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A new approach to the synthesis of the 5′-deoxy-5′-methylphosphonate linked thymidine oligonucleotide analogues

Tomas Szabó, Annika Kers and Jacek Stawinski*

Department of Organic Chemistry, Arrhenius Laboratory, Stockholm University, S-106 91 Stockholm, Sweden

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ABSTRACT

A new synthetic method for the preparation of the 5′-deoxy-5′-methylphosphonate linked thymidine oligonucleotides (5′-methylenephosphonate analogues) was developed. The method is based on the use of a phosphonate protecting group, 4-methoxy-1-oxido-2-picolyl, enabling intramolecular nucleophilic catalysis which together with the condensing agent, 2,4,6-triisopropylbenzenesulfonyl chloride, secures fast and efficient formation of the 5′-methylenephosphonate internucleosidic bonds. The produced protected oligomers were treated with thiophenol and triethylamine to remove the phosphonate protecting groups, cleaved from the solid support using concentrated aqueous ammonia, and purified by HPLC. Several thymidine oligonucleotide analogues with the chain length of up to 20 nucleotidic units, in which all internal 5′-oxygen atoms have been replaced by methylene groups directly bound to phosphorus, were synthesised using this methodology.

INTRODUCTION

Since the first demonstration by Zamecnik and Stephenson (1,2) that synthetic oligonucleotides complementary to some strategic regions of viral DNA can inhibit the viral replication, an impressive number of oligonucleotides analogues (3) has been developed as potential therapeutics for the antisense (4) (modulation of the translation process) and the antigene (5,6) (modulation of the transcription process) approach to the selective control of genes expression.

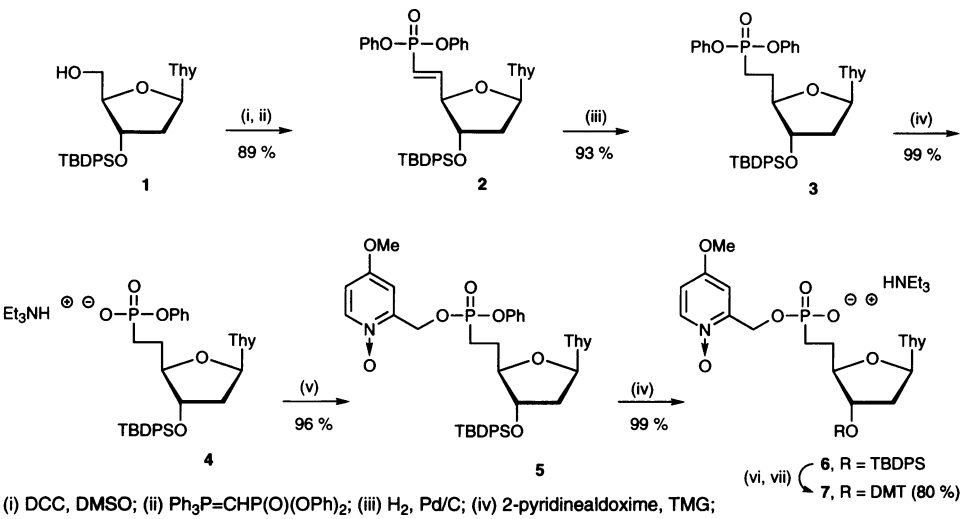
One of many challenges to be met for the development of oligonucleotides as therapeutics is the susceptibility of the natural phosphodiester bonds to degradation by cellular and serum nucleases. This problem has been successfully tackled by using chemically synthesized oligonucleotide analogues having modified internucleotidic bonds [e.g., phosphorothioates (7), methylphosphonates (8)], modified sugar residues [e.g., 2′-*O*-alkyl ribofuranosides (9), L-deoxyribonucleosides (10), oligoarabinonucleotides (11)] or those in which the phosphate group or the sugar–phosphate backbone was replaced by a neutral functionality (12) [e.g., oxyamide group (13), 'peptide nucleic acids', PNAs (14)]. To exert a biological effect, oligonucleotide analogues have to cross the external cell membranes, and this, apart from the problems connected with maintenance of the binding specificity *in vivo*, is considered to be an important factor limiting the bioefficacy of the antisense (antigene) approach (15,16). To improve the uptake and to facilitate the cytoplasmic transport, the antisense oligonucleotide analogues are often coupled to some carrier molecules, e.g., poly(L-lysine) (17), polyethylene glycol (18) or phospholipids (19), or can be encapsulated into antibody targeted liposomes (20).

Although some analogues with little resemblance to natural oligonucleotides [*casus* PNAs (14)] have potentially useful therapeutic properties, a more rational approach seems to be searching for effective DNA surrogate therapeutics among those having modifications which retain or strengthen the properties of the phosphate diesters. Among such chemically modified oligonucleotides, phosphorothioate and methylphosphonate derivatives have been most thoroughly investigated (21). Unfortunately, due to the presence of chiral phosphorus centers, chemical syntheses of these analogues usually lead to a pool of diastereomers, among which physicochemical and biological properties may vary significantly. This stereochemical problem in the instance of oligonucleotide analogues containing phosphorus–carbon bonds may be circumvented by placing the phosphonate function in the bridging position of an internucleosidic bond. This will produce achiral (at the phosphorus centre) analogues with a high resemblance to natural oligonucleotides but which still retain non-hydrolyzable, nuclease resistant P–C bond attached to the C3′ or C5′ atom [(nucleosid-5′ (or 3′)-yl)methylphosphonates]. Although this kind of analogues was prepared for the first time some 25 years ago by Jones and Moffatt (22), they did not receive much attention (23–25), probably due to some inconveniences in their preparation (26–28).

In this paper we present an efficient synthesis of thymidine oligonucleotide analogues with chain length of up to 20 units, in which the all 5′-oxygen atoms are replaced by methylene groups bound to phosphorus (5′-deoxy-5′-methylphosphonate linked oligonucleotides or 5′-methylenephosphonate analogues). The method makes use of a significant rate enhancement exerted by an intramolecular catalytic phosphonate protecting group [4-methoxy-1-oxido-2-picolyl (29)] and is compatible with the

* To whom correspondence should be addressed

Scheme 1.



(i) DCC, DMSO; (ii) $Ph_3P{=}CHP(O)(OPh)_2$; (iii) H_2, Pd/C; (iv) 2-pyridinealdoxime, TMG; (v) 4-methoxy-1-oxido-2-pyridinemethanol, 2-chloro-5,5-dimethyl-2-oxo-1,3,2-dioxaphosphinane; (vi) Bu_4NF; (vii) DMT-Cl, DMAP

requirements for solid-phase synthesis of oligonucleotide analogues.

RESULTS AND DISCUSSION

Presence of the P–C bond in phosphonic acid and its derivatives diminishes a partial positive charge at the phosphorus centre and makes these compounds, in general, less reactive than the corresponding phosphate congeners in reactions involving nucleophilic attack at the P-center. This may pose some serious problems in the esterification reactions promoted by condensing agents. As it was noted for the phosphate esters (30) with alkoxy versus aryloxy groups bound to phosphorus, a slower rate of the condensation in the case of alkyl phosphates always results in lower yield of the desired product due to competing side-reactions involving a condensing agent and a nucleosidic component. This phenomenon is even more pronounced in the instance of methylphosphonate esters (31) where condensations under the standard reaction conditions for phosphate esters (32) give usually 30–40% lower yields. To overcome these problems, methods based on reactive *C*-phosphonates [P(V)] (33,34) or methylphosphonite derivatives [P(III)] (35,36) have been developed. Preliminary studies from this laboratory (37) showed that in a condensing agents promoted synthesis of methylphosphonate esters, the yields varied considerably depending on the reaction conditions, and the best results can be achieved when a mild condensing agent (e.g., 2-chloro-2-oxo-5,5-dimethyl-1,3,2-dioxaphosphinane) is used in combination with a powerful nucleophilic catalyst (e.g., pyridine *N*-oxide derivatives).

In contradistinction to nucleoside methylphosphonates, in 5′-deoxy-5′-phosphonomethyl nucleoside derivatives (e.g., **3**) the P–C bond is a part of a nucleoside system and thus two hydroxyl groups bound to phosphorus are available for esterification. To form a 5′-deoxy-5′-methylphosphonate linked dimer one can envisage using (nucleosid-5′-yl)methylphosphonic acid [Jones and Moffatt's approach (26)] or its monoesters as nucleotidic components for the condensation. In the latter instance, aryl protecting groups seem to be preferred (partial activation of the phosphonate centre) and two communications appeared concerning synthesis of dinucleosides analogues with 3′-methylene- (38,39) or 5′-methylenephosphonate (28) internucleosidic linkages using such an approach.

Recently, we have proposed another way to overcome the problems connected with an inherent low reactivity of some *C*-phosphonate derivatives (27) by using, as it was suggested for the phosphotriester synthesis (29,40), a protecting group which could enhance the rate of esterification of the phosphonate function via neighbouring group participation. We chose for our studies the 4-methoxy-1-oxido-2-picolyl group and compared its influence on the rate of condensation with phenyl as the phosphonate protecting group.

Synthesis of nucleotidic units with phenyl or 4-methoxy-1-oxido-2-picolyl phosphonate protection

To evaluate usefulness of two kinds of phosphonate protecting groups, aryl and intramolecular catalytic ones, in the synthesis of oligonucleotide analogues having in the 5′-positions methylene groups, a synthetic scheme for the preparation of the nucleotidic units **4** (with phenyl as a phosphonate protecting group) and **6** (with 4-methoxy-1-oxido-2-picolyl group) was designed (Scheme 1). The phosphonate **4**, which served also as a starting material for the other nucleotidic unit (6), was prepared from thymidine by modification of the Jones and Moffatt procedure (22). The *tert*-butyldiphenylsilyl (TBDPS) group (41), which should provide the proper stability throughout the synthesis, was chosen for the protection of the 3′-hydroxyl function of thymidine. This group, owing to its susceptibility to fluoride treatment should also be relatively easy to replace at the end of synthesis by 4,4′-dimethoxytrityl group, a more useful one in the planned solid phase synthesis of the corresponding oligonucleotide analogues.

The preparation of **4** commences with a Pfitzner–Moffatt oxidation (42) of the free 5′-hydroxyl of **1**. The resulting 5′-aldehyde was subjected *in situ* to the reaction with an appropriate Wittig reagent to yield the vinylphosphonate **2**, which was hydrogenated over Pd/C to the diphenyl phosphonate **3**.

Finally, the selective removal of one phenyl group from **3** to produce the monophenyl phosphonate **4** was effected by treatment with 2-pyridinealdoximate (43).

The nucleotidic unit **6** with an intramolecular catalytic group was prepared from the phosphonate **4** in two steps. First, **4** was condensed with a slight excess of 4-methoxy-1-oxido-2-pyridinemethanol (44) in the presence of 2-chloro-2-oxo-5,5-dimethyl-1,3,2-dioxaphosphinane (NEP) as a condensing agent and 4-methoxypyridine *N*-oxide as a nucleophilic catalyst to produce the phosphonate diester **5**, and then the phenyl group was selectively removed with pyridinealdoxime to give **6**.

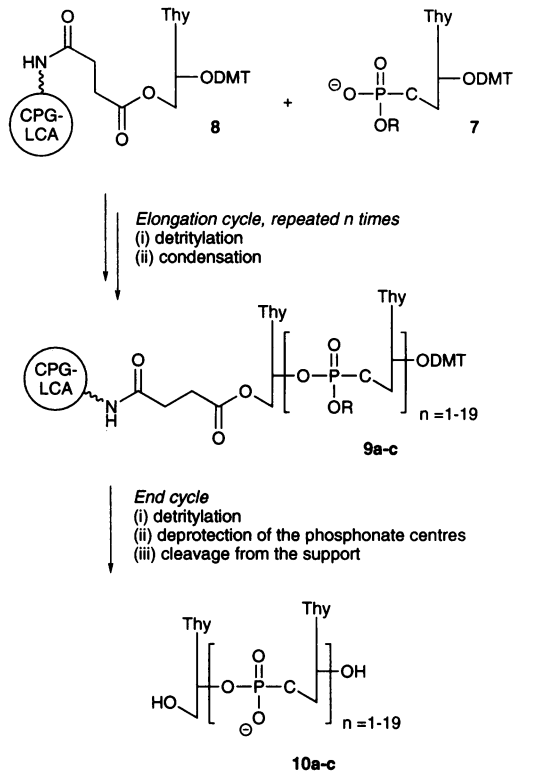
For the planned solid-phase syntheses, a more labile protection for the 3′-hydroxyl in **6** was needed. To this end, the phosphonate **6** was treated with tetrabutylammonium fluoride (TBAF) in THF, followed by DMAP-catalyzed dimethoxytritylation in pyridine. The phosphonate **7**, which was used as a substrate for the solid phase synthesis of the 5′-methylenephosphonate analogues of thymidine oligonucleotides, was obtained in 62% overall yield as calculated on the starting 3′-*O*-*tert*-butyldiphenylsilylthymidine.

Comparison of phenyl versus 4-methoxy-1-oxido-2-picolyl as phosphonate protecting groups

From a point of view of electronic density at the phosphorus centre, the phosphonate **4** with the phenyl group should react faster in the condensation reaction with a nucleosidic component to form a 5′-deoxy-5′-methylphosphonate linked dimer than the nucleotide **6** (or **7**), having a substituted alkyl as a phosphonate protecting group. However, it was possible that the importance of an electronic factor of the phenyl group in **4** might be exceeded by the rate enhancement exerted by the picolyl function in **6**, due to neighbouring group participation effect.

To get a reliable comparison between efficiency of both protecting groups in the phosphonate esters synthesis (the rate enhancement and possible side-products formation), first the optimal conditions for the condensation of **4** with a nucleosidic component were investigated. For this purpose the nucleotidic (4) and nucleosidic (5′-*O*-*tert*-butyldiphenylsilylthymidine) components were subjected in pyridine to a condensation promoted by three different coupling agents [2-chloro-2-oxo-5,5-dimethyl-1,3,2-dioxaphosphinane (NEP), 2,4,6-triisopropylbenzenesulfonyl chloride (TPS-Cl) and diphenylphosphorochloridate (DPPC)] in the presence of two types of efficient nucleophilic catalysts [4-methoxypyridine *N*-oxid (MPNO) and *N*-methylimidazole (NMI)]. As judged from TLC analyses, using TPS-Cl in combination with MPNO or using DPCP with any of the catalysts investigated, resulted in a fast reaction (the nucleosidic component disappeared within 1–2 min) but the desired dimer was usually a minor reaction product (mainly due to extensive side reactions involving the condensing agents and the nucleoside). The condensations promoted by TPS-Cl or NEP in the presence of NMI proceeded rather slowly (completion within 2 h) but were rather clean (0–10% side products). The optimal results were obtained when the cyclic chlorophosphate (NEP) was used as a condensing agent together with the pyridine *N*-oxide derivative as a catalyst. Under these conditions formation of the phosphonate dimer was complete within 30 min without noticeable formation of side-products. These results are in agreement with our previous findings concerning synthesis of dinucleoside methylphosphonates (37,45). Similar results were obtained when *o*-chlorophenyl was used as a phosphonate protecting group.

Scheme 2.



Abbreviations: CPG-LCA: Controlled Pore Glass-Long Chain Alkyl DMT: 4,4'-Dimethoxytrityl, Thy: Thymin-1-yl, R: 4-Methoxy-1-oxido-2-picolyl Notes: a: n = 4; b: n = 9; c: n = 19

Rates of removal of phenyl and 4-methoxy-1-oxido-2-picolyl phosphonate protecting groups were also evaluated. The 1-oxido-4-methoxy-2-picolyl group was cleanly removed within 30 min using standard dealkylation conditions (46), whereas the phenyl protecting group required ~20 h oximate (43) treatment.

Solid-phase synthesis of thymidine oligonucleotide analogues 10

As a final stage of these investigations we evaluated utility of the nucleotidic unit **7** with an intramolecular catalytic phosphonate protecting group as a building block for the solid-phase synthesis of the 5′-deoxy-5′-methylphosphonate linked thymidine oligonucleotide analogues. For this purpose, long chain alkylamine CPG was functionalized according to Pon *et al.* (47), which involved, *inter alia*, conversion of 3′-*O*-dimethoxytritylthymidine (48) to its 5′-*O*-succinate ester and condensation of the crude product with the acid pre-treated solid support to give a loading of the support of 45 μmol/g. The chain elongation (Scheme 2) was performed manually in a gas-tight syringe as reaction vessel by adding a pyridine solution of the phosphonate **7** and a condensing agent to the support-bound thymidine with a free 3′-OH group.

Concerning the choice of a coupling reagent for the solid-phase preparations of thymidine oligonucleotide analogues **10**, no obvious candidate could be singled out from the comparative studies on the dimers formation in solution syntheses. However, from a limited comparison of a coupling efficiency on a solid

support, TPS-Cl was selected as the condensing agent by virtue of the higher yields obtained in the pentamer syntheses (~96% versus ~92% per step for NEP). It should be noted that reliable determination of the optimal conditions for the preparations of analogues **10** depends on reproducible ambient conditions, which may vary when a manual syringe-based technique is used. At the present stage of investigations the effect of capping on the quality of the products **10** has not been checked.

The synthetic protocol, which includes two chemical steps in the elongation cycle and two steps in the end cycle (deprotection), is shown in Table 1. Using this protocol, several thymidine oligonucleotide analogues **10**, with chain lengths up to 20 units have been synthesized. An average coupling yield per condensation was 95–98% throughout the syntheses. The fully protected, solid-support bound oligonucleotides **10** were treated with thiophenolate (29,46) to remove the catalytic phosphonate protecting groups, and then with a mixture of concentrated ammonia and ethanol to cleave the oligomers from the support. After evaporation and lyophilization the crude oligonucleotidic products were obtained as white solids.

Table 1. The protocol for the solid phase syntheses of thymidine oligonucleotide analogues **10**

Description of a cycle	Volume	Time	
Elongation cycle			
1.	Dichloroethane wash	5 × 1 ml	
2.	Detritylation[a]	5 × 1 ml	5 × 1 min
3.	Dichloroethane wash	5 × 1 ml	
4.	Pyridine wash	5 × 1 ml	
5.	Condensation step[b]		5 min
6.	Pyridine wash	5 × 1 ml	
7.	Repeat steps 1–6		
End cycle			
8.	Dichloroethane wash	5 × 1 ml	
9.	Detritylation[a]	5 × 1 ml	5 × 1 min
10.	Dioxane wash	5 × 1 ml	
11.	Dealkylation[c]	1 ml	60 min
12.	Methanol wash	5 × 1 ml	
13.	Diethyl ether wash	5 × 1 ml	
14.	Cleavage from the support[d]	1 ml	overnight

[a]1% Trifluoroacetic acid in dichloroethane.
[b]2,4,6-Triisopropylbenzenesulfonyl chloride (50 μmol) was added to a solution of **7** (30 μmol) in pyridine (500 μl) and the resulting mixture drawn into the syringe containing the solid support.
[c]Thiophenol–triethylamine–dioxane (1:1:2, v/v).
[d]Conc. NH_4OH–ethanol (3:1, v/v).

Purification and analysis of thymidine oligonucleotide analogues 10

To evaluate the efficiency of the internucleosidic 5′-deoxy-5′-*C*-methylphosphonate bonds formation, the crude oligonucleotide analogues **10** (obtained as described above) were analyzed by HPLC. Figure 1 [traces (a), (c) and (e)] shows the elution profiles obtained from a chromatography of crude oligonucleotides with four, five and nineteen 5′-methylenephosphonate internucleosidic bonds (**10 a–c**). The desired full-length oligomers eluted as the major products preceded by truncated sequences. In following preparative runs, fractions containing the oligomers with the desired chain length were collected, and after checking their purity by HPLC [Fig. 1, traces (b), (d) and (f)], the oligomers were subjected to enzymatic digestion with phosphodiesterases from snake venom (SVPDE) (which cleaves P–O–C3′ linkages) and from bovine spleen (BSPDE) (which cleaves P–O–C5′ linkages). As anticipated, due to the presence of the P–C–C5′ bonds in the oligonucleotide analogues **10**, they were completely resistant to the BSPDE treatment [Fig. 1, trace (j)] but were fully digested with SVPDE [Fig. 1, traces (g), (h) and (i)] affording (thymidin-5′-yl)methylphosphonate and thymidine as sole products in the expected ratios.

Conclusions

A new method for the synthesis of the 5′-deoxy-5′-methylphosphonate linked thymidine oligonucleotide analogues, based on a building block **7** containing an intramolecular catalytic phosphonate protecting group, was developed. The synthetic transformations leading to the phosphonate **7** were performed in 62% overall yield starting from the 3′-protected thymidine derivative **1**. Presence of a catalytic group at the phosphorus centre in **7** made the condensations significantly faster than those when aryl protecting groups were used. Also, removal of the picolyl group via thiophenolate promoted dealkylation was found to be faster than deprotection of aryl phosphonates with pyridinealdoxime. Efficiency of the chain-assembly of the oligothymidylic acids analogues **10** approaches that of unmodified oligonucleoside phosphotriesters. The synthesized oligothymidine 5′-methylenephosphonates analogues were completely resistant towards bovine spleen phosphodiesterase but underwent degradation by snake venom phosphodiesterase (49). Studies are in progress in this laboratory to optimize and to extend the methodology to the preparation of heterosequences and to implement it to a machine-assisted synthesis of oligonucleotides in order to assess the potential of 5′-methylenephosphonate DNA analogues as antisense/antigene therapeutical agents.

EXPERIMENTAL

Materials and methods

Pyridine was dried by refluxing with CaH_2 overnight followed by distillation, re-distillation from *p*-toluenesulfonyl chloride and storage over molecular sieves (4 Å). Dioxane was dried by distillation from $LiAlH_4$ and stored over Na-wire. Tetrahydrofuran was dried by distillation from $LiAlH_4$ directly before use. Chloroform was passed through basic Al_2O_3 prior to use. DMF and DMSO were made anhydrous by distillation from CaH_2 at reduced pressure (~10 mm Hg) and stored over molecular sieves (4 Å).

3′-*O*-*tert*-Butyldiphenylsilylthymidine (50), 2-chloro-5,5-dimethyl-2-oxo-1,3,2-dioxaphosphinane (51) and 4-methoxy-1-oxido-2-pyridinemethanol (44) were prepared by the published procedures. Oxalic acid dihydrate, 2-pyridinealdoxime and tetrabutylammonium fluoride trihydrate were commercial grades (Aldrich). 4-Methoxypyridine-1-oxide hydrate (Aldrich) was dried over P_2O_5 overnight at 70°C at 0.2 mm Hg and 1,3-dicyclohexylcarbodiimide (Aldrich) and 1,1,3,3-tetramethylguanidine (Aldrich)

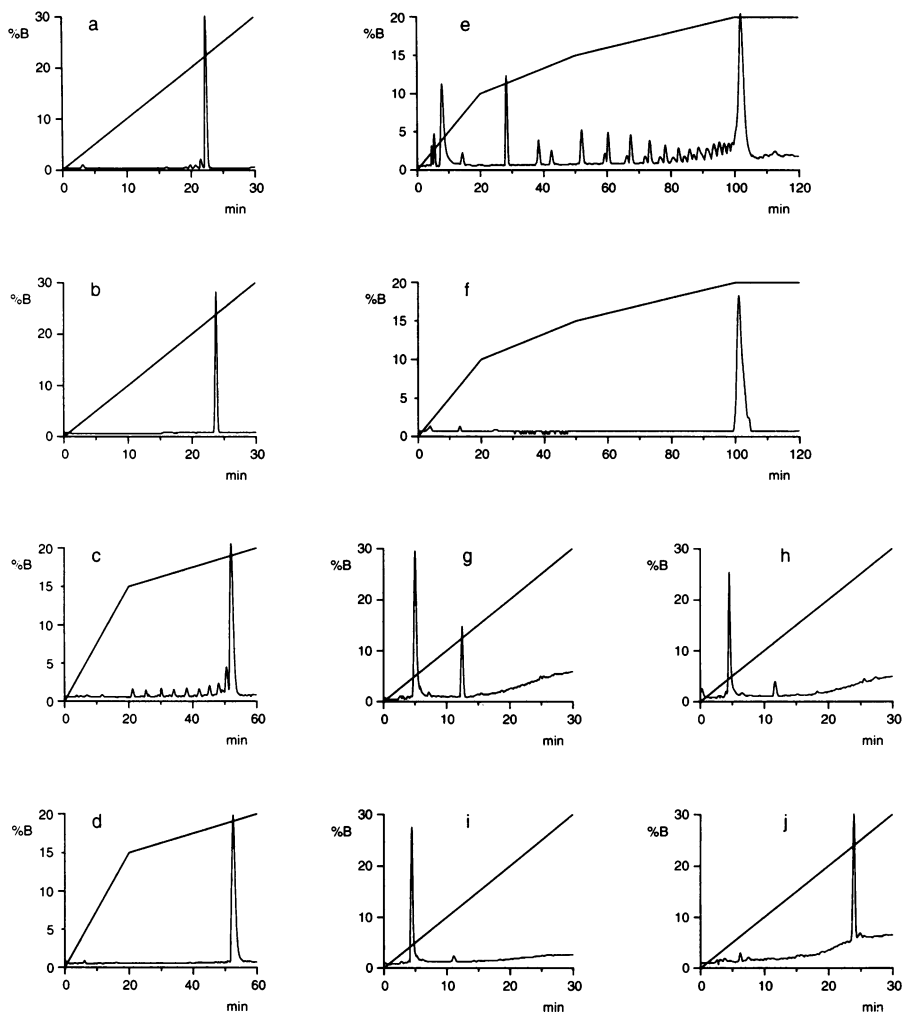


Figure 1. The HPLC chromatograms (Supelcosil LC 18 column, see Experimental for details): (**a**) crude 5mer **10a**; (**b**) purified 5mer **10a**; (**c**) crude 10mer **10b**; (**d**) purified 10mer **10b**; (**e**) crude 20mer **10c**; (**f**) purified 20mer **10c**; (**g**) digestion of the 5mer **10a** with SVPDE; (**h**) digestion of the 10mer **10b** with SVPDE; (**i**) digestion of the 20mer **10c** with SVPDE; (**j**) digestion of the 5mer **10a** with BSPDE.

were vacuum distilled. 1 M triethylammonium bicarbonate buffer (~pH 7) (TEAB) was prepared by passing carbon dioxide through an aqueous solution containing the appropriate amount of triethylamine. For column chromatography, silica gel (35–70 μm) from Amicon Europe was used, and the columns were run in the flash mode. All evaporations were carried out under reduced pressure using rotatory evaporator, unless stated otherwise. Yields reported refer to products obtained after drying at p < 1 mm Hg for at least 24 h.

Solid-phase synthesis, purification and analysis of the oligonucleotide analogues 10

Controlled-pore glass long-chain alkylamine solid support (Aldrich) was functionalized with the succinylated 3′-*O*-dimethoxytritylthymidine (48) according to a published procedure (47). Reversed phase HPLC analysis and purifications were done on a Gilson instrument equipped with a Supelcosil® LC 18 column. Snake venom phosphodiesterase (*Crotalus adamanteus*) and spleen phosphodiesterase (*bovine*) were purchased from Sigma. Buffer substances used were *pro analysis*.

NMR and MS analysis. ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded on a Jeol GSX-270 FT spectrometer. Chemical shifts are given in p.p.m. relative to tetramethylsilane (^{1}H, $CDCl_3$, 25°C) or $CDCl_3$ ($\delta = 77.17$ p.p.m., ^{13}C, $CDCl_3$, 25°C) or 2% H_3PO_4 in H_2O (^{31}P, $CDCl_3$, 25°C). High resolution FAB mass spectra were recorded on a Jeol SX-102 instrument.

Diphenyl (triphenylphosphoranylidene)methylphosphonate

Diphenyl chloromethylphosphonate (52) (63.4 g, 224 mmol) and triphenyl phosphine (58.8 g, 224 mmol) were stirred at 160°C for 16 h. Heating was discontinued and the reaction mixture solidified. The crude phosphonium salt was dissolved in dichloromethane (400 ml), transferred to a separatory funnel and treated with 2 M NaOH (200 ml). After separation of the layers, the organic phase was dried over Na_2SO_4 and evaporated to leave the ylide as a light yellow oil. The crude ylide was dissolved in ethyl acetate (500 ml) with heating and allowed to cool slowly to room temperature. Crystals obtained were recrystallized from ethyl acetate (500 ml) to give the title compound as off-white crystals. Yield 91.4 g (80.2%).

Diphenyl [1′-(2′,5′,6′-trideoxy-3′-*O*-*tert*-butyldiphenylsilyl-β-D-*ribo*-hex-5-enofuranosyl)-1-thymin]-6′-phosphonate (2)

To a solution of 3′-*O*-*tert*-butyldiphenylsilylthymidine (7.21 g, 15.0 mmol), 1,3-dicyclohexylcarbodiimide (9.28 g, 45.0 mmol) in DMSO (60 ml) containing pyridine (1.2 ml, 15 mmol), trifluoroacetic acid (0.58 ml, 7.5 mmol) was added and the reaction mixture stirred at room temperature for 18 h. After that time, diphenyl (triphenylphosphoranylidene)methylphosphonate (11.44 g, 22.5 mmol) was added and the stirring was continued for 5 h. Excess carbodiimide was hydrolysed by a careful addition of oxalic acid dihydrate (3.78 g, 30.0 mmol) dissolved in methanol (15 ml). The urea formed was removed by filtration and washed with toluene (200 ml). The combined filtrates were extracted with water (4 × 100 ml), dried over Na_2SO_4, and evaporated. Silica gel column chromatography using toluene–ethyl acetate (2:1, v/v) as eluent afforded the title compound as a white foam. Yield 9.44 g (88.8%). $\delta_H(CDCl_3)$ 1.09 (9 H, s), 1.75 (1 H, ddd, *J* –13.6, 8.1, 5.7), 1.84 (3 H, d, 1.1), 2.28 (1 H, ddd, *J* -13.6, 5.7, 2.7), 4.21 (1 H, m), 4.48 (1 H, m), 5.93 (1 H, ddd, *J* –21.3, 17.2, 1.8), 6.46 (1 H, dd, 8.1, 5.7), 6.61 (1 H, ddd, *J* 23.5, 17.2, 4.4), 6.9–7.6 (21 H, m) and 8.19 (1 H, s); $\delta_C(CDCl_3)$ 12.7, 19.2, 27.0, 39.3, 76.1, 85.2, 86.0, 86.3, 111.8, 116.0, 118.8, 120.5(6), 120.6(4), 120.7, 125.5, 128.2, 128.3, 129.2, 129.9, 130.4(6), 130.5(4), 132.7, 134.8, 135.8, 150.3, 150.6, 150.7 and 163.6; $\delta_P(CDCl_3)$ 9.84; HRMS Found: $(M–H)^-$, 707.2328. $C_{39}H_{41}N_2O_7PSi$ requires *M*–H, 707.2342

Diphenyl (3′-*O*-*tert*-butyldiphenylsilylthymidin-5′yl) methylphosphonate (3)

To a solution of the vinylphosphonate **2** (9.21 g, 13.0 mmol) in ethanol (260 ml), Pd/C (10%) (2.6 g) was added and the reaction mixture stirred under hydrogen at atmospheric pressure. After 24 h when the calculated amount of hydrogen had been consumed, the catalyst was removed by filtration through Celite® and the solvent evaporated. The title compound was obtained as a white foam. Yield 8.58 g (92.8%). $\delta_H(CDCl_3)$ 1.08 (9 H, s), 1.82 (3 H, d, 1.1), 1.7–2.4 (6 H, m), 3.93 (1 H, m), 4.09 (1 H, m), 6.35 (1 H, dd, *J* 7.7, 5.9), 6.9–7.7 (21 H, m) and 8.31 (1H, s); $\delta_C(CDCl_3)$ 12.6, 19.1, 21.4, 23.5, 26.4, 26.5, 27.0, 39.9, 75.6, 84.7, 85.9, 86.2, 111.6, 120.5, 120.6, 125.4, 128.1, 129.9, 130.2(9), 130.3(5), 132.9, 133.0, 134.9, 135.8, 150.2, 150.3 and 163.6; $\delta_P(CDCl_3)$ 24.50; HRMS Found: $(M–H)^-$, 709.2527. $C_{39}H_{43}N_2O_7PSi$ requires *M*–H, 709.2499.

Phenyl (3′-*O*-*tert*-butyldiphenylsilylthymidin-5′-yl) methylphosphonate, triethylammonium salt (4)

To the phosphonate diester **3** (7.82 g, 11.0 mmol) dissolved in dioxane–water (3:1, v/v, 110 ml), 2-pyridinealdoxime (4.03 g, 33.0 mmol) and 1,1,3,3-tetramethylguanidine (4.14 ml, 33.0 mmol) were added. The reaction mixture was stirred at room temperature for 18 h and then partitioned between 1 M TEAB (200 ml) and $CHCl_3$ (2 × 200 ml). The combined organic phases were evaporated and the residue was purified by silica gel chromatography using a stepwise gradient of MeOH (0–12%) in $CHCl_3$–Et_3N (995:5, v/v). The title compound was obtained as a white foam. Yield 8.05 g (99.4%). $\delta_H(CDCl_3)$ 1.06 (9 H, s), 1.19 (9 H, t, *J* 7.0), 1.5–1.8 (5 H, m), 1.84 (3 H, s), 2.21 (1 H, m), 2.87 (6 H, q, *J* 7.0), 3.96 (1 H, m), 4.08 (1 H, m), 6.34 (1 H, dd, *J* 8.5, 5.5), 7.0–7.6 (16 H, m) and 8.13 (1 H, br s); $\delta_C(CDCl_3)$ 9.2, 12.6, 19.1, 23.0, 25.0, 27.0, 28.6(9), 28.7(4), 39.9, 45.4, 76.0, 84.9, 87.7, 88.0, 111.0, 120.6(6), 120.7(4), 122.8, 127.9, 129.2, 130.1, 133.1, 133.3, 135.2, 135.8, 150.3, 153.3 and 163.8; $\delta_P(CDCl_3)$ 21.57; HRMS Found: M^-, 633.2275. $C_{33}H_{38}N_2O_7PSi$ requires *M*–, 633.2186.

4-Methoxy-1-oxido-2-picolyl phenyl (3′-*O*-*tert*-butyldiphenylsilylthymidin-5′-yl)methylphosphonate (5)

The phosphonate monoester **4** (7.36 g, 10.0 mmol), 4-methoxy-1-oxido-2-pyridinemethanol (1.71 g, 11.0 mmol) and 4-methoxypyridine-1-oxide (3.75 g, 30.0 ml) were rendered anhydrous by evaporation of added pyridine (2 × 50 ml) and then dissolved in the same solvent (100 ml). 2-Chloro-5,5-dimethyl-2-oxo-1,3,2-dioxaphosphinane (5.54 g, 30.0 mmol) was added and the reaction mixture was stirred at room temperature. When the condensation was complete (~1 h, TLC analysis), the reaction mixture was concentrated and the residue partitioned between 0.5 M sodium bicarbonate (250 ml) and $CHCl_3$ (2 × 250 ml). The combined organic phases were dried over Na_2SO_4 and evaporated. Purification by silica gel column chromatography using a stepwise gradient of MeOH (0–10%) in $CHCl_3$ afforded the title compound as a white foam. Yield 7.40 g (95.9%). $\delta_P(CDCl_3)$ 29.17; HRMS Found: $(M–H)^-$, 770.2556 $C_{40}H_{46}N_3O_9PSi$ requires *M*–H, 770.2663.

4-Methoxy-1-oxido-2-picolyl (3′-*O*-*tert*-butyldiphenylsilylthymidin-5′-yl)methylphosphonate, triethylammonium salt (6)

To the phosphonate diester **5** (6.95 g, 9.0 mmol) dissolved in dioxane–H_2O (3:1, v/v, 90 ml), 2-pyridinealdoxime (2.20 g, 18.0 mmol) and 1,1,3,3-tetramethylguanidine (2.26 ml, 18.0 mmol) were added. The reaction mixture was stirred at room temperature for 22 h and then partitioned between 1 M TEAB (200 ml) and $CHCl_3$ (2 × 200 ml). The combined organic phases were evaporated and the residue was purified by silica gel chromatography using a stepwise gradient of MeOH (0–28%) in $CHCl_3$–Et_3N (995:5, v/v). The title compound was obtained as a white foam. Yield 7.10 g (99.0%). $\delta_H(CDCl_3)$ 1.06 (9 H, s), 1.28 (9 H, t, *J* 7.3), 1.5–1.8 (5 H, m), 1.86 (3 H, s), 2.20 (1 H, m), 3.02 (6 H, q, *J* 7.3), 3.84 (3 H, s), 3.97 (1 H, m), 4.13 (1 H, m), 5.09 (2 H, d, *J* 6.2), 6.30 (1 H, dd, *J* 8.1, 5.5), 6.73 (1 H, dd, *J* 7.3, 3.3), 7.21 (1 H, d, *J* 3.3), 7.1–7.6 (11 H, m), 8.13 (1 H, d, *J* 7.3) and 8.39 (1 H, br s); $\delta_C(CDCl_3)$ 9.2, 12.6, 19.2, 22.6, 24.7, 27.0, 28.7, 39.7, 45.5, 56.3, 60.8, 76.1, 85.2, 87.7, 88.0, 108.5, 110.5, 111.1, 128.0, 130.1, 133.2, 133.3, 135.4, 135.8, 139.8, 150.5, 151.4, 151.5, 158.4 and 163.8; $\delta_P(CDCl_3)$ 24.44; HRMS Found: M^-, 694.2404. $C_{34}H_{41}N_3O_9PSi$ requires *M*, 694.2350

4-Methoxy-1-oxido-2-picolyl [3′-*O*-(4,4′-dimethoxytrityl) thymidin-5′-yl]methylphosphonate, triethylammonium salt (7)

The 3′-*O*-silylated phosphonate **6** (6.38 g, 8.0 mmol) was dissolved in freshly distilled THF (64 ml) and treated with tetrabutylammonium fluoride trihydrate (10.1 g, 32.0 mmol) for 4 h. Water (16 ml) was then added and the reaction mixture concentrated to an oil. Tetrabutylammonium ions were removed by passing the residue dissolved in water through an ion-exchange column (Dowex® 50W, pyridinium form). Fractions containing the product were pooled and lyophilised. Repeated

lyophilization from water (100 ml) afforded crude desilylated product. Half of this material and 4-dimethylaminopyridine (49 mg, 0.40 mmol) were dried by evaporation of added pyridine (40 ml) and dissolved in the same solvent (40 ml). 4,4′-Dimethoxytrityl chloride (2.03 g, 6.0 mmol) was added and the reaction mixture stirred at 70°C for 5 h. After cooling to room temperature, MeOH (8 ml) was added and the reaction mixture evaporated to near dryness. The residue was partitioned between 1 M TEAB (200 ml) and $CHCl_3$ (2 × 200 ml). The combined organic phases were evaporated and the residue purified by silica gel chromatography using a stepwise gradient of MeOH (0–20%) in $CHCl_3$–Et_3N (995:5, v/v). The title compound was obtained as a white foam. Yield 2.74 g (79.6%). $\delta_H(CDCl_3)$ 1.23 (9 H, t, *J* 7.3), 1.5–1.8 (6 H, m), 1.85 (3 H, s), 2.92 (6 H, q, *J* 7.3), 3.71 (1 H, m), 3.77 (3 H, s), 3.77 (3 H, s), 3.82 (3 H, s), 4.00 (1 H, m), 5.11 (2 H, d, *J* 6.6), 6.22 (1 H, pt, *J* 7.1), 6.72 (1 H, dd, *J* 7.3, 3.3), 7.25 (1 H, d, *J* 3.3), 6.8–7.4 (14 H, m), 8.09 (1 H, d, *J* 7.3) and 8.57 (1 H, br s); $\delta_C(CDCl_3)$ 9.2, 12.6, 19.9, 23.1, 25.1, 29.0(9), 29.1(5), 37.9, 45.5, 55.3, 56.2, 60.8, 60.8, 76.7, 85.1, 86.1, 86.4, 87.2, 108.5, 110.5, 111.1, 113.3, 113.4, 127.1, 128.0, 128.4, 130.3, 130.4, 135.4, 136.2, 136.5, 139.7, 145.2, 150.5, 151.5, 151.6, 158.2, 158.7, 158.8 and 163.9; $\delta_P(CDCl_3)$ 24.51; HRMS Found: M^-, 758.2515. $C_{39}H_{41}N_3O_{11}P$ requires *M*, 758.2479.

Comparison of the formation of the thymidine dimer analogue using nucleotidic units with phenyl (4) or with intramolecular catalytic group (6) at the phosphonate centre

A nucleosidic component (5′-*O*-*tert*-butyldiphenylsilylthymidine, 0.144 g, 0.30 mmol), the phosphonate **4** (1.2 equiv.) and 4-methoxypyridine *N*-oxide or *N*-methylimidazole (4 equiv.) were rendered anhydrous by evaporation of added pyridine and dissolved in the same solvent (3 ml). The solution was divided into three equal volumes, and to each of them the appropriate condensing agent was added (2,4,6-triisopropylbenzenesulfonyl chloride, diphenyl phosphorochloridate or 2-chloro-2-oxo-5, 5-dimethyl-1,3,2-dioxaphosphinane, 3 equiv.) After different time intervals (1–120 min) aliquots (10 µl) were removed, quenched with 2 M TEAB (25 µl), and after evaporation analyzed by TLC on silica gel using chloroform–methanol (9:1, v/v). The coupling reactions in which the phosphonate **6** with an intramolecular catalytic group was used, were performed analogously, with the exception that no external nucleophilic catalyst was added. For the results and the comparison, see the text.

Comparison of removal of the phosphonate protecting groups from the thymidine dimers

The thymidine dimer (50 µmol) with phenyl or with 4-methoxy-1-oxido-2-picolyl phosphonate protecting group (prepared as described above and purified by silica gel chromatography) was treated with 2-pyridinealdoxime (400 µmol) and 1,1,3,3-tetramethylguanidine (400 µmol) in dioxane–water (3:1, v/v, 1.0 ml) or with thiophenol–triethylamine–dioxane (1:1:2, 1.0 ml), respectively. Aliquots were removed at different time intervals and analyzed by TLC on silica gel. For the results, see in the text.

Solid-phase syntheses of the 5′-deoxy-5′-methylphosphonate linked thymidine oligonucleotide analogues

A gas-tight Hamilton® syringe equipped with a sintered glass filter at the base was charged with 22 mg of a solid support (LCAA-GPG 500 Å) functionalized (45 µmol/g) with 3′-*O*-(dimethoxytrityl)-5′-*O*-succinylthymidine according to Pon *et al.* (47). Reagents and solvents for the manual solid-phase syntheses were kept over molecular sieves in the septa-capped bottles. The assemble of oligonucleotidic chains was carried out by drawing into the syringe the appropriate amounts of reagents as specified in the protocol in Table 1, with a continuous swirling the contents of the syringe. All washings from the detritylation step were combined and used for the colorimetric trityl assays. Averaged coupling yields per condensation step were in the range 95–98% as judged from the trityl reading. After the desired number of the elongation cycles, the support-bound fully protected oligomers were treated with thiophenol–triethylamine–dioxane (1:1:2) to remove the phosphonate protecting groups, followed by ammonolysis of the succinate linkage. The crude products from the syntheses were lyophilized (white solids) and subjected to HPLC analysis.

Reversed-phase HPLC analysis and purification of 10

Homogeneity of the crude oligomers **10** obtained from the syringe syntheses was analyzed by the reversed-phase HPLC using a Supelcosil® LC 18 column (15.0 cm × 4.6 mm) and a stepwise gradient of 0.1 M NH_4OAc in H_2O–MeCN (1:1, v/v) (solvent B) in 0.1 M NH_4OAc in H_2O (solvent A) at a flow rate of 1 ml/min. When a satisfactory resolution of an oligomer with the desired chain-length and truncated sequences was attained, the same gradient was used for the preparative run. Representative chromatograms together with the gradients used are shown in Figure 1.

Enzymatic characterization of the 5′-deoxy-5′-methylphosphonate thymidine oligonucleotide analogues

Approximately 0.1 OD of the purified thymidine oligonucleotide analogues **10a–c** were subjected to enzymatic digestion using snake venom (SVPDE) or bovine spleen (BSPDE) phosphodiesterases and the reaction mixtures were analyzed by HPLC. The digestions were performed at 37°C using an oligomer concentration of 1.0 OD/ml. The enzyme concentrations and corresponding buffers were (a) 0.4 U/ml, Tris–HCl (50 mM, pH 8.0), $MgCl_2$ (10 mM); (b) 0.5 U/ml, 2-(*N*-morpholino)ethanesulfonic acid (10 mM, pH 6.5). Incubation times were 3 and 12 h, respectively. Representative chromatograms from the enzymatic digests are shown in Figure 1.

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