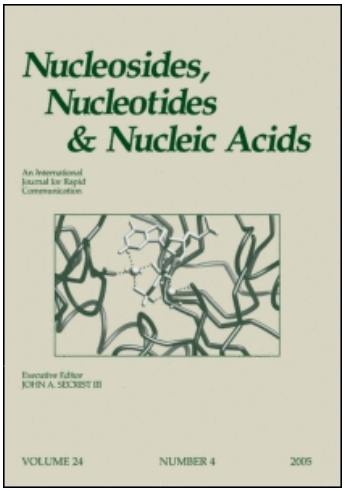

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Fpmp-Protected α-Hydroxyphosphonate Diesters for The Synthesis of Pro-Oligonucleotides

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FPMP-PROTECTED α-HYDROXYPHOSPHONATE DIESTERS FOR THE SYNTHESIS OF PRO-OLIGONUCLEOTIDES

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ABSTRACT: The synthesis of Fpmp-protected α-hydroxybenzylphosphonate diesters as building blocks for the oligonucleotide synthesis is described. The modified oligonucleotides obtained after cleavage from the solid support and deprotection of the acid-labile Fpmp group should lead to potential *pro-oligonucleotides.*

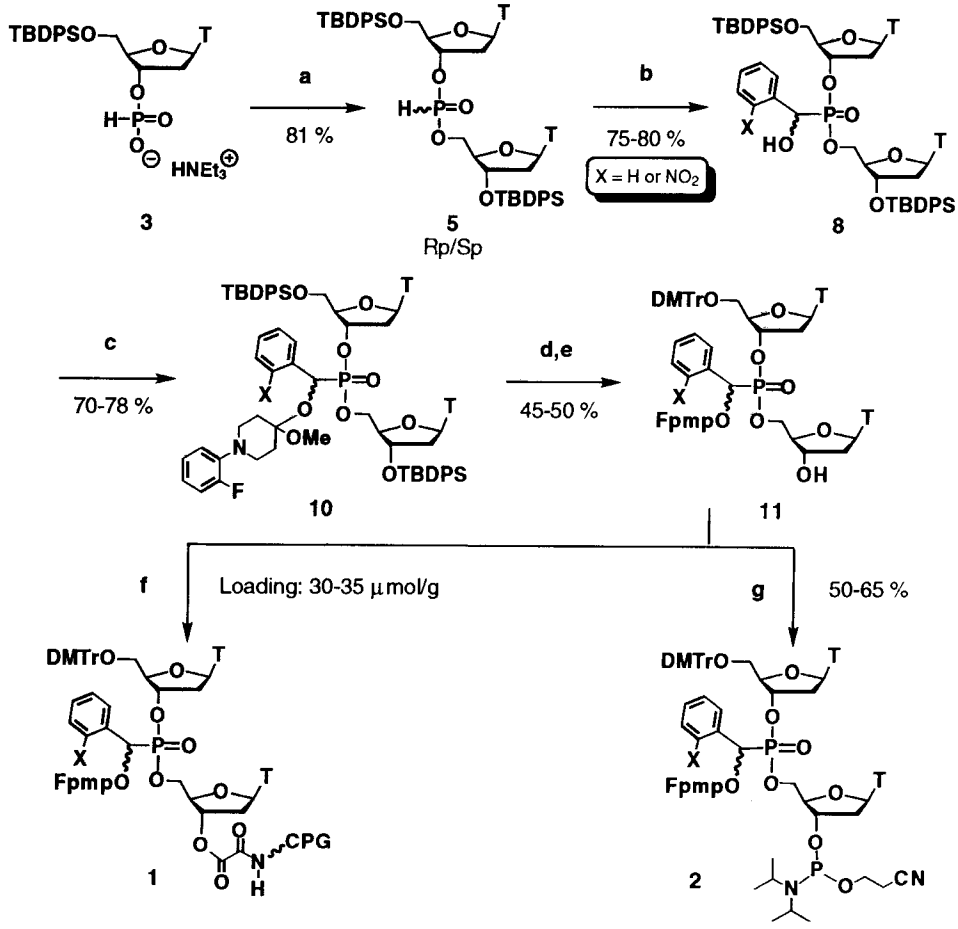
Antisense oligonucleotides on the basis of DNA or RNA have become an important possibility to treat viral diseases[1]. The mode of action is the hybridization of an antisense oligonucleotide with a complementary sequence of the sense-RNA target strand. Beside the selective recognition of their target, oligonucleotides have to be taken up by cells and have to be nuclease-resistant. Natural oligonucleotides showed no biological activity because of low penetration through biomembranes and high instability against cellular endo- and exonucleases. Methylphosphonate-, phosphorothioate-, phosphorodithioate- and phosphotriester-modified oligonucleotides have been synthesized to circumvent these limitations[2]. These modifications are more lipophilic than natural phosphodiester oligonucleotides and more stable against nucleases. But 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 previous studies, we presented the α-hydroxybenzylphosphonate moiety as a new lipophilic phosphodiester-backbone modification, which could additionally act as a ***pro-oligonucleotide-concept***[4]. It was shown, that α-hydroxybenzylphosphonates bearing strong electron-withdrawing substituents in the aromatic ring rearrange into the corresponding benzylphosphotriesters[5]. On the other hand, introduction of an electron-donating substituent resulted the direct cleavage reaction. Therefore we decided to synthesize two different derivatives of dimer building blocks: one dimer contains a α-hydroxy-2-nitrobenzyl residue (predominantly rearrangement) whereas the second is the unsubstituted α-hydroxybenzyl moiety (exclusively direct cleavage). In our earlier work, we used different silyl-protected α-hydroxyphosphonates as building blocks for the synthesis of $(T)_{15}$-oligonucleotides[4]. To

cleave the oligomers from the solid support, we used a cold saturated solution of ammonia in dry methanol within 5 min. This method is suitable for the preparation of polythymidine oligonucleotides, but unlikely to synthesize mixed oligonucleotides because we have to use aqueous ammonia to cleave the base-protecting groups if we use the standard amidites for oligonucleotide synthesis (dA^{bz}, dG^{ibu} and dC^{bz}). Herein we make use of the 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl (Fpmp) group, which was first introduced by Reese for the protection of 2'-hydroxy functions in oligo*ribo*nucleotide synthesis[6]. Using this acid-labile group, α-hydroxyphosphonate modified oligonucleotides should be cleaved still Fpmp-protected from the solid support by aqueous ammonia. After desalting and purification, the Fpmp unblocked oligonucleotides can be isolated after treatment with a solution of 0.01M hydrochloric acid at pH 2.0-2.5 or with a solution of sodium acetate at pH 3.25 as described by Rao[7]. Under these acidic conditions, no cleavage or rearrangement of the α-hydroxyphosphonate moiety occurs. The Fpmp-protected dimer building blocks were prepared as 3'-oxalyl-linked to the AP-CPG-support **1** and as 3'-phosphoamidites **2**.

The syntheses of **1** and **2** are shown in scheme 1. The syntheses start from thymidyl-3'-H-phosphonate **3** which was coupled with 3'-*O*-*t*-butyldiphenylsilylthymidine **4** [3'-(*O*TBDPS)T]. **4** was synthesized via 5'-*O*-dimethoxytritylthymidine with *t*-butyldiphenyl-silylchloride and deprotection of the trityl group with a solution of 2% benzoic acid in CH_2Cl_2/MeOH 7:3 to yield the 3'-silyl masked thymidine. We use the TBDPS group to protect the 5'- and 3'-positions of the dimer because these are stable against trifluoroacetic acid (TFA) used to incorporate the Fpmp group and for a better separation of the stereoisomers of **5** due to this bulky silyl group. The H-phosphonate diesters **5** were obtained as a 1:1 diastereomeric mixture with respect to the chiral phosphorus center[8]. The separation of the stereoisomers was achieved by silica gel chromatography using ethyl acetate/CH_2Cl_2 3:1 + 0.1% acetic acid. The ^{31}P-NMR chemical shifts in $CDCl_3$ are 7.54 ppm for the "fast"- and 9.41 ppm for the "slow"-diastereomer. Diesters **5** were reacted with benzaldehyde **6** or 2-nitrobenzaldehyde **7** to give the α-hydroxybenzylphosphonates **8** again in a 1:1 diastereomeric mixture with respect to the new chiral center at the α-C-atom[9]. The stereoisomers **8** were inseparable using flash chromatography and consequently the further syntheses were carried out with the diastereomeric mixtures. For the introduction of this acetalic Fpmp-protecting group 1-(2-fluorophenyl)-4-methoxy-1,2,5,6-tetrahydropyridine **9** was synthesized as described by Reese[6]. The fully blocked dimers **10** could be prepared in 70-78% yield by slightly changing the literature conditions using 3 eq. of **9** and 3 eq. of TFA in small amounts of CH_2Cl_2 as the solvent. Compound **11** is the key intermediate for the building blocks **1** and **2**. Compounds **11** were isolated after deprotection of the TBDPS groups in **10** using triethylamine trihydrofluoride (NEt_3•3HF; 60-70% yield) and subsequent selective 5'-protection with 4,4'-dimethoxytritylchloride (70-75% yield). We used NEt_3•3HF to cleave the TBDPS groups because the purification of the products was easier achieved than in the case of the tetrabutylammonium salt of the products when using TBAF in THF.

The yields of the two steps are not optimized. **1** could be prepared using the procedure of Letsinger[10] with an average loading of 30-35 μmol/g. The phosphoamidites **2** were obtained



a) 3'-(*O*TBDPS)T **4**, pivaloylchloride, pyridine, rt, 8 min; b) benzaldehyde **6** (or 2-nitrobenzaldehyde **7**), CH_2Cl_2, NEt_3 (cat), rt, 8h; c) 1-(2-fluorophenyl)-4-methoxy-1,2,5,6-tetrahydropyridine **9**, CH_2Cl_2, trifluoroacetic acid, rt, 6-18h; d) triethylamine trihydrofluoride, triethylamine, rt, 12h; e) 4,4'-dimethoxytrityl-chloride, DMAP, NEt_3, pyridine, 35°C, 10-15h; f) oxalylchloride, 1,2,4-triazole, CH_3CN, pyridine, rt, 1h, then AP-CPG-support (550 Å), rt, 16h; g) bis(di*iso*propylamino)-β-cyanoethylphosphine, CH_2Cl_2/CH_3CN (1:1), 1H-tetrazole, rt, 3h

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

from **11** after reaction with bis(di*iso*propylamino)-β-cyanoethylphosphine in the presence of 1H-tetrazole.

With the dimer building blocks **1** we will be able to synthesize oligonucleotides containing one α-hydroxyphosphonate modification at the 3'-terminus. Using the phospho-amidites **2** we modify oligonucleotides at the 5'-end as well as within the backbone. Deprotection of the β-cyanoethyl groups and cleavage from the solid support using aqueous ammonia result the oligomers with the Fpmp group still at the α-hydroxy function. Acidic

cleavage of the Fpmp group will finally yield the fully unblocked, modified oligonucleotides. Furthermore, the Fpmp-protected α-hydroxyphosphonate dimers should allow the synthesis of oligonucleotides with mixed sequences containing standard base-protecting groups.

In summary, the successful synthesis of the dimer building blocks **1** and **2** demonstrates the incorporation of the Fpmp protecting group into α-hydroxybenzylphosphonates. The use of these building blocks in the oligonucleotide synthesis should improve the yields of protected α-hydroxyphosphonate modified oligomers. Further studies are in progress in our laboratory in order to prove their ability to act as potential *pro-oligonucleotides*. The results will be published in due course.

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