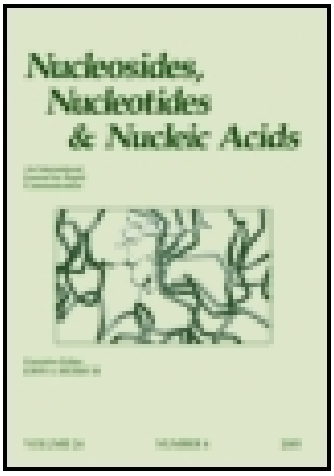

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Synthesis, Base Pairing Properties and Nuclease Resistance of Oligothymidylate Analogs Containing Methoxyphosphoramidate Internucleoside Linkages

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SYNTHESIS, BASE PAIRING PROPERTIES AND NUCLEASE RESISTANCE OF OLIGOTHYMIDYLATE ANALOGS CONTAINING METHOXYPHOSPHORAMIDATE INTERNUCLEOSIDE LINKAGES.

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Abstract: A dithymidine N-methoxyphosphoramidate was synthesized and incorporated into oligothymidylate analogs. The analogs form less stable duplexes with poly dA and poly rA than the corresponding "all diester" oligomer. The instability is more pronounced with poly rA than with poly dA. The analogs were found to be highly resistant to nucleases.

INTRODUCTION

Modulation of the expression of genetic information by antisense oligodeoxynucleotides is of great interest because of their potential use as therapeutic agents[1-4]. However, unmodified oligonucleotides are rapidly degraded by extra- and intracellular nucleases and are unable to penetrate efficiently inside the cell due to their polyanionic character[2]. These difficulties could be circumvented by uncharged modifications of the phosphodiester backbone, such as phosphotriesters[5-10], methylphosphonates[11-15] and phosphoramidates[16-20] which provide stability to enzymatic cleavage and may enhance cellular uptake. However, the lower binding affinity of these analogs for their DNA and RNA targets and their inability to direct RNAse H cleavage[21] seems to limit their use as chemotherapeutics agents. Nevertheless, these apparent disadvantages may be exploited in antisense technology. It has been

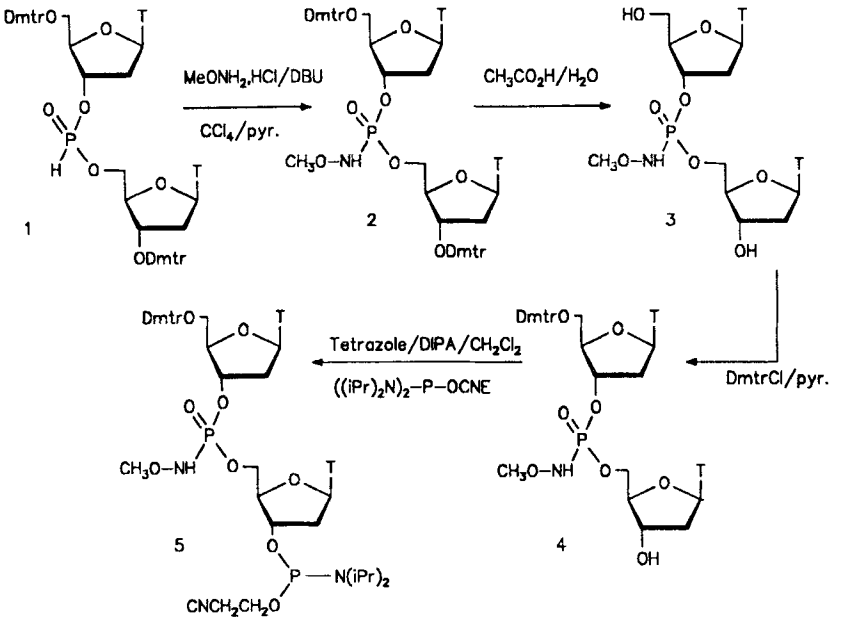
shown recently that chimeric oligonucleotides combining methylphosphonate[22,23] or phosphoramidate[24,25] portions with a central phosphodiester section, considerably reduce undesired RNase H cleavages at RNA sites of partial complementary without significantly disturbing activity at the target site. These considerations opened the way to the discovery and the synthesis of new backbone-modified oligonucleotide analogs.

Although a number of phosphoramidate oligonucleotides[16-20] have been prepared and their biophysical and biological properties studied, the synthesis of hydroxy or alkoxyphosphoramidate oligonucleotide derivatives has not yet been reported. These compounds might be interesting since it has been shown that dialkylesters of O-methylhydroxylamido[26] and of hydroxylamido[27] phosphoric acid are water soluble. Thus, these modifications would circumvent the solubility problem, in aqueous biological media, found with neutral phosphorus oligonucleotides such as methylphosphonates. Herein, we would like to report the first synthesis of oligothymidylate analogs containing methoxyphosphoramidate combined with phosphodiester internucleoside linkages. Resistance of these derivatives to nucleases and their hybridization properties with poly dA and poly rA were also examined.

RESULTS and DISCUSSION

Synthesis of 3',5'-dithymidine N-methoxyphosphoramidate

While the amides of phosphoric acid esters have been widely studied, there are few reports in the literature describing the synthesis of dialkyl N-methoxy (or alkoxy) phosphoramidates[26-28]. Most often, these compounds were obtained by the reaction of the free base of methoxyamine with the corresponding dialkyl phosphite in the presence of carbon tetrachloride[27]. However, due to the weakly basic properties of methoxyamine (pKb = 9.4)[29], these reactions are sluggish when compared to those of primary or secondary amines with hydrogenophosphonate diesters[16-18]. Furthermore, free O-alkylhydroxylamines are often volatile and are neither readily prepared and nor easy to handle [29]. To bypass these problems, Zwierzak and Brylikowska[30] obtained satisfactory results with methoxyamine hydrochloride by using a phase transfer technique. The reaction of methoxyamine with diethylphosphite was carried out in carbon tetrachloride, in the presence of triethylbenzylammonium chloride, by addition of a concentrated aqueous solution of potassium hydroxide. This process,



T= Thymin-1-yl; Dmtr= 4,4'-dimethoxytrityl.

SCHEME 1

unfortunately, is not appropriate for the synthesis of oligonucleotide derivatives especially on solid support.

Here we present the synthesis of a dinucleoside N-methoxy-phosphoramidate in a one-phase reaction. Reaction of the hydrogeno phosphonate diester **1** with methoxyamine was unsuccessful when triethylamine was utilised to generate the free base from methoxyamine hydrochloride. Replacing triethylamine by the stronger base 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) in a carbon tetrachloride / pyridine medium (5/1; v/v) afforded N-methoxy phosphoramidate **2** in 50 to 80% yield.

Deprotection in acetic acid / water (8/2,v:v) during 3.5 hours at room temperature gave the deprotected dimer **3** as a mixture of diastereoisomers in 95 % yield. This reaction was carefully followed (by HPLC), as longer acidic treatment could result in phosphorous-nitrogen cleavage, generating a phosphodiester dimer[16-18]. As expected, dimer **3** was easily soluble in water (this was not the case with dithymidinyl methylphosphotriester analog[31]).

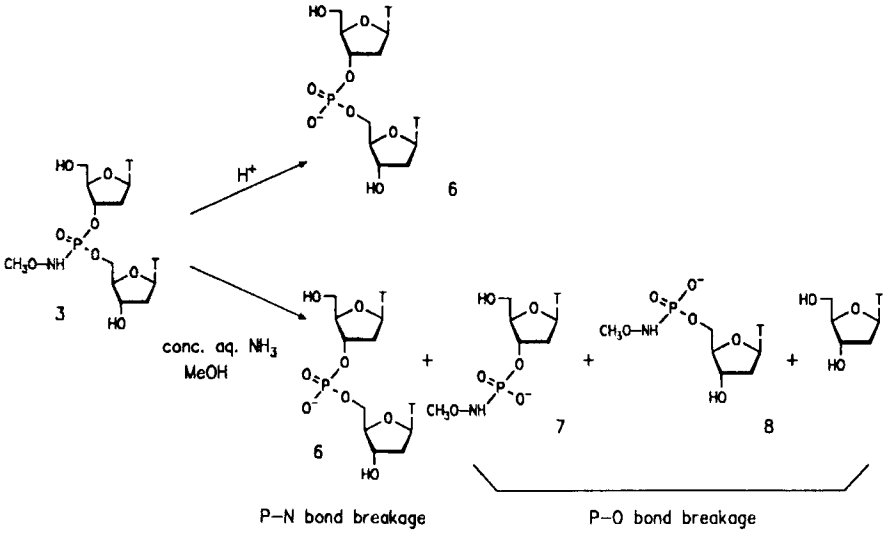
Compound **3** was fully characterized by ^{1}H-NMR, ^{31}P-NMR and FAB-MS. From the ^{1}H-NMR spectrum, a coupling constant $J^{1}_{H-}{}^{31}_{P}$ of 17.9 Hz resulting from the spin-spin interaction between the proton of the amido and the P nucleus was measured[26,30]. Then, sequential dimethoxytritylation and phosphitylation of **3** following standard protocols provided dimer **5** in an overall yield of 30%.

Stability of the N-methoxyphosphoramidate link.

It is recognized that the chemical properties of O-alkylhydroxylamides of phosphoric acid are analogous to those of the corresponding amides[26]. In particular, they undergo hydrolytic cleavage of the P-N bond in acidic medium. Thus, as with regular phosphoramidates[16-18], treatment of **3** with formic acid, at 90°C, resulted in complete P-N bond breakage within five minutes to give the phosphodiester dimer **6**. The identity of this compound was confirmed by a HPLC co-injection with an authentic sample. The stability of compound **3** was checked in 3% CCl_3CO_2H in CH_2Cl_2. This treatment is required to remove transient DmTr protecting group in the 5'-O-position of an oligonucleotide during chain elongation, in a synthesis on solid support (about one minute treatment per cycle). Compound **3** was slowly degraded at room temperature ($t_{1/2}$ = 6.7 hours) to give the corresponding phosphodiester dimer **6**. The stability of the P-N bond in this medium is sufficient to use the fully protected dimer **5** in an oligonucleotide synthesis on solid support.

The stabilty of the internucleoside linkage of compound **3** was studied under various basic conditions. Standard synthesis of oligonucleotides on solid support require a terminal treatment with concentrated ammonium hydroxide solution, which releases the oligonucleotide from the solid support and removes all the base labile protecting groups. Compound **3** was not stable under such basic conditions. After 24 hours in concentrated aqueous ammonia / methanol (1/1, v/v) at 55°C, HPLC analysis showed that dimer **3** was completely degraded ($t_{1/2}$= 4 hours), with 57% of P-N bond breakage giving rise to phosphodiester **6** and 43% of P-O bond breakage resulting in the formation of thymidine and of two other compounds which were assumed to be the methoxyphosphoramidate monoesters (3' and 5') of thymidine **7** and **8**, (*Scheme 2*). The same result was observed when a 0.1 M aqueous sodium hydroxyde solution was employed instead of concentrated aqueous ammonia.

These results are inconsistent with literature data[26], describing a slow degradation of alkoxyphosphoramidate compounds under basic conditions, with



SCHEME 2

exclusive P-O bond cleavage. Fortunately, a methanol saturated with ammonia treatment gave appreciably less degradation of compound **3**. These mild basic conditions have been used to deprotect base-sensitive oligonucleotides such as RNA and methylphosphonate backbone-modified oligonucleotides[32]. After two hours, at room temperature, less than 1% degradation of the internucleoside link of **3** was detected. Using this treatment, the synthesis of oligonucleotide analogs bearing methoxyphosphoramidate internucleoside links became feasible.

Synthesis of dodecathymidylate analogs.

The methoxyphosphoramidate dimer block **5** was incorporated into dodecathymidylates in one, four and five positions by standard solid phase DNA chemistry using phosphoramidite methodology[33], except that an oxalyl anchor[34] between the growing oligonucleotide and a long chain alkyl amine-controlled pore glass (LCAA-CPG) solid support, was employed instead of a succinyl linker. Indeed, initial attempts to release efficiently oligomers from a succinyl-LCAA-CPG support with satured methanolic ammonia were unsuccessful.

No difference in reactivity between the dimer **5** and a regular phosphoramidite was observed, as estimated by the trityl assay. Oligomer **9** was synthesized with

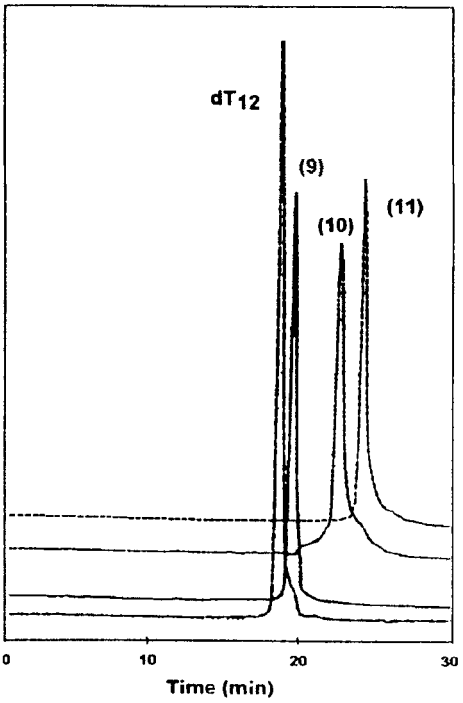


FIG.1: Reverse-phase HPLC analysis of oligothymidylate analogs obtained after purification (Cf experimental section). UV detection at 260 nm.

one modification placed in the middle of the sequence (see TABLE 1). In compound **10**, two modified dimers **5** were incorporated at both 3'- and 5'-end. First internucleoside bonds at the 5'-end and at the 3'-end of the oligomer were respectively a methoxyphosphoramidate and a phosphodiester. In oligonucleotide **11**, modified linkages were fully alternated with normal phosphodiesters, such that the oligomer had five methoxyphosphoramidate and six phosphodiester internucleoside bonds.

These oligonucleotides were deblocked and removed from the solid support by treatment with saturated methanolic ammonia and purified by reverse phase HPLC. The integrity of the isolated oligomers **9**, **10**, **11** was confirmed by hydrolysis of the methoxyphosphoramidate to phosphodiester linkages. Thus, a treatment with 85% formic acid at 90°C[16-18] for 30 minutes and subsequent reverse-phase HPLC analyses of the corresponding mixtures revealed quantitative conversion of the modified oligonucleotides into dodecathymidylate dT_{12}. FIG.1 shows the chromatograms of **9**, **10**, **11** and of dT_{12}.

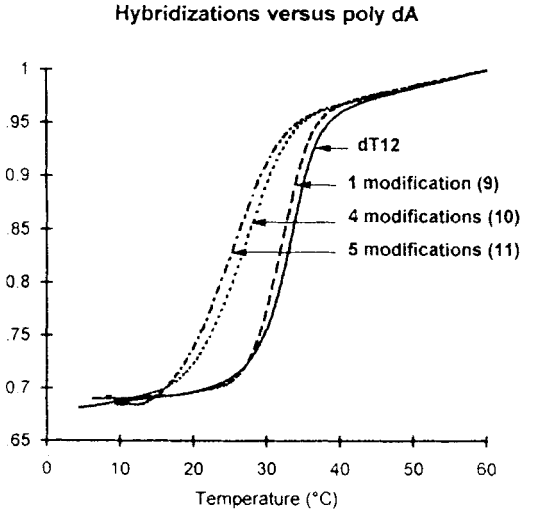


FIG.2

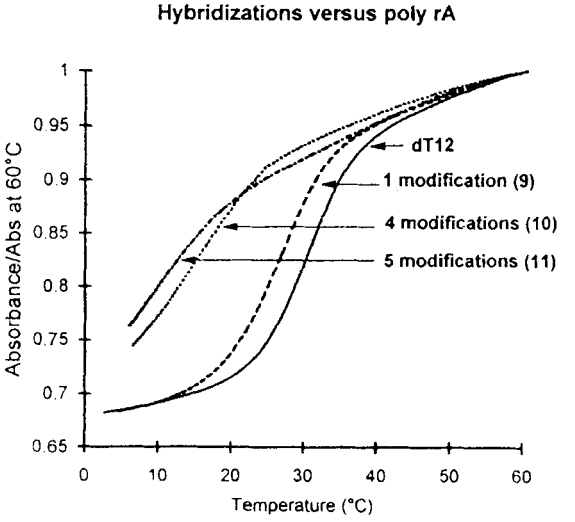


FIG.3

TABLE 1: Thermal stability (T_m)[a] of duplexes formed between oligothymidylate analogs and poly dA or poly rA targets.

Compounds		versus poly dA T_m(°C)	versus poly dA ΔT_m/mod(°C)	versus poly rA T_m(°C)	versus poly rA ΔT_m/mod(°C)
$d(T)_{12}$		33.5		30.5	
$d(T_6 \blacklozenge T_6)$ [b]	(**9**)	32	-1.5	27.5	-3
$d(T \blacklozenge T)_2T_3(T \blacklozenge T)_2T)$	(**10**)	27.5	-1.5	16.5	-3.5
$d(T(T \blacklozenge T)_5T)$	(**11**)	25.5	-1.6	11.5	-3.8

(a) Melting temperatures (T_m) measured at $6x10^{-5}$M nucleotide concentration in 10 mM sodium cacodylate, 100 mM sodium chloride buffer solution (pH 7). (b) ♦ means presence of a methoxyphosphoramidate internucleoside linkage.

Retention times of those analogues on reverse-phase HPLC increase with the number of modifications: dT_{12}, 18.52 min, **9**, 19.41 min, **10**, 22.45 min, **11**, 24.01 min.

Hybridization studies with poly dA and poly rA

Complex formation between dodecathymidylate analogs **9**, **10**, **11** with poly dA and poly rA was investigated by both thermal denaturation and renaturation (T_m) analyses using changes in absorbance at 260 nm as a function of temperature. The experiments were carried out at an equal nucleotide concentration ($6x10^{-5}$M) of either **9**, **10**, **11**, or dT_{12} and poly dA or poly rA. FIGURES 2 and 3 show the melting curves of **9**, **10**, **11** and dT_{12} respectively with poly dA and with poly rA. TABLE 1 summarizes the melting temperatures (T_m) for the complexes. No significant differences were observed between thermal association and dissociation curves.

As shown in FIGURES 1 and 2, the absorbances of solutions of oligomers with poly dA or poly rA in 0.1M NaCl at low temperatures (5 - 10°C) were strongly reduced relative to those calculated for non-interacting oligomers. All samples showed similar hypochromicity, the same as for the natural duplexes

dT_{12} with poly dA and poly rA. Thermal association curves were sigmoidal, monophasic and fairly sharp, indicative of duplexes[35] with ordered stacking of bases. Additionally, it can be seen that incorporation of one, four and five modifications leads to a progressive decrease in the melting temperature of the complexes compared to the "parent" duplexes with dT_{12}. This decrease is a linear function of the number of modifications. The average destabilisation is 1.5°C per modification with poly dA as the complementary strand. A lowered affinity for duplex formation with poly rA was observed. In this case, a decrease in T_m of about 3.4°C per modification indicates an appreciable distorsion of the structure of the DNA-RNA duplex compared to the "parent" duplex.

Enzymatic degradation of dodecathymidylate analogs.

Nuclease resistance of oligomers **10** and **11** was investigated in comparison with the resistance of unmodified dT_{12} by use of the purified S1 nuclease, snake venom phosphodiesterase (SVPDE) and calf spleen phosphodiesterase (CSPD). Because of the chemical instability of the methoxyphosphoramidate link under acidic and basic conditions, the behavior of **10** and **11** in the media used for enzymatic degradations was also studied without the enzymes.

TABLE 2 summarizes the half-lives of the oligomers. As can be seen, two modifications at both the 5'-end and the 3'-end of the oligomer **10**, reduced the degradation of the phoshodiester "window" towards the action of the endonuclease S1 ($t_{1/2}$ = 39 min instead of 16 min for dT_{12}). A greater stabilizing effect ($t_{1/2}$ = 2.1 days) was observed when the phosphodiester link was surrounded by modifications (compound **11**). In comparison, compound **10** was only slowly degraded in the buffer solution (pH 4.5, 37°C) without the enzyme ($t_{1/2}$ = 9 days).

When **10** and **11** were incubated with the 3'-exonuclease SVPDE, the corresponding undecamers resulting from thymidine 5'-monophosphate release from the 3'-end, were obtained. The rate of hydrolysis was reduced approximately by half compared to that of unmodified dT_{12} ($t_{1/2}$ >21 min for **10** and **11** instead of 12 min for dT_{12}). Due to the presence of a methoxyphosphoramidate dimer at the 3'-end of the resulting undecamers, a subsequent stabilization of the 5'-vicinal phosphodiester linkage was observed ($t_{1/2}$ > 11 hours instead of 12 min for dT_{11}). It should be noted that compounds **10** and **11** were degraded in the buffer solution used in this experiment with a half life of approximatively 20 hours (pH 9, 37°C).

TABLE 2: Half-lives of oligonucleotide analogs in presence of purified enzymes.

	$d(T)_{12}$	$d(T\blacklozenge T)_2T_3(T\blacklozenge T)_2T)$ (**10**)	$d(T(T\blacklozenge T)_5T)$ (**11**)
S1 nuclease [a]	16 min	39 min	2.1 days
Snake venom phosphodiesterase[b]	14 min (12 mer)	21 min (12 mer)	26.5 min (12 mer)
	12 min (11 mer)	11.4 hrs (11 mer)	16.2 hrs (11 mer)
Calf spleen phosphodiesterase[c]	22 min (12 mer)	10 days (12 mer)	2 hrs (12 mer)
	21 min (11 mer)		12 days (11 mer)

[a] 50 mM sodium acetate buffer (pH 4.5) containing 300 mM sodium chloride and 100 mM zinc acetate, 37°C. [b] 100 mM Tris HCl buffer (pH 9.0) containing 10 mM magnesium chloride, 37°C. [c] 125mM ammonium acetate buffer (pH 6.8) containing 2.5 mM EDTA, 37°C.

Against the action of the 5'-exonuclease CSPDE, the stabilizing effect on phosphodiester bonds was even more prononced. Thus, the 5'-terminal phosphodiester link of the oligomer **11** was five times more stable than that of dT_{12} ($t_{1/2}$ = 2 hours instead of 22 min for dT_{12}). Hydrolysis of methoxyphosphoramidite links in the buffer solution (pH 6.8, 37°C) was then responsible of the degradation of the resulting undecamer and of the dodecamer **10**. Therefore, the half-life of oligomer **10** was the same with or without the enzyme.

CONCLUSION

Oligothymidylate analogs containing one, four and five N-methoxy phosphoramidate internucleoside linkages have been synthesized. These modifications moderately weaken duplex stability of oligonucleotide analogs when hybridized with poly dA, but display a considerably lower binding affinity with poly rA. The replacement of the negatively charged phosphodiester linkage by N-methoxyphosphoramidate significantly improves the stability of the oligomers against exonucleases and endonucleases. This novel internucleoside linkage is a promising phosphodiester analog for oligonucleotides used as nuclease resistant probes or therapeutic agents.

EXPERIMENTAL PART

Material and methods. NMR spectra were recorded at 250 MHz for ^{1}H and at 100 MHz for ^{31}P on a Brücker AC250 spectrometer. δ values are in ppm relative to tetramethylsilane as internal standard (^{1}H NMR) and relative to 85% H_3PO_4 as external standard (^{31}P NMR). FAB Mass spectra were recorded on a JEOL JMS 300 DX spectrometer. The silica gel (0.040-0.063 mm) used for column chromatography was purchased from Merck. Snake venom phosphodiesterase (*Crotalus durissus*), Nuclease S1 (from *Aspergillus oryzae*) and Phosphodiesterase II (Calf spleen) were purchased from Boehringer (Mannheim, Germany). HPLC was performed on a Waters-Millipore instrument (Bedford, MA), equipped with two M510 solvent delivery systems, a M680 solvent programmer, a M712 autosampler, and a M990 diode-array UV-detector. Precolumns and columns were purchased from SFCC/Shandon (Eragny, France). Distilled water was purified on a Milli-Q system (Millipore).

5'-O-(4,4'-dimethoxytrityl)-3'-thymidine-3'-O-(4,4'-dimethoxytrityl)-5'-thymidine-N-methoxyphosphoramidate (2)

A solution of methoxyamine hydrochloride (4.18g, 50 mmoles) and DBU (7.61g, 50 mmoles) in anhydrous pyridine (50 ml), was stirred for 10 min then added to a suspention of **1** (5.67g, 5 mmoles) in carbon tetrachloride (200 ml). After 2 hours, the reaction mixture was diluted with dichloromethane (100 ml) and washed with water (2 x 100 ml). The organic layer was evaporated, and the residue purified by column chromatography on silica gel using a gradient of methanol in dichloromethane (0 to 5%) to give **2**. Yield: 2.96g (2.51 mmoles, 50%). ^{31}P NMR (DMSO-d_6): δ 8.14, 8.04.

3'-thymidine-5'-thymidine-N-methoxyphosphoramidate (3)

A solution of the dinucleoside **2** (2.96g, 2.51 mmoles) in acetic acid / water (50 ml, 8/2, v:v), was stirred for 3.5 hours at room temperature. Then, the reaction mixture was frozen and the solvent was removed by lyophilization. The residue was dissolved in dioxan / water (150 ml, 1/2, v:v) and the resulting mixture was washed with diethyl ether (100 ml). The aqueous layer was lyophilized to give **3** as a white solid material. Yield: 1.35 g (2.35 mmoles, 94%).

^{31}P NMR (DMSO-d_6): δ 8.90, 8.83. ^{1}H NMR (DMSO-d_6): δ 11.34 and 11.30 (2 s, 2H exchangeables, 2 NH-3), 9.12 (d, 1H exchangeable, J_{PNH} = 17.9 Hz, P-NH-O), 7.70 (s, 1H, H-6), 7.54 and 7.50 (2s, 1H, H-6, 2 isomers in a 45/55 ratio), 6.21 (m, 2H, 2 H-1'), 5.44 and 5.23 (2 br s, 2H exchangeables, OH-3' and

-5'), 4.98 (s, 1H, H-3' of 5'-unit of the dimer), 4.28-3.10 (m, 7 H, H-3', 2 H-4', 2 H-5',5''), 3.55 and 3.54 (2s, 3H, N-O-CH_3, 2 isomers in a 55/45 ratio), 2.07-2.37 (m, 4H, 2 H-2',2''), 1.80 and 1.78 (2s, 6H, 2 CH_3-5). FAB $^+$ (3-nitrobenzyl alcohol as matrix): 576 $(M+H)^+$.

H.P.L.C. : Two diastereoisomers, R_T = 18.30, 18.39 min, column: Hypersil SDS C18 (3 µm), Elution : from A (50 mM $AcONH_4$ pH 7) to B (A + 25% CH_3CN) in 20 min at a flow rate of 1 ml/min.

5'-O-(4,4'-dimethoxytrityl)-3'-thymidine-5'-thymidine-N-methoxy phosphoramidate (4)

To a solution of **3** (0.76g, 1.35 mmoles) in anhydrous pyridine (30 ml) was added 4,4'-dimethoxytrityl chloride (0.68g, 2 mmoles) and the resulting mixture was stirred for 4 hours at room temperature, then partitionned between dichloromethane (50 ml) and a saturated $NaHCO_3$ aqueous solution (50 ml). The organic layer was dried over Na_2SO_4, then evaporated. The residue was chromatographied on a silica gel column, using a gradient of methanol in dichloromethane (0 to 10%) to give **4**. Yield : 0.79 g (0.9 mmole, 66%).

^{31}P NMR (DMSO-d_6): δ 8.85. ^1H NMR (DMSO-d_6): δ 11.40 and 11.32 (2s, 2H exchangeables, 2 NH-3), 9.13 (d, 1H exchangeable, J_{PNH} = 17.9 Hz, P-NH-O), 6.86-7.52 (m, 15H, 2 H-6, 13 H trityl), 6.20 (m, 2H, 2 H-1'), 5.44 (t, 1H exchangeable, OH-3'), 5.05 (m, 1H, H-3' of 5' unit of the dimer), 4.30-3.00 (m, 7 H, H-3', 2 H-4' and 2 H-5',5''), 3.70 (s, 6H, 2 O-CH_3 trityl), 3.42 and 3.50 (2s, 3H, N-O-CH_3), 2.55-2.00 (m, 4H, 2 H-2',2''), 1.75 and 1.42 (2s, 6H, 2 CH_3-5).

5'-O-(4,4'-dimethoxytrityl)-3'-thymidine-3'-O-(O-cyanoethyl-N,N-diisopropyl-phosphoramidite)-5'-thymidine-N-methoxyphosphoramidate (5)

A mixture of **4** (0.741g, 0.845 mmole) and diisopropyl ammonium tetrazolide (0.072g, 0.42 mmole) was dried by coevaporation with anhydrous acetonitrile (3 times) and dissolved in freshly distilled dichloromethane (8 ml) before N,N-bis (diisopropylamino)-O-cyanoethyl phosphite was added under argon. After 12 hours at room temperature, ethyl acetate (25 ml) was added and the resulting solution was washed with brine (30 ml). The organic layer was dried (Na_2SO_4) and evaporated. The residue was purified by chromatography on silica gel using a gradient of dichloromethane (0 to 99%) in cyclohexane containing 1% of NEt_3 to give **5** . Yield : 0.45 g (0.43 mmole, 50%).

^{31}P NMR (DMSO-d_6) : δ 149.09, 149.02, 8.84, 8.80.

Synthesis of the oligodeoxynucleotides. The oligodeoxynucleotides **9**, **10**, **11** and dT_{12} were synthesized on an Applied Biosystems DNA-synthesizer (model 381A) using commercial dTβ-cyanoethyl phosphoramidite and compound **5**. First nucleoside (3'-OH) was linked to regular LCAA-CPG by means of an oxalyl anchor. Oligomers were prepared on a 1.0 μmol scale employing the standard synthesis cycle. The efficiency of each coupling step was monitored by the release of the dimethoxyltrityl cation. The coupling average efficiency of dimer **5** was of 99 %. Removal of the oligonucleotides from the solid support and deprotection was carried out at 20°C in methanolic ammonia for 2 hours. The oligomers were purified by reverse phase H.P.L.C. on a Spherisorb C18 preparative column (250mm x 10mm) with a gradient of acetonitrile from 10% to 30% in 0.05M triethylammonium acetate buffer (pH 7) in 40 min at a flow rate of 2 ml/min. Purity of the samples was checked by H.P.L.C. on a Nucleosil C18, 5μ column using a gradient of acetonitrile from 5% to 30% in 0.05M triethylammonium acetate buffer (pH 7) in 30 min at a flow of 1 ml/min.

UV Melting experiments. Melting curves were recorded on a UVIKON 810 spectrophotometer (Kontron) interfaced to an IBM PC compatible computer. The temperature control was through a HUBER PD 415 temperature programmer connected to a refrigerated water bath (Huber ministat). The cell compartement was flushed with dry nitrogen during measurements below ambient temperature. The heating rate was 0.5°C/min. Tm values were determined from the maxima of first derivates plots of absorbance versus temperature.

Enzymatic hydrolysis. The oligonucleotides (2 A_{260} units) were incubated at 37°C in either :

- 10 μl of 50 mM sodium acetate buffer (pH 4.5), 300 mM sodium chloride, 100 mM zinc acetate, 70 μl of water containing 2 units of nuclease S1.

- 100 μl of 100 mM Tris HCl buffer (pH 9.0), 10 mM magnesium chloride containing 2 μl of the commercial solution of SVPDE (3 units/ml).

-80 μl of 125mM ammonium acetate buffer (pH 6.8), 2.5 mM EDTA containing 2 μl of the commercial solution of CSPDE (0.013 units/ml final concentration).

Aliquots were analysed by analytical HPLC on a Nucleosil C18 5μm column using a gradient of acetonitrile from 5% to 30% in 50 mM $AcONH_4$ buffer (pH 7) in 30 min for **10** and **11** degradations, and from 5% to 20% in 50 mM $AcONH_4$ buffer (pH 7) in 45 min for dT_{12} hydrolysis, at a flow rate of 1 ml/min.

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