STUDIES ON TRANSFORMATIONS OF H-PHOSPHONATES INTO DNA ANALOGUES CONTAINING P-S OR P-C BONDS

by

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Abstract

In this thesis, mechanistic and synthetic studies on transformations of H-phosphonates into DNA analogues containing P-S or P-C bonds are described. Configurational stability of dinucleoside H-phosphonates and the stereochemical course of their sulfurisation in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) were investigated. In light of these studies, the reported stereoselective sulfurisation of dinucleoside H-phosphonates and benzoylphosphonates in the presence of DBU was proved to be incorrect.

Efficient protocols for the synthesis of new nucleotide analogues with non-ionic C-phosphonate internucleotide linkages were developed. The synthesis of dinucleoside 2-pyridylphosphonates was successfully performed by a DBU-promoted reaction of H-phosphonate diesters with N-methoxypyridinium salts. The thio analogues, 2-pyridyl- and 4-pyridyl phosphonothioate diesters, could be obtained by modifying the reactions developed for their oxo counterparts. Dinucleoside 3-pyridylphosphonates were prepared via a palladium(0)-catalysed cross coupling strategy that could be extended also to the synthesis of nucleotide analogues with metal-complexing properties, i.e. terpyridyl- and bipyridylphosphonate derivatives.

Oligonucleotides modified with pyridylphosphonate internucleotide linkages have been prepared and preliminary studies on their hybridisation properties and resistance towards enzymatic degradation were performed.

Finally, nucleotidic units for the incorporation of pyridylphosphonate groups at the 5’-terminus of oligonucleotides were designed. Condensations of such units with a suitably protected nucleoside afforded after oxidation the expected dinucleoside (3’-5’)-phosphates with pyridylphosphonate monoester functions at the 5’-ends.
List of Papers

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. The Case for Configurational Stability of H-Phosphonate Diesters in the Presence of Diazabicyclo[5.4.0.]undec-7-ene
   Tommy Johansson and Jacek Stawinski
   *Bioorganic and Medicinal Chemistry, 2001, 9, 2315-2322.

II. 2-Pyridylphosphonates: A New Type of Modification for Nucleotide Analogues
   Tommy Johansson, Annika Kers and Jacek Stawinski

III. Nucleoside H-phosphonates. Part 19: Efficient Entry to Novel Nucleotide Analogues with 2-Pyridyl- and 4-Pyridylphosphonothioate Internucleotide Linkages
    Tommy Johansson and Jacek Stawinski

IV. Synthesis of Dinucleoside Pyridylphosphonates Involving Palladium(0)-catalysed Phosphorus-carbon Bond Formation as a Key Step
    Tommy Johansson and Jacek Stawinski
    #Chemical Communications, 2001, 2564-2565.

V. Towards Nucleotide Analogues with Metal Complexing Properties. Synthesis of Dinucleoside Terpyridyl- and Bipyridylphosphonates
   Tommy Johansson and Jacek Stawinski
   In manuscript.

VI. Novel DNA Analogues with 2-, 3- and 4-Pyridylphosphonate Internucleotide Bonds: Synthesis and Hybridisation Properties
    Katarzyna Zmudzka, Tommy Johansson, Marzena Wojcik, Magdalena Janicka, Marian Nowak, Jacek Stawinski and Barbara Nawrot.

VII. Synthesis of a Nucleotide Unit for the Incorporation of a Terminal 5’-Pyridylphosphonate Moiety into Oligonucleotides
    Tommy Johansson and Jacek Stawinski
    In manuscript.

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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>A</td>
<td>Adenosine</td>
</tr>
<tr>
<td>AcOH</td>
<td>Acetic acid</td>
</tr>
<tr>
<td>B</td>
<td>a Nucleobase</td>
</tr>
<tr>
<td>BDT</td>
<td>1,3-Benzodithiol-2-yl</td>
</tr>
<tr>
<td>BINAP</td>
<td>2,2'-Bis(diphenylphosphino)-1,1'-binaphtyl</td>
</tr>
<tr>
<td>BSA</td>
<td>N,N-Bis(trimethylsilyl)acetamide</td>
</tr>
<tr>
<td>C</td>
<td>Cytidine</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
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<tr>
<td>DBPB</td>
<td>2-(Di-tert-butylphosphino)biphenyl</td>
</tr>
<tr>
<td>DBU</td>
<td>1,8-Diazabicyclo[5.4.0]undec-7-ene</td>
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<tr>
<td>DDQ</td>
<td>2,3-Dichloro-5,6-dicyanobenzoquinone</td>
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<td>DMT</td>
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</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPHP</td>
<td>Diphenyl H-phosphonate</td>
</tr>
<tr>
<td>DPPE</td>
<td>1,2-Bis(diphenylphosphino)ethane</td>
</tr>
<tr>
<td>DPPF</td>
<td>1,1'-Bis(diphenylphosphino)ferrocene</td>
</tr>
<tr>
<td>DPPP</td>
<td>1,3-Bis(diphenylphosphino)propane</td>
</tr>
<tr>
<td>G</td>
<td>Guanosine</td>
</tr>
<tr>
<td>MDS</td>
<td>Mesitylene-1,3-disulfonyl chloride</td>
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<tr>
<td>Me-Oligos</td>
<td>Methylphosphonate oligonucleotides</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<td>Pivaloyl chloride</td>
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<tr>
<td>T</td>
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</tr>
<tr>
<td>TBAF</td>
<td>Tetrabutylammonium fluoride</td>
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<tr>
<td>TBDMS</td>
<td>tert-Butyldimethylsilyl</td>
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<tr>
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<td>Triethylamine</td>
</tr>
<tr>
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<td>Trifluoroacetic acid</td>
</tr>
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<td>Tetrahydrofuran</td>
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<tr>
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<td>Thymine</td>
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<tr>
<td>T_m</td>
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</tr>
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<td>TMS-Cl</td>
<td>Trimethylsilyl chloride</td>
</tr>
<tr>
<td>Tr-Cl</td>
<td>Triphenylmethyl chloride</td>
</tr>
<tr>
<td>U</td>
<td>Uridine</td>
</tr>
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1. Oligonucleotides as Therapeutic Agents

1.1 General Introduction

Until now, most drugs have been discovered by a trial-and-error approach, although rational design would be ideal. This is mainly due to complicated interactions between the active substances and the target molecules (usually proteins) that are often poorly understood. In this context, therapeutics acting at the level of nucleic acids and preventing formation of defective proteins appears to be an attractive approach to drug design.

In 1978 Zamecnik and Stephenson first proposed the use of short fragments of DNA as antisense agents for therapeutic purposes. The concept of the antisense approach is relatively straightforward: an oligonucleotide complementary to the target mRNA strand by virtue of Watson-Crick base pair hybridisation (Figure 1), can inhibit the translation process and thereby block the transfer of genetic information from DNA to protein. This inhibition of gene expression seems more attractive than targeting the resulting protein, since an antisense drug can be rationally designed on the basis of the known sequence of RNA. Gene expression can also be regulated at the level of DNA transcription and this approach is referred to as the antigene strategy. In this approach, the antigene oligonucleotide is hybridised to the target double stranded DNA (via Hoogsteen base pairing), forming a triplex and thus inhibiting transcription by blocking the gene from RNA polymerase.

Figure 1.1 Watson-Crick base pairing.

Whether the mRNA (antisense approach) or DNA (antigene approach) should be the preferred target is a matter of discussion since both approaches have their own merits. By blocking the DNA, gene expression is most efficiently suppressed. However, the antigene oligonucleotide has to not only cross the cell membrane, but the nuclear membrane as well to exert its biological effect. At the present stage, the antisense strategy seems to be a more promising therapeutic technique. Hence, the main focus has been on the antisense rather than the antigene approach.
Provided that the base sequence of a target mRNA is known, the design of a potential antisense drug should in theory be simple according to the rules of base pairing. However, secondary and tertiary structures and bound proteins make only a few of the potential binding sites in a specific mRNA available in practice. There are also several basic properties required for an oligonucleotide to be useful as an antisense therapeutic agent. Such an oligonucleotide must, for example, i) efficiently penetrate through the cell membrane, ii) be resistant towards chemical and enzymatic degradation, iii) bind specifically to the complementary target sequence, and iv) form stable complexes with the target sequence. In addition, an antisense drug should target the specific tissues and/or organs where it is supposed to act. As natural oligonucleotides undergo rapid degradation by intracellular endo- and exonucleases, the use of modified oligonucleotides became a necessity in the development of drugs based on the antisense/antigene methodology.

After the first reports by Zamecnik and Stephenson1,2 on inhibition of viral infections by synthetic oligonucleotides, a wide array of modifications positioned at different regions of oligonucleotides have been proposed (Figure 1.2).4,5 A crucial feature for an antisense/antigene oligonucleotide is to maintain good hybridisation properties, i.e. there should be no interference in hydrogen bonding with the target nucleic acid sequence. Hence, modifications are usually made in the phosphate backbone rather than in the sugars or bases. Numerous modifications of the internucleotidic bond have been studied, e.g. phosphorothioates,6 phosphorodithioates,7 methylphosphonates,8 phosphoroamidates,9 as well as analogues with non-phosphorus internucleosidic linkages, like siloxanes10 and carbamates.11

Figure 1.2 Possible positions for modifications in oligonucleotides.
As to the synthesis of oligonucleotides, there are in principle four types of methods available. Apart from the phosphodiester\textsuperscript{12} and phosphotriester\textsuperscript{13} approaches that are seldom used nowadays, there are the phosphoramidite and the H-phosphonate methods.

The phosphoramidite method according to Caruthers and Beaucage,\textsuperscript{14–16} that originates from Letsinger's phosphite triester method,\textsuperscript{17} is the most commonly used approach for automated solid phase synthesis of oligonucleotides. The standard protocol involves a tetrazole-catalysed coupling of a nucleoside 3’-β-cyanoethyl N,N-diisopropylphosphoramidite with the 5’-end of the growing oligonucleotide, and a subsequent oxidation by iodine/water (Scheme 1.1). The β-cyanoethyl group is cleaved off at the end of the synthetic cycle.

In recent years, the H-phosphonate method,\textsuperscript{18–23} first described in 1957 by Todd,\textsuperscript{24} has been established as a versatile complement to the phosphoramidite approach. It can even be argued as the preferred method of choice, since the nucleoside H-phosphonate synthons are easier to handle and no phosphate protective groups are needed during the synthesis. The method consists of a pivaloyl chloride promoted condensation between a nucleoside 3’-H-phosphonate and the 5’-hydroxyl residue of the oligonucleotide (Scheme 1.2). The H-phosphonate linkages can, in contrast to phosphoramidites, be oxidised with iodine/water at the end of the synthetic cycle, i.e. when the oligomer construction is complete.

\begin{center}
\textbf{Scheme 1.1} The phosphoramidite method.
\end{center}
Phosphorothioate Nucleic Acids

Probably the most conservative modification of natural phosphate linkages is the phosphorothioate bond, where one of the non-bridging oxygens has been substituted by a sulfur atom. Phosphorothioate oligonucleotides (S-oligos) were studied early in the history of antisense methodology and their properties have been well examined. S-oligos combine several features that are unique for this type of modification. These are, e.g. (i) high solubility in water (the negative charge is preserved in the phosphorothioate bond), (ii) increased stability towards nuclease degradation, (iii) efficient and selective hybridisation properties with target mRNA, and (iv) the formed duplexes are substrates for RNase-H, i.e. the target mRNA is enzymatically cleaved. The fact that S-oligos render the target mRNA susceptible to degradation by RNase-H is a significant advantage since the mRNA is irreversibly destroyed and the antisense oligonucleotide is released. Hence, a catalytic cycle is achieved.

It was thus hardly surprising that the first antisense drug on the market, Vitravene®, was an oligonucleotide with a phosphorothioate-modified backbone. Vitravene® is a 21 unit long oligonucleotide that was released in 1998 for the treatment of cytomegalovirus induced retinitis in the eyes of immunodeficient patients. The drug efficiently targets cytomegalovirus mRNA and is administrated as eyedrops. Several other antisense drugs with phosphorothioate backbone are in various stages of clinical trials.

Although S-oligos have several advantages, the chirality of the phosphorothioate linkage that results in formation of \(2^n\) diastereomers for \(n\) introduced phosphorothioate bonds, may pose some problems. S-Oligos have been synthesized by using various protocols, e.g. the H-phosphonate approach and the phosphoramidite approach (in both cases, the iodine/water system is replaced by sulfur in the oxidation step). These methods produce S-oligos in good yields, but generate racemic phosphorothioate linkages. In the case of Vitravene®, which is synthesized by the
phosphoramidite method, $2^{20}$ diastereomers are formed. Of course it is not desirable for a drug to contain over 1 million isomers, as each isomer in principle may have different properties and only some of them might be highly active. To overcome these shortcomings, the search for stereocontrolled synthesis of S-oligos has been in focus of many research groups during the last decades.\textsuperscript{33}

In 1986, Hata \textit{et al.} reported on a stereoselective synthesis of R\textsubscript{P} diastereomeric phosphorothioate dimers T\textsubscript{S}T and dA\textsubscript{S}T\textsuperscript{34} (Scheme 2.1, Chapter 2). In this reaction, a diastereomeric mixture of dinucleoside benzoylphosphonate was treated with sulfur in the presence of $n$-butylamine and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), producing exclusively the R\textsubscript{P} phosphorothioate in \textit{ca} 60\% yields. A complementary method, where a preferential formation of the S\textsubscript{P} phosphorothioate diastereomer could be achieved, was published by Cossticks and Williams in 1987.\textsuperscript{35} This reaction involved condensation of a $\beta$-cyanoethyl S-protected nucleoside 3'-phosphorothioate with a 3'-protected nucleoside, using 1-(mesitylenesulfonyl)-5-(2-pyridyl)tetrazole as coupling agent. After deprotection, the S\textsubscript{P} isomer was obtained in 80\% excess, but the overall yield was poor. More recently, a number of stereoselective methods for the formation of P-chiral internucleotide linkages were developed.\textsuperscript{36-41} All of these methods make use of nucleoside phosphoramidites in which the phosphorus atom is part of a cyclic system that usually bears chiral, bulky auxiliaries attached to the phosphorus centre (Scheme 1.3).

![Scheme 1.3](image)

\textit{Scheme 1.3} Stereoselective approach for the synthesis of dinucleoside phosphorothioates.

As to stereospecific formation of internucleotide phosphorothioate linkages, only a few methods are available.\textsuperscript{42-44} The most recent method,\textsuperscript{44} developed in this lab, consists of a stereospecific coupling of a diastereomerically pure nucleoside-3'-yl 1-oxido-4-methoxy-2-picoly1 phosphorothioate with a 3'-deprotected nucleoside (Scheme 1.4). The picoline $N$-oxide residue acts as an intramolecular nucleophilic catalyst, hence a stereospecific conversion is obtained.
Scheme 1.4 Stereospecific synthesis of internucleotidic phosphorothioate linkages.

In Chapter 2 of this thesis, stereochemical and mechanistic investigations on the stereoselective synthesis of phosphorothioates reported by Hata et al., are presented.

1.3 Non-ionic C-phosphonate Nucleic Acids

A class of phosphate analogues that also has received considerable attention are non-ionic C-phosphonate derivatives, where one of the non-bridging oxygens has been replaced with an alkyl or aryl group (Figure 1.3).

By eliminating the negative charge at the phosphorus centre, this class of oligonucleotide analogues was expected to have some favourable properties; e.g. enhanced cellular uptake (the cell membrane is a barrier to polyanionic molecules, whereas neutral molecules can cross it via passive diffusion) and increased binding
affinity to a complementary RNA strand (charge repulsion between the strands is alleviated). Oligonucleotides with these features should be particularly useful as antisense/antigene therapeutics.

The most extensively studied non-ionic C-phosphonate analogues are the methylphosphonate derivatives. Miller et. al. first demonstrated the potential of methylphosphonate oligonucleotides (Me-oligos) as antisense/antigene agents in the early 1980’s, and since then, many research groups have contributed to this field. It was found that Me-oligos (i) form stable duplexes with complementary RNA strands. Melting temperatures ($T_m$ values) of duplexes formed by Me-oligos are usually similar to those formed by the corresponding natural oligonucleotides at high salt concentration but significantly higher than the latter at low salt concentration. The methylphosphonate linkages are (ii) quite stable under physiological conditions and resistant to degradation by both endo- and exonucleases. Me-oligos (iii) penetrate very efficiently through the membrane of some cells, while uptake through the membrane of *E. coli* B cells turned out to be restricted to Me-oligos shorter than five nucleotide units. In contrast to S-oligos, (iv) duplexes formed between Me-oligos and target RNA strands are not substrates for RNase-H. The chirality at the phosphorus centre of methylphosphonates is another complication that gives rise to $2^{n-1}$ diastereomers for an *n* unit long Me-oligo.

Me-Oligos have been prepared by a variety of methods. The most efficient procedure is based on the phosphoramidite approach and involves a tetrazole-promoted coupling of 5’O-dimethoxytritylated nucleoside-3’-yl *N,N*-diisopropylmethylphosphonamidite with the 5’-hydroxyl group of an oligonucleotide and a subsequent oxidation by iodine/water (Scheme 1.5). In 1998, Engels achieved a stereoselective synthesis of a methylphosphonate dimer (84% excess of the $R_P$ diastereomer) by replacing tetrazole with sterically hindered imidazole derivatives.

![Scheme 1.5](image-url)  
**Scheme 1.5** Phosphoramidite approach for the synthesis of methylphosphonates.
Recently, several other non-ionic C-phosphonate DNA analogues, e.g. oligo(nucleoside hydroxymethylphosphonate)s\textsuperscript{54} oligo(nucleoside cyanomethylphosphonate)s\textsuperscript{55} oligo(nucleoside octylphosphonate)s\textsuperscript{56} oligo(nucleoside phenylphosphonate)s\textsuperscript{57,58} and oligo(nucleoside arylalkylphosphonate)s\textsuperscript{59,60} have been prepared and their properties evaluated. It turned out that oligonucleotides modified with phenylphosphonate or arylalkylphosphonate linkages at selected positions have good hybridisation properties, and these types of analogues appear to be the most promising candidates as new antisense/antigene drugs. Interestingly, partially benzylphosphonate-modified oligonucleotides, when used as antisense agents against hepatitis C virus (HCV), were more effective and specific inhibitors of viral replication than the corresponding phosphorothioate or methylphosphonate derivatives.\textsuperscript{61}

Studies on the synthesis of a new class of non-ionic C-phosphonates, pyridylphosphonate derivatives, as well as evaluation of their properties, will be discussed in Chapter 3-8 of this thesis.
2. Investigations on the Configurational Integrity of H-Phosphonate Diesters in the Presence of DBU (Paper I)

2.1 Background

Sulfurisation of configurationally stable tetracoordinated P(III) compounds, e.g. dioxaphosphinanes\textsuperscript{62} and dinucleoside H-phosphonates,\textsuperscript{63-65} is known to proceed stereospecifically with retention of configuration. This constitutes the basis for synthetic applications of these compounds as chiral precursors in various stereospecific transformations.

\[ \text{pseudo-rotation} \]

\[ \text{Scheme 2.1 The mechanism proposed by Hata et al.} \]

In this context, a stereoselective\textsuperscript{66} rather than stereospecific, sulfurisation of dinucleoside H-phosphonates reported by Hata \textit{et al.}\textsuperscript{34,67} attracted our attention as an exception to the commonly observed stereoretentive course of sulfurisation of P(III) compounds. If true, this could open a possibility for a stereocontrolled synthesis of phosphorothioate oligonucleotides based on the H-phosphonate methodology. The authors claimed that when a diastereomeric mixture of dinucleoside benzoylphosphonates of type 3 was treated with \textit{n-}butylamine, 1,8-
Diazabicyclo[5.4.0]undec-7-ene (DBU) and elemental sulfur in pyridine, the exclusive formation of the \( R_P \) phosphorothioate diastereomer 2b was observed (Scheme 2.1).

Hata et al.\(^{34,67} \) explained this unexpected course of the reaction by assuming a DBU-mediated epimerisation of H-phosphonate diester 1, formed via deacylation of benzoylphosphonate 3 by \( n \)-butylamine. According to this mechanism, DBU made a nucleophilic attack on H-phosphonate 1 to form a pentacoordinated intermediate that underwent epimerisation via pseudorotation,\(^{68} \) and after departure of DBU afforded predominantly the \( S_P \) H-phosphonate isomer 1b from which, upon sulfurisation with elemental sulfur, \( R_P \) phosphorothioate 2b was formed. This stereoselectivity was only observed for dinucleoside benzoylphosphonates with 5'-O-dimethoxytrityl (5'-O-DMT) and 3'-O-(1,3-benzodithiol-2-yl) (3'-O-BDT) protecting groups in combination with the nucleoside sequence thymidine-thymidine (T-T) or adenosine-thymidine (A-T). When other hydroxyl protecting groups or nucleoside sequences were used, almost no stereoselectivity was found. It was thus claimed that apparently certain types of interactions between the nucleoside residues in the pentacoordinated DBU-intermediate were responsible for the stereoselectivity of the epimerisation process.

\[
\text{Scheme 2.2 i) General mechanism of pseudorotation; ii) Pseudorotation mechanism applied to Hata's example.}
\]

After a closer look at the mechanistic explanation proposed by Hata et al.,\(^{34,67} \) we found that epimerisation via a pseudorotation pathway should not be possible in this case. According to the pseudorotation mechanism of pentacoordinated phosphorus intermediates,\(^{68} \) two substituents in apical positions are exchanged for two substituents in equatorial positions as illustrated in Scheme 2.2 (path i). These rules, when applied to Hata’s example, show that after one pseudorotation, a DBU molecule would appear in the equatorial position from which it cannot depart (Scheme 2.2, path ii). The intermediate must thus undergo a second pseudorotation to place DBU in an apical position from which it can depart. But this gives back the original configuration at the phosphorus centre.
Although the mechanism proposed by Hata\textsuperscript{34,67} seems to be highly unlikely, epimerisation of H-phosphonate 1 (and a stereoselective formation of one H-phosphonate diastereomer) might have occurred in another way, e.g. via a ligand exchange reaction of 1 mediated by DBU or during the conversion of benzoylphosphonate 3 into H-phosphonate 1. This prompted us to investigate this phenomenon in more detail.

### 2.2 Sulfurisation of H-Phosphonate Diesters in the Presence of DBU

In our initial studies we investigated the sulfurisation of H-phosphonate diester 1 (in the original procedure by Hata et al.\textsuperscript{34,67} produced \textit{in situ} from benzoylphosphonate 3) under the experimental conditions reported by the authors. Thus, we chose dithymidinyl H-phosphonate 1 with the 5’-O-dimethoxytrityl and the 3’-O-(1,3-benzodithiol-2-yl) protecting groups, since it was claimed that the presence of these two bulky protecting groups together with the nucleoside sequence T-T (alt. A-T) were essential for the occurrence of stereoselective sulfurisation of 1 in the presence of DBU.

\begin{center}
\includegraphics[width=\textwidth]{scheme2.png}
\end{center}

\textbf{Scheme 2.3} Stereospecific sulfurisation of H-phosphonate 1.

A diastereomeric mixture of dinucleoside H-phosphonate 1 (ca 1:1) in pyridine was treated with \textit{n}-butylamine, DBU and sulfur (Scheme 2.3). The reaction was rapid and clean, affording within 5 minutes dinucleoside phosphorothioate 2 as a 1:1 mixture of $R_P$ and $S_P$ diastereomers. Since the ratio of the produced diastereomers of phosphorothioate 2 was practically the same as that of the starting material 1, it was
clear that under the investigated reaction conditions, none of the diastereomers of 2 was formed preferentially \( i.e. \) the reaction did not show any stereoselectivity.

However, to find out if any epimerisation might occur during the course of sulfurisation, separate diastereomers of dinucleoside H-phosphonate 1 were sulfurised under the reaction conditions described above. It was found that 1a \( (\delta_p = 7.24 \text{ ppm}, R_P) \) was converted exclusively to the diastereomer 2a \( (\delta_p = 57.42 \text{ ppm}, S_P) \), and 1b \( (\delta_p = 8.38 \text{ ppm}, S_P) \) afforded exclusively the 2b \( (\delta_p = 57.37 \text{ ppm}, R_P) \).

These experimental data showed that sulfurisation of H-phosphonate diester 1 under the reported conditions was stereospecific (>98% retention, \( ^{31} \text{P NMR} \)).

### 2.3 Configurational Stability of H-Phosphonate Diesters in the Presence of DBU

Although these results clearly demonstrated a stereospecific course of sulfurisation of H-phosphonate diesters in the presence of DBU, there was still a possibility that P-chiral H-phosphonates may undergo epimerisation when exposed to DBU for an extended period of time. Since sulfurisation of 1 in the presence of DBU to produce phosphorothioate 2 in our hands was completed before the first \( ^{31} \text{P NMR} \) spectra was recorded (<5 min), while that reported by Hata \textit{et al.} required ca 1 hour, we wanted to see if treatment of H-phosphonate 1 in pyridine with DBU for at least 1 hour would cause any epimerisation. One should note that the chemical and configurational stability of H-phosphonate diesters in the presence of DBU is an important issue, since this particular base is frequently used in various transformations involving dinucleoside H-phosphonates.\(^{69-71}\)

In preliminary experiments, upon the addition of DBU to H-phosphonate 1 in pyridine, a significant broadening of the \( ^{31} \text{P NMR} \) resonances occurred, which prevented any reliable analysis using this spectroscopic method. Since sulfurisation of 1 under these conditions was found to be stereospecific, we attempted to make a “snapshot” of the reaction mixture by the addition of elemental sulfur, assuming that the composition of the phosphorothioate diesters formed would correspond to the composition of H-phosphonate diesters before sulfurisation. To get a sufficient resolution in the \( ^{31} \text{P NMR} \) spectra, the reaction mixture was worked up before recording the spectra.

To this end, a 1:1 diastereomeric mixture of H-phosphonate diester 1 in pyridine was treated with DBU and after 2 hours, an excess of elemental sulfur was added. \( ^{31} \text{P NMR} \) analysis revealed the presence of several products in the reaction mixture (Scheme 2.4). These were identified as a 1:1 diastereomeric mixture of
dinucleoside phosphorothioate 2 (δ_p = 57.37 and 57.42 ppm, ~40%), symmetrical dinucleoside phosphorothioates 8 (δ_p = 56.9 ppm, ~20%) and 9 (δ_p = 58.3 ppm, ~20%) and two nucleoside H-phosphonate monoesters 6 (δ_p = 3.1 ppm, ~10%) and 7 (δ_p = 4.7 ppm, ~10%). The ratio of products varied slightly from experiment to experiment, but a general tendency observed was that the amount of symmetrical phosphorothioates (8 and 9) correlated with the amount H-phosphonate monoesters (6 and 7) formed. Essentially the same product distribution was observed when running the reaction in dichloromethane, which confirmed that pyridine had not a notable influence on the reaction outcome.

![Scheme 2.4](image)

**Scheme 2.4** Reaction of H-phosphonate 1 with DBU and subsequent sulfurisation.

These results showed that H-phosphonate diester 1 in the presence of DBU underwent significant degradation and, prior to the addition of sulfur, the reaction mixture contained along with 1, symmetrical dinucleoside H-phosphonates (4, 5) and nucleoside H-phosphonate monoesters (6, 7). This degradation of H-phosphonate 1 was most likely due to traces of water that, in the presence of a strong base, caused hydrolysis of 1 producing H-phosphonate monoesters 6, 7. The subsequent transesterification of 1 by the released nucleoside residues produced symmetrical H-phosphonate diesters 4, 5.

An alternative pathway to the formation of the symmetrical H-phosphonate diesters 4 and 5 would be a DBU-promoted ligand exchange reaction. However, since
the amount of symmetrical phosphorothioates 8 and 9 paralleled the amount of H-phosphonate monoesters 6 and 7 formed, a hydrolytic pathway seemed more likely.

To find out if DBU can cause any epimerisation of H-phosphonate diesters, separate $S_P$ and $R_P$ diastereomers of dinucleoside H-phosphonate 1 in pyridine were treated with DBU for 2 hours and then sulfurised with elemental sulfur (Scheme 2.5). In these experiments, along with the formation of symmetrical phosphorothioate diesters (8:9, ca 1:1) and H-phosphonate monoesters (6:7, ca 1:1), we also observed a variable degree of epimerisation of 1. When starting from the pure $R_P$ H-phosphonate diastereomer 1a, sulfurisation yielded phosphorothioate 2 as a 3:2 $S_P$/ $R_P$ diastereomeric mixture, while starting from the pure $S_P$ isomer 1b, the $S_P$/ $R_P$ ratio of phosphorothioate 2 was 1:3.

![Scheme 2.5](image)

**Scheme 2.5** Treatment of the $R_P$ H-phosphonate 1a with DBU, followed by sulfurisation.

The most likely explanation for the observed epimerisation was transesterification of H-phosphonate diester 1 with small amounts of nucleosides arising from hydrolysis of 1 (Scheme 2.6, path i). In agreement with this explanation, the degree of epimerisation paralleled the degree of hydrolysis of 1 and formation of symmetrical diesters 4 and 5. Furthermore, the ratio of diastereomers of H-phosphonate 1 approached the value of 1:1 if hydrolysis was extensive.

However, since DBU exhibits a pronounced nucleophilicity towards the phosphorus centre, we could not exclude that a DBU-catalysed ligand exchange was, at least partly, responsible for the findings above (Scheme 6, path ii). Such a mechanism could in principle also be in agreement with the fact that extended hydrolysis gave more symmetrical diesters (4, 5) and epimerisation products, since the increased amount of nucleosides in the reaction mixture should speed up the rate of the ligand exchange process.

To exclude a DBU-catalysed ligand exchange mechanism, we had to completely eliminate the hydrolytic decomposition of 1. Since traces of water seemed to be virtually impossible to get rid of, we tried to use trimethylsilyl chloride (TMS-Cl) to secure anhydrous reaction conditions.
Scheme 2.6 Possible pathways for epimerisation of dinucleoside H-phosphonate diesters: i) epimerisation involving hydrolysis and transesterification; ii) epimerisation via a DBU catalysed ligand exchange mechanism.

When DBU (up to 6 equiv.) was added to separate $R_P$ and $S_P$ diastereomers of dinucleoside H-phosphonate 1 in pyridine in the presence of TMS-Cl (1 equiv.), no hydrolysis and no epimerisation was observed within 2 hours. On the basis of these results we could tentatively conclude that dinucleoside H-phosphonates of type 1 are chemically and configurationally stable in the presence of DBU, provided that the hydrolytic path of their decomposition is eliminated.

2.4 Investigation on the Stereochemical Course of the Sulfurisation of Benzoylphosphonates in the Presence of DBU

2.4.1 Synthesis of Dinucleoside Benzoylphosphonates

Since it became clear that no epimerisation (within the time required for the sulfurisation) took place at the level of the H-phosphonate diester 1, the stereoselectivity reported by Hata et al.\textsuperscript{34,67} could be due to an epimerisation process of benzoylphosphonate 3 in the presence of $n$-butylamine and DBU.

To investigate this reaction we first had to prepare benzoylphosphonate 3. Hata et al.\textsuperscript{74} produced this compound via a stepwise coupling reaction of benzoylphosphonic acid with the 5'-O-DMT protected thymidine and 3'-O-BDT protected thymidine, to produce the desired dinucleoside benzoylphosphonate 3 (Scheme 2.7).
Scheme 2.7 Hata's synthesis of dinucleoside benzoylphosphonate 3.

However, this synthetic scheme did not seem to be versatile enough for our purposes. Encouraged by reports on efficient P-acylations of P(III) compounds, we set out to develop a method to produce benzoylphosphonate 3 via P-benzoylation of the H-phosphonate diester 1 that we already had. In the development of this synthetic procedure, we used as a model compound the commercially available diethyl H-phosphonate 10 (Scheme 2.8). The progress of all reactions was followed by $^{31}$P-NMR spectroscopy.

Scheme 2.8 Formation of the desired benzoylphosphonate 12 along with some by-products.

The reaction of diethyl H-phosphonate 10 with benzoyl chloride was initially carried out in pyridine in the presence of triethylamine (TEA). Unfortunately, the formation of the desired benzoylphosphonate 12 was sluggish and several by-products were also formed. When N,O-bis(trimethylsilyl)acetamide (BSA) was used instead of TEA, the generated diethyl trimethylsilyl phosphite 11 reacted rapidly with benzoyl chloride to afford the desired benzoylphosphonate 12 as a main product along with dihydropyridine-2-phosphonate 14 (20%), dihydropyridine-4-phosphonate 13 (5%) and an additional by-product (5%, $\delta_P=16.0$ ppm). The dihydropyridine derivatives 13
and 14 were probably formed via N-activation of pyridine by benzoyl chloride, followed by nucleophilic attack of phosphite 11 on the pyridine ring. To avoid these side reactions, the solvent was changed to dichloromethane.

When benzoyl chloride, BSA and TEA were added to diethyl H-phosphonate 10 in dichloromethane, a rapid conversion into the desired product 12 (85%) occurred. However, along with 12, a substantial amount of the by-product resonating at 16.0 ppm (15%) was observed (31P NMR). Our guess was that the by-product was bisphosphonate 15, formed by the reaction of benzoylphosphonate 12 with trimethylsilyl phosphite 11. This undesired formation of 15 could be rationalised by the fact that silylation of H-phosphonate 10 was significantly faster than the subsequent reaction of trimethylsilyl phosphite 11 with benzoyl chloride and thus the accumulated phosphite 11 reacted also with the formed benzoylphosphonate 12 to afford bisphosphonate 15. To substantiate this assumption, diethyl H-phosphonate 10 was silylated separately and an equimolar amount of diethyl benzoylphosphonate 12 was added. As expected, under these conditions bisphosphonate 15 was formed as a major product.

To suppress the formation of 15, we tried to slow down the rate of formation of phosphite 11 by carrying out the silylation reaction in the absence of base. In addition, benzoyl chloride was added to the reaction mixture before BSA to further avoid accumulation of 11. With these modifications, we significantly improved the formation of diethyl benzoylphosphonate 12, although generation of bisphosphonate 15 could not be fully eliminated.

This synthetic protocol was then applied to the synthesis of dinucleoside benzoylphosphonate 3 (Scheme 2.9). When dinucleoside H-phosphonate 1 in dichloromethane was allowed to react with benzoyl chloride in the presence of BSA, an exclusive formation of benzoylphosphonate 3 was achieved (completion within 2 hours). The slightly acidic reaction conditions resulted, however, in some detritylation. To circumvent this problem, the relatively weak base N,N-diethylaniline was used. This prevented detritylation without affecting the exclusive formation of dinucleoside benzoylphosphonate 3.

Dinucleoside benzoylphosphonate 3 proved to be much more labile than expected on the basis of literature reports. Attempted purification of 3 by extraction or column chromatography resulted in extensive debenzoylation. However, precipitation of the reaction mixture into petroleum ether gave the desired product 3 as a white solid and most of the excess reagents could be washed away.

By reacting separate diastereomers of 1 with benzoyl chloride under these reaction conditions, dinucleoside benzoylphosphonate 3 could be obtained in a
stereospecific manner. The $R_P$ and $S_P$ diastereomers of 3 were isolated in 82% and 86% yields, respectively.

![Diagram of compounds 1 and 3 with chemical structures and P chemical shifts](attachment:image.png)

1a $R_P$, $\delta_P = 7.24$ ppm  
1b $S_P$, $\delta_P = 8.38$ ppm  
3a $R_P$, $\delta_P = -1.35$ ppm, yield: 82%  
3b $S_P$, $\delta_P = -1.56$ ppm, yield: 86%

Scheme 2.9 Synthesis of dinucleoside benzoylphosphonate 3.

### 2.4.2 Sulfurisation of Dinucleoside Benzoylphosphonates in the Presence of DBU

Now, as a final stage of our studies, we could investigate the stereochemical course of sulfurisation of benzoylphosphonate 3 under the reaction conditions reported by Hata.\textsuperscript{34,67} A diastereomeric mixture of benzoylphosphonate 3 (1:1) was thus treated with elemental sulfur in the presence of $n$-butylamine and DBU. The reaction was fast and only one broad peak could be detected in $^{31}$P NMR in the region of chemical shifts of phosphorothioates. However, after work up, two well resolved signals appeared ($\delta_P = 57.37$ and 57.42 ppm; ratio 1:1), which proved that sulfurisation of benzoylphosphonate 3 did not lead to stereoselective formation one diastereomer of 2.

To elucidate a possible influence of the amounts of $n$-butylamine and DBU on the stereochemical outcome of this reaction, some additional experiments were carried out. It was found that regardless of the ratio of $n$-butylamine and DBU used for the reaction, the sulfurisation invariably led to the same equimolar amounts of phosphorothioate 2 diastereomers. However, if the reaction was carried out with DBU alone or if DBU was added before $n$-butylamine, a varying amount of an unknown by-product in addition to phosphorothioate 2, was found. The use of excess of DBU (10 equiv.) in the absence of $n$-butylamine gave almost exclusively this unexpected by-product. After some model experiments with diethyl benzoylphosphonate 12 in the presence of DBU and isolation of the corresponding by-product, we could assign its structure as tetranucleoside phosphonate-phosphate 17 (Scheme 2.10).
This somewhat unexpected pathway of the reaction was apparently due to a slow debenzoylation of 3 into the phosphite salt of 1 that could react with benzoylphosphonate 3 to afford bisphosphonate 16. However, bisphosphonate 16 was never observed under these reaction conditions since it rapidly underwent rearrangement\textsuperscript{78} into phosphonate-phosphate 17. With excess of \textit{n}-butylamine, the debenzoylation reaction was apparently much faster than the subsequent side reactions and hence no phosphonate-phosphate 17 was observed under the reaction conditions used by Hata \textit{et al.}\textsuperscript{34,67}

Although the experiments so far showed that sulfurisation of benzoylphosphonate 3 into the corresponding phosphorothioate 2 was not stereoselective, we could not exclude a possibility of epimerisation of 3 occurring in the presence of \textit{n}-butylamine and DBU. To investigate this possibility, we had to use separate \textit{R} and \textit{S} isomers of benzoylphosphonate 3. By using the developed protocol, separate diastereomers of benzoylphosphonate 3 could be obtained directly from separate diastereomers of dinucleoside H-phosphonate 1 (Scheme 2.9).

Sulfurisation of separate diastereomers of benzoylphosphonate 3 in the presence of \textit{n}-butylamine and DBU showed that also this transformation was stereospecific (>98\%, \textsuperscript{31}P NMR) (Scheme 2.11).
Scheme 2.11  Stereospecific sulfurisation of benzoylphosphonate 3.

2.5 Conclusions

We have shown that sulfurisation of H-phosphonate diester 1 in the presence of n-butylamine and DBU, as well as the generation of 1 from the corresponding benzoylphosphonate 3, occurred stereospecifically, most likely with retention of configuration. Furthermore, we found that H-phosphonate diesters of type 1 are chemically and configurationally stable in the presence of DBU even upon prolonged treatment, provided that the hydrolytic path of their decomposition is eliminated.
3. Studies Directed Towards the Synthesis of Nucleotide Analogues with a 2-Pyridylphosphonate Internucleotide linkage (Paper II)

Simple dialkyl pyridylphosphonate derivatives show a wide range of biological activities\(^ {79-82}\) and a pyridine ring is found in many natural products and drugs.\(^ {83}\) Thus, the introduction of the pyridylphosphonate moiety into nucleotides seemed to be a potentially useful modification. As a non-ionic C-phosphonate analogue, it may provide the modified oligonucleotide with i) increased stability towards enzymatic degradation, ii) enhanced cellular uptake and iii) increased affinity to complementary antisense/antigene strand. But apart from just being another C-phosphonate analogue, pyridylphosphonates have several other interesting features. These are e.g. iv) the presence of a nitrogen that could potentially modulate stability of formed duplexes or triplexes by hydrogen bonding, solvation or coordination to metal ions and (v) a possibility to introduce additional functionalities to the pyridyl ring by quaternisation or substitutions.

Since we previously developed a method for producing dinucleoside 4-pyridylphosphonates,\(^ {71}\) an obvious task was to get access to the isomeric 2-pyridylphosphonate derivatives. 2-Pyridylphosphonates are special members of the pyridylphosphonate family due to their ability to form bidentate complexes with metal ions.\(^ {84,85}\) In the field of nucleic acids, this feature can be exploited, e.g. for the development of new artificial nucleases\(^ {86}\) or specific probes for investigation of electron transfer (ET) phenomena in nucleic acids.\(^ {87}\)

3.1 Introduction to the Synthesis of 2- and 4-Pyridylphosphonates

There are only a few synthetic methods available to produce simple dialkyl 2-pyridyl-\(^ {88-90}\) and 4-pyridylphosphonates,\(^ {90-92}\) but none of them can be applied to the synthesis of nucleotide analogues, due to rather harsh reaction conditions and low to moderate yields. Most of these methods are based on reactions of dialkyl sodium/lithium phosphites or trialkyl phosphites with \(N\)-substituted pyridinium salts and the regioselectivity obtained depends mainly on the nature of the \(N\)-substituent (Scheme 3.1).
Previously, inspired by Redmore’s work, a convenient and efficient method to produce dinucleoside 4-pyridylphosphonate 20 was developed in our lab. In this procedure, dinucleoside H-phosphonate 18 is dissolved in pyridine and the addition of trityl chloride and DBU promotes a conversion of 18 into the corresponding 1,4-dihydropyridine intermediate 19, which collapses into the desired 4-pyridylphosphonate 20 during work up (Scheme 3.2). This reaction turned out to be stereospecific and the diastereomers of 20 were isolated in good yields ($R_p$: 76%; $S_p$: 87%).

As a continuation, we wanted to extend the scope of this type of chemistry in order to develop a method to incorporate 2-pyridylphosphonate moieties into nucleic acids.

Scheme 3.2 Synthesis of dinucleoside 4-pyridylphosphonate 20.

**3.2 Reactions of H-Phosphonate Diesters with N-Aroylpyridinium Salts**

1,2-Dihydropyridine phosphonate 14 and 1,4-dihydropyridine phosphonate 13, formed as by-products in our attempted synthesis of diethyl benzoylphosphonate 12 (Scheme 2.8, Chapter 2), now attracted our attention as a possible entry to pyridylphosphonate derivatives (Scheme 3.3). Although the dihydropyridine
derivatives 13 and 14 were formed as minor products, the favourable regioselectivity (20% 4-isomer 13, 80% 2-isomer 14) encouraged us to try to tune the reaction conditions to improve the yields as well as the regioselectivity. In the development of this method, diethyl H-phosphonate 10 was initially used as a model compound. Progress of these reactions was followed by $^{31}$P NMR spectroscopy.

Previously, in the attempted synthesis of benzoylphosphonate 12, benzoyl chloride and BSA were added to diethyl H-phosphonate 10 in pyridine, forming diethyl benzoylphosphonate 12 as the main product (70%). By changing the solvent to dichloromethane and adding only 8 equiv. of pyridine, the formation of 12 was slightly reduced (60%) while the amount of dihydropyridine phosphonates 13 and 14 increased (~40%). Unfortunately, it was not possible to further optimise this reaction to favour the formation of dihydropyridine phosphonates 13 and 14.

![Scheme 3.3 Reaction pathway to pyridylphosphonates 21 and 22.](image)

As an alternative approach, benzoyl chloride was replaced with the sterically hindered mesityl chloride (2,4,6-trimethylbenzoyl chloride) with the aim that it would still activate pyridine towards a nucleophilic attack mainly at the 2-position, but not acylate H-phosphonate 10. Indeed, with this reagent only 1,2-dihydropyridine phosphonate 14 (80%) and 1,4-dihydropyridine phosphonate 13 (20%) were formed. Dihydropyridine derivatives 13 and 14 were found to be very stable and were isolated
as a mixture by silica gel column chromatography in 78% yield. 1,2-Dihydropyridine derivative 14 could be isolated in 60% yield.

The isolated 1,2-dihydropyridylphosphonate 14 was subjected to various reaction conditions to find an efficient approach to convert 14 into 2-pyridylphosphonates 22. Finally, 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ), which recently has been reported\textsuperscript{94} as an efficient oxidising reagent of similar systems, turned out to promote a rapid conversion of 14 into the 2-pyridylphosphonate 22 (isolated in \textit{ca} 70% yield).

This two step procedure, when applied on dinucleoside H-phosphonate 18, was not an efficient method to produce dinucleoside 2-pyridylphosphonates although it may be useful in the synthesis of other pyridylphosphonate derivatives.

### 3.3 Reactions of H-Phosphonate Diesters with N-Methoxypyridinium salts

Since activation of the pyridine ring towards a nucleophilic attack by the formation of N-methoxypyridinium salts has shown the desired regioselectivity in reactions with various nucleophilic species\textsuperscript{95} including P(III) nucleophiles,\textsuperscript{88} this approach seemed attractive for the preparation of 2-pyridylphosphonate analogues of nucleotides. To explore this avenue, N-methoxypyridinium tosylate 23 was prepared\textsuperscript{96,97} (Scheme 3.4) and its reaction with diethyl H-phosphonate 10 was initially investigated.

\begin{equation}
\text{N} \text{Me}_{\text{O}} \text{pTs}^{-} \text{N} \text{O} \quad \text{Scheme 3.4} \quad \text{Preparation of N-methoxypyridinium tosylate 23. i) AcOH, H}_{2}\text{O}_{2}; \text{ ii) methyl p-toluenesulfonate.}
\end{equation}

Diethyl H-phosphonate 10 in dichloromethane was silylated by TMS-Cl in the presence of TEA to produce the nucleophilic trimethylsilyl phosphite 11, which was allowed to react with N-methoxy pyridinium tosylate 23. When the reaction mixture was analysed by \textsuperscript{31}P NMR spectroscopy (after 3 hours), numerous phosphorus-containing species, along with unreacted starting material 10, were detected. The replacement of TMS-Cl by BSA afforded a much cleaner reaction mixture containing a species resonating at 22.1 ppm as the major product (>90%). The chemical shift suggested a dialkoxyphosphinoyl group bound to an aliphatic carbon, which would be in agreement with a formed 1,2-dihydropyridine intermediate like 27 (Scheme 3.6). However, intermediates of type 27 have been reported to be very labile\textsuperscript{88} and the
species resonating at 22.1 ppm did not decompose into 2-pyridylphosphonate 22, even when subjected to various harsh conditions. Fortunately, the unknown product could be isolated by silica gel chromatography and was identified by analysis of $^1H$, $^{13}C$ and $^{31}P$ NMR data as silylated hydroxymethylphosphonate 25 (Scheme 3.5). This unexpected course of the reaction could be traced back to the known instability of $N$-alkoxypyridinium salts, which under basic conditions might undergo $\beta$-elimination producing the corresponding aldehyde and pyridine. In the case of $N$-methoxy pyridinium salt 23, the generated formaldehyde reacted apparently with silyl phosphite 11 to produce hydroxymethylphosphonate 24, which under the reaction conditions was silylated to give silylated hydroxymethylphosphonate 25.

BSA, or possibly the formed silyl phosphite 11, could apparently promote decomposition of $N$-methoxypyridinium tosylate, since the formation of silylated hydroxymethylphosphonate 25 was observed also in absence of an external base. Thus, the use of silylating agents to increase nucleophilicity of 10 turned out to be counterproductive in this reaction, since the desired nucleophilic attack on the pyridine ring was slower than decomposition of the pyridinium salt 23.

Thus, we investigated the reaction of diethyl H-phosphonate 10 with $N$-methoxypyridinium salt 23 using only base as an activator. It was found that diethyl H-phosphonate 10 reacted rapidly with $N$-methoxypyridinium salt 23 in acetonitrile in the presence of DBU affording within 5 minutes the desired 2-pyridylphosphonate 22 ($\sim$80% formed, isolated in 74% yield) and some hydroxymethylphosphonate 24 ($\sim$20%) (Scheme 3.6).
Scheme 3.6 Reaction of phosphite salt 26 with N-methoxypyridinium salt 23.

These findings indicated that, under the reaction conditions, nucleophilic attack of diethyl phosphite anion 26 on the pyridine ring was significantly faster than decomposition of the pyridinium salt 23 in the presence of DBU. In agreement with this, we found that DBU and N-methoxypyridinium tosylate 23 could not be mixed before the addition of H-phosphonate 10, since the pyridinium salt then decomposed and hence no 2-pyridylphosphonate 22 was formed. The efficacy of this approach in the synthesis of a new type of nucleotide analogue with a 2-pyridylphosphonate internucleotide linkage could now be explored.

A diastereomeric mixture (ca 1:1) of 5',3'-O,O-bis(dimethoxytrityl) protected dinucleoside H-phosphonate 18 was dissolved in acetonitrile and N-methoxypyridinium tosylate 23 and DBU were added. The reaction went to completion within 5 min affording, as a sole nucleotidic material, dinucleoside 2-pyridylphosphonate 29 (1:1 diastereomeric mixture) (Scheme 3.7). In contrast to 4-pyridylphosphonates, no intermediacy of the corresponding 1,2-dihydropyridine 28 could be detected in this reaction using 31P NMR spectroscopy. Dinucleoside 2-pyridylphosphonate 29 was isolated in ca 85% yield.

To establish the stereochemical course of the formation of 2-pyridylphosphonate 29, the reaction was carried out on the separate diastereomers of dinucleoside H-phosphonate 18. It was found that the $R_P$ isomer 18a ($\delta_P = 6.9$ ppm) afforded exclusively 2-pyridylphosphonate 29a resonating at higher field ($\delta_P = 10.6$ ppm, $R_P$, isolated in 86% yield), while the $S_P$ isomer 18b ($\delta_P = 8.6$ ppm) afforded exclusively 2-pyridylphosphonate 29b resonating at lower field ($\delta_P = 11.0$ ppm, $S_P$, isolated in 85% yield). The reaction was hence established as a stereospecific transformation that proceeded most likely with retention of configuration at the phosphorus centre.
Dinucleoside 2-pyridylphosphonates 29a and 29b were deprotected by a mild acid treatment (80% AcOH aq.) and the unprotected 2-pyridylphosphonates 30a and 30b could be isolated by extraction.

### 3.4 Conclusions

A mild and efficient method to produce nucleotides with a novel 2-pyridylphosphonate internucleotide linkage was developed. Easily available dinucleoside H-phosphonates could be converted into the target 2-pyridylphosphonates in a fast and stereospecific reaction that proceeds, most likely, with retention of configuration.
4. Synthesis of Nucleotide Analogues with 2- and 4-Pyridylphosphonothioate Internucleotide Linkages (Paper III)

In contrast to simple dialkyl pyridylphosphonates, pyridylphosphonothioates are rare phosphorus compounds. The only method of synthetic value affords the target pyridylphosphonothioates in mediocre yields and requires lengthy thiation of their oxygen congeners with P$_2$S$_5$ in toluene under reflux. Since we now had efficient protocols to produce dinucleoside 2-pyridyl and 4-pyridylphosphonates, we decided to investigate the possibility to synthesize their phosphonothioate counterparts. By replacement of the non-bridging oxygen by sulfur in the pyridylphosphonate internucleotidic linkage, we hope to alter its properties to be useful in the development of new antisense and antigen therapeutics.

In the approach previously developed in our group to synthesize dinucleoside 4-pyridylphosphonates, N-tritylpyridinium chloride is produced in situ by addition of trityl chloride to pyridine. The trityl group both activates the pyridine ring for nucleophilic attack and blocks the more reactive 2- and 6-positions by sterical hindrance to promote a nucleophilic attack exclusively at the 4-position. Considering the synthesis of dinucleoside 4-pyridylphosphonothioate via a reaction of H-phosphonothioate in pyridine (Scheme 4.1), the known ligand exchange process of 31 in pyridine could in principle cause a problem, but we anticipated that the P-C bond formation should be significantly faster than this side reaction.

4.1 Results and Discussion

When a diastereomeric mixture of H-phosphonothioate in pyridine was subjected to treatment with trityl chloride (Tr-Cl) and DBU, the desired 1,4-dihydropyridine derivative was indeed formed as the major nucleotidic species and no ligand exchange was observed. However, under these reaction conditions, formation of phosphorothioate occurred to a significant degree (20%). Use of more base (up to 8 equiv. DBU), speeded up the desired reaction and reduced the amount of oxidation product to 7%, but simultaneously unknown by-products (6% at 87 and 88 ppm, $^{31}$P NMR) started to form.

We assumed that formation of phosphorothioate under the reaction conditions was probably due to air oxidation of the highly reactive phosphite form generated from 31 in the presence of a strong base (DBU). To solve this problem,
DBU was replaced by TEA, since a weaker base would produce a lower concentration of the phosphite salt of H-phosphonothioate 31 and hence oxidation should be suppressed.

![Chemical structures](image)

**Scheme 4.1** Synthesis of 4-pyridylphosphonothioate 33.

Indeed, when a diastereomeric mixture of dinucleoside H-phosphonothioate 31 was treated in pyridine with Tr-Cl in the presence of TEA, a rapid reaction (<10 min) produced a diastereomeric mixture of 1,4-dihydropyridine derivative 32 quantitatively. However, the 1,4-dihydropyridylphosphonothioate intermediate 32 turned out to be rather stable and, in contrast to its oxo counterpart that collapsed spontaneously to the corresponding pyridylphosphonate, it underwent only a slow conversion into 4-pyridylphosphonothioate 37 (ca 20% conversion after a few hours).

To facilitate the conversion of 1,4-dihydropyridine intermediate 32 into the desired product 33, oxidation with iodine was tried out. When 2 equiv. iodine were added to the reaction mixture containing intermediate 32, a fast formation of the desired product 33 (ca 50% after 10 min) was observed, but the desired reaction did not proceed further and with time a substantial formation of phosphorothioate 35 was found. This undesired reaction pathway was probably due to a possible reversibility of the formation of 1,4-dihydropyridine intermediate 32, which can lead to the formation small concentrations of starting material 31. H-Phosphonate 31 can then in the presence of iodine and traces of water, be oxidized to phosphorothioate 35.

A higher concentration of iodine should speed up the desired rearomatisation reaction and thereby suppress the formation of by-product 35. Indeed, the addition of 4 equiv. iodine furnished a complete conversion of dihydropyridylphosphonothioate 32 into 4-pyridylphosphonothioate 33 within 15 minutes. In a preparative run, using the
developed conditions, 4-pyridylphosphonothioate 33 was isolated in ca 80% yield as a mixture of diastereomers.

The stereochemistry of this reaction was investigated by performing the transformation on separate diastereomers of dithymidine H-phosphonothioate 31. It was found that H-phosphonothioate 31a (“fast” isomer) afforded 4-pyridylphosphonothioate 33a with the intermediacy of 32a, while the H-phosphonothioate 31b (“slow” isomer) gave product 33b with the intermediacy of 32b. Thus, the transformation was found to be stereospecific and proceeding, most likely, with overall retention of configuration. The separate diastereomers were isolated in 82% (33a, Rp) and 77% (33b, Sp) yields and deprotected, producing 34a and 34b, respectively.

In the synthesis of dinucleoside 2-pyridylphosphonothioate 37, the method previously developed for oxo pyridylphosphonates\textsuperscript{100} was applied. Following this protocol, \textit{N}-methoxy pyridinium tosylate 23 (2 equiv.) and DBU (4 equiv.) were added to dinucleoside H-phosphonothioate 31 in acetonitrile (Scheme 4.2). The reaction was rapid (<5 min), but along with the desired 2-pyridylphosphonothioate 37 (55%), \textsuperscript{31}P NMR revealed the presence of 4-pyridylphosphonate 33 (15%) and also by-products resonating close to 2-pyridylphosphonate 37 (30%; two peaks at ca 78 ppm and two peaks at ca 80 ppm). When the order of addition was changed, \textit{i.e.} DBU was added before \textit{N}-methoxy pyridinium tosylate, the regioselectivity was improved (65% 2-pyridylphosphonothioate; 5% 4-pyridylphosphonothioate) but the amount of unidentified by-products was still unacceptably high (30%).

To be able to improve this reaction, it was necessary to identify the by-products formed. On the basis of their chemical shifts that were very close to those of 2-pyridylphosphonothioate 37 and by considering possible reaction pathways, we assumed that these side products were, most likely, isomeric methyl nucleoside 2-pyridylphosphonothioates 39 and 40 (Scheme 4.2). This could be consistent with a possible decomposition pathway of 1,2-dihydropyridylphosphonothioate 36 that along with 2-pyridylphosphonothioate 37 also should generate methoxide anions. Since H-phosphonothioate diesters are susceptible to nucleophilic substitution at the phosphorus centre,\textsuperscript{101} the released methoxide anions might replace either the 5’- or 3’-nucleosidic unit in H-phosphonothioate 31, and this would ultimately lead to the formation of the observed by-products 39 and 40.
In the final protocol, DBU was added before N-methoxypyridinium tosylate and the amount of the latter one was decreased to 1.2 equiv. Under these reaction conditions, the formation of methyl nucleoside pyridylphosphonothioates \(39\) and \(40\) was completely eliminated and a high regioselectivity was obtained (95% 2-pyridylphosphonothioate \(37\); 5% 4-pyridylphosphonothioate \(33\)). When carried out on a preparative scale under the optimised conditions, the reaction of H-phosphonothioate \(31\) with N-methoxypyridinium tosylate in the presence of DBU afforded 2-pyridylphosphonothioate \(37\) in ca 80% isolated yield.

In the context of regioselectivity of the above reaction, \textit{i.e.} formation of 2-pyridyl- \textit{versus} 4-pyridylphosphonothioate derivatives, some interesting observations were made. In all cases, when reactions of H-phosphonothioate \(31\) and N-methoxypyridinium tosylate were promoted by DBU, the major product formed was 2-pyridylphosphonothioate \(37\). The other positional isomer, 4-pyridylphosphonothioate \(33\), was formed only in small amounts (5-15%). In contrast, by the use of TEA instead of DBU, 4-pyridylphosphonothioate \(33\) was afforded as a major product (ca 80%). Although it is not fully clear how these changes of reaction conditions influence the reaction outcome, it seems that kinetic \textit{versus} thermodynamic control may be the most important factor. Since the base probably facilitates the collapse of N-methoxy-1,2-dihydropyridylphosphonothioate \(36\) by proton abstraction, this process should be fast in the presence of the strong base DBU and thus formation of the kinetic product, 2-
pyridylphosphonothioate 37, should be favoured. However, in the presence of the weaker base TEA, the collapse of 1,2-dihydropyridine intermediate 32 is expected to be slower, hence this initially formed intermediate may isomerise to the thermodynamically more stable 1,4-dihydropyridylphosphonothioate which ultimately will lead to the formation of 4-pyridylphosphonothioate 33.

The stereochemical course of the formation of 2-pyridylphosphonothioate 37 (Scheme 4.2) was then elucidated. The exclusive formation of 2-pyridylphosphonothioate 37a from H-phosphonothioate 31a (“fast” isomer), and 37b from H-phosphonothioate 31b (“slow” isomer), established this reaction as stereospecific. Also in this case, the reaction most likely proceeded with overall retention of configuration. The separate diastereomers were isolated in 79% (37a, R<sub>p</sub>) and 80% (37b, S<sub>p</sub>) yields and deprotected to form 38a and 38b, respectively.

4.2 Conclusions

Simple and efficient methods for the preparation of new types of nucleotide analogues bearing 2-pyridyl- and 4-pyridylphosphonothioate internucleotide linkages were developed. The target pyridylphosphonothioates were obtained stereospecifically and in high yields starting from easily available dinucleoside H-phosphonothioates.
5. Studies on the Synthesis of Dinucleoside 3-Pyridylphosphonates (Paper IV)

5.1 Introduction to Pd(0)-Catalysed Cross Couplings

Palladium-catalysed reactions of aryl and vinyl halides with organometallic nucleophiles are widely used for the purpose of C-C bond formation. Variants of these type of reactions have found widespread applications in modern organic chemistry, e.g., Suzuki (boron-mediated),\textsuperscript{102} Stille (tin-mediated),\textsuperscript{103} Negishi (zinc-mediated),\textsuperscript{104} Corriu-Kumada-Tamao (magnesium-mediated),\textsuperscript{105} and Sonogashira (copper-mediated)\textsuperscript{106} coupling reactions. In recent years, palladium-catalysed reactions with nitrogen,\textsuperscript{107,108} oxygen,\textsuperscript{109} sulfur,\textsuperscript{110} phosphorus,\textsuperscript{111} and boron-based\textsuperscript{112} nucleophiles have gained an increasing interest. The pioneering studies by Buchwald\textsuperscript{108} and Hartwig\textsuperscript{107} (1995) on palladium-catalysed cross couplings of nitrogen nucleophiles with aryl halides have contributed significantly to this field.

\[
\text{Scheme 5.1} \quad \text{Palladium(0)-catalysed cross couplings of aryl halides with: i) organometallic species; ii) non-organometallic nucleophiles.}
\]

Transition metal-catalysed cross couplings of phosphorus-based nucleophiles with aryl halides have been known since the early 1980s when Hirao\textsuperscript{111} reported on Pd(0)-catalysed reactions of dialkyl H-phosphonates with aryl halides. Using this approach, various simple P-aryl compounds have been prepared.\textsuperscript{113-115}

This route attracted our attention as a possible entry to new dinucleoside pyridylphosphonate derivatives that could not be obtained by existing methods. However, under the reaction conditions reported by Hirao,\textsuperscript{111} dialkyl H-phosphonates were used in excess, often in the absence of solvents and high temperature was required (\textit{ca} 90 °C). These conditions are, unfortunately, not applicable to nucleic acid components. Thus, we wanted to develop a reaction that would be compatible with the relatively sensitive dinucleoside H-phosphonates derivatives.
5.2 Pd(0)-Catalysed Cross Couplings of Dinucleoside H-Phosphonates with Halopyridines

Our previously developed methods for the synthesis of dinucleoside pyridylphosphonates were restricted to the 2- and 4-pyridyl isomers. Since the position of the phosphonyl group on the pyridine ring may be important for biological activity, it was desirable that all three isomeric compounds were accessible for chemical and biological studies. This prompted us to look for an alternative method in order to get access to dinucleoside 3-pyridylphosphonates.

Introduction of a phosphonyl group into the 3-position of a pyridine ring is generally rather difficult, and the few methods reported for simple dialkyl derivatives are usually low yielding. Inspired by the pioneering work of Hirao, we decided to investigate the chemistry and stereochemistry of a palladium(0)-catalysed cross coupling of dinucleoside H-phosphonate 18 with bromopyridines 41-43 as a viable way for preparing dinucleoside pyridylphosphonates (Scheme 5.2). Initially, we focused on the synthesis of dinucleoside 3-pyridylphosphonate 44 with the hope that later on, the developed method could be extended to the synthesis of 2- and 4-pyridyl phosphonates (29 and 20) as well.

The palladium(0)-mediated coupling of dinucleoside H-phosphonate 18 with 3-bromopyridine 42 required some experimentation to develop the best conditions of solvents and reagents to promote the desired transformation into 3-pyridylphosphonate 44. THF was chosen as solvent since it has been widely used in these kinds of reactions and refluxes at a moderate temperature (66 °C). Pd(PPh₃)₄ turned out to catalyse this transformation, although a quite high catalyst loading was required (20 mol%). Eventually, the use of equimolar amounts of dinucleoside H-phosphonate 18 and 3-bromopyridine 42 in the presence of Pd(PPh₃)₄ and triethylamine (TEA) under reflux in THF, was found to quantitatively produce dinucleoside 3-pyridylphosphonate 44 (³¹P NMR). The reaction was completed in ca 4 hours and 44 was isolated in ca 85% yield (Scheme 5.4, Table 5.1).
A catalytic cycle expected for this type of reaction\textsuperscript{115} is shown in Scheme 5.3, and we could confirm its validity by $^{31}$P NMR spectroscopy. It seems that the rate-determining step is a ligand substitution, \textit{i.e.} the formation of \textit{trans}-adduct B via nucleophilic attack of the phosphorus nucleophile on the initial intermediate, \textit{trans}-adduct A. Since the only intermediate observed by $^{31}$P NMR spectroscopy during the course of the reaction was that of \textit{trans}-adduct A ($\delta_P = 24.2$ ppm), the two consecutive steps in which B collapses into 3-pyridylphosphonate \textit{44} (\textit{trans-cis} isomerisation, reductive elimination\textsuperscript{117}) and the oxidative addition of 3-bromopyridine \textit{42} to palladium, all have to be fast. Intermediate A could actually be prepared separately, isolated and characterised.\textsuperscript{118} The same course of the reaction was observed when the isolated intermediate A was reacted under reflux in THF with H-phosphonate \textit{18} in the presence of TEA.

To investigate the stereochemistry of this palladium(0)-catalysed cross coupling, separate diastereomers of H-phosphonate diester \textit{18} were subjected to the reaction with 3-bromopyridine \textit{42} (Scheme 5.4). It was found that the P-C bond formation in this reaction was fully stereospecific as the $R_P$ H-phosphonate \textit{18a} ($\delta_P = 6.9$ ppm) exclusively afforded the diastereomer \textit{44a} ($\delta_P = 16.3$ ppm, isolated in 85%
yield), while the $S_P$ diastereomer 18b ($\delta_P = 8.6$ ppm) produced the other diastereomer of 3-pyridyolphosphonate 44b ($\delta_P = 17.2$ ppm, isolated in 80% yield).

To find out if the formation of the P-C bond in this reaction occurred with retention or inversion of configuration at the phosphorus centre, we synthesized separate diastereomers of 2-pyridyolphosphonate 29 and 4-pyridyolphosphonate 20 using palladium(0) chemistry (Scheme 5.4) and compared them with the compounds obtained via the previously developed methods, i.e. by reactions with $N$-trityl-71 or $N$-methoxypyridinium salts.

Dinucleoside 4-pyridyolphosphonate 20 could be prepared using the procedure developed for 3-pyridyolphosphonate 44. By reacting separate diastereomers of dinucleoside H-phosphonate 18 with 4-bromopyridine 43 in the presence of Pd(PPh$_3$)$_4$ and TEA, the diastereomers of 4-pyridyolphosphonate 20 were produced with the same stereochemistry as observed using the pyridine/trityl chloride/DBU system.$^{71}$ Since the latter conversion proceeds, most likely, with retention of configuration, we could tentatively conclude that the palladium(0)-catalysed cross coupling of H-phosphonate 18 with 4-bromopyridine 43 and 3-bromopyridine 42 also proceeds in a stereoretentive fashion.

![Scheme 5.4](image)

**Scheme 5.4** Synthesis of dinucleoside 2-, 3- and 4-pyridyolphosphonates.

Attempted preparation of dinucleoside 2-pyridyolphosphonate 29 by a palladium(0)-catalysed cross coupling of H-phosphonate 18 with 2-bromopyridine 41 (Scheme 5.4) turned out to be a more difficult task as the protocol developed for the 3-pyridyl isomer 44 afforded the desired product in less than 30% (5 h, $^{31}$P NMR). The lower reactivity of 2-bromopyridine 41 compared to 3-bromopyridine 42 in our
reaction was somewhat surprising, since palladium(0) cross couplings of amines with 2-bromopyridine 41 have been reported to be at least as effective as with the 3-isomer 42. A possible explanation for the inefficient coupling of H-phosphonate 18 with 2-bromopyridine 41 could be inactivation of the catalyst by the formed product, 2-pyridylphosphonate 29 (Scheme 5.5).

2-Pyridylphosphonates are known to be $O,N$-bidentate chelating ligands with good metal complexing properties and could thereby inhibit the oxidative addition step by the formation of inactive palladium(0) complexes. To circumvent this problem, bidentate phoshine ligands where utilised in order to prevent the ligand replacement. Neither of the catalyst systems initially investigated (1,2-bis(diphenylphosphino)ethane (DPPE) or 1,3-bis(diphenylphosphino)propane (DPPP) and Pd(OAc)$_2$) could efficiently catalyse the desired transformation. However, inspired by the recent findings by Hartwig et al. that sterically hindered bidentate phosphines are highly efficient ligands in palladium(0)-catalysed $N$-arylation of amines, 1,1'-bis(diphenylphosphino)ferrocene (DPPF) was tried out. When dinucleoside H-phosphonate 18 and equimolar amounts of 2-bromopyridine 41 were refluxed in THF in the presence of Pd(OAc)$_2$, DPPF and TEA, the efficiency of 2-pyridylphosphonate 29 formation increased to ca 80% ($^{31}$P NMR). It seems that the rigid and sterically hindered DPPF in the presence of Pd(OAc)$_2$ forms a relatively stable palladium(0) complex and the ligands are not displaced by the formed 2-pyridylphosphonate 29.

By carrying out this reaction on separate diastereomers of H-phosphonate 18, the transformation was found to occur with the same stereochemistry as that of the DBU/$N$-methoxypyridinium promoted reaction, i.e. probably with retention of configuration at the phosphorus centre. The diastereomers of 2-pyridylphosphonate 29 were isolated in 54% (29a, $R_p$) and 51% (29b, $S_p$) yields (Scheme 5.4, Table 5.1).
Table 5.1  Experimental conditions for selected reactions\(^a\)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Bromopyridine</th>
<th>Catalyst</th>
<th>Product</th>
<th>Conversion(^b)</th>
<th>yield(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 (mixed)</td>
<td>41</td>
<td>Pd(PPh(_3))(_4)</td>
<td>2-pyridyl 29</td>
<td>&lt;30</td>
<td>-</td>
</tr>
<tr>
<td>18 (mixed)</td>
<td>41</td>
<td>Pd(OAc)(_2) + DPPE</td>
<td>2-pyridyl 29</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>18 (mixed)</td>
<td>41</td>
<td>Pd(OAc)(_2) + DPPP</td>
<td>2-pyridyl 29</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>18 (mixed)</td>
<td>41</td>
<td>Pd(OAc)(_2) + DPPF</td>
<td>2-pyridyl 29a (R(_P))</td>
<td>~80</td>
<td>54</td>
</tr>
<tr>
<td>18 (mixed)</td>
<td>41</td>
<td>Pd(OAc)(_2) + DPPF</td>
<td>2-pyridyl 29b (S(_P))</td>
<td>~80</td>
<td>51</td>
</tr>
<tr>
<td>18 (R(_P))</td>
<td>42</td>
<td>Pd(PPh(_3))(_4)</td>
<td>3-pyridyl 44a (R(_P))</td>
<td>&gt;95</td>
<td>85</td>
</tr>
<tr>
<td>18 (S(_P))</td>
<td>42</td>
<td>Pd(PPh(_3))(_4)</td>
<td>3-pyridyl 44b (S(_P))</td>
<td>&gt;95</td>
<td>80</td>
</tr>
<tr>
<td>18 (R(_P))</td>
<td>43</td>
<td>Pd(PPh(_3))(_4)</td>
<td>4-pyridyl 20a (R(_P))</td>
<td>&gt;95</td>
<td>66</td>
</tr>
<tr>
<td>18 (S(_P))</td>
<td>43</td>
<td>Pd(PPh(_3))(_4)</td>
<td>4-pyridyl 20b (S(_P))</td>
<td>&gt;95</td>
<td>69</td>
</tr>
</tbody>
</table>

\(^a\)Reaction conditions: 20 mol % Pd(0), 1.2 equiv. TEA, reflux in THF for ca 5 hours. \(^b\)Conversion according to \(^{31}\)P NMR spectroscopy. \(^c\)Isolated yields.

5.3 Conclusions

An efficient method for the preparation of dinucleoside 3-pyridylphosphonate 44, and also for 2- and 4-pyridylphosphonates 20 and 29, based on a palladium(0)-catalysed cross coupling of dinucleoside H-phosphonate 18 with the corresponding bromopyridines 41-43, was developed. These reactions were stereospecific and occurred most likely with retention of configuration at the phosphorus centre.
As we now had the tools to produce various pyridylphosphonates, we were aiming towards the synthesis of polypyridylphosphonate derivatives with metal complexing properties. An efficient method to incorporate 2,2':6',2''-terpyridine, 2,2'-bipyridine and 1,10-phenanthroline moieties directly onto the phosphorus centre would be of great interest, since oligonucleotides appended with these moieties have shown promising properties as artificial nucleases in the presence of metal ions.\textsuperscript{86,120-123} Also, these metal-complexing nucleotidic compounds could be useful in the development of chemical probes for investigation of electron transfer phenomena in nucleic acids.\textsuperscript{87,124-126} Since these systems contain more than one pyridine nitrogen, \textit{N}-activation did not seem viable to achieve regioselective reactions. Instead, the most straightforward method for the synthesis of polypyridylphosphonates appeared to be a palladium(0)-catalysed cross coupling strategy (Scheme 6.1).

\begin{center}
\begin{tikzpicture}
\node[above] at (3,4) {$\text{RO'-P-OR'}$};
\node[below] at (3,4) {$18$};
\node[above] at (7,4) {$\text{PO R' OR}$};
\node[below] at (7,4) {$45$};
\node[above] at (11,4) {$\text{PO R' OR}$};
\node[below] at (11,4) {$46$};
\node[above] at (15,4) {$\text{PO OR'}$};
\node[below] at (15,4) {$47$};
\draw[->] (3,3) -- (7,3);
\draw[->] (3,2) -- (7,2);
\draw[->] (3,1) -- (7,1);
\draw[->] (7,3) -- (11,3);
\draw[->] (7,2) -- (11,2);
\draw[->] (7,1) -- (11,1);
\draw[->] (11,3) -- (15,3);
\draw[->] (11,2) -- (15,2);
\draw[->] (11,1) -- (15,1);
\end{tikzpicture}
\end{center}

\textit{R} = 5'-O-DMT-thymidyn-3'-yl; \textit{R'} = 3'-O-DMT-thymidin-5'-yl

\textbf{Scheme 6.1} Target polypyridylphosphonates in the Pd(0)-catalysed cross coupling.

\section{6.1 Results and discussion}

In the first attempts to produce dinucleoside terpyridylphosphonate 45, commercially available 4’-chloroterpyridine 48 was reacted with equimolar amount of dinucleoside H-phosphonate 18 in the presence of TEA and various palladium(0) catalysts under reflux in THF for 5 hours (Scheme 6.3). With \textit{Pd(PPh}_3)_4, no product was formed. By using a catalyst system consisting of \textit{Pd(OAc)}_2 and 1,1’-bis(diphenylphosphino)ferrocene (DPPF), only a small amount of the desired product 45 was obtained (<10\%, \textsuperscript{31}P NMR). However, recent reports on efficient arylaminations of chloroarenes using sterically hindered ligands\textsuperscript{108} encouraged us to try a \textit{Pd(OAc)}_2 and 2-(di-\textit{tert}-butylphosphino)biphenyl (DBPB) system. This led to a reasonably high conversion into 45 (\textit{ca} 50\%, \textsuperscript{31}P NMR), but the isolated yields were poor (28%).
**Scheme 6.2** Preparation of bromoarenes 49-51.

**Scheme 6.3** Synthesis of dinucleoside polypyridylphosphonates 45-47.

Since we could not achieve an efficient coupling of H-phosphonate 18 with 4’-chloroterpyridine 48, the more reactive 4’-bromoterpyridine$^{127}$ 49 was prepared (5-bromo-2,2’-bipyridine$^{128}$ 50 and 3-bromo-1,10-phenanthroline$^{129}$ 51 were obtained similarly, Scheme 6.2). When standard conditions, as described above, were applied using 4’-bromoterpyridine 49 and Pd(Ph$_3$)$_4$, exclusive formation of dinucleoside 4’-
terpyridylphosphonate 45 was observed (³¹P NMR). As the separate diastereomers of H-phosphonate 18 were subjected to these reaction conditions, a fully stereospecific conversion into the diastereomers of 4'-terpyridylphosphonate 45 was achieved. The separate diastereomers of 45 were isolated in 73% and 66% yield, respectively (Scheme 6.3, Table 6.1).

Attempted preparation of dinucleoside 5-bipyridylphosphonate 46 in a reaction of H-phosphonate 18 with 5-bromobipyridine 50 in the presence of TEA and Pd(PPh₃)₄ under reflux in THF, resulted in a conversion of only 60% of 18 into bipyridylphosphonate 46 within 5 hours (³¹P NMR). Fortunately, by changing the catalyst system to Pd(OAc)₂ and DPPF, almost quantitative conversion could be obtained. The reaction was carried out on separate diastereomers of 18 and proved to be stereospecific. The diastereomers of bipyridylphosphonate 46 were isolated in 64% and 61% yield, respectively (Scheme 6.3, Table 6.1).

A comparison between some additional ligands revealed that 2,2'-bis(diphenylphosphino)-1,1'-binaphthyl (BINAP) was as efficient as DPPF, while neither 1,2-bis(diphenylphosphino)ethane (DPPE) nor 1,3-bis(diphenylphosphino)propane (DPPP) could promote any formation of 5-bipyridylphosphonate 46. Since 2,2'-bipyridines are known to form stable complexes with palladium(0),¹³⁰,¹³¹ a possible inactivation pathway of the catalyst via complexation could explain the reduced reactivity in the oxidative addition step (Scheme 6.4). This would also provide a rational to the increasing reactivity of rigid and sterically demanding bidentate ligands (DPPF, BINAP), that might prevent the bipyridine moiety of 5-bipyridylphosphonate 50 or 5-bromobipyridine 46 from replacing these ligands and inhibit the oxidative addition.

As expected, the preparation of dinucleoside 3-(1,10-phenantrolyl)phosphonate 47 turned out to be the most difficult task. When the coupling between dinucleoside H-phosphonate 18 and 3-bromo-1,10-phenanthroline 51 was catalysed by Pd(PPh₃)₄ or Pd(OAc)₂–BINAP, no desired product 47 was formed.
A Pd(OAc)$_2$–DPPF catalyst system did promote the coupling, but only 10 % conversion was observed after 5 hours ($^{31}$P NMR). The reduced reactivity of this system is however in line with the even more pronounced complexing properties of 1,10-phenantroline$^{132}$ compared to 2,2’-bipyridine, as the rigid structure forces the two nitrogens into juxtaposition (Scheme 6.4).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Haloarene</th>
<th>Catalyst</th>
<th>Product</th>
<th>Conversion$^b$</th>
<th>Yield$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 (mixed)</td>
<td>48</td>
<td>Pd(PPh$_3$)$_4$</td>
<td>-</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>18 (mixed)</td>
<td>48</td>
<td>Pd(OAc)$_2$ + DPPF</td>
<td>45</td>
<td>&lt;10</td>
<td>-</td>
</tr>
<tr>
<td>18a (R$_p$)</td>
<td>48</td>
<td>Pd(OAc)$_2$ + DBPB</td>
<td>45a (R$_p$)</td>
<td>~50</td>
<td>28</td>
</tr>
<tr>
<td>18a (R$_p$)</td>
<td>49</td>
<td>Pd(PPh$_3$)$_4$</td>
<td>45a (R$_p$)</td>
<td>&gt;95</td>
<td>73</td>
</tr>
<tr>
<td>18b (S$_p$)</td>
<td>49</td>
<td>Pd(PPh$_3$)$_4$</td>
<td>45b (S$_p$)</td>
<td>&gt;95</td>
<td>66</td>
</tr>
<tr>
<td>18 (mixed)</td>
<td>50</td>
<td>Pd(PPh$_3$)$_4$</td>
<td>46</td>
<td>~60</td>
<td>-</td>
</tr>
<tr>
<td>18 (mixed)</td>
<td>50</td>
<td>Pd(OAc)$_2$ + DPPE/DPPP</td>
<td>-</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>18a (R$_p$)</td>
<td>50</td>
<td>Pd(OAc)$_2$ + DPPF</td>
<td>46a (R$_p$)</td>
<td>&gt;95</td>
<td>64</td>
</tr>
<tr>
<td>18b (S$_p$)</td>
<td>50</td>
<td>Pd(OAc)$_2$ + DPPF</td>
<td>46b (S$_p$)</td>
<td>&gt;95</td>
<td>61</td>
</tr>
<tr>
<td>18 (mixed)</td>
<td>51</td>
<td>Pd(PPh$_3$)$_4$</td>
<td>-</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>18 (mixed)</td>
<td>51</td>
<td>Pd(OAc)$_2$ + BINAP</td>
<td>-</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>18 (mixed)</td>
<td>51</td>
<td>Pd(OAc)$_2$ + DPPF</td>
<td>47</td>
<td>&gt;10</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$Reaction conditions: 20 mol % Pd(0), 1.2 equiv. TEA, reflux in THF for 5 hours. $^b$Conversion according to $^{31}$P NMR spectroscopy. $^c$Isolated yields. $^d$Isolation not attempted.

6.2 Conclusions

We have designed efficient synthetic protocols for the attachment of two types of metal complexing functionalities, terpyridyl and bipyridyl groups, to an internucleotide bond. These consisted of the palladium(0)-catalysed cross coupling of dinucleoside H-phosphonates 18 with the corresponding arene bromides using either Pd(PPh$_3$)$_4$ (for terpyridyl derivatives) or Pd(OAc)$_2$ and sterically rigid DPPF or BINAP ligands (for bipyridyl derivatives). The reactions were found to be efficient (>95%, $^{31}$P NMR spectroscopy), completely stereospecific (most likely retention of configuration at the phosphorus centre), and afforded the desired dinucleoside 4’-terpyridylphosphonate 45 and dinucleoside 5-bipyridylphosphonate 46 in 61-73% isolated yields. The synthesis of dinucleoside 3-(1,10-phenanthryl)phosphonate 47 is still under investigation in this laboratory.
7. Oligonucleotides with 2-, 3- and 4-Pyridylphosphonate Internucleotide Linkages: Synthesis and Properties (Paper VI)

As part of our collaborative research with Dr. Barbara Nawrot’s group (Centre of Molecular and Macromolecular Research, Lodz, Poland), we set out to synthesize oligonucleotides with pyridylphosphonate linkages and evaluate their properties. For this purpose, P-stereodefined dinucleoside building blocks with 2-, 3- and 4-pyridylphosphonate internucleosidic bonds were produced, phosphitylated in the free 3’-OH position and incorporated into oligonucleotides by automated solid phase synthesis. Binding affinity of the produced oligonucleotides towards complementary single stranded RNA and DNA fragments, as well as towards complementary double stranded DNA was studied. Also, preliminary CD-studies and evaluation of stability of these constructs against nucleases were carried out.

7.1 Synthesis

For this project, suitably protected dinucleoside pyridylphosphonates with defined stereochemistry at the phosphorus centre were required. In Scheme 7.1 the synthesis of building blocks of type 59, bearing a 4,4’-dimethoxytrityl group at the 5’-O position and a free 3’-OH function, and the subsequent phosphitylation to produce phosphoramidites 60, is shown. The synthesis commences with the preparation of 3’-O-TBDMS-thymidine 55 that was obtained from thymidine 52 by some simple protecting group manipulations. This nucleosidic unit was then coupled with 5’-O-DMT-thymidine H-phosphonate 56 (also prepared from 52), affording dinucleoside H-phosphonate 57. The diastereomers of 57 (ca 1:1 mixture) were separated by silica gel column chromatography and converted stereospecifically into the corresponding pyridylphosphonates 58 using the methods earlier described in this thesis. The 3’-O-TBDMS protecting group from compounds 58 was removed using TBAF in THF. In this manner six isomeric dinucleoside pyridylphosphonates of type 59 (2-, 3- or 4-pyridylphosphonates with $R_P$ or $S_P$ configuration) were prepared. These building blocks were treated with $O$-2-cyanoethyl $N,N$-diisopropylphosphoramidochloridite and ethyl-$N,N$-diisopropylamine in THF to produce phosphoramidites 60.
Reagents and conditions: i) DMT-Cl, pyridine; ii) imidazole, TB DMS-Cl, pyridine; iii) AcOH, THF; iv) DMT-Cl, pyridine; v) a: DPHP, pyridine; b: H2O, TEA; vi) 55, Pv-Cl, pyridine; vii) 2-pyridyl: N-methoxypyridinium tosylate, DBU, acetonitrile; 3-pyridyl: Pd(PPh3)4, TEA, 3-bromopyridine, reflux in THF; 4-pyridyl: Tr-Cl, DBU, pyridine; viii) TBAF, THF; ix) O-2-cyanoethyl-N,N-diisopropylphosphoramidochloridite, N,N-diisopropyl ethylamine, THF.

Scheme 7.1 Preparation of dinucleoside pyridylphosphonate building blocks 60.

3’-O-Phosphoramidite building blocks 60 were used for the incorporation of pyridylphosphonate moieties into oligonucleotides by automated solid phase synthesis. As shown in Scheme 7.2, twelve oligonucleotides (oligothymidylate 20-mers) bearing various pyridylphosphonate units (2-, 3- or 4-pyridylphosphonates with R_p or S_p configuration), either as single (T9T_9TT_9) 61 or double modifications (T8T_8TT_8TT_8) 62, were obtained in this way (x represents the corresponding pyridylphosphonate linkage). The non-modified linkages were all natural phosphate bonds.
Scheme 7.2 Oligonucleotides with a single pyridylphosphonate modification 61 or with double pyridylphosphonate modifications 62.

7.2 Properties

Binding affinity of the produced oligo(deoxyribonucleoside pyridyl-phosphonate)s 61 and 62 to complementary oligonucleotides dA_{19} and A_{19}, and to the double-stranded hairpin oligomer d(A_{21}C_{4}T_{21}) were determined by UV melting temperature measurements at pH 7.4. In addition, stability of triplexes of oligomers 62 with d(A_{21}C_{4}T_{21}) at pH 5.0 were studied. As shown in Table 7.1, in most cases stability of the corresponding duplexes and triplexes strongly depends on the sense of chirality at the modified phosphorus centre as well as the position of the nitrogen in the pyridyl ring.
Table 7.1 Binding affinity of oligonucleotides 61 and 62 towards single stranded DNA, RNA and towards double stranded DNA. All melting temperatures (T\textsubscript{m} \textsuperscript{a}) were determined at pH 7.4 \textsuperscript{b} and, in addition, for triplexes of 62 with hairpin oligonucleotide d(A\textsubscript{21}C\textsubscript{4}T\textsubscript{21}) at pH 5.0.\textsuperscript{c}

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>X</th>
<th>dA\textsubscript{19} T\textsubscript{m}</th>
<th>∆T\textsubscript{m}</th>
<th>A\textsubscript{19} T\textsubscript{m}</th>
<th>∆T\textsubscript{m}</th>
<th>d(A\textsubscript{21}C\textsubscript{4}T\textsubscript{21}) T\textsubscript{m} pH 7.4</th>
<th>∆T\textsubscript{m} pH 7.4 (pH 5.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>61-2R T\textsubscript{9}T\textsubscript{9}TT\textsubscript{9}</td>
<td></td>
<td>51.4</td>
<td>+0.4</td>
<td>40.2</td>
<td>-1.2</td>
<td>28.7</td>
<td>+1.1</td>
</tr>
<tr>
<td>62-2R T\textsubscript{8}TT\textsubscript{9}TT\textsubscript{8}</td>
<td></td>
<td>49.5</td>
<td>-0.7</td>
<td>37.7</td>
<td>-1.8</td>
<td>28.1 (48.9\textsuperscript{c})</td>
<td>+0.2 (-1.6)</td>
</tr>
<tr>
<td>61-2S T\textsubscript{8}TT\textsubscript{9}</td>
<td></td>
<td>49.1</td>
<td>-1.9</td>
<td>37.3</td>
<td>-4.1</td>
<td>28.7</td>
<td>+1.1</td>
</tr>
<tr>
<td>62-2S T\textsubscript{8}TT\textsubscript{9}TT\textsubscript{8}</td>
<td></td>
<td>45.4</td>
<td>-2.8</td>
<td>32.3</td>
<td>-4.5</td>
<td>28.5 (47.8\textsuperscript{c})</td>
<td>+0.4 (-2.2)</td>
</tr>
<tr>
<td>61-3R T\textsubscript{9}T\textsubscript{9}TT\textsubscript{9}</td>
<td></td>
<td>51.8</td>
<td>+0.8</td>
<td>40.0</td>
<td>-1.4</td>
<td>28.4</td>
<td>+0.8</td>
</tr>
<tr>
<td>62-3R T\textsubscript{8}TT\textsubscript{9}TT\textsubscript{8}</td>
<td></td>
<td>50.7</td>
<td>-0.1</td>
<td>38.4</td>
<td>-1.5</td>
<td>28.5 (50.7\textsuperscript{c})</td>
<td>+0.4 (-0.7)</td>
</tr>
<tr>
<td>61-3S T\textsubscript{9}T\textsubscript{9}TT\textsubscript{9}</td>
<td></td>
<td>48.5</td>
<td>-2.5</td>
<td>37.0</td>
<td>-4.4</td>
<td>28.3</td>
<td>+0.7</td>
</tr>
<tr>
<td>62-3S T\textsubscript{8}TT\textsubscript{9}TT\textsubscript{8}</td>
<td></td>
<td>42.7</td>
<td>-4.1</td>
<td>31.6</td>
<td>-4.9</td>
<td>28.3 (43.7\textsuperscript{c})</td>
<td>+0.3 (-4.2)</td>
</tr>
<tr>
<td>61-4R T\textsubscript{9}T\textsubscript{9}TT\textsubscript{9}</td>
<td></td>
<td>53.0</td>
<td>+2.0</td>
<td>42.5</td>
<td>+1.1</td>
<td>26.5</td>
<td>-0.9</td>
</tr>
<tr>
<td>62-4R T\textsubscript{8}TT\textsubscript{9}TT\textsubscript{8}</td>
<td></td>
<td>52.9</td>
<td>+0.9</td>
<td>41.1</td>
<td>-0.1</td>
<td>32.1 (51.5\textsuperscript{c})</td>
<td>+2.2 (-0.3)</td>
</tr>
<tr>
<td>61-4S T\textsubscript{9}T\textsubscript{9}TT\textsubscript{9}</td>
<td></td>
<td>47.9</td>
<td>-3.1</td>
<td>38.8</td>
<td>-2.6</td>
<td>31.9</td>
<td>+4.3</td>
</tr>
<tr>
<td>62-4S T\textsubscript{8}TT\textsubscript{9}TT\textsubscript{8}</td>
<td></td>
<td>43.8</td>
<td>-3.6</td>
<td>34.2</td>
<td>-3.6</td>
<td>31.6 (45.1\textsuperscript{c})</td>
<td>+2.0 (-3.5)</td>
</tr>
<tr>
<td>T\textsubscript{20}</td>
<td></td>
<td>51.0</td>
<td></td>
<td>27.6 (52.2\textsuperscript{c})</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}T\textsubscript{m} values determined with the accuracy of ±0.5 °C. \textsuperscript{b}T\textsubscript{m} measurements in 10 mM Tris-HCl, pH 7.4, 100 mM NaCl and 5 mM MgCl\textsubscript{2} buffer. \textsuperscript{c}T\textsubscript{m} measurements in 10 mM Tris-HCl, pH 5.0, 100 mM NaCl and 5 mM MgCl\textsubscript{2} (values in parentheses). \textsuperscript{d}The T\textsubscript{m} differences per each of the introduced pyridylphosphonate linkages (ΔT\textsubscript{m}). \textsuperscript{e}Reference oligothymidylate 20-mer containing phosphate linkages.

The DNA/DNA and DNA/RNA duplexes containing oligothymidylate strands with R\textsubscript{p}-pyridylphosphonate units differed only slightly from unmodified reference complexes, and the most stable among them were those containing 4-pyridylphosphonate linkages. The difference in T\textsubscript{m} values (ΔT\textsubscript{m}) for these complexes were in the range of -1.8 to +2.0 °C per each introduced pyridylphosphonate modification. In the case of oligomers containing S\textsubscript{p}-pyridylphosphonate moiety, every 2-, 3- or 4-pyridylphosphonate modification introduced caused a significant lowering of the T\textsubscript{m} values of the corresponding DNA/DNA and DNA/RNA duplexes by 1.9 to 4.9 °C per modification. It was, however, not surprising that the S\textsubscript{p} isomer had a more pronounced destabilizing effect on the double helixes since this phenomena has been observed earlier for other modifications, e.g. methylphosphonates.\textsuperscript{134} The pyridyl residue of the S\textsubscript{p} isomer is in fact oriented “inward”, towards the minor groove and might interfere with the base stacking.

For triplexes between hairpin oligonucleotide d(A\textsubscript{21}C\textsubscript{4}T\textsubscript{21}) and oligomers 61 and 62 at pH 7.4, dissociation at equal or slightly higher temperatures than the reference complexes were observed (ΔT\textsubscript{m} up to +2.2°C) in all cases except for the triplex from S\textsubscript{p}. 

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4-pyridylphosphonate modified 61-4S, where a remarkable stabilising effect was found ($\Delta T_m +4.3 \, ^\circ C$). For the triplexes investigated at pH 5.0, a significant increase in $T_m$ values was observed for both the unmodified complex and the complexes containing two pyridylphosphonate units (Table 7.1, values in parentheses). However, the pyridylphosphonate modifications had a more or less pronounced destabilising effect on the formed complexes compared to the reference triplex ($\Delta T_m -0.3 \text{ to } -4.2^\circ C$).

It seems that the differences in stability of the formed complexes (duplexes and triplexes), in comparison to the unmodified counterparts, are due to various favourable/unfavourable interactions involving the pyridylphosphonate moieties. Apart from reducing electrostatic repulsions, the presence of a pyridine ring may provide an alternative mode of hydration and can be an additional source of stabilizing stacking interactions with nucleobases.

Oligonucleotides 61 (T$_9$T$_9$TT$_9$) and reference oligothymidylate T$_{20}$ were used to study the influence of a pyridylphosphonate modification on the stability of the modified oligonucleotides against 3’- and 5’-exonucleases.

![Figure 7.1 Degradation of 61-2R and reference T$_{20}$ oligomers in 50% human plasma.](image)

For human plasma 3’-exonuclease assay, 5’-radiolabeled oligonucleotides were incubated with 50% human plasma. Products of degradation were analysed by PAGE (polyacrylamide gel electrophoresis) under denaturing conditions. Reference oligomer T$_{20}$ afforded a ladder of products ranging from T$_{19}$ to T$_8$ after incubation for 4 hours (Figure 7.1). The modified oligonucleotides 61 were degraded only partially under these conditions. For instance, degradation of oligonucleotide 61-2R proceeded from the 3’-terminus and was arrested by the presence of the 2-pyridylphosphonate...
internucleotide linkage and thus producing, within 8 hours, the $T_9T_xT$ fragment as the major species within 8 hours.

The progress of degradation of modified oligonucleotides $61$ by snake venom phosphodiesterase (a 3’-exonuclease) and calf spleen phosphodiesterase (a 5’-exonuclease) was followed by MALDI-TOF MS. All oligonucleotides of type $61$ were found to be resistant to exonucleolytic hydrolysis at the modification site. For example, the digestion of 2-pyridylphosphonate derivative $61\text{-}2R$ with 5’-exonuclease resulted in a set of peaks ranging from $T_9T_xTT_9$ ($m/z$ 6079) to $T_xTT_9$ ($m/z$ 3344). The other modified oligonucleotides $61$ gave similar sets of signals when digested by the investigated enzymes.

The influence of pyridylphosphonate-modified oligonucleotides $62$ ($T_8T_xTT_xTT_8$) on the geometry of the duplexes formed with complementary DNA or RNA strands was evaluated by CD (circular dichroism) spectroscopy. From the shape of these spectra one could conclude that duplexes formed by pyridylphosphonate-modified oligonucleotides adopt B (for DNA/DNA) and A (for DNA/RNA) helical structures, similar to their unmodified counterparts. Although some minor differences in signal intensities were observed at certain frequencies, it was clear that the pyridylphosphonate linkages did not cause any severe disturbance of the tertiary helical structures.

### 7.3 Conclusions

Our preliminary data show that the introduction of pyridylphosphonate linkages at specific positions in oligonucleotides provides tunable properties that can be useful in the design of new antisense/antigene agents. The replacement of natural phosphate bonds by P-chiral 2-, 3- or 4-pyridylphosphonate bonds in oligodeoxyribonucleotides did not introduce significant geometric alterations to the double-helical complexes formed by these modified oligonucleotides. However, the modification had a remarkable influence on the stability of the formed double stranded complexes, depending on stereochemistry of the modified unit and the mode of pyridine ring substitution. The observed differences in $T_m$ values for duplexes formed by oligonucleotides $61$ and $62$ with complementary DNA or RNA strands and those of unmodified reference complexes suggest that only $S_p$-pyridylphosphonate units significantly destabilize double-helical complexes (up to 4.9 °C per modification). In most cases of triple-helical structures investigated at neutral conditions (pH 7.4), pyridylphosphonate moieties slightly stabilize the complexes, presumably due to favourable interactions involving the pyridine ring. Finally, degradation by both 3’-
and 5’-exonucleases is arrested at the modification site of pyridylphosphonate-modified oligonucleotides.
8. Synthesis of a Nucleotidic Unit for the Incorporation of Terminal 5’-Pyridylphosphonate Moieties into Oligonucleotides (Paper VII)

As we now had produced oligonucleotides with internucleotidic pyridylphosphonate linkages and evaluated some of their properties, a protocol for the synthesis of terminally pyridylphosphonate-modified oligonucleotides became desirable to get a more complete picture of this type of modification. In this project we were aiming towards the development of a nucleotidic unit suitable for introduction of a pyridylphosphonate monoester function to the 5’-terminal position of oligonucleotides. The initial strategy was to synthesize building blocks of type 67 (Scheme 8.1), with a methyl-protected pyridylphosphonate unit in the 5’-position and an H-phosphonate in the 3’-position. These units were designed to be incorporated into oligonucleotides via H-phosphonate methodology,18-23 and to subsequently be oxidised and deprotected to produce 5’-pyridylphosphonate-modified oligonucleotides. Unfortunately, a number of problems arose in the synthesis of pyridylphosphonate building blocks 67. Hence, this approach was eventually modified and instead building blocks of type 69 (Scheme 8.3) were developed in order to achieve a practical and efficient synthetic protocol.

8.1 Towards the Synthesis of a key Nucleotidic Unit

Methyl nucleoside H-phosphonate 64 could be produced by a pivaloyl chloride promoted coupling of nucleoside H-phosphonate monoester 63 with methanol in pyridine (Scheme 8.1). Methyl nucleoside H-phosphonate 64 was isolated in 72% yield. Starting from this suitably protected H-phosphonate diester, we assumed that the corresponding 2-, 3- and 4-pyridylphosphonates 65a-c could be produced under the conditions previously developed for the synthesis of dinucleoside pyridylphosphonates.71,100,133 Methyl nucleoside 2-pyridylphosphonate 65a could indeed be obtained by a DBU promoted reaction of methyl nucleoside H-phosphonate 64 with N-methoxypyridinium tosylate, and was isolated in 83% yield. The protocol previously developed for the synthesis of dinucleoside 4-pyridylphosphonates71 worked also for H-phosphonate 64. The corresponding methyl nucleoside 4-pyridylphosphonate was thus produced via a DBU promoted reaction of methyl nucleoside H-phosphonate 64 with triphenylmethyl chloride in pyridine, followed by the addition of iodine to
facilitate rearomatisation of the 1,4-dihydropyridine intermediate. The desired product 65c was isolated in 92% yield.

In an attempted synthesis of 3-pyridylphosphonate 65b, the desired product was obtained as the major species via a Pd(PPh₃)₄ catalysed reaction of methyl nucleoside H-phosphonate 64 with 3-bromopyridine in the presence of triethylamine, but extensive demethylation was observed. By replacing TEA with N,N-diethylaniline, α-picoline, 2,6-lutidine or sym-collidine, demethylation was suppressed but neither of these bases could promote full conversion into product 65b. Propylene oxide, a proton scavenger that has been used in a similar palladium(0)-catalysed coupling,¹³⁵ did not promote full conversion either. Finally, N,N-diisopropylethylamine was found to provide the best compromise regarding nucleophilicity vs. basicity, since demethylation was significantly suppressed while a full conversion was still achieved. Under these conditions, the produced methyl nucleoside 3-pyridylphosphonate 65b could be isolated in 61% yield.

Scheme 8.1 Retrosynthesis of H-phosphonate building blocks 67.

The next step in the synthesis was deprotection of the 3’-O-position (Scheme 8.1). By dissolving methyl nucleoside 2-pyridylphosphonate 65a in 80% aq. AcOH/THF 2:1 (v/v) at 50°C, complete detritylation was achieved. The deprotected product 66a was isolated in 85% yield. 3-Pyridylphosphonate 65b and 4-pyridylphosphonate 65c were treated in the same manner and efficient conversion into deprotected compounds 66b and 66c, respectively, were observed. However, after purification, products 66b and 66c decomposed upon standing. A similar pattern of peaks was observed in both cases although 4-pyridylphosphonate 66c seemed to be slightly more labile than 3-pyridylphosphonate 66b.
Since the tritylated counterparts of these compounds were completely stable, we assumed that the observed instability of 3-pyridylphosphonate 66b and 4-pyridylphosphonate 66c could be due to the presence of the free hydroxyl group that via an intramolecular attack on the phosphorus labilises these compounds (this assumption turned out to be incorrect; see later in this Chapter). To circumvent this problem, we decided to phosphorylate the 3’-hydroxyl groups of 66b-c immediately after deprotection of 65b-c, to produce synthetic intermediates 67b-c that were expected to be more stable (Scheme 8.1).

To this end the deprotection protocol was changed to get 3- and 4-pyridylphosphonates 66b-c with a minimal number of synthetic manipulations. Thus, 3’-O-dimethoxytritylated pyridylphosphonate 65b was applied on the top of a short silica gel column, and detritylation with a simultaneous elution of dimethoxytritanol was performed with 1% TFA in methylene chloride containing 1% of methanol. Product 66b, eluted with a stepwise gradient of THF in methylene chloride, was concentrated, and immediately subjected to phosphorylation with diphenyl H-phosphonate in pyridine. After work-up and silica gel chromatography, the phosphorylated 3’-O-(H-phosphonylated) 3-pyridylphosphonate 67b was isolated in 63% yield. Using an analogous protocol, 4-pyridylphosphonate derivative 67c was obtained in 58% yield, and 2-pyridylphosphonate 67a in 65% yield, by phosphorylation of the previously isolated precursor 66a.

Somewhat unexpectedly, however, the isolated 3’-O-(H-phosphonyl) 3-pyridylphosphonate 67b and 4-pyridylphosphonates 67c, also turned out to be labile, similarly to the corresponding parent compounds 66a and 66c. By this time we thought that the lability of compounds 67b and 67c might be due to the negatively charged monoester moiety in the 3’-position that could make an intramolecular attack on the phosphorus centre. Based on this assumption, the synthesis of neutral, non-nucleophilic phosphoramidite building blocks 68 seemed to be an attractive way to circumvent these stability problems (Scheme 8.2).

2-Pyridylphosphonate phosphoramidite 68a was thus prepared via a reaction of 66a with O-methyl N,N-diisopropylphosphoramidochloridite in the presence of N,N-diisopropylethylamine and was isolated in 48% yield. Phosphoramidite 68a was stable upon storage. 4-Pyridylphosphonate phosphoramidite 68c was produced in a similar way, although the deprotection had to be done immediately before the phosphitylation. The phosphoramidite 68c could be isolated, but decomposed upon standing.
Since phosphoramidite 68c decomposed as readily as H-phosphonate 67c, we considered the option of making the pyridylphosphonate moiety ionised, via removal of the methyl protecting group from compounds 67 (Scheme 8.3). Since preliminary experiments were encouraging, we appended our synthetic scheme with the demethylation step that was carried out on H-phosphonate derivatives 67 immediately after the purification. Standard protocol for demethylation of phosphate triesters using thiophenol in the presence of TEA turned out to be inefficient when applied to our compounds, and required lengthy reaction time (days). However, by replacing TEA by DBU, a clean conversion of pyridylphosphonate diesters 67 into the corresponding bis-phosphonic acids 69 was achieved within 3 h. Compounds 69a-c were stable solids and were obtained in total (3 steps) yield of 55-65%.

**Scheme 8.3** Final protocol for the synthesis of H-phosphonate building blocks 69.

*Reagents and conditions:* i) pivaloyl chloride, methanol, pyridine; ii) 2-pyridylphosphonate: N-methoxypyridinium tosylate, DBU, acetonitrile; 3-pyridylphosphonate: Pd(PPh₃)₄, N,N-diisopropylethylamine, 3-bromopyridine, reflux in THF; 4-pyridylphosphonate: a: trityl chloride, DBU, pyridine; b: iodine; iii) detritylation on column, eluent: dichloromethane +1% TFA, +1% methanol; iv) a: DPHP, pyridine; b: triethylamine, H₂O; v) thiophenol, DBU, pyridine.
8.2 Coupling of the key Nucleotide Unit with a Nucleosidic Component

With these stable nucleotide building blocks 69, we could finally check if a selective H-phosphonate coupling was possible in the presence of a pyridylphosphonate monoester moiety. Pivaloyl chloride was chosen as coupling agent since it is known to promote fast and efficient coupling of H-phosphonates with alcohols. 23 3'-O-Dimethoxytritylthymidine was used as a model compound for a 5'-deprotected oligonucleotide.

Indeed, pivaloyl chloride promoted a fast coupling of H-phosphonates 69 with 3'-O-DMT-thymidine producing intermediates 70 (Scheme 8.4). The pyridylphosphonate moiety did get O-pivaloylated, but no C-phosphonate coupling product was observed. The addition of iodine-triethylamine-water resulted in immediate oxidation of the P-H bond and simultaneous deprotection of the pivaloylated pyridylphosphonate, yielding the final products 71. These reactions were all quantitative according to $^{31}$P NMR studies and the 2-pyridylphosphonate 71a, 3-pyridylphosphonate 71b and 4-pyridylphosphonate 71c were isolated in 80-88% yields.

![Scheme 8.4](image)

Reagents and conditions: i) 3'-O-dimethoxytritylthymidine, pivaloyl chloride, pyridine; ii) iodine, triethylamine, H$_2$O.

Scheme 8.4 Incorporation of H-phosphonate building blocks 69 to the 5'-terminus of a nucleoside unit.

8.3 Possible Decomposition Pathways for Nucleoside Methyl Pyridylphosphonates

The observed instability of nucleotide units of type 66b-c, 67b-c and 68b-c (vide supra) was a bit puzzling in light of the fact that the parent 3'-O-dimethoxytritylated derivatives 65 did not show any noticeable tendency to
decompose. Taking into account differences in chemical structures between compounds 65b-c and 66b-c, the most appealing explanation was the involvement of the 3’-hydroxyl function as an intramolecular catalytic group in the decomposition pathway. However, compounds 67b-c bearing the 3’-O-(H-phosphonate) functionality and also the corresponding 3’-O-(methyl N,N-diisopropylphosphoramidites) 68b-c showed a similar lability in solution, and thus we had to look for another explanation.

By analysing the pattern of the $^{31}$P NMR signals of the decomposition products formed from pyridylphosphonate 66c, and by identifying one of them as a demethylated product of 66c, led us to a tentative decomposition pathway for this compound as depicted in Scheme 8.5. This pathway, which features two demethylation reactions, was substantiated by $^{31}$P NMR spectroscopy study on isolated nucleoside 4-pyridylphosphonate 66c. To this end the $^{31}$P NMR spectra of compound 66c that has been kept as a solid in a freezer at –25°C were recorded at different time intervals. After few hours of storage, the spectra revealed the presence of two sets of signals. A singlet at $\delta_P = 8.70$ ppm (compound 72c, the demethylation product of 66c), and two singlets at $\delta_P = 10.71$ and 9.79 ppm (ratio ca 1:1:1; total ca 4 %) became apparent. The latter two resonances we tentatively assigned to the expected N-methylated compound 73c (two diastereomers of one-phosphorus spin system). The signals due to 72c and 73c were growing in time, but simultaneously a new resonance, a singlet at $\delta_P = 5.05$ ppm, appeared. This signal was gradually increasing in intensity and after several hours it became the major resonance of the decomposition products. To this species resonating at 5.05 ppm, we tentatively assigned a zwitterionic structure 74c. In a separate experiment, when 4-pyridylphosphonate 66c was refluxed in methanol/acetonitrile, a high conversion into the decomposition product 74c was achieved, and after isolation its identity could be confirmed by $^1$H NMR analysis.

![Scheme 8.5 Decomposition pathway of compound 66c.](image-url)
Since the other pyridylphosphonate derivates 66b, 67b, and 67c showed a similar pattern of signals in the $^{31}$P NMR spectra upon decomposition in solution, the above mechanism can also be safely extended to these compounds.

To rationalise the observed susceptibility of pyridylphosphonates 65, 66 and 67 towards demethylation, both electronic and steric factors should be considered. For derivatives 65, it seems that a bulky 3’-O-dimethoxytrityl group effectively prevents self-demethylation of the methyl pyridylphosphonates via steric hindrance, irrespective of the position of the nitrogen atom in the pyridine ring. For derivatives 66 (and perhaps 67), the demethylation occurs predominately by nucleophilic attack of nitrogen of the less sterically hindered 4-pyridyl- or 3-pyridylphosphonate moiety. Another factor that might increase the resistance of methyl 2-pyridylphosphonate derivatives towards self-demethylation is the fact that the nitrogen atom in 2-pyridylphosphonates should be less nucleophilic than those in the 3-pyridyl-, and perhaps also in the 4-pyridylphosphonates.

### 8.4 Conclusions

Efficient protocols for the syntheses of nucleotidic units of type 69, designed for the incorporation of 2-pyridyl-, 3-pyridyl- or 4-pyridylphosphonate monoester groups into oligonucleotides, were developed. The reaction of 69 with a nucleosidic component showed that the pivaloyl chloride promoted condensations were completely chemoselective, and after oxidation with iodine-water, afforded the desired dinucleoside (3’-5’)-phosphates with pyridylphosphonate monoester functions at the 5’-ends in high yields.
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(66) A reaction is here referred to as a stereoselective one only in cases where one diastereomer is preferentially formed from a 1:1 diastereomeric mixture of starting material.


