Novel DNA analogues with 2-, 3- and 4-pyridylphosphonate internucleotide bonds: synthesis and hybridization properties

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Received (in Montpellier, France) 21st May 2003, Accepted 15th July 2003
First published as an Advance Article on the web 15th September 2003

Oligothymidylates modified with stereodefined 2-pyridyl-, 3-pyridyl- and 4-pyridylphosphonate moieties at one or two juxtaposed internucleotide positions were prepared, and their avidity towards complementary single stranded DNA and RNA, as well as toward double stranded DNA were evaluated by UV melting temperature and CD studies. It was found that the sense of chirality at the phosphorus centre and the position of the nitrogen atom in the pyridyl ring of a pyridylphosphonate moiety are important factors governing stability of double- and triple-stranded complexes formed by these oligonucleotides. DNA/DNA and DNA/RNA duplexes containing oligothymidylate strands with R_P-pyridylphosphonate units differed only slightly from unmodified reference complexes. In contrast to this, the S_P-pyridylphosphonate derivatives exhibited lower binding affinity than both their R_P-counterparts and the unmodified reference oligonucleotide T_{20}. Triplexes of oligo(thymidyl pyridylphosphonate)s with hairpin oligomer d($A_{21}C_4T_{21}$) were found mostly to be thermodynamically slightly more stable in pH 7.4 and less stable in pH 5.0 than non-modified complexes. As expected, oligonucleotides with pyridylphosphonate internucleotide bonds were recognised by 3′- and 5′-exonucleases but the chimeric oligonucleotide chains were not cleaved at the modification sites.

Introduction

DNA analogues with enhanced stability against nucleolytic degradation and higher binding affinity toward complementary DNA or RNA strands are of interest due to their potential application as therapeutics in the antisense or antigene strategies.[1] Among the backbone modified oligonucleotides in which therapeutic potential has been recognised, are methylphosphonate analogues.[2,3] These DNA analogues are widely reported to efficiently inhibit selected genes expression in cellular systems.[4] Thermodynamic stability of duplexes of oligonucleotides possessing the R_P-methylphosphonate internucleotide bonds in alternate positions is usually increased in comparison to racemic and S_P-chiral methylphosphonate analogues.[5,6] Neutralization of negative charge of a phosphate backbone by introduction of a methylphosphonate moiety has been shown to influence the DNA structure,[7–9] duplex flexibility[10] and protein binding affinity.[11] Recently several other non-ionic analogues of DNA, closely related to methylphosphonates, *e.g.* oligo(nucleoside methanephosphonamidate)s,[12] oligo(nucleoside benzylphosphonate)s,[13] hydroxymethyl phosphonate)s,[14] and cyanomethylphosphonate)s[15] have been synthesized and their hybridization properties were evaluated. Interestingly, benzylphosphonate congeners, when used as antisense agents against hepatitis C virus (HCV), turned out to be more effective and more specific inhibitors of viral replication than the corresponding phosphorothioate or methylphosphonate derivatives.[16,17]

Thus, as part of our program on developing new antisense/antigene agents, we have recently embarked on investigations of pyridylphosphonates as a novel type of non-ionic nucleic acid analogues. Although the most striking feature of these compounds, similarly to methyl- and benzylphosphonates, is a lack of negative charge at the phosphorus centre, there is much more to pyridylphosphonates than being only the uncharged analogues of nucleic acids. A pyridyl ring, which is an essential part of these analogues, is also frequently present in biologically active natural products and constitutes an indispensable structural element of various drugs.[18] Recently, pyridine *N*-oxide derivatives were found to be potent, non-nucleoside inhibitors of reverse transcriptase active against HIV-1 and HIV-2 replication.[19] Also other low molecular phosphorus compounds bearing a pyridyl moiety connected to the phosphorus centre *via* a P–C bond (pyridylphosphonate derivatives) exhibit a broad spectrum of biological activity.[20–28]

The added value of pyridylphosphonates as a new type of modification of a phosphorus centre in biologically important DNA phosphate esters is that they combine structural features that can be instrumental in tuning chemical and biological properties of potential antisense/antigene agents. These are, *e.g.* (i) a chirality of the phosphorus centre that permits to control the orientation of the pyridyl ring in double-stranded complexes (major *versus* minor groove); (ii) the presence of a nitrogen atom that potentially can modulate stability of double- or triple-stranded complexes *via* formation of hydrogen

 DOI: 10.1039/b305689a

bonds or coordination of metal ions; (iii) a possibility of introducing additional functionalities to a pyridyl ring (*e.g.* quaternisation of the nitrogen atom, substitutions in the pyridyl ring) that can modify further chemical, biological, and therapeutic properties of drugs bearing a pyridylphosphonate moiety.

In this paper we describe the synthesis of pyridylphosphonate building blocks with defined stereochemistry at the phosphorus centre and their incorporation into oligothymidylic acids. We also present preliminary results on the influence of the stereochemistry at the phosphorus centre and position of the phosphonyl group in the pyridine ring on stability of the formed double- and triple-stranded complexes with complementary oligodeoxy- and oligoribonucleotide targets.

Results and discussion

Modification of internucleotide bonds in nucleic acids is a proven strategy when searching for potential novel constructs useful as antigene[29,30] and antisense[31] therapeutics. Since non-ionic DNA analogues containing dinucleoside R_P-methylphosphonates are known to possess favourable properties as antisense agents,[6] we assumed that the presence of P-chiral pyridylphosphonate internucleotide linkages in oligonucleotides may amplify some of these features and also bring an additional, useful functionality to an antisense oligonucleotide *e.g.* metal ions complexing properties. To investigate this possibility, P-stereodefined dinucleoside building blocks **3** with isomeric 2-, 3-, and 4-pyridylphosphonate moieties were prepared and converted into the phosphoramidite units **4** (Scheme 1). These, together with standard nucleoside 3′-phosphoramidite monomers, have been used for the assembly of the oligonucleotide chains.

Synthesis of pyridylphosphonate building blocks

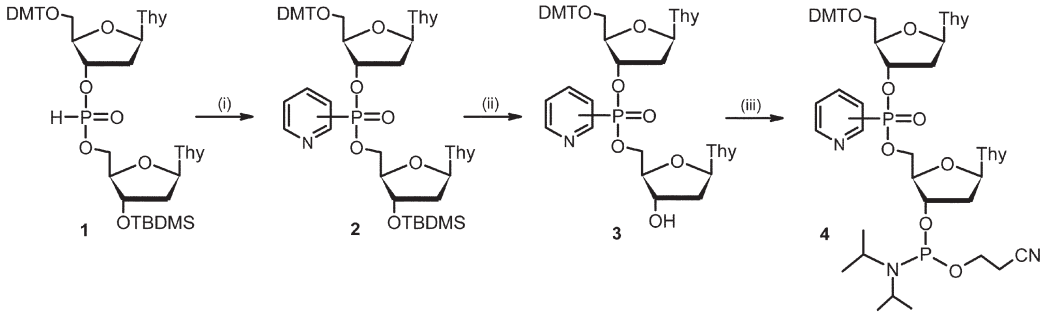
As a synthetic strategy for the preparation of oligonucleotide pyridylphosphonate analogues we chose the incorporation of dinucleoside building blocks, containing modified internucleotide linkages with stereodefined P-chiral centres, to the growing oligonucleotide chain. This called for the synthesis of six suitably protected dinucleoside 3′,5′-pyridylphosphonates of type **3** (Scheme 1) which, after conversion into the corresponding phosphoramidite derivatives **4**, could be used as substrates in an automated solid phase synthesis of oligonucleotides.

The synthesis of pyridylphosphonate building blocks of type **3**, bearing a 4,4′-dimethoxytrityl group at the 5′-*O*-position and a free 3′-OH function, is presented in Scheme 1. The synthesis commenced with the preparation of 3′-*O*-TBDMS-thymidine that was obtained from thymidine using simple protecting group manipulation protocols. This nucleosidic unit was then coupled with 5′-*O*-DMT-thymidine H-phosphonate to produce dinucleoside H-phosphonate **1**. The P-diastereomers of **1** (*ca.* 1 : 1 mixture) were separated by silica gel column chromatography and transformed into the corresponding pyridylphosphonate derivatives **2** using methods developed previously in our laboratories.[32–34] Specifically, treatment of H-phosphonates **1-R** and **1-S** with *N*-methoxypyridinium tosylate[33] in acetonitrile in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) afforded 2-pyridylphosphonate derivatives **2-2R** and **2-2S**, respectively. The reaction of **1** with *N*-tritylpyridinium cation[32] (generated *in situ* from trityl chloride and pyridine) led in the presence of DBU to corresponding 4-pyridylphosphonates **2-4R** and **2-4S**, while the palladium-catalysed reaction of **1-R** and **1-S** with 3-bromopyridine[34] afforded dinucleoside 3-pyridylphosphonates **2-3R** and **2-3S**, respectively. All these reactions were completely stereospecific and occurred most likely with retention of configuration at the phosphorus centre.[32–34] The 3′-*O*-TBDMS protecting groups from pyridylphosphonates **2** were then removed using a solution of TBAF in tetrahydrofuran. In this way, six isomeric dinucleoside pyridylphosphonates of type **3** (2-pyridyl-, 3-pyridyl- and 4-pyridyl- derivatives) with R_P and S_P configurations were prepared.

The absolute configurations at the phosphorus centre of dinucleoside pyridylphosphonates **3** were tentatively assigned as R_P and S_P on the basis of known stereochemistry of the parent diastereomers of dinucleoside H-phosphonate diesters **1**[35,36] used as substrates for the reactions, and the assumption that the conversions of **1** into pyridylphosphonates **2** occurred with retention of configuration.[32–34]

Synthesis of phosphoramidite derivatives 4

P-Stereodefined dithymidyl-3′,5′-pyridylphosphonates **3**, bearing 2-pyridyl (**3-2R** and **3-2S**), 3-pyridyl (**3-3R** and **3-3S**), and



Thy = thymin-1-yl
TBDMS = *t*-butyldimethylsilyl
DMT = 4,4'-dimethoxytrityl

1-R, R_P configuration at the phosphorus centre
1-S, S_P configuration at the phosphorus centre
2-2R, 3-2R, 4-2R, R_P 2-pyridyl derivatives
2-2S, 3-2S, 4-2S, S_P 2-pyridyl derivatives
2-3R, 3-3R, 4-3R, R_P 3-pyridyl derivatives
2-3S, 3-3S, 4-3S, S_P 3-pyridyl derivatives
2-4R, 3-4R, 4-4R, R_P 4-pyridyl derivatives
2-4S, 3-4S, 4-4S, S_P 4-pyridyl derivatives

Scheme 1 Synthesis of phosphoramidite derivatives **4**. The first part of the numbering system refers to the type of a compound (*e.g.* **2**, **3** and **4**), while the second one indicates a position of the nitrogen atom in the pyridine ring (*e.g.* **2**, **3** or **4**) and *R* and *S* corresponds to the stereochemistry at the phosphorus centre *R* or *S*, respectively. *Reagents and conditions:* i) For 2-pyridyl derivatives: *N*-methoxypyridinium tosylate, DBU, acetonitrile; for 3-pyridyl derivatives: $Pd(PPh_3)_4$, TEA, 3-bromopyridine, reflux in THF; for 4-pyridyl derivatives: Tr-Cl, CBU, pyridine; ii) TBAF, THF; iii) 2-cyanoethyl-*N*,*N* -diisopropylchlorophosphoramidite, *N*,*N*-diisopropyl ethylamine, THF.

4-pyridyl (**3-4R** and **3-4S**) moieties, with *R* or *S* absolute configuration at phosphorus atom, respectively, were used as substrates for the preparation of the corresponding phosphoramidite units **4** (Scheme 1). A successful phosphitylation was achieved by treatment of pyridylphosphonates **3** in THF[37] with 2-cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite (1.5 eq.) and ethyl-*N*,*N*-diisopropylamine (3 eq.). The reaction was carried out at room temperature under argon and its progress was monitored by TLC analysis. When the formation of product **4** levelled off (usually overnight), the reaction mixture was applied on a silica gel column. The column was washed with hexane–ethyl acetate (1 : 1, v/v) and then pure phosphoramidites **4** were eluted using a gradient of methanol (0–3%) in chloroform. Yields of the isolated products were *ca.* 60%. Phosphoramidites **4** were rather stable and could be stored as foams for a prolonged time under argon at −20 °C.

Synthesis of chimeric oligonucleotides

3′-*O*-Phosphoramidite building blocks **4** were used for the incorporation of pyridylphosphonate moieties into oligodeoxyribonucleotides by automated solid phase synthesis.[38] Two types of chimeric oligonucleotides, oligothymidylates T_9TxTT_9 (**5**) and $T_8TxTTxTT_8$(**6**), carrying one or two modified internucleotide linkages (*x* represents the corresponding 2-, 3- or 4-pyridylphosphonate linkage), were designed for these studies. The syntheses of oligomers of type **5** and **6** were performed using succinyl- or oxalyl-linked LCA-CPG solid support. The coupling efficiency for both R_P and S_P diastereomers of **4** in the series of 2- and 3-pyridylphosphonates, was moderate only (in the range of 40–42%, as determined by DMT-ion assay), but it was remarkably higher (97–98%) for phosphoramidites **4-4R** and **4-4S** (for the numbering system adopted in this paper, see Scheme 1). Since relatively low yields for incorporation of 2- and 3-pyridylphosphonates into oligonucleotides are most likely not due to the presence of adventitious water, more studies on chemical reactivity of this type of compounds are required.

A standard ammonia treatment (30% ammonium hydroxide, 1 h, RT) to cleave the oligomers from the succinyl-linked LCA-CPG solid support, followed by a two-step purification–deprotection procedure,[39] worked well for 2- and 3-pyridylphosphonate modified-oligonucleotides and produced the 5′-dimethoxytrityl protected oligonucleotides of type **5** and **6** as major products. However, for the oligonucleotides bearing 4-pyridylphosphonate moieties, an extensive cleavage at the site of modification occurred during ammonia treatment and the desired oligonucleotides **5-4R**, **5-4S**, **6-4R** and **6-4S** were only the minor products of the reaction. We managed, however, to significantly suppress this degradation process by using oxalyl-linked LCA-CPG solid support.[40] Due to higher lability of the oxalyl linker, a removal of 2-cyanoethyl protecting groups with a simultaneous cleavage of 5′-DMT-protected oligomers **5-4R**, **5-4S**, **6-4R** and **6-4S** from the solid support could be achieved by treatment with 1% aqueous triethylamine for 30 min at room temperature with only negligible degradation of the oligonucleotide chains. Studies on stability of pyridylphosphonate diesters under various experimental conditions are in progress.

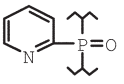
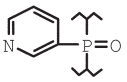
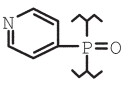
The 5′-*O*-DMT protected oligomers of type **5** and **6** were finally subjected to a two-step purification–deprotection procedure,[39] and the structure and purity of unprotected modified oligonucleotides of type **5** and **6** were confirmed by MALDI-TOF mass spectrometry, reverse phase HPLC analysis (Table 1) and polyacrylamide gel electrophoresis (PAGE).

Hybridization properties of oligomers 5 and 6

Binding affinity of chimeric oligo(deoxyribonucleoside pyridylphosphonate)s **5** and **6** to the complementary oligonucleotides dA_{19} and A_{19}, and to the double-stranded hairpin oligomer $d(A_{21}C_4T_{21})$ was determined by UV melting temperature measurements at pH 7.4. In addition, stability of triplexes of oligonucleotides **6** with dsDNA at pH 5.0 was studied. As it is shown in Table 2, stability of the corresponding duplexes and triplexes strongly depends on the sense of chirality at the phosphorus atom of the pyridylphosphonate unit and the mode of the pyridyl ring substitution. The DNA/DNA and DNA/RNA duplexes containing oligothymidylate strands with R_P-pyridylphosphonate units differ only slightly from unmodified reference complexes, and the most stable among them are those bearing 4-pyridylphosphonate linkages. The differences in *Tm* values (ΔTm) for these complexes are in the range of +2.0 to −1.8 °C per each introduced pyridylphosphonate modification, with the estimated measurement error ±0.5 °C.

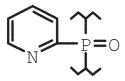
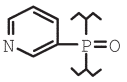
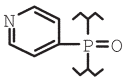
The oligomers containing S_P-pyridylphosphonate moieties bind to complementary DNA and RNA strands with lower affinity than their R_P-counterparts and the unmodified reference oligonucleotide T_{20}. In this case every 2-, 3-, and 4-pyridylphosphonate modification introduced into an oligonucleotide chain caused lowering of the *Tm* value of the

Table 1 Spectral and chromatographic characteristics of oligonucleotides **5** and **6**

Number	ODN	X	Calc. MW		MALDI TOF		HPLC[a]
			DMT ON	DMT OFF	DMT ON	DMT OFF	(Rt/min.)
5-2R	$T_9T_xTT_9$		6381	6079	6386	6081	23.3
6-2R	$T_8T_xTT_xTT_8$		6443	6140	6448	6140	24.9
5-2S	$T_9T_xTT_9$		6381	6079	6384	6082	26.4
6-2S	$T_8T_xTT_xTT_8$		6443	6140	6445	6142	25.3
5-3R	$T_9T_xTT_9$		6381	6079	6383	6084	23.2
6-3R	$T_8T_xTT_xTT_8$		6443	6140	6445	6147	24.8
5-3S	$T_9T_xTT_9$		6381	6079	6386	6080	23.3
6-3S	$T_8T_xTT_xTT_8$		6443	6140	6444	6140	24.5
5-4R	$T_9T_xTT_9$		6381	6079	6381	6088	24.2
6-4R	$T_8T_xTT_xTT_8$		6443	6140	6444	6150	24.9
5-4S	$T_9T_xTT_9$		6381	6079	6383	6088	24.1
6-4S	$T_8T_xTT_xTT_8$		6443	6140	6451	6143	24.4

[a] Buffer A: aqueous 0.1 M ammonium bicarbonate pH 7.5; buffer B: 0.1 M ammonium bicarbonate pH 7.5 in water–acetonitrile, 6 : 4. HPLC buffer system: Time 0 → 20 min: 15 → 50%; 20 → 23 min: 50 → 100%; 23 → 32 min: 100%; 32 → 34 min: 100 → 15% of buffer B. According to HPLC data purity of all compounds **5** and **6** was > 95%.

Table 2 Binding affinity of oligonucleotides **5** and **6** toward single stranded DNA, RNA and toward double stranded DNA. All melting temperatures (*Tm*[a]) were determined at pH 7.4[b] and, in addition, for triplexes of **6** with hairpin oligonucleotide d($A_{21}C_4T_{21}$) at pH 5.0[c]

		dA_{19}		A_{19}		d($A_{21}C_4$ T_{21})	
Oligomer	Pyridyl phosphonate unit	*Tm*	Δ*Tm*[d]	*Tm*	Δ*Tm*	*Tm* in pH 7.4 (in pH 5.0)	Δ*Tm* in pH 7.4 (in pH 5.0)
5-2R		51.4	+0.4	40.2	−1.2	28.7[e]	+1.1
6-2R		49.5	−0.7	37.7	−1.8	28.1 (48.9)	+0.2 (−1.6)
5-2S		49.1	−1.9	37.3	−4.1	28.7[e]	+1.1
6-2S		45.4	−2.8	32.3	−4.5	28.5 (47.8)	+0.4 (−2.2)
5-3R		51.8	+0.8	40.0	−1.4	28.4[e]	+0.8
6-3R		50.7	−0.1	38.4	−1.5	28.5 (50.7)	+0.4 (−0.7)
5-3S		48.5	−2.5	37.0	−4.4	28.3[e]	+0.7
6-3S		42.7	−4.1	31.6	−4.9	28.3 (43.7)	+0.3 (−4.2)
5-4R		53.0	+2.0	42.5	+1.1	26.5[e]	−0.9
6-4R		52.9	+0.9	41.1	−0.1	32.1 (51.5)	+2.2 (−0.3)
5-4S		47.9	−3.1	38.8	−2.6	31.9[e]	+4.3
6-4S		43.8	−3.6	34.2	−3.6	31.6 (45.1)	+2.0 (−3.5)
T_{20}		51.0		41.4		27.6 (52.2)	

[a] *Tm* values determined with an accuracy of ±0.5 °C [b] *Tm* measurements in 10 mM Tris-HCl, pH 7.4, 100 mM NaCl and 5 mM $MgCl_2$ buffer and the details are given in Experimental [c] *Tm* measurements in 10 mM Tris-HCl, pH 5.0, 100 mM NaCl and 5 mM $MgCl_2$ (*Tm* values are given in parentheses) [d] The *Tm* differences per each of the introduced pyridylphosphonate linkage (Δ*Tm*) [e] *Tm* at pH 5.0 not determined

corresponding DNA/DNA duplexes by 1.9 to 4.1 °C, and for the DNA/RNA duplexes by up to 4.9 °C per modification (Table 2).

A possible reason why only the S_P-pyridylphosphonate units significantly destabilize the double-helical structure, can be the sterically unfavorable positioning of the pyridine ring of the S_P pyridyl residues in the formed duplexes. Molecular dynamics simulations performed for oligo(nucleoside methylphosphonate)s[7–9] demonstrated that neutralization of charges in the DNA chain due to the presence of the P-methyl groups affects the electrostatic environment around the minor groove of the helix – a region in which the localisation of structural water molecules and metal cations depends on the sequence.[41] Such oligonucleotides show lower affinity toward their complementary sequences than unmodified oligomers, although the duplexes bearing R_P-methylphosphonate linkages usually exhibit higher thermal stability than their S_P-counterparts. It was postulated that the latter phenomena can be due to a more favourable solvation effect in R_P methylphosphonate derivatives.[5] In contrast to these, modified oligonucleotides containing R_P-pyridylphosphonate moieties bind stronger to the complementary DNA sequences than their S_P-counterparts but their complexes are usually as stable as those derived from the corresponding unmodified oligonucleotides (Δ*Tm* within the range of ±2.0 °C).

Since homo-oligothymidylates can form triple helical structures of T*dA-T type[29,30] by binding to poly(dA)/poly(T) duplexes *via* the Hoogsteen-type hydrogen bonds, we investigated also this phenomenon for pyridylphosphonate-modified oligonucleotides. For the triplex melting experiments performed at pH 7.4, the hairpin oligonucleotide d($A_{21}C_4T_{21}$) and oligomers **5** and **6** were used. All the triplexes dissociated at equal or slightly higher temperatures than the reference (unmodified) complexes (Δ*Tm* up to +2.2 °C), apart from the triplex of **5-4S** which melted remarkably higher than its parent congener (Δ*Tm* +4.3 °C). For the triplexes investigated at lower pH (pH 5.0), a significant increase in *Tm* values was observed for both the unmodified and the two pyridylphosphonate units-containing complexes (Table 2, values in parentheses), while the hairpin duplex was equally stable in neutral and acidic conditions (*Tm* 70 °C). The reason of increase of the thermal stability of triplexes in acidic pH may be due to release of sodium counterions and thermodynamic uptake of protons.[42] Although there are noteworthy variations in *Tm* values of the triplexes at the lower pH, depending on the configuration at the phosphorus center in pyridylphosphonate residues and the mode of substitution of the pyridine ring (*e.g.* Table 2, oligomer **6-3R** *vs.* **6-3S** or **6-2S** *vs.* **6-3S**), these differences are somewhat difficult to interpret at the present stage of investigations.

It seems that the differences in stability of the formed complexes (triplexes and duplexes), in comparison to the unmodified counterparts, are the result of various favourable/unfavorable interactions involving the pyridylphosphonate moieties. Apart from reducing electrostatic repulsions, the presence of the pyridine ring may provide an alternative mode of hydration for the system and can be the additional source of stabilizing stacking interactions with nucleobases. This is in contrast to methylphosphonate oligonucleotides which form more stable triplexes than their unmodified counterparts only when the phosphonate internucleotide linkage has R_P-configuration.[43]

Enzymatic digestion of oligo(thymidine pyridylphosphonate)s

Oligonucleotides **5** and the reference oligothymidylate T_{20} were used to study the influence of a pyridylphosphonate modification on the stability of the modified oligonucleotides against 3′- and 5′-exonucleases. For human plasma 3′-exonuclease assay, 5′-radiolabeled oligonucleotides were incubated with 50% human plasma. Products of degradation were analysed by PAGE under denaturing conditions (20% polyacrylamide, 7 M urea). The results showed that the reference oligomer after incubation for 4 hours at 37 °C afforded a ladder of products ranging from T_{19} to T_8 (Fig. 1). Longer incubation time led to the dimers TpT (results not shown). The modified oligomers were degraded only partially under these conditions. For example, degradation of oligonucleotide **5-2R** proceeded from the 3′-terminus and was arrested by the presence of the 2-pyridylphosphonate internucleotide linkage. Slightly different polyacrylamide gel mobility of the cleavage products from modified oligonucleotides **5** was probably caused by the presence of a non-ionic pyridylphosphonate residue in these molecules and their higher molecular weight, compared to those released from the unmodified oligonucleotide T_{20} (Fig. 1). The formation of minute amounts of low molecular weight products (faster migrating oligomers than that of T_9TxT) could be due to a partial chemical cleavage of a P–O linkage

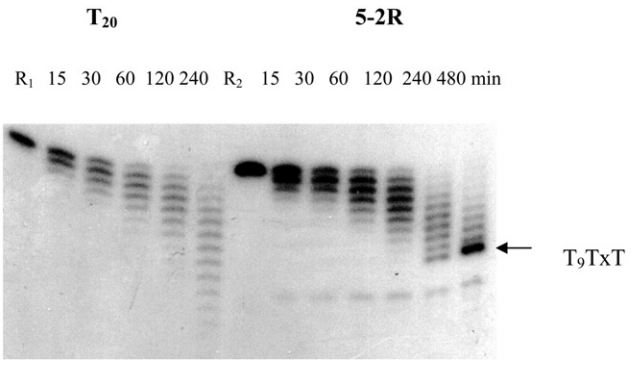


Fig. 1 Degradation of T_{20} and **5-2R** oligomers in 50% human plasma at different time points. Conditions are as indicated in Materials and Methods.

at the modification site, followed by enzymatic degradation of the produced fragments. Since a non-ionic pyridylphosphonate internucleotide bond, analogously to a methylphosphonate bond, is highly resistant to endonucleases, it seems unlikely that these phenomena can be caused by a residual endonucleolytic activity of human plasma, observed for certain hairpin loop oligodeoxyribonucleotides in the presence of magnesium ions.[44]

The progress of degradation of modified oligonucleotides **5** by snake venom phosphodiesterase (a 3′-exonuclease) and calf spleen phosphodiesterase (a 5′-exonuclease) was investigated by MALDI-TOF MS technique. It was found that all oligonucleotides of type **5** were resistant to exonucleolytic hydrolysis at the modification site. For example, the digestion of 2-pyridylphosphonate derivative **5-2R** with the 5′-exonuclease (Fig. 2) resulted in the appearance of a set of peaks ranging from T_9TxTT_9 (negative ion m/z 6079) to $TxTT_9$ (m/z 3344). The other modified oligonucleotides **5** gave similar sets of MALDI-TOF MS signals when digested by all investigated enzymes (results not shown).

CD spectra of duplexes containing pyridylphosphonate-modified oligonucleotides

The influence of pyridylphosphonate-modified oligonucleotides on the geometry of the duplexes formed with unmodified counterparts was investigated using CD spectroscopy. The CD spectra of **6**/dA_{19} (DNA/DNA type of a duplex) and **6**/A_{19} (DNA/RNA type of a duplex) are shown in Fig. 3a and b, respectively. From the shape of the spectra one can infer that the duplexes formed by pyridylphosphonate-modified oligonucleotides adopt similar to their unmodified counterparts form

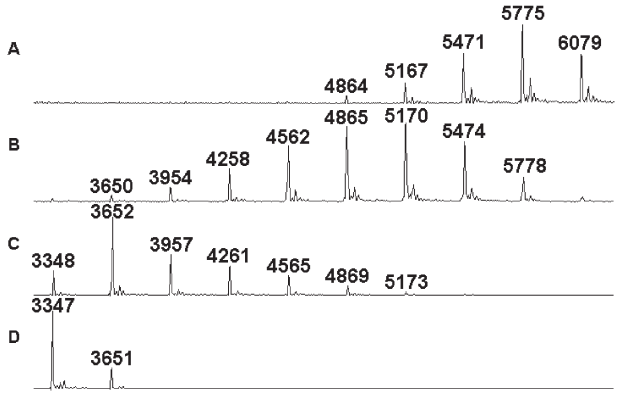


Fig. 2 Digestion of **5-2R** with calf spleen phosphodiesterase (5′-exonuclease). MALDI-TOF spectra were collected after 15, 45, 90 and 180 min of cleavage reaction.

B (for DNA/DNA complexes) and A (for DNA/RNA complexes) types of helical structures. For the **6-3R**/DNA duplex and for all other complexes bearing the RNA template, however, an additional band of medium intensity at around 230 nm, not present in the CD spectra of the referenced duplexes, was observed. For the **6-3S**/RNA duplex we observed slightly reduced signal intensity in the range of 215–245 nm (Fig. 3b). In addition, for the **6-3R**/DNA duplex, the disappearance of a positive band in the region of 260–270 nm was observed, while for the duplex **6-3S**/RNA this band was present, although with a slightly reduced intensity.

These minor changes observed in the CD spectra of the duplexes bearing 3-pyridylphosphonate modification may result from the presence of an electronic transition oriented along the helical axis with polarization perpendicular to the base plane. Theoretical calculations using the CNDO/Cl method[45] suggested that one of the n–π transitions of the purine ring is probably responsible for the observed changes in the CD spectra at 220–230 and around 250 nm. The disappearance of a positive band in the 260–270 nm region for the **6-3R**/DNA duplex and its presence for the duplex **6-3S**/RNA, can be due to some duplex deformations, as the CD spectra around 270 nm (n–π transitions) are highly sensitive to the changes of angle between neighbouring bases. This is especially important for RNA, where the second and third nearest neighbours contribute together more to intensity of the CD bands than do the nearest neighbours in DNA.[46]

Conclusions

Our preliminary data show that oligo(nucleoside pyridylphosphonate)s offer a novel type of constructs in designing new antigene/antisense agents. The replacement of the native phosphodiester bond by the P-chiral 2-, 3- or 4-pyridylphosphonodiester bond in oligodeoxyribonucleotides does not introduce significant geometric alterations to the structure of double-helical complexes formed by these modified oligonucleotides. The modification has, however, remarkable influence on the

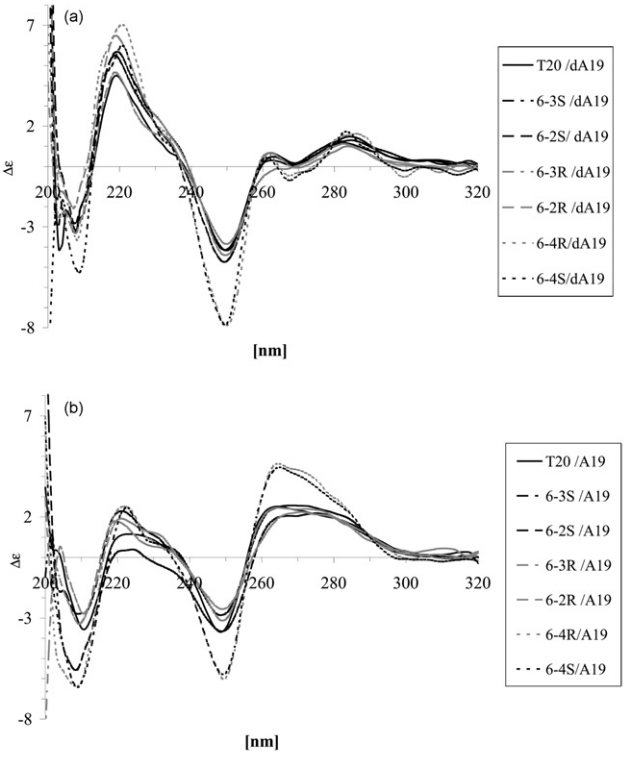


Fig. 3 CD spectra of duplexes of **6** with DNA (a) and with RNA (b) templates.

stability of the respective double-stranded complexes, depending on the stereochemistry of the modified unit and the mode of the pyridine ring substitution. The observed differences in *Tm* values for the duplexes formed by oligonucleotides **5** and **6** with complementary DNA and RNA sequences and those of unmodified reference complexes suggested that only S_P-pyridylphosphonate units destabilize significantly (up to 4.9 °C per modification) double-helical structures. In most cases of triple-helical structures investigated under neutral conditions (pH 7.4), a pyridylphosphonate moiety slightly stabilizes this type of complexes, probably due to favourable interactions involving the pyridine ring. Finally, a pyridylphosphonate moiety confers also certain resistance to nucleases of the modified oligonucleotides that can be useful when designing new antisense/antigene drugs.

Further work on the preparation of pyridylphosphonate oligonucleotides of mixed sequences and assessment of their properties from a point of view of potential drugs, is in progress in our laboratories.

Experimental

Materials and methods

Pyridine was dried by refluxing with CaH_2 overnight followed by distillation, redistillation from *p*-toluenesulfonyl chloride and stored over molecular sieves (4 Å). Tetrahydrofuran was dried by distillation from $LiAlH_4$ directly before use. Anhydrous acetonitrile (LabScan) was stored over molecular sieves (4 Å). Starting material, dinucleoside H-phosphonate **1** was obtained by condensation of 5′-O-dimethoxytritylthymidine 3′-H-phosphonate[47] with 3′-O-*tert*-butyldimethylsilylthymidine,[48] analogously to the published procedure.[49] Tetrabutylammonium fluoride trihydrate (TBAF), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), and 4,4′-dimethoxytrityl chloride were commercial grades (Aldrich). 2 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 (40–60 μm) from Scharlau was used, and the columns were run in the flash mode. Compounds were made anhydrous by co-evaporation with anhydrous pyridine. ^{1}H, and ^{31}P NMR spectra were recorded on a Varian-400 FT spectrometer. For ^{31}P NMR spectroscopy, 2% H_3PO_4 in D_2O was used as an external standard (inner tube). Subscripts "a" and "b" in the ^{1}H NMR data refer to the protons in nucleosid-3′-yl and nucleosid-5′-yl units, respectively.

Syntheses

Preparation of 5′-O-dimethoxytritylthymidin-3′-yl thymidin-5′-yl 2-pyridylphosphonates 3-2R and 3-2S[32–34]. Separate diastereomers of dinucleoside H-phosphonate **1** (**1R** or **1S**; 0.800 g, 0.844 mmol) in acetonitrile (16 mL) were treated with *N*-methoxypyridinium tosylate (0.474 g, 1.688 mmol) and DBU (0.504 mL, 3.38 mmol). After 5 min the reaction mixtures were concentrated and partitioned between 10% aq. $NaHCO_3$ and CH_2Cl_2. The organic layers were dried over anh. Na_2SO_4, evaporated and the residue were purified by silica gel chromatography using a stepwise gradient of methanol (0–4%) in toluene–ethyl acetate (1 : 1, v/v) containing 0.01% TEA.

2-2R (from **1-R**), 0.538 g (62%); white solid. δ_P ($CDCl_3$) 10.76 ppm.

2-2S (from **1S**), 0.752 g (87%); white solid. δ_P ($CDCl_3$) 11.20 ppm.

A 1 M solution of tetrabutylammonium fluoride (1 mL, THF) was added to separate diastereomers of pyridylphosphonates **2** (**2-2R** or **2-2S**; 0.490 g, 0.478 mmol) in THF (4 mL). After 20 min the reaction mixtures were concentrated and partitioned between 10% aq. $NaHCO_3$ and CH_2Cl_2. The organic layers were dried over anh. Na_2SO_4, evaporated and the residues were purified by silica gel chromatography using a stepwise gradient of methanol (0–8%) in CH_2Cl_2 containing 0.01% TEA. Yields of isomeric pyridylphosphonates **3**: **3-2R** (from **2-2R**) 0.411 g (84%); white solid. Purity > 98% (^{1}H NMR spectroscopy). Anal. Calcd for $C_{46}H_{48}N_5O_{13}P$: C, 60.72; H, 5.32; N, 7.70. Found: C, 60.43; H 5.21; N 7.50. ^{31}P NMR: (δ in ppm $CDCl_3$) 11.46. ^{1}H NMR: (δ in ppm $CDCl_3$) 8.62 (d, 1H, J = 4.7 Hz, pyrH-6), 7.90 (m, 1H, pyrH-3), 7.77 (m, 1H, pyrH-4), 7.54 (s, 1H, H_a6), 7.43 (m, 1H, pyrH-5), 7.38 (s, 1H, H_b6) 7.33–7.13 (m, 9H, ArH), 6.81 (m, 4H, 4 × Ar-H ortho to CH_3O group), 6.48 (m, 1H, H_a1′), 6.30 (m, 1H, H_b1′), 5.31 (t, 1H, J = 6.0 Hz, H_a3′), 4.63 (m, 1H, H_b3′), 4.57–4.35 (m, 2H, H_b5′), 4.24 (s, 1H, H_a4′), 4.17 (m, 1H, H_b4′), 3.79 (s, 6H, 2 × CH_3O), 3.44–3.18 (m, 2H, H_a5′), 2.81–2.74 (m, 2H, H_a2′), 2.45–2.23 (m, 2H, H_b2′), 1.72 (s, 3H, C_b5-CH_3), 1.42 (s, 3H, C_a5-CH_3).

3-2S (from **2-2S**) 0.406 g (83%); white solid. Purity > 98% (^{1}H NMR spectroscopy). Anal. Calcd for $C_{46}H_{48}N_5O_{13}P$: C, 60.72; H, 5.32; N, 7.70. Found: C, 60.49; H 5.28; N 7.53. ^{31}P NMR: (δ $CDCl_3$) 10.75 ppm. ^{1}H NMR: (δ in ppm $CDCl_3$) 8.72 (d, 1H, J = 4.7 Hz, pyrH-6), 7.96 (t, 1H, J = 7.3 Hz, pyrH-3), 7.83 (m, 1H, pyrH-4), 7.55 (s, 1H, H_a6), 7.48 (m, 1H, pyrH-5), 7.32–7.17 (m, 10H, ArH, H_b6), 6.81 (d, 4H, J = 8.1 Hz, 4 × Ar-H ortho to CH_3O group), 6.44 (m, 1H, H_a1′), 6.21 (t, 1H, J = 6.4 Hz, H_b1′), 5.28 (t, 1H, J = 5.6 Hz, H_a3′), 4.55 (m, 1H, H_b3′), 4.39 (s, 1H, H_a4′), 4.39–4.25 (m, 2H, H_b5′), 4.04 (m, 1H, H_b4′), 3.77 (s, 6H, 2xCH_3O), 3.50–3.38 (m, 2H, H_a5′), 2.61–2.30 (m, 2H, H_a2′), 2.40–2.14 (m, 2H, H_b2′), 1.79 (s, 3H, C_b5-CH_3), 1.38 (s, 3H, C_a5-CH_3).

Preparation of 5′-O-dimethoxytritylthymidin-3′-yl thymidin-5′-yl 3-pyridylphosphonates 3-3R and 3-3S. Separate diastereomers of dinucleoside H-phosphonate **1** (**1R** or **1-S**; 0.900 g, 0.950 mmol), $Pd(PPh_3)_4$ (0.220 g, 0.190 mmol), triethylamine (0.158 mL, 1.14 mmol) and 3-bromopyridine (0.094 mL, 0.96 mmol) in freshly distilled and degassed THF (20 mL) were refluxed under an atmosphere of argon. After 5 h the reaction mixtures were concentrated and partitioned between 10% aq. $NaHCO_3$ and CH_2Cl_2. The organic layers were dried over anh. Na_2SO_4, evaporated and purified by silica gel chromatography using a stepwise gradient of methanol (0–4%) in toluene–ethyl acetate (1 : 1, v/v) containing 0.01% TEA.

2-3R (from **1-R**), 0.780 g (80%); white solid. δ_P ($CDCl_3$) 16.75 ppm.

2-3S (from **1-S**), 0.700 g (72%); white solid. δ_P ($CDCl_3$) 17.35 ppm.

Fully protected 3-pyridylphosphonates **2** (**2-3R** or **2-3S**; 0.490 g, 0.478 mmol) in THF (4 mL) were treated separately with 1 M solution of tetrabutylammonium fluoride (1 mL, THF) for 20 min. Further work-up and purification as described above for the 2-pyridylphosphonate derivatives.

3-3R (from **2-3R**) 0.410 g (84%); white solid. Purity > 98% (^{1}H NMR spectroscopy). Anal. Calcd for $C_{46}H_{48}N_5O_{13}P$: C, 60.72; H, 5.32; N, 7.70. Found: C, 60.53; H 5.20; N 7.44%. ^{31}P NMR: (δ $CDCl_3$) 16.65 ppm. ^{1}H NMR: (δ in ppm $CDCl_3$) 10.18, 9.78 (2s, 2H, 2 × NH), 8.95 (d, 1H, J = 5.8 Hz, pyrH-2), 8.79 (m, 1H, pyrH-6), 8.03 (m, 1H, pyrH-4), 7.52 (s, 1H, H_a6), 7.36 (m, 1H, pyrH-5), 7.31–7.16 (m, 10H, ArH, H_b6), 6.80 (d, 4H, J = 9.1 Hz, 4 × Ar-H ortho to CH_3O group), 6.46 (m, 1H, H_a1′), 6.20 (m, 1H, H_b1′), 5.21 (m, 1H, H_a3′), 4.58 (m, 1H, H_b3′), 4.42–4.33 (m, 2H, H_b5′), 4.16 (m, 1H, H_a4′), 4.13 (s, 1H, H_b4′), 3.78 (s, 6H, 2 × CH_3O), 3.42–3.17 (m, 2H, H_a5′), 2.86–2.41 (m, 2H, H_a2′), 2.45–2.16 (m, 2H, H_b2′), 1.74 (s, 3H, C_b5-CH_3), 1.43 (s, 3H, C_a5-CH_3).

3-3S (from **2-3S**) 0.410 g (84%); white solid. Purity > 98% (^{1}H NMR spectroscopy). Anal. Calcd for $C_{46}H_{48}N_5O_{13}P$: C, 60.72; H, 5.32; N, 7.70. Found: C, 60.37; H 5.19; N 7.41%.

^{31}P NMR: (δ $CDCl_3$) 17.28 ppm. ^{1}H NMR: 9.65, 9.60 (2s, 2H, 2 × NH), 8.99 (d, 1H, J = 5.6 Hz, pyrH-2), 8.82 (m, 1H, pyrH-6), 8.07 (m, 1H, pyrH-4), 7.52 (s, 1H, H_a6), 7.43 (m, 1H, pyrH-5), 7.35–7.20 (m, 9H, ArH), 7.08 (s, 1H, H_b6), 6.82 (d, 4H, J = 7.7 Hz, 4 × Ar-H ortho to CH_3O group), 6.38 (m, 1H, H_a1'), 6.12 (m, 1H, H_b1'), 5.17 (m, 1H, H_a3'), 4.39 (m, 2H, H_b3', H_a4'), 4.23 (m, 2H, H_b5'), 4.02 (m, 1H, H_b4'), 4.13, 3.78 (s, 6H, 2xCH_3O), 3.53–3.40 (m, 2H, H_a5'), 2.52–2.31 (m, 2H, H_a2'), 2.37–2.09 (m, 2H, H_b2'), 1.75 (s, 3H, C_b5-CH_3), 1.42 (s, 3H, C_a5-CH_3).

Preparation of 5′-O-dimethoxytritylthymidin-3′-yl thymidin-5′-yl 4-pyridylphosphonates 3-4R and 3-4S. Separate diastereomers of dinucleoside H-phosphonate **1** (**1R** or **1-S**; 0.900 g, 0.950 mmol) in pyridine (30 mL) were treated with trityl chloride (0.318 g, 1.14 mmol) and DBU (0.342 mL, 1.14 mmol) for 15 min, followed by the addition of iodine (0.482 g, 1.90 mmol). After 5 min the reaction mixtures were concentrated and partitioned between sat. aq. $NaHCO_3$-sat. aq. $Na_2S_2O_3$ (1 : 1, v/v; 30 mL)) and CH_2Cl_2 (50 mL). The organic layers were dried over anh. Na_2SO_4, evaporated and the residues were purified by silica gel chromatography using a stepwise gradient of methanol (0–4%) in toluene-ethyl acetate (1 : 1, v/v) containing 0.01% TEA.

2-4R (from **1-R**) 0.734 g (75%); white solid. δ_P ($CDCl_3$) 15.25 ppm.

2-4S (from **1-S**) 0.642 g (66%); white solid. δ_P ($CDCl_3$) 16.09 ppm.

Fully protected 4-pyridylphosphonates **2** (**2-4R** or **2-4S**; 0.490 g, 0.478 mmol) in THF (4 mL) were treated separately with 1 M solution of tetrabutylammonium fluoride (1 mL, THF) for 20 min. Further work-up and purification as described above for the 2-pyridylphosphonate derivatives.

3-4R (from **2-4R**) 0.404 g (83%); white solid. Purity >98% (^{1}H NMR spectroscopy). Anal. Calcd for $C_{46}H_{48}N_5O_{13}P$: C, 60.72; H, 5.32; N, 7.70. Found: C, 60.79; H 5.41; N 7.78%. ^{31}P NMR: (δ $CDCl_3$) 15.43 ppm. ^{1}H NMR: (δ in ppm $CDCl_3$) 10.34, 10.06 (2s, 2H, 2 × NH), 8.73 (m, 2H, pyrH-2/6), 7.58 (m, 2H, pyrH-3/5), 7.51 (s, 1H, H_a6), 7.30–7.16 (m, 10H, ArH, H_b6), 6.79 (m, 4H, 4 × Ar-H ortho to CH_3O group), 6.44 (m, 1H, H_a1'), 6.21 (t, 1H, J = 6.4 Hz, H_b1'), 5.21 (m, 1H, H_a3'), 4.54 (m, 1H, H_b3'), 4.44–4.31 (m, 2H, H_b5'), 4.16 (m, 1H, H_a4'), 4.10 (s, 1H, H_b4'), 3.76 (s, 6H, 2 × CH_3O), 3.41–3.16 (m, 2H, H_a5'), 2.82–2.40 (m, 2H, H_a2'), 2.48–2.18 (m, 2H, H_b2'), 1.74 (s, 3H, C_b5-CH_3), 1.43 (s, 3H, C_a5-CH_3).

3-4S (from **2-4S**) 0.431 g (88%); white solid. Purity >98% (^{1}H NMR spectroscopy). Anal. Calcd for $C_{46}H_{48}N_5O_{13}P$: C, 60.72; H, 5.32; N, 7.70. Found: C, 60.84; H 5.38; N 7.81%. ^{31}P NMR: (δ $CDCl_3$) 15.54 ppm. ^{1}H NMR: 9.73, 9.67 (2s, 2H, 2 × NH), 8.78 (m, 2H, pyrH-2/6), 7.63 (dd, 2H, J = 5.5 Hz, $^3J_{PH}$ = 13.7 Hz, pyrH-3/5), 7.51 (s, 1H, H_a6), 7.35–7.21 (m, 9H, ArH), 7.09 (s, 1H, H_b6), 6.82 (d, 4H, J = 8.8 Hz, 4 × Ar-H ortho to CH_3O group), 6.38 (m, 1H, H_a1'), 6.12 (m, 1H, H_b1'), 5.16 (m, 1H, H_a3'), 4.38 (m, 2H, H_b3', H_a4'), 4.23 (m, 2H, H_b5'), 4.04 (m, 1H, H_b4'), 3.77 (s, 6H, 2 × CH_3O), 3.53–3.39 (m, 2H, H_a5'), 2.49–2.32 (m, 2H, H_a2'), 2.39–2.07 (m, 2H, H_b2'), 1.77 (s, 3H, C_b5-CH_3), 1.42 (s, 3H, C_a5-CH_3).

Preparation of phosphoramidite building blocks 4. 2-Cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite (29.6 μL, 0.15 mmol) was added under argon to a solution of dimer **3** (91 mg, 0.1 mmol) and ethyl-*N*,*N*-diisopropylamine (52 μL, 0.3 mmol) in freshly distilled THF (3 mL). The reaction mixture was stirred overnight at room temperature and its progress was monitored by means of TLC (9 : 1, $CHCl_3$: MeOH). When no further increase in the concentration of product **4** was observed (12–16 h), the reaction mixture was applied under argon on a silica gel column. The product was eluted with hexane–ethyl acetate (1 : 1, v/v) and then with gradient of methanol (up to 3%) in chloroform. Fractions containing pure phosphoramidites **4** were concentrated *in vacuo* and stored as foams under argon at −20 °C.

4-2R: yield 60%, R_f = 0.60; ^{31}P NMR (δ in ppm): 12.0, 149.5, 149.9; LSIMS: 1108 $[M-H]^-$, 1109.0 $[M+H]^+$, 1132.5 $[M+Na]^+$; **4-2S**: yield 59%, R_f = 0.53; ^{31}P NMR (δ in ppm): 12.1, 149.6, 150.1; LSIMS: 1109.0 $[M+H]^+$; **4-3R:** yield 48%, R_f = 0.62; ^{31}P NMR (δ in ppm): 17.7, 17.8, 150.0, 150.2; LSIMS: 1109.0 $[M+H]^+$; **4-3S**: yield 45%, R_f = 0.57; ^{31}P NMR (δ in ppm): 17.9, 18.1, 149.5, 149.9; LSIMS: 1109.0 $[M+H]^+$; **4-4R**: yield 63%, R_f = 0.65; ^{31}P NMR (δ in ppm): 15.8, 15.9, 149.6, 149.7; LSIMS: 1108.5 $[M-H]^-$, 1132.5 $[M+Na]^+$; **4-4S**: yield 55%, R_f = 0.58. ^{31}P NMR (δ in ppm): 16.3, 16.5, 149.7; 149.9; LSIMS: 1109.0 $[M+H]^+$;

Oligonucleotide synthesis

The 3′-*O*-phosphoramidite building units **4** were used for the synthesis of modified oligomers **5** and **6** by automated solid phase methodology.[38] The 1 μmole scale synthesis of oligomers was performed on an ABI 394 synthesizer (Applied Biosystems Inc., Foster City, CA) using succinyl-linked LCA-CPG solid support. The only difference in the manufacturer's recommended protocol was a prolonged coupling time (up to 90 s). The coupling efficiency of **4** was determined by DMT-ion assay. The 5′-terminal DMT group was left at the oligonucleotide. Oligomers **5-2R**, **5-2S**, **5-3R**, **5-3S**, **6-2R**, **6-2S**, **6-3R** and **6-3S** were cleaved from the solid support by treatment with 30% ammonium hydroxide (1 mL) for 1 h at room temperature. Oligomers **5-4R**, **5-4S**, **6-4R** and **6-4S** were synthesized analogously on oxalyl-linked LCA-CPG solid support.[40] These oligomers were removed from the solid support by treatment with 1% aqueous triethylamine for 30 min at room temperature. The 5′-O-DMT protected oligomers **5** and **6** were purified by a standard RP HPLC method. The removal of 5′-DMT group was achieved by treatment with 50% acetic acid for 30 min at room temperature, followed by the RP HPLC purification on PRP-1 Hamilton column (305 × 7 mm) to produce fully deprotected oligonucleotides **5** and **6** in 15–80% yield. The structure and purity of oligomers were confirmed by MALDI-TOF mass spectrometry, 20% polyacrylamide/7 M urea gel electrophoresis (PAGE), and RP HPLC analysis. Reverse phase HPLC was performed on a Gilson apparatus (Middleton, WI, USA) with ThermoQuest Hypersil ODS 5 μ column (250 × 4.6 mm) in 0.1 M triethylamine bicarbonate pH 7.5/acetonitrile buffer system.

Melting temperature measurements

Samples for the melting temperature measurements (duplexes or triplexes) were prepared by hybridization of modified oligomers with the dA_{19}, A_{19} or $d(A_{21}C_4T_{21})$ hairpin oligonucleotide at a concentration 1×10^{-6} M of each oligomer in 10 mM Tris-HCl, pH 7.4, 100 mM NaCl and 5 mM $MgCl_2$ or in 10 mM Tris-HCl, pH 5.0, 100 mM NaCl and 5 mM $MgCl_2$. Melting profiles were recorded after heating to 70 °C followed by annealing to 5 °C with a temperature gradient of 0.5 °C min^{-1}. Oligonucleotides were kept for 5 min at 5 °C then heated at temperature gradient of 0.2 °C min^{-1} to 86 °C. The melting temperatures were calculated using the first order derivative method. Measurements were done on Cintra 40 instrument (GBC Australia).

Assay for digestion of oligo(thymidine pyridylphosphonate)s with 3′-exonuclease from human plasma

The samples of oligonucleotides **5** and the reference oligothymidylate T_{20} were radiolabeled at the 5′-end with [γ-^{32}P]ATP and T4 polynucleotide kinase according to standard procedure.[50] Samples of each oligonucleotides **5** (concentration 5 μM) were incubated in phosphate-buffered saline (PBS)

containing 50% human plasma. At specified time intervals (15, 30, 60, 120, 240 and 480 min) 10 μl aliquots were withdrawn, and the reaction was stopped by heating at 95 °C for 5 min. After addition of 100 μl of water the samples were vigorously shaken, the precipitated proteins were spun down, and the aqueous solutions were lyophilized in a SpeedVac rotary evaporator. The remaining pellet was dissolved in formamide containing 0.03% bromophenol blue and 0.03% xylene cyanol (10 μl) and analysed by 20% polyacrylamide/7 M urea gel electrophoresis. The radioactive products were visualized by autoradiography. Reference samples R_1 and R_2 containing the parent 5′-^{32}P-labeled oligonucleotides dissolved in PBS were incubated at 3 °C for 240 and 480 min, respectively.

Assay for digestion of oligo(thymidine pyridylphosphonate)s with snake venom phosphodiesterase (svPDE) or calf spleen phosphodiesterase (PDE II)

4 μl samples of oligonucleotides **5** (0.1 A_{260} in 100 μL of water) were mixed with svPDE (1 μL, 0.1 mU) or with PDE II (1 μL, 0.4 mU) and incubated at 37 °C. After 15, 45, 90 and 180 min 1 μL aliquots were withdrawn, mixed with 1 μL of the matrix [2,4,6-trihydroxyacetophenone (10 mg μL^{-1} in water–acetonitrile 1 : 1)–ammonium dicitrate (50 mg mL^{-1} in water), 8 : 1, v/v] and applied directly to the sample plate. After 10 min of drying/crystallization the samples were analysed by MALDI-TOF mass spectrometry using Voyager Elite instrument (PerSeptive Biosystems, USA).

CD spectra of duplexes 6 with dA_{19} and A_{19}

CD measurements of duplexes **6**/$\mathbf{dA}_{19}$ and **6**/$\mathbf{A}_{19}$ were performed on Jobin Yvon CD6 (France) apparatus. Samples of duplexes (1.46×10^{-6} M) were dissolved in 10 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$ buffer pH 7.4 (1 mL) and the spectra (200–320 nm) were recorded at 25 °C.

Acknowledgements

Financial support from The State Committee for Scientific Research in Poland (grant PBZ-KBN-059/T09/09), the Swedish Research Council and the Swedish Foundation for Strategic Research is gratefully acknowledged. We are grateful to Professor Wojciech J. Stec for his inspiration for this project and fruitful discussion.

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