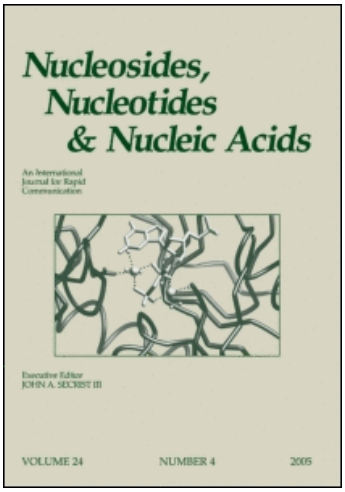

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SYNTHESIS AND BIOPHYSICAL PROPERTIES OF OLIGOTHYMIDYLATES CONTAINING ALKOXYPHOSPHORAMIDATE INTERNUCLEOSIDE LINKAGES.

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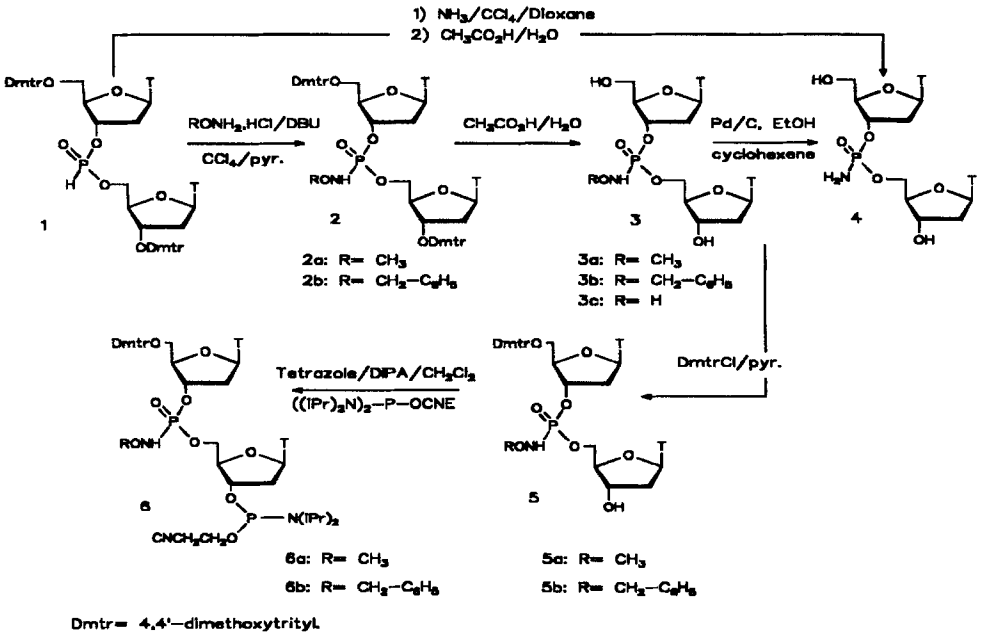
Abstract: The synthesis and the biophysical properties of oligothymidylate analogs containing N-methoxy- and N-benzyloxyphosphoramidate combined with phosphodiester internucleoside linkages are reported.

Antisense strategy is restricted by the nuclease degradation and the insufficient cellular uptake of the natural charged phosphodiester oligonucleotides. Therefore, chemical modifications of oligonucleotides are required for their effective use as potential antisense drugs. Here, we report a new internucleosidic modification in which the phosphodiester bond is replaced by an N-alkoxyphosphoramidate link.

The N-alkoxyphosphoramidate dimers **2a** and **2b** were obtained by oxidation of the dinucleoside H-phosphonate **1** with a solution of the corresponding alkoxyamine hydrochlorides in CCl_4/pyridine medium (5/1, v/v) in the presence of 1,8-diazabicyclo [5.4.0] undec-7-ene (DBU), (80% yield). Then, subsequent detritylation gave the deprotected dimers **3a** and **3b** as mixtures of diastereoisomers. From the ^{1}H-NMR spectra (DMSO-d_6) exchangeable protons resonating as well resolved doublets (δ=9.12 ppm, $J^1_{HN-}{}^{31}_P$=17.9 Hz) were in accord with the literature data[1]. An attempt to obtain the N-hydroxyphosphoramidate dimer (**3c**, R=H) from **3b** under catalytic transfer hydrogenation in ethanol using 10% Pd/C and cyclohexene as the hydrogen donor[2] was unsuccessful, and resulted exclusively in the formation of the phosphoramidate **4**. Structure of this compound was confirmed by comparison with an authentic sample of **4** obtained by oxidation of the H-phosphonate **1** with saturated ammonia solution in CCl_4/dioxane[3], followed by acidic detritylation. In order to incorporate the N-alkoxyphosphoramidate link into oligonucleotides, dimers **3a** and **3b** were

dimethoxytritylated and phosphytilated to provide phosphoramidite synthons **6a** and **6b**, (overall yield of 25% for **6a** and 50% for **6b**).



The dimer blocks **6a** and **6b** were then incorporated into dodecathymidylate analogs using phosphoramidite chemistry on solid support according to a standard 1 µmole synthesis cycle. Due to the observed instability of the alkoxyphosphoramidate linkage in concentrated aqueous ammonia solution ($t_{1/2}$ hydrolysis~ 1 hr at 55°C) the more labile oxalyl anchor[4] was employed instead of the usual succinyl linker between the solid support and the growing oligonucleotides. Then, oligonucleotides were deprotected and removed from the solid support by treatment with methanolic ammonia[5]. Analogs combining natural phosphodiester and alkoxyphosphoramidate internucleoside links (up to five) were obtained (See sequences **6a,b**; **7a**, and **8a,b**).

TpTpTpTpTpT♦TpTpTpTpTpT **6a, b**	*p*= phosphodiester link
T♦TpT♦TpTpTpTpT♦TpT♦TpT **7a**	♦ = modified internucleoside link
TpT♦TpT♦TpT♦TpT♦TpT♦TpT **8a, b**	a=N-methoxyphosphoramidate
	b=N-benzylhydroxyphosphoramidate

The stability of duplexes of dodecathymidylates analogs with poly (dA) and poly (rA) was investigated by thermal denaturation experiments. The affinity of the N-

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

Compounds		versus poly (dA) Tm (°C)	versus poly (dA) ΔTm/mod (°C)	versus poly (rA) Tm (°C)	versus poly (rA) ΔTm/mod (°C)
$d(T)_{12}$		33.5		30.5	
$d(T_6\blacklozenge T_6)$	**(6a)**	32	- 1.5	27.5	- 3
	(6b)	29	- 4.5	23	- 7.5
$d(T\blacklozenge T)_2T_3(T\blacklozenge T)_2T$	**(7a)**	27.5	- 1.5	16.5	- 3.5
$d(T(T\blacklozenge T)_5T)$	**(8a)**	25.5	- 1.6	11.5	- 3.8
	(8b)	12	- 4.3	<0	**nd**

(a) Melting temperatures (T_m) measured at 5 μM oligomer concentration in 10 mM sodium cacodylate, 100 mM sodium chloride buffer solution (pH 7).

TABLE 2: Half-lives of oligothymidylate analogs towards nuclease action.

	$d(T)_{12}$	$d(T\blacklozenge T)_2T_3(T\blacklozenge T)_2T$ **(7a)**	$d(T(T\blacklozenge T)_5T)$ **(8a)**
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.

methoxyphosphoramidate oligonucleotides for poly (dA) was slightly decreased compared to the affinity of the natural oligomer (ΔTm/mod~1.5°C) (TABLE 1). This decrease was more important with poly (rA) as the target (ΔTm/mod~3°C). Likely due to the steric hindrance around the phosphorus atom, an important destabilization was observed with N-benzyloxyphosphoramidate compounds [ΔTm/mod~4.5°C with poly (dA) and ΔTm/ mod~7.5°C with poly (rA)].

Nuclease resistance of N-methoxyphosphoramidate oligonucleotides **7a** and **8a** towards the action of purified enzymes was investigated in comparison with the resistance of unmodified $d(T)_{12}$. It was shown (TABLE 2) that N-

methoxyphosphoramidate oligonucleotides were resistant to exo- and endonuclease hydrolysis. Due to the modified linkage, a stabilizing effect was observed on both 5'- and 3'-vicinal phosphodiester bonds.

N-alkoxyphosphoramidate oligonucleotides represent a new class of backbone modified DNA analogs. Dodecathymidylates with alternating phosphodiester and alkoxyphosphoramidate linkages were synthesized with one to five modifications and their biophysical properties were studied. The replacement of the negatively charged phosphodiester linkage by the N-methoxyphosphoramidate link moderatly weaken duplex stability of the oligonucleotides analogs when hybridized with poly (dA), and display a lower binding affinity with poly (rA). Destabilization is more pronounced with N-benzyloxyphosphoramidate compounds. The resistance of N-metoxyphosphoramidate oligonucleotides towards nuclease action is significantly improved. Work is currently in progress to obtain less hindered N-hydroxyphosphoramidate oligonucleotides.

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