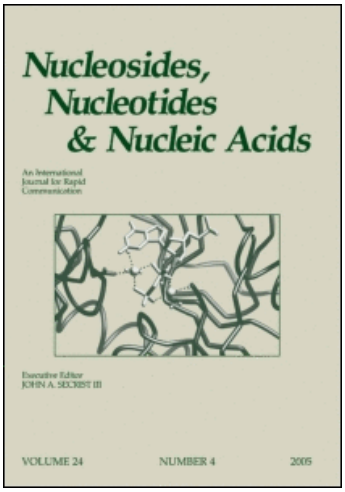

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Synthesis and Enzymatic Studies of Modified Oligonucleotides with Abiological Monomer Units

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SYNTHESIS AND ENZYMATIC STUDIES OF MODIFIED OLIGONUCLEOTIDES WITH ABIOLOGICAL MONOMER UNITS

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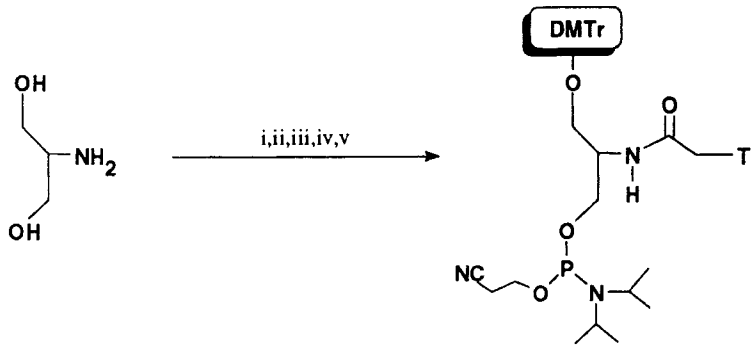
ABSTRACT: The synthesis and the enzymatic studies of modified oligonucleotides containing a PNA modified PNA-DNA dimer block and a new acyclic racemic serinol nucleoside is described. We show that both, the PNA-DNA dimer block[1] and the modified PNA-spacer (acyclic serinol nucleoside)[2] can be used as modified templates for the enzymatic generation of single stranded DNA. Degradation studies of the oligonucleotides containing the PNA-DNA dimer block with snake venom phosphodiesterase show that the modified oligonucleotides are stable towards exonucleolytic degradation.

Introduction

One of the most important features of chemically synthesized modified oligonucleotides is the possibility to create specific tailor-made molecules for a specific application. One group of modified oligonucleotides (antisense drugs) are designed to interrupt the translation process by which pathogenic proteins are produced[3]. Different backbone modifications of oligonucleotides have been used in the antisense strategy[4] to stabilize oligonucleotides against degradation in biological media. One of the most interesting constructs in this field of work is certainly the Peptide nucleic acid (PNAs)[5]. Peptide nucleic acids are known to be nuclease resistant analogues, which capable of stable and discriminating hybridization[6] to biological nucleic acids. Recently we reported on the synthesis of PNA-DNA chimers as a new class of PNA-containing building blocks[1]. We showed that they can be incorporated into DNA chains in a nucleic acid synthisizer without having to use peptide chemistry and discussed their hybridization properties. PNA-DNA chimeric oligonucleotides of different structure have been reported from other laboratories[7,8].

Here we describe the synthesis of a PNA-Spacer (scheme 1) and its incorporation into oligonucleotides[2]. In enzymatic studies the modified oligonucleotides (Tab. 1) containing



Scheme 1.: Synthesis of the PNA-spacer B (acyclic serinol nucleoside);

(i) DMTrCl/DMAP in pyridine, 24h,r.t.; (ii)TMSCl in pyridin, 24h, r.t.; (iii) DCC, DhbtOH (3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine), thyminyl acetic acid in DMF, 1h 0°C, 3h r.t.; (iv) conc. aq. ammonia in pyridine 2h (v) diisopropylethylamin, β-cyanoethoxy-chloro-(N,N-diisopropyl)-amino-phosphane in dichlormethane, 2h,r.t.

Table 1. OLIGONUCLEOTIDE STRUCTURES

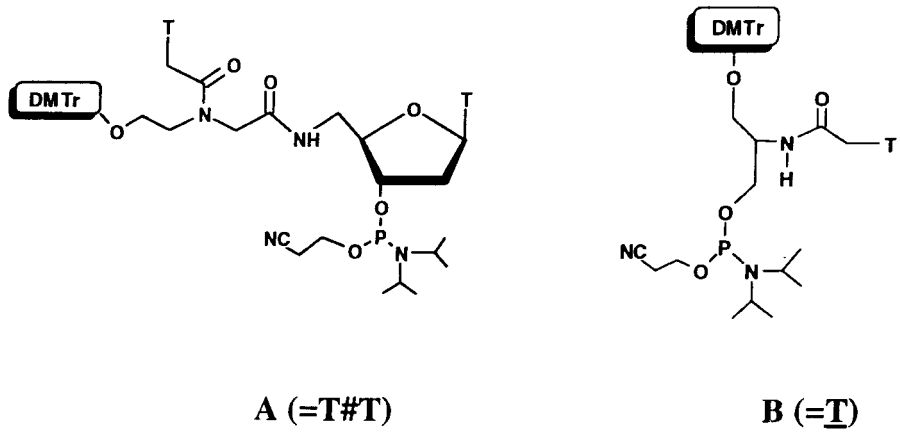
Name	Structure (5'-3')
PD-1-DB-17	TTAACTTCTTCACA**T#T**C
PD-2-DB-17	CACCAAC**T#T**C**T#T**CCACA
PD-1-DB-28	CGTATTGCCG**T#T**ATTCGTGCTCCCGCTG
OR-17-1	TTAACTTCTTCACATTC
OR-17-2	CACCAACTTCTTCCACA
OR-28	CGTATTGCCGTTATTCGTGCTCCCGCTG
ORC-28	CAGCGGGAGCACGAATAACGGCAATACG
P-10	CAGCGGGAGC
PS-AN-28	CGTATTGCCG<u>T</u>TATTCGTGCTCCCGCTG
PS-AN-17	CACCAAC<u>TT</u>C<u>TT</u>CCACA

T#T= PNA-DNA dimer block; Fig 1.; A

<u>T</u>= PNA-Spacer-(Acyclic serinol nucleoside); Fig. 2.; B

the PNA-DNA dimer block (Fig. 1, A) and the PNA-Spacer (Fig. 1, B) were examined with respect to their stability to snake venom phosphodiesterase (Fig. 2 and 3) and their effectivity as templates for the enzymatic synthesis of single stranded copies by DNA Polymerase I Klenow Large Fragment (Fig 4).

The oligonucleotides prepared in this way were sequenced by the method of Maxam and Gilbert (Fig 5).



A (=T#T) **B (=T)**

Fig 1.: Monomer units prepared for the incorporation into chimeric oligonucleotides; PNA-DNA dimer block (**A**) and PNA-Spacer (acyclic serinol nucleoside) (**B**)

Results and Discussion

Synthesis of the monomer units

The general synthesis of different PNA-DNA dimer blocks was described recently[1]. These dimer units were assembled by coupling a 5'-modified deoxythymidine a modified PNA monomer. This dimer block A (Fig.1; A) was used in the same way as nucleoside phosphoramidites. In contrast to previously reported work a combination of nucleoside with peptide chemistry is not necessary to synthesize PNA-DNA chimeric oligonucleotides. The synthesis of the PNA-Spacer (Fig.1; B) is described in scheme 1. The preparation of construct B started from serinol (2-amino-1,3-propanediol). We selectively protected one hydroxyl-group with 4,4'-dimethoxytriphenylmethylchloride, the second one with trimethylsilylchloride. In the next step we coupled the thyminyl aceticacid by an amide bond to the primary amino function. After selective deprotection of the silylated hydroxyl-group with conc. aq. ammonia the hydroxyl-group was phosphorylated by β-cyanoethoxy-chloro-(N,N-diisopropyl)-amino-phosphane. This PNA-Spacer was used in the same way as the dimer block A in an ordinary DNA synthesizer.

Oligonucleotide synthesis

Oligonucleotides were prepared on a Pharmacia Gene Assembler 4Primers, using the phosphoramidite method and following the standard coupling cycles. The coupling efficiencies of the PNA-DNA dimer block and the acyclic serinol nucleoside were similar as for commercially available phosphoramidites. The modified oligonucleotides obtained

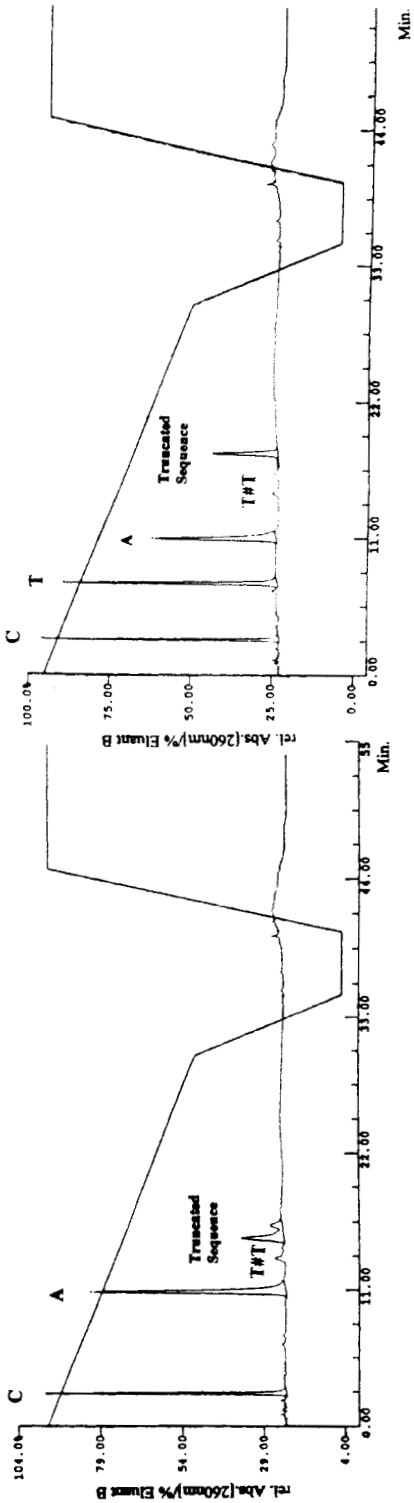


Fig 2.: Snake venom phosphodiesterase (and alkaline Phosphatase) degradation studies with PNA-oligonucleotide PD-1-DB-17 and PD-2-DB-17. T=Thymidine; C=Cytosin; A=Adenosin; **T#T**= PNA-DNA dimer block. Eluant A: 0,1 M TAEE, pH 7, 3% acetonitrile; Eluant B: 75% acetonitrile, 25% 0,1 M TAEE, (Triethylammoniumacetate) pH 7

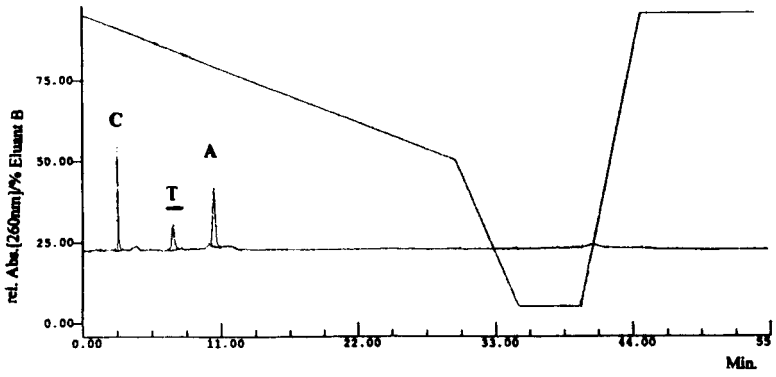


Fig. 3.: Snake venom phosphodiesterase (and alkaline Phosphatase) degradation studies with PNA-spacer (acyclic serinol nucleoside) oligonucleotide AN-17.; A= Adenosine; T= PNA-spacer (acyclic serinol nucleoside); Eluant A and B see Fig 2.

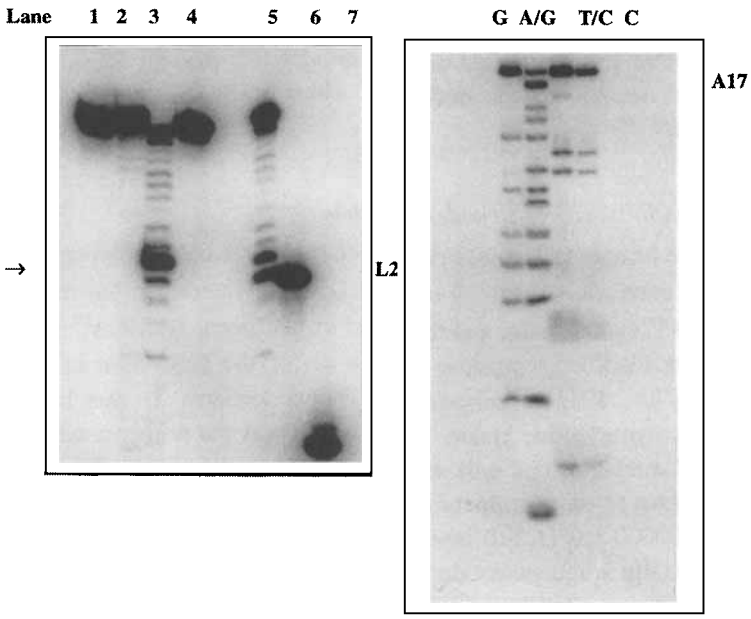


Fig. 4.: Generation of single stranded DNA strands by Klenow Polymerase I

Lane: 1: Template complementary sequence;

2: Polymerisation with template OR-28;

3: Polymerisation with template PD-1-DB-28;

4: see lane 1; 5: Polymerisation with template PS-AN-28; 6: oligonucleotide for comparison, length L2; 7: Primer sequence

Fig. 5.

Fig. 5.: Autoradiogram of the electrophoretic separation of the Maxam and Gilbert sequencing reaction on 20% polyacrylamide gel. Sequencing of the truncated sequence Fig. 5 ; lane 3 (→).

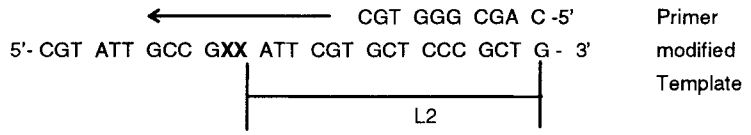
from these synthesis were purified by polyacrylamide gel electrophoresis. The sequences of the synthesized oligonucleotides are given in Table 1.

Nucleolytic degradation studies

The behavior towards snake venom phosphodiesterase has often been used as a model in degradation studies. The enzyme is known to cleave biological oligonucleotides from their 3' end within 5-15 min at 37°C [9]. Digestion with snake venom phosphodiesterase was performed by incubating 1 OD modified- or native oligonucleotide (in 50µl aq.) with 4µl snake venom solution, 4µl alkaline phosphatase and 42µl 10x alkaline phosphatase reaction buffer for 90 min at 37°C. After this time the enzyme was inactivated by boiling for 2 min. The unmodified oligonucleotides (OR-17-1 and OR-17-2) show no resistance against snake venom at all. The PNA-DNA dimer block modified oligonucleotides (PD-1-DB-17 and PD-2-DB-17) were sufficient to confer resistance against nucleolytic degradation in biological systems (Fig 2). The PNA-oligonucleotides (PD-1-DB-17 and PD-2-DB-17) were only slowly degraded within 90 min. The appearance of truncated chains (Fig. 2) suggests that the resistance of the modified oligonucleotide is at the site of PNA modification. On the other hand modified oligonucleotides (PS-AN-17; PS-AN-28) containing the PNA-Spacer (serinol acyclic nucleoside) show no stability against exonucleolytic cleavage (Fig. 3).

Replication of modified single-stranded DNA templates

DNA Polymerase I Large (Klenow) Fragment consists of a single polypeptide chain which lacks the 5'→3' exonuclease activity of intact DNA polymerase I, but retains the 5'→3' polymerase, 3'→5' exonuclease, and the strand displacement activities[10]. We used in our enzymatic studies modified templates (scheme 2) for the generation of single stranded DNA strands by the Klenow enzyme. The Primer (scheme 2) was labelled with [γ-^{32}P]ATP by T4 polynucleotide kinase. The first step was the primer annealing by heating the template and a labelled and unlabelled primer in a microcentrifuge tube at 90°C and slowly cooling down to room temperature (2µg (2µl) oligonucleotide; 0,7µg (0,7µl) unlabelled Primer, 100000 cps (1,3µl) labeled Primer). After this we prepared the following reaction mixture: 4µl oligonucleotide/Primer solution, 4,3µl bidest. water, 10xreaction buffer (Promega information sheet 525), 10µl of a 0,5mM solution of dNTPs and 1µl (5u) of the Klenow enzyme. The reaction mixture was incubated at room temperature for 2 hours. The enzyme reaction was stopped by heating the mixture at 75°C for 10 minutes. The samples were lyophilized, resuspended in loading buffer (80% formamide, 50mM tris-borate, pH 8,3, 1mM EDTA, 0,1% wt/vol xylene cyanol, 0,1% wt/vol bromphenolblue) and applied to a 20% polyacrylamide gel containing 7 M urea. By using modified template PD-1-DB-28 (oligonucleotide containing PNA-DNA dimer block) the enzyme reaction stopped partly at the DNA-part of the PNA-DNA dimer block (see Fig. 4 lane 3 and Fig. 5), but also full length product was obtained. In the case of template PS-AN-28 the enzymatic polymerisation stopped one base before the PNA-Spacer and directly at the modification, but also in this case full length product was obtained (Fig 5, lane 5). So in these



Template	XX	
OR-28	T T	(unmodified deoxythymidines)
PD-1-DB-28	T#T	(PNA-DNA-dimer block)
PS-AN-28	T T	(PNA-spacer; acyclic nucl.)

Scheme 2.: Generation of single stranded DNA strands by using modified templates by Klenow Polymerase I.

cases the Klenow enzyme partly accepts the PNA modifications and generates a single stranded DNA strand. We found that in the case of modified templates the unusually long reaction time of two hours and a very high concentration of dNTPs are necessary for the enzyme reaction. In the case of a reaction time of 0.5 hours and a lower concentration of dNTPs the enzyme reaction showed only truncated sequences.

Sequencing of the generated single stranded DNA

The sequence analysis clarified, that in the case of oligonucleotides containing the PNA-DNA dimer block (Fig.1; A; oligonucleotide PD-1-DB-28), the enzymatic reaction stopped partly at the DNA side of the dimer block by correctly incorporating a deoxyadenosin-5'-triphosphate. Sequence analysis was done by the method of Maxam and Gilbert with an Oligonucleotide Sequencing Kit (Boehringer-Mannheim) after gel isolation of the main truncated sequence in Fig. 5; lane 3; (→).

Experimental

Syntheses were controlled by ^{1}H, ^{13}C, ^{31}P, and 200MHz and 500MHz-NMR (Bruker AC 200, Bruker AM 500) and by MS (Varian MAT 711, Finnigan MRT, Finnigan TSU 7000). The synthesis of the oligonucleotides was done on a DNA synthesizer (Pharmacia Gene Assembler 4 Primers). The oligonucleotides were purified by a RP-HPLC system (Applied Biosystem Modell 152 A, LiChroCART 125-4 Merck). The SVPDE were purchased from Boehringer Mannheim and the Polymerase I Klenow Large Fragment from USB and Promega. All other experiments were performed as described in „Results".

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