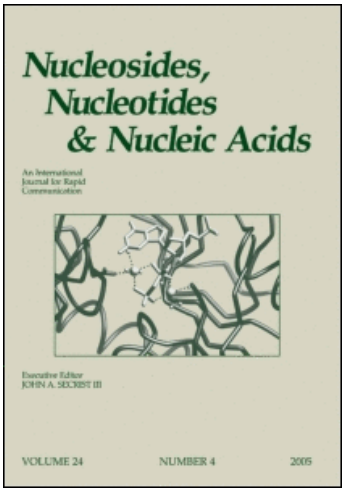

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Enzymatic and Hybridization Properties of Oligonucleotide Analogs Containing Novel Phosphoramidate Internucleotide Linkages

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ABSTRACT

In line with the paradigm, that antisense oligonucleotides should contain minimal structural modifications, in order to minimize the risk of toxicity and antigenicity, we describe here the preparation and the properties of oligonucleotides modified to contain, in addition to phosphodiester bonds, a small number of phosphoramidate internucleotide linkages substituted with aminoethoxyethyl groups in order to convey protection against exo- and endonucleases. Prolonged stability was, in fact, found in model experiments with respective enzymes, as well as in studies done in human blood serum. Regardless of number and position of phosphoramidate linkages, the modified oligonucleotides showed only a slight decrease of T_m in hybridization studies with complementary oligonucleotides.

Key Words: Oligonucleotide analogs; Phosphoramidate internucleotide; Oligoethylene glycol branches; Hybridization; Exo- and endonucleolytic stability.

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INTRODUCTION

The engineering of oligonucleotide hybridization, the increase of stability towards enzymatic hydrolysis, and the fast and automatic synthesis of backbone modified oligonucleotide analogs is of great interest in biochemical research.

Oligonucleotides and their analogs have been investigated for more than 20 years as specific gene inhibitory agents within cells on the basis of the ''antisense'' principle, where an oligonucleotide complementary to a segment of biologically important mRNA is targeted to inhibit the function of that mRNA intracellularly.[1] The development and applications of antisense technology are now well documented.[2–4] Because unmodified phosphodiester oligonucleotides are rapidly degraded by serum or intracellular nucleases, a number of analogs of oligonucleotides have been proposed.[5,6] Phosphorothioate oligodeoxynucleotides (PS-ODNs, often named ''first generation oligonucleotides'') have been extensively studied and tested in human clinical trials.[7] Nevertheless, PS-ODNs show various hematologic toxicities mainly due to non-specific effects related to their binding to proteins on the cell surface or in the serum.[8,9] Also, due to their polyanionic nature and polarity, uptake by targeted cells is not very efficient. Other oligonucleotide analogs, with morpholino backbones or methoxyethyl modifications, were developed and are actually used in clinical trials. However, this ''second generation'' of antisense oligonucleotides still exhibits some toxicity.[10,11] In view of these problems, we have taken interest in looking at analogous structures that deviate minimally from biological oligonucleotides, but still have the desirable properties of antisense oligonucleotides (resistance to nucleases).[12] The H-phosphonate method[13,14] allows to introduce different backbone modifications in a very fast and efficient way.[15] We used this strategy for the fully automated preparation[16] of oligonucleotides with phosphoramidate internucleotide linkages substituted with 2-(2-aminoethoxy)ethyl (AEE) group. Previously we described the preparation of oligonucleotides with triester internucleotide linkages substituted with α,ω-dihydroxy-(3,6-dioxa)-octan-1-yl groups (''triethyleneglycol triester linkages'') in the antisense strategy.[17] Several researchers have recently synthesized oligonucleotides modified with aminoalkyl phosphoramidates, in order to obtain cationic oligomers.[18,19] In the course of our research on minimally modified oligonucleotides for potential therapeutic applications we have previously described the preparation of oligonucleotides with terminal sense inversion exhibiting high resistance to exonucleolytic degradation in biological media.[12] In order to provide also for stabilization against endonucleolytic attack, we have now introduced a new kind of backbone DNA modification (see Fig. 1) which, although applied only to one, two or three internucleotide linkages of 15 mer oligonucleotides, does not hinder stable selective hybridization to complementary oligonucleotides and provide for high exo- and endonucleolytic stability. After synthesis, purification by preparative PAGE (20%) and characterization by MALDI MS, T_m values of the modified oligonucleotides with their complementary DNA analogues were determined and compared to native DNA–DNA duplexes. In enzymatic studies the modified oligonucleotides containing aminoethoxyethyl (AEE) phosphoramidate internucleotide linkages were examined with respect to stability towards snake venom phosphodiesterase and S1 nuclease as well as their degradation in human blood serum. The enzymatic degradation was followed by

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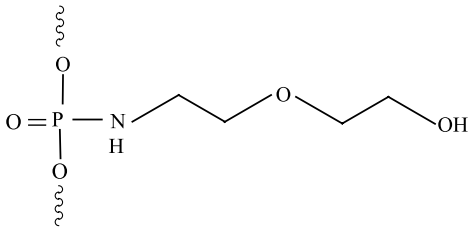

Figure 1. Incorporated AEE-phosphoramidate-internucleotide linkage.

analytical PAGE (20%) (Figs. 2 and 3). The properties of these compounds in gene inhibition are described in a forthcoming publication.[20]

RESULTS AND DISCUSSION

AEE-phosphoramidate modified oligonucleotide analogues were synthesized by a fully automatic solid phase DNA synthesis by using a combination of phosphoramidite and H-phosphonate methods.[21] The phosphoramidate internucleotide linkages were introduced by oxidation of H-phosphonate linkages with a solution containing 0.1 M I_2 and 1.0 M 2-(2-aminoethoxy)ethanol in acetonitrile (1.2 ml oxidation solution for 1.2 min.) followed by the capping step. The formation of the phosphoramidate indicated

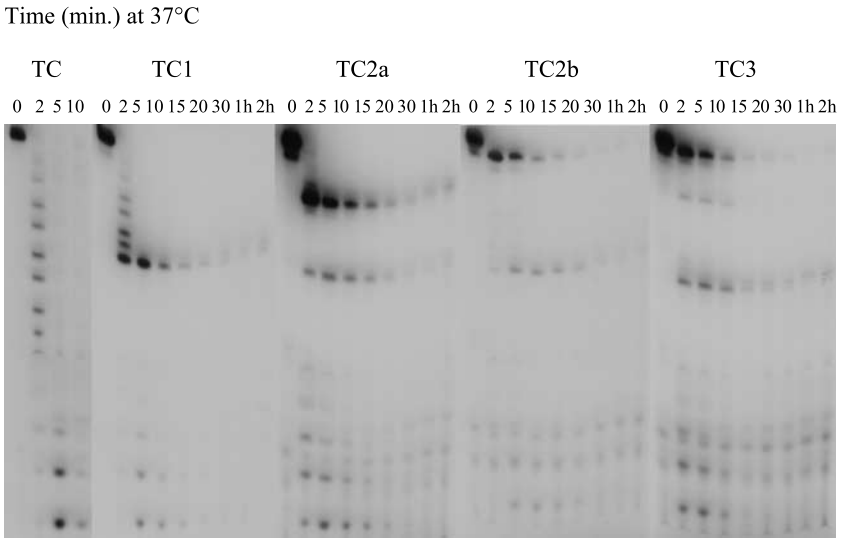

Figure 2. Snake venom phosphodiesterase (SVPDE1) degradation of the unmodified (TC) and the modified (TC1, TC2a, TC2b and TC3) oligonucleotides on a 20% polyacrylamide gel.

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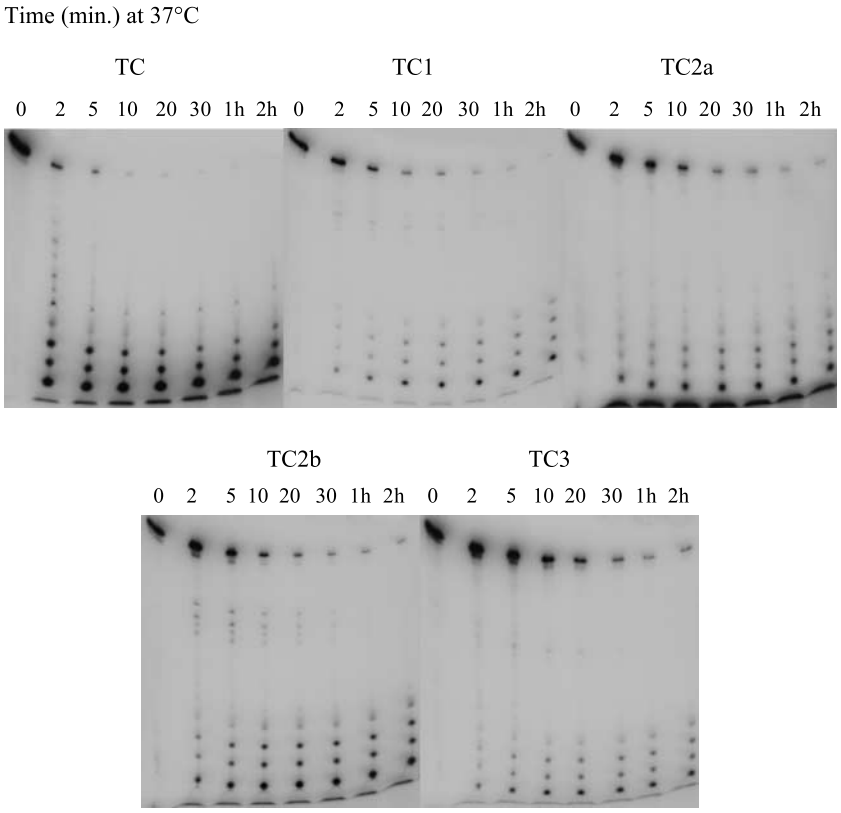

Figure 3. S1-endonuclease degradation of the unmodified (TC) and modified (TC1, TC2a, TC2b and TC3) oligonucleotides on a 20% polyacrylamide gel.

that under the reaction conditions the amino function was a better nucleophile than the hydroxyl group of the amino alcohol, since protecting the amino group of the amino alcohol produces phosphotriesters instead of phosphoramidates, but in a slower rate.[22] All oligonucleotides synthesized in this way were purified by preparative PAGE (20%) and characterized by MALDI–TOF mass spectrometry (Table 1).

Table 1. MALDI–TOF mass spectrometry of the unmodified (TC) and modified (TC1, TC2a, TC2b, TC3) oligonucleotides.

Abbreviation	Structure	Calculated mass	Observed mass
TC	5′-TTTTTCTCTCTCTCT-3′	4426.0	4424.9
TC1	5′-TTTTTCT*CTCTCTCT-3′	4514.0	4512.0
TC2a	5′-TTTTTCT*CT*CTCTCT-3′	4602.0	4603.4
TC2b	5′-TTTTTCT*CTCTCT*CT-3′	4602.0	4602.9
TC3	5′-TTTTTCT*CTCT*CT*CT-3′	4690.0	4691.3

* = Aminoethoxyethyl-phosphoramidate-internucleotide linkage (see Fig. 1).

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Hybridization Properties of the AEE-Phosphoramidate Modified Oligonucleotides

The melting temperatures of the modified oligonucleotides with their complementary DNA and RNA sequences were investigated under physiological conditions (150 mM NaCl, 10 mM NaH_2PO_4, 2 mM $MgCl_2$, pH 7.4) and compared to the corresponding melting temperatures of the native DNA–DNA and DNA–RNA duplexes (Table 2). All modifications at different positions of the sequences lowered the T_m values in a range of 0.7–3.0°C. There was no difference regarding the position and the number of AEE modifications of the oligonucleotides. This very low decrease of T_m indicates that there is nearly no influence of the AEE modification on the stability of the DNA–DNA and DNA–RNA double helices. However, in the case of a mismatch situation (sequence M1, Table 2) we observed a high drop of the T_m value of the DNA–DNA duplex (13°C for only one mismatch base, *T* instead of *G*).

Determination of the Exonucleolytic and Endonucleolytic Stability by Snake Venom Phosphodiesterase and S1 Nuclease

Snake venom phosphodiesterase (SVPDE) is known to cleave unmodified oligonucleotides from their 3′ end at 37°C.[23] The stability towards enzymatic degradation is an important factor for using modified oligonucleotides in different biological systems.

Enzymatic degradation studies were performed by incubating 5′-^{32}P-labelled oligonucleotides (500 000 c.p.m.), using γ-^{32}P-ATP and T4 polynucleotide kinase, and 0.012 mU SVPDE at 37°C in a pH 7.5 buffer (70 mM Tris-borate) containing 10 mM $MgCl_2$, in a total volume of 40 µl. Aliquots (4 µl) were removed at the desired time points, stopped by addition of 2 µl stop solution (30 mM EDTA, 8 M urea) followed by heating at 90°C for 10 min. Loading buffer (80% formamide, 10 mM NaOH, 1.0 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue) was added and the reaction products were then analyzed by denaturing PAGE (20%). Electrophoresis was performed at a constant voltage of 2000 V for 2 h. The polyacrylamide gel was then dried and exposed to X-ray film. The unmodified oligonucleotide (TC) exhibited no stability towards the enzymatic degradation at all. The AEE modified oligonucleotides (TC1, TC2a, TC2b and TC3) showed a resistance to exonucleolytic degradation at the AEE backbone modification. The analysis by PAGE showed, that the enzymatic degradation stopped exactly at the phosphoramidate modification (Fig. 2).

S1 nuclease is known to cleave internal phosphodiester internucleotide linkages. The reaction conditions were similar as in SVPDE degradation studies. The modified sequences, TC1, TC2a, TC2b and TC3, showed a higher stability towards S1-endonucleolytic degradation than the unmodified sequence, TC, (Fig. 3).

Figure 4 presents the percentages of degradation of the unmodified and modified sequences as a function of time in S1-endonuclease solutions. The degradation percentages have been determined by densitometric measurements. In the case of the unmodified sequence (TC), degradation is faster compared with the degradation of the modified sequences (TC1, TC2a, TC2b and TC3). Thus, in the case of S1-endonuclease attack, complete degradation of TC is about 5 minutes, and for TC1 is about 15 minutes and 30 minutes for TC2a and TC2b and about 1 hour for TC3. These results can be

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Table 2. T_m values of the unmodified (TC) and modified (TC1, TC2a, TC2b, TC3) oligonucleotides with their complementary nucleic acids.

	Oligonucleotide	T_m (°C) deoxyribo (ribo)	ΔT_m (°C) deoxyribo (ribo)	ΔT_m (°C) per modification deoxyribo (ribo)
	3′-AAAAAGAGAGAGAGA-5′ (sense match)			
TC	5′-TTTTTCTCTCTCTCT-3′	47.3 (47.2)	–	–
TC1	5′-TTTTTCT*CTCTCTCT-3′	46.6 (46.3)	− 0.7 (− 0.9)	− 0.7 (− 0.9)
TC2a	5′-TTTTTCT*CT*CTCTCT-3′	46.1 (45.8)	− 1.2 (− 1.4)	− 0.6 (− 0.7)
TC2b	5′-TTTTTCT*CTCTCT*CT-3′	46.3 (46.0)	− 1.0 (− 1.2)	− 0.5 (− 0.6)
TC3	5′-TTTTTCT*CTCT*CT*CT-3′	44.6 (44.2)	− 2.7 (− 3.0)	− 0.9 (− 1.0)
M1	3′-AAAAA*T*AGAGAGAGAGA-5′ (sense mismatch)			
TC	5′-TTTTTCTCTCTCTCT-3′	34.3	− 13.0	

* = Aminoethoxyethyl-phosphoramidate-internucleotide linkage (see Fig. 1).
Buffer (150 mM NaCl, 10 mM NaH_2PO_4, 2 mM $MgCl_2$); pH 7.4.
DNA–DNA (DNA–RNA); $\Delta T_m = T_m$ modified oligonucleotide—T_m unmodified oligonucleotide.

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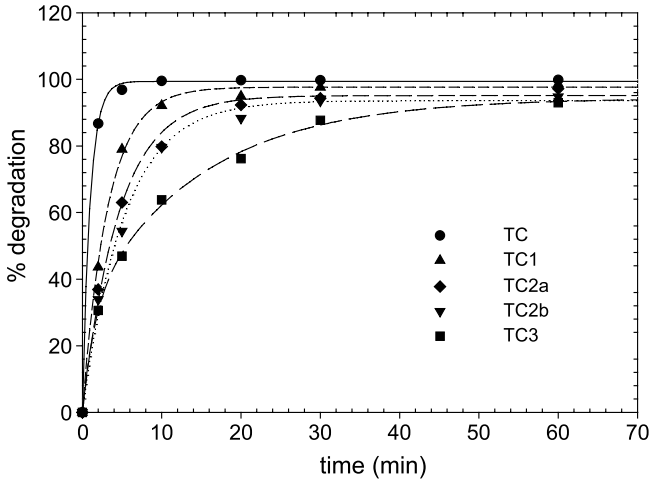

Figure 4. Kinetics of S1-endonuclease degradation of the unmodified (TC) and modified (TC1, TC2a, TC2b and TC3) oligonucleotides.

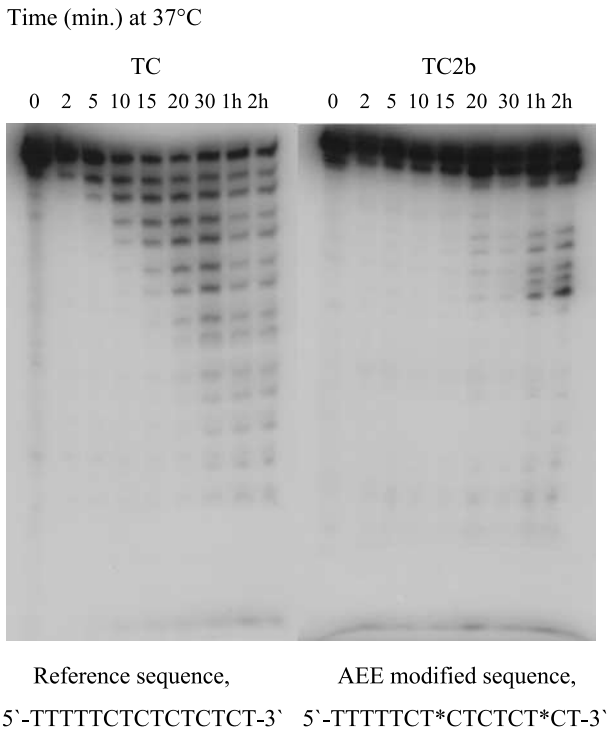

Figure 5. Stability of unmodified (TC) and modified (TC2b) oligonucleotides in human blood serum.

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explained by the protection provided by the AEE-phosphoramidate internucleotide linkages during S1-endonuclease attack.

Test of Stability of the AEE Modified Oligonucleotides Towards Enzymatic Degradation in Human Blood Serum

To investigate, if the few AEE modifications are sufficient to convey resistance against nucleolytic degradation in biological fluids, a serum test was performed by incubating 5′-^{32}P-labeled oligonucleotide TC2b in human blood serum. As shown in Fig. 5, the unmodified oligonucleotide (TC) was strongly degraded after 10 min., whereas the modified oligonucleotide containing AEE internucleotide linkages (at positions two and eight in the sequence) was quickly shortened by one nucleotide at the 3′ end, but the residual molecule was very stable towards nucleolytic degradation for a very long period. This was indicated by PAGE (Fig. 5).

CONCLUSIONS

Melting temperatures of the modified oligonucleotides containing AEE phosphoramidate internucleotide linkages with their complementary DNA and RNA sequences were determined and compared to the corresponding T_m values of natural DNA–DNA and DNA–RNA duplexes. Regardless of the number and the position of the AEE phosphoramidate units the modification leads only to a negligible drop of T_m. Furthermore we found that the modified oligonucleotides were more stable towards exo- and endonucleases and also against enzymatic degradation in human blood serum (mainly 3′-exonucleases.[24]) The degradation was monitored by denaturing PAGE (20%). AEE modification can easily be combined with 3′-inversion[12] to convey enhanced stability.

EXPERIMENTAL

Oligonucleotides

The oligonucleotides listed in Table 1 were synthesized on a Gene Assembler Special DNA synthesizer (Pharmacia) by a fully automatic solid phase DNA synthesis using a combination of phosphoramidite and H-phosphonate methods.[25] The phosphoramidate internucleotide linkages were introduced by oxidation of H-phosphonate linkages with a freshly prepared solution of 0.1 M I_2 and 1.0 M 2-(2-aminoethoxy)ethanol (commercial grade, distilled prior to use) in dry acetonitrile using following protocol:

1.	Acetonitrile—washing	60 s
2.	0.1 M H-phosphonate/acetonitrile—pyridine 1:1	100 μL
3.	Acetonitrile (reagents separation segment)	20 μL
4.	0.3 M pivaloyl chloride/acetonitrile—pyridine 1:1	100 μL
5.	Coupling	120 s

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6.	Acetonitrile—washing	30 s
7.	Oxidation (I_2/amino alcohol/acetonitrile)	1 mL/60 s
8.	Acetonitrile—washing	60 s
9.	Detritylation (3% trichloroacetic acid in dichloroethane)	60 s
10.	Continuation of the synthesis	

Yields of 97% for the H-phosphonate coupling steps were observed, whereas yields of internucleotide bond formation were higher (>98%) with phosphoramidite intermediates. At the end of the synthesis, the protecting groups were removed in 30% ammonia at 55°C, overnight, and the product was desalted on NAP-10 gel filtration columns (Pharmacia) and lyophilized in vacuo. Purification of oligonucleotides was performed on 20% preparative PAGE. The introduction of phosphoramidate internucleotide linkages slightly reduced yields of oligonucleotides. Isolated yields of modified oligonucleotides were 92–96% of those of the unmodified oligonucleotide. After purification the oligonucleotides were desalted by gel filtration on NAP-10 columns and characterized by MALDI–TOF mass spectrometry.

UV Thermal Denaturation Studies

Thermal denaturation studies were performed by mixing modified or unmodified oligonucleotides with the DNA or RNA target strands in equimolar ratios (0.5:0.5 OD) in 1 ml (150 mM NaCl, 10 mM NaH_2PO_4, 2 mM $MgCl_2$; pH 7.4) buffer. Spectrophotometric measurements were performed at 260 nm using 1 cm path length quartz cuvettes at a heating rate of 0.5°C/min. Melting temperatures were taken as the temperature of half-dissociation and were obtained from first derivative plots.

Nuclease Degradation Studies

For the snake venom phosphodiesterase (SVPDE1) degradation studies, 5′-^{32}P-labelled oligonucleotides (500 000 c.p.m.) and 0.012 mU SVPDE1 (from *Crotalus adamanteus* venom, Sigma) were incubated at 37°C in a pH 7.5 buffer (70 mM Tris-borate) containing 10 mM $MgCl_2$, in a total volume of 40 μl. For the S1 nuclease degradation, reactions were carried out as above, but in 0.5 M NaOAc, pH 4.5, 2.8 M NaCl, 45 mM $ZnSO_4$ buffer and with 0.5 U S1 nuclease (Promega). The samples were incubated at 37°C. Aliquots were removed at the desired time and quenched by addition of 2 μl stop solution (30 mM EDTA, 8 M urea). After denaturation (90°C, 10 min.) the samples were analyzed by denaturing PAGE. Percentages of cleaved oligonucleotides and degradation were evaluated after densitometric scanning of the bands.

Serum Degradation Kinetics

Serum degradation was performed by suspending oligonucleotide (5′-terminally labelled with ^{32}P) in fresh human blood serum (100 μl). The mixture was incubated at 37°C, and at various time intervals an aliquot (10 μl) was lyophilized, resuspended in the proper loading buffer and separated electrophoretically by using a 20% PAGE and visualized by autoradiography.

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