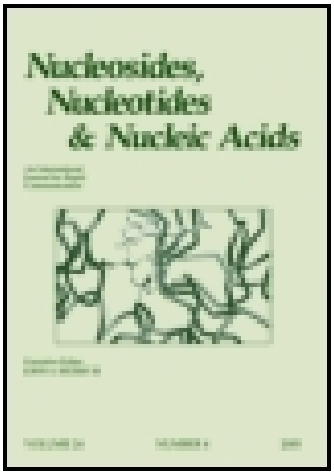

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Synthesis of 3′,5′-Dithymidylyl-α-hydroxyphosphonate Dimer Building Blocks for Oligonucleotide Synthesis—A New Pro-oligonucleotide

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SYNTHESIS OF 3',5'-DITHYMIDYLYL-α-HYDROXYPHOSPHONATE DIMER BUILDING BLOCKS FOR OLIGONUCLEOTIDE SYNTHESIS

- A NEW PRO-OLIGONUCLEOTIDE APPROACH -

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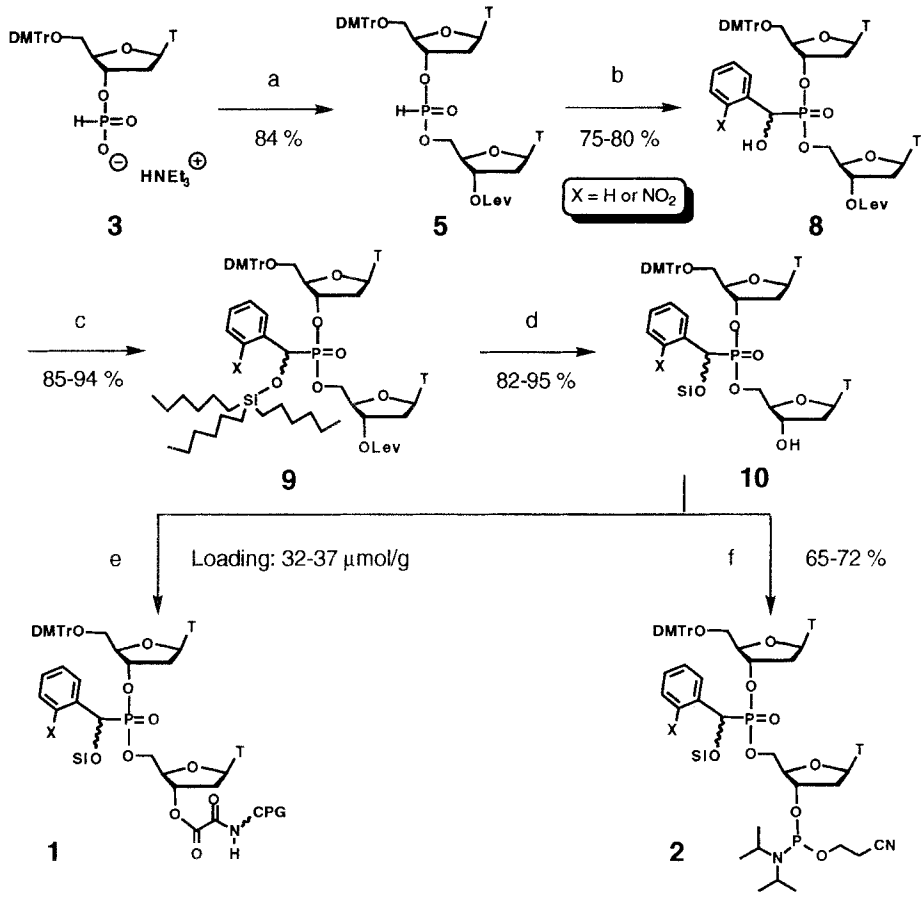
ABSTRACT: The synthesis of the dimer building blocks **1** and **2** and their introduction into $(T)_{15}$-oligonucleotides is described. The stability against 3'-exonuclease digestion (SVP) as well as the hybridization properties (T_m values) were examined.

DNA or RNA antisense oligonucleotides are an important possibility to treat viral diseases. The mode of action is the hybridization of an antisense oligonucleotide with a complementary sequence of the sense-RNA target strand[1]. Unfortunately natural oligonucleotides showed no biological activity because of low penetration through biomembranes and of high instability against cellular endo- and exonuclease activity. In order to circumvent these limitations different chemically modified oligonucleotides have been synthesized: methylphosphonates, phosphorothioates, phosphorodithioates and phosphotriesters as backbone modifications were introduced[2]. All these modifications are more lipophilic than the natural phosphodiester oligonucleotide and much more stable against exonucleases: Most of them are not degradable which is not always desired because of toxic side effects. The consequence for this purpose is the use of ***Pro-oligonucleotides***[3].

In this work we present the α-hydroxybenzylphosphonate moiety as a new lipophilic phosphodiester-backbone modification, which could also act as a ***Pro-oligonucleotide-concept***[4]. It was shown before, that α-hydroxybenzylphosphonates bearing strong electron-withdrawing substituents in the aromatic ring rearrange to yield benzylphosphotriesters[5]. On the other hand, introduction of an electron-donating substituent leads to the direct cleavage reaction[5]. Therefore two different derivatives of the dimer building blocks were synthesized: one dimer contains a α-hydroxy-2-nitrobenzyl residue (predominantly rearrangement) whereas the second is the unsubstituted α-hydroxybenzyl moiety (exclusively direct cleavage). Both dimer building blocks were prepared as 3'-oxalyl-linked on CPG-support **1** and as 3'-phosphoamidites **2**. These dimers should allow the incorporation of the new backbone modi-

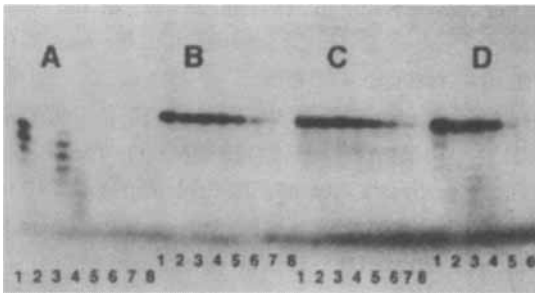


a) 3'-(OLev)T **4**, pivaloylchloride, pyridine, rt, 8 min.; b) benzaldehyde **6** (or 2-nitrobenzaldehyde **7**), CH_2Cl_2, NEt_3 (cat.), rt, 8 h; c) $(n\text{-Hex})_3SiCl$, pyridine, rt, 7 h; d) hydrazine hydrate, pyridine/HOAc (3:2), pyridine, rt, 3 min.; e) oxalylchloride, 1,2,4-triazole, CH_3CN, pyridine, rt, 1 h, then AP-CPG-support (550 Å), rt, 16 h; f) bis(diisopropylamino)-β-cyanoethylphosphine, CH_2Cl_2/CH_3CN (1:1), 1H-tetrazole, rt, 3 h

SCHEME 1: Synthesis of the dimer building blocks **1** and **2**

fication into an oligonucleotide following the phosphoamidite chemistry at different positions: at the 3'-, at the 5'-terminus as well as mixed modified oligonucleotides containing internal and terminal modifications. Here we concentrate on the synthesis of 3'-modified oligo's.

The syntheses of **1** and **2** are summarized in scheme 1. The syntheses, which uses H-phosphonate chemistry, starts from thymidyl-3'-H-phosphonate **3** which was coupled with 3'-levulinylthymidine **4** [3'-(OLev)T] to yield the H-phosphonate diester **5**[6]. Diester **5** was reacted with benzaldehydes (X=H **6** or NO_2 **7**) to give the corresponding α-hydroxybenzyl-phosphonates **8**[7]. The α-hydroxyl groups of **8** were protected using the trihexylsilyl group to give **9**. Compound **10** is the key intermediate for **1** and **2**, which could be isolated after deprotection of the levulinyl group from **9** with hydrazine hydrate. **1** could be prepared using



Probes 1-8: **1**: 0 min., **2**: 2 min., **3**: 5 min., **4**: 10 min., **5**: 30 min., **6**: 60 min., **7**: 120 min., **8**: 300 min.; Test: 1 OD of each oligonucleotide

FIGURE 1: PAGE-Gel (15% acrylamide, 10 M urea) of the digestion of the $(T)_{15}$-oligonucleotides with 0.009 U of SVP in 32 mM TRIS buffer with 15 mM $MgCl_2$ at 37 °C

TABLE 1: Melting temperatures (T_m values) of the different modified $(T)_{15}$-oligonucleotides on a 1.0 μM-scale in 10 mM HEPES buffer with 140 mM NaCl (pH 6.8)

Oligonucleotide	5'-(T)13TpT-3'	5'-(T)13TpT-3'	5'-(T)13TpT-3'	5'-(T)13TpT-3'
T_m value against $(dA)_{15}$ [°C]	38.1	38.4	37.0	38.2
T_m value against $(rA)_{15}$ [°C]	33.6	33.9	33.1	33.0

the procedure of Letsinger[8] with an average loading of 32-37 μmol/g. The phosphoamidites **2** were obtained from **10** in 65-72% yield after reaction with bis(di*iso*propylamino)-β-cyanoethylphosphine in the presence of 1H-tetrazole.

3'-modified $(T)_{15}$-oligonucleotides were synthesized with the dimer building blocks **1** on a 1.0 μM-scale. After deprotection of the β-cyanoethyl groups with di*iso*propylamine at room temperature for 14 h, the oligomers were liberated from the solid support within 5 min. with a cold saturated solution of ammonia in dry methanol. It is necessary to avoid any moisture in this step. Then the cleavage was stopped by dropwise addition of 50% HOAc until pH≤7. After desalting on a Bond Elut C18-column the purity of the oligonucleotides were examined by HPLC and electrospray (ESI) mass spectrometry.

The stability of the synthesized 3'-modified $(T)_{15}$-oligonucleotides was tested against snake venom phosphodiesterase (SVP) in a 32 mM TRIS buffer containing 15 mM $MgCl_2$

(pH 6.8) at 37 °C (as shown in Figure 1). The stability of the modified oligonucleotides against 3'-exonucleases (lane B, C and D) in contrast to the natural $(T)_{15}$-mer (lane A) was six fold enhanced from ≈ 8 min. to ≈ 60 min.

The hybridization properties of the 3'-modified $(T)_{15}$-oligonucleotides were measured against $(dA)_{15}$ and $(rA)_{15}$ as references (see table 1). The melting temperatures were determined in a 1.0 μM-scale in a 10 mM HEPES buffer with 140 mM NaCl (pH 6.8) in a range from 10 °C to 80 °C. The observed T_m values of the unmodified and the modified oligonucleotides were in a range of 0.5 °C in all cases.

In summary, the use of the dimer building blocks **1** and **2** allows efficient incorporation of the α-hydroxybenzylphosphonate moiety into oligonucleotide conjugates. These should act as potential ***pro-oligonucleotide***, releasing the unmodified oligonucleotide after rearrangement and hydrolytic cleavage of the resulting phosphotriester or after direct cleavage. The stability of the modified $(T)_{15}$-oligonucleotides against exonucleases showed a significant enhancement in contrast to the natural $(T)_{15}$-oligonucleotide. The melting temperatures (T_m values) of the modified oligonucleotides against $(dA)_{15}$ and $(rA)_{15}$ were in the same range as the unmodified oligonucleotide.

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