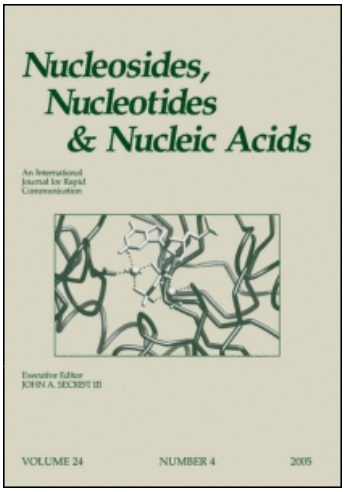

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ENZYMATIC AND HYBRIDIZATION PROPERTIES OF OLIGONUCLEOTIDE ANALOGUES CONTAINING NOVEL PHOSPHOTRIESTER INTERNUCLEOTIDE LINKAGE

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ABSTRACT: Enhanced cellular uptake, stable and discriminating hybridization and increased stability in biological media are of particular interest for oligonucleotides of potential therapeutic application. Additionally, toxicity or immunogenicity of the oligonucleotide analogues and their biodegradation products should be minimized by minimal alteration of the biological structure and effort and cost of bulk production should be as low as possible by using a standard automated synthesis protocol. Oligonucleotide phosphotriesters with oligoethyleneglycol substituents show promise to ideally combine all these advantages. Here we describe the hybridization properties and the stability of modified oligonucleotides containing triester internucleotide linkages substituted with α,ω-dihydroxy-(3,6-dioxa)-octan-1-yl group ("triethyleneglycol triester linkages") towards enzymatic degradation. The triester linkages are stable towards exo- and endonucleases. Regardless of number and position of triester linkages, the modified oligonucleotides showed practically no decrease of T_m in hybridization studies with complementary biological oligonucleotides. In further enzymatic studies the modified oligonucleotides were highly stable towards nucleases in human blood serum.

Introduction

The engineering of oligonucleotide hybridization, the increase of stability towards enzymatic hydrolysis and the fast and automatic synthesis of backbone modified

oligonucleotide analogues is of great interest in biochemical research. In recent years many different backbone modifications were synthesized and used in chemical and pharmaceutical research (e.g. antisense strategy)[1,2]. Especially the more recently developed H-phosphonate method[3,4] allows to introduce different backbone modifications in a very fast and efficient way[5]. We used this strategy for the fully automated preparation[6] of oligonucleotides with triester internucleotide linkages substituted with α,ω-dihydroxy-(3,6-dioxa)-octan-1-yl group ("triethyleneglycol triester linkages"). Previously other laboratories described the preparation and the application of polyethyleneglycol (PEG) modified oligonucleotides in the antisense strategy[7] by adding PEG to the terminus of oligonucleotides resp. insertion of oligo ethyleneglycol bridges into the main chain. In the course of our research on minimally modified oligonucleotides[8] 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. 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 or two internucleotide linkages of 15mer oligonucleotides provide for stable hybridization to complementary oligonucleotides and high exo- and endonucleolytic stability.

The synthesis and the characterization by MALDI MS were recently reported[9]. After purification by RP-HPLC T_m values of the modified oligonucleotides with their complementary DNA and RNA analogues were determined and compared to native DNA-DNA and DNA-RNA duplexes. In enzymatic studies the modified oligonucleotides containing "triethyleneglycol triester" internucleotide linkages were examined with respect to their stability towards snake venom phosphodiesterase and S1 nuclease as well as their degradation in human blood serum. The enzymatic degradation was followed by RP-HPLC (see fig. 2a,b).

Results and Discussion

"Triethyleneglycol-Triester" modified oligonucleotide analogues were synthesized by a fully automatic solid phase DNA synthesis by using a combination of phosphoramidite and H-phosphonate method. The triester internucleotide linkages were introduced by an

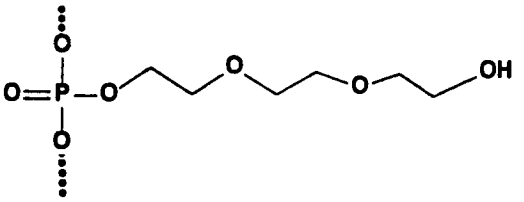


Fig. 1: Incorporated triethyleneglycol-triester-internucleotide linkage

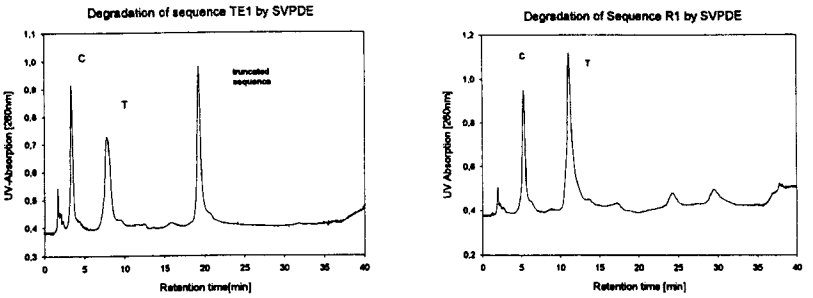
oxidation step with iodine (in triethyleneglycol/pyridine/tetrahydrofuran) followed by a special capping step. All oligonucleotides synthesized in this way were purified by RP-HPLC.

Hybridization properties of the Triethyleneglycol-Triester modified oligonucleotides

The melting temperatures of the modified oligonucleotides with their complementary DNA and RNA sequences were investigated under physiological conditions (165mM, pH 7,0; Na^+, see table 1) and compared to the corresponding melting temperatures of the native DNA-DNA and DNA-RNA duplexes (see table 2). All modifications at different positions of the sequences lowered the T_m values in a range of 0,5-1,2°C. There was no difference regarding the position and the number of triethyleneglycol modifications of the oligonucleotides. This very low decrease of T_m indicates that there is nearly no influence of the triethyleneglycol modification on the stability of the DNA-DNA and DNA-RNA double helix. In the case of a mismatch situation (sequence M1, tab.1) we observed a high selectivity and a higher drop of the T_m value of the triethyleneglycol modified DNA-DNA duplex in comparison to a native DNA-DNA duplex.

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 within 5-15 min at 37°C[10]. The stability towards enzymatic degradation is an important factor for using modified oligonucleotides in different biological systems. Enzymatic degradation studies were performed by



Sequence TE1: 5'- TTTTTC-*-TCTCTCTCT -3' Sequence R1: 5'- TTTTTCTCTCTCTCT -3'

Fig. 2a: SVPDE degradation of the modified and unmodified oligonucleotides

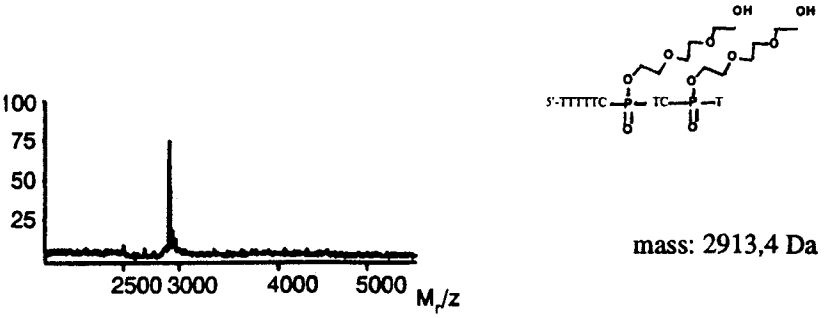
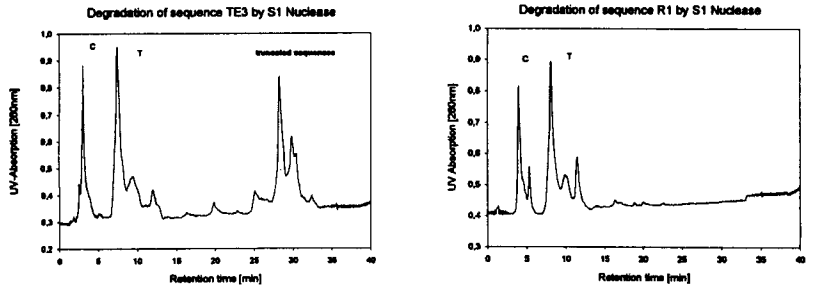


Fig. 2b: MALDI mass spectrometry of the enzymatic degradation of sequence TE2 by SVPDE



Sequence TE3: 5'- TTTTTC-*-TCTCTC-*-TCT -3' Sequence R1: 5'- TTTTTCTCTCTCTCT -3'

Fig. 2c: S1-nuclease degradation of the modified and unmodified oligonucleotides

Tab. 1: T_m values of the modified oligonucleotides with complementary nucleic acids

T_m values with DNA#

Modified oligonucleotide (5'-3') TE1/TE2/TE3		oligonucleotide [°C]	Δ T_m [°C]
SD1	3'-AAAAAGAGAGAGAGA (sense match, deoxyribo)	47,2 (47,5)	
XR	TTTTTCTCTCTCTCT		
TE 1	TTTTTC-*-TCTCTCTCT		- 0,5 (- 1,2)
TE 2	TTTTTC-*-TC-*-TCTCTCT		- 0,6 (- 0,6)
TE 3	TTTTTC-*-TCTCTC-*-TCT		- 0,3 (- 1,0)
M1	3'-AAAAA*T*AGAGAGAGA (sense mismatch)		
XR	TTTTTCTCTCTCTCT	32,5 (32,6)	
TE2	TTTTTC-*-TC-*-TCTCTCT		-1,8 (-2,1)

Tab. 2

T_m values with RNA#

Modified oligonucleotide (5'-3') [°C]		oligonucleotide	Δ T_m [°C]
SR1	3'-rAAAAAGAGAGAGAGA (sense match/ribo)	48,2 (48,5)	
XR	TTTTTCTCTCTCTCT		
TE 1	TTTTTC-*-TCTCTCTCT		- 2,1 (-2,0)
TE 2	TTTTTC-*-TC-*-TCTCTCT		- 2,5 (-2,7)
TE 3	TTTTTC-*-TCTCTC-*-TCT		- 0,8 (- 0,9)

 # -*- = Triethyleneglycol-triester-internucleotide linkage, see scheme I; TE = treiethyleneglycol

Buffer 1xSSC (sodium chloride, sodium citrate; 165mM Na^+); pH 7,0

ramp: up (down), ramp rate: 0,5°C/min

heating (cooling); Δ T_m = T_m modified oligonucleotide - T_m unmodified oligonucleotide

incubating 1 OD_{260} modified or reference oligonucleotide (in 50µl aq.) with SVPDE (in glycerol), alkaline phosphatase and 10x alkaline phosphatase reaction buffer for 60min at 37°C. After this time SVPDE was inactivated by heating (90°C) for 2min. The unmodified oligonucleotide R1 exhibited no stability towards the enzymatic degradation at all (see fig. 1a,). The triethyleneglycol modified oligonucleotide TE1 showed a high stability against the exonucleolytic degradation. Even after one hour at 37°C no further degradation was observed. The appearance of one truncated sequence in the HPLC diagram in figure 2a suggests that the site of resistance to enzymatic degradation of the modified oligonucleotide is at the triethyleneglycol backbone modification.

That the enzymatic degradation stopped exactly at the triester modification was verified by MALDI MS (see fig. 2b)

S1 nuclease is known to cleave internal phosphodiester internucleotide linkages. The reaction conditions were similar as in SVPDE degradation studies. The reaction time was 30 min at 37°C. The unmodified sequence R1 (fig 2c) was completely degraded. In the case of the triester modified oligonucleotides TE3 we found in the HPLC diagram two peaks corresponding to truncated sequences. This result indicates, that the triethyleneglycol triester modified oligonucleotides showed a high stability towards endonucleolytic degradation by S1 nuclease.

Test of stability of the triethyleneglycol modified oligonucleotides towards enzymatic degradation in human blood serum

To investigate, if the triethyleneglycol-triester modified oligonucleotides are sufficient to convey resistence against nucleolytic degradation in biological fluids, a serum test was performed by incubating ^{32}P-labelled oligonucleotide TE3 (see table 1) in human blood serum (fig 3). As shown in fig. 3, the unmodified oligonucleotide R1 was strongly degraded after 15 min.. The experiment in Fig 3 indicates, that the modified oligonucleotide containing triethyleneglycol internucleotide linkages was very stable towards nucleolytic degradation for a very long period. This was indicated by PAGE (fig.3)

Conclusion

Melting temperatures of the modified oligonucleotides containing "triethyleneglycol triester" internucleotide linkages with their complementary DNA and RNA sequences

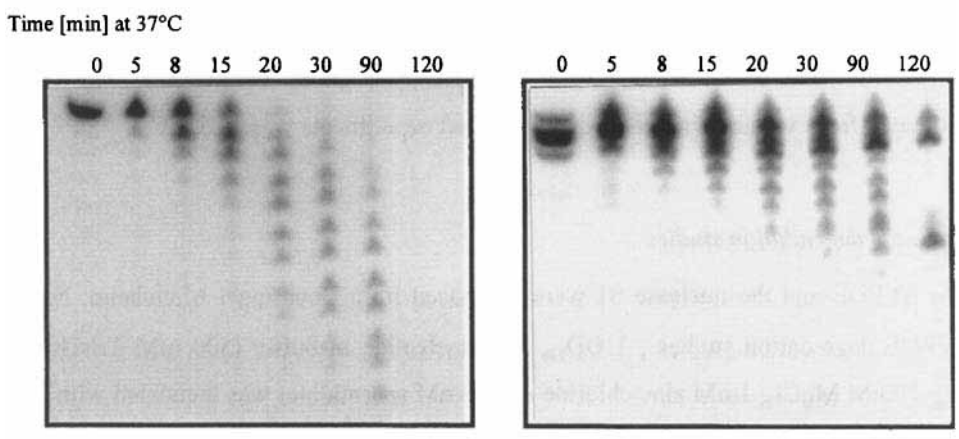


Fig. 3 A

Sequence R1: 5'- TTTTTCTCTCTCTCT -3'

Reference sequence

Fig. 3 B

Sequence TE3: 5'- TTTTTC-*- TCTCTC-*-TCT -3'

Triethyleneglycol modified sequence

Fig. 3: Stability of modified and unmodified oligonucleotides in human blood serum

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 triethyleneglycol triester units the modification leads only to a negligible drop of T_m. Furthermore we found that the modified oligonucleotides were very stable towards exo- and endonucleases and also against enzymatic degradation in human blood serum. The degradation was monitored by RP-HPLC and MALDI mass spectrometry. Triethyleneglycol modification can easily be combined with 3'-inversion[9] to convey extreme stability.

Experimental

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 in 1xSSC (sodium chloride and sodium citrate, 165 mM Na^+; pH 7,0) buffer. Spectrophotometric measurements were performed at 260nm 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. Precision in Tm values, estimated from variance in two or three repeated experiments was +- 0,5°C.

Nuclease degradation studies

The SVPDE and the nuclease S1 were purchased from Boehringer Mannheim. For the SVPDE degradation studies , 1 OD_{260} oligonucleotide in buffer (500 mM TrisHCl pH 9.0; 10mM $MgCl_2$, 1mM zinc chloride and 10mM spermidine) was incubated with 0.1 U snake venom phosphodiesterase at 37°C for 1h. The 5'-end phosphate groups were removed by alkaline phosphatase. For the S1 nuclease degradation, reactions were carried out as above, but in 100mM sodium acetate, pH 5,0, 10mM zinc acetate buffer and with 5 U S1 nuclease. The samples were incubated at 37°C for 30 min. After denaturation (90°C, 2 min) the samples were analyzed by RP-HPLC.

serum degradation kinetics

Serum degradation was performed by resuspending oligonucleotide (terminally labeled with [γ-^{32}P]ATP) in fresh human blood serum. The mixture was incubated at 37°C, and at various time intervals an aliquot (5µl) was lyophilized and resuspended in the proper loading buffer and separated electrophoretically by using a 20% polyacrylamide, 7 M urea gel and visualized by autoradiography.

Acknowledgments

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