9. 5′-O-(4,4′-Dimethoxytrityl)-3′-O-(6-methylthiohexoxy)-(N,N-diisopropylamino)phosphinyl-2′-deoxythymidine (**32**).* ^{31}P NMR (121 MHz, C_6D_6): δ 150.9, 150.5. $^+$ESI-TOF MS: calcd for $C_{44}H_{60}N_3O_8PS$ $(M+H)^+$ 822.3917, found 822.4568.

*3.2.10. 5′-O-(4,4′-Dimethoxytrityl)-3′-O-(5-methylthiopentoxy)-(N,N-diisopropylamino)phosphinyl-2′-deoxythymidine (**33**).* ^{31}P NMR (121 MHz, C_6D_6): δ 148.5, 147.9. $^+$ESI-TOF MS: calcd for $C_{43}H_{58}N_3O_8PS$ $(M+H)^+$ 808.3760, found 808.3686.

*3.2.11. 5′-O-(4,4′-Dimethoxytrityl)-3′-O-(5-isopropyl-thiopentoxy)-(N,N-diisopropylamino)-phosphinyl-2′-deoxythymidine (**34**).* ^{31}P NMR (121 MHz, C_6D_6): δ 149.5, 148.8. $^+$ESI-TOF MS: calcd for $C_{45}H_{62}N_3O_8PS$ $(M+H)^+$ 836.4073, found 836.3935.

*3.2.12. 5′-O-(4,4′-Dimethoxytrityl)-3′-O-(4-trifluoromethylthiobutoxy)-(N,N-diisopropylamino)phosphinyl-2′-deoxythymidine (**35**).* ^{31}P NMR (121 MHz, C_6D_6): δ 147.7, 147.1. $^+$ESI-TOF MS: calcd for $C_{42}H_{53}F_3N_3O_8PS$ $(M+H)^+$ 848.3321, found 848.3354.

*3.2.13. 5′-O-(4,4′-Dimethoxytrityl)-3′-O-(4-methoxymethylthiobutoxy)-(N,N-diisopropylamino)phosphinyl-2′-deoxythymidine (**36**).* ^{31}P NMR (121 MHz, C_6D_6): δ 148.0, 147.3. $^+$ESI-TOF MS: calcd for $C_{43}H_{58}N_3O_9PS$ $(M+H)^+$ 824.3710, found 824.0285.

*3.2.14. 5′-O-(4,4′-Dimethoxytrityl)-3′-O-[2-(2-methylthioethoxy)-ethoxy]-(N,N-diisopropylamino)phosphinyl-2′–deoxythymidine (**37**).* ^{31}P NMR (121 MHz, C_6D_6): δ 149.5, 149.1. $^+$ESI-TOF MS: calcd for $C_{42}H_{56}N_3O_9PS$ $(M+H)^+$ 810.3553, found 810.3558.

*3.2.15. 5′-O-(4,4′-Dimethoxytrityl)-3′-O-(3-methylthiomethoxypropoxy)-(N,N-diisopropylamino)phosphinyl-2′-deoxythymidine (**38**).* ^{31}P NMR (121 MHz, C_6D_6): δ 148.4, 147.9. $^+$ESI-TOF MS: calcd for $C_{42}H_{56}N_3O_9PS$ $(M+H)^+$ 810.3553, found 810.4007.

*3.2.16. 5′-O-(4,4′-Dimethoxytrityl)-3′-O-(2-methylthiomethoxyethoxy)-(N,N-diisopropylamino)phosphinyl-2′-deoxythymidine (**39**).* ^{31}P NMR (121 MHz, C_6D_6): δ 149.2, 148.7. $^+$ESI-TOF MS: calcd for $C_{41}H_{54}N_3O_9PS$ $(M+H)^+$ 796.3397, found 796.3536.

*3.2.17. N^4-Benzoyl-5′-O-(4,4′-dimethoxytrityl)-3′-O-[2-(N-formyl-N-methylamino)ethoxy]-(N,N-diisopropylamino)phosphinyl-2′-deoxycytidine (**40**).* ^{31}P NMR (121 MHz, C_6D_6): δ 149.0, 148.9, 148.5, 148.4.[5b] $^+$ESI-TOF MS: calcd for $C_{39}H_{51}N_4O_8P$ $(M+H)^+$ 838.3707, found 838.3692.

*3.2.18. N^2-Isobutyryl-5′-O-(4,4′-dimethoxytrityl)-3′-O-[2-(N-formyl-N-methylamino)ethoxy]-(N,N-diisopropylamino)phosphinyl-2′-deoxyguanosine (**41**).* ^{31}P NMR (121 MHz, C_6D_6): δ 149.0, 143.9, 143.7. FAB-HRMS: calcd for $C_{45}H_{58}N_7O_9P$ $(M+Na)^+$ 894.3933, found 894.3978.[5b]

3.3. General procedure for the manual solid-phase synthesis of the thermosensitive dinucleoside phosphorothioate triesters 42–57

The conversion of commercial 5′-*O*-DMTr-TsuccCPG (0.2 μmol) to TsuccCPG (Scheme 2) was effected by syringe injection of 3% TCA in CH_2Cl_2 (2 mL) through the synthesis column containing the support, which was manually agitated over a period of 1 min. After extensive washing of TsuccCPG with CH_2Cl_2 (10 mL) and MeCN (10 mL), the coupling reaction was performed by syringe injection of a premixed solution of a deoxyribonucleoside phosphoramidite [Tp(OR)N(*i*-Pr)$_2$, 20 mg, 0.03 mmol], selected from **24**–**39** (Scheme 1), and 0.45 M 1*H*-tetrazole in MeCN (0.3 mL) through the synthesis column and by agitating the support over a period of 5 min. Excess reagents were expelled from the column with MeCN (2×10 mL). The support was then exposed to 0.05 M 3*H*-1,2-benzodithiol-3-one 1,1-dioxide[30] in MeCN (1 mL) for 2 min. Excess oxidant was washed off the support with MeCN (2×10 mL). Removal of the DMTr group was performed by suspending the support in a solution of 3% TCA in CH_2Cl_2 (2 mL) for 1 min. After carefully washing the support with MeCN (2×10 mL), the dinucleoside thiophosphate triester (Tps(OR)T, **42**–**57**) was released from the support upon brief exposure (3 min) to pressurized (∼2.5 bar) methylamine gas,[19] and was purified by RP-HPLC prior to being subjected to thermolytic deprotection conditions.

3.4. Solid-phase synthesis of the thermosensitive dinucleoside phosphorothioate triester 58

The preparation of **58** was performed as described above for the synthesis of **48** with the exception of performing an additional oxidation step using 5 M *tert*-butyl hydroperoxide in decane for 20 min prior to removing the 5′-DMTr group and releasing the dinucleoside thiophosphate triester from the support. The identity of **58** was confirmed by $^{-}$MALDI-TOF MS: calcd for $C_{23}H_{33}N_4O_{13}PS_2$ $(M-H)^-$ 667, found 668.

3.5. Thermolytic deprotection of the dinucleoside phosphorothioate triesters 42–58

An RP-HPLC-purified dinucleoside phosphorothioate triester (~50 nmol) was dissolved in PBS (1×, pH 7.4, 300 μL) and was heated to the desired temperature (37 °C or 90 °C). Aliquots (75 μL) were taken out at predetermined time points for analysis by RP-HPLC. The formation of TpsT was monitored in order to determine the half time of the thiophosphate deprotection reaction.

3.6. Automated solid-phase synthesis of heat-sensitive DNA sequences

Solid-phase synthesis of 5′-$dC_{PS(MOE)}C_{PS(MOE)}T_{PS(FMA)}T_{PS(FMA)}T_{PS(FMA)}T_{PS(FMA)}C_{PS(MOE)}G_{PS(FMA)}T_{PS(FMA)}T_{PS(FMA)}T_{PS(FMA)}T_{PS(FMA)}C_{PS(MOE)}C_{PS(MOE)}T$ (**59**) and 5′-$dC_{PS(MOE)}C_{PS(MOE)}T_{PS}T_{PS}T_{PS}T_{PS}C_{PS(MOE)}G_{PS}P_{S}T_{PS}T_{PS}T_{PS}C_{PS(MOE)}C_{PS(MOE)}T$ (**60**), where PS stands for a phosphorothioate diester function, PS(FMA) stands for a thermolytic 2-(*N*-formyl-*N*-methylamino)ethyl phosphorothioate triester function, and PS(MOE) stands for a thermostable 2-methoxyethyl phosphorothioate triester function, was performed on a 0.2 μmol scale using a commercial long chain alkylamine controlled-pore glass support loaded with 5′-*O*-DMTr-dT as the leader nucleoside. The syntheses were carried out using a DNA/RNA synthesizer and phosphoramidites **25**, **40** (R=FMA), and **41** (R=FMA), or commercial 2-cyanoethyl deoxyribonucleoside phosphoramidites as 0.1 M solutions in dry MeCN. The reaction time for each phosphoramidite coupling step was 180 s. All ancillary reagents necessary for the preparation of the oligonucleotides were purchased and utilized as recommended by the instrument's manufacturer. The iodine oxidation step of the synthesis cycle was replaced with a sulfurization step employing 0.05 M 3*H*-1,2-benzodithiol-3-one 1,1-dioxide in MeCN, as suggested in the literature.[30] The sulfurization step was performed before the capping step, and the reaction time for these steps was 120 s and 60 s, respectively. The synthesis columns containing the oligonucleotides were placed into a stainless steel pressure vessel and exposed to pressurized ammonia (~10 bar at 25 °C) for 12 h.[19] Upon removal of residual ammonia from the pressure container, the 5′-*O*-DMTr-oligonucleotides were eluted off the synthesis column with 40% MeCN in 0.1 M triethylammonium acetate (TEAA, pH 7.0) (500 μL).

3.7. Purification of DNA sequences

Oligonucleotide purification was accomplished by RP-HPLC employing a UV–vis diode array detection system and an analytical 5 μm Supelcosil LC-18S column (4.6 mm×25 cm). The elution gradient for the purification of 5′-*O*-DMTr-$dC_{PS(MOE)}C_{PS(MOE)}T_{PS(FMA)}T_{PS(FMA)}T_{PS(FMA)}T_{PS(FMA)}C_{PS(MOE)}G_{PS(FMA)}T_{PS(FMA)}T_{PS(FMA)}T_{PS(FMA)}T_{PS(FMA)}C_{PS(MOE)}C_{PS(MOE)}T$ was performed as follows: starting from 0.1 M TEAA (pH 7.0), a linear gradient of 3.3% MeCN/min was pumped at a flow rate of 1 mL/min for 30 min.

The elution gradient for the purification of the 5′-*O*-DMTr-$dC_{PS(MOE)}C_{PS(MOE)}T_{PS}T_{PS}T_{PS}T_{PS}C_{PS(MOE)}G_{PS}T_{PS}T_{PS}T_{PS}T_{PS}C_{PS(MOE)}C_{PS(MOE)}T$ was optimized as follows: starting from 0.1 M TEAA (pH 7.0), a linear gradient of 1.3% MeCN/min was pumped at a flow rate of 1 mL/min for 30 min.

The chromatographic peaks corresponding to the product were collected and the eluates were concentrated to a film using a stream of air without heating. Each oligonucleotide was dissolved in 80% AcOH (500 μL) and each solution was left standing at ambient temperature for 30 min in order to completely remove the DMTr group. Excess AcOH was removed by evaporation using a stream of air without a heat source. Each DNA oligomer was then dissolved in a solution of 40% MeCN in 0.1 M TEAA (pH 7.0) (400 μL) and purified by RP-HPLC using the same chromatographic conditions as those used for the purification of 5′-*O*-DMTr-$dC_{PS(MOE)}C_{PS(MOE)}T_{PS}T_{PS}T_{PS}T_{PS}C_{PS(MOE)}G_{PS}T_{PS}T_{PS}T_{PS}T_{PS}C_{PS(MOE)}C_{PS(MOE)}T$.

The identity of the RP-HPLC-purified DNA sequences was confirmed by mass spectrometry.

5′-$dC_{PS(MOE)}C_{PS(MOE)}T_{PS(FMA)}T_{PS(FMA)}T_{PS(FMA)}T_{PS(FMA)}C_{PS(MOE)}G_{PS(FMA)}T_{PS(FMA)}T_{PS(FMA)}T_{PS(FMA)}T_{PS(FMA)}C_{PS(MOE)}C_{PS(MOE)}T$ (**59**)$^{-}$MALDI-TOF MS: calcd for $C_{196}H_{283}N_{47}O_{97}P_{14}S_{14}$ $(M-H)^-$ 5727, found 5716, 5′-$dC_{PS(MOE)}C_{PS(MOE)}T_{PS}T_{PS}T_{PS}T_{PS}C_{PS(MOE)}G_{PS}T_{PS}T_{PS}T_{PS}T_{PS}C_{PS(MOE)}C_{PS(MOE)}T$ (**60**)$^{-}$ESI-MS: calcd for $C_{160}H_{220}N_{38}O_{88}P_{14}S_{14}$ $(M-H+K)^-$ 5001, found 5003.

When **60** is obtained from the control DNA sequence **61**. $^{-}$ESI-MS: calcd for $C_{160}H_{220}N_{38}O_{88}P_{14}S_{14}$ $(M-H)^-$ 4962, found 4966.

3.8. Thermal thiophosphate deprotection of the DNA sequence 59

The RP-HPLC-purified oligonucleotide **59** (5 OD_{260}) was dissolved in PBS, pH 7.4, (300 μL) and placed in a heat block pre-heated at 90±2 °C in order to thermolytically cleave the FMA thiophosphate protecting groups. Two fractions (100 μL each) of the solution were analyzed by RP-HPLC; one fraction was analyzed at half time of the FMA thiophosphate deprotection reaction (23 min) and the second after complete FMA thiophosphate deprotection (3 h). 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 TEAA (pH 7.0), a linear gradient of 1.3% MeCN/min was pumped at a flow rate of 1 mL/min for 30 min. The RP-HPLC profiles of each analysis are presented in Figure 1B and C.

3.9. PAGE analysis of the thermal thiophosphate deprotection of the DNA sequence 59

The FMA-free oligonucleotide **60** and the control DNA sequence $dC_{PS(MOE)}C_{PS(MOE)}T_{PS}T_{PS}T_{PS}T_{PS}C_{PS(MOE)}G_{PS}T_{PS}T_{PS}T_{PS}T_{PS}C_{PS(MOE)}C_{PS(MOE)}T$ that was originally synthesized from the use of phosphoramidite **40** (R=MOE) and 2-cyanoethyl deoxyribonucleoside phosphoramidites were analyzed (0.5 OD_{260} each) by PAGE under denaturing conditions (20% polyacrylamide–7 M urea, 1× TBE buffer, pH 8.3). The 40 cm×20 cm×0.75 mm gel was prepared as described by Maniatis et al.[31] Electrophoresis was carried out at 350 V until the bromophenol blue dye of the loading buffer had traveled ~80% of the gel's length. The gel was then stained by soaking in a solution of Stains-all, as reported elsewhere.[32] A photography of the gel is shown in Figure 2.

3.10. Relative time-dependent solubility of the DNA sequence 59 in PBS at 90 °C

The fully protected oligonucleotide **59** (1 OD_{260}) was dissolved in a minimal amount of MeCN (300 μL) and 100 μL of this solution was added to 900 μL of pre-heated (90 °C) PBS (1×, pH 7.4) in a quartz cuvette sealed with a Teflon cap. The temperature of the

mixture was maintained at 90 °C using a Peltier temperature control unit and the absorbance of the solution was measured at different time points at 268 nm.

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