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Oligodeoxynucleoside phosphoramidates (P-NH₂): synthesis and thermal stability of duplexes with DNA and RNA targets

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ABSTRACT

Syntheses of non ionic oligodeoxynucleoside phosphoramidates (P-NH₂) and mixed phosphoramidate–phosphodiester oligomers were accomplished on automated solid supported DNA synthesizer using both H-phosphonate and phosphoramidite chemistries, in combination with *t*-butylphenoxyacetyl for N-protection of nucleoside bases, an oxalyl anchored solid support and a final treatment with methanolic ammonia. Thermal stabilities of the hybrids formed between these new analogues and their DNA and RNA complementary strands were determined and compared with those of the corresponding unmodified oligonucleotides, as well as of the phosphorothioate and methylphosphonate derivatives. Dodecathymidines containing P-NH₂ links form less stable duplexes with DNA targets, d(C₂A₁₂C₂) (ΔT_m /modification -1.4°C) and poly dA (ΔT_m /modification -1.1°C) than the corresponding phosphodiester and methylphosphonate analogues, but the hybrids are slightly more stable than the one obtained with phosphorothioate derivative. The destabilization is more pronounced with poly rA as the target (ΔT_m /modification -3°C) and could be compared with that found with the dodecathymidine methylphosphonate. The modification is less destabilizing in an heteropolymer–RNA duplex (ΔT_m /modification -2°C). As expected, the P-NH₂ modifications are highly resistant towards the action of various nucleases. It is also demonstrated that an all P-NH₂ oligothymidine does not elicit *Escherichia coli* RNase H hydrolysis of the poly rA target but that the modification may be exploited in chimeric oligonucleotides combining P-NH₂ sections with a central phosphodiester section.

INTRODUCTION

The use of natural oligonucleotides as therapeutic agents in an antisense approach in which the target is RNA or DNA, suffers from drawbacks such as their inherent instability towards degradation by

extra and intra-cellular nucleases and their relatively poor cellular uptake (1). Attempts to produce nuclease resistant oligonucleotides and to improve their cellular uptake while keeping the specificity of binding to their targets, has resulted in the preparation of several phosphodiester backbone modified analogues (2,3). Of these, phosphorothioates (4) are the most widely applied derivatives of oligodeoxynucleotides for an antisense approach, but they remain somewhat susceptible to degradation by nucleases (5), are taken up by cells more slowly than phosphodiesters and present a strong tendency to bind with numerous proteins (6). Replacement of the negatively charged oxygen atom in the phosphodiester backbone by uncharged groups to produce non-ionic oligonucleotide analogues such as methylphosphonates (7) or phosphoramidates (8) would allow them to enter cells by an alternative mechanism and confer increased nuclease resistance. However, methylphosphonates suffer from the disadvantage of being relatively insoluble in water, aqueous buffers and biological media (9). Furthermore, these modifications induce chirality and the binding properties of the resulting diastereoisomers to a complementary target are clearly dependent on the orientation of the substituted groups around the phosphorus atom (9–11). Despite the effort directed towards the stereocontrolled synthesis of phosphorothioates (12) and methylphosphonates (13), mixtures of heterogeneous oligomers are largely used which lower the binding to the target (14). To bypass the chirality problem, a number of non-phosphate, neutral and achiral linkages have been prepared (15,16). However, the syntheses of such backbones are in general difficult and versatile. Reported preparations are not particularly suitable for automated synthesizers to the level at which all linkages are modified. The most frequently used strategy is to prepare a modified nucleoside dimer, to convert it in a phosphoramidite synthon and to incorporate it into an oligonucleotide sequence where modified linkages are alternated with normal phosphodiesters (15). In such oligomers, the polyanionic character is reduced, but not suppressed and these modifications do not necessarily provide increased cellular uptake as well as a total nuclease resistance. For that reason and although the automatic synthesis of non-ionic phosphoro oligonucleosides cannot be rendered stereospecific for the time being, these phosphoro analogues stay attractive because they can be prepared in a relatively

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straightforward manner by minor modifications to the existing automated DNA synthetic methods.

If it has been clearly demonstrated that the negative impact of stereoisomerism on hybridization is enhanced with bulky substituents (8). It is assumed that the influence of a modification on the stabilities of duplexes also results from the disturbance of the spin of hydration around the modified phosphate groups (8,17). The amides of phosphorus acid are known to have good solvating properties (18). Furthermore, in phosphoramidates (P-NH₂), the fact that the NH₂ group is small, uncharged in aqueous solution and forms hydrogen bonds readily (19), gives them favourable solubility characteristics required for hydration, with a minimum steric hindrance around the phosphorus atom, and makes them attractive potential candidates for non-anionic analogues of oligonucleotides. The syntheses of *N*-alkyl (8,20) and *N*-alkoxy (21) phosphoramidate derivatives of DNA have been described and their biophysical properties studied. Unfortunately, due to the reported decomposition of dithymidine phosphoramidate under the base conditions necessary for removal of the common heterocyclic *N*-acyl protecting groups and release of an oligonucleotide from the regular succinyl anchored solid support (20) (concentrated ammonia, 55°C, 5–16 h), syntheses of phosphoramidate (P-NH₂) oligonucleotides have been limited to a dimer level (19,20,22). However, recent developments in oligonucleotide chemistry on a more labile oxalyl anchored solid support (23) combined with the use of monomers possessing highly labile protecting groups (24), of mild and rapid deprotection conditions (25) have enabled us to synthesise these base-sensitive oligonucleotides. In this regard, the use of pent-4-enoyl (26) as nucleobase protective group (27) appears very promising, and recently allowed the synthesis of various dinucleosides containing a P-NH₂ linkage (28). We would like to report here, the first synthesis of partially or fully modified oligonucleotide phosphoramidates (P-NH₂) of length sufficient to explore the antisense potential of these analogues. The nuclease resistance properties of the modified oligonucleotides and the thermal stabilities of duplexes formed with their DNA or RNA complements have been evaluated and compared with those of unmodified oligonucleotide, as well as those of the phosphorothioate and methylphosphonate analogues.

MATERIALS AND METHODS

Oligonucleotide synthesis

Syntheses of the oligodeoxynucleotide analogues were carried out on 1 μmol scale using a 381A Applied Biosystems DNA-synthesizer. First, nucleoside was linked to LCAA-CPG (500 Å, from Sigma) by means of an oxalyl linker (23). Analogues combining phosphodiester and phosphoramidate internucleoside links were prepared by using both phosphoramidite and hydrogen phosphonate chemistries. After each coupling step using commercially available nucleoside-cyanoethyl phosphoramidites and appropriate reagents (from Millipore), the intermediate phosphite triesters were oxidized by a 1 min treatment with a 1.1 M solution of *t*-butylhydroperoxide in dichloromethane. H-phosphonate couplings were carried out using deoxynucleoside H-phosphonate derivatives (24) and standard reagents from Glen Research. At the end of the chain elongation, oxidative amidation was performed manually by treating CPG linked oligonucleotide with a saturated solution of ammonia in carbon tetrachloride/dioxan (4/1, v/v) for 30 min at 0°C (20). After filtration, the oligomers were released from the

solid support and deprotected with a treatment with saturated methanolic ammonia at 20°C over 2 h for phosphodiester/phosphoramidate oligomers **1**, **2**, **3** and **7**, and 5 min for the fully modified P-NH₂ oligomers **4** and **6** (Table 1).

HPLC methods

HPLC was performed on a Waters-Millipore instrument equipped with two M510 solvent delivery systems, a M680 solvent programmer, a U6K injector and a M990 diode array UV detector. Oligonucleotides were purified by reverse-phase HPLC on a Delta-pack preparative column (7.8 × 300 mm, C18, 5 μm, Millipore) using a gradient of acetonitrile from 10 to 25% in 0.05 M triethylammonium acetate buffer (pH 7) in 40 min at a flow rate of 2 ml/min. The desired fractions were combined, evaporated, dissolved in water and lyophilised. Purity of the samples was checked by HPLC at 260 nm on a Nucleosil analytical column (4.6 × 150 mm, C18, 5 μm, Macherey-Nagel) using a gradient of acetonitrile from 0 to 30% in 0.05 M triethylammonium acetate buffer (pH 7) in 30 min at a flow rate of 1 ml/min. Using this procedure, 7–20 A₂₆₀ units of purified oligomers were isolated in each case.

Mass spectrometry

Mass spectra were recorded on a SSQ 7000 quadrupole mass spectrometer (Finnigan MAT, San Jose, USA) fitted with an electrospray interface. Samples (final concentration 30 μM) were dissolved in water-methanol (v/v, 1:1) containing triethylamine (1.5%) and introduced into the mass spectrometer at a 10 μl/min flow-rate with a Harvard Apparatus 22 pump model. The negative ion electrospray mass spectra were transformed into real mass spectra, using the DEC-Finnigan software.

NMR measurements

³¹P-NMR spectra were recorded on a Brüker AC 250 spectrometer at 100 MHz. Samples were dissolved in D₂O. Chemical shifts values are in p.p.m. relative to external 85% H₃PO₄.

Formic acid mediated degradation of d(ACACCCAATTCT) analogues (29)

One A₂₆₀ unit of oligonucleotide was dissolved in 90% aqueous formic acid (1 ml) and the resulting solution was heated at 120°C for 12 h. After cooling, the solution was evaporated to dryness under reduced pressure, and the residue was dissolved in 0.1 M triethylammonium acetate buffer, pH 7 (0.2 ml) for HPLC analysis (Nucleosil analytical column, 4.6 × 150 mm, C18, 5 μm, Macherey-Nagel, eluent: 0.05 M triethylammonium acetate, pH 6.9, flow rate of 1 ml/min). The base composition of the oligonucleotides **6**, **7** and **8** was confirmed after applying this degradation, which cleanly liberated the purine and pyrimidine bases (Cyt, 3.03 min; Thy, 7.59 min; Ade, 17.63 min) and quantification of the peak areas at 260 nm, using an equimolar mixture of the three bases as standard.

Melting temperatures

Melting curves were recorded on a UVIKON 931 spectrophotometer (Kontron). The temperature control was through a HUBER PD 415 temperature programmer connected to a refrigerated water bath (Huber Ministat). Typical experiments were carried out with

equimolar ratio of modified oligomers and their targets in a 10 mM sodium cacodylate buffer (pH 7 or pH 5.45) containing 10 mM, 100 mM or 1 M sodium chloride. The samples were preheated at 75°C, and the change in absorbance at 260 nm as a function of the temperature was recorded. The cooling or heating rate was 0.5°C/min. The cell compartment was continuously flushed with dry nitrogen for temperatures below room temperature. T_m values were determined from the maxima of the first derivative plots of absorbance versus temperature.

Enzymatic hydrolysis experiments

Snake venom phosphodiesterase (SVPDE) (*Crotalus durissus*), Nuclease S1 (*Aspergillus oryzae*) and Calf spleen Phosphodiesterase II (CSPDE) were purchased from Boehringer. The oligothymidines (2 A₂₆₀ U) were incubated at 37°C in either: 10 µl 50 mM sodium acetate buffer (pH 4.5), 300 mM sodium chloride, 100 mM zinc acetate added with 70 µl of water containing 2 U nuclease S1; 100 µl of 100 mM Tris-HCl buffer (pH 9), 10 mM magnesium chloride added with 2 µl of the commercial solution of SVPDE (3 U/ml); or 80 µl of 125 mM ammonium acetate buffer (pH 6.8), 2.5 mM EDTA containing 2 µl of the commercial solution of CSPDE (0.5 U/ml). Aliquots were analysed using the analytical conditions described in HPLC methods.

RNase H digestion of poly rA/oligothymidine analogues duplexes

Escherichia coli RNase H was purchased from Pharmacia. These experiments were performed with oligothymidylate analogues and poly rA (240 µM in nucleotide concentration for each strand) in 10 mM Tris-HCl buffer (pH 7), 10 mM magnesium chloride and 100 mM sodium chloride (500 µl). The samples were preheated at 70°C, allowed to slowly cool down to 0°C, then the temperature was stabilised at 20°C before adding the enzyme (5 U). Digestion curves were obtained by plotting the absorbance at 260 nm as a function of time.

RESULTS AND DISCUSSION

Preparation of oligonucleosides containing phosphoramidate (P-NH₂) linkages

Standard synthesis of oligonucleotides anchored by a succinyl linker to controlled pore glass (CPG) support, requires an ultimate chemical step with concentrated ammonium hydroxide at 55°C for 5–16 h to release the oligonucleotides from the solid support and completely remove nucleobase and phosphodiester protecting groups. Although a number of *N*-alkylphosphoramidate oligonucleotide derivatives (8) have been prepared using this current methodology, the synthesis of less hindered P-NH₂ analogues has been limited to the preparation of dinucleotides (19,20,22) or trinucleotide (28). This is due to the fact that the P-NH₂ linkage could not survive the drastic ammonia treatment. Indeed, it has been shown that a dithymidine phosphoramidate (P-NH₂) was rapidly hydrolysed under such alkaline treatment ($t_{1/2}$ 15 min) (20) affording a mixture of thymidine and corresponding 3'- and 5'-phosphoramidic acid monoesters (22,30). Fortunately, we found that a treatment with saturated methanolic ammonia gave appreciably less degradation of this dimer ($t_{1/2}$ 3.8 days). This mild

treatment has been used to deprotect base-sensitive oligonucleotides such as RNA and methylphosphonates (24) as well as oligonucleoside *N*-alkoxyphosphoramidates (21). Under these conditions, the release of an oligonucleotide from an oxalyl anchored solid support is quantitative after 5 min at room temperature (23), and the complete removal of cyanoethyl phosphate protecting groups is achieved within 2 h at room temperature (21,24). We estimated that <1.5% of a P-NH₂ linkage will be degraded (after 2 h treatment with methanolic ammonia) on an oligonucleotide containing both P-NH₂ and P-O⁻ (initially protected with cyanoethyl) linkages, which is quite acceptable.

To examine the effect of the modification on binding capacities, several analogues of dodecathymidylate 5 were prepared (Table 1). Oligomer 1 was synthesized with one modification placed in the middle of the sequence. In compound 2, modified linkages were alternated with normal phosphodiesters, in such a way that the oligomer had six phosphoramidate and five phosphodiester internucleoside bonds. In the oligothymidine 3, an internal part of five adjacent phosphodiesters was surrounded by two terminal sections of three contiguous phosphoramidates. In dodecathymidine 4, natural phosphodiester linkages were completely replaced by phosphoramidate ones. In addition, two analogues of the mixed-base dodecanucleoside 8 complementary to the splice acceptor site of mRNA coding for HIV-1 tat protein (31) were synthesized (Table 1). In the oligomer 7, as for compound 3, an internal part of five adjacent phosphodiesters was surrounded by two terminal sections of three contiguous phosphoramidates. Finally, oligonucleotide 6 was fully modified. In order to obtain phosphodiester and phosphoramidate linkages in the same oligomer, cyanoethyl phosphoramidite and H-phosphonate chemistries were respectively employed. As it has been demonstrated that *t*-butylhydroperoxide is able to selectively oxidize phosphite triester intermediates into phosphate triesters without affecting H-phosphonate linkages (32), this reagent was used instead of the common iodine treatment after each phosphoramidite coupling step. Then, the H-phosphonate diesters were oxidized into phosphoramidate linkages, by treatment with a saturated solution of ammonia in dioxan/CCl₄ (20). As classical amino protecting groups are routinely removed under drastic conditions (concentrated aqueous ammonia, 5–16 h at 55°C), *N*-*t*-butylphenoxyacetyl protected nucleoside phosphoramidites and H-phosphonates (24) were employed during the synthesis of heteropolymers 6 and 7, in combination with *t*-butylphenoxyacetic anhydride as capping reagent. In our hands and in contrast to what has been reported in the literature (24), the coupling efficiency of the H-phosphonate salts of deoxyadenosine and deoxycytidine was much lower (<90%) than the one of the corresponding phosphoramidites (>98%) and may account for the low yields of purified compounds (Table 2). Finally, the oligonucleotides were deprotected and removed from the solid support by treatment with saturated methanolic ammonia at room temperature (2 h for oligomers 1, 2, 3 and 7; 5 min for compounds 4 and 6).

The chromatograms of 3 and 4 (Fig. 1A) are representative analyses of the products released from the solid support after the ammonia treatment. The oligonucleotides were purified by reverse phase HPLC. Figure 1B depicts the chromatograms of compounds 3 and 4 obtained after purification. Owing to the absence of charge on phosphoramidate linkages, retention times of those analogues on reverse phase HPLC increase with the number of modifications (Table 2).

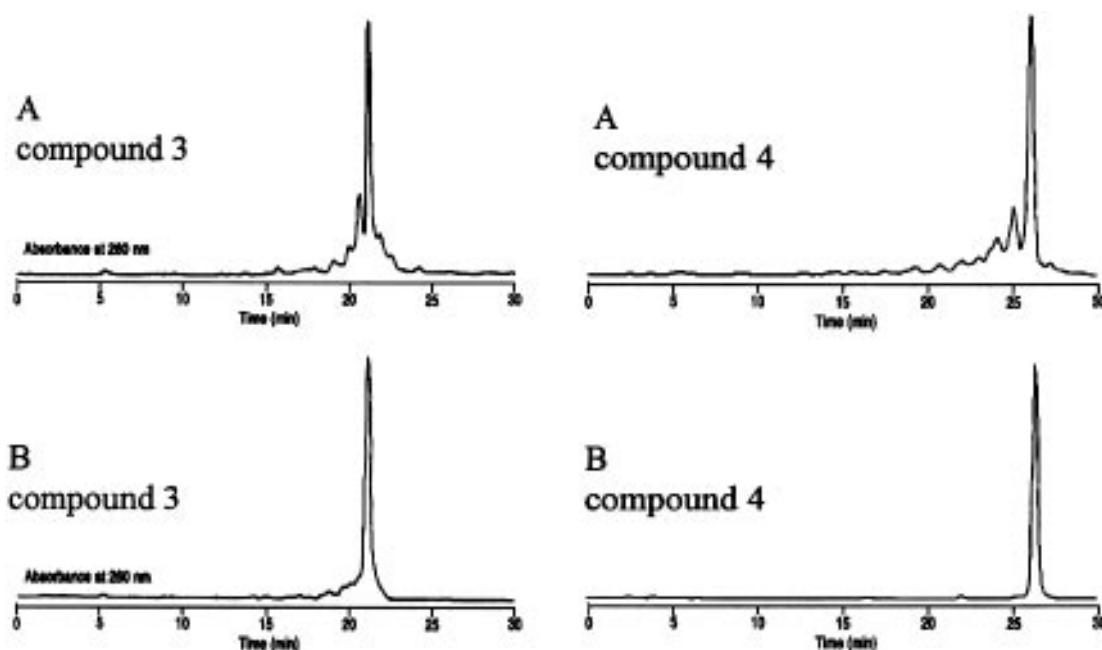


Figure 1. HPLC profiles at 260 nm of **3** and **4**. (A) Crude product mixtures recovered from solid support. (B) After purification of products obtained from (A).

Table 1. Oligonucleotides synthesized

Oligonucleotide sequences	
1	(dTp) ₅ dTp _n (dTp) ₅ dT
2	(dTp _n dTp) ₅ dTp _n dT
3	(dTp _n) ₃ (dTp) ₅ (dTp _n) ₃ dT
4	(dTp _n) ₁₁ dT
5	(dTp) ₁₁ dT
6	d-Ap _n Cp _n Ap _n Cp _n Cp _n Cp _n Ap _n Ap _n Tp _n Tp _n Cp _n T
7	d-Ap _n Cp _n Ap _n Cp _n Cp _n Ap _n Ap _n Tp _n Tp _n Cp _n T
8	d-ApCpApCpCpCpApApTpTpCpT
Targets	
9	d-CpCpApApApApApApApApApApApCpC d-ApGpApApTpTpGpGpTpGpT r-ApGpApApUpUpGpGpGpUpGpU

p refers to a phosphodiester internucleoside link, and pn to a phosphoramidate linkage.

³¹P-NMR spectroscopy was used for the characterization of the modified oligonucleotides (Table 2). The spectrum of the alternating oligomer **2** (Fig. 2) consists of two sets of peaks in a ~5:6 ratio; one set due to the phosphodiester resonances at -0.5 p.p.m. and a downfield set at 12.7 p.p.m. in agreement with the literature data concerning the phosphoramidate resonances (22,30). Similar ³¹P-NMR spectra were obtained for **1**, **3** and **7**, with the ratio of the integration for peaks at -0.5 and 12.7 p.p.m. varying according to the expected ratio of P-O⁻ to P-NH₂ internucleoside bonds and in agreement with electrospray mass spectrometry data (Table 2). NMR resonances at 12.7 p.p.m. were observed for compounds **4** and **6**. These spectra provide evidence that the phosphoramidate P-NH₂

linkages survived ultimate treatment (saturated methanolic ammonia at room temperature for 2 h). In order to further confirm the integrity of the phosphoramidate linkages, dodecathymidine analogues **1**, **2**, **3** and **4** were quantitatively hydrolysed to phosphodiester by a formic acid treatment at 90°C for 5 min (8) giving rise to (dTp)₁₁dT, as ascertained by HPLC in comparison with an authentic sample (Table 2). The base composition of the oligonucleotides **6**, **7** and **8** was established by a formic acid degradation (29), which cleanly released adenine, thymine and cytosine in a 4:3:5 ratio as determined by HPLC.

Table 2. Oligonucleoside phosphoramidates physical data^a

Oligomer	R _T , min	δ ³¹ P-NMR, p.p.m.	Calculated mass	Observed mass	Isolated yield ^b
5	17.51				
1	18.29	12.65, -0.27	3587.4	3587.6	60%
2	21.18	12.67, -0.53			18%
3	21.91	12.64, -0.46	3582.5	3582.0	12%
4	26.24	12.66			19%
8	22.90				
7	24.74	12.73, -0.53			6%
6	28.84	12.73			5%

^aSee Materials and Methods.

^bCalculated from support bound nucleoside.

Base-pairing of oligonucleotide analogues with DNA and RNA complementary strands

Interactions between dodecathymidylate analogues with **9**, poly dA and poly rA were investigated by both thermal denaturation

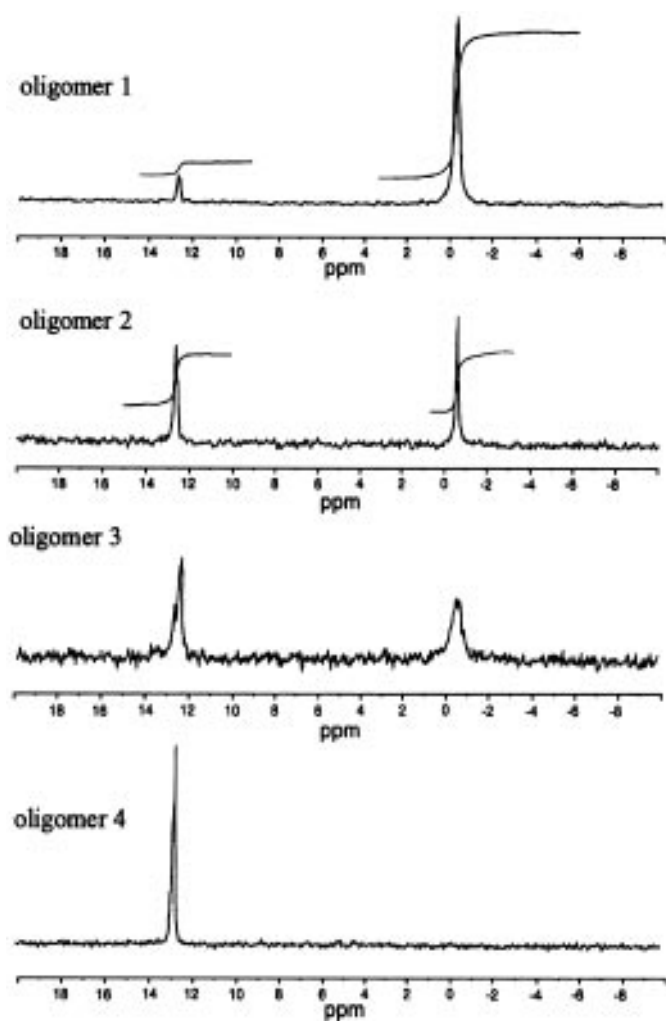


Figure 2. ^{31}P -NMR spectra of oligomers 1, 2, 3 and 4 in D_2O .

and renaturation analyses using changes in absorbance at 260 nm versus temperature. The experiments were carried out at an equal nucleotide concentration ($60\ \mu\text{M}$) of the thymidylate analogues and the target compounds. At the end of the experiment, a sample was examined by HPLC to confirm that no significant degradation of the modified oligonucleotide had occurred. Melting temperatures are presented in Table 3. No differences were observed between thermal association and dissociation curves. It can be seen from these results that the introduction of phosphoramidate internucleoside linkages into the dodecathymidylate affects the thermal stability of the duplexes formed with either DNA or RNA complementary strands. Thus, incorporation of one, six and eleven modifications leads to a progressive decrease in the melting temperature of the complexes compared to the 'parent' unmodified duplexes. T_m value is almost a linear function of the number of modifications. The average destabilisation is 1.4°C and 1.1°C per modification in duplexes obtained with **9** and poly dA, respectively. As generally observed for oligo (dT) and its analogues (32), the affinity for duplex formation with poly rA is lower than that observed with poly dA. However, in this case, the

difference of stability between the oligothymidine–poly dA and the oligothymidine–poly rA hybrids is enhanced (1.8°C) when compared with natural complexes (0.2°C). Moreover, the average decrease in T_m of $\sim 2.9^\circ\text{C}/\text{modification}$ compared with $(\text{dTp})_{11}\text{dT}$ –poly rA hybrid, did not allow us to observe the binding of the uniformly modified $(\text{dTpn})_{11}\text{dT}$ with poly rA, indicating an appreciable distortion of the structure of the modified oligothymidine–RNA duplex compared with the 'parent' duplex. Nevertheless, at a four times higher nucleotide concentration ($240\ \mu\text{M}$), T_m values of 16°C and of 38°C were observed for the modified, **4**, and the natural hybrids ($\Delta T_m/\text{mod} = -2^\circ\text{C}$), respectively. So, even if the modification is really destabilizing, one can admit that it does not compromise Watson–Crick base pairing with a ribonucleotide complementary strand. Furthermore, the T_m value (13.3°C) of the duplex formed between oligonucleotide **3** and poly rA supports this conclusion. Indeed, this T_m value cannot be attributed only to the binding with poly rA of the internal non-modified section $(\text{dTp})_5\text{dT}$ of the oligomer **3** under the same conditions, no transition above 0°C was observed with $(\text{dTp})_5\text{dT}$ and poly rA.

Table 3. Thermal stabilities ($^\circ\text{C}$) of duplexes formed by oligonucleoside phosphoramidates^a

Oligomer	Complementary strand					
	9		poly dA		poly rA	
	T_m	$\Delta T_m/\text{mod}$	T_m	$\Delta T_m/\text{mod}$	T_m	$\Delta T_m/\text{mod}$
5	27.0	–	31.5	–	29.5	–
1	25.2	–1.8		nd	26.4	–3.1
2	17.7	–1.6	24.2	–1.2	11.3	–3.0
3	18.9	–1.4		nd	13.3 ^b	–2.7
4	13.0	–1.3	20.5	–1.0	$<0^\circ\text{C}$	<-2.7
average $\Delta T_m/\text{mod}$		–1.4		–1.1		–2.9

^aExperiments were carried out in a buffer (pH 7) containing 10 mM sodium cacodylate, 0.1 M sodium chloride, at $60\ \mu\text{M}$ nucleotide concentration in each strand.

^bIn the same experimental conditions, no transition was observed for $(\text{dTp})_5\text{dT}$ with poly rA ($T_m < 0^\circ\text{C}$).

^cIn 10 mM Tris–HCl, 10 mM MgCl_2 , 0.1 M NaCl buffer solution (pH 7) at a nucleotide concentration of $240\ \mu\text{M}$, a T_m value of 16°C was observed for fully modified oligomer **4** with poly rA target and a T_m value of 38°C was found for the natural duplex.

Experiments at different NaCl concentrations (Table 4) show that the T_m value for the hybrid involving the fully modified oligonucleotide $(\text{dTpn})_{11}\text{dT}$ **4** and **9** is weakly modified by changes in the ionic strength of the medium either at pH 7 or 5.45. This behaviour most likely reflects the absence of charge repulsion between a non-ionic oligonucleotide and the negatively charged phosphodiester backbone of the target strand as observed for other neutral backbone analogues (8). Consequently, the modification P– NH_2 is not protonated between pH 5.45 and 7. This result is in accord with the known low basicity of phosphoric amide diesters (18,33,34). In contrast, the stability of the 'parent' duplex $(\text{dTp})_{11}\text{dT}$ –**9** increases with increasing NaCl concentration as expected. Thus, under low salt conditions (10 mM NaCl), the modified duplex is as stable as the 'parent' one (Table 4).

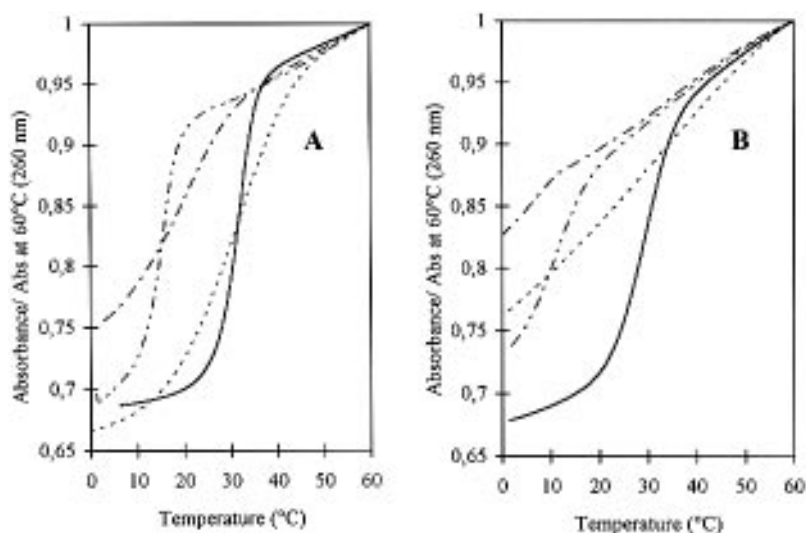


Figure 3. Thermal dissociation curves of the hybrids formed with uniformly modified dodecathymidylate analogues and (A) poly dA, (B) poly rA. (—), all phosphodiester; (.....), all methylphosphonate; (· · — · ·), all phosphorothioate; (— — —), all phosphoramidate. Experimental conditions, as in Table 5.

Table 4. Salt concentration dependence of the T_m ($^{\circ}\text{C}$) of the fully modified phosphoramidate oligomer with **9** compared with the natural duplex

Oligomer		NaCl concentration		
		0.01 M	0.1 M	1 M
5	pH 7	15	27	42
4	pH 7	13	13	16
4	pH 5.45	14	14	16

Experiments were carried out in a 10 mM sodium cacodylate buffer, at 60 μM nucleotide concentration for each strand.

The affinity of the phosphoramidate oligomer **4** was also compared with that of two other backbone modified dodecathymidines: a phosphorothioate and a methylphosphonate analogues. Data on the interactions with DNA and RNA targets are reported in Table 5 and some typical dissociation curves are shown in Figure 3. It appears, when considering duplexes formed with single stranded DNA, that phosphoramidate linkage is slightly less destabilizing ($\Delta T_m/\text{mod} = -1.3^{\circ}\text{C}$ with **9** and -1°C

with poly dA) than the phosphorothioate modification ($\Delta T_m/\text{mod} = -1.5^{\circ}\text{C}$). Comparatively, the introduction of methylphosphonate linkages into the oligodeoxyribonucleotide does not perturb its ability to bind to the DNA target. However, Figure 3 clearly shows broader melting transitions for neutral methylphosphonate and phosphoramidate analogues than for ionic phosphorothioate and phosphodiester oligomers. These broad curves are most likely due to significant differences in the binding properties of the diastereoisomers within these oligomers (35). In contrast to the behaviour of the neutral phosphoramidate and methylphosphonate oligomers with DNA targets, duplex formation between these oligonucleotides and the RNA target was not detected under the conditions used for these experiments (Fig. 3). Only the upper portion of an apparent melting transition was observed with the phosphoramidate analogue, and the transition with the methylphosphonate compound was so broad that it was not possible to ascertain unambiguously that the oligomer hybridized to the poly rA target. Finally, the ionic phosphorothioate modification was the less destabilizing of the studied modifications ($\Delta T_m/\text{mod} = -1.7^{\circ}\text{C}$) when considering duplexes formed with the ribonucleotide target.

Table 5. Comparison of hybridization properties^a of several uniformly modified dodecathymidylates with **9**, poly dA and poly rA

Modification at phosphorus	Complementary strand					
	9		poly dA		poly rA	
	T_m	$\Delta T_m/\text{mod}$	T_m	$\Delta T_m/\text{mod}$	T_m	$\Delta T_m/\text{mod}$
phosphodiester 5	27	—	31.5	—	29.5	—
phosphorothioate	10.6	-1.5	15.6	1.5	10.4	-1.7
methylphosphonate	27	0	30.5	-0.1	<0 ^b	<-2.7
phosphoramidate 4	13	-1.3	20.5	-1.0	<0	<-2.7

^aExperiments were carried out in a buffer (pH 7) containing 10 mM sodium cacodylate and 0.1 M sodium chloride, at 60 μM nucleotide concentration for each strand.

^bNo evidence for hybridization from the melting curve data (Fig. 3).

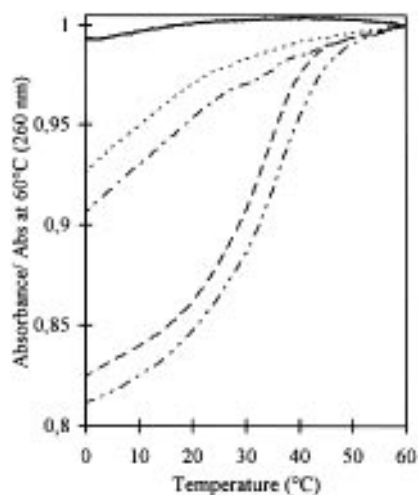


Figure 4. Thermal dissociation curves of the hybrids formed with modified heteropolymers **6** and **7** with DNA or RNA complementary strands. (—), **6** alone; (.....), **6** with ribonucleotide target; (·-·-·), **6** with deoxyribonucleotide target; (- - -), **7** with ribonucleotide target; (·-·-·), **7** with deoxyribonucleotide target.

Although the results on the hybridization of the oligothymidine phosphoramidate with poly rA seem disappointing, they deserve comment. First, we have shown that even if the modified oligomer does not form detectable duplexes with the RNA target at 60 μ M nucleotide concentration for each strand, it does at higher concentration. In addition, it had been shown recently (35) that a methylphosphonate oligomer linked to a psoralen moiety, which was not able to bind to its target under melting experiment conditions, was effective in cross linking the same target at higher concentration. Secondly, the observed behaviour of the oligonucleotide analogues results from the AT base pairing and could be different with more favourable GC base pairs, as was demonstrated with phosphorothioate analogues (36). Nevertheless, T_m experiments performed with the heteropolymers **6** and **7** (Fig. 4) showed that only the oligomer **7** containing a central section of phosphodiester was able to hybridize above 0°C with its DNA (36.5°C) or RNA (34°C) complementary strand. T_m values were however $\sim 10^\circ\text{C}$ lower than those of the phosphodiester parent duplexes (47.6 and 46.1°C, respectively, with DNA and RNA targets) (31). From this experiment, it can be pointed out that the difference in stability between duplexes with a deoxyribonucleotide and a ribonucleotide targets is less pronounced with a modified dodecamer of mixed-base sequence ($\Delta T_m/\text{mod} = -2^\circ\text{C}$ in both cases) than it was for dodecathymidine analogues. With the fully modified oligonucleoside **6**, only the upper portions of the melting transitions were observed with both DNA and RNA targets. The low binding affinity of these analogues for their RNA target may be exploited in antisense technology. Indeed, it has been shown that chimeric oligonucleotides combining methylphosphonate (37,38) or phosphoramidate (39,40) portions with a central phosphodiester section considerably reduce undesired RNase H cleavage at RNA sites of partial complementarity without significantly disturbing activity at the targeted site.

Substrate activity of duplexes formed between phosphoramidate oligomers **2**, **3** or **4** and poly rA for *E. coli* RNase H was evaluated by measuring the UV absorption at 260 nm as a function of time

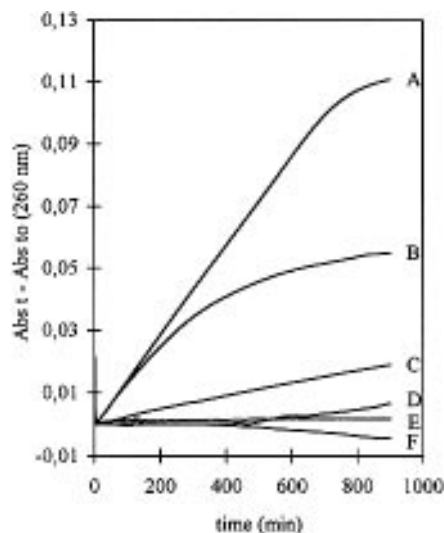


Figure 5. RNase H digestion of poly rA-dodecathymidylate duplexes. Plotted are normalized absorbance at 260 nm versus time. Experiments were carried out at 20°C in a 10 mM Tris-HCl buffer (pH 7) containing 10 mM MgCl_2 , 0.1 M NaCl, at a 240 μ M nucleotide concentration for each strand. (A) **5**-poly rA, (B) **3**-poly rA, (C) **2**-poly rA, (D) poly rA alone, (E) $\alpha(\text{dTp})_{11}\text{dT}$ -poly rA, (F) **4**-poly rA. All samples were preheated at 70°C, then allowed to slowly cool down and stabilized at 20°C before RNase H was added.

(Fig. 5) as described by Stein *et al.* (36). Duplexes of poly rA with the 'parent diester' $(\text{dTp})_{11}\text{dT}$ and with its α -anomeric analogue (41) as well as poly rA alone were used respectively as positive and negative controls. It was shown that the chimeric oligonucleotide **3** with a central phosphodiester section was able to elicit RNase H hydrolysis of poly rA, as well as the natural phosphodiester (Fig. 5). This result is in accord with the described behaviour of chimeric oligonucleotides constituted of a central section of phosphodiester surrounded by modified sections (37,38). The fully modified oligonucleotide **4** does not elicit RNase H activity (in the conditions used we obtained a T_m for this duplex at 16°C). Surprisingly, the oligonucleotide **2** with alternated phosphodiester and phosphoramidate internucleoside linkages, is able to induce RNase H cleavage of poly rA, despite the fact that only 50% of the hybrid was present under the conditions used in this experiment. This hydrolysis, however, proceeds far more slowly than with the oligomer **3**.

Enzymatic degradation of oligonucleoside phosphoramidates

Stability of phosphodiester links present in oligonucleotide analogues **2** and **3** was investigated in comparison with that of unmodified $(\text{dTp})_{11}\text{dT}$ towards the action of purified S1 nuclease, calf spleen phosphodiesterase (CSPDE) or snake venom phosphodiesterase (SVPDE). Table 6 summarises the half-lives of the oligomers. As can be seen, the rate of hydrolysis by nuclease S1 of the alternated oligonucleotide **2** ($t_{1/2} = 20.7$ h) is greatly reduced compared with that of unmodified **5** ($t_{1/2} = 7$ min). In contrast, the phosphodiester 'window' of the oligomer **3** is not protected by P-NH₂ sections present at 3'- and 5'-ends. Towards the action of the 5'-exonuclease CSPDE, one modification at the 5'-end of the oligomer **2** prevents vicinal phosphodiester link from the degradation ($t_{1/2} = 26$ h). The stabilizing effect of three

consecutive phosphoramidate modifications at the 5'-end on a neighbouring phosphodiester bond (oligomer **3**, $t_{1/2}$ = 12 days) is more pronounced. When **2** or **3** was incubated with 3'-exonuclease SVPDE, hydrolysis of the phosphodiester bonds was lower when compared with those obtained with **5** ($t_{1/2}$ = 14 min). No significant difference was observed between the two modified oligomers ($t_{1/2}$ = 9 h for compound **2**, and 8.5 h for compound **3**). This could result, at least in part, from the chemical instability of the phosphoramidate link in base medium (pH 9) used in these experiments ($t_{1/2}$ of ~20 h for **2** and **3**). Data reported in Table 6 clearly indicate a pH dependence for the hydrolysis of the phosphoramidate links with a maximum of stability ~pH 7.

Table 6. Half-lives of oligonucleotide analogues in the presence of purified enzymes

	(dTp) ₁₁ dT (5)	(dTpdTp) ₅ dTpndT (2)	(dTpn) ₃ (dTp) ₅ (dTpn) ₃ dT (3)
S1 nuclease ^a	7 min	20.7 h (11 days)	7 min (14 days)
CSPDE ^b	22 min	26 h (19 days)	12 days (20 days)
SVPDE ^c	14 min	9 h (23 h)	8.5 h (20 h)

Half-lives were determined by measuring the disappearance of starting oligonucleotide. Reactions were performed at 37°C in:

^a50 mM sodium acetate buffer (pH 4.5) containing 300 mM sodium chloride and 100 mM zinc acetate;

^b125 mM ammonium acetate buffer (pH 6.8) containing 2.5 mM EDTA;

^c100 mM Tris-HCl buffer (pH 9) containing 10 mM magnesium chloride.

Values indicated in brackets refer to half-lives in absence of nuclease.

Phosphoramidate (P-NH₂) represents an interesting oligonucleotide backbone modification because the NH₂ group is small, uncharged in aqueous solution and has favourable solubility characteristics. We have shown that the combination of H-phosphate and phosphoramidate chemistries, with the use of the easily cleaved oxalyl anchor, labile exocyclic amino protecting groups, and a final deprotection with methanol saturated with ammonia had enabled us to synthesize partially or totally modified dodecadeoxynucleotides. These compounds are able to form hybrids with complementary DNA or RNA strands. The resulting duplexes are significantly less stable than those of the 'wild-type' species, especially with RNA targets. We demonstrated that a modified oligomer combining an internal phosphodiester section surrounded by phosphoramidate sections at 5'- and 3'-ends was resistant to exonuclease hydrolysis and able to elicit RNase H cleavage of the RNA target. These properties make the modification attractive for constructing potential candidates for the antisense approach.

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