Studies on Oxidative Couplings in H-Phosphonate Chemistry

by

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Abstract

In this thesis oxidative coupling of H-phosphonate and H-phosphonothioate diesters with different alcohols and amines are presented. Since the reactions with alcohols previously have been particularly unfavourable due to competing side reactions, a modified protocol leading to high coupling yields of structurally diverse hydroxylic components was developed. The phosphorylation reaction was studied using $^{31}$P NMR spectroscopy and for the first time the previously only postulated reactive intermediate involved in these reactions was observed.

The use of iodine in combination with a bulky chlorosilane in pyridine was found to have a profound effect on both the suppression of side reactions and the rate of the oxidative couplings, and led to a clean formation of phosphorylated products in high yields. This synthetic protocol was then extended to include coupling reactions with bifunctional reagents containing hexamethylene linkers to provide handles for derivatisations of oligonucleotides.

A synthetic protocol consisting of the stereospecific oxidative coupling of amines with H-phosphonate diesters to produce phosphoroamidates was designed in such a way that it permitted control of the stereochemical outcome of the reactions.

Based on a silylation-mediated reaction utilising phenyl H-phosphonothioate monoester as a thiophosphonyl transferring agent, a method was developed and used for the preparation of H-phosphonothioate building blocks for the synthesis of DNA analogues.
## Table of contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Papers</td>
<td>IV</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>V</td>
</tr>
<tr>
<td><strong>1. General Introduction</strong></td>
<td>1</td>
</tr>
<tr>
<td>1.1 Oligonucleotide Therapeutics</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Modifications at the Phosphorus Centre</td>
<td>4</td>
</tr>
<tr>
<td><strong>2. The Concept of Oxidative Coupling</strong></td>
<td>6</td>
</tr>
<tr>
<td>2.1 Stereochemistry of Coupling Reactions</td>
<td>6</td>
</tr>
<tr>
<td><strong>3. Nucleophilic Catalysis in Phosphorylation Reactions (Paper I)</strong></td>
<td>8</td>
</tr>
<tr>
<td>3.1 Phosphorylation of Alcohols in Pyridine</td>
<td>8</td>
</tr>
<tr>
<td><strong>4. Problem of Hydrolysis by Adventitious Water (Paper II)</strong></td>
<td>14</td>
</tr>
<tr>
<td>4.1 Suppressing Hydrolysis with Silylating Reagents</td>
<td>15</td>
</tr>
<tr>
<td>4.2 Iodine as a Catalyst in Oxidative Phosphorylations</td>
<td>18</td>
</tr>
<tr>
<td>4.2.1 Oxidative Coupling of Alcohols with H-Phosphonates</td>
<td>19</td>
</tr>
<tr>
<td>4.2.2 Oxidative Coupling of Alcohols with H-Phosphonothioates</td>
<td>20</td>
</tr>
<tr>
<td><strong>5. Oxidative Coupling of Amines with H-Phosphonates (Paper III)</strong></td>
<td>21</td>
</tr>
<tr>
<td>5.1 Controlling the Stereochemical Outcome</td>
<td>22</td>
</tr>
<tr>
<td><strong>6. Oxidative Coupling of N- and O-Binucleophiles with</strong></td>
<td>25</td>
</tr>
<tr>
<td>H-Phosphonates and H-Phosphonothioates (Paper IV)</td>
<td>27</td>
</tr>
<tr>
<td>6.1 Reaction with Amino Nucleophiles</td>
<td>27</td>
</tr>
<tr>
<td>6.1.1 Reaction with 6-Aminohexanol</td>
<td>27</td>
</tr>
<tr>
<td>6.1.2 Reaction with 1,6-Diaminohexane</td>
<td>28</td>
</tr>
<tr>
<td>6.2 Reaction with Oxygen Nucleophiles</td>
<td>29</td>
</tr>
<tr>
<td>6.2.1 Reaction with 6-Aminohexanol Hydrochloride</td>
<td>29</td>
</tr>
<tr>
<td>6.2.2 Reaction with (Monosilylated) 1,6-Hexanediol</td>
<td>30</td>
</tr>
<tr>
<td><strong>7. Silylation-Mediated Thiophosphonylations (Paper V)</strong></td>
<td>32</td>
</tr>
<tr>
<td><strong>8. Conclusions</strong></td>
<td>35</td>
</tr>
<tr>
<td><strong>9. Acknowledgements</strong></td>
<td>36</td>
</tr>
<tr>
<td><strong>10. References and Notes</strong></td>
<td>37</td>
</tr>
<tr>
<td>Appendix A: Supplementary Experimental Data for Paper II</td>
<td>40</td>
</tr>
</tbody>
</table>
List of Papers

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

I. Reinvestigation of the $^{31}$P NMR Evidence for the Formation of Diorganyl Phosphoropyridinium Intermediates

II. Oxidative Coupling of H-Phosphonate and H-Phosphonothioate Diesters. Iodine as a Reagent and a Catalyst

III. Controlling Stereochemistry During Oxidative Coupling. Preparation of Rp or Sp Phosphoramidates from One P-Chiral Precursor.
     Nilsson, J.; Stawinski, J. *Submitted*.


V. Silylation-Mediated Transesterification of Phenyl H-Phosphonothioate. A New Entry to Nucleoside H-Phosphonothioate Monoesters.
   Lavén, G.; Nilsson, J.; Stawinski, J. *Submitted*. 
Abbreviations

ATP  Adenosine triphosphate
B    A nucleobase
Cat  Catalyst
CBTU S-(p-Chlorobenzyl)isothiuronium
DMT  4,4´-Dimethoxytrityl
DNA  Deoxyribonucleic acid
DIPEA N,N-Diisopropylethylamine (Hünig’s base)
DMAP 4-Dimethylaminopyridine
DPCP Diphenyl chlorophosphate
DPHP Diphenyl H-phosphonate
ee   Enantiomeric excess
HMDS 1,1,1,3,3,3-Hexamethyldisilazane
mRNA Messenger ribonucleic acid
n.d. Not detected
NMR Nuclear Magnetic Resonance
Nu   Nucleophile
o.n. Overnight
RNA Ribonucleic acid
T    Thymidine
Thy Thymin-1-yl
TBDMS tert-Butyldimethylsilyl
TBDPS tert-Butyldiphenylsilyl
TEA Triethylamine
THF Tetrahydrofurane
Thy Thymine
TMS Trimethylsilyl
sON Synthetic oligonucleotide
Ph   Phenyl
Py   Pyridine
1. General Introduction

Phosphorus-containing compounds (Figure 1) play an important role in nature where they often are involved as substrates for enzymes\(^1\), bioenergy mediators\(^1\) or as part of a structural backbone of biomolecules that carry genetic information\(^1\). They are also used as potent pesticides (e.g. herbicides, insecticides or fungicides)\(^2\), warfare agents\(^3\) or therapeutics. Organic phosphates are primarily abundant in the biosphere in the form of negatively charged phosphorodiester linkages between sugar moieties in DNA or RNA, in phospholipids and as metabolic energy carriers in the form of triphosphates (e.g. ATP).

![Figure 1. Examples of biologically active phosphorus compounds.](image)

1.1 Oligonucleotide Therapeutics

It is well known that viruses, and especially retroviruses, infect their hosts at the level of the genome. In 1978 Zamecnik and Stephenson discovered that complementary base pairing occurring between an externally added 13-mer oligonucleotide and viral mRNA may inhibit viral gene expression.\(^4,5\) Their findings were a milestone that gave birth to a new concept of treatment of viral diseases by utilising synthetic oligonucleotides (sON) that can form duplexes with complementary RNA (antisense approach, via Watson-Crick base pairing) or triplexes with complementary DNA (antigene approach, via Hoogsteen base pairing).\(^6\) The RNA-DNA duplexes are enzymatically degraded by RNase H into fragments in the cytosol, resulting in cleavage of the mRNA and release of the sON for additional duplex formations.\(^7\) In antigene approach, the sON is instead coordinated to a double stranded DNA and physically inhibits transcription by blocking the access of RNA polymerase (Figure 2). In this way the production of a detrimental or defective protein can be stopped or at least significantly slowed down, and the effect of the viral infection is extinguished. If only a sufficiently long sequence of a viral DNA is known, then it is possible to use this
information to produce the corresponding antisense or antigene sON. This implies that antisense or antigene oligonucleotides can be synthesized by principle of rational drug design, which is a holy grail of organic medicinal chemists.

Figure 2. Schematic principles of the antigene and antisense approach.

Classical drugs usually target the harmful protein that causes the disease, and since a single molecule of mRNA can be responsible for the production of thousands of protein molecules, the potency of antisense drugs is by far greater than that of traditional drugs that would require a higher dose in order to reach the same effect. In this context the antigene approach should be even more effective since one molecule of DNA in turn gives rise to several copies of mRNA.6

In order to be an efficient antisense or antigene drug, the sON must be altered in such a way that it still can hybridise with high selectively to the desired RNA or DNA
sequence, but at the same time to be structurally modified enough to become resistant to nucleolytic enzymes. To achieve selective base pairing between a target sequence and a sON, the oligonucleotide has been calculated to contain at least 17-21 nucleotides. Statistically, a 17-nucleotides long sequence appears only once in the human genome. In addition, since only approximately 20% of the total cell DNA is transcribed simultaneously and viral transcription occur to a higher extent than that for the host genes, this should further increase specific hybridisation.

Numerous types of modifications of oligonucleotides, bearing alterations at nucleobases, the ribose/deoxyribose moieties or at the phosphate linkages have been evaluated for the purpose of antisense or antigen therapy in recent years. Oligonucleotides having exchanged the phosphorodiester linkage for other functional groups (dephospho-analogues), as well as derivatives in which both the ribose and phosphate linkages have been replaced for alternative structures, have also been reported. Especially nucleic acid analogues having the sugar-phosphate backbone replaced by a peptide skeleton, known as peptide nucleic acids (PNA), are gaining attention (Figure 3).

![Figure 3. Potential sites for introduction of modifications in a DNA oligonucleotide (A) in comparison to a PNA analogue (B).](image)

Since phosphorothioate diesters bear a negative charge and thus are isoelectronic with ordinary phosphates, it is not surprising that the first approved antisense drug on the market was an oligomer containing phosphorothioate internucleotidic linkages. When a few of the phosphate linkages in the sON are replaced by phosphorothioates, the in vivo
half life of the oligomer is strongly enhanced as natural endo- and exonucleases much faster degrade the viral mRNA than the modified sON. The presence of sulfur also introduces chirality and increases the size of the phosphate group.

One of the main problems of the contemporary antisense technology is the delivery of the drug to the cells where it is supposed to exert its biological effect. The polyanionic nature of oligonucleotides makes it unfavourable to be transported through biological membranes into the cytosol. The only antisense drug available on the market today, the 21-mer Vitravene®, is active against cytomegalovirus induced retinitis in the eyes of immunodeficient patients, and is administrated as eyedrops.

Oligonucleotides, bearing chemically attached reporter groups to be detected by chemofluorescence, radioactivity, with antibodies, or with substrates of coupled enzymes are also commonly used in biotechnology as hybridisation probes and diagnostic tools in medicine.

1.2 Modifications at the Phosphorus Centre

There are numerous modifications of the internucleosidic phosphate linkages and these usually consist of a bridging or non-bridging oxygen atom being replaced by another atom (or molecule) e.g. phosphorothioates, phosphoroamidates, phosphorotriesters or methylphosphonates (Figure 4). If one of the two non-bridging oxygen atoms is replaced by other heteroatoms the phosphate group becomes chiral, but usually only one of the formed P-diastereomers demonstrates desired biological properties. It was found that the most stable complexes are usually formed when a modification is pointing away from the duplex, towards the solvent. By removing the negative charge at the phosphorus centre, the electrostatic repulsions between two oligonucleotide strains are reduced which generally favours hybridisation.
Figure 4. Phosphate analogues; a methylphosphonate (A), a phosphorotriester (B), a phosphoramidate (C) and a phosphorothioate (D).

The use of H-phosphonate chemistry as a means to introduce different modifications at the phosphorus centres has several advantages. The most important is the possibility of using the same starting material to produce various products of choice, which would be difficult to obtain using other synthetic methods. The H-phosphonate monoester synthons are also usually more stable and easier to handle than, for instance, the corresponding building blocks used in the phosphoramidite method.\textsuperscript{21-23}

This thesis will primarily focus on the oxidative couplings of dinucleoside H-phosphonates and H-phosphonothioates with amines and alcohols. The products of these reactions are phosphoroamidates and phosphotriesters, respectively, which both constitute stable and chiral products that potentially can show high affinity for target RNA or DNA fragments. The uncharged nature of these phosphate analogues also should alleviate problems of their passage through biological membranes if used as antisense or antigene agents. Furthermore, the chirality at the phosphorus centre offers a possibility of using only one diastereomer as a drug, either via stereospecific synthesis or a post-synthetic chromatographic separation.
2. The Concept of Oxidative Couplings

Oxidative coupling of H-phosphonates consists of two steps, namely the oxidation of the P-H bond into a halophosphate, followed by a nucleophilic displacement of the halide by alcohols or amines (Scheme 1). The reaction was first developed during the 40’s last century by Atherton and Todd, who oxidised dibenzyl H-phosphonate with carbon tetrachloride in the presence of different amines as nucleophiles.\(^{24,25}\)

![Scheme 1. The oxidative coupling reaction. Alcohols or primary and secondary amines can act as nucleophiles.](image)

Although the reaction appears deceptively simple, there are several problems that should be addressed. If the nucleophile does not react fast enough with the formed phosphorohalidate, usually a nucleophilic catalyst (often pyridine) is required to accelerate the reaction. However, as phosphorohalidates become much more reactive in the presence of catalysts, they will not only react with the desired substrate but may be attacked by any nucleophile present. Nucleoside H-phosphonates may contain various amounts of water that can interfere in this reaction, causing hydrolysis and, as a result, formation of various side products. This is the major problem when oxidative coupling with alcohols is attempted. Amines are far more reactive \textit{per se} and usually do not require nucleophilic catalysts for reactions with phosphorohalidates.

2.1 Stereochemistry of Coupling Reactions

The oxidation of H-phosphonates is usually stereospecific, \textit{i.e.} one diastereomer of the substrate affords only one diastereomer of phosphorohalidate. Depending on the type of oxidising agent used (usually iodine\(^ {26,27}\), carbon tetrachloride\(^ {24}\) or N-halosuccinimide\(^ {28,29}\)), different phosphorohalidates are obtained. The reactivity of phosphorohalidates towards nucleophiles is decreasing in the order I>Br>Cl>>F. Due to the ease of handling, most commonly iodine or carbon tetrachloride are used in oxidative coupling reactions. The oxidation pathway using carbon tetrachloride (the Atherton-Todd reaction) has been the subject of discussion in the past\(^ {25}\) and although nowadays it is
generally believed to involve an X-philic attack of phosphorus on a halide atom, still publications appear that claim other reaction pathways.\textsuperscript{30}

Since phosphorohalidates are only moderately reactive, reactions with alcohols usually require nucleophilic catalysts to effect substitution at the phosphorus centre. The penalty for using nucleophilic catalysts is that stereomerically pure phosphorohalidates are epimerised by the catalyst and a mixture of diastereomers of the product is formed (Scheme 2).

\textbf{Scheme 2.} Epimerisation of diastereomerically pure phosphorohalidates by nucleophilic catalysts and the subsequent alcoholysis.

The use of less nucleophilic bases such as Hünig’s base (DIPEA) or triethylamine, usually ensures that the reaction proceed in a stereospecific manner. The base deprotonates the alcohol and thereby increases its reactivity towards halophosphates while the steric bulk of the base prevents it from attacking (and epimerising) the phosphorus centre. Unfortunately, base catalysis is usually less efficient than nucleophilic catalysis, and requires the use of a larger excess of alcohol for suppressing the competing hydrolysis. In contradistinction to this, primary and secondary amines react swiftly to produce phosphoroamidates in a stereospecific manner.\textsuperscript{31}

A high rate of reaction with nucleophiles in oxidative couplings is imperative. Alcohols react slowly and require either base or nucleophilic catalysts. In both cases excess of alcohol is needed in order to diminish the competing hydrolysis. In some publications, an excess of H-phosphonates rather than the alcohol has been used to achieve high coupling yields.\textsuperscript{28,32}

Amines, on the other hand, display high enough reactivity to directly react with phosphorohalidates, and the rates exceed that of reaction with water. For this reason
reactions with amines are usually fast, stereospecific, quantitative and proceed with a total inversion of configuration (Scheme 3).\textsuperscript{31}

\begin{align*}
\text{Oxidation} & \quad \text{RNH}_2 \\
\text{Phosphorylation} & \quad \text{OR'}
\end{align*}

\textbf{Scheme 3.} The oxidative coupling reaction with amines is stereospecific.

Oxidative coupling is primarily used in conjunction with amines due to the pronounced inefficiency of the reactions with alcohols. Phenols are an exception from this rule and couple more efficiently due to the generally higher acidity than for alcohols. In this sense, hydrolysis problems are lessened since the formed phenolate reacts faster than water.\textsuperscript{33,34} Owing to the stereospecificity of coupling reactions with amines, a method has been developed for a spectroscopic determination of the ratio of D and L forms of amino acids (ee determination) by quantifying the ratio of the obtained diastereomeric phosphoroamidates using \textsuperscript{31}P NMR spectroscopy.\textsuperscript{35,36}

\section*{3. Nucleophilic Catalysis in Phosphorylation Reactions \textit{(Paper I)}}

In organic chemistry, nucleophilic catalysis play an important role in improving reaction rates and often enables reactions that otherwise would be difficult to carry out.\textsuperscript{37} One of the most commonly used catalysts is pyridine. This unhindered aromatic amine demonstrates high nucleophilicity and good leaving group properties. Pyridine can easily be removed from the reaction mixtures by the means of extraction or evaporation. It may be used as solvent or cosolvent since it also displays good solubility for organic compounds and is easy to handle, although toxic.\textsuperscript{38} In phosphorus and nucleotide chemistry, pyridine is often used both due to its ability to dissolve polar compounds (that show low solubility in other organic solvents), and due to its nucleophilic catalytic properties.

\subsection*{3.1 Phosphorylation of Alcohols in Pyridine}

A reaction that has been subject of several mechanistic investigations is phosphorylation of alcohols.\textsuperscript{39-41} Phosphorylated alcohols are an important class of
compounds that perform a variety of different tasks in an organism. For example they are required in the biosynthesis of nucleotides, act as precursors of biological energy carriers, and are important regulators of various metabolic pathways.\textsuperscript{42}

In synthetic nucleotide chemistry, as well as in nature, the first step towards synthesis of oligonucleotides begins with a phosphorylation reaction of a ribose unit.\textsuperscript{1} For this reason the phosphorylation reaction is of high importance and interest. When investigating reactions at a phosphorus centre, $^{31}$P NMR is a very useful tool to elucidate reaction pathways and in conjunction with incremental addition of reagents, $^{31}$P NMR spectroscopy often allows observations of reactive intermediates.

Although pyridine plays a central role in phosphorylation reactions \textit{in vitro}, the reactive intermediates containing pyridine as an integral part (e.g. a pyridinium adduct of metaphosphate) have until recently only been detected for phosphate monoester derivatives $1$. Intermediates of this type have also on many occasions been postulated for the more reactive phosphate diester derivative $2$ (Chart 1).\textsuperscript{43,44} The greater reactivity of intermediates of type $2$ is generally believed to be due to the lack of betaine-like stabilisation compared to $1$. In 1995 Perich \textit{et al.} reported on a spectroscopic detection of this elusive pyridinium diester adduct $2$.\textsuperscript{45}

![Chart 1. Reactive intermediates containing a pyridine moiety.](image)

In contrast to our observations, the publication by Perich \textit{et al.} claimed that a reaction between halophosphates (3 or 4, Chart 2) and pyridine resulted in the formation of a detectable amount of the formerly only postulated pyridinium adduct $2$. This, in conjunction with many reactions that proceed via this putative intermediate, prompted us to closely reinvestigate these claims. Perich observed, using $^{31}$P NMR spectroscopy, that diphenyl chlorophosphate (3a) in THF reacted upon addition of pyridine to form a compound resonating at -25.5 ppm (25\% of the total phosphorus amount, the remaining 75\% being the starting material 3a) and to which they assigned the structure of the postulated intermediate 2a. They repeated the experiment with diphenyl bromophosphate 4a and observed the same peak (at ca -25.5 ppm), but now representing 83\% of the total
phosphorus compounds. This was rationalised by the better leaving group ability of bromide over chloride anion and an equilibrium occurring between the halide anions and the pyridinium adduct. The authors also went further to investigate different dialkyl chlorophosphates and observed analogous intermediates, this time resonating at ca -12 ppm.

Since these highly hydrolytically sensitive pyridinium adducts normally produce side products due to unavoidable hydrolysis\textsuperscript{46}, which Perich \textit{et al.} did not observe at all, we suspected that their data most likely were misinterpreted. We therefore repeated their experiments on diphenyl and diethyl derivatives and even though we got similar spectroscopic results (signals at -25.5 ppm and -12.6 ppm, respectively), we noticed that these peaks were conspicuously close to those of the tetrasubstituted pyrophosphates \textit{7a} and \textit{7b} (that are formed as a result of hydrolysis). Perich \textit{et al.} neither commented on that, nor did they make any attempt to differentiate between these potential products.

![Chart 2](chart2.png)

\textbf{Chart 2.} Intermediates and products detected by \textsuperscript{31}P NMR spectroscopy.

To clarify this issue we repeated the same experiments, with different amounts of water present in the THF solution. When adding diphenyl halophosphate \textit{3a} or \textit{4a} to the wet THF solutions, no reaction was observed. Since pyridine is required to effect the displacement at the phosphorus centre, the formation of hydrolysis product became apparent first upon the addition of pyridine. Varying the amount of added water from nil up to 0.5 equiv. the product resonating at ca -25 ppm increased from ca 20\% to becoming the major one. Further addition of water (0.5-1 equiv.) consumed this product stepwise and quantitatively resulted in a new peak at -9.6 ppm after a total of 1 equiv. of water had
been added. This peak was assigned to phosphate diester 6a after spiking the reaction mixture with an authentic sample.

Both pyridinium adduct 2a and pyrophosphate 7a are known to react with nucleophiles such as amines and alcohols. However, while 2a would result in only one product, pyrophosphate 7a should afford two in equimolar amounts, a phosphoroamidate (or phosphorotriester) and phosphate diester 6a. Addition of 10 equiv. of ethanol or aniline to a pyridine solution of DPCP in THF (consisting of 80% 3a and 20% of 7a) resulted in a 9:1 ratio of 8a:6a (or 9a:6a), consistent with our hypothesis. In order to show that pyridine was not an integral part of the intermediate resonating at -25 ppm, we generated this same species by adding TEA to a solution of DPCP 3a in wet THF.

All experiments were also carried out in MeCN in order to exclude any possibility that the solvent would interfere with our results. Generally the hydrolysis always was more extensive in MeCN than in THF, but this probably only reflected the content of water in these solvents.

Since Perich et al. also included alkyl derivatives in his studies, we used diethyl derivative 3b as another representative model compound. By adding 0.5 equiv. of water to 3b we managed to quantitatively produce the product resonating at -13.7 ppm, assigned by Perich as pyridinium adduct 2b. The product was completely resistant towards reactions with water, ethanol or aniline for several hours, which clearly would not be the case for the pyridinium adduct. Spiking the reaction mixture with a genuine sample of tetraethyl pyrophosphate 7b gave a signal that resonated at exactly the same chemical shift as the compound in question.

The reason why pyridinium adducts of type 2 not have been observed previously in the reactions of halophosphates with pyridine is probably due to the fact that chloride and bromide are good nucleophiles that can react with 2 and strongly drive the equilibrium towards the halophosphate 3 or 4 (Scheme 4).

![Scheme 4. The position of the equilibrium reaction of pyridinium adduct 2 with halides depends on the type of halide.](image-url)
The concentration of the pyridinium adduct 2 is therefore apparently very low and usually below the level of detection by means of $^{31}$P NMR spectroscopy, although the phosphorylation pathway goes via this intermediate. In the instance of phosphoroiodidates, due to the low nucleophilicity of iodide towards phosphorus centres, pyridine was found to be able to form a detectable amount of pyridinium adduct 2 by displacing the iodide in iodophosphate 5.

However, while being a weak nucleophile towards phosphoryl centres, iodide is a good nucleophile for soft electrophilic centres such as sp$^3$ carbons in alkyl groups. A general side reaction for alkyl substituted pyridinium adducts are therefore dealkylation of pyridinium adduct 2, to form the stabilised betaine structure 1. This may, in turn, react to produce additional side products that may undergo further dealkylations and decompositions.$^{39,45,47}$ Dealkylation reactions take place on a slower timescale than halogen displacement by pyridine of halophosphates, but in the absence of other nucleophiles, dealkylation usually result in complex reaction mixtures (Scheme 5).

Scheme 5. Dealkylation and decomposition of pyridinium intermediates 2.

In order to observe the desired pyridinium adduct and to avoid side reactions, we decided to use a diaryl iodophosphate. Diphenyl H-phosphonate 10a was therefore reacted with iodine in MeCN using non-nucleophilic 2,6-lutidine as a base. Within a few minutes, a major peak at -48.6 ppm was observed ($^{31}$P NMR) and it was tentatively assigned to iodophosphate 5a. Addition of 50 equiv. pyridine to the reaction mixture consumed the originate peak and gave rise to two new signals at -14.4 ppm (65%) and -25.5 ppm (25%) respectively.$^{48}$ The latter was identified as pyrophosphate 7a, most likely as a result of unavoidable hydrolysis. The signal at -14.4 ppm was not observed
before for derivatives 3a or 4a, and its chemical shift was in agreement with the postulated structure of a pyridinium adduct of type 2a (Scheme 6).

To check the reactivity of this new intermediate, an excess of aniline or ethanol was added to the reaction mixture. This gave rise to products 8a and 6a or 9a and 6a respectively, in a ratio of ca 8:1 which was the expected outcome for a reaction with the postulated structure of pyridinium adduct 2a.

To obtain additional proof for the structure of the intermediate 2a, we reacted diphenyl phosphoric acid 6a with triflic anhydride to form the mixed anhydride 11 (-25.7 ppm). Since triflates display extremely low nucleophilicity, we assumed that upon addition of pyridine, the same intermediate as previously described in the reaction of iodophosphate should be observed. This proved to be the case as the compound resonating at -14.4 ppm was the major product also in this reaction after addition of pyridine. This gave further structural evidence for the identity of 2a.

Since the $^{31}$P NMR chemical shift of pyridinium adduct 2a should be sensitive to the electronic effect of substituents on the pyridine ring, we carried out an additional experiment.

To this end we replaced pyridine by DMAP (12b), which we expected to have increased electron density in the aromatic ring that should stabilise the pyridinium intermediate. The reactivity of 12b and the stability of complex 13 exceeded our
expectations as DMAP also managed to form the same pyridinium adduct (-12.6 ppm), not only with iodophosphate 5a, but with chlorophosphate 3a and bromophosphate 4a as well. Resolution enhanced coupled $^{31}$P NMR spectra showed a triplet for the $^3$J-couplings of P-N-C-H in the produced 4-dimethylaminopyridinium adduct 13.

4. Problem of Hydrolysis by Adventitious Water (Paper II)

Spurious water in pyridine and as absorbed to hygroscopic nucleosides is unavoidable and highly unfavourable in oxidative coupling reactions. For other coupling reactions, for instance those carried out in the presence of a condensing agents (e.g. pivaloylchloride or DPCP), this does not cause much problem since hydrolysis easily could be suppressed by using an excess of a coupling reagent.\textsuperscript{21} Thus, in these reactions the moisture is consumed prior to the coupling and does not interfere with the condensation. In the oxidative coupling, however, the limiting reagent and the one of highest significance is the \textit{in situ} formed phosphorohalidate and every percentage of hydrolysis at this stage will result in an unavoidable loss of product and in lower yields.

The first step of hydrolysis gives a phosphorodiester 6 that reacts fast with another equivalent of phosphorohalidate 5, forming tetrasubstituted pyrophosphate 7. Thus one equivalent of water will eliminate two equivalents of 5. Utilising a strong nucleophilic catalyst such as DMAP (12b) or N-methylimidazole may regain one equivalent of the lost molecules by attacking the phosphoryl centres of the pyrophosphate, forming a reactive species, 14, that is stable enough to be formed in appreciable amount and to react with other nucleophiles. Despite this, at least one equivalent of nucleotidic material will be lost due to hydrolysis (Scheme 7).
In principle, when only a slight excess of alcohol is used, the yields are lowered by the competing hydrolysis by 30-40%. Generally, a coupling reaction with hydroxylic components requires a large excess (>5 equiv.) of the alcohol in order to successfully suppress the competing hydrolysis reaction. This works well for simple alcohols, but if the reaction is to be extended to more complex alcohols, then this procedure is unsatisfactory. Furthermore, the intermediate pyridinium adduct dealkylates readily and forms several side products if it is not intercepted by a nucleophile fast enough (vide supra).

4.1 Suppressing Hydrolysis with Silylating Reagents

In an attempt to suppress hydrolysis and eliminate formation of side products, we tried several approaches to limit the amount of adventitious water. Dried, freshly distilled solvents kept over molecular sieves in conjunction with repeated coevaporation of the starting material with pyridine, were not very effective, and 20-40% of the spectroscopically observed phosphorus content of the reaction mixtures was still found as pyrophosphates. Reactions carried out in the presence of powdered molecular sieves did not either improve the yield of oxidative coupling.

Previously in our group, we used TMS-Cl (15a) to suppress side reactions due to interfering moisture (Chart 3).49,50
Trimethylsilyl chloride (15a) would potentially bring at least two beneficial characteristics to the reaction; a silyl group for consuming water and a chloride anion to stabilise and secure the intermediate from potential dealkylation (Scheme 8). Unfortunately 1 equiv. of 15a proved to be inadequate for trapping water, though the chloride ions intervened directly with the iodophosphate 5, displacing the iodide and yielded the chlorophosphate 3 together with the undesired pyrophosphate 7. The formed chlorophosphates demonstrated an impractically high stability even in pyridine and required prolonged alcoholysis to be converted into phosphorotriesters (several hours).

For the analogous reaction of H-phosphonothioates 10c, the corresponding thiophosphorochloridates 3c formed were even more stable than their oxo congener. Although dealkylation in these instances was completely eliminated, a competing hydrolysis still posed a problem.

Since TMS-Cl 15a previously in our hands has worked very nicely in respect of trapping water from reaction mixtures, the lack of effect in the investigated reactions was somewhat puzzling. We reasoned that this could be due to the fact that the product of hydrolysis of 15a, the trimethylsilanol 16a per se still could attack the phosphoryl centre,
yielding a silylated phosphate diester. The silyl phosphate 18 could become desilylated under the reaction conditions by chloride or pyridine and the resulting phosphate diester 6 might then attack another molecule of halophosphate, ultimately resulting in the formation of pyrophosphates (Scheme 9).

Scheme 9. Reaction of silanols with chlorophosphates.

Thus, we assumed that a sterically hindered silyl species, e.g. TBDPS-Cl (15b) as a water scavenger would be advantageous for various reasons. Firstly, it would yield a more sterically hindered silanol (16b) that should be less nucleophilic and secondly, undesired silylation of the alcohol would also be suppressed. It was gratifying to observe that the presence of 1 equiv. of TBDPS-Cl 15b (added to the reaction mixture 20 seconds prior to the reaction with iodine), furnished a clean formation of the desired phosphorotriesters (17 or 17c), although the reaction still proceeded rather slowly (several hours to reach completion; the reaction with the thio derivative was even slower, <24 h). Addition of stronger nucleophilic catalysts, for instance DMAP or N-methylimidazole did not provide any additional rate enhancements. These catalysts are commonly used in silylation reactions of alcohols51-53 and, unfortunately, a competing silylation became rather pronounced. Since slow oxidative coupling is undesirable from the point of view of its compatibility with solid phase synthesis of oligonucleotides, we searched for other types of catalysts for these reactions.
4.2 Iodine as a Catalyst in Phosphorylations

Iodine has a long history of being a catalyst in various organic reactions, in acylation or isopropylidination in carbohydrate chemistry, in Friedel-Craft acylations or in silylation reactions with HMDS. In light of these, we turned our attention to an investigation of phosphorylation of alcohols using chlorophosphates in the presence of iodine or iodide anions that has been carried out in our group in the past. It was evident from these studies that an increased concentration of iodine had a profound effect on the rate of alcoholysis of chlorophosphates.

We found that in oxidative couplings, by simply adding a supplementary amount of iodine to the reaction mixture containing TBDPS-Cl (15b) resulted in a significant shortening of the reaction time. Depending on the amounts of both iodine and the added TBDPS-Cl, the reaction time varied from several hours to a few seconds. During these syntheses, we discovered that iodine not only improved the rate of phosphorylations but also accelerated silylation reactions.

Since alcohols are the nucleophiles to be coupled in the presence of silyl chloride 15b, it might be expected that an undesired silylation of the alcohol can become a competing reaction. However, in neat pyridine, in the presence of excess of iodine, the increase in rates was more pronounced for chlorophosphates than for silyl chlorides. As a consequence, phosphorylation of the alcohol was the only reaction observed.

Using an excess of chlorosilane 15b unavoidably introduced chloride anions into the reaction mixture. This resulted in an extended reaction time as a consequence of the lower reactivity of the formed chlorophosphophate, but nevertheless secured anhydrous reaction conditions otherwise unattainable. A higher chloride concentration demanded more iodine in order to achieve a similarly high reaction rate as for the corresponding reactions with lower chloride concentrations. Since a relatively large amount of iodine was necessary to complete the reaction within five minutes (1.5-3 equiv. for oxophosphorylations and 3-6 equiv. for thiophosphorylations, respectively), it was evident that consumption of iodine somehow must have occurred. Presumably, the mechanism through which iodine catalysis operates is the ability of iodine to form complexes with halides (trihalide anions), (Scheme 10). The equilibrium between a phosphorohalidate and the corresponding pyridinium adduct apparently depends on the kind of halide present (vide supra). For iodophosphates the equilibrium is shifted towards the pyridinium adduct, whereas for other phosphorohalidates, it is shifted toward the halophosphates (Scheme 4). In the presence of iodine, free chloride anions may form diiodochloride anions which due to charge dispersion should be less nucleophilic than...
chloride anions, thus the equilibrium should be shifted towards the pyridinium adduct. Consequently, a higher concentration of the reactive pyridinium adduct is built up when iodine is present, and since this species is responsible for the phosphorylation of alcohols, the overall rate of phosphorylation is increased.

The previously mentioned dealkylation reactions of pyridinium adducts 2 (Scheme 5) did not pose a problem under these reaction conditions, presumably because the overall concentrations of pyridinium adduct 2 still is low. Furthermore, the trihalide anion may react with the pyridinium adduct, giving back the more stable chlorophosphate in the absence of other nucleophiles, thereby saving pyridinium intermediate 2 from decomposition.

\[
\begin{align*}
\text{R'O} & \text{Cl} \quad \overset{\text{I}_2}{\rightleftharpoons} \quad \text{R'O} \quad \text{Cl} \\
\text{Pyridine} & \quad \overset{\text{I}_2}{\rightleftharpoons} \quad \text{R'O} \quad \text{N} \quad \text{Cl}
\end{align*}
\]

\[X=\text{O or S}\]

**Scheme 10.** Iodine catalysis by entrapping the chloride ion.

### 4.2.1 Oxidative Coupling of Alcohols with H-Phosphonates

To find scope and limitations of the developed protocols of oxidative couplings, we selected alcohols with diverse structural features. Testing structurally different butanols \((n\text{-BuOH, } i\text{-BuOH and } t\text{-BuOH})^{62}\) (1.5 equiv) as model alcohols using 1 equiv. of TBDPS-Cl and 3 equiv. of iodine showed that coupling with either diethyl or dithymidinyl H-phosphonate yielded the desired butyl triesters as the only products in moderate to high yields (80-90%) (Scheme 11, Table 1). The reactions with 3’- and 5’-protected nucleosides and 2-pyridinone as alcohols also produced the corresponding phosphate triesters as the sole products and in good yields (75-90%).

\[
\begin{align*}
\text{R'O} & \text{P} \quad \text{Cl} \quad \overset{\text{I}_2}{\rightleftharpoons} \quad \text{R'O} \quad \text{Cl} \\
\text{TBDPS-Cl} & \quad \overset{\text{I}_2}{\rightleftharpoons} \quad \text{R'O} \quad \text{OR} \\
\text{Pyridine} & \quad \overset{\text{I}_2}{\rightleftharpoons} \quad \text{R'O} \quad \text{OR}
\end{align*}
\]

\[X=\text{O or S}\]

**Scheme 11.** Checking the limitations of the modified reaction protocol.
4.2.2 Oxidative Coupling of Alcohols with H-Phosphonothioates

The protocols described earlier for oxidative coupling reactions between the chosen model alcohols and H-phosphonates are with a few modifications applicable also to H-phosphonothioates. There are, however, some notable differences.

Firstly, a larger amount of iodine (3-6 equiv.) is required in order for the reaction to be complete within the desired, reasonable time (<5 min). This is probably due to generally lower reactivity of thiochlorophosphates.\(^\text{63}\)

Secondly, it is important that the reaction mixture is sufficiently dried, since iodine otherwise will desulfurise thiophosphoric acid derivatives and thus further complicate the reaction mixture.\(^\text{64}\)

Thirdly, a larger van der Waals radius of sulfur than that of oxygen may cause steric hindrance at the thiophosphoryl centre. Due to this, reactions with primary and simple secondary alcohols work well, but attempted coupling of H-phosphonothioate diesters with the 3´-hydroxyl functions of nucleosides failed. Longer reaction time or additional amounts of iodine did not improve the reactions with sterically hindered alcohols. The only detectable products of such reactions were the corresponding phosphorothiochloridates. Presumably this is due to the thiophosphoryl centre being too crowded for an attack by sterically hindered alcohols.

It seems that the overall steric hindrance at the thiophosphoryl group is important in these reactions as Diethyl H-phosphonothioate is oxidatively coupled to \(\text{t-BuOH}\) in high yields (>80%) whereas dithymidinyl H-phosphonothioate gave no reaction with this tertiary alcohol at all.
H-phosphonothioates with alcohols. These reactions can be carried out stereospecifically (i.e., one P-diastereomer of H-phosphonate diesters gives rise to only one P-diastereomer of resulting phosphoroamidates). The reactions proceed with an overall inversion of configuration at the phosphorus centre. Due to the stereospecificity in the coupling and the high coupling yields, the reactions of halophosphates with amines are the most frequently used in oxidative couplings. Due to the stereospecificity in the coupling and the high coupling yields, the reactions of halophosphates with amines are the most frequently used in oxidative couplings.

Even in the absence of nucleophilic catalysts, water usually cannot compete with amines for halophosphoryl centres and the yields are therefore consistently high (95-100%).

### Table 1

<table>
<thead>
<tr>
<th>R’=</th>
<th>R”=</th>
<th>Ethyl</th>
<th>Ethyl</th>
<th>5’-O-DMT-thymidin-3-yl</th>
<th>3’-O-DMT-thymidin-5-yl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ROH</td>
<td>Entry (%)</td>
<td>Entry (%)</td>
<td>Entry (%)</td>
<td>Entry (%)</td>
</tr>
<tr>
<td>n-BuOH</td>
<td>19a</td>
<td>84</td>
<td>19b</td>
<td>86</td>
<td>25a</td>
</tr>
<tr>
<td>i-BuOH</td>
<td>20a</td>
<td>85</td>
<td>20a</td>
<td>82</td>
<td>26a</td>
</tr>
<tr>
<td>t-BuOH</td>
<td>21a</td>
<td>83</td>
<td>21a</td>
<td>81</td>
<td>27a</td>
</tr>
<tr>
<td>5’-DMT-T</td>
<td>22a</td>
<td>79</td>
<td>22a</td>
<td>n.d.</td>
<td>28a</td>
</tr>
<tr>
<td>3’-DMT-T</td>
<td>23a</td>
<td>85</td>
<td>23a</td>
<td>84</td>
<td>29a</td>
</tr>
<tr>
<td>2-Pyridinone</td>
<td>24a</td>
<td>93</td>
<td>24a</td>
<td>90</td>
<td>30a</td>
</tr>
</tbody>
</table>

Isolated yields for oxidative coupling of diethyl and dinucleoside H-phosphonate and H-phosphonothioates with alcohols. Reactions are carried out in pyridine using 1.5 equiv. of alcohol, 1 equiv. TBDPS-Cl and 1.5 equiv. (X=O) or 3 equiv. (X=S) of iodine. All reactions that worked were finished within 5 min.

### 5. Oxidative Coupling of Amines with H-Phosphonates (Paper III)

The high reactivity of amines towards halophosphoryl centres can be of great advantage in synthetic bioorganic chemistry. Primary and secondary amines react instantly (within a few seconds) with both oxo and thiohalophosphates. Since these reactions are fast, there is no need for nucleophilic catalysts (vide supra), and thus the problem of epimerisation is eliminated. The oxidative coupling of amines is the reaction of highest synthetic potential as it can be carried out stereospecifically (i.e. one P-diastereomer of H-phosphonate diesters gives rise to only one P-diastereomer of resulting phosphoroamidates). The reactions proceed with an overall inversion of configuration at the phosphorus centre. Due to the stereospecificity in the coupling and the high coupling yields, the reactions of halophosphates with amines are the most frequently used in oxidative couplings. Even in the absence of nucleophilic catalysts, water usually cannot compete with amines for halophosphoryl centres and the yields are therefore consistently high (95-100%).
5.1 Controlling the Stereochemical Outcome

For biological and therapeutic applications, usually stereochemically pure compounds are required. Although the use of synthetic methods based on stereospecific reactions provide an accessible route to these substances, the inherent disadvantage is that only one diastereomer of the substrate can be used to form the desired product with the correct stereochemical disposition. This strongly limits the generality of the procedures as the other diastereomer of the substrate, which even may be the major one, then lacks synthetic value. If there would be a method of converting either diastereomer of the precursor into the other, or a possibility to transform them into reactive intermediates sharing common stereochemistry, it should be feasible to produce any diastereomer of the product starting from either diastereomer of the substrate. This would imply that all of the substrate, regardless of the stereochemical configuration, may be used for the synthesis of only one diastereomer of the product.

Since amines display stereospecificity and high reactivity towards not only iodophosphates but also towards chlorophosphates, we assumed that this feature could be used to control the stereochemical outcome in oxidative coupling reactions.

Iodophosphates are known to be extremely reactive and to undergo dealkylation by iodide anions in the absence of nucleophiles and, as we observed earlier, in the presence of chloride ions and pyridine they are rapidly converted into phosphorochloridates (vide supra). However, of high synthetic value is the fact that this phenomenon also occurs in the absence of pyridine and thus the exchange of halogen is likely to proceed with an inversion of configuration at the phosphorus centre, resulting in a chlorophosphate having the opposite configuration to that of the parent iodophosphate. In contrast to a direct coupling reaction of iodophosphate, yielding phosphoroamidate with an inverted stereochemistry, a pathway via a chlorophosphate intermediate would instead lead to a double inversion and thus should be formed with a total retention of configuration (Scheme 12).
This concept would theoretically allow any single diastereomer of H-phosphonate diesters to be coupled stereospecifically to produce phosphoroamidates with $R_p$ or $S_p$ configuration, depending on whether chloride ions are present during the reaction or not.

To substantiate the legitimacy of our assumption, separate diastereomers of dinucleoside H-phosphonate were subjected to oxidation with iodine (1.5 equiv.) in MeCN, in the presence of 3 equiv. of triethylammonium hydrochloride and 6 equiv. of TEA (Scheme 13). Indeed, the stereochemically inverted chlorophosphate 3 was generated within 5 min ($^{31}$P NMR) and a successive addition of $n$-butylamine or aniline (3 equiv.) afforded the stereoinverted phosphoroamidate 31 or 32 as the single product in high yields (79-84%). Carrying out the corresponding control experiments without chloride ions present gave rise to the other diastereoisomers of the phosphoroamidates (31, 32) via a direct coupling reaction (Table 2). In the latter reaction the amine had to be present in the reaction mixture from the beginning to avoid dealkylation and detritylation.
Table 2. Separate diastereomers of phosphoramidates 31 and 32 obtained via direct and stereoinverted oxidative coupling reactions.
In theory, this procedure could also be applicable for the oxidative coupling of alcohols. Unfortunately, the lower reactivity of halophosphates in the absence of nucleophilic catalysts presently makes the procedure less efficient. Prolonged reaction times or base catalysis proved to be of little use for this purpose since this mainly caused formation of other side products.72

6. Oxidative Coupling of N- and O-Binucleophiles with H-Phosphonates and H-Phosphonothioates (Paper IV)

Oxidative coupling reactions constitute an excellent possibility of attaching various molecules to synthetic oligonucleotides. Via an oxidative coupling of a bifunctionalised polymethylene chain with H-phosphonates, an oligonucleotide carrying a chemical handle for further derivatisation is obtained. Through this new functionality, a variety of reporter groups carrying a chemofluorescence moiety73, haptens for antibodies15, intercalating agents6 or others74 may be attached to oligonucleotides. Also artificial nuclease moieties could be connected via these kinds of linkers. Previously binucleophiles have been used for increasing water solubility of sONs, where the connected additional polar functionality increases the hydrophilicity of the oligomer.75

For various reasons bifunctional linkers have been attached to oligonucleotides using oxidative coupling procedures in the past.76 For instance, they have been connected at one end of the oligonucleotide chain via phosphoroamidate functionalities originating from oxidative couplings between 1,6-diaminohexane and hexamethylene polyphosphonate linkers.77,78 The rationale of locating them at the end of the oligonucleotide chain was that the destabilising effect on hybridisation then should be minimised.

For our part, we investigated the feasibility of achieving efficient coupling reactions of internucleosidic H-phosphonates and H-phosphonothioates with binucleophiles that not have been explored much but for the reactions with amines.77

A hexamethylene chain is considered to be of sufficient length to keep the attached group away from the duplex and thereby minimising interference in hybridisation. A shorter chain would be more likely to destabilise the internucleosidic phosphate linkage due to possible cyclisations and unfavourable interactions. The most common types of linkers used for derivatisation of oligonucleotides are those derived from 1,6-diaminohexane79 rather than 1,6-hexanediol.
Since amines are usually more reactive than alcohols, it would be desirable to have a free amino function at the end of the linker rather than a hydroxyl group. From the point of view of efficient hybridisation properties, a triester modification at the phosphorus would also be more advantageous than an amidate, since it closer resembles natural phosphate. Even though some simple alkyl dinucleoside triesters are known to undergo dealkylation \textit{in vivo}\textsuperscript{6}, we decided to synthesise various combinations of binucleophilic hexamethylene linkers oxidatively coupled to dinucleoside H-phosphonates and H-phosphonothioates (Scheme 14).

![Scheme 14](image)

\textbf{Scheme 14.} Oxidative couplings of binucleophiles with dithymidinyl H-phosphonate and H-phosphonothioate.

The aim of these studies was thus to investigate chemoselectivity and stereochemical aspects of oxidative coupling of unprotected binucleophiles with H-phosphonate and H-phosphonothioate diesters, to get a deeper understanding of the underlying H-phosphonate chemistry.

Apart from the previously discussed general aspects of oxidative coupling reactions there are some problems that are specific for binucleophiles. Firstly, there are more potential side reactions feasible (\textit{e.g.} potential bisphosphorylation of the binucleophiles). Secondly, the polar nature of the additional functionality of the linker might complicate the purification.
6.1 Reaction with Amino Nucleophiles

Phosphorylations of amines either by using H-phosphonates and oxidative coupling protocols or starting from commercially available halophosphates are well known reactions. The most common way for attaching linkers used for biodiagnostics is via a phosphoroamidate bond, for example, by oxidative coupling of an H-phosphonate with monoprotected diamines. The protective group is subsequently removed from the amino function after the coupling reaction is complete.

We decided to investigate the coupling reactions of unprotected 6-aminohexanol and 1,6-diaminohexane as binucleophiles with H-phosphonate or H-phosphonothioate diesters.

6.1.1 Reaction with 6-Aminohexanol

The reactions between 6-aminohexanol and dinucleoside H-phosphonate or H-phosphonothioate diesters were carried out in MeCN, using 3 equiv. of aminohexanol and 1.2 equiv. of iodine (Scheme 15). Both reactions went smoothly and yielded the phosphoroamidates 33a or 33b in high yields. The greater reactivity of the amino group in comparison to the hydroxyl function gave a chemoselective and stereospecific reaction affording the phosphoroamidate 33, bearing a free hydroxyl function at the end of the linker, in acceptable yields (>84%).

![Scheme 15. Oxidative coupling of aminohexanol with dithymidinyl H-phosphonate and H-phosphonothioate.](image-url)
6.1.2 Reaction with 1,6-Diaminohexane

The analogous reaction with diaminohexane was, as expected, also stereospecific but nevertheless posed more challenges (Scheme 16).

The reaction was first carried out in MeCN with 3 equiv. of diamine and 1.2 equiv. of iodine. This, unfortunately, resulted in 20-30% of the undesired bisphosphorylated product.

It appeared almost impossible to avoid bisphosphorylations of 1,6-diaminohexane. Regardless of the kind of H-phosphonate or H-phosphonothioate diesters that was used (dinucleoside or diethyl derivatives) in the reaction, a statistical amount of bisphosphorylated products was formed. The difference in reactivity of the amino functions of the free linker in comparison to that of monophosphorylated linker was evidently low since the second phosphorylation apparently was independent of the first. Though mono protection of one amino function is possible and even customarily used, we found that unprotected diamines could be used in oxidative coupling reactions, provided a large enough excess of this nucleophile is used. Indeed, using 15 equiv. of the added diamine reduced the level of bisphosphorylation to below 3%. To be able to dissolve the highly polar diamine, ethanol was used as a cosolvent. This was possible due to the high preference for N- over O-nucleophiles in oxidative couplings.

![Scheme 16. Oxidative coupling of diaminohexane with dithymidinyl H-phosphonate and H-phosphonothioate.](image)

The polar nature of the amino linker had an unfavourable effect on the otherwise simple chromatographic purification of uncharged dinucleoside products. It was evident that compounds bearing a protonated aminoalkyl group could not be obtained in
acceptable yields using conventional eluent systems for silica gel chromatography due to micelle formations that resulted in excessive smearing and coelution with other substances. After thorough testing of different mobile phase compositions, we finally arrived to a 5-component solvent system containing a combination of water-triethylamine-chloroform-acetone-methanol (13:2:210:150:42 v/v) that permitted chromatographic purification of the phosphoroamidate 34a. H-phosphonothioate diesters did not differ much from H-phosphonates in oxidative coupling and thus a similar protocol to that of H-phosphonates was used for the synthesis and purification of the thiophosphoroamidate 34b. The isolated yields of the pure diastereomers of phosphoroamidates 34 were >80%.

6.2 Reaction with Oxygen Nucleophiles

Linkers attached via phosphorotrriester linkages that are formed by oxidative coupling reactions are rarely found in the literature because of lower efficacy in oxidative coupling and lower stability in comparison to the phosphoroamidates (vide supra). In the light of this, our previously developed protocols for oxidative couplings of alcohols constituted basis for the coupling of hydroxyl functionalised hexamethylene linkers with dinucleoside H-phosphonate and H-phosphonothioates. We considered that the reactions should work reasonably well also for these compounds. As linkers we chose 1,6-hexanediol and 6-aminohexanol. Since protonation of amino alcohols has successfully been used in the past for inverting the chemoselectivity towards P(V) phosphoryl centres (from that of amino to the hydroxyl function preference), we decided to use the hydrochloride salt for masking the amino function.

6.2.1 Reaction with 6-Aminohexanol Hydrochloride

Having a good system for purification of nucleotidic compounds with a free amino end, we decided to apply our modified procedure of oxidative couplings to include a reaction previously published by our group. Back then, protonation with hydrogen chloride of amino alcohols to form ammonium alcohols was discovered to completely deactivate the amine as a nucleophile and thereby inverting the chemoselectivity of the phosphorylation reaction of binucleophiles from N to O. The reaction time was, however, prolonged due to the presence of chloride ions (>30 min) and the yields were generally modest (~50%) because of the inability of preventing hydrolysis and presumably also due to some losses at work-up.
The use of our developed procedure with iodine for improving the reaction rates of chlorophosphate made it possible to once again use the hydrochloride salt as substrate. The presence of chloride ions introduced with the ammonium salt to the reaction mixture, required a slight modification to the protocol, namely, an increase of the amount of iodine to keep the reaction time within a few minutes. By using a combination of TBDPS-Cl 15b (1.5 equiv.) and excess of iodine (5 equiv. X=O or 10 equiv X=S) in pyridine, a clean formation of the desired triesters 35a or 35b (as hydrochloride salts) in unprecedented yields (ca 90%) within 5 min, was achieved (Scheme 17).

![Scheme 17. Oxidative coupling of aminohexanol hydrochloride with dithymidinyl H-phosphonate and H-phosphonothioate.](image)

In this reaction, pyridine was required not only to secure a reasonably short reaction time, but also for dissolving the ammonium salt. As expected, because of pyridine mediated epimerisation of the halophosphates, the coupling was overall non-stereospecific and led to a mixture of diastereomeric phosphorotriesters 35 in a ca 1:1 ratio.

6.2.2 Reaction with (Monosilylated) 1,6-Hexanediol

Attempted oxidative coupling reactions of H-phosphonate diesters with hexanediol resulted not only in the desired product but also in the formation of some side products. We discovered that iodine in pyridine not only had a pronounced effect on the rate of phosphorylations, but on the silylation reaction as well. During the course of the condensation (a few minutes), the free hydroxyl end of the coupled linker was partially silylated (15-40%) by the added TBDPS-Cl. According to literature, these reactions are
slow in the absence of strong nucleophilic catalysts (DMAP or N-methylimidazole), and usually require several hours at room temperature even when carried out in DMF.\textsuperscript{51-53}

It was thus obvious that our former protocol of suppressing bisphosphorylation by employing excess of the nucleophile in this case would be of little use. Furthermore, an excessive amount of the diol might introduce spurious water to the reaction mixture. The diol was therefore monosilylated using a TBDMS group that easily could be removed after the coupling reaction. By protecting one hydroxyl function, the diol was regarded as an ordinary alcohol and the usual protocol for oxidative couplings (1 equiv. of TBDPS-Cl and 1.5 (oxo) or 3 equiv. (thio) of iodine in pyridine) afforded triesters 36\textit{a} or 36\textit{b} in high yields (>80%), (Scheme 18).

The reaction was not stereospecific and yielded a ca 1:1 mixture of diastereomeric products, regardless of the original diastereomeric ratio of the halophosphate intermediate. Desilylation of phosphorotriesters 36 were carried out using tetrabutylammonium fluoride in THF, and yielded the target compounds, phosphorotriesters 39, bearing free hydroxyl functions in acceptable yields (>74%).

\textbf{Scheme 18.} Oxidative coupling of monosilylated hexandiol with dithymidinyl H-phosphonate and H-phosphonothioate.
7. Silylation-Mediated Thiophosphonylations (*Paper V*)

Suitably protected nucleoside H-phosphonothioate monoesters are important synthons for the construction of oligonucleotides bearing sulfur modifications at the phosphorus centres\(^{81,82}\) (*e.g.* phosphorothioates and phosphorodithioates and analogues thereof) using H-phosphonate methodology. The tervalent P(III) nature of the intermediates involved in procedures relying on H-phosphonate and H-phosphonothioate chemistry allows for further manipulations otherwise unattainable by means of synthetic approaches based on P(V) compounds and thereby offers a broader variety of feasible products\(^{83-86}\).

Although there are a few reaction protocols available for synthesising H-phosphonothioate monoesters, in spite of good yields, they all suffer from different degrees of limitations and drawbacks.\(^{50,87-89}\) To expand the array of available synthetic methods for the preparation of the H-phosphonothioate monoesters we have developed an inexpensive thiophosphonylating reagent that is easy to prepare on large scale and displays favourable characteristics such as high stability against air oxidation and decomposition.

Aryl H-phosphonate diesters are known to easily undergo hydrolysis\(^{90}\), alcoholysis\(^{91,92}\) and sulphonylation\(^{50}\) and are therefore commonly used as electrophilic reagents for producing compounds containing H-phosphonate\(^{91,92}\) and H-phosphonothioate\(^{50}\) functionalities. Similarly to aryl H-phosphonate monoesters, H-phosphonothioate monoesters are stable compounds that require an activating reagent for promoting condensation reactions. Having this in mind, we sought to make use of phenyl H-phosphonothioate monoester as a thiophosphonylating reagent by catalysis of a silylating agent. Similarly to a reaction carried out by Jones *et al.*, we reasoned that an *O*-silylation of phenyl H-phosphonothioate (thereby mimicking a diester), would increase the susceptibility of this compound towards alcoholysis and result in the displacement of the phenyl group.\(^{93}\)

Phenyl H-phosphonothioate 40 is easily accessible via sulphonylation of commercially available DPHP analogously to a published procedure\(^{50}\) (Scheme 19) and is usually obtained as the triethylammonium salt, which *per se* is a sticky oil that is difficult to purify. However, in order to present a viable route towards nucleoside H-phosphonothioates it would be desirable to avoid a cumbersome chromatographic purification of the thiophosphonylating agent and thus facilitate its large scale synthesis. To this end, we searched for a suitable counter ion to obtain our compound in a crystalline form. After evaluating several salts we finally ended up with the S-(p-
chlorobenzyl) isothiuronium (CBTU) derivative 40 which cleanly produced a stable, crystalline solid with good solubility in organic solvents and that was possible to recrystallise out of the reaction mixture in large scale and in an acceptable yield.

![Scheme 19](image)

Scheme 19. Sulfhydrolysis of DPHP into the thiophosphonylating reagent 40.

The inconveniences of having to prepare freshly saturated hydrogen sulfide in dioxane prior to the preparation of nucleoside H-phosphonothioates is here diminished since this operation has to be carried out only once for the purpose of synthesis of 40, which can be stored for prolonged time (months) without any noticeable decomposition.

Having an easy access to reagent 40, we investigated which reaction conditions that would be most suitable for transferring the thiophosphonyl moiety to the hydroxyl function of a protected nucleoside (Scheme 20). Since the reaction was found to be rather slow (over night) the choice of solvent system and type of silylation reagent was crucial as the hydroxyl group of the nucleoside under the reaction conditions also may undergo silylation. The presence of pyridine was evidently unavoidable as it facilitated both the formation of silyl ester 41 as well as its alcoholysis. However, since the competing silylation process of the nucleoside also increases in the presence of nucleophilic catalysts, it was found that a limited amount of pyridine would be appropriate for improving the P vs. Si selectivity. The solvent system that we finally arrived to consisted of toluene-pyridine 4:1 (v/v). Furthermore, since it was obvious that the silylating agent must be carefully chosen on the basis of preventing the competing silylation of the nucleoside 42, hence TBDPS-Cl was selected due to the steric bulkiness of this compound. The increased size of the TBDPS group in comparison to the smaller TBDMS functionality not only decreased the reactivity towards alcohols, but it also contributed to the stability towards desilylation of 41. This minimised the concentration of free silylating reagents present in the reaction mixture and as a consequence, the undesired silylation of nucleosides was suppressed.
All attempts to speed up this reaction were unsuccessful, as elevated temperatures, addition of base or stronger nucleophilic catalysts all contributed to increased silylation of nucleosides due to loss of selectivity. Thus the reaction conditions were set to be mild (ambient temperature), although this resulted in a prolonged reaction time.

To evaluate the synthetic protocol, the reaction conditions were applied to preparative syntheses involving the protected nucleosides 42a-d, and after aqueous workup followed by silica gel chromatography, the desired H-phosphonothioate monoesters 43a-d were isolated as white foams in 72-77% yield. This approach can probably be extended to other hydroxylic compounds and thus expands array of synthetic methods for the preparation of H-phosphonothioate monoesters.
8. Conclusions

The pathway through which the oxidative couplings occur has been investigated using $^{31}$P NMR and on this basis we have developed mild and general procedures for the synthesis of various structurally diverse products in moderate to high yields. The formerly only postulated pyridinium adduct 2, which plays a crucial role as the reactive intermediate in phosphorylation reactions carried out in pyridine, was spectroscopically observed for the first time and its identity was strengthened.

Generally, phosphoroamidates could be produced stereospecifically and we found that chloride anions could be used to steer the stereochemical outcome of these reactions.

The formation of phosphorotriesters in oxidative coupling with alcohols required nucleophilic catalysis, which caused epimerisation at the phosphorus centre. Although these reactions also may be carried out in a stereospecific manner using base catalysis, the lower efficiency generally gave more complex reaction mixtures and required a higher excess of the alcohol.

TBDPS-Cl in conjunction with iodine was found to work excellent in respect of trapping interfering water, while iodine was discovered to strongly increase the reactivity of not only phosphorochloridates and thiophosphorochloridates, but also of silyl chlorides. A tentative mechanism for this iodine catalysis was proposed.

Finally a synthetic method based on a silylation-mediated pathway utilising phenyl H-phosphonothioate monoester as a thiophosphonyl transferring agent has been developed and used to produce H-phosphonothioate monomer building blocks for oligonucleotide analogue synthesis.
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- Past and present roommates and the rest of the Department of Organic Chemistry for giving this place its very nice and pleasant atmosphere.

- My friends in the outside world.

- My family and relatives.

- Johanna
10. References and Notes

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A base is required for neutralising hydrogeniodide that is formed during the oxidation reaction with iodine. Iodide might also be present from partial hydrolysis of the parent phosphoroiodidate.

After the formation of phosphoroiodidate was complete, EtSH was added to decompose the excess of iodine in order to suppress side reactions arising from oxidised side products.

The product appears to be a putative phosphoroamidate, presumably formed as a consequence of a side reaction where iodine reacts with tertiary amines to form secondary amines (via dealkylation). The secondary amine may in turn attack the chlorophosphate, and thus form the undesired phosphoroamidate.

Thiohalophosphates are usually less reactive than oxohalophosphates. This is most probably a direct result of the higher electron withdrawing properties of oxygen in comparison to the less electronegative sulfur.

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Thiohalophosphates are usually less reactive than oxohalophosphates. This is most probably a direct result of the higher electron withdrawing properties of oxygen in comparison to the less electronegative sulfur.
Appendix A:

Supplementary Experimental Data for Paper II
(The numbering refers to Paper II)

5'-O-(4,4'-dimethoxytrityl)thymidin-3'-yl bis 3'-O-(4,4'-dimethoxytrityl)thymidin-5'-yl phosphate 7a

$^1$H NMR: (δ in ppm CDCl$_3$), 9.08 (s, 1H, NH), 8.97 (s, 1H, NH), 8.73 (s, 1H, NH), 7.49 (s, 1H, H$_a$6), 7.43-7.14 (m, 27 H, ArH), 7.09-7.03 (s x2, H$_b$6, H$_c$6), 6.87-6.75 (m, 12H, ArH para to methoxy), 6.32 (m, 1H, H$_a$1'), 6.20-6.09 (m, 2H, H$_b$1', H$_c$1'), 5.05 (m, 1H, H$_a$3'), 4.15 (m, 2H, H$_b$3', H$_c$3'), 4.03 (m, 1H, H$_a$4'), 4.00-3.90 (m, 2H, H$_b$4', H$_c$4') 3.78-3.52 (m, 4H, H$_b$5', H$_c$5'), 3.82-3.68 (s x3, 18H, 6 x CH$_3$O), 3.48-2.22 (m, 2H, H$_a$2'), 1.92-1.52 (m, 4H, H$_b$2', H$_c$2'), 1.78 (s, 6H, C$_b$5-CH$_3$, C$_c$5-CH$_3$), 1.34 (s, 3H, C$_a$5-CH$_3$).

$^{31}$P NMR: (δ in ppm CDCl$_3$) -2.04

$^{13}$C NMR: (δ in ppm CDCl$_3$) 163.82, 163.69 (3 x C4), 159.06 (6C of DMT), 150.60, 150.36, 150.30 (3 x C2), 145.00, 144.94, 144.18 (3C of DMT), 136.17, 136.09 (2 x C$_b$6), 135.39 (C$_a$6), 135.17, 135.08 (6C of DMT), 130.40, 130.30, 128.55, 128.44, 128.36, 128.29, 127.58, 127.46 (27C of DMT), 113.66, 113.57 (12C of DMT), 111.97, 111.34 (3 x C5), 87.65, 87.55 (3 x C$_{DMT}$), 86.78, 86.13 (2 x C$_b$1'), 84.52 (C$_a$4'), 84.18 (C$_a$1', 2x C$_b$4'), 79.95 (C$_a$3'), 74.17, 74.05 (2 x C$_b$3'), 67.03 (2 x C$_b$5'), 63.58 (C$_a$5'), 55.48 (6 x CH$_3$O), 38.82 (3 x C2'), 12.50 (2 x C$_b$5-CH$_3$), 11.81 (C$_a$5-CH$_3$).

5'-O-(4,4'-dimethoxytrityl)thymidin-3'-yl bis 3'-O-(4,4'-dimethoxytrityl)thymidin-5'-yl phosphorothioate 7b

$^1$H NMR: (δ in ppm CDCl$_3$), 9.15 (s, 1H, NH), 9.02 (s, 1H, NH), 8.76 (s, 1H, NH), 7.51 (s, 1H, H$_a$6), 7.46-7.14 (m, 27 H, ArH), 7.07-7.00 (s x2, H$_b$6, H$_c$6), 6.87-6.76 (m, 12H, ArH para to methoxy), 6.28 (m, 1H, H$_a$1'), 6.23-6.07 (m, 2H, H$_b$1', H$_c$1'), 5.25 (m, 1H, H$_a$3'), 4.18 (m, 2H, H$_b$3', H$_c$3'), 3.98 (m, 2H, H$_b$4', H$_c$4'), 3.90 (m, 1H, H$_a$4'), 3.88-3.55 (m, 4H, H$_b$5', H$_c$5'), 3.81-3.68 (s x3, 18H, 6 x CH$_3$O), 3.45-3.20 (m, 2H, H$_a$5'), 2.48-2.22 (m, 2H, H$_a$2'), 1.94-1.56 (m, 4H, H$_b$2', H$_c$2'), 1.82-1.76 (s, 6H, C$_b$5-CH$_3$, C$_c$5-CH$_3$), 1.44 (s, 3H, C$_a$5-CH$_3$).

$^{31}$P NMR: (δ in ppm CDCl$_3$) 67.6

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40
$^{13}$C NMR: (δ in ppm CDCl$_3$) 163.88, 163.84, 163.73 (3 x C4), 159.05 (6C of DMT), 150.56, 150.39, 150.33 (3 x C2), 145.03, 144.96, 144.30 (3C of DMT), 136.18, 136.07 (2 x C$_b$6), 135.93, 135.37 (6C of DMT), 135.20 (C$_a$6), 130.49, 130.40, 130.32, 130.26, 128.55, 128.46, 128.32, 127.49 (27C of DMT), 113.67, 113.62 (12C of DMT), 111.91, 111.38, 111.31 (3 x C5), 87.64, 87.62, 87.51(3 x C$_{DMT}$), 87.02, 86.17 (2 x C$_b$1'), 84.67 (C$_a$1'), 84.26, 84.18 (C$_a$4', 2 x C$_b$4'), 80.32 (C$_a$3'), 74.44, 74.25 (2 x C$_b$3'), 68.38 (2 x C$_b$5'), 63.56 (C$_a$5'), 55.48, 55.45 (6 x CH$_3$O), 39.09, 38.91 (3 x C2'), 12.60 (2 x C$_b$5-CH$_3$), 11.97 (C$_a$5-CH$_3$).

5'-O-(4,4'-dimethoxytrityl)thymidin-3'-yl 3'-O-(4,4'-dimethoxytrityl)thymidin-5'-yl 2-pyridonyl phosphate 8a (mixture of diastereomers)

$^1$H NMR: (δ in ppm CDCl$_3$) 8.93 (s, 1H, NH), 8.86 (s, 1H, NH), 8.77 (s, 1H, NH), 7.95, 7.64 (d, 1H, Hc6'), 7.74-7.64 (t, 1H, Hc4'), 7.57, 7.54 (s, 1H, H$_a$6), 7.46-7.39 (s, 1H, Hb6), 7.38-7.18 (m, 18 H, ArH), 7.10, 7.01 (t, 1H, Hc5'), 6.78, 6.63 (d, 1H, Hc3'), 6.87-6.78 (m, 8H, ArH para to methoxy), 6.44 (m, 2H, H$_a$1', H$_b$1'), 5.55, 5.49 (m, 1H, H$_a$3'), 4.38 (m, 1H, H$_b$3'), 4.18-4.16 (m, 1H, H$_a$4'), 4.14-3.98 (m, 1H, H$_b$4') 3.90-3.68 (m, 2H, H$_a$2'), 2.11-1.80 (m, 2H, H$_b$2'), 1.77, 1.74 (s x2, 3H, C$_b$5-CH$_3$), 1.34, 1.33 (s x2, 3H, C$_a$5-CH$_3$).

$^{31}$P NMR: (δ in ppm CDCl$_3$) -8.08, -8.12

$^{13}$C NMR: (δ in ppm CDCl$_3$) 163.84, 163.80, 163.76 (2 x C4), 159.05, 158.99 (4C of DMT), 157.33 (C$_c$1'), 150.65, 150.61, 150.56, 150.31 (2 x C2), 147.92, 147.74 (C$_c$6'), 145.06, 144.21, 144.14 (2C of DMT), 140.59, 140.50 (C$_c$4'), 136.23 (C$_b$6), 135.79, 135.49, 135.21 (4C of DMT), 135.16, (C$_a$6), 130.37, 128.52, 128.48, 128.42, 128.37, 128.30, 128.26, 127.52, 127.42 (18C of DMT), 121.48, 121.20 (C$_c$5'), 113.63, 113.55 (8C of DMT), 113.15, 112.83 (C$_c$3'), 111.99, 111.89, 111.45 (2 x C5), 87.69, 87.57, 87.47 (2 x C$_{DMT}$), 85.38 (C$_b$1'), 85.26, 84.98 (C$_a$4'), 84.77 (C$_a$1'), 84.54, 84.35 (C$_b$4'), 80.69, 80.47 (C$_a$3'), 74.59 (C$_b$3'), 68.51 (C$_b$5'), 63.64 (C$_a$5'), 55.46 (4 x CH$_3$O), 39.44 (2 x C2'), 12.55 (C$_b$5-CH$_3$), 11.74 (C$_a$5-CH$_3$).
5'-O-(4,4´-dimethoxytrityl)thymidin-3'-yl 3'-O-(4,4´-dimethoxytrityl)thymidin-5'-yl 2-pyridonyl phosphorothioate **8b** (mixture of diastereomers)

**1H NMR:** (δ in ppm CDCl₃), 8.92 (s, 1H, NH), 8.82 (s, 1H, NH), 8.75 (s, 1H, NH), 7.75, 7.67 (d, 1H, H₆'), 7.75-7.61 (t, 1H, H₄'), 7.60, 7.58 (s, 1H, HA6), 7.50-7.18 (m, 19 H, ArH, H₆), 7.11, 7.03 (dd, 1H, H₅'), 6.79, 6.62 (d, 1H, H₄'), 6.92-6.88 (m, 8H, ArH para to methoxy), 6.48 (m, 1H, H₁'), 6.41(m, 1H, Ha₁'), 5.69, 5.61 (m, 1H, Ha₃'), 4.44 (m, 1H, H₂'), 4.36, 4.16 (m, 1H, Ha₄'), 4.15-3.90 (m, 1H, H₄'), 3.86-3.62 (m, 2H, H₅'), 3.82-3.69 (s x3, 12H, 4 x CH₃O), 3.52-3.36 (m, 2H, H₃'), 2.74-2.32 (m, 2H, H₄'), 2.20-1.78 (m, 2H, H₂'), 1.78, 1.76 (s x2, 3H, Cb₅-CH₃), 1.44, 1.42 (s x2, 3H, Cb₅-CH₃).

**31P NMR:** (δ in ppm CDCl₃) 61.1, 60.5

**13C NMR:** (δ in ppm CDCl₃) 163.89, 163.85 (2 x C₄), 158.99 (4C of DMT), 157.51, 157.44 (C₃', 150.66, 150.53 (2 x C₂), 147.90, 147.80 (C₆'), 145.12, 144.39, 144.34 (2C of DMT), 140.41, 140.29 (C₄'), 136.38, 136.34 (Cb₆), 136.29, 135.85, 135.77, 135.44, 135.40, 135.29 (C₆, 4C of DMT), 130.41, 130.32, 130.25, 128.52, 128.49, 128.30, 127.45 (18C of DMT), 121.58, 121.43 (C₅'), 114.17, 114.07 (C₃'), 113.65, 113.59 (8C of DMT), 111.89, 111.78, 111.50, 111.46 (2 x C₅), 87.69, 87.47 (2 x CDMT), 85.52 (Cb₁'), 85.35, 85.20 (Cb₄'), 84.71 (Ca₁'), 84.63, 84.52 (Ca₄'), 80.83, 80.63 (Ca₃'), 74.94, 74.89 (Cb₃'), 68.69 (Cb₅'), 63.79, 63.68 (Cₐ₅'), 55.46 (4 x CH₃O), 39.53, 39.36 (2 x C₂'), 12.59, 12.53 (Cb₅-CH₃), 11.91, 11.87 (Ca₅-CH₃).