Synthesis and triplex forming properties of pyrrolidino-DNA

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List of Publications

During the present thesis, the following articles have been published:

“Pyrrolidino-DNA”

“A short, efficient synthesis of 2’-deoxypseudoisocytidine based on Heck-chemistry”

“Synthesis and triplex forming properties of pyrrolidino pseudoisocytidine containing oligodeoxynucleotides”
“Where nature finishes producing its own species, man begins, using natural things and in harmony with this very nature, to create an infinity of species.”

Leonardo da Vinci
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Summary

Sequence specific binding of triplex forming oligonucleotides (TFOs) in the major groove of double stranded DNA can control and modulate gene expression at the level of transcription and is the basis of antigene technology. Unfortunately, the use of TFOs as antigene agents suffers from several limitations, one of the major being the low thermal stability of the triplexes. As part of our current research we are interested in increasing TFO affinity to a DNA target by applying the ‘dual recognition’ approach. In this approach, a target dsDNA is not only recognised by selective base-base interactions but also via an additional, non-specific salt bridge between an appropriately placed positive charge in the TFO and a negatively charged phosphate oxygen in the target DNA backbone.

Based on a recent X-ray data on a parallel DNA triplex, molecular modelling showed that replacement of the 4’-oxygen in a deoxynucleoside residue of a TFO by a basic nitrogen would potentially place a positive charge next to a non-bridging pro-R-phosphate oxygen of the purine strand of a target dsDNA. In preliminary studies, pyrrolidino-pseudonucleosides containing the base uracil (dpψU) and N-1-methyl uracil (dpψT) for A-T base-pair recognition as well as the base isocytosine (dpψiC) for GC base-pair recognition within the parallel triplex binding motif were explored (Figure 1).

Figure 1: First generation of pyrrolidino C-nucleoside.

In the first part of this work, the first generation of pyrrolidino C-nucleoside were further investigated in order to confirm and complete the data obtained from the preliminary results. The pyrrolidino analogues of T, dpψU and dpψT, exhibited triplex destabilizing effects in a strong sequence dependent manner. Both the base and the sugar modifications are responsible for loss in triplex stability. On the other side, TFOs containing the dpψiC unit were shown to produce more stable triplexes compared to the natural system. The stabilizing property was
proved to arise exclusively from the pyrrolidino-sugar modification and to be independent of the sequence context.

In the second part of the research project, new pyrrolidino pseudonucleosides were designed, synthesized and incorporated into TFOs. Pyrrolidino 2-pyridone (dp2P) and 2-aminopyridine (dp2AP), analogues of T and C, respectively, are deletion mutants displaying only the minimum functionalities required for target recognition via Hoogsteen base pairing (Figure 2).

![Figure 2: Second generation of pyrrolidino C-nucleoside.](image)

This second generation of pyrrolidino analogues was used to test hypotheses proposed to explain the discrepancy between dpψiC and dpψiU(T), and, in case of success, to provide access to efficient T and C analogues for stable and selective triplex formation within the pyrimidine binding motif. However, in both cases, the TFOs containing the new pyrrolidino units were binding to their target duplex with less affinity than the parent systems or the TFOs containing the first generation of pyrrolidino C-nucleosides. With these experiments it could be excluded that differences in the syn/anti conformation at the glycosidic angle in dpψiU and dpψiC are responsible for the differential behaviour of the first generation pyrrolidino C-nucleosides.
Chapter 1: Introduction

1.1 Nucleic Acids

1.1.1 Structure of DNA and RNA \[^{[1, 2]}\]

Nucleic acids are biopolymers built from nucleotides, the phosphate esters of nucleosides. Nucleosides are formed by condensation of a nucleobase derived from the pyrimidine or purine chromophore, and a pentose. The nature of the pentose distinguishes ribonucleic acid (RNA) from deoxyribonucleic acid (DNA). RNA is composed from D-ribose linked in its furanose form to N9 of the purine bases, adenine (A) or guanine (G), or N1 of the pyrimidine bases, cytosine (C) or uracil (U). In DNA the 2’-deoxy-D-ribofuranose is joined in the same way to the four bases, with the exception that uracil (U) is replaced by thymine (T) (figure 1.1).

![Nucleobases](image)

**Figure 1.1: Structure of the nucleobases and composition of a nucleotide.**

The diphosphate units are strong acids and exist as anions at neutral pH. The ‘bases’ are in reality only very weakly basic, and A, C, and G become protonated only below pH 4. The imide NHs of G, T and U are deprotonated at pHs above 9.

Two nucleotides can be connected via reaction between the phosphate residue of one nucleotide and the 3’ hydroxyl of a second one forming in this way a dinucleotide. This dimer bears a free phosphate residue at its 5’ end and a free hydroxyl group at the 3’ end allowing incorporation of additional nucleotide through the formation of new phosphodiester bonds. The elongation process leads to a single strand of nucleic acid or oligonucleotide.
Two strands of complementary sequence can assemble in a double helical structure. This secondary structure is held together mainly by base-pair stacking between adjacent pairs and by hydrogen bonds between the major amino-keto tautomers of the bases to link A with T and C with G in Watson-Crick base-pairing (Figure 1.2). \[^{3, 4}\] Thereby the amount of purine bases (G, A) is equal to the amount of pyrimidine bases (C, T) for all double stranded nucleic acids.

**Figure 1.2:** Watson-Crick base pairs for A-T and G-C.

DNA and RNA double helices show two grooves between successive turns of the sugar-phosphate backbone with different depth and width. One is called minor groove in which O2 of pyrimidine and N3 of the purine bases are located, and the other is the major groove, which is located on the opposite side, and contains N7 and N6 of the purine bases, as well as O4 or N4 of the pyrimidine bases (Figure 1.3 left and centre). On either side (especially the major groove side) there is potential for the base pairs to H-bond with external groups like water or proteins.

**Figure 1.3:** Major and minor groove of DNA (left), translocational movements of base-pairs relative to the helix axis (center) and sugar puckering modes (right).
There exists a wide variety of right handed double helical DNA conformations, and all of these can be classified in two families: A and B. These are associated with the sugar pucker C3’-endo (N) for the A family and C2’-endo (S) for the B family (Figure 1.3, right and Table 1.1). A-DNA form is favoured at low humidity and high salt concentrations, while B-DNA form is predominant at high humidity and low salt concentrations.

### Table 1.1: Comparison of helix parameters.

<table>
<thead>
<tr>
<th>Entry</th>
<th>A-DNA</th>
<th>B-DNA</th>
<th>Z-DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helicity</td>
<td>Right-handed</td>
<td>Right-handed</td>
<td>Left-handed</td>
</tr>
<tr>
<td>Sugar puckering</td>
<td>C(3’)-endo</td>
<td>C(2’)-endo</td>
<td>C(2’)-endo in pyrimidine and C(3’)-endo in purine</td>
</tr>
<tr>
<td>Number of bases per turn</td>
<td>11</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>Distance between neighboring base-pairs (Å)</td>
<td>2.9</td>
<td>3.3 - 3.4</td>
<td>3.7</td>
</tr>
<tr>
<td>Dislocation of base-pairs from the axis (Å)</td>
<td>4.5</td>
<td>-0.2 to -1.8</td>
<td>-2 to -3</td>
</tr>
<tr>
<td>Tilt of bases (°)</td>
<td>20</td>
<td>-6</td>
<td>7</td>
</tr>
</tbody>
</table>

An example of a left-handed helical structure is the Z-DNA. The Z-helix is also an antiparallel duplex, but its two backbone strands run downward at the left of the minor groove and upward at the right, and this is opposite from those of A- and B-DNA. This structure is stabilized by high concentrations of MgCl₂ or NaCl. It is most favoured for alternating G-C sequences. The helix parameters for A, B and Z-DNA are listed in Table 1.1.

The most important parameter distinguishing an A-type from a B-type helix is the shift dislocation (Figure 1.3, center), which means the displacement of base-pairs away from the helix axis. In B-DNA, the base pairs are located near to the axis while in A-DNA, the axis is pushed away from the base pair. When purine bases and pyrimidine bases are located one after the other continuously, pyrimidine bases tend to move away from the minor groove to form the Z-DNA.

On the other hand, RNA helical structures are restricted to A-form duplexes with 11-12 residues per turn. RNA-DNA duplexes have a structure that is globally an A-form helix but with modified conformation in the sugars in the DNA strand. This leads to a minor groove which is wider than A-RNA but narrower than B-DNA.

### 1.1.2 From DNA to RNA to Protein

DNA is the most important biomolecule since it carries the genetic information of a cell. It consists of thousands of genes and each gene serves as a recipe on how to build a protein molecule. Proteins perform important tasks for the cell functions or serve as building blocks. The flow of
information from the genes determines the protein composition and thereby the functions of the cell (Figure 1.4).

The DNA is situated in the nucleus, organized into chromosomes. Every cell must contain the whole genetic information and the DNA is therefore duplicated before a cell divides (replication). To be able to replicate, the cell has to unfold and unwind the DNA, and also has to separate the two strands from each other. When proteins are needed, the corresponding genes are transcribed into mRNA (messenger RNA, transcription). In eukaryotes the mRNA is first processed so that non-coding parts are removed (splicing) and is then transported out of the nucleus (transport). Outside the nucleus, the proteins are synthesized from an mRNA template (translation) based upon the original code passed on from DNA to RNA.

During this process the nucleotide sequence of a mRNA is translated into the amino acid sequence of a protein. The protein synthesis takes place at the ribosome, which provides the basic machinery for the translation process. The major role of the ribosome is to catalyze the coupling of amino acids into protein according to the sequence specified by the mRNA. The amino acids are brought to the ribosome by tRNA (transfer RNA) molecules.

The flow of information leading from gene to protein can theoretically be manipulated at any level, targeting DNA, RNA or protein. Triplex forming oligonucleotides (TFOs), peptide nucleic acids (PNAs) and polyamides (PAs) can be designed to recognize specific targets within the promoter or coding region of a target gene and inhibit transcription (antigene strategy, see below). Oligonucleotides can also be used to bind specifically to mRNA and inhibit translation (antisense and ribozyme strategies). It is also possible to use oligonucleotides to interfere with or selectively stimulate the function of particular proteins (aptamer strategy). Recently a new strategy of gene targeting, based on the use of small interfering RNA duplexes (siRNA), has drawn the attention of many researchers for its powerful capacity of silencing genes. [6]
1.2 The Triple Helix

1.2.1 General Properties

The polymorphic nature of DNA manifests itself in a variety of multistranded architectures that depart from canonical B-DNA structures, mostly in purine-rich sequences. The double helix itself has become a complex and dynamic structure, sensitive to its sequence but also capable of accommodating a remarkable range of chemical modifications. The accumulation of data on multistranded conformations (like triplexes, quadruplexes and quintuplexes), loops, bulges and junctions has helped in understanding the multitude of ways in which a given collection of oligonucleotides may be arranged in space.

The possibility that DNA might form triple helices was suggested in 1953 by Pauling and Corey. Four years later Felsenfeld et al. reported the formation of a three-stranded polynucleotide molecule (Figure 1.5). Based on optical density of various mixtures of one poly(A) and two poly(U) RNA strands, the formation of a stable complex was proven. They suggested two additional hydrogen bonds between a uracil of the third strand and an adenine of the A-U Watson-Crick base pair. In 1968, a potential biological role for these structures was identified when it was found that in vitro transcription mediated by Escherichia coli RNA polymerase was inhibited by a third RNA strand. The apparent restriction of such structures to homopolymers and the absence of facile methods for synthesizing mixed oligonucleotides hindered further development for more than a decade. Interest in triplexes revived with the finding that intramolecular triplexes (H-DNA) can apparently form in cells. DNA with adjacent segments of homopurine and homopyrimidine runs can melt and refold into a three-stranded structure. Structural studies of the triple-helical portions of various H-DNAs revealed two basic structural motifs and identified the common base triads used in intermolecular triplexes formed with standard DNA bases, as described below. Several roles have been proposed for naturally occurring H-DNA in DNA replication, transcription and recombination. However it remains to establish unambiguously that these intramolecular DNA triple helical structures can act as molecular switches to modulate gene expression and other DNA metabolism events in a structure dependent manner, in addition to the well established sequence-specific regulation.

Interest in triplexes increased dramatically in the late 1980s with the realization that TFOs could be used as DNA sequence

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*Figure 1.5: Triple Helix of DNA.*
reading agents,\textsuperscript{[16]} with potential uses as antigene agents and tools in molecular biology.\textsuperscript{[17-30]} In these complexes the third strand lies in the major groove of the target DNA duplex, where it forms base triplets with the \textit{Watson-Crick} base-pairs, through \textit{Hoogsteen} or reverse \textit{Hoogsteen} hydrogen bonding with the oligopurine bases (Figure 1.6).\textsuperscript{[31]} Conformational information about triplexes is available from many spectroscopic studies in solution\textsuperscript{[32,33]} and from fiber diffraction,\textsuperscript{[34]} but there is no comparison with the detailed crystallographic structural data available for oligonucleotide duplexes. The x-ray structure of a 2:1 peptide nucleic acid-DNA triplex has been reported,\textsuperscript{[35]} but crystals formed by nucleic acid triplexes are invariably disordered, at best giving rise to fiber-like diffraction.\textsuperscript{[36]} However, crystal structures of triple helix fragments are known in the literature and enabled the construction of models of both parallel and antiparallel triplexes.\textsuperscript{[37,38]}

Two stable DNA triple-helical motifs are known that differ according to the orientation and base composition of the third strand. In the parallel or pyrimidine binding motif, the homo-pyrimidine triplex-forming oligonucleotide is parallel oriented to the duplex purine strand forming H-bonded \textit{Hoogsteen} C\textsuperscript{+}-GC and T-AT base triplets. The antiparallel or purine motif is characterized by G-GC, A-AT and T-AT reverse \textit{Hoogsteen} base triplets between a purine rich TFO that is antiparallel oriented to the purine strand of the DNA duplex (Figure 1.6). In both motifs, the third strand requires a target DNA homopurine–homopyrimidine sequence tract, ideally of 15–30 nucleotides.

\begin{itemize}
\item (a) Parallel Motif
\item (b) Antiparallel Motif
\end{itemize}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1_6.png}
\caption{Molecular structures of the canonical \textit{Hoogsteen} (a) and reverse \textit{Hoogsteen} (b) base triplets in the known parallel and antiparallel triple helical binding motifs.}
\end{figure
1.2.2 Antigene Strategy \[39\]

Selective artificial control of gene expression is a longstanding dream in biotechnology and human therapy. Oligonucleotides seem perfectly suited for this purpose because of their unique base-base recognition properties. In the antisense approach, \[40\] short oligonucleotides are designed to bind to specific sequences of a mRNA of interest via *Watson–Crick* duplex formation, to block gene expression on the level of translation according to various molecular mechanisms. This approach is widely explored not only in the therapeutic area, where approximately 70–80 oligonucleotides targeted to various diseases are currently in clinical trials and only one phosphorothioate DNA-oligomer, namely Vitravene, has been approved for treatment of cytomegalovirus-induced retinitis, \[43\] but also as tools to downregulate gene expression in the area of functional genomics. In the antigene approach (Figure 1.7), oligonucleotides bind sequence selectively to genomic double stranded DNA and interfere with transcription and the DNA processing machinery via triple helix formation. Some intrinsic advantages of the antigene over the antisense principle can be identified. First, there are only two target copies of DNA per diploid cell as compared with the hundreds to thousands of mRNA copies that have to be targeted in the antisense approach. This should dramatically reduce the amount of oligonucleotide needed for activity. Moreover, as shown in Figure 1.7, not only transcriptional activation and deactivation but also gene knockout as well as targeted mutagenesis, targeted recombination and sequence-selective manipulation of genomic DNA can be achieved.

![Figure 1.7: Basic antigene concepts and corresponding applications. Gene expression can be up regulated or down regulated on the level of transcription via selective triple-helix formation, preferably at promoter sites. Selective modification of the genome includes site-specific mutagenesis via triplex delivered mutagens (e.g. psoralen), or homologous recombination through triplex delivered donor DNA via DNA repair. Further applications arise from triplex-targeted chemical modification of the gene (e.g. cleavage, site-specific cross-linking or alkylation).](image)

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\[7\]
1. **Modulation of Gene expression.** Many recent reports have identified novel ways to inhibit expression of specific genes or the activation of some other genes. For example, a TFO targeted to the 3′-untranslated region (3′-UTR) of the bcl-2-proto-oncogene effectively inhibited synthesis of bcl-2 protein. [44] Another novel approach, worthy to mention, is the generation of TFOs inside a cell to specifically suppress a gene’s function. [45]

2. **Site specific mutagenesis.** Since the discovery of triplex-forming sequences, a major effort has been using them as specific reagents to deliver DNA damaging chemicals that will induce site-specific damage in a target sequence in the genome. [22] For example, several groups used psoralen-linked TFOs to stimulate homologous recombination in yeast and in mammalian targets and to increase mutation frequencies up to 2500-fold compared to the control non-triplex-forming oligonucleotides. [46] It should be pointed out that TFOs themselves can induce mutation frequency. [47] In 2000 Vasquez and coworkers [48] presented the first study describing a triplex mediated mechanism of site directed mutagenesis induced in animals, therefore opening a new field of investigation aimed at probing the potential of the triplex approach in vivo. [49]

3. **Modification of genomic DNA.** Double-strand breaks in the target gene could be induced by TFOs containing radioactive isotopes, [50] non-radioactive chemicals [51] and antitumor drugs. [52] Despite the potential benefits for biotechnology in general, the investment of resources in TFOs has been limited, and therewith progress in this field has been slow. Consequently, there is still no antigene oligonucleotide in clinical trials.

1.2.3 Limitation in the Antigene Strategy [53, 54]

There are many difficulties in using TFOs as a tool in molecular genetics:

- **Delivery and cellular uptake of TFOs.** An adequate amount of third strand has to reach the nucleus, and thus needs to cross two membrane barriers.

- **In vivo stability.** The stability of unmodified oligonucleotides in cells, serum and whole animals depends on their resistance to cellular nucleases. It has been demonstrated that various backbone modifications significantly enhance in vivo stability.

- **Unexpected secondary effect,** like the toxicity or immunogenicity of the modified oligonucleotides, as well as non-antisense activities arising from unspecific binding to elements of the cellular matrix.

- **Low thermal stability.** The binding affinity of TFOs is not very high and is usually weaker than that of the underlying DNA duplex. In large part this is due to the charge repulsion resulting from bringing together the three polyanionic DNA strands.
G-rich TFOs. In the context of antiparallel triplexes, G-rich TFOs are known to adopt unusual structures, namely G-quartets, which compete for triplex formation. Those structures are favoured in the presence of high concentration of monovalent cations \[55\] and since intracellular K\(^+\) concentration in most eukaryotic cells is around 140 mM this may create problems with some potentially useful TFOs.

pH dependence. For the parallel motif, the severest restriction is the pH sensitivity of the C\(^+\)-GC base triplet, which is intrinsically unstable under physiological conditions due to the low pK\(_a\) of the third strand cytosines.

Restriction to homopurine tracts. The classical triplets described in Figure 1.6 require strand wise separation of purine and pyrimidine in the target duplex. Recognition of the pyrimidine residues is harder to achieve and usually restricts triplex formation to homopurine-homopyrimidine tracts.

1.2.4 Approaches to increase the efficacy of antigene agents [39, 53, 54]

Many different approaches have been developed in order to increase nuclease resistance and thermal stability, to overcome pH limitations and the restriction to homopurine tracts. Parallel triplexes are expected to be more stable than the antiparallel ones, and are better known from a structural and functional point of view. Therefore most of the modifications which will be described here concern parallel triplexes.

Delivery and cellular uptake of TFOs. Many effective methods have been applied in order to induce efficient uptake, like generating oligonucleotides in vivo, [56] use of cationic lipid [57] or nanoparticles formed from TFOs in the presence of dendrimers, [58] condensation of TFOs to short peptides. [59] So far there is no consensus on the best method.

In vivo stability. An easy RNA-modification consists in the methylation of O2’. In addition to form triplexes with increased stability, 2’-O-methylated TFOs display a fairly good resistance against nucleases. [60] The same result is obtained with the family of 2’-aminoalkyloxy derivative especially those bearing an aminoethoxy or aminopropoxy side chain. [61] Replacement of the phosphate group by a phosphorothioate or phosphorodithioate confers nuclease resistance as well. However this type of molecules is more efficient in antisense than in antigene therapy. [62, 63] They lead to unstable antiparallel triplexes and inhibit formation of parallel triplexes when present in a TFO. More drastic modifications of the sugar-phosphate backbone result as well in nuclease resistant TFOs. Peptide nucleic acid (PNA), based on a neutral amide backbone composed of an N-(2-aminoethyl)-glycine linkage, [64] and morpholino DNA are representatives of this class of
compounds. Most of the modifications mentioned in this part exhibit additional properties interesting for triplex formation that will be described below.

**pH dependence.** The main issue associated with the formation of parallel triple helices is the protonation of the C residues in the Hoogsteen strand. Many authors have stated that C\(^+\)-GC triplets are more stable than T-AT triads, but, as expected, their stability varies depending on the pH. The pK\(_a\) of isolated cytosine is 4.3 \[^{[65]}\] and can increase when incorporated into oligonucleotides, however optimum C\(^+\)-GC triplet stability still requires acidic conditions. Many efforts of the synthetic chemists have been addressed to obtain either: i) neutral C-analogues mimicking protonated cytosine, with two hydrogen bond donor groups, in order to achieve pH-independent triplex formation; or ii) more basic C-analogues.

Neutral analogues have been designed as derivatives of either the pyrimidine or the purine ring (Figure 1.8 left). The first group should in principle give rise to the most stable triplexes, since they cause little or no distortion in the sugar phosphate backbone. Pseudoisocytosine (ψC), \[^{[66-68]}\] 6-oxo-5-alkylecytosines (C\(_{\text{o xo}}\) and C\(_{\text{Me oxo}}\) \[^{[69, 70]}\] and pyrazine base \[^{[66]}\] lead to triplets that are isomorphus to the canonical T-AT triplet. Those heterocycles already have a hydrogen atom at the N3 position which allows them to bind to the guanine base in a pH-independent fashion. Triplexes containing these modified bases are more stable at neutral pH than cytidine-containing triplexes but generally do not achieve the stability of 5-methyl-cytidine containing triplexes (see below). In the second group, purine analogues with the required hydrogen bonding pattern have been employed to replace C\(^+\) in the parallel motif. At neutral pH, N\(^7\)-deoxyguanosine (N\(^7\)-G) binds contiguous G-C base-pairs with higher affinity than \(^{5\text{Me}}\)C\(^+\), while the contrary was observed for isolated G-C base-pairs. This difference is probably caused by the lack of structural isomorphism between N\(^7\)-G•G-C and T•A-T. \[^{[71]}\]

![Figure 1.8: Cytosine analogues. Neutral analogues (left) and analogues with increased acidity (right).](image)
Different C-nucleosides with 2-aminopyridine rings (P) (Figure 1.8 right) have been prepared and evaluated as cytosine analogues with a higher pKₐ and the same pattern of hydrogen donor groups.[^72-75] α (α P) and β (β P) anomers as well as methyl derivatives (MeP) of P-nucleosides form stable triplexes at higher pHs than cytosine or methyl-cytosine whereas 2’-OMe-derivatives are less stable.

5-methyl-cytosine (MeC or C) (Figure 1.9) has been substituted for cytosine in the third strand of triple helices for many years.[^76] This modification enhances considerably the stability of triplexes at neutral pH, even though the pKₐ of MeC is only 0.2 units higher than that of C. This stabilizing effect has been related to the extra spine of methyl groups within the DNA major groove. These methyl groups increase stability by either inducing the release of water molecules, thereby contributing a positive entropy change,[^77] or by improving the base stacking,[^78] or both. In order to introduce hydrophobicity to the triplex, favoring both desolvation and stacking interactions, the methyl group of both thymine and 5-methylcytosine has been exchanged by a propynyl group (Figure 1.9).[^79] Positive results in parallel triplex formation are observed when 5-propynyl-U replaces T, but not when 5-propynyl-C replaces C. 5-propynyl-C was found to be less basic than C (pKa 3.3). On the other hand, 5-halopyrimidine derivatives were shown to destabilize triplex formation and this behavior was attributed to a decrease in stacking interactions.[^80]

### Restriction to homopurine tracts

An important limitation of the antigene technology comes from the restriction of TFOs to bind to homopurine/homopyrimidine DNA target sequences. Although such sequences are statistically over-represented in the genome it would be desirable to have nucleobases at hand that specifically recognize pyrimidine bases or whole pyrimidine/purine Watson–Crick base-pairs. Recognizing pyrimidine bases is hampered by the fact that only one H-bond donor or acceptor site of C and T is available for binding in the major groove. Recognizing T in a TA base-pair is even more complicated because of the presence of the methyl group, which leads to steric interference with the TFO backbone, especially in the parallel binding motif.

Many strategies have been employed in the last 10 years for recognizing pyrimidine interruptions, but a general solution is still elusive.[^81] Among the natural DNA base pairs, T-CG and G-TA (Figure 1.10) emerge as the best triplets for pyrimidine recognition. The T-CG triplet can be accommodated within both parallel and antiparallel triplexes, while G-TA is limited to parallel
structures. However these triplets are much less stable than T-AT or C'-GC and multiple inversions are strongly destabilizing.

\[ \text{T-CG Parallel} \hspace{1cm} \text{T-CG Antiparallel} \hspace{1cm} \text{G-TA Parallel} \]

\textit{Figure 1.10:} Chemical structures of the T-CG triplet in both parallel and antiparallel orientations and the G-TA triplet.

An alternative less selective strategy is to avoid the offending pyrimidine residue by skipping a base in the third strand (abasic site) or opposing it with a non selective residue (universal base). Among the abasic analogues, only few gave satisfying results. The 4-(aminobutyl)-propane-1,3-diol (Figure 1.11 top) \cite{82} is one of the least destabilizing abasic residues due to positive interactions between the protonated amino group and the phosphate backbone.

The anthraquinone unit A (Figure 1.11 top), proposed by Richert and co-workers, \cite{83} was also found to give high affinity for pyrimidine sites over the purine ones.

\textit{Figure 1.11:} Abasic sites (top) and universal bases (bottom).

Azole derivatives have been used as universal bases in the context of antiparallel triplexes and were shown to bind to both TA and CG inversion sites. The lack of hydrogen bonding sites of the small aromatic base residues explains their ability to stack within the duplex and form unspecific contact with the different base pairs (Figure 1.11 bottom). \cite{84-86}

Another approach for the sequence-specific recognition of double-stranded DNA is the use of heterocyclic compounds which form hydrogen bond contacts with substituents on both bases of the Watson-Crick base pair.
Within the parallel motif, the base S (Figure 1.12), which is the best representative of this class of base analogues so far, was found to bind to a TA inversion site with an affinity that almost matches that of a T-AT canonical base triplet, and with reasonable selectivity. According to the proposed model, the S-TA triplet is characterized by three hydrogen bonds involving the N7 atom, the 6-amino group of adenine and the 4-oxo group of thymine (Figure 1.12). [87-89] At the beginning of this year, the 2’-aminoethoxy derivative of the base S has been prepared and shows greater affinity than G or S for a single TA interruption. Although it still interacts with other base pairs, in particular CG, it is described as the best nucleoside so far for recognition of TA within the parallel binding motif. [90]

Another example of a synthetic base analogue which has been successfully employed for recognizing thymine of a TA base pair is the 3-oxo-2,3-dihydropyridazine (E) (Figure 1.13). [91, 92] This analogue, which was incorporated into the *Hoogsteen* strand of a bis-PNA, contains a CH$_2$CH$_2$NH linker to avoid steric clash with the 5-methyl group of thymine and a hydrogen bond donor positioned to bind to the 4-oxo group of thymine. Although the E-TA triplet is less stable than the canonical triplets C$^+$-GC and T-AT, it forms complexes with higher T$_m$ values than positioning a G opposite to a TA pair.

**Targeting TA**

![S nucleoside designed to recognize the whole Watson-Crick base pair.](image)

**Targeting CG**

![Nucleobase analogues for TA and CG recognition.](image)

Deoxynucleosides with acceptor or double acceptor bases like the pyridone (P$^B$) [93] or pyrimidone (4HT) [94] have been investigated as units to target CG inversion sites in parallel TFOs (Figure 1.13). They are essentially C4 carbonyl deletion mutants of thymine, which disable their
binding to an AT base pair while maintaining the binding profile to CG sites. Both bases were shown to be highly selective for CG inversion sites but have compromised affinity when compared to a canonical base-triplet. Combination of the pyrimidone base with a 2'-aminoethoxy side chain has recently proved to be an excellent system for CG interruption. The selectivity arising from the base is conserved whereas the affinity is dramatically increased due to the positively charged side chain.\[95, 96\]

Earlier this year, Brown and Fox have achieved recognition of all four base pairs by triple helix formation at physiological pH, using triplex forming oligonucleotides that contain four different synthetic nucleotides (Figure 1.14).\[97\] They demonstrate successful triplex formation at a 19-mer oligopurine sequence that contains two CG and two TA interruptions. The complexes are pH dependent, but are still stable at pH 7.0. BAU (see low thermal stability section below), \(^{\text{Me}}\)P and \(^{\text{AP}}\)P\[98\] retain considerable selectivity, and single base pair changes opposite these residues cause a large reduction in affinity. In contrast, S is less selective and tolerates CG pairs as well as TA.

\[\text{Figure 1.14: Synthetic nucleotides used to recognise all four base pairs.}\]

If several approaches allow stable and selective binding of a CG interruption in a homopurine strand, a solution for recognizing TA base pairs with high selectivity over other base pairs, especially CG, still needs to be achieved.

\[\text{Low thermal stability.}\] The strategies examined to date to increase the triplex stability rely either on the addition of external compounds (like binding ligands or intercalators\[99-102\]) which will not be detailed here or on the modification of the TFO. There are a few basic concepts in improving the binding of TFOs to duplex targets that have been successfully applied in the past few years: i) first, modification of the sugar-phosphate backbone in order to obtain conformationally adapted or constrained TFOs; ii) second, the introduction of positive charges in the TFO, designed to make additional binding contacts with the phosphodiester residues of the target \textit{Watson–Crick} duplex besides the base–base interactions (dual recognition concept).
Different changes in the phosphate moiety (Figure 1.15) have enlarged the triplex stability. For instance, replacement of the O3’ → P5’ phosphodiester linkage by a N3’ → P5’ phosphoramidate linkage in TFOs is very successful, yielding stable parallel triplexes under physiological conditions. More recently, Imanishi and coworkers found that the pyrimidine motif TFO partially including 2’,5’-phosphodiester linkages forms a very stable triplex under near physiological conditions.

Another interesting approach involves the use of TFOs containing conformationally constrained nucleoside units. As with duplex formation, less entropy loss upon triplex formation and thus higher triplex stability is expected. Given the preference of RNA-over DNA-TFOs in the parallel binding motif, oligonucleotide analogues that are constrained in a C3’-endo (RNA-like) conformation should be advantageous. Imanishi and coworkers investigated triplex formation with the conformationally constrained RNA analogue LNA (locked nucleic acid, figure 1.16) in the pyrimidine motif and found substantial increases in thermal and thermodynamic stability of corresponding triplexes at neutral pH (the abbreviation BNA, bridged nucleic acid, synonymous for LNA is used by the Imanishi group). There exist an optimal number of LNA-residues within a DNA-TFO that exert a stabilizing effect. Interestingly, fully modified LNA-TFOs do not form triplexes with DNA targets at all. A kinetic analysis of triplex formation with LNA revealed that most of the observed increase in the binding constant at neutral pH arises from a decreased dissociation rate. Giovannangeli and coworkers evaluated the capacity of G-rich TFO/LNAs to interfere with biological processes in vitro and in cells. They were able to demonstrate that those oligonucleotides are active and capable of inhibiting transcription elongation at submicromolar concentrations. Moreover, a number of recent reports show the wide applicability of LNA oligonucleotides for gene silencing and their use for diagnostic purposes.
Data also exist on triplex binding with the LNA-analogue ENA, containing a 2’, 4’-ethylene bridge (Figure 1.16). ENA is able to stabilize triplex formation at neutral pH roughly to the same extent as LNA. Notably, fully modified ENA-TFOs do bind to their DNA target while fully modified LNA-TFOs do not. This phenomenon is not completely understood. Lately the synthesis and duplex binding properties of 2’-O-aminoethyl modified 2’-amino-LNA have been described. This structure perfectly combines the features of conformational rigidity and dual binding and may be an interesting candidate as high affinity TFO. However, triplex binding data are yet missing.

![Figure 1.16: Structure of TFOs with conformationally constrained or adapted sugar analogues.](image)

Recently it has been shown that tricylo-DNA purine sequences are able to form remarkably stable Watson-Crick and Hoogsteen duplexes as well as triplexes with DNA and RNA in the parallel binding motif, while being unable to form triplexes in the purine motif (Figure 1.16). Another RNA-modification for triplex stabilization is the methylation of O2’. Triplexes having 2’-O-methylated third strands are thermally very similar to unmodified DNA triplexes, but they are more stable than pure DNA and RNA-DNA/DNA triplexes.

Representatives of the class of neutral backbone oligonucleotide analogues with interesting antigene properties are PNA and morpholino-DNA (Figure 1.17).

![Figure 1.17: Chemical structures of TFOs with uncharged backbone structures.](image)
PNA is an RNA/DNA mimic in which the sugar phosphate backbone is replaced by an achiral, acyclic, non-ionic, dipeptide-like backbone attached to the purine and pyrimidine bases via a methyl carbonyl linker. The unique chemical make up provides PNA with unique properties:

1. Base pairing in PNAs is not affected by interstrand electrostatic repulsion and occurs with high affinity\cite{120} and enhanced rates of association.\cite{121}

2. PNAs are particularly resistant to nuclease and protease degradation, thus increasing the lifetime \textit{in vitro} and \textit{in vivo}.\cite{64}

3. Absence of a repetitive charged backbone also prevents PNAs from binding to proteins that normally recognize polyanions, avoiding a major source of non-specific interactions.\cite{122}

PNAs hybridize to complementary DNA and RNA in a sequence dependant manner in either parallel or antiparallel fashion, indicating that its backbone is more flexible than the one of DNA. Homopyrimidine PNAs bind to complementary DNA sequences to form highly stable (PNA)$_2$-DNA triple helices. In these complexes one PNA strand invades the duplex by displacing the homopyrimidine DNA strand (P-loop formation, Figure 1.18) thus offering the possibility for site specific modulation of gene expression or modification of the gene.\cite{40,123} A more recent approach relies on the use of olefinic peptide nucleic acids (OPAs),\cite{124} which seem not to be able to improve the characteristics of normal PNA. The morpholino oligonucleotides bind to target DNA in the parallel but not in the antiparallel motif. Binding in the parallel motif requires low pH and takes place in the absence of Mg$^{2+}$ or K$^+$ ions (salt free conditions). A 25-mer morpholino oligonucleotide targeted to the HER-2/neu promoter region in the presence of 140 mM K$^+$ with and without 10 mM Mg$^{2+}$ at pH 5.0 shows ca. two-fold increased binding as compared to unmodified DNA-TFO.\cite{125} Thus, it is not stability that excels in this system. The advantages are more related to cellular uptake and distribution due to the non-charged backbone.

\textit{Figure 1.18:} Schema of PNA binding modes for targeting double stranded DNA. PNA oligomers are drawn in bold. (1) Standard duplex invasion complex formed with some homopurine PNAs. (2) Double-duplex invasion complex, very stable but only possible with PNAs containing modified nucleobases. (3) Conventional triple helical structure (triplex) formed with cytosine-rich homopyrimidine PNAs binding to complementary homopurine DNA targets. (4) Stable triplex invasion complex, leading to the displacement of the second DNA strand into a “D-loop”.

\[ 	ext{Duplex invasion} \quad \text{Double duplex invasion} \quad \text{Triplex} \quad \text{Triplex invasion} \]

1

D-loop
As mentioned at the beginning of this section dealing with triplex thermal stability, a successful approach for neutralizing the charge repulsion between the three negatively charged phosphodiester backbones in a triplex is to modify the sugar-phosphate backbone and/or the base of the TFO so as to incorporate positive charges. In addition, the target dsDNA can be recognised not only by selective base-base interactions but also via an additional, non-specific salt bridge between these appropriately placed positive charges in the TFO and negatively charged phosphate oxygens in the target DNA backbone. This approach is known as the “dual recognition” concept. The best explored and most promising parallel motif TFOs of this family of cationic oligonucleotides are those containing an aminooxy (AE) side-chain at C2’ of the ribonucleoside units, developed by Cuenoud and colleagues (Figure 1.19). Under physiological conditions, the amino function of this side-chain is protonated and leads to an enhanced kinetic of triplex formation and a greater stability of the resulting complex of ca. 3.5°C per modification at pH 7.0 and low Mg$^{2+}$ concentration. Fully modified 2’-aminoethoxy TFOs (AE-TFOs) can lead to triplexes that are more stable than the target duplex alone. The gain in thermodynamic stability (ΔG) of ca. 0.5 kcal/mol for each modified unit is mostly due to a 1000-fold enhanced association rate compared to that of a DNA–TFO. This is an important feature knowing that association kinetics for triplex formation is substantially lower as compared with duplex formation. The stabilizing effect was shown by NMR to arise from specific hydrogen bonds of the protonated amino function to the pro-R non-bridging phosphate oxygen of the purine strand of the underlying target duplex. The 2’-amino side-chain occurs in a gauche$^+$ conformation and shows astonishingly low conformational mobility in the triplex. Variations in the length of the side chain did not further improve binding efficiency. AE-TFOs have recently been shown to vastly broaden in vitro and in vivo applications of antigene oligonucleotides.

![Figure 1.19: Dual (base and phosphate) recognition candidates.](image)

In the same line as 2’-AE-nucleosides, 2’-O-[2-(guanidinium)ethyl] modified TFOs (2’-O-GE, figure 1.19) were shown to have high affinity to dsDNA with an increase in stability of 3.2°C per
Furthermore these modified TFOs exhibited an exceptional exonuclease resistance.

Another point of attachment of alkyl amino chains pointing towards the same spatial direction as in AE-TFOs is the position C4’ of the deoxyribose unit (Figure 1.19). Matsuda and co-workers recently synthesized the corresponding 4’-amino-TFOs but they found only marginal thermal triplex stabilization by less than 1°C per modification. Shortening or lengthening the tether between C4’ and the amino function consistently reduced affinity relative to the two-carbon linker. There is no doubt, however, that the efficiency of this system does not match that of the AE-TFOs.

Since triplexes are known to be stabilised by spermine, several groups have covalently attached this chain, or similar groups like tetraethyleneoxyamine to the 4-N position of 5-methylcytosine (Figure 1.20). Stable triplexes at pH 7.4 indicated that the lack of the second hydrogen bond of the C-GC triplet can be compensated by favorable electrostatic and hydrophobic interactions. A substitute for T bearing a basic amino group, 5-(aminopropargyl)-uracil, leads to an increase in triplex melting temperatures of 2°C per replaced T at the Hoogsteen strand. Quantitative footprinting experiments showed that triple helices containing several C⁺-GC triplets could be formed even at pH 6 when T was replaced by 5-(aminopropargyl)-U in the third strand. A supplementary increase in thermal stability was reported when 5-(aminopropargyl)-U was combined with the 2’-aminoethoxy group resulting in bis-amino-U (BAU). The high selectivity and the enhanced stability of the BAU-AT triplet relative to T-AT and its discrimination against other base pair indicate that BAU is a very useful base analogue for the sequence-specific formation of stable triple helices at pH 7.0.

Positive charges were also added as modified phosphate linkages in both parallel and antiparallel triplexes. Oligomers containing cationic phosphoramidate linkages in purine TFOs resulted in efficient triplex formation (Figure 1.21a). Moreover, these TFOs specifically inhibited the expression of a plasmid DNA injected into Xenopus.
Introduction

Recently cationic dimethylaminopropyl phosphoramidate (PNHDMAP) linkages have been introduced into oligonucleotides with α-anomeric configurations (Figure 1.21 b). These cationic pyrimidine α-TFOS were shown to bind to duplex target with high stability and more efficiently than non-ionic phosphoramidate α-oligonucleotides or cationic β-TFOS. More drastic substitution of the entire phosphate group by cationic linkages, like guanidinium or methylated thioureas, leads also to stabilization of the triplexes.

1.3 Pyrrolidino nucleoside

1.3.1 Preliminary results

As part of our current research we are interested in increasing TFO affinity to a DNA target by applying the ‘dual recognition’ approach. Molecular modelling analysis based on recent X-ray data on a parallel DNA triplex showed that replacement of the 4’-oxygen in a deoxynucleoside residue of a TFO by a basic nitrogen would potentially place a positive charge next to a non-bridging pro-R-phosphate oxygen of the purine strand of a target dsDNA (Figure 1.22 top). Therefore imino-sugar-based nucleosides represent excellent candidates for ‘dual recognition’ capable of selective base-base interactions as well as additional, non-specific salt bridge formations. Since N, N-glycosidic bonds are not stable, it was planned to connect the aglycon via a C-C-bond.

The pyrrolidino analogue of deoxyuridine (dpψU, Figure 1.22 bottom) bears the base known from the naturally occurring nucleoside pseudouridine and has the same hydrogen bonding pattern as U or T. Pseudouridine, also called the 'fifth base', is the most abundant modified nucleoside in natural RNA. Its N-1-methyl-2'-deoxy derivative has been incorporated into triplex forming oligonucleotides. Since the substitution of C⁺ by 5MeC⁺ in the third strand of a triplex leads to a gain in stability, it was also planned to synthesize the N-1'-methyl derivative of dpψU (= dpψT, pyrrolidino pseudothymidine).

The C-nucleoside dpψiC (Figure 1.22 bottom) exhibits the same hydrogen bonding pattern as C⁺ and is known from the artificial nucleoside pseudoisocytidine, which was designed to substitute C⁺ in the third strand of a triplex. It was shown that pseudoisocytidine and its 2-N-methyl derivative form Hoogsteen type base pairings through hydrogen bonding with the G-C base-pair in neutral and even basic conditions.

1 For all pyrrolidine C-nucleosides, a different atom numbering of the bases and sugar units was applied than for natural nucleosides (see Exp. Part).
Introduction

The pyrrolidino-C-nucleoside project was first started as part of the Ph.D. work of Dr A. Häberli [147] and the preliminary results obtained at that time were both exciting and intriguing. The pyrrolidino C-nucleosides were prepared via an efficient regio- and stereospecific synthesis based on a Palladium-catalysed Heck coupling as key step. [148] The phosphoramidate building blocks of these modified nucleosides were incorporated into TFOs by standard oligonucleotide synthesis and the melting temperatures (Tm) of the corresponding triplexes were measured and compared to the Tm of the natural triplex. The Tm values for triplex dissociation from UV-melting curve experiments of the strands containing pyrrolidino pseudoU and its N-1’-methyl derivative units are significantly lower compared to the natural oligomer. Relative destabilization of triplex formation by ca. -13 to -1 °C per modification in a strongly sequence-dependent manner was observed. [149] In the case of pyrrolidino pseudo-iso-C, the protonated pyrrolidine unit leads in the contrary to triplex stabilization by ca. 2 °C per modification at pH 6.

1.3.2 Aim of the work

The positive results obtained with the first series of experiments were promising but additional data was needed to validate the project. Therefore further investigations on the binding properties of the pyrrolidino pseudo nucleosides containing TFOs were considered necessary in order to get more information and confirm the conclusions drawn so far. Two points were still essential to be explored:
Stability contribution from the pyrrolidino ring. Compared to natural nucleosides, the pyrrolidino analogues display both modified sugar and bases. To determine the exact role played by each in the properties exhibited by the pyrrolidino pseudonucleosides, their deoxyribo equivalents were required (Figure 1.22 bottom). Pseudouridine is commercially available and has therefore already been investigated showing that in the case of \( \text{dp}\psi\text{U} \) and \( \text{dp}\psi\text{T} \) both sugar and nucleobase are responsible for destabilization.\(^{[149]} \) Pseudoisocytidine on the other side was not easily accessible and was therefore not studied yet. The corresponding phosphoramidate monomer was synthesized, incorporated into TFOs and the denaturation experiments were carried out in order to complete the set of data.

Sequence dependence. Pyrrolidino-pseudonucleosides containing the base uracil (\( \text{dp}\psi\text{U} \)) and N-1’-methyl uracil (\( \text{dp}\psi\text{T} \)) showed a strongly sequence dependent behavior. In the case of \( \text{dp}\psi\text{iC} \), little was known about the influence of the neighbouring residues on the triplex stability. TFOs containing \( \text{dp}\psi\text{iC} \) were therefore explored in a different sequence context. Moreover, it was also interesting to see the difference between TFO hybrids containing natural and modified nucleotides, and a TFO composed only of pyrrolidino moieties. It was surmised that the protonated pyrrolidine or the \( C-C \)-glycosidic bond might lead to an altered sugar conformation and consequently the incorporation of only few pyrrolidino units in an otherwise natural DNA strand might create distortions of the regular helix. To answer these questions a fully modified sequence was prepared and investigated.

In a second part of the work, a new generation of pyrrolidino nucleosides with simplified nucleobase analogues was designed, synthesized and incorporated into DNA. The idea was to improve the binding properties of the previous pyrrolidino generation and to shed some light on the unexplained difference in behavior between \( \text{dp}\psi\text{U} \) or its N-1-methyl derivative and \( \text{dp}\psi\text{iC} \).

Besides the unclear difference in properties between the T and the C analogue, another striking result was the further loss in stability arising from the addition of a methyl group to the N-1’ position of pseudoU. Although pyrimidines are essentially found in the anti conformation, it is not excluded that in this particular case the base adopts a syn conformation and the pyrrolidino pseudouridine excerts a hydrogen bond to the \( N-7 \) of the adenosine of the purine strand via \( N-1' \)- and not via \( N-3' \)-hydrogen (Figure 1.23 left). The \( N-1' \) methyl group would thus prevent one of the hydrogen contact in the *Hoogsteen* base pairing resulting in destabilization.
The proposed explanation for the change in base conformation is based on a possible intramolecular hydrogen bond formation between C4’ carbonyl and N1H leading to a pseudo syn conformation of the nucleobase. The energy necessary to bring the hydrogen bond participants in the right conformation for optimal *Hoogsteen* base pairing may in part contribute to the destabilisation observed with dpψU. In order to verify this hypothesis, a pyrrolidino C-nucleoside bearing 2-pyridone as nucleobase (dp2P) was designed as an analogue of T lacking the C4’ carbonyl and therefore unable to perform the hydrogen bond mention above (Figure 1.23 right).

2-pyridone represents actually the most simplified base keeping only the minimum of elements required for the T-AT base pairing pattern. Based on the same idea, 2-aminopyridine already known as a successful cytosine analogue in parallel triplexes was chosen as nucleobase for the new pyrrolidino analogue of C (dp2AP, see Figure 1.24). The second part of the work consisted then in the synthesis of the phosphoramidite building blocks dp2P and dp2AP, their incorporation into TFOs as substitutes for T and C, the evaluation of the triplex pairing properties and finally comparison with the results obtained with the first generation of pyrrolidino pseudonucleosides.
1.4 References


Introduction


Introduction

Introduction


Introduction
Chapter 2: First generation of pyrrolidino C-nucleosides

2.1 Synthesis and pairing properties of oligodeoxynucleotides containing drψiC

2.1.1 Building block synthesis

The C-Nucleoside pseudoisocytidine (ψiC) and its 2’-deoxyribo derivative (drψiC) were first prepared by Watanabe and coworkers in the context of their potential use as therapeutic agents with antileukemic properties. [1, 2] Starting in the early nineties of the last century, the interest in pseudoisocytidine and its 2’O-alkylated derivatives grew due to its function as a neutral replacement for protonated cytidine in triplex forming oligonucleotides within the parallel binding motif. [3-5] Current strategies for the synthesis of 2’-deoxypseudoisocytidine rely on pseudouridine as starting material. In these approaches, the uracil base is converted into isocytosine via an N-methylation-guanidinylation pathway. The 2’-OH function is then removed via standard Barton-McComby reaction. Especially the conditions for remodeling the base require high temperatures in a basic, protic environment, which has been shown in the past to lead to partial isomerization at the pseudoanomeric center. There is clearly need for an improved synthesis.

A novel synthesis of 2’-deoxypseudoisocytidine, and its building block for DNA synthesis, has been designed. This new route is based on Heck-coupling of an N-protected pseudoisocytosine base with a corresponding furanoid glycal. Heck chemistry has successfully been applied in the past for the synthesis of C-nucleosides with β-configuration at the pseudoanomeric center using a variety of natural and non-natural bases. [6-9] We recently contributed to this field by providing access to pyrrolidino pseudonucleosides via Heck chemistry. [10]

The halogenated, N-benzoylated pseudoisocytosine 3 was obtained in two steps starting from isocytosine (1) as depicted in Scheme 2.1. [11, 12] Reaction of 1 and N-iodosuccinimide (NIS) in hot acetic acid, followed by protection of the exocyclic amino group with benzoic anhydride led to iodide 3. Both products (2 and 3) could be isolated as pure solids by simple filtration. The furanoid glycal 4, known for its use in Heck chemistry based synthesis of C-nucleosides, was easily prepared according to literature procedures. [13, 14] The synthesis of the N-benzoyl protected 2’-deoxypseudoisocytidine 6, and the corresponding phosphoramidite building block 8 for DNA synthesis is outlined in Scheme 2.2.
Due to poor solubility of the non silylated base 3, a one-pot two-step procedure for the Heck coupling was applied: the base was solubilized first via in situ O-silylation with BSA, followed by subsequent addition of the glycal 4 and the palladium-based catalyst. The catalyst system was previously shown to work efficiently in the pyrrolidino-pseudonucleoside synthesis. Intermediate 5 was obtained in a good yield of 68%. The next step involved the desilylation of the hydroxyl functions which was carried out using HF-pyridine in THF. The resulting ketone was subjected without further characterization to a diastereoselective reduction with NaBH(OAc)$_3$. A yield of 65% over the two steps was obtained. The relative configurations of the newly installed chiral centers at C(1’) and C(3’) in 6 were rigorously confirmed by $^1$H-NMR-NOE experiments (Figure 2.1). A mutual NOE between H(4’) and H(1’) proved the β-configuration at the pseudoanomeric center,
while the strong NOE between H(3’) and H(2’β) is in agreement with the ribo- and not the xylo-configuration of the furanose unit.

![Diagram of pyrrolidino C-nucleoside](image)

**Figure 2.1**: Relevant, mutual NOE signals in compound 6.

The remaining synthesis of the building block 8 for DNA synthesis was finished in two steps and 64% yield via tritylation of 6 and subsequent phosphitylation of 7 according to standard procedures.

In conclusion a novel, mild and stereoselective synthesis of 2’-deoxypseudoisocytidine was successfully elaborated. The phosphoramidite derivative 8, useful for applications in DNA triple helix chemistry, is thus available in five steps and an overall yield of 28% starting from the glycal 4 and base 3. These starting materials in turn can be obtained in two steps each from commercially available compounds.

### 2.1.2 Oligodeoxynucleotide synthesis

To determine the contribution of the pyrrolidino ring to the increased triplex stability in TFOs containing dpψiC, TFOs with similar sequence but bearing the deoxyribo-equivalent monomer drψiC instead of dpψiC were synthesized. Synthesis was performed on a 1.0 μmol scale on an automated DNA synthesiser using standard solid-phase phosphoramidite chemistry (see Annexe 1). Minor modifications to the synthesis cycle were introduced for the incorporation of the non-natural building blocks. More precisely, the coupling time was extended from 1.5 to 6 min and the standard activator tetrazole was replaced by the more powerful (S-benzylthio)-1H-tetrazole. Coupling efficiencies for the modified units were typically > 97%, according to trityl assay. Standard ammonia deprotection led to cleavage of the crude oligonucleotides from the solid support and removal of all protecting groups. Crude oligomers were purified by ion exchange HPLC and characterised by ESI mass spectrometry. More details concerning the oligonucleotides synthesis,
purification and characterisation are given in the experimental part. The thermal stability of the triplexes formed between the TFOs and the target duplex are measured by UV-melting curve experiments (see Annexe 2).

The previously synthesized oligonucleotides as well as those newly prepared for direct comparison and the T<sub>m</sub> data of TFO dissociation from the 21-mer dsDNA target are summarized in Table 2.1. Since protonation of the pyrrolidino ring nitrogen is pH-dependent, the melting experiments were carried out at different pH values. NMR titration experiments with the monomer dψU revealed a pK<sub>a</sub> of 7.9 for the pyrrolidino ring nitrogen. Therefore the triplex formation was investigated in the pH range from 6 to 9.

Table 2.1: Sequence of TFOs and T<sub>m</sub> data (°C) of third strand dissociation from UV melting curves (260 nm).

<table>
<thead>
<tr>
<th>TFO</th>
<th>Sequence</th>
<th>Mod.</th>
<th>pH</th>
<th>T&lt;sub&gt;m&lt;/sub&gt;*</th>
<th>ΔT&lt;sub&gt;m&lt;/sub&gt;/mod.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ref1</td>
<td>5'-d(TTTTCTCTCTCTCT)</td>
<td>/</td>
<td>6.0</td>
<td>43.1</td>
<td>0</td>
</tr>
<tr>
<td>dp1</td>
<td>5'-d(TTTTCTXTCTCTCT)</td>
<td>X=dψiC</td>
<td>6.0</td>
<td>45.5</td>
<td>+2.4</td>
</tr>
<tr>
<td>dr1</td>
<td>5'-d(TTTTCTCTCTCTCT)</td>
<td>X=drψiC</td>
<td>6.0</td>
<td>39.9</td>
<td>-3.2</td>
</tr>
<tr>
<td>dp2</td>
<td>5'-d(TTTTCTXTCTCTCT)</td>
<td>X=dψiC</td>
<td>6.0</td>
<td>46.9</td>
<td>+1.9</td>
</tr>
<tr>
<td>dr2</td>
<td>5'-d(TTTTCTCTCTCTCT)</td>
<td>X=drψiC</td>
<td>6.0</td>
<td>37.9</td>
<td>-2.6</td>
</tr>
<tr>
<td>dp3</td>
<td>5'-d(TTTTXTXTXTXTXT)</td>
<td>X=dψiC</td>
<td>6.0</td>
<td>53.0</td>
<td>+2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7.0</td>
<td>44.4</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8.0</td>
<td>37.1</td>
<td>--</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>9.0</td>
<td>28.0</td>
<td>--</td>
</tr>
<tr>
<td>dr3</td>
<td>5'-d(TTTTXTXTXTXTXT)</td>
<td>X=drψiC</td>
<td>6.0</td>
<td>16.4</td>
<td>-5.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7.0</td>
<td>11.2</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>8.0</td>
<td>13.5</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>9.0</td>
<td>10.0</td>
<td>--</td>
</tr>
</tbody>
</table>

* Single strand concentration = 1.2 µM in 140 mM KCl, 7 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.5 mM MgCl<sub>2</sub>. T<sub>m</sub> of target duplex = 57.0 ± 1.0°C.

UV-melting curves were monitored at 260 nm and a heating-cooling-heating cycle in the temperature range 0-90°C was applied with a linear gradient of 0.5°C min<sup>-1</sup>. The depicted curve obtained for TFO dr2 with target duplex at pH 6.0 (Figure 2.2) is representative of a typical triplex melting profil. All TFOs examined here gave similar thermal melting curves. The curves present two transitions, the one at lower temperature corresponding to the triplex-to-duplex melting whereas the one at higher temperature reflects duplex-to-single-strands transition. While the heating cycles were superimposable, the cooling cycles usually showed slight hysteresis for the association of the third strands.
First generation of pyrrolidino C-nucleosides

Figure 2.2: UV-melting curves (λ = 260 nm) of ternary mixtures of TFO dr2 with its duplex target. Experimental conditions as in Table 2.1.

At pH 6, where the pyrrolidino ring nitrogen is fully protonated, the triplexes with TFOs dp1-3, bearing dpψiC residues (Table 2.1), were more stable than the one formed with the reference TFO Ref1, in which the modified units were replaced by deoxycytidine. Each modification contributed to triplex stability by 1.9 to 2.4°C. TFO dp3 with five dpψiC units was found to form the most stable of all investigated triplexes with a Tm that was ca. 10°C higher than that of the corresponding unmodified triplex. The TFOs dr1-3, containing pseudoisocytidine units (Table 2.1), were used to identify affinity differences arising exclusively from the change of the sugar. A decrease in Tm/mod by ca. 2.5-5.0°C for these TFOs relative to the DNA control Ref1 was found. This clearly shows that the pyrrolidino modification is contributing significantly to stability, as it is able to overcompensate for the generally destabilising nature of the pseudobases under the given conditions.

Of special interest was TFO dp3 in which all deoxycytidines were replaced by dpψiC units. In contrast to deoxycytidine, the isocytidine pseudonucleosides need no protonation at the bases for binding to their target G-C base-pairs. Thus, any dependence of target binding of dp3 from pH in the interval of pH 6-9 must be due to a change of the protonation state of the pyrrolidine ring nitrogen. Indeed, raising the pH from 6.0 to 9.0 leads to a reduction in triplex Tm by 25°C, corresponding to 3°C/mod (Figure 2.3 left). In contrast, and as expected, dr3, containing deoxypseudoisocytidine residues instead of the pyrrolidino-pseudoisocytidines essentially shows no pH dependence in target binding, indicating that no reversible protonation events are involved in target binding (Figure 2.3 right).
These results unambiguously highlight the importance of the protonated state of the pyrrolidino units in target binding and is in agreement with the ‘dual recognition’ principle by specific base-base interactions and unspecific salt bridge formation between the protonated pyrrolidino nitrogens of the TFO and phosphate units of the target DNA.

2.2 Sequence effect on pyrrolidino C-nucleosides

2.2.1 New sequence context for \( \psi iC \)

The natural TFO associated to the first target duplex used to study pyrrolidino C-nucleosides (Table 2.1) contains nine Ts located in various different neighbourhoods. Therefore, judicious substitutions permit to place the \( \psi iU \) unit in a large variety of adjacent base combination (between two Cs, two Ts, a T and a C, two \( \psi U U \)s…) and the effects involved by the position of the modification could be investigated. An extreme sequence dependence of the triplex stability was observed.

On the other hand, this target duplex allowed \( \psi iC \) to be surrounded only by two Ts wherever the modification was incorporated in the TFO. It was obviously of high interest to check whether \( \psi iC \) was subjected to similar sequence dependence as displayed by \( \psi U \). Therefore it was necessary to choose a new triplex system in which the TFO offered multiple \( \psi iC \) incorporation sites each presenting a different set of nearest neighbours. For comparison, the strands containing \( \psi iC \) at the same positions were prepared as well. The corresponding modified 15-mer TFOs, their DNA target and the \( T_m \) for third strand melting at pH values between 6 and 8 are summarized in Table 2.2. These data were again compared with those of a reference TFO, \textbf{Ref2}, bearing 5-methyldeoxycytidine (\( \text{MeC} \)) instead of deoxycytidine at the X positions.
The oligonucleotide dp5 contains five dpψiC units placed in a way so that they are found between two Ts, between a T and another dpψiC and between two dpψiC residues. At pH 6, dp5 binds to the target duplex with considerably higher affinity than the reference TFO Ref2. The Tm is ca. 13°C higher and the ∆Tm/mod of 2.6°C is on the same order as in the previous sequence context. Again, the Tms drop upon increasing the pH from 6.0 to 8.0 due to partial deprotonation of the pyrrolidino nitrogen. Remarkably, even at pH 8 where Ref2 has no detectable affinity anymore to its target, dp5 still binds dsDNA with a Tm of ca. 30°C. This Tm is even higher than that of Ref2 at pH 6. The TFO dr5, containing five drψiC units, was again used for dissecting the role of the sugar modification. As expected, the Tms between pH 6.0 and 8.0 are similar ruling out protonation events upon target binding. In addition, their values between 11-16°C are considerably lower than those of dp5, again demonstrating the superiority of the dpψiC units.

TFOs dp5 and dp3 both contain five dpψiC modifications but display a totally different sequence context. However they show identical triplex stabilizing properties. This result indicates that contrary to dpψU, dpψiC containing TFOs do not suffer from sequence dependency. Whatever residues are adjacent to dpψiC in the TFO, the pyrrolidino pseudonucleoside contributes to the same extent to an increase in triplex stability with a ∆Tm/mod in the range of 2-2.5°C.

2.2.2 Fully modified pyrrolidino C-nucleosides containing TFO

Still in the context of sequence effects, another interesting point needed to be elucidated. In the case of the pyrrolidino pseudoU nucleoside analogue, the incorporation of a single modified unit leads to a larger destabilization per modification than the incorporation of two or five consecutive
units. Similar results have already been reported in the literature. \[16\] If the pyrrolidino C-nucleosides present a sugar conformationally different from that of natural deoxynucleosides, incorporation of only few modifications can lead to distortions in the TFO backbone resulting in diminished triplex stability. On the other hand a TFO containing only pyrrolidino units with all identical sugar conformation should have a regular backbone and exhibit the full abilities of the pyrrolidino pseudonucleosides. To test this hypothesis, a fully modified pyrrolidino oligonucleotide was synthesized.

Oligonucleotide synthesis was performed on a 1.0 µmol scale on an automated DNA synthesiser as described before (see section 2.1.2). The TFO contains only pyrrolidino pseudonucleosides except for a deoxyribonucleoside unit at the 3’ end for practical reasons. Under pH conditions of the HPLC phosphate buffers the strand is in a zwitterionic form with a neutral total charge. As a consequence the strand has no retention in ion exchange HPLC and could not be purified using classical buffers. Finally ion exchange purification was achieved by FPLC with a MonoQ column using a sodium hydroxyde buffer at pH 12.0. The high pH was necessary to deprotonate the pyrrolidino units in order to get a negatively charged oligonucleotide. The obtained modified 15-mer TFOs, the natural reference TFO, their DNA target and the $T_m$ for third strand melting are summarized in Table 2.3.

### Table 2.3: Sequence of TFOs and $T_m$ data (°C) of third strand dissociation from UV melting curves (260 nm).

<table>
<thead>
<tr>
<th>TFO</th>
<th>Target</th>
<th>Sequence</th>
<th>Mod.</th>
<th>pH</th>
<th>$T_m^a$</th>
<th>$\Delta T_m$/mod.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ref1</td>
<td>5'-d(TTTTTTCCTCTCTCT)</td>
<td>/</td>
<td>6.0</td>
<td>43.1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>dp4</td>
<td>5'-d(YYYYYYYYYYYYYYXT)</td>
<td>$X=\text{dp}\psi\text{iC}, Y=\text{dp}\psi\text{U}$</td>
<td>6.0</td>
<td>n.d. $^b$</td>
<td>n.d. $^b$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Single strand concentration = 1.2 µM in 140 mM KCl, 7 mM NaH$_2$PO$_4$, 0.5 mM MgCl$_2$. $T_m$ of target duplex = 57.0 ± 1.0°C.

$^b$ No $T_m$ detectable.

The fully modified, dpψU and dpψiC containing TFO dp4 seems not to show any triplex formation with its target. The curves from thermal melting experiments present a monophasic UV-melting transition that could be attributed to the melting of the Watson-Crick duplex (Figure 2.4). The heating and cooling curves are superimposable, no hysteresis phenomenon, usually accompanying the triplex formation during cooling process, was observed. This result is in line with the previous observations with TFOs containing only dpψU units that were found to strongly destabilise triplexes. Full replacement of the natural residues resulting in a uniform iminosugar-phosphate backbone did not have the expected effect and failed to improve the binding properties.
induced by dpψU. Obviously the destabilising effect of the nine dpψU units cannot be compensated by the five dpψiC units in dp4.

![Figure 2.4: UV-melting curves (heating and cooling, λ = 260 nm) of ternary mixtures of TFO dp4 with its duplex target. Experimental conditions as in Table 2.3.](image)

In order to definitely rule out that the observed single transition arises from a triplex-to-single-strands melting and to confirm the negative binding result of the fully modified dp4, a gel retardation experiment (see Annexe 3) was performed. TFOs dp3 and dp5 were used as reference (Figure 2.5).

![Figure 2.5: UV shadowing of a non-denaturing 20% polyacrylamide gel at pH 7.1. Lanes 1 and 2: purine rich and pyrimidine rich single strands of the DNA target for dp3 and dp4; lane 3-5: dp3, dp5 and dp4 (single strands); lane 6 and 7: target duplexes; lane 8: ternary mixture of dp5 with its target duplex strands; lane 9: ternary mixture of dp3 with its target duplex strands; lane 10: ternary mixture of dp4 with its target duplex strands. 0.7 nmol of each strand was used with equal stoichiometry in mixtures.](image)

From the ternary mixtures of the TFOs dp3, dp5 and dp4 with their duplex targets it clearly emerges that no triplex is formed in the latter case (lane 10) while stable triplexes occur in the two former cases (lane 8 and 9). These observations are in full agreement with the results obtained from UV-melting curve experiments.
First generation of pyrrolidino C-nucleosides

All together, the analyses performed so far provide enough information to draw conclusions concerning the properties of pyrrolidino C-nucleosides containing TFOs. However these conclusions highlight an unexpected and unexplained discrepancy between the pyrrolidino analogue of C and the pyrrolidino analogues of T: on one side dpψiC containing TFOs exhibit increase in triplex thermal stability in a sequence independent fashion. The gain in stability has been proven to arise from the positively charged pyrrolidino unit. On the other side, dpψU and dpψT induce both a loss in triplex stability when incorporated in a TFO. Moreover the destabilization happens in a strong sequence specific manner. The next part of this work will focus on the study of this discrepancy.

2.3 References

Chapter 3: Second generation of pyrrolidino C-nucleosides

3.1 Introduction

In the previous chapter, pyrrolidino pseudoisocytosine was demonstrated to be a successful positively charged substitute for cytidine in TFOs, conducing to an increase in triplex thermal stability of about 2-2.5°C per replaced C. The TFOs containing the corresponding pyrrolidino nucleosides developed to supersede T, dpψU and dpψT, show however lower target binding affinity then the natural reference. An efficient pyrrolidino-based substitute for T would provide, together with dpψiC, a powerful system for highly stable and selective triplex formation within the pyrimidine motif.

The stabilising effect of dpψiC has been proven to result from the positive charge at the pyrrolidine nitrogen. The understanding of which phenomenon is competing with the favourable electrostatic effect in the case of dpψU and dpψT would be of considerable help in order to develop an improved pyrrolidino analogue of T. A possible reason might be a preference for a pseudo-syn conformation of the base in these pyrrolidino nucleosides due to intramolecular H-bond formation between the pyrrolidine NH and the C4’ carbonyl. The energy penalty arising from a change of this base orientation into that favourable for Hoogsteen base pairing might result in the observed loss of triplex stability.

In order to corroborate this hypothesis, pyrrolidino 2-pyridone (dp2P), lacking the C4’ carbonyl moiety and presenting only the minimum required hydrogen bonding abilities to perform Hoogsteen base pairing, was designed as potential T analogue (Figure 3.1 left). Based on the same strategy, 2-amino-pyridine was developed as corresponding C substitute (Figure 3.1 right).

![Figure 3.1: Structure of pyrrolidino C-nucleosides dp2P (left) and dp2AP (right).](image_url)

Compared to cytosine, this nucleobase conserves only the functional groups necessary for C+-GC triplet and has already been successfully used as a more basic C analogue. Even if the incorporation of a dpψiC unit results in global triplex stabilization, this positive effect arises only
from the pyrrolidino modification. The isocytosine modification alone was shown to contribute negatively to triplex stability. Pyrrolidino 2-amino-pyridine (dp2AP) could be a better alternative if the nucleobase provides an additional increase in duplex binding affinity.

Among the different methodologies available for preparation of C-nucleosides \[5\] especially imino-sugar based C-nucleosides, \[6\] our efforts were first concentrated on palladium-catalysed *Heck* mediated C-C bond formation between the pyrrolidine and the corresponding aglycon since this reaction was successfully applied for the synthesis of the first generation of pyrrolidino pseudo-nucleosides.

### 3.2 Synthesis of phosphoramidite building blocks

#### 3.2.1 First strategy: *Heck* coupling-based synthesis

The synthesis of these new pyrrolidino nucleosides was expected to be straightforward by following the route developed earlier for the first pyrrolidino generation. \[7\] The retrosynthetic path for dp2P is depicted in scheme 3.1. The key step of this synthesis was the stereoselective *Heck* coupling between enamine 9 and the corresponding iodinated pyridone 10.

![Scheme 3.1: Retrosynthetic analysis for phosphoramidite 13 using Heck Chemistry.](image)

CBz protected enamine 9 could be obtained in seven steps and 52% overall yield starting from *trans*-3-hydroxy-L-proline. \[7\] 10 was prepared in a single iodination step starting from 2-pyridone (14, Table 3.1). In a first attempt, a mixture of products differentiated by the number and position of the introduced iodine atoms was obtained (Entry 1). \[8\] Several methods were applied in order to improve the yield and/or the regioselectivity. The use of NIS in acetic acid at rt gave the best result and the desired 5-iodo-2-pyridone could be isolated as the major product from a mixture with 3-iodo- and 3,5-diiodo-2-pyridone in 46% yield (Entry 3). Similar yields were reported for the preparation of 10 by an oxidative method using NaI and NaOCl which was not tried here. \[9\] Another method described in the literature allowed iodination of an N-alkyl-pyridin-2-one in 79% yield \[10\] but these conditions did not prove efficient in the present case (Entry 2).
Second generation of pyrrolidino C-nucleosides

Table 3.1: Reactions conditions and yields of the iodination of 14.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Conditions</th>
<th>% (10)</th>
<th>% (15)</th>
<th>% (16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NIS, AcOH, 70 to 90°C, 3h</td>
<td>22</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>I2, Ph(OCOCF3)2, py, CH2Cl2, 1h</td>
<td>10</td>
<td>/</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>NIS, AcOH, rt, 4h</td>
<td>46</td>
<td>6</td>
<td>26</td>
</tr>
<tr>
<td>4</td>
<td>1) BSA, CH2Cl2 2) NIS 3) H2O, 4d</td>
<td>28</td>
<td>/</td>
<td>/</td>
</tr>
</tbody>
</table>

Once both precursors for the Heck reaction 9 and 10 were obtained, the coupling was tried under the conditions described for dpψU or dpψiC. Unfortunately in this case the use of Pd(OAc)2 as catalyst, AsPh3 as a soft ligand and diisopropylethylamine as base proved to be unsuccessful and no coupling products could be isolated (Table 3.2, entry 1).

Table 3.2: Reactions conditions of the Heck coupling between 9 and 10, 17 or 18.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Aglycon</th>
<th>Catalyst</th>
<th>Ligand</th>
<th>Base</th>
<th>Other</th>
<th>Solvent</th>
<th>T (°C)</th>
<th>% Prod</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10a</td>
<td>Pd(OAc)2</td>
<td>Ph3As</td>
<td>Bu3N</td>
<td>/</td>
<td>DMF</td>
<td>65</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>10a</td>
<td>Pd(OAc)2</td>
<td>Ph3As</td>
<td>Bu3N</td>
<td>/</td>
<td>DMF</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>10a</td>
<td>Pd(OAc)2</td>
<td>Ph3P</td>
<td>Bu3N</td>
<td>/</td>
<td>DMF</td>
<td>65</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>10a</td>
<td>(Dppf)PdCl2</td>
<td>/</td>
<td>Bu3N</td>
<td>/</td>
<td>DMF</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>17</td>
<td>Pd(dba)2</td>
<td>dppp</td>
<td>Bu3N</td>
<td>/</td>
<td>DMF</td>
<td>65</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>17</td>
<td>Pd(OAc)2</td>
<td>Ph3As</td>
<td>Bu3N</td>
<td>/</td>
<td>DMF</td>
<td>75</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>10b</td>
<td>Pd(OAc)2</td>
<td>Ph3As</td>
<td>Bu3N</td>
<td>/</td>
<td>DMF</td>
<td>65</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>18</td>
<td>Pd(OAc)2</td>
<td>/</td>
<td>/</td>
<td>HBF4, NaN3</td>
<td>MeOH/H2O</td>
<td>55</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>18</td>
<td>Pd(OAc)2</td>
<td>/</td>
<td>/</td>
<td>tBuNO2, BF3, Et3O</td>
<td>EtOH</td>
<td>55</td>
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</tr>
<tr>
<td>10</td>
<td>18</td>
<td>Pd(dba)2</td>
<td>/</td>
<td>/</td>
<td>tBuNO2</td>
<td>DCM/ CICH2CO2H</td>
<td>rt</td>
<td>0</td>
</tr>
</tbody>
</table>

Not discouraged by the first negative attempt, a series of different conditions was tried out in order to attain the desired Heck product. Initially the temperature was increased from 65 to 80°C but with the only effect of decomposing the starting material (entry 2). The more common ligand Ph3P was used instead of Ph3As with no better result (entry 3). The complete change of the catalytic system did no show any improvement; no trace of the product was observed (entry 4). Protection of
the aglycon either as a silyl ether (17) or as the N-CBz (10b) derivative in order to modify the electronic properties did not prove successful (entries 5-7). It is noteworthy that the conditions described in entry 5 have already been used with O-protected 2-pyridone derivatives and yielded 90% of C-nucleoside. \textsuperscript{[11]} As a consequence of these unsuccessful experiments, further modifications were envisaged: the iodine in 10a could be replaced by a more reactive leaving group like trifluoromethanesulfonate \textsuperscript{[12]} or \textsubscript{N_{2}}. \textsuperscript{[13]} Our interest was on the diazonium salt derivative. Rather than isolating the diazonium intermediate, it was formed \textit{in situ} during the Heck reaction. However, several attempts in aqueous (entry 8) or in organic solvents (entries 9 and 10) did not produce the expected pyrrolidino C-nucleoside.

Given the number of parameters that can influence the reactivity in Heck chemistry (catalyst, ligands, base, solvent, temperature, additives, aglycon leaving group…) the number of possible combinations is almost infinite. According to our experience in this field and to procedures described for similar systems in the literature, the conditions expected to work best have been selected and tried out, however, without success. At this point, instead of carrying out random attempts of Heck couplings, a modified strategy based on the 2-amino-pyridine nuclobase was applied (Figure 3.2). Succeeding in the Heck coupling between enamine 9 and 2-amino-pyridine derivative 19 would give access to dp2AP and its phosphoramidite building block. In addition, the amino pyridine could be converted into the desired pyridone dp2P via diazotation followed by hydrolysis.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure32.png}
\caption{Modified Heck based strategy giving access to dp2P through dp2AP.}
\end{figure}

A variety of aglycons useful for Heck coupling-based synthesis of dp2AP was prepared starting from commercially available 5-bromo-2-amino-pyridine (20, Scheme 3.2). Direct treatment of 20 with 2.2 eq of Buthyllithium followed by addition of I\textsubscript{2} did not yield the desired product 19. The first equivalent of base deprotonates obviously the exocyclic amine and leads to an anionic intermediate deactivated for halogen-metal exchange. Protection of the primary amine was therefore
Second generation of pyrrolidino C-nucleosides

required. Installation of the “stabase” protecting group followed by the halogen exchange was performed in one pot. The obtained intermediate was subjected without purification to acidic hydrolysis to yield unprotected product 19 in 63% over the three steps. Compound 19 was then easily converted in its N-Bz derivative 21 by treatment with BzCl in pyridine. Alternatively, the starting material 20 could also be oxidized using a mixture of hydrogen peroxide and sulphuric acid to prepare the nitro compound 22. [14]

Several attempts of Heck coupling using these aglycons were performed and the conditions are summarized in Table 3.3. Direct coupling of the commercially available aglycon 20 with the enamine 9 under the conditions developed for the first generation of pyrrolidino (Entry 1) failed. Better results were expected with the more reactive iodo aglycon 19 (Entry 2). However in this case again no product formation was observed. The Heck coupling between a derivative of 5-iodo-2-amino-pyridine and a furanoid glycal was described using Pd(dba)2 as catalyst and P(C6F5)3 as the best out of several ancillary ligand examined. [11] However in our hands these conditions did not produce any observable C-nucleoside (Entry 3).

Table 3.3: Reactions conditions of the Heck coupling between 9 and 19, 20, 21 or 22.

<table>
<thead>
<tr>
<th>Entry</th>
<th>X/R</th>
<th>Aglycon</th>
<th>Catalyst</th>
<th>Ligand</th>
<th>Base</th>
<th>Solvent</th>
<th>T</th>
<th>% Prod</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Br/ NH2</td>
<td>20</td>
<td>Pd(OAc)2</td>
<td>AsPh3</td>
<td>EtNiPr2</td>
<td>DMF</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>I/ NH2</td>
<td>19</td>
<td>Pd(OAc)2</td>
<td>AsPh3</td>
<td>EtNiPr2</td>
<td>DMF</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>I/ NH2</td>
<td>19</td>
<td>Pd(dba)2</td>
<td>P(C6F5)3</td>
<td>EtNiPr2</td>
<td>CH3CN</td>
<td>reflux</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>I/ NHBz</td>
<td>21</td>
<td>Pd(OAc)2</td>
<td>AsPh3</td>
<td>EtNiPr2</td>
<td>DMF</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>I/ NHBz</td>
<td>21</td>
<td>Pd(dba)2</td>
<td>P(C6F5)3</td>
<td>NBu3</td>
<td>CH3CN</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>Br/ NO2</td>
<td>22</td>
<td>Pd(OAc)2</td>
<td>AsPh3</td>
<td>EtNiPr2</td>
<td>DMF</td>
<td>80</td>
<td>0</td>
</tr>
</tbody>
</table>

The unprotected aglycons may not undergo palladium-mediated coupling under these conditions presumably because of nitrogen complexation with palladium. A similar problem was
Second generation of pyrrolidino C-nucleosides

reported in the literature and solved by masking the exocyclic amine. Application of the conditions tried so far to the benzoylated 5-iodo-2-amino-pyridine 21 remained vain (Entries 4 and 5). Disappearance of the starting material was observed but not accompanied by formation of the Heck product. In a last attempt the nitro derivative 22 was used with the Pd(OAc)$_2$/AsPh$_3$ catalytic system but again only degraded starting material was detected (Entry 6).

The coupling of the N-protected enamine 9 and the poorly functionalized halogeno-heterocyclic derivatives of 2-pyridone and 2-amino-pyridine under a variety of Heck-type conditions failed to yield significant amounts of product. The reactions led either to unreacted starting material or in most of the cases to decomposition of the enamine. Under the basic conditions in which the coupling reaction was performed, aromatisation of the pyrrolidino ring was often observed presumably via syn-β-elimination (scheme 3.3). In our case Heck chemistry did not prove suitable for the formation of the desired C-C bond and there was clearly need for an alternative to achieve the coupling.

![Scheme 3.3: Aromatisation side reaction of enamine 9 to yield 23.](image)

3.2.2 Second strategy: lactam chemistry based synthesis

A widely used method for the preparation of C-nucleosides consists in the addition of the organometallic derivative of the nucleobase to the corresponding lactone followed by reduction of the resulting hemiacetal. Similarly, Yokoyama and co-workers synthesized different stereoisomers of pyrrolidino C-nucleosides, pyrrolidino 3-deoxy-C-nucleosides, and pyrrolidino 3,4-dideoxy-C-nucleosides as glycosidase inhibitors. The C-C bond formation was performed by the addition of the lithium or Grignard reagents of the heterocycles to the corresponding substituted γ-lactams or related compounds. In the second synthetic strategy developed for the preparation of dp2P and dp2AP, the nucleosidation step was based on this chemistry as an alternative to Heck coupling.

The established synthesis of enamine 9 was taken as starting point and appropriately modified for the preparation of lactam 29. The lactam could be obtained in six steps and 65 % overall yield starting from trans-3-hydroxy-L-proline 24 as shown in Scheme 3.4. After Fmoc-protection of the ring nitrogen of the starting material, the carboxylic acid was selectively reduced to the primary alcohol to yield almost quantitatively diol 26. The two hydroxyl functions were then masked as silyl
ethers and the Fmoc was cleaved off with piperidine to give intermediate 27. Protection of the pyrrolidine nitrogen as tert-butoxy carbamate 28 was followed by oxidation of the C2 position by RuO4.\textsuperscript{[17]} The latter was formed \textit{in situ} with catalytic RuO\textsubscript{2} in the presence of an excess of sodium periodate.

![Scheme 3.4: Synthesis of lactam 29](image)

\textbf{Scheme 3.4: Synthesis of lactam 29.} a) Fmoc-Cl, dioxane, 5 \% NaHCO\textsubscript{3} soln., 0°C to rt, 9 h, 98\%; b) BH\textsubscript{3}⋅(CH\textsubscript{3})\textsubscript{2}S, THF, reflux, 2 h, 97\%; c) TBDMS-Cl, imidazole, THF, rt, 2 h; d) piperidine, THF, rt, 12 h, 90\% over 2 steps; e) Boc\textsubscript{2}O, 1M aq NaOH/THF (1/3), 0°C to rt, 12h, 98\%; f) RuO\textsubscript{2}, 10\% aq NaIO\textsubscript{4}, AcOEt, 4h, 77%.

The use of a variety of metal derivatives of heterocycles has been reported from the more common organolithium or magnesium to cerium, copper derivatives, etc. In an initial experiment the easily accessible lithium derivative was chosen. For the \textit{Heck} reaction, it was important to use the iodo derivative of the aglycon. In this case, the corresponding bromo analogue suits perfectly well and proved to be easier to prepare. 5-bromo-pyridin-2-one (30) was obtained in 77\% yield under mild regioselective conditions by treatment of pyridone 14 with NBS in acetonitrile at rt (Scheme 3.5).\textsuperscript{[18]} The corresponding iodination occurred in only 46\% yield.

![Scheme 3.5: Reaction conditions of bromination of 14](image)

\textbf{Scheme 3.5: Reaction conditions of bromination of 14.} a) NBS, CH\textsubscript{3}CN, rt, 3d, 77\%.

The 2-pyridone functionality of 30 was protected \textit{in situ} with BSA (Scheme 3.6). The halogen-metal exchange was performed by addition of Butylithium and the resulting lithio-pyridine 31 reacted with lactam 29 to give the expected product, however in moderate yields (42\%). Even though the reduction step is usually perform directly on the crude intermediate obtained from the addition step, in our case the hydroxylactam intermediate 32 was isolated. The trimethylsilyl ether does not require a deprotection step; it is cleaved spontaneously during the silica gel column chromatography purification.
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Scheme 3.6: Coupling of lactam 29 with lithio derivative 31 followed by reduction of the hemiaminal 32. a) THF, BSA, rt, 30 min; b) Butyllithium, -78°C, 30 min; c) 29, THF, -78°C, 2h, 42%; d) See table 3.3.

Classical conditions used to carry out the reduction of a hemiacetal were applied to hydroxylactam 32 but after two and a half hours of reaction at -78°C only starting material could be observed (Table 3.4, entry 1). Neither the extension of the reaction time up to 12h nor the addition of a larger excess of *Lewis* acid helped the reaction to occur (Entries 2 and 3 resp.). The change of boron trifluoride etherate to tin tetrachloride as *Lewis* acid did not show any effect even with a large excess (Entry 4). On the other side replacement of triethyl silane by the more powerful sodium borohydride remained unsuccessful as well (Entry 5).

### Table 3.4: Reaction conditions of reduction of hemiaminal 32.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Lewis Acid</th>
<th>Eq.</th>
<th>Reducing Agent</th>
<th>Eq.</th>
<th>Reaction Time</th>
<th>Yield 33</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BF₃·Et₂O</td>
<td>3</td>
<td>Et₃SiH</td>
<td>3</td>
<td>2h30</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>BF₃·Et₂O</td>
<td>3</td>
<td>Et₃SiH</td>
<td>3</td>
<td>12h</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>BF₃·Et₂O</td>
<td>6</td>
<td>Et₃SiH</td>
<td>3</td>
<td>12h</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>SnCl₄</td>
<td>6</td>
<td>Et₃SiH</td>
<td>3</td>
<td>12h</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>BF₃·Et₂O</td>
<td>3</td>
<td>NaBH₄</td>
<td>1</td>
<td>20h</td>
<td>0</td>
</tr>
</tbody>
</table>

In parallel to the reduction attempts, modifications were applied to the addition step in order to increase the yield. The possibility that BSA or some side products arising from the protection step might interfere with the addition led to the preparation of a protected derivative of 30. The benzyl group was a good candidate due to its stability under strong basic and nucleophilic conditions. However, the protection conditions needed to be tuned up to avoid a mixture of N-Bz and O-Bz protected products. Two modified literature procedures were finally found leading selectively either to the O-protected compound 34 or to the N-protected compound 35 (Scheme 3.7).[^19] Surprisingly the O-benzylated pyridone proved to be more stable under butyllithium treatment than the N-benzylated pyridone and was therefore used for the experiment. On the other side, the primary amine of pyridine 20 was bis-protected with paramethoxybenzyl chloride according to a literature procedure affording 36 (Scheme 3.7).[^20]
Scheme 3.7: Synthesis of compound 34, 35 and 36. a) BnBr, Ag₂CO₃, THF, reflux, 4h, 93%; b) K₂CO₃, nBu₄NBr, BnBr, CH₂Cl₂, rt, 3 h, 68%; c) PMBCl, NaH, THF, 0°C to rt, 6 h, 88%.

Treatment of the halogeno pyridines 34 and 36 with butyllithium under the conditions used previously resulted in both cases in the corresponding 5-lithio derivative. Reaction of either of these nucleophiles with lactam 29 failed in producing the desired product. Instead the major products isolated were the results of hydrolysis of the lithio-pyridines. The latter seem to behave rather like a base than a nucleophile and perform reversible abstraction of an acidic proton at C3 instead of addition to the carbonyl (Figure 3.3). When the reactions with either the 2-pyridone or the 2-amino-pyridine derivatives were quenched almost the whole amount of lactam was recovered together with the dehalogenated pyridines. The products of addition could not be detected.

Figure 3.3: Competition between nucleophilic and basic reactivity of the lithio-pyridine derivatives.

The low yields obtained for the addition of the 5-lithio-pyridines and the difficulties encountered during reduction of the hemiaminal dramatically decreased the chances of success of this second strategy. Solutions exist but would probably lead to longer synthesis and lower yields. Instead a new approach based on similar starting materials was envisioned.

3.2.3 Third strategy: N-acyliminium chemistry based synthesis

Hanessian[21-23] and others[24] have shown the possibility to introduce an alkyl or an aryl group at the α-carbon to the nitrogen of substituted pyrrolidines via N-acyliminium chemistry. [25] The C-
C bond formation occurs through the reaction between a nucleophilic carbon and the N-acyliminium ion which exhibit higher reactivity than the corresponding imine or iminium ion (Figure 3.4).

![N-acyliminium](image)

**Figure 3.4: Representation of the N-acyliminium chemistry.**

Instead of nucleophilic addition to the lactam 29 followed by reduction of the resulting hemiaminal, conversion of the lactam to the aminal 37 (Table 3.5, general scheme) and nucleophilic reaction on the acyliminium ion formed in situ by Lewis acid activation would yield the same product in an equivalent pathway only with inverted steps.

Selective reduction of the lactam moiety of 29 was performed efficiently with Super-Hydride (LiBEt3H) and the resulting hemiaminal was pure enough to be used for the next step without further purification (Table 3.5 step a). Reaction of the intermediate in methanol in presence of catalytic amounts of p-toluenesulfonic acid is a widely used method \(^{[23, 24, 26]}\) to access the aminal; however, it failed in the present case (Table 3.5, step b, entry 1). These conditions led only to decomposed material. On the other side treatment with 2,2-dimethoxypropane and camphor sulfonic acid (CSA) \(^{[27, 28]}\) gave no reaction and all the hemiaminal was recovered (entry 2). The methoxy analogue 37 was finally obtained by treatment with methyl orthoformate, a catalytic amount of BF3-etherate and powdered molecular sieves \(^{[29, 30]}\) in 60% yield over two steps (entry 3).

<table>
<thead>
<tr>
<th>Entry</th>
<th>a</th>
<th>b</th>
<th>Yield 37</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LiBEt3H, THF, -78°C</td>
<td>pTsOH, MeOH</td>
<td>/</td>
</tr>
<tr>
<td>2</td>
<td>LiBEt3H, THF, -78°C</td>
<td>Dimethoxypropane, CSA, CH2Cl2</td>
<td>/</td>
</tr>
<tr>
<td>3</td>
<td>LiBEt3H, THF, -78°C</td>
<td>CH(OMe)3, BF3-etherate, 4Å MS, Et2O</td>
<td>60%</td>
</tr>
</tbody>
</table>
Second generation of pyrroldino C-nucleosides

As aglycon, the organocopper derivative of pyridine-2-one was used. Compound 30 was TMS-protected in situ with BSA (Scheme 3.8). Addition of Butyllithium at -78°C led to halogen metal exchange and subsequent treatment with CuBr-Me₂S, still at -78°C, afforded the cuprate derivative of silylated 30. Boron trifluoride-etherate was then added followed by 37 and the temperature was allowed to reach rt. One major product was isolated from the crude mixture; however, spectral characterisation revealed that it was not the desired product 33 but the isomer 38.

\[ \text{Scheme 3.8: Coupling reaction of aglycon 30 with aminal 37 under acyliminium chemistry. a) BSA, THF, rt, 30 min; b) Butyllithium, -78°C, 30 min; CuBr-Me₂S, -78°C, 1h; c) BF₃·Et₂O, -78°C, 30 min; d) 37, -78°C to rt, 2h, 60% overall yield.} \]

Cleavage of the TMS protective group by the Lewis acid followed by rearrangement of the anion resulting in a change of the nucleophilic centre to N-1 explains the obtained product. Clearly, a new protecting group, stable under the reaction conditions needed to be found for the pyridone moiety of 30. Several groups were introduced and the stability was tested against both butyllithium and BF₃-etherate under the conditions used for the coupling. Surprisingly, neither the TBDMS nor the TBDPS silyl ethers were stable in the presence of the Lewis acid in THF at -78°C. Rapidly the free pyridone was formed. On the other side, the THP protected analogue proved stable against BF₃-etherate but when treated with one equivalent of butyllithium at -78°C and quenched with water after one hour, 50% of the starting material was recovered together with the expected dehalogenated compound and some side-products. Finally benzyl-protected bromo-pyridin-2-one 34 previously used (Scheme 3.7) emerged as the best candidate: no BF₃-etherate-mediated deprotection occurred and clean and quantitative conversion to the dehalogenated derivative was obtained upon butyllithium treatment followed by hydrolysis.

Treatment of 37 with a mixture of 34, butyllithium, copper bromide-dimethyl sulfide complex and boron trifluoride diethyl etherate in similar conditions as before (Scheme 3.8) did unfortunately not produce any detectable amount of nucleoside. Detailed analysis of the reaction showed that the organometallic derivative of 34 decomposes above -78°C, before it reacts with the acyliminium ion. The coupling was therefore repeated and the temperature was kept at -78°C. In this case, no progression of the reaction was detected even after 24h. A test was then performed with the aminal 37 in presence of BF₃·Et₂O and Et₃SiH as nucleophile. While maintaining the temperature at -78°C,
no reaction could be observed after several hours. As soon as the temperature was allowed to rise, the reaction proceeded and ended up with complete conversion of 37 to reduced 28 (Scheme 3.4). This indicates that at -78°C the aminal is stable and is converted to the reactive N-acyliminium derivative only upon increase of the temperature.

The aminal 37 and the organometallic derivative of pyridone 34 display incompatible reactivity for coupling through acyliminium chemistry. At low temperature, the acyliminium ion does not form and no reaction occurs. When the temperature rises, the organometallic species decomposes before formation of or reaction with the iminium derivative. A possible alternative is the use of the corresponding imine. Even if the imine is less reactive, it does not require activation and can react directly with a nucleophile.

3.2.4 Fourth strategy: Imine based synthesis

Several pyrrolidino C-nucleosides with aromatic (hetero)-cycles, such as substituted phenyls, imidazoles or 9-deazapurines, as aglycons were synthesized as transition state inhibitors for nucleoside hydrolases or nucleoside phosphatases by Schramm and co-workers. The aglycon was introduced via addition of the corresponding aryl-lithium or aryl-Grignard reagents to the imine function of substituted 3,4-dihydro-2H-pyrroles. [31-34]

This strategy proved completely unsuccessful in the case of the first generation of pyrrolidino pseudonucleosides and has therefore not been considered yet. However, after the inabilities of the former synthetic methodologies to furnish the desired product, this approach was the last attempt to attain the nucleosides through a synthesis containing an iminosugar-nucleobase coupling step. In case of failure, more complicated, long, linear routes like incorporation of a functional group in pseudo-anomeric position and construction of the base around this group or modification of a molecule already possessing a scaffold similar to that of the target molecule including the wanted C-C bond must be employed.

The new strategy relies on the coupling of imine 39 (Scheme 3.9) and an organometallic derivative of 34. The suitable imine was an intermediate in the known synthesis of CBz-protected enamine 9. It is available in six steps and 62% overall yield starting from trans-3-hydroxy-L-proline. Treatment of intermediate 27, whose synthesis was shown previously (Scheme 3.4) with NCS in hexane resulted in the N-chloro analogue (Scheme 3.9). Subsequent LTMP-mediated elimination of HCl at -78°C afforded imine 39 in a good yield. [32] Even if similar imines have been reported to decompose easily, [31] 39 was isolated by column chromatography and proved to be stable at rt.
Second generation of pyrrolidino C-nucleosides

Scheme 3.9: Synthesis of imine 40 from intermediate 27. a) NCS, hexane, rt, 1h; b) LTMP, THF, -78°C, 2h, 74% over two steps.

Addition of butyllithium to benzyl-protected pyridone 34 gave the lithio derivative 40 (Scheme 3.10). This intermediate can again react either as a nucleophile and attack the imine yielding the desired product 41 or as a base and reversibly deprotonate the starting material. In practice, half of the starting material was recovered and half was converted to the product in a 1:10 mixture α/β. The separation of the two anomers at this stage presented some difficulties. Therefore they were usually collected together and separated in the next step. Finally the desired nucleoside analogue 41 was synthetically accessible.

Scheme 3.10: Possible pathways during the reaction between imine 39 and lithio derivative of 34. a) Buthyllithium, THF, -78°C, 1h; b) 39, THF, -78°C, 50% of 41 over two steps.

The benzyl protective group was removed via catalytic hydrogenation and the pure β anomer 43 was isolated in 35% yield after two steps (Scheme 3.11). The right configuration at the pseudo-anomeric centre was proved by ¹H-NMR-NOE experiments: a strong mutual NOE was observed between H2 and H5 indicating the β anomic form. The Fmoc group suitable for oligonucleotide synthesis was used to protect the pyrrolidino nitrogen and the silyl ethers were cleaved off with acidic methanol leading to diol 45 in 88% yield from 43. Standard tritylation of the primary alcohol (→46) followed by phosphitylation afforded phosphoramidite building block 13.
Second generation of pyrrolidino C-nucleosides

Scheme 3.11: Synthesis of phosphoramidite 13. a) 10% Pd/C, H₂, MeOH, rt, 30 min., 35% over two steps; b) Fmoc-OSu, THF, dioxane, 1M aq. NaHCO₃, rt, 1 h 30; (c) 1M aq HCl soln./MeOH 1:10, rt, 20 h, 88% over two steps; (d) DMTCl, pyridine, rt, 5 h, 80%; (e) CEPCl, iPr₂NEt, THF, rt, 2h, 85%.

When 13 was tested for oligonucleotide synthesis, the pyridone functionality turned out to give side-reactions decreasing dramatically the synthesis efficiency as it will be shown in more detail in the next section. Therefore the protection of the pyridone was required. Bevers et al have already prepared DNA strands containing C-nucleosides bearing the pyridin-2-one base and they reported the necessity of protecting the 2-pyridone carbonyl without giving further details about the reasons. [35] Since it proved successful in their case, the p-nitrophenylethyl group (NPE) was chosen to mask the pyridone moiety.

Scheme 3.12: Synthesis of phosphoramidite 50. a) PPh₃, DIAD, p-nitrophenylethanol, dioxane, rt, 8 h, 35%; b) 1M aq HCl/MeOH 1:10, rt, 24 h, 67%; c) DMTCl, pyridine, rt, 5 h, 74%; d) CEPCl, iPr₂NEt, THF, rt, 2h, 82%.

The synthesis of this O-protected building block started from intermediate 44. The NPE group was incorporated via Mitsunobu reaction (Scheme 3.12). Even if all the starting material was consumed and only one new product seemed to be formed, the fully protected compound 47 was
obtained in a rather low yield of 35%. The remaining steps were performed under similar conditions as in the previous route. The diol 48 was attained via treatment with HCl in methanol and converted to 49 by reaction with DMTCl in pyridine. Finally phosphitylation of 49 afforded the fully protected monomeric unit 50 in 82% yield.

A similar pathway was applied for the synthesis of dp2AP and its phosphoramidate derivative (Scheme 3.13). The previously used PMB-protected amino-pyridine 36 was subjected to bromine-lithium exchange followed by reaction with imine 39. The desired β anomer 51 could be separated from the α anomer at this stage. Again the β configuration was undoubtedly demonstrated using 1H-NMR-NOE experiments carried out on 51. The free pyrrolidine nitrogen was again Fmoc protected leading to fully protected compound 52. Since the PMB group is not compatible with solid phase-based oligonucleotide synthesis, replacement for a suitable protecting group was required. However, neither palladium-catalyzed hydrogenation nor oxidative cleavage with ceric ammonium nitrate (CAN) removed the electron rich benzyl groups. Deprotection under acidic conditions (trifluoroacetic acid) resulted in desilylation of the hydroxyl groups. Another solution was to change the protecting group strategy for the coupling step.

Scheme 3.13: Synthesis of intermediate 52. a) Buthyllithium, THF, -78°C, 1h; b) 39, THF, -78°C, 2h, 26%; c) Fmoc-OSu, THF, dioxane, 1 M aq. NaHCO3, rt, 4h, 63%.

The “stabase” adduct of 5-bromo-2-aminopyridine 53 was prepared according to a literature procedure (Scheme 3.14). [1, 2] Formation of the 5-lithio derivative of 53 with buthyllithium at -78°C and in situ reaction with imine 39 furnished after purification the β-pyrrolidino pseudonucleoside 54 as showed by 1H-NMR-NOE experiments. The yield of coupling was slightly lower than for the PMB-protected analogue but cleavage of the stabase protecting group during silica gel column chromatography saves one deprotection step.

Scheme 3.14: Synthesis of intermediate 54. a) Buthyllithium, Cl(Me)2SiCH2CH2Si(Me)2Cl, THF, -78°C, 2.5h, 79%; b) Buthyllithium, THF, -78°C, 1h; b) 39, THF, -78°C, 2h, 19%.
Fmoc protection of compound 54 was performed selectively at the pyrrolidino nitrogen (Scheme 3.15). For the exocyclic amine, classical protective groups suitable for oligonucleotide synthesis (acetyl, benzoyl, etc) were reported to exhibit an increased stability against ammonolysis and were not removed under classical oligonucleotide deprotection conditions. In order to avoid resorting to harsher deprotection conditions, the more labile phenoxyacetyl (Pac) group was incorporated on the primary amine and intermediate 56 was formed in excellent yield. The diol 57 was obtained quantitatively from 56 via acidic methanolysis of the silyl ethers. However the reaction should be checked regularly and stopped as soon as both hydroxyls are free. An extended reaction time would lead to partial deprotection of the Pac group. DMT protection afforded compound 58 and introduction of the phosphoramidite moiety yielded the building block 59.

Scheme 3.15: Synthesis of phosphoramidite 59. a) Fmoc-OSu, THF, dioxane, 1 M aq. NaHCO₃, rt, 30 min, 75%; b) Pac₂0, pyridine, rt, 2h, 99%; c) 1M aq HCl/MeOH 1:10, rt, 24 h, 99%; d) DMTCl, pyridine, rt, 2 h, 88%; e) CEPCI, iPr₂NEt, THF, rt, 1h30, 90%.

The fraction of Pac-deprotected product 60 formed once during the TBDMS deprotection of compound 56 was isolated and converted to the fully deprotected dp2AP (61) by treatment with piperidine to remove the Fmoc group (Scheme 3.16). Pyrrolidino C-nucleoside 61 was fully characterized and the β configuration at the anomeric centre was confirmed by ¹H-NMR-NOE experiments indicating that no anomerisation happened during any step of the synthesis, in particular during the TBDMS deprotection performed under strong acidic conditions.

Scheme 3.16: Synthesis of dp2AP. a) 1M aq HCl/MeOH 1:10, rt, 30 h, 76% of 57 and 23% of 60; b) piperidine, DMF, rt, 12h, 91%.
The phosphoramidite building blocks of dp2P and dp2AP (13, 50 and 59 respectively) were now available for incorporation into nucleic acids strands via automated solid phase oligonucleotide synthesis. The preparation of the TFOs and the evaluation of their duplex binding affinities as well as the comparison of these results with those obtained for the first generation of pyrrolidino containing oligonucleotides will be presented in the next part of this work.

### 3.3 Oligonucleotides synthesis and pairing properties

#### 3.3.1 Dp2P containing TFOs

In an initial experiment, phosphoramidite building block 13 was incorporated into oligonucleotides. Oligonucleotide synthesis was performed on a 1.0 μmol scale on an automated DNA synthesizer using standard solid-phase phosphoramidite chemistry (see Annexe 1). Small changes were made to the standard procedures for the coupling of the non-natural monomers (see section 2.1.2). The prepared sequences are listed in Table 3.6.

<table>
<thead>
<tr>
<th>TFO</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ref1</td>
<td>5'-d(TTTTTTCTCTCTCTCT)</td>
</tr>
<tr>
<td>dp6</td>
<td>5'-d(TTTTTTCXCTCTCTCT)</td>
</tr>
<tr>
<td>dp7</td>
<td>5'-d(TTTXXCTCTCTCTCT)</td>
</tr>
<tr>
<td>dp8</td>
<td>5'-d(TTTTTCXCTCTCTCT)</td>
</tr>
<tr>
<td>dp9</td>
<td>5'-d(TTTTTCXCTCTCTCT)</td>
</tr>
<tr>
<td>dp10</td>
<td>5'-d(XXXXXCTCTCTCTCT)</td>
</tr>
<tr>
<td>dp11</td>
<td>5'-d(TTTTXCTCTCTCTCT)</td>
</tr>
<tr>
<td>dp12</td>
<td>5'-d(TTTXTCTCTCTCTCT)</td>
</tr>
</tbody>
</table>

TFOs dp8 and dp11, bearing two and one modifications respectively, are used here as representatives of this first series of modified oligonucleotides for the description of the problems encountered during their preparation. According to the detritylation assay, the coupling efficiencies seemed high and regular throughout the syntheses leading to excellent overall yields. However, the crude mixture obtained after ammonolysis of dp8 exhibited an unexpectedly messy HPLC trace (Figure 3.5). Furthermore, isolation and characterization of the main peak revealed a mass significantly lower than the right mass.
Figure 3.5: IE-FPLC chromatogram for oligonucleotide dp8.

Mass spectrometric analysis of the crude mixture revealed the presence of the expected oligonucleotide. Extensive study of the crude mixture allowed the identification and isolation of the oligonucleotide out of a mixture of numerous low intensity peaks with many of them at a higher retention time compared to the main peak (Figure 3.6).

Figure 3.6: IE-FPLC chromatogram of the crude oligonucleotide dp8.
This situation did not correspond at all to the obtained detritylation assay. The first hypothesis was the degradation of the oligonucleotide during ammonolysis. To verify this hypothesis, the deprotection time was reduced from 18h at 60°C to 4h at the same temperature. However, no improvement was observed and the HPLC chromatograms obtained in both cases were superimposable indicating that the problem probably occurs already during the synthesis.

An easier situation was expected in the case of monomodified sequences therefore the attention was focused on oligonucleotide dp11 (Table 3.6) in order to identify the problem. To confirm that no decomposition occurs during the treatment with ammonia, two batches were separately deprotected under different conditions. Crude mixtures obtained after 4h at 60°C or 5h at room temperature displayed identical HPLC chromatograms thereby ruling out oligonucleotide degradation during the ammonolysis. Again the HPLC trace, presenting several peaks with similar intensity, was in complete disagreement with the detritylation assay that suggested a clean synthesis and one major product. The most important peaks were all isolated and analyzed by mass spectrometry. The desired mass was found together with several products exhibiting surprisingly higher masses (Figure 3.7). A careful analysis of the found masses led to the conclusion that the unprotected pyridone functionality of dp2P reacted with the incoming monomer during the coupling step of the oligonucleotide synthesis generating branched strands. Some examples of side-products possibly formed are depicted in Figure 3.8.

![Figure 3.7: 1E-HPLC chromatogram of the crude oligonucleotide dp11.](image)
With the incorporation of only a single dp2P modification the oligonucleotide synthesis gave rise to a variety of side-products. In the case of two modifications, the desired sequence was hardly identified among a complicated mixture of products. The TFO containing five pyrrolidino units has not been studied but the expected oligonucleotide would have probably not been detected. It was obvious that a successful preparation of the dp2P containing TFOs required protection of the pyridone moiety. Therefore the NPE-protected phosphoramidite building block 50 described previously was used.

![Figure 3.8: Structure of possible side-products observed during oligonucleotide synthesis.](image)

The synthesis of oligonucleotide dp11 was repeated using the fully protected monomeric unit 50 and in this case the detritylation assay reflected faithfully the synthesis efficiency. A drop in the synthesis efficiency of about 60% was observed upon coupling of the dp2P unit (Figure 3.9 left). A two-step deprotection procedure involving first treatment with 0.5 M DBU in pyridine to remove the NPE group followed by ammonolysis to cleave the strand from the solid support and remove the remaining protective groups was applied. Analytical HPLC injection of the crude mixture showed two major peaks which according to the detritylation assay could correspond one to the desired sequence the other to the shorter sequence that failed to couple with the pyrrolidino monomer (Figure 3.9 right). The HPLC trace was matching the detritylation assay.
Figure 3.9: Detritylation assay and IE-HPLC chromatogram for oligonucleotide dp11.

As expected, the most important peak was identified as the sequence lacking the last bases starting from the modified nucleoside. The second most intense peak, however, proved to be the oligonucleotide dp11 still bearing the NPE group. Apparently the DBU deprotection step failed to reveal the pyridone functional group.

Table 3.7: Reaction conditions for removal of the NPE group in oligonucleotide dp11.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Reagent</th>
<th>Conditions</th>
<th>Extent of deprot.(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5 M DBU in Py</td>
<td>rt, 8h</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0.5 M DBU in Py</td>
<td>55°C, 20h</td>
<td>33</td>
</tr>
<tr>
<td>3</td>
<td>1 M DBU in CH$_3$CN</td>
<td>rt, 18h</td>
<td>33</td>
</tr>
<tr>
<td>4</td>
<td>1 M TBD in CH$_3$CN</td>
<td>rt, 20h</td>
<td>66</td>
</tr>
<tr>
<td>5</td>
<td>1 M TBD in CH$_3$CN</td>
<td>rt, 20h + 60°C, 2h</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>1 M TBD in CH$_3$CN</td>
<td>60°C, 8h</td>
<td>100</td>
</tr>
</tbody>
</table>

Figure 3.10: IE-FPLC chromatogram showing the efficacy of the different NPE deprotection conditions.
Several conditions were tested varying the reaction time, temperature or base (Table 3.7). The deprotection efficacy associated to the different conditions applied was determined by analytical HPLC (Figure 3.10). As mentioned above, treatment with 0.5 M DBU in pyridine at room temperature proved inefficient (Entry 1). Increasing both the temperature and the reaction time led only to partial deprotection with the protected form as major compound (Entry 2). Similar results were obtained when 1 M DBU in acetonitrile was used (Entry 3). Treatment with the stronger base TBD as a 1 M solution in acetonitrile at room temperature for 20h also conducted to incomplete deprotection but the conversion exceeded 50% (Entry 4). An additional treatment at 60°C for 2h completed the reaction (Entry 5). Finally the procedure that proved successful required 1 M TBD in acetonitrile at 60°C for 8h. In contrary to the initial conditions employed, this procedure also leads to partial cleavage of the oligonucleotide from the solid support. Subsequent treatment with ammonia for 18h at 60°C resulted in the fully deprotected strand. TFO dp11 was isolated by HPLC and its integrity was confirmed by mass spectrometry.

Preparation of TFO dp11 served to confirm that the protection of the pyridone moiety of dp2P is necessary to avoid formation of branched oligonucleotides as by-products during the solid phase synthesis. In addition, the appropriate deprotection and purification conditions for dp2P containing oligonucleotides were established. The triplex forming ability of TFO dp11 was then investigated using melting-curve experiments and the results were compared to the data obtained for the first generation of pyrrolidino C-nucleosides. All these data are compiled in Table 3.8.

### Table 3.8: Sequence of TFOs and $T_m$ data (°C) of third strand dissociation from UV melting curves (260 nm).

<table>
<thead>
<tr>
<th>Entry</th>
<th>TFO</th>
<th>Sequence</th>
<th>$T_m$</th>
<th>$\Delta T_m$/mod.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Refl</td>
<td>5'-d(TTTTCTCTCTCTCT)</td>
<td>43.1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>dp13</td>
<td>5'-d(TTTTCCTCTCTCT)</td>
<td>38.4</td>
<td>-4.7</td>
</tr>
<tr>
<td>3</td>
<td>dpm13</td>
<td>5'-d(TTTTYCTCTCTCT)</td>
<td>37.0</td>
<td>-6.1</td>
</tr>
<tr>
<td>4</td>
<td>dp11</td>
<td>5'-d(TTTTZCTCTCTCT)</td>
<td>32.0</td>
<td>-11.1</td>
</tr>
</tbody>
</table>

*Single strand concentration = 1.2 µM in 140 mM KCl, 7 mM NaH2PO4, 0.5 mM MgCl2, pH = 6.0. $T_m$ of target duplex = 57.0 ± 1.0°C.

Compared to the natural triplex, the triplex containing TFO dp11 exhibited a significant loss in stability of about 11°C per modification (Entry 4). This corresponds to further destabilization compared to dpψU and even dpψT containing triplexes (Entries 2 and 3, resp.). This unexpected result was confirmed by circular dichroism spectroscopy (CD, Annexe 3). The CD spectra of the
Second generation of pyrrolidino C-nucleosides

triplexes were measured at different temperature below and above the third strand melting temperature. The resulting curves exhibited almost identical shape except in the region below 235 nm (Figure 3.11 left). Changes in the spectra appeared upon an increase of the temperature from 20 to 40°C. The major difference in the CD spectral properties was observed at 220 nm. Consequently the variation of the CD intensity at a fixed wavelength of 220 nm was monitored between 10 and 50°C and a sigmoidal curve was obtained (Figure 3.11 right). The first derivative of this curve gave a $T_m$ value of 32°C which is in perfect agreement with that from the UV-melting results.

![Figure 3.11: CD spectra of the triplex containing TFO dp11; left: CD spectra measured at various temperatures; right: temperature scan CD spectra from 10 to 50°C at 220 nm; single strand concentration = 1.2 µM in 140 mM KCl, 7 mM NaH$_2$PO$_4$, 0.5 mM MgCl$_2$, pH 6.0.](image)

The missing carbonyl group seems to play an important role in triplex stability. It may positively influence the stacking interactions or the solvation properties rather than to lead to a destabilization due to hydrogen bond formation with the pyrrolidino nitrogen as initially postulated.

Another explanation that could be put forward is a partial isomerisation at the anomeric centre during the strong basic TBD treatment. The anionic intermediate could cause the pyrrolidino ring to open and then close back in a non-stereospecific manner leading to anomerisation (Figure 3.12).

![Figure 3.12: Proposed anomerisation mechanism during NPE deprotection.](image)
As a control, the conditions used to remove the NPE group after oligonucleotide synthesis were applied to the free nucleoside. A pure sample of β anomer was subjected to treatment with 1 M TBD in acetonitrile at 60°C for 8h. 1H-NMR of the resulting crude mixture showed retention of the anomeric configuration, no traces of the α anomer were detected. This experiment allowed to reject the hypothesis of anomerisation during the p-(nitrophenyl)ethyl group removal.

The triplex destabilization observed for all the pyrrolidino analogues of thymidine might also be a consequence of a preference of the nucleobases for their enol tautomeric form. The natural nucleobases T (or U), C and G exist in both the keto and the enolic form, however with a strong predominance of the keto tautomer. If on the contrary dpψU, dpψT and dp2P exist preferentially in the enolic form, their base pairing would be found modified in a way that they would be complementary to a G instead of an A in the Hoogsteen motif (Figure 3.13). These enolic bases placed in front of an A would correspond to a mismatch situation leading to a decrease in triplex stability. The deletion of the carbonyl or amine groups in dp2P should not prevent this tautomerism modifying the Hoogsteen base pairing. Therefore no improvement would be observed with the new pyrrolidino nucleoside.

![Figure 3.13: Tautomeric forms of dpψU (left) and dp2P (right) and the corresponding Hoogsteen base pairing.](image)

In order to verify this hypothesis, a new target duplex (Target 2, Table 3.9) having a guanosine instead of an adenosine as antagonist for the pyrrolidino analogues of T was prepared. The data obtained from melting curve experiments are summarized in Table 3.9 together with the Tm values measured with the usual duplex (Target 1) for comparison. The unmodified TFO Ref1 used as reference led to a strongly destabilized triplex with a Tm of about 30°C and a ΔTm of -12.6 relative to the Target 1 as expected for a mismatch situation (Entry 1). The dpψU containing TFO dp13 formed with the duplex target 2 a triplex also substantially less stable than with the duplex target 1 (Entry 2). The Tm value was in the same range as for the reference triplex indicating that this system presented a mismatch as well. A mismatch was also encountered in the case of the TFO dp11 containing triplex which exhibited a thermal stability similar to the ones measured with Ref1.
or dp13 (Entry 3). The Tm was however slightly lower; an additional destabilization could arise from a loss in stacking interactions or solvation effects due to the missing carbonyl function. It is noteworthy that in the case of TFO dp11 the ΔTm observed between the two triplex systems is rather low. The pyrrolidino pyridin-2-one unit recognizes the AT base pair within the parallel binding motif with very low affinity almost comparable to a case of base mismatch.

Table 3.9: Sequence of TFOs and Tm data (°C) of third strand dissociation from UV melting curves (260 nm).

<table>
<thead>
<tr>
<th>Entry</th>
<th>TFO</th>
<th>X</th>
<th>Tm° (Target 1)</th>
<th>Tm° (Target 2)</th>
<th>ΔTm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ref1</td>
<td>T</td>
<td>43.1</td>
<td>30.5</td>
<td>-12.6</td>
</tr>
<tr>
<td>2</td>
<td>dp13</td>
<td>dpψU</td>
<td>38.4</td>
<td>30.5</td>
<td>-7.9</td>
</tr>
<tr>
<td>3</td>
<td>dp11</td>
<td>dp2P</td>
<td>32</td>
<td>29.5</td>
<td>-2.5</td>
</tr>
</tbody>
</table>

a Single strand concentration = 1.2 µM in 140 mM KCl, 7 mM NaH2PO4, 0.5 mM MgCl2, pH = 6.0. Tm of target duplex 1 = 57.0 ± 1.0°C. Tm of target duplex 2 = 61.0 ± 1.0°C.

The nucleobases of the dpψU and dp2P nucleotides proved to be in the expected tautomeric form to recognize an AT base pair via Hoogsteen base pairing. No gain in stability was attained when these units were placed in front of a G base; on the contrary a strong loss in triplex thermal stability characteristic of a base mismatch situation was observed.

None of the experiments involving the nucleobase could account for the triplex destabilization associated with the different pyrrolidino analogues of T. This could indicate a sugar implication in the negative results. The imino-sugar is presumably in a C2'-endo (S) pucker like a natural 2'-deoxynucleoside. Preparation of the 2'-hydroxypyrrrolidino series should result in nucleoside with a C3'-endo (N) sugar conformation. It would be interesting to see the effect of this modification on the triplex thermal stability. In addition RNA TFOs are known to give rise to more stable triplexes because of a backbone conformation closer to the one adopted by the triple helical structure.[36] In a preliminary experiment the dpψU monomer was incorporated in a RNA strand, expecting that the general RNA-like conformation of the oligonucleotide would force the 2'-deoxy-pyrrolidino monomer in a C3'-endo sugar pucker. The natural RNA TFO was prepared as well to serve as reference and the Tm values of third strand dissociation form the target duplex 1 for these two TFOs are shown in Table 3.10. The data concerning the corresponding DNA sequences are presented as well to allow direct comparison.
Second generation of pyrrolidino C-nucleosides

Table 3.10: Sequence of TFOs and Tm data (°C) of third strand dissociation from UV melting curves (260 nm).

<table>
<thead>
<tr>
<th>Entry</th>
<th>TFO</th>
<th>Sequence</th>
<th>Tm°</th>
<th>ΔTm/Mod.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ref1</td>
<td>5'-d(TTTTCTCTCTCTCTCT)</td>
<td>43.1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>dp13</td>
<td>5'-d(TTTTXCTCTCTCTCTCT)</td>
<td>38.4</td>
<td>-4.7</td>
</tr>
<tr>
<td>3</td>
<td>Ref3</td>
<td>5'-r(UUUUUCUCUCUCUCUCUCU)</td>
<td>47.0°C</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>p1</td>
<td>5'-r(UUUUUCUCUCUCUCUCUCU)</td>
<td>38.0°C</td>
<td>-9°C</td>
</tr>
</tbody>
</table>

*Single strand concentration = 1.2 µM in 140 mM KCl, 7 mM NaH₂PO₄, 0.5 mM MgCl₂, pH = 6.0. Tm of target duplex = 57.0 ± 1.0°C.*

As expected, the natural RNA TFO Ref3 was found to bind the target duplex with higher affinity than the DNA counterpart Ref1 (Entries 3 and 1, respectively). The modified RNA strand p1 and its DNA equivalent dp13, however, formed triplexes of comparable stability revealing a stronger destabilization when dpyU is incorporated in a RNA strand than in a DNA strand (Entries 4 and 2, respectively). Indeed, a decrease of 9°C per modification was observed in the RNA series against 5°C in the DNA series. This result could be explained by the fact that the pyrrolidino C-nucleotide did not adopt the C3'-endo sugar pucker under the influence of the RNA backbone. It is therefore obvious that the introduction of a DNA-like unit (dpyU) in an otherwise RNA oligonucleotide leads to a perturbation of the helix in addition to the usual destabilization associated with this modification. A positive outcome of this preliminary experiment would have been encouraging and would have supported the idea that the change in the sugar conformation can solve the destabilization problem. A negative result, however, does not exclude that this is still the case since it is impossible to predict the sugar conformation of the 2'-deoxypyrrolidino unit in the RNA strand. The only way to surely verify this assertion requires the preparation of the fully RNA TFO containing a 2'-hydroxypyrrolidino pseudoU. The synthesis of the corresponding phosphoramidite building block is currently ongoing.

### 3.3.2 Dp2AP containing TFOs

The phosphoramidite building block 59 prepared as previously described was incorporated into TFOs as a cytidine analogue. As for the TFOs containing dp2P, the oligonucleotide synthesis was carried out on a 1.0 µmol scale on an automated DNA synthesiser using standard solid-phase phosphoramidite chemistry (see Annexe 1). In addition to the minor changes usually made to the standard procedures for the non-natural monomers (see section 2.1.2), monomeric unit 59 also required modification of the capping solution. The Pac-protected amino group of 2-aminopyridine has been reported to be partially acetylated during the capping step of the oligonucleotide synthesis.
These acetyl groups were not cleaved under standard ammonolysis and harsher conditions are necessary. Acetylation can be prevented by the use of phenoxyacetic anhydride as capping agent. The synthesis proceeded with high efficiency and the modified capping step allowed for successful postsynthetic deprotection under classical conditions (33% ammonia, 60°C, 18h). The sequences prepared for this study and their Tm values for third strand dissociation are listed in Table 3.11.

All the modified TFOs led to triple helical structures exhibiting diminished stability compared to the natural system at pH 6.0. However, the destabilization decreases with an increased number of modifications from -5°C/mod in the case of monomodified TFO dp14 to -3°C/mod for TFO dp16 containing five modifications (Entries 2 and 6, resp.). In a different sequence context, runs of four contiguous dp2AP seemed to lead to further destabilization (dp17, Entry 9). This phenomenon is well known for cytosines: the relative stabilities of triple helices containing C+-GC triplets drops as the number of adjacent C+-GC triplets increases due to further decrease in the pK value of cytosine N3 and local repulsion of contiguous positive charges in the pile of protonated cytosines. However these composition effects have been reported to be less pronounced for 2-aminopyridine C-nucleosides. This appears to be true in this case since the additional loss in stability is rather low with only about 1°C per modification.

More interesting is the pH variation study of the dp2AP unit. Triplexes containing TFOs dp14 and dp15 were strongly affected by the pH value: an increase in pH from 6.0 to 7.0 led to a significant drop in Tm (Entries 2-3 and 4-5) which was consistent with the presence of pH dependent functionalities in the TFO (cytosine and 2-aminopyridine bases as well as pyrrolidino units). However the pH dependency seemed to arise mainly from the cytosine since replacement of a natural C with a dp2AP unit (dp14 → dp15) resulted in a lower Tm decrease when the pH rises from 6.0 to 7.0. This suggestion was confirmed with the TFOs dp16 and dp17 having all natural cytosine replaced by dp2AP moiety. They produced triple helical structures that showed no drop in the Tm value in the same pH range despite the five pyrrolidino 2-aminopyridine units known to be pH-dependent (Entries 6-7 and 13-14). The triplexes lost in stability only starting from pH 8.0 and at pH 9.0 none of the systems were found to form triplexes anymore (Entries 8-9 and 15-16). Although the dp2AP led to less stable triplexes compared to the references at pH 6.0, the weaker pH-dependence gave the advantage at pH 7.0. TFO dp17 for instance exhibited a loss in stability of 4.3°C per modification at pH 6.0. But a pH 7.0 it formed a triplex with similar Tm whereas the reference Ref2 had no longer any affinity to the target duplex (Entries 10-11 and 13-14).
Table 3.11: Sequence of TFOs and \(T_m\) data (°C) of third strand dissociation from UV melting curves (260 nm).

<table>
<thead>
<tr>
<th>Entry</th>
<th>TFO</th>
<th>Sequence</th>
<th>(pH)</th>
<th>(T_m^a)</th>
<th>(\Delta T_m/\text{mod.})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ref1</td>
<td>5'-d(TTTTTCCTCTCTTCT)</td>
<td>6.0</td>
<td>43.1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>dp14</td>
<td>5'-d(TTTTCTXTCTCTCTCT)</td>
<td>6.0</td>
<td>38.0</td>
<td>-5.1</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td>7.0</td>
<td>19.1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>dp15</td>
<td>5'-d(TTTTXXCTCTCTCTCT)</td>
<td>6.0</td>
<td>36.1</td>
<td>-3.5</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td>7.0</td>
<td>22.1</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>dp16</td>
<td>5'-d(TTTTXXTXTXXTXT)</td>
<td>6.0</td>
<td>26.9</td>
<td>-3.2</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td>7.0</td>
<td>27.0</td>
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</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td>8.0</td>
<td>22.1</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td>9.0</td>
<td>n.d. (^b)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Ref2</td>
<td>5'-d(TTTCCCCCTTTCTTT)</td>
<td>6.0</td>
<td>27.2</td>
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<tr>
<td>11</td>
<td></td>
<td></td>
<td>7.0</td>
<td>n.d. (^b)</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td>8.0</td>
<td>n.d. (^b)</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>dp17</td>
<td>5'-d(TTTXXXXTTTTXTTT)</td>
<td>6.0</td>
<td>5.7</td>
<td>-4.3</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td></td>
<td>7.0</td>
<td>7.0</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td>8.0</td>
<td>n.d. (^b)</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td></td>
<td></td>
<td>9.0</td>
<td>n.d. (^b)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Single strand concentration = 1.2 \(\mu\)M in 140 mM KCl, 7 mM NaH\(_2\)PO\(_4\), 0.5 mM MgCl\(_2\), \(pH = 6.0\). \(T_m\) of target duplex 1 = 57.0 ± 1.0°C, target duplex 2 = 60°C ± 1.0°C.  
\(^b\) No \(T_m\) detectable.

Incorporation of dp2AP into TFOs gives rise to triplexes displaying an astonishingly low pH-dependence in the range considered here compared to corresponding systems containing cytidines, methyl-cytidines or any pyrrolidino nucleoside studied so far. Both the pyrrolidino and the amino-pyridine nitrogen atoms seem to benefit from a high protonation state up to pH 8 generating triplexes that would not suffer from pH effects under physiological conditions. This remarkable property is however compromised by the triplex destabilization properties induced by dp2AP. The missing carbonyl group might play a role in these negative effects for the same reasons given for dp2P. Furthermore, the two protonated sites present in the nucleoside at a rather close distance could cause charge repulsion resulting in modification of the imino-sugar or base conformation and thereby contributing to diminished target binding affinity.

3.4 References

Second generation of pyrrolidino C-nucleosides


Chapter 4: Conclusions and outlook

4.1 Conclusions

4.1.1 First generation of pyrrolidino pseudonucleosides

Preliminary results obtained with pyrrolidino modified TFOs showed that the analogues of T, dpψU and its N1-methyl derivative dpψT, led to triplex destabilization whereas the analogue of C, dpψiC, significantly enhanced triplex stability. In the first part of the presented work, further experiments have been carried out to confirm the preliminary results. It has also been demonstrated that in the case of dpψiC the positive stabilizing effect was entirely due to the pyrrolidino modification which even compensates for a slight loss in stability caused by the base modification. As expected, the basic imino-sugar amine is protonated at physiological pH and the positive charge forms a salt bridge with a negatively charged oxygen of the phosphate backbone of the underlying duplex. The favourable electrostatic interaction helps to overcome charge repulsion arising from the assembly of three polyanionic strands and therefore leads to triplex stabilization. During the course of this study, a short and efficient synthesis of 2’-deoxy pseudoisocytidine based on Heck chemistry has been developed. A sequence effect study revealed that unlike the pyrrolidino T analogues, which exhibit strong sequence dependent properties, dpψiC contributes always to the same extent to triplex stability, independently of the sequence context. A fully modified TFO containing only pyrrolidino units was finally prepared but failed to improve the triplex binding properties especially in the case of dpψU.

4.1.2 Second generation of pyrrolidino pseudonucleosides

In the second part of the work, pyrrolidino-2-pyridone (dp2P) and -2-amino-pyridine (dp2AP), deletion mutants of dpψU and dpψiC, respectively, were developed in order to both shed light on the differential behaviour between dpψU and dpψiC and improve triplex stability. The synthesis of the new pyrrolidino C-nucleosides and their phosphoramidite building blocks turned out to be more complicated than expected. The Heck coupling-based route used for the first generation failed with these new nucleobases. Addition of the organometallic derivative of the heterocycles to either the acyliminium ion or the corresponding lactam proved inefficient as well. As a last resort nucleophilic addition of the lithio-derivative of the nucleobases onto the required imine produced the desired C-nucleosides. The yields were moderate but predominantly the β-anomers were obtained. After the
nucleosidation, no major problems were encountered in the remaining steps leading to the phosphoramidites 13, 50, and 59.

The synthesis of a first series of modified TFOs using monomer 13 failed due to side reactions between the pyridone functionality and the incoming building blocks during oligonucleotide synthesis. The necessary protection of the pyridone group led to building block 50. The latter as well as monomeric unit 59 were both successfully incorporated into TFOs.

In the case of dp2P containing TFOs, UV-melting curve and CD spectroscopy experiments showed a strong destabilization of the modified triplex compared to the unmodified system. The carbonyl and nitrogen groups missing in dp2P compared to dpψU seem to play an important role in triplex stability by favourable stacking interactions and/or solvation effects. The hypothesis of an inappropriate base conformation caused by a hydrogen bond between the C4' carbonyl function of dpψU and a pyrrolidino NH was invalidated by these results. Alternatively, a predominance in the pyrrolidino T analogues of the enolic tautomeric form of the nucleobase, different from the one required for a T-AT triplet formation but suitable to recognize GC within the parallel binding motif, would also lead to strong triplex destabilization. A new target duplex, placing a G instead of an A opposite to the pyrrolidino T analogues, was used to verify this theory. However, the study was in agreement with the nucleobases existing in the expected keto tautomeric form. No base-related argument could support a hypothesis explaining the discrepancy between dpψiC and dpψU or dpψT. A different imino-sugar conformation between the pyrrolidino analogue of C and T and/or local structural differences between the dpψiC-GC and the dpψU-AT (or dpψT-AT) triplets could result in the loss of the favourable electrostatic contact and therefore in the drop of the triplex stability. To investigate these effects, RNA third strands, bringing more homogeneity in the sugar conformation, was prepared. The TFO containing the 2'-deoxypyrrolidino unit dpψU produced, however, a less stable triplex than the unmodified counterpart. The result obtained suggests that the changes in the sugar conformation may not be the reason for triplex destabilization. It has to be stated though that this finding is not fully conclusive since it is impossible to guarantee that the 2'-deoxypyrrolidino unit adopted the RNA-like C-3'-endo sugar pucker under the influence of the RNA backbone. The preparation and incorporation into RNA TFOs of the 2'-hydroxypyrrolidino C-nucleoside is required to obtain a final answer on this question.

All dp2AP containing TFOs were found to bind their target duplexes with lower affinity than the reference TFOs at pH 6. Several factors like the lacking carbonyl moiety or the proximity of two positive charges in the pyrrolidino C-nucleoside might account for the reduced stability. However, the pH dependence, resulting from the required protonation at the imino-sugar and the base, proved
Conclusions and outlook

to be considerably low in the range of pH investigated. The Tm values stayed constant when the pH rose from 6.0 to 7.0 and diminished weakly from 7.0 to 8.0. Only at pH 9.0 the TFOs target affinity dropped dramatically and no triplex formation was observed anymore. This suggests that the basicity of the pyrrolidino ring nitrogen and of the 2-aminopyridine are similar in an oligonucleotide as in the monomeric units (pKa of pyrrolidino nitrogen in dpψU = 7.9, pKa of 2-aminopyridine = 6.9).

4.2 Outlook

4.2.1 Pyrrolidino analogue of T

To complete the data acquired with the monomodified dp2P containing strand, the preparation of TFOs containing multiple substitutions as well as a sequence effect study could be carried out and might attenuate the strong negative result obtained so far.

To provide easier access to the monomer necessary for the preparation of the above mentioned TFOs, an improvement in the phosphoramidite synthesis, especially of the coupling step, would be of great interest. Less basic organometallic derivatives could be used (grignard, Ce, Cu, etc) resulting in a higher addition/base abstraction ratio and thus a higher yield, whereas the use of a more bulky protective group (TBDPS for instance) for the 3’-hydroxyl group could influence the $\alpha/\beta$ ratio more in favour of the desired $\beta$ anomer.

The importance of the pyrrolidino pseudo-sugar conformation for the triplex stability still needs to be clarified. The RNA third strand containing a single 2′-deoxy-pyrrolidino unit gave inconclusive results. The synthesis of the 2′-hydroxy-pyrrolidino pseudoU phosphoramidite building block is ongoing and subsequent incorporation into TFOs is planned. These strands, displaying a conformationally uniform RNA-like sugar-phosphate backbone, will prove whether the C3′-endo pyrrolidino-sugar pucker is more favourable to effect dual recognition and will lead to triplex stabilizing TFOs. An X-ray structure of a pyrrolidino nucleoside would also considerably help to obtain structural information especially about the sugar conformation. Several crystallization attempts have been carried out but they unfortunately proved unsuccessful so far. In the best case, amorphous material was obtained.

4.2.2 Pyrrolidino analogue of C

The two main reasons proposed for the destabilizing effect observed with dp2AP containing triplexes were the missing carbonyl functionality and the two positive charges placed at short distance in the nucleoside. This hypothesis could be verified with compound 62 (Scheme 4.1). This
C analogue, which is structurally close to dpψiC, displays the right hydrogen bonding pattern for a GC recognition within the parallel binding motif without requirement of protonation. In addition, the nucleobase lacks the N-H-1/N-H-3 tautomerism which might play a role in target GC recognition in the case of dpψiC. Replacement of the N-H-1 by CH enforces the required N-H-3 form and might thus still improve the triplex binding properties of dpψiC. Some preliminary experiments have been performed on intermediate 55 in order to introduce the carbonyl group by oxidation of the pyridine nitrogen to the nitrone followed by rearrangement. The first step worked efficiently but the rearrangement conditions tried so far proved unsuccessful.

In the continuation of the project, a synthesis might be developed in order to generate the new pyrrolidino C-nucleoside 62 and its phosphoramidite building block 63 from the known intermediate 55. The monomeric unit 63 will then be incorporated into TFOs and their triplex forming properties will be tested and compared to those obtained for dpψiC and dp2AP.

![Scheme 4.1: Retrosynthetic scheme leading to phosphoramidite 63.](image)

In the long term, if the research described above leads to efficient pyrrolidino analogues of both thymidine and cytidine, showing higher triplex binding affinity within the parallel motif than the natural counterparts, pyrrolidino modified TFOs could be used in biological experiments and tested as antigene agents.
Chapter 5: Experimental Part

5.1 General

5.1.1 Instrumentation

Melting Points

A Büchi Melting Point B-545 apparatus was used. The melting points were measured in glass capillaries and are indicated in °C without correction.

Chromatography

Silica gel thin layer chromatography (TLC) was performed using SIL G-25 UV254 from Macherey-Nagel. Visualization was done using UV-light (254 nm) or staining solutions (Cerium reagent: 10.5 g cerium(IV) sulfate, 21 g phosphomolybdic acid hydrate, 60 ml sulfuric acid, 900 ml H2O; anisaldehyde reagent: 50 ml p-anisaldehyde, 10 ml acetic acid, 50 ml sulfuric acid, 900 ml ethanol; ninhydrin reagent: 0.3 g ninhydrin, 100 mL of 1-butanol, 3 mL of acetic acid).

Flash chromatography (FC) was performed with Silica Gel 60 (particle size 40-63 µm) from Fluka.

HPLC was performed either on an Äkta Basic 10/100 system from Amersham Pharmacia Biotech or on a HP Series 1100 system from Hewlett-Packard and FPLC was performed on a FPLC system from Pharmacia biotech.

NMR-Spectroscopy

1H NMR spectra were recorded at 300 MHz on a Bruker AC-300 spectrometer. Chemical shifts are reported in ppm relative to the residual undeuterated solvent (CDCl3: 7.26 ppm, DMSO: 2.50 ppm, CD3OD: 3.35 ppm, D2O: 4.65 ppm). Coupling constants J are in Hz. Multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet, br = broad.

13C NMR spectra were recorded at 75 MHz on a Bruker AC-300 spectrometer. Chemical shifts are reported in ppm relative to the residual undeuterated solvent (CDCl3: 77.00 ppm, DMSO: 39.70 ppm, CD3OD: 49.30 ppm).

1H/1H and 1H/13C correlation experiments (COSY) were used for signal assignments.

31P NMR spectra were recorded at 161.9 MHz on a Bruker DRX-500 spectrometer. Chemical shifts are reported in ppm relative to 85 % H3PO4 as an external standard.

Difference NOE experiments were recorded on a Bruker DRX-500 spectrometer.
**Mass Spectrometry**

Electron impact (EI) spectra were recorded on an *AutoSpec Q VG* with an ionization energy of 70 eV.

Fast atom bombardment (FAB⁺) spectra were recorded on an *AutoSpec Q VG*.

Electrospray ionization mass spectra (ESI) were recorded either on a *Fisons Instrument VG Platform* (oligonucleotides) or on an *Applied Biosystems, Sciex QSTAR Pulsar* (monomers).

**DNA Synthesis**

The chemical synthesis of the oligonucleotides was performed either on the 1.3 µmol scale on a *Pharmacia LKB Gene Assembler Special* DNA-synthesizer or on the 1.0 µmol scale on a *PerSeptive Biosystems Expedite Nucleic Acid Synthesis System* using standard phosphoramidite chemistry. The phosphoramidite building blocks of the natural nucleosides and the nucleosides bound to CPG-solid support were from *Glen Research*.

**Melting Curves**

Thermal denaturation experiments were carried out on a *Varian Cary 3E UV/Vis* spectrophotometer. Absorbances were monitored at 260 nm and the heating rate was set to 0.5°C/min. A heating-cooling-heating cycle in the temperature range 20-90°C or 0-90°C was applied. The absorbance melting curves were smoothed and the first derivative curves obtained using the *Varian WinUV* or *Microcal Origin* softwares. For temperatures < 20°C, nitrogen was passed through the spectrophotometer to avoid condensation on the cuvettes. To avoid evaporation of the solutions, 6-8 drops of dimethylpolysiloxane were added at the top of the samples in the cell.

**Circular Dichroism Spectroscopy**

CD spectra were recorded on a *Jasco J-715* spectropolarimeter equipped with a *Jasco PFO-350S* temperature controller. The temperature was measured directly in the sample. The graphs were subsequently smoothed with a noise filter. The phosphate buffer was used as blank.
### 5.1.2 Abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
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<tr>
<td>2AP</td>
<td>2-aminopyridine</td>
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<tr>
<td>A</td>
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<td>Ac</td>
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5.2 Synthesis of pyrrolidino C-nucleoside phosphoramidites

5.2.1. Nomenclature

The compounds based on C-nucleosides \textbf{dp2P} and \textbf{dp2AP} were named as substituted pyrrolidines and numbered as described below.
5.2.2. Syntheses

1. 2-Amino-5-iodo-4-oxo-3,4-dihydropyrimidine (2)

![Reaction Scheme]

To a soln. of 2-amino-4-oxo-3,4-dihydropyrimidine (1, 5.20 g, 46.8 mmol) in AcOH (84 ml) at 70°C was added N-iodosuccinimide (11.6 g, 51.6 mmol). The suspension was warmed to 100°C. After 1 h, the suspension was cooled to rt and H₂O (84 ml) was added. The solid was separated by filtration over a filter paper (Whatman 1PS), washed with H₂O and dried under HV to give 9.68 g (87 %, 40.8 mmol) of 2 as a white solid.

Mp 245-250°C (decomp.)

\(^1\)H-NMR (300 MHz, d₆-DMSO): 6.70 (br s, 2 H, H₂N); 7.93 (s, 1 H, H-C(6)); 11.26 (br s, 1 H, HN).

\(^{13}\)C-NMR (75 MHz, d₆-DMSO): 71.31 (C5); 156.84 (C6); 159.97, 161.35 (C2, C4).

MS (EI⁺): 237 (100, M⁺), 196 (16), 182 (28), 127 (20).

$^1$H-NMR-spectrum of 2
Experimental Part

2. 2-(N-Benzoylamino)-5-iodo-4-oxo-3,4-dihydropyrimidine (3)

A suspension of 2 (5.00 g, 21.1 mmol) and benzoic anhydride (12.6 g, 55.7 mmol) in dry DMF (80 ml) was warmed to 100°C. After 1.5 h, a clear, slightly yellow soln. was obtained. Most of the DMF was evaporated and the residue suspended in EtOH (150 ml). The solid was separated by filtration over a filter paper (Whatman 1PS) and washed with cold EtOH (150 ml). Drying under HV afforded 5.46 g (76%, 16.0 mmol) of 3 as a white powder.

Mp 296-299°C (decomp.).

$^1$H-NMR (300 MHz, d$_6$-DMSO): 7.54 (t, 2 H, J = 7.5, Bz); 7.67 (t, 1 H, J = 7.4, Bz); 8.03 (d, 2 H, J = 7.4, Bz); 8.31 (s, 1 H, H-C(6)); 12.25 (br s, 2 H, 2 HN).

$^{13}$C-NMR (75 MHz, d$_6$-DMSO): 81.22 (C5); 128.70 (Bz); 132.63 (Bz); 133.36 (Bz); 152.52 (C6); 158.96, 160.71 (C2, C4); 169.80 (C=O Bz).

MS (EI$^+$): 341 (67, M$^+$), 105 (100), 77 (73).

HR-MS (ESI$^+$, [M + H]$^+$): 341.9752 (C$_{11}$H$_9$N$_3$O$_2$I requires: 341.9739).
$^1$H-NMR-spectrum of 3
3. 2-benzoylamino-5-[2'-deoxy-3',5'-bis-O-(tert-butylidemethyisilyl)-β-D-erythro-pent-2-enofuranosyl]-3H-pyrimidin-4-one (5)

To a suspension of 3 (1.83 g, 5.37 mmol) in dry DMF (11 ml) was added dropwise N,O-bis(trimethylsilyl)acetamide (BSA, 1.66 ml, 6.77 mmol). After 1 h, N,N-diisopropylethylamine (1.27 ml, 7.39 mmol) and 4 (778 mg, 2.26 mmol) were added to the clear solution. To a solution of triphenylarsine (262 mg, 0.86 mmol) in dry DMF (32 ml) in a separate flask was added Pd(OAc)$_2$ (105 mg, 0.47 mmol). After 30 min, this solution was added dropwise to the first solution and the mixture was heated to 80°C for 22 h. The reaction was quenched by addition of H$_2$O (15 ml), and most of the solvents were evaporated. The residue was diluted with AcOEt, washed with H$_2$O, the organic phase separated, dried (MgSO$_4$) and evaporated. FC (AcOEt/hexane 3:7) gave a yellow solid. Crystallization from hexane afforded 857 mg (68 %) of 5 as a white solid.

$^1$H-NMR (300 MHz, CDCl$_3$): 0.03, 0.05, 0.16, 0.21 (4 s, 12 H, 4 CH$_3$-Si); 0.86, 0.92 (2 s, 18H, 2 (CH$_3$)$_3$C-Si); 3.72 (dd, $J_1 = 3.5$, $J_2 = 11.2$, 1 H, H-C(5')); 3.85 (dd, $J_1 = 2.0$, $J_2 = 11.2$, 1 H, H-C(5')); 4.56 (m, 1 H, H-C(4')); 4.97 (m, 1 H, H-C(2')); 5.80 (m, 1 H, H-C(1')); 7.49-7.55 (m, 2 H, Bz); 7.61-7.66 (m, 1 H, Bz); 7.98-8.00 (m, 2 H, Bz); 8.02 (s, 1 H, H-C(6)).

$^{13}$C-NMR (75 MHz, CDCl$_3$): -5.36, -5.13, -4.89 (4 CH$_3$-Si); 17.99, 18.43 (2 (CH$_3$)$_3$C-Si); 25.53, 25.90 (2 (CH$_3$)$_3$C-Si); 63.59 (C5'); 78.05, 83.99 (C4', C1'); 100.20 (C2'); 128.03, 128.89 (2 Bz); 133.47 (C6); 150.15 (C3').

$^1$H-NMR-spectrum of 5
4. N^4-benzoyl-2'-deoxypseudoisocytidine (6)

A solution of HF-pyridine (70 % HF, 0.66 ml, ca. 25 mmol HF) in THF (10 ml) was added dropwise to a solution of 5 (756 mg, 1.36 mmol) in THF (28 ml). In intervals of 10 h, additional portions of HF-pyridine (0.33 ml, ca. 13 mmol HF) were added until the starting material had completely disappeared (TLC control). After 28 h the obtained suspension was diluted with AcOH (8 ml) and evaporated. The residue was dissolved without further purification in a mixture of AcOH (25 ml) and MeCN (25 ml), cooled to –15°C, and NaBH(OAc)_3 (718 mg, 3.39 mmol) was added in portions (288 mg followed by 430 mg after 30 min). After 2 h the mixture was distributed between AcOEt and H_2O. The organic layer was dried (MgSO_4) and evaporated. FC (CH_2Cl_2/MeOH 9:1) resulted in 289 mg (65 %, 0.87 mmol) of 6 as a white solid.

^1H-NMR (300 MHz, CD_3OD): 2.09 (ddd, J_1 = 5.9, J_2 = 10.1, J_3 = 12.9, 1 H, Hβ-C(2')); 2.33 (ddd, J_1 = 1.8, J_2 = 5.9, J_3 = 12.9, 1 H, Hα-C(2')); 3.68 (dd, J_1 = 4.6, J_2 = 11.8, 1 H, H-C(5')); 3.73 (dd, J_1 = 4.1, J_2 = 11.8, 1 H, H-C(5')); 3.96-4.00 (m, 1 H, H-C(4')); 4.36-4.38 (m, 1 H, H-C(3')); 5.12 (dd, J_1 = 5.9, J_2 = 9.9, 1 H, H-C(1')); 7.56-7.61 (m, 2 H, Bz); 7.68-7.72 (m, 1 H, Bz); 7.98 (s, 1 H, H-C(6)); 8.05-8.08 (m, 2 H, Bz).

Difference-NOE (500 MHz, CD_3OD): 2.09 (Hβ-C(2')) → 2.33 (11.05 %, Hα-C(2')); 2.33 (Hα-C(2')) → 2.09 (8.42 %, Hβ-C(2')); 3.68-4.00 (H-C(4')) / 4.36-4.38 (3.21 %, H-C(3')) / 7.98 (0.52 %, H-C(6)); 3.64-3.76 (H2-C(5')) / 3.96-4.00 (5.62 %, H-C(4')) / 4.36-4.38 (3.21 %, H-C(3')) / 7.98 (0.52 %, H-C(6)); 3.64-4.00 (H-C(4')) / 3.96-4.00 (3.95 %, H2-C(5')) / 4.36-4.38 (2.21 %, H-C(3')) / 5.12 (2.75 %, H-C(1')); 3.64-3.76 (H2-C(5')) / 3.96-4.00 (2.85 %, H-C(4')) / 3.96-4.00 (2.85 %, H-C(4')) / 3.96-4.00 (2.85 %, H-C(4')) / 3.96-4.00 (2.85 %, H-C(4')); 5.12 (H-C(1')) → 2.33 (1.17 %, Hα-C(2')); 3.64-3.76 (2.49 %, H2-C(5')) / 3.96-4.00 (2.85 %, H-C(4')) / 7.98 (3.94 %, H-C(6)); 7.98 (H-C(6)) → 2.09 (0.84 %, Hβ-C(2')) / 3.64-3.76 (0.79 %, H2-C(5')) / 5.12 (4.96 %, H-C(1')).
\[ ^{13}\text{C-NMR (75 MHz, CD}_3\text{OD): 42.09 (t, C(2')); 64.33 (t, C(5')); 74.74, 76.74 (2 d, C(1'), C(3')); 89.2 (d, C(4')); 129.66, 130.11 (2 d, Bz); 134.67 (d, C(6)).} \]

HR-MS (FAB\(^+\), [M + H]\(^+\)): 332.1261 (calc. 332.1246).

\[ ^{1}\text{H-NMR-spectrum of 6} \]
5. N^4-benzoyl-5'-O-(dimethoxytrityl)-2'-deoxypseudoisocytidine (7)

To a solution of 6 (76 mg, 0.23 mmol) in dry pyridine (1 ml) was added 4,4'-dimethoxytrityl chloride (94 mg, 0.27 mmol) and DMAP (2.8 mg, 0.02 mmol). Additional portions of 4,4'-dimethoxytrityl chloride (39 mg, 0.11 mmol, each) were added in intervals of 1 h until no more starting material was detected by TLC (total reaction time of 6 h). The solution was diluted with AcOEt, washed with water, the organic phase dried (MgSO₄) and evaporated. The residue was purified by FC (CH₂Cl₂/MeOH 2%, conditioned with 1% Et₃N) to give 121 mg (84 %, 0.19 mmol) of 7 as a white foam.

¹H-NMR (300 MHz, CDCl₃): 1.93 (ddd, J₁ = 6.3, J₂ = 9.2, J₃ = 13.2, 1 H, Hβ-C(2')); 2.48 (ddd, J₁ = 2.9, J₂ = 6.3, J₃ = 13.2, 1 H, Hα-C(2')); 3.22 (dd, J₁ = 5.3, J₂ = 9.9, 1 H, H-C(5')); 3.31 (dd, J₁ = 4.4, J₂ = 9.9, 1 H, H-C(5')); 3.78 (s, 6 H, 2 OCH₃); 4.04 (m, 1 H, H-C(4')); 4.39 (m, 1 H, H-C(3')); 5.17 (dd, J₁ = 6.1, J₂ = 9.0, 1 H, H-C(1')); 6.81-6.84 (m, 4 H, DMT); 7.18-7.33 (m, 7 H, DMT); 7.42-7.45 (m, 2 H, DMT); 7.48-7.53 (m, 2 H, Bz); 7.59-7.64 (m, 1 H, Bz); 7.81 (s, 1 H, H-C(6)); 7.93-7.96 (m, 2 H, Bz).

¹³C-NMR (75 MHz, CDCl₃): 41.16 (t, C(2')); 55.20 (q, 2 OCH₃); 64.30 (t, C(5')); 73.85, 74.19 (2 d, C(1'), C(3')); 85.49 (d, C(4')); 113.13, 126.81, 127.82, 127.92 (4 d, DMT); 128.15, 128.95 (2 d, Bz); 130.02, 130.04 (2 d, DMT); 133.57 (d, C(6)); 135.93, 144.73, 158.49 (3 s, DMT).

HR-MS (FAB⁺, [M + H]⁺): 634.2525 (calc. 634.2553).
$^1$H-NMR-spectrum of 7
6. N⁴-benzoyl-5'-O-(dimethoxytrityl)-2'-deoxypseudoisocytidine-3'-O-(2-cyanoethyl-N,N-diisopropyl)phosphoramidite (8)

To a solution of 7 (107 mg, 0.17 mmol) in dry THF (4 ml) was added EtNiPr₂ (145 µl, 0.84 mmol) followed by 2-cyanoethyl diisopropylchlorophosphoramidite (94 µl, 0.42 mmol). After 70 min, 5 % aq. NaHCO₃ was added and the mixture extracted twice with AcOEt. The combined organic layers were dried (MgSO₄) and evaporated. FC (AcOEt/hex ane 6:4, conditioned with 1% Et₃N) gave 107 mg (76 %, 0.13 mmol) of 8 as a white foam.

¹H-NMR (300 MHz, CDCl₃): 1.06-1.33 (m, 12 H, 4 CH₃-C); 1.86-1.96 (m, 1 H, Hβ-C(2')); 2.45 (t, J = 5.0, 1 H, -CH₂CN); 2.55-2.67 (m, 2 H, Ha-C(2'), -CH₂CN); 3.21-3.29 (m, 2 H, H-C(5')); 3.778, 3.785 (2 s, 6 H, 2 OCH₃); 3.50-3.86 (m, 4 H, 2 H-C(CH₃)₂, OCH₂); 4.18 (m, 1 H, H-C(4')); 4.49 (m, 1 H, H-C(3')); 5.16 (m, 1 H, H-C(1')); 6.80-6.84 (m, 4 H, DMT); 7.18-7.34 (m, 7 H, DMT); 7.43-7.45 (m, 2 H, DMT); 7.49-7.53 (m, 2 H, Bz); 7.60-7.63 (m, 1 H, Bz); 7.83, 7.86 (2 s, br, 1 H, H-C(6)); 7.93-7.95 (m, 2 H, Bz).

¹³C-NMR (75 MHz, CDCl₃): 20.16, 20.34 (2 dt, J(C, P) = 6.68, OCH₂CH₂CN); 24.53, 24.58, 24.63, 24.68 (4 q, 2 (CH₃)₂CH); 29.68 (t, C(2')); 43.10 (dd, J(C, P) = 5.80, (CH₃)₂CH); 43.27 (dd, J(C, P) = 6.07, (CH₃)₂CH); 55.20, 55.21 (2 q, 2 OCH₃); 58.25, 58.32 (2 dt, J(C, P) = 18.19, OCH₂CH₂CN); 63.80, 63.95 (2 t, C(5')); 74.14, 74.20 (2 d, C(1')); 75.19, 75.55 (2 dd, J(C, P) = 17.29, C(3')); 85.03, 85.25 (2 dd, J(C, P) = 4.86, C(4')); 86.07 (s, C(5')-O-C); 113.07 (d, DMT); 117.50, 117.63 (2 s, CN); 126.75, 126.79, 127.78, 127.82 (4 d, DMT); 128.19, 128.25, 129.03 (3 d, Bz); 130.07, 130.11 (2 d, DMT); 133.65 (d, C(6)); 135.97, 144.75, 158.42 (3 s, DMT).

³¹P-NMR (161.9 MHz, CDCl₃): 149.04, 149.56.
HR-MS (FAB$, [M + H]^+$): 834.3635 (calc. 834.3631).

$^1$H-NMR-spectrum of 8
7. 2-Hydroxy-5-iodopyridine (10)

To a solution of 2-hydroxypyridine (14, 5.00 g, 52.6 mmol) in acetic acid (75 mL) was added N-iodosuccinimide (11.83 g, 52.6 mmol) and the mixture was stirred at r.t. for 4h. The red solution was then concentrated by rotary evaporation and the residue was purified by FC eluting with AcOEt/hexane (6:4) to (1:0). The obtained yellow product was crystallized from MeOH/hexane to yield 5.06 g (46 %) of 10 as a white solid. The spectroscopic data measured for 10 were compared and found similar to the one described in the literature. [1]

TLC (AcOEt): Rf 0.32

$^1$H-NMR (300 MHz, d$_6$-DMSO): 6.22 (d, J = 9.6, 1H, H3); 7.56 (dd, J$_1$ = 2.6, J$_2$ = 9.6, 1H, H4); 7.65 (d, J = 2.6, 1H, H6); 11.68 (br s, 1H, NH).

$^{13}$C-NMR (75 MHz, d$_6$-DMSO): 66.17 (C5); 121.61 (C3); 141.99, 148.10 (C4, C6); 161.39 (C2).

1H-NMR-spectrum of \textbf{10}
8. 2-Amino-5-iodopyridine (19)

\[
\begin{align*}
\text{Br} & \quad \text{NH}_2 \\
C_5\text{H}_5\text{BrN}_2 & \quad \text{Mol. Wt.: 173,01} \\
\text{20} & \\
\end{align*}
\]

\[
\begin{align*}
\text{I} & \quad \text{NH}_2 \\
C_5\text{H}_5\text{IN}_2 & \quad \text{Mol. Wt.: 220,01} \\
\text{19} & \\
\end{align*}
\]

To a solution of 2-amino-5-bromopyridine (20, 1.5 g, 8.7 mmol) in dry THF (24 mL) at -78°C was added dropwise BuLi (). After 1 h a solution of 1,2-Bis-(chlorodimethylsilyl)ethane (1.9 g, 8.7 mmol) in THF (4.5 mL) was slowly added and the solution was stirred for additional 90 min at -78°C. Another dropwise addition of BuLi (1.6 M in hexane, 5.7 mL, 9.1 mmol) was then performed and the temperature was allowed to reach rt over a period of 2 h. The reaction mixture was cooled down again at -78°C and a third amount of BuLi (1.6 M in hexane, 5.7 mL, 9.1 mmol) was added followed after 1 h by a solution of iodine (2.4 g, 9.5 mmol) in THF (20 mL). The temperature was slowly risen to rt over 1 h and the solution diluted with AcOEt (20 mL). A solution of 20 % aq. Na$_2$S$_2$O$_3$ (60 mL) was added, the phases were separated and the aqueous phase was extracted with 40 mL AcOEt. The combined organic phases were dried (MgSO$_4$) and evaporated. The brown oily residue was dissolved in 10 mL ether, 2 M aq HCl (30 L) was added and the biphasic solution was vigorously stirred for 45 min. The mixture was washed with ether (40 mL), the water phase was neutralized with aq sat NaHCO$_3$ to pH 7 and then extracted with 3x40 mL AcOEt. The organic phase was dried (MgSO$_4$) and evaporated to yield a yellowish solid. FC (AcOEt/hexane 4:6) followed by crystallization of the collected yellowish solid afforded 1.3 g (69 %) of 19 as off-white crystals.

TLC (AcOEt/hexane 1:1): R$_f$ 0.44

$^1$H-NMR (300 MHz, CDCl$_3$): 4.47 (br s, 2H, NH$_2$); 6.36 (dd, J$_1$ = 0.7, J$_2$ = 8.5, 1H, H3); 7.63 (dd, J$_1$ = 2.3, J$_2$ = 8.5, 1H, H4); 8.22 (dd, J$_1$ = 0.7, J$_2$ = 2.3, 1H, H6).

$^{13}$C-NMR (75 MHz, CDCl$_3$): 77.88 (C5); 110.84 (C3); 145.35 (C4); 153.75 (C6); 157.21 (C2).

MS (EI$^+$): 221 (23, [M + H]$^+$), 220 (100, M$^+$), 193 (52), 93 (48), 66 (94).

$^1$H-NMR-spectrum of 19

![NMR Spectrum Image]
9. **2-Benzylamino-5-iodopyridine (21)**

![Chemical Structures](image)

To a suspension of 19 (430 mg, 2.0 mmol) in CH₂Cl₂ (2 mL) was added pyridine (173 µL, 2.2 mmol) and the mixture was cooled down to 0°C. BzCl (249 µL, 2.2 mmol) was then added slowly and the temperature was allowed to reach rt over 2h. The reaction was stirred at rt for 20 h, the suspension was then distributed between CH₂Cl₂ (15 mL) and water (10 mL), the organic phase washed with 5 % aq HCl (10 mL), 5 % aq NaOH and water (10 mL). The organic phase was subsequently dried (MgSO₄) and evaporated. The brown solid residue was subjected to FC (AcOEt/hexane 1:15) and the obtained white solid was crystallized from CHCl₃/MeOH 1:1 to yield 21 (591 mg, 93 %) as white needles.

TLC (AcOEt/hexane 15:85): Rₜ 0.42

¹H-NMR (300 MHz, CDCl₃): 7.47-7.62 (m, 3H, Bz); 7.89-7.93 (m, 2H, Bz); 8.01 (dd, J₁ = 2.1, J₂ = 8.7, 1H, H4); 8.26 (d, J = 8.7, 1H, H3); 8.44 (d, J = 1.9, 1H, H6); 8.67 (br s, 1H, NH).

¹³C-NMR (75 MHz, CDCl₃): 85.80 (C5); 115.96 (C3); 127.20, 128.90 (CH Bz); 132.48 (C Bz); 133.89 (C Bz); 146.43 (C4); 150.66 (C2); 153.65 (C6); 165.66 (C=O).

MS (EI⁺): 324 (12, M⁺), 105 (100), 77 (59).

HR-MS (EI⁺, M⁺): 323.9760 (C₁₂H₉N₂OI requires: 323.9760).
$^1$H-NMR-spectrum of 21
10. 2-Nitro-5-bromopyridine (22)

A solution of 2-amino-5-bromopyridine (20, 585 mg, 3.4 mmol) in conc. sulphuric acid (8 mL) was added dropwise to a mixture of 10 % aq H₂O₂ (5.5 mL) and conc. sulphuric acid (6 mL) at 0°C. After the addition was complete, the temperature was risen to rt over 5 h. The green solution was then slowly poured into an ice-water mixture (100 mL). A first fraction of product (274 mg) precipitated and was collected by filtration. The filtrate was basified to pH 8 with solid KOH and extracted with 3x50 mL AcOEt. The organic layers were combined, washed with water and brine (50 mL each), dried (MgSO₄) and evaporated. The brown solid was purified by FC (AcOEt/hexane 1:9) and the slightly yellow solid obtained (114 mg) was combined to the first fraction to give 388 mg (57 %) of 22.

\[ \text{C}_5\text{H}_5\text{BrN}_2 \quad \text{Mol. Wt.: 173.01} \]

\[ \text{C}_5\text{H}_3\text{BrN}_2\text{O}_2 \quad \text{Mol. Wt.: 202.99} \]

\( ^1\text{H}-\text{NMR} \) (300 MHz, CDCl₃): 8.18 (d, J = 1.5, 2H, H₃, H₆); 8.71 (t, J = 1.5, 1H, H₄).

\( ^{13}\text{C}-\text{NMR} \) (75 MHz, CDCl₃): 119.31 (C₃); 127.01 (C₅); 142.36 (C₄); 150.10 (C₆); 155.23 (C₂).

MS (EI⁺): 202/204 (15/15, M⁺), 156/158 (100/100), 76 (81), 50 (40).

HR-MS (EI⁺, M⁺): 201.9375 (C₅H₃N₂O₂Br requires: 201.9378).
Experimental Part

$^1$H-NMR-spectrum of 22
11. *trans*-N-[(9-Fluorenylmethoxy)carbonyl]-3-hydroxy-L-proline (25)

A soln. of 9-fluorenylmethyl chloroformate (11.0 g, 42.5 mmol) in dioxane (150 ml) was added to a suspension of *trans*-3-hydroxy-L-proline (24, 5.5 g, 41.9 mmol) in dioxane (300 ml) and 5 % NaHCO₃ (150 ml) at 0°C. After 2 h, the cooling bath was removed and stirring was continued for 7 h. Most of the dioxane was evaporated, the aq. layer washed twice with Et₂O, acidified with 2 N aq. HCl to pH 3.0 and extracted with AcOEt (3 x). The combined org. layers were dried (MgSO₄) and evaporated to give 14.7 g (98 %) of 25 as a white powder.

\[
\begin{align*}
\text{H-NMR} (300 \text{ MHz, } d_6-\text{DMSO}) & : 1.80-1.98 (m, 2H, H4); 3.48-3.54 (m, 2H, H5); 4.05-4.37 (m, 5H, H2, H3, Fmoc CH, Fmoc CH₂); 5.52, 5.59 (2 \text{ br s, 1H, HO}); 7.29-7.44 (m, 4H, arom. Fmoc); 7.62-7.69 (m, 2H, arom. Fmoc); 7.88-7.91 (m, 2H, arom. Fmoc); 12.91 (br s, 1H, HO₂C). \\
\text{C-NMR} (75 \text{ MHz, } d_6-\text{DMSO}) & : 34.38, 35.37 (2 t, C(4)); 47.33, 47.97 (2 t, C(5)); 49.61 (d, Fmoc CH); 69.70, 69.98 (2 t, Fmoc CH₂); 70.91, 71.18 (2 d, C(3)); 75.81, 76.91 (2 d, C(2)); 123.15 (d, arom. Fmoc); 128.14, 128.24 (2 d, arom. Fmoc); 130.16 (d, arom. Fmoc); 143.66, 143.75 (2 s, arom. Fmoc); 146.69, 146.82 (2 s, arom. Fmoc); 157.03, 157.21 (2 s, NCO₂); 174.79, 175.09 (2 s, CO₂H).
\end{align*}
\]

MS (FAB⁺): 354 (26, [M + H]⁺), 338 (24), 309 (100).
HR-MS (FAB\(^+\), [M + H]\(^+\)): 354.13574 (calc. 354.13415).

\(^1\)H-NMR-spectrum of 25
12. (2R,3S)-N-[(9-Fluorenylmethoxy)carbonyl]-3-hydroxy-2-hydroxymethyl pyrrolidine (26)

To a soln. of 25 (13.0 g, 36.8 mmol) in dry THF (130 ml) were added BH₃-(CH₃)₂S (2 M in THF, 96.2 ml, 192.4 mmol). The soln. was heated at reflux for 2 h, cooled to rt and quenched by addition of MeOH. The soln. was evaporated, the residue dissolved in AcOEt, washed with H₂O, dried (MgSO₄) and evaporated. FC (toluene/THF 1:1 to 5:6 gave 12.1 g (97%) of 26 as a white solid.

\[ 25 \quad \text{C}_{20}\text{H}_{19}\text{NO}_5 \quad \text{Mol. Wt.: 353.37} \]
\[ 26 \quad \text{C}_{20}\text{H}_{21}\text{NO}_4 \quad \text{Mol. Wt.: 339.39} \]

TLC (toluene/THF 1:1): Rₜ 0.24

\(^1\)H-NMR (300 MHz, d₆-DMSO): 1.66-1.75 (m, 1H, H₄); 1.94-2.07 (m, 1H, H₄); 3.08-3.20 (m, 1H, CH₂-OH); 3.28-3.57 (m, 4H, H₂, H₅, CH₂-OH); 4.17-4.21 (m, 1H, H₃); 4.27 (s, 3H, Fmoc CH, Fmoc CH₂); 4.76-4.82 (m, 1H, HO-CH₂); 4.92-4.94 (m, 1H, HO-C(3)); 7.33 (t, 2H, J = 7.2, arom. Fmoc); 7.42 (t, 2H, J = 7.0, arom. Fmoc); 7.66 (d, 2H, J = 7.4, arom. Fmoc); 7.90 (d, 2H, J = 7.4, arom. Fmoc).

MS (FAB⁺): 340 (100, [M + H]⁺), 309 (22), 179 (60), 155 (52), 119 (83).

$^1$H-NMR-spectrum of 26
13. \( (2R,3S)-3-[(\text{tert-Butyl})\text{dimethylsilyl}]\text{oxy}-2-[(\text{tert-butyl})\text{dimethylsilyl}]\text{oxymethyl pyrrolidine} \) (27)

To a soln. of 26 (12.0 g, 35.4 mmol) and imidazole (8.4 g, 123 mmol) in dry THF (150 ml) was added \( \text{tert-butyldimethylsilyl} \)chloride (11.7 g, 77.6 mmol). After 2 h, piperidine (23 ml, 233 mmol) was added and stirring was continued for 12 h. The soln. was evaporated and the residue distributed between AcOEt and H\(_2\)O. The org. layer was dried (MgSO\(_4\)) and evaporated. FC (CH\(_2\)Cl\(_2\)/MeOH/25 % aq. NH\(_3\) 50:1:1 to 25:1:1) afforded 11.0 g (31.8 mmol, 90 %) of 27 as a yellow oil.

\(^{1}\)H-NMR (300 MHz, CDCl\(_3\)): 0.06 (s, 12 H, 4 CH\(_3\)-Si); 0.88, 0.89 (2 s, 18 H, 2 (CH\(_3\))\(_3\)C-Si); 1.63-1.72 (m, 1 H, H-C(4)); 1.83-1.95 (m, 1 H, H-C(4)); 2.22 (s, 1 H, HN); 2.90-3.11 (m, 3 H, H\(_2\)-C(5)); 3.54-3.64 (m, 2 H, CH\(_2\)-OTBDMS); 4.10-4.15 (m, 1 H, H-C(3)).

\(^{13}\)C-NMR (75 MHz, CDCl\(_3\)): −5.50, −5.45, −4.76, −4.59 (4 q, 4 CH\(_3\)-Si); 18.02, 18.27 (2 s, 2 (CH\(_3\))\(_3\)C-Si); 25.82, 25.90 (2 q, 2 (CH\(_3\))\(_3\)C-Si); 35.28 (t, C(4)); 44.82 (t, C(5)); 63.12 (t, CH\(_2\)-OTBDMS); 68.14 (d, C(2)); 73.77 (d, C(3)).

MS (FAB\(^{+}\)): 346 (100, [M + H]\(^{+}\)), 288 (8), 200 (17).

HR-MS (FAB\(^{+}\), [M + H]\(^{+}\)): 346.25998 (calc. 346.25976).
Experimental Part

$^1$H-NMR-spectrum of 27
To an ice cold solution of 27 (1.12 g, 3.2 mmol) in 90 mL of a 1:3 mixture of 1 M aqueous NaOH/THF (22.5 mL/67.5 mL) was added di-tert-butyl-dicarbonate (2.13 g, 9.7 mmol). After addition the ice bath was removed and the mixture was stirred vigorously overnight. The volatiles were then removed and the residue was distributed between Et₂O (50 mL) and water (50 mL). The phases were separated and the aqueous phase was subsequently extracted twice with Et₂O (50 mL). The combined organic phases were dried (MgSO₄) and evaporated. The crude product was subjected to FC in AcOEt/hexane (1:19) to yield 1.42 g (98 %) of pure 28 as yellow oil.

TLC (AcOEt/hexane 1:9): R_f 0.66

H-NMR (300 MHz, CDCl₃): 0.02, 0.03, 0.04, 0.06 (4s, 12H, 4 CH₃-Si); 0.86, 0.88 (2s, 18H, 2 (CH₃)₃C-Si); 1.45, 1.46 (2s, 9H, (CH₃)₃C-O); 1.65-1.76 (m, 1H, H₄); 1.90-2.06 (m, 1H; H₄); 3.28-3.75 (m, 5H, H₂, 2H₅, CH₂O); 4.34-4.37 (m, 1H, H₃).

C-NMR (75 MHz, CDCl₃): -5.53, -5.45, -5.39, -4.73, -4.64 (4 CH₃-Si); 17.98, 18.22 (2 (CH₃)₃C-Si); 25.76, 25.79, 25.87 (2 (CH₃)₃C-Si); 28.52, 28.56 ((CH₃)₃C-O); 31.92, 33.01 (C₄); 44.72, 45.22 (C₅); 61.81, 62.58 (CH₂O); 67.60, 67.78 (C₂); 73.55, 73.89 (C₃); 78.91, 79.26 ((CH₃)₃C-O); 154.74, 154.94 (C=O).

MS (ESI⁺): 446 (35, [M + H]⁺), 390 (40), 346 (100), 214 (38).

HR-MS (ESI⁺, [M + H]⁺): 446.3120 (C₂₂H₄₈NO₄Si₂ requires: 446.3121).
$^1$H-NMR-spectrum of 28
15. 3-(tert-Butyl-dimethyl-silanyloxy)-2-(tert-butyl-dimethyl-silanyloxymethyl)-5-oxo-pyrrolidine-1-carboxylic acid tert-butyl ester (29)

\[
\begin{align*}
\text{TBDMSO} & \quad \text{N} \\
\text{TBDMSO} & \quad \text{Boc} \\
\text{28} & \quad \text{C}_{22}\text{H}_{47}\text{NO}_{4}\text{Si}_{2} \\
\text{Mol. Wt.: 445.78} \\
\text{TBDMSO} & \quad \text{N} \\
\text{TBDMSO} & \quad \text{Boc} \\
\text{29} & \quad \text{C}_{22}\text{H}_{45}\text{NO}_{5}\text{Si}_{2} \\
\text{Mol. Wt.: 459.77}
\end{align*}
\]

RuO₂ (74 mg, 0.6 mmol) was added to an aqueous solution of 10% NaIO₄ (14.8 mL, 6.9 mmol NaIO₄). After 5 min a solution of 28 (1.23 g, 2.8 mmol) in AcOEt (8.2 mL) was added to the yellow solution. The obtained dark green solution was vigorously stirred till no starting material was detected anymore by TLC (typically after 4h.). The phases were separated and the water phase was extracted with 3x30 mL AcOEt. The combined organic phases were treated with 10 mL of iPrOH for 30 min then the black precipitate was filtered off thru celite, the filtrate was dried (MgSO₄) and evaporated. The resulting brown oil was purified by FC in AcOEt/hexane (1:20) to yield 981 mg of pure 29 (77%) as a white solid.

TLC (AcOEt/hexane 1:9): \(R_f 0.43\)

\(^1\)H-NMR (300 MHz, CDCl₃): 0.02, 0.04, 0.07, 0.08 (4s, 12H, 4 CH₃-Si); 0.87 (s, 18H, 2 (CH₃)₃C-Si); 1.53 (s, 9H, (CH₃)₃C-O); 2.30 (d, \(J = 17.50\), 1H, H₃); 2.85 (dd, \(J_1 = 5.7, J_2 = 17.5\), 1H, H₃); 3.77-3.79 (m, 2H, CH₂O); 3.94-3.97 (m, 1H, H₅); 4.29 (d, \(J = 5.7\), 1H, H₄).

\(^1\)C-NMR (75 MHz, CDCl₃): -5.62, -4.77, -4.73 (4 CH₃-Si); 17.96, 18.17 (2 (CH₃)₃C-Si); 25.68, 25.80 (2 (CH₃)₃C-Si); 28.06 (((CH₃)₃C-O); 42.88 (C3); 62.09 (CH₂O); 67.63, 68.79 (C4, C5); 82.87 (((CH₃)₃C-O); 150.10 (C=O); 173.29 (C2).

MS (ESI⁺): 460 (18, \([M + H]^+\)), 404 (25), 360 (100).

HR-MS (ESI⁺, \([M + Na]^+\)): 482.2731 (C₂₂H₄₅NO₅NaSi₂ requires: 482.2734).
$^1$H-NMR-spectrum of 29
16. 2-Hydroxy-5-bromopyridine (30)

To a solution of 2-hydroxypyridine (14, 10.00 g, 105 mmol) in CH$_3$CN (500 mL) was added NBS (18.72 g, 105 mmol) and the reaction mixture was stirred at rt protected from light. After 3 days, the solvent was removed by rotary evaporation to yield yellow oil. Purification by FC (AcOEt/hexane 8:2 to 1:0) yielded 14.17 g (77 %) of 30 as white solid.

TLC (AcOEt): R$_f$ 0.20

$^1$H-NMR (300 MHz, d$_6$-DMSO): 6.34 (d, J = 9.5, 1H, H3); 7.54 (dd, J$_1$ = 2.9, J$_2$ = 9.5, 1H, H4); 7.68 (d, J = 2.9, 1H, H6); 11.75 (br s, 1H, NH).

$^{13}$C-NMR (75 MHz, d$_6$-DMSO): 98.63 (C5); 120.36 (C3); 137.91 (C4); 143.39 (C6); 161.50 (C2).

MS (EI$^+$): 173/175 (97/97, $M^+$), 145/147 (100/100), 94 (21), 66 (79), 39 (80).
Experimental Part

$^1$H-NMR-spectrum of 30
17. 4-(tert-Butyl-dimethyl-silyloxy)-5-(tert-butyl-dimethyl-silyloxymethyl)-2-hydroxy-2-((6-oxo-1,6-dihydro-pyridin-3-yl)-pyrrolidine-1-carboxylic acid tert-butyl ester (32)

![Chemical Structure](image)

To a suspension of 10 (433 mg, 2.0 mmol) in dry THF (3 mL) was added BSA (532 µL, 2.2 mL). After 30 min, the obtained clear solution was cooled down to -78°C and BuLi (1.22 mL, 2.0 mmol) was added dropwise. The solution was stirred for another 30 min and was added to a solution of 29 (500 mg, 1.1 mmol) in dry THF (4 mL) at -78°C. After 2 h the reaction was quenched with sat aq NaHCO₃ (7 mL) and the temperature was allowed to reach rt. The layers were separated and the aqueous phase was extracted with 2x10 mL Et₂O. The combined organic phases were dried (MgSO₄) and evaporated to produce a yellow solid. FC (AcOEt/hexane 1:9 to 2:8) yielded 251 mg (42 %) of 32 as a white solid.

TLC (AcOEt/hexane 2:8): Rf 0.41

¹H-NMR (300 MHz, CDCl₃): 0.00, 0.02, 0.04, 0.06 (4s, 12H, 4 CH₃-Si); 0.83, 0.88 (2s, 18H, 2 (CH₃)₃C-Si); 1.25 (s, 1H); 1.53, 1.54 (2s, 18H, ((CH₃)₃C-O); 1.96 (dd, J₁ = 1.9, J₂ = 18.5, 1H, H3); 2.64 (dd, J₁ = 9.8, J₂ = 18.5, 1H, H3); 3.31 (d, J = 9.4, 1H); 3.68-3.73 (m, 1H); 3.89 (dd, J₁ = 4.1, J₂ = 10.3, 1H); 4.04-4.13 (m, 2H, CH₂O); 4.33-4.35 (m, 1H); 6.21 (d, J = 6.2, 1H); 6.91 (d, J = 6.2, 1H).

¹³C-NMR (75 MHz, CDCl₃): -5.69, -5.61, -5.59, -5.56 (4 CH₃-Si); 18.01, 18.12 (2 (CH₃)₃C-Si); 25.64, 25.77 (2 (CH₃)₃C-Si); 28.08 ((CH₃)₃C-O); 33.38 (C3); 36.51; 59.98; 62.48; 63.58; 64.18, 64.90; 73.18; 82.97; 83.35; 83.51; 127.09; 129.24; 148.74; 149.51; 149.71; 149.78; 168.70; 172.45

MS (ESI⁺): 555 (82, [M + H⁺]), 455 (100), 360 (60), 228 (68).
Experimental Part

$\textsuperscript{1}$H-NMR-spectrum of 32
18. 2-Benzylcyloxy-5-bromopyridine (34)

\[
\begin{align*}
&\text{30} \\
&\text{C}_8\text{H}_4\text{BrNO} \\
&\text{Mol. Wt.:} 174.00
\end{align*}
\]

\[
\begin{align*}
&\text{34} \\
&\text{C}_{12}\text{H}_{10}\text{BrNO} \\
&\text{Mol. Wt.:} 264.12
\end{align*}
\]

To a solution of 30 (5.00 g, 28.7 mmol) in dry THF (250 mL) was added BnBr (4.1 mL, 34.5 mmol) and Ag$_2$CO$_3$ (4.75 g, 17.2 mmol). The mixture was refluxed in darkness for 5h and then filtered through celite, washed with ether and evaporated. The residue was purified by FC (AcOEt 2 to 5 % in hexane) to yield 7.04 g (93 %) of 34 as white solid.

TLC (AcOEt/hexane 2:98): $R_f$ 0.26

$^1$H-NMR (300 MHz, CDCl$_3$): 5.35 (s, 2H, CH$_2$ Bn); 6.73 (d, $J = 8.7$, 1H, H3); 7.29-7.46 (m, 5H, Bn); 7.66 (dd, $J_1 = 2.5$, $J_2 = 8.7$, 1H, H4); 8.21 (d, $J = 2.5$, 1H, H6).

$^{13}$C-NMR (75 MHz, CDCl$_3$): 67.98 (CH$_2$ Bn); 111.89 (C5); 112.93 (C3); 127.97, 128.00, 128.49, 136.89 (Bn); 141.18 (C4); 147.44 (C6); 162.40 (C2).

MS (EI$^+$): 263/265 (56/56, $M^+$), 184 (8), 157/159 (15/15), 91 (100), 65 (55)

HR-MS (EI$^+$, $M^+$): 262.9941 (C$_{12}$H$_{10}$BrNO requires: 262.9946)
$^1$H-NMR-spectrum of 34
19. 1-Benzyl-5-bromo-1H-pyridin-2-one (35)

To a suspension of 30 (1.26 g, 7.2 mmol), K$_2$CO$_3$ (2.00 g, 14.5 mmol) and nBu$_4$NBr (10% w/w, 126 mg) in CH$_2$Cl$_2$ stirred at rt was added BnBr (860 µL, 7.2 mmol). After 3h, all the starting material has reacted and the solids were removed by filtration. The filtrate was evaporated and the resulting slightly yellowish solid was purified by column chromatography in AcOEt/hexane 2:3 to give 1.31 g (68%) of 35 as white solid.

TLC (AcOEt/hexane 2:3): R$_f$ 0.24

$^1$H-NMR (300 MHz, CDCl$_3$): 5.10 (s, 2H, CH$_2$ Bn); 6.53 (d, J = 9.6, 1H, H3); 7.28-7.40 (m, 7H, H4, H6, Bn).

$^{13}$C-NMR (75 MHz, CDCl$_3$): 52.07 (CH$_2$ Bn); 98.09 (C5); 122.39 (C3); 128.23, 128.33, 129.02, 135.63 (Bn) 136.95 (C4); 142.44 (C6); 161.05 (C2).

MS (EI$^+$): 263/265 (18/18, M$^+$), 91 (100), 57 (63)

HR-MS (EI$^+$, M$^+$): 262.9940 (C$_{12}$H$_{10}$BrNO requires: 262.9946)
$^1$H-NMR-spectrum of 35
Experimental Part

20. 5-Bromo-2-(bis-(4-methoxy-benzyl)-amino)-pyridine (36)

\[
\begin{align*}
\text{Br} & \quad \text{N} \\
\text{NH}_2 & \quad \text{NPMB}_2 \\
\text{C}_6\text{H}_5\text{BrN}_2 & \quad \text{C}_{21}\text{H}_{21}\text{BrN}_2\text{O}_2 \\
\text{Mol. Wt.:} & \quad 173.01 \\
\text{Mol. Wt.:} & \quad 413.31 \\
\end{align*}
\]

To a solution of 2-amino-5-bromo-pyridine (20, 1 g, 5.8 mmol) in dry THF (20 mL) under argon was added sodium hydride (60% in oil, 578 mg, 14.4 mmol) followed by 4-methoxy-benzylchloride (1.97 mL, 14.4 mmol). The disappearance of the starting material was monitored by TLC in AcOEt/hexane (2:8). The reaction was quenched by slow addition of sat aq NH₄Cl (6 mL) to the brown suspension. Water (20 mL) was then added and the organic phase was separated. The aqueous phase was extracted with twice 30 mL of ether. The combined organics were washed with brine (20 mL), dried (MgSO₄) and evaporated to yield dark yellow oil. 2.15 g (90%) of pure 36 were obtained as white solid after F.C. using AcOEt/hexane (1:19) for elution.

TLC (AcOEt/hexane 2:8): Rf 0.66

\(^1\)H-NMR (300 MHz, CDCl₃): 3.80 (s, 6H, 2 CH₃O); 4.68 (s, 4H, 2 CH₂ PMB); 6.36 (dd, J₁ = 0.4, J₂ = 9.0, 1H, H₃); 6.86 (m, 4H, CH arom.); 7.15 (m, 4H, CH arom.); 7.42 (dd, J₁ = 2.7, J₂ = 9.0, 1H, H₄); 8.22 (dd, J₁ = 0.4, J₂ = 2.7, 1H, H₆).

\(^{13}\)C-NMR (75 MHz, CDCl₃): 50.38 (2 CH₂ PMB); 55.20 (2 CH₃O); 106.36 (C5); 107.49 (C3); 113.97 (4 CH arom.); 128.23 (4 CH arom.); 129.75 (2 C arom.); 139.56 (C4); 148.33 (C6); 157.07, 158.71 (C2, C arom).

MS (EI\(^+\)): 412/414 (28/28, [M + H\(^+\)]\(^\ddagger\)), 291/293 (82/82), 121 (100), 77 (39).

HR-MS (EI\(^+\), \(M^+\)): 412.0788 (C\(_{21}\)H\(_{21}\)BrN\(_2\)O\(_2\) requires: 412.0786).
1$^1$H-NMR-spectrum of 36
21. 3-(tert-Butyl-dimethyl-silanyloxy)-2-(tert-butyl-dimethyl-silanyloxymethyl)-5-methoxy-pyrrolidine-1-carboxylic acid tert-butyl ester (37)

To a solution of 29 (578 mg, 1.3 mmol) in dry THF (10 mL) at -78°C was added a solution of 1M super hydride in THF (LiBEt₃H, 1.5 mmol, 1.51 mL). After 30 min, the reaction was quenched with 3.5 mL of aq sat NaHCO₃ and the mixture was warmed up to 0°C. 8 drops of 30% aq H₂O₂ were added and the mixture was stirred 20 min. The organic layer was then isolated and the aq phase was extracted with 3x10 mL of Et₂O. The combined organics were dried (MgSO₄) and evaporated. The residue was dissolved in dry Et₂O (5 mL) and 4Å powder molecular sieves (35.5 mg) were added. While stirring, methyl orthoformiate (275 µL, 2.5 mmol) and BF₃•Et₂O (41 µL) were added. After 2 h the reaction was quenched with 5 mL of aq sat NaCl and 20 drops of Et₃N. The phases were separated and the aq phase was extracted with 2x10 mL of Et₂O. The organic phases were joined, dried (MgSO₄), evaporated and the crude was purified by FC (AcOEt/hexane 1:24). Compound 37 was obtained as colorless oil in 52% yield (309 mg) over 2 steps.

TLC (AcOEt/hexane 1:9): Rf 0.57

¹H-NMR (300 MHz, CDCl₃): 0.05, 0.06 (2s, 12H, 4 CH₃-Si); 0.86, 0.90 (2s, 18H, 2 (CH₃)₃C-Si); 1.47 (s, 9H, (CH₃)₃C-O); 1.93-2.09 (m, 2H, H3); 3.32 (br s, 3H, OCH₃); 3.60-3.79 (m, 3H, H5, OCH₂); 4.56 (br s, 1H, H4); 5.13-5.27 (m, 1H, H2).

¹³C-NMR (75 MHz, CDCl₃): -5.44, -4.85, -4.72 (4 CH₃-Si); 17.87, 18.31 (2 (CH₃)₃C-Si); 25.72, 25.90 (2 (CH₃)₃C-Si); 28.35 ((CH₃)₃C-O); 40.87, 41.44 (C3); 55.68, 55.90 (OCH₃); 60.57, 61.64 (CH₂O); 67.33, 67.84, 70.85, 71.90 (C4, C5); 79.96 ((CH₃)₃C-O); 88.68 (C2); 155.61 (C=O).

MS (ESI⁺): 498 (18, [M + Na]⁺), 476 (10, [M + H]⁺), 444 (100), 344 (35).
HR-MS (ESI⁺, [M + Na]⁺): 498.3034 (C₂₃H₄₉NO₅NaSi₂ requires: 498.3047).
$\textsuperscript{1}$H-NMR-spectrum of 37
22. 4-(tert-Butyl-dimethyl-silyl oxy)-5-(tert-Butyl-dimethyl-silyloxymethy l)-2-(2-oxo-2H-pyridin-1-yl)-pyr rolide-1-carboxylic acid tert-buty l ester (38)

To a suspension of 30 (186 mg, 0.8 mmol) in dry THF (1 mL) was added BSA (226 µL, 0.9 mmol). After 30 min the obtained clear solution was cooled down to -78°C and BuLi (1.6 M in hexane, 525 µL, 0.8 mmol) was added. The solution was stirred for 30 min before being added to a suspension of CuBr-Me2S (173 mg, 0.8 mmol) in THF (1 mL) at -78°C. The resultant green solution was stirred one hour at -78°C. BF3•Et2O (106 µL, 0.8 mmol) was then added and the mixture was stirred for further 30 min. At last a solution of 37 (100 mg, 0.2 mmol) in THF (350µL) was added and the stirring was continued for one hour at -78°C. The temperature was then allowed to rise to rt over an hour. After 1h30 stirring at rt, another portion of BF3•Et2O (106 mL, 0.8 mmol) was added. An hour later, the reaction was quenched with 1 mL of a 1:1 mixture of 25% ammonia/aq sat NH4Cl. The layers were separated and the aq. phase was extracted with 3x3 mL AcOEt. The combined organics were washed with brine, dried (MgSO4) and concentrated to give green solid. FC (AcOEt/hexane 15:85 to 2:8) afforded 70 mg (62 %) of 38 as colorless oil.

TLC (AcOEt/hexane 1:9): Rf 0.57

1H-NMR (300 MHz, CDCl3): -0.08, 0.05, 0.06, 0.07 (4s, 12H, 4 CH3-Si); 0.78, 0.90 (2s, 18H, 2 (CH3)3C-Si); 1.25 (s, 9H, (CH3)3C-O); 1.90-2.12 (m, 1H, H3); 2.65-2.74 (m, 1H, H3) 3.73-3.84 (m, 2H, OCH2); 4.05-4.08 m, 1H, H5); 4.37-4.39 (m, 1H, H 4); 6.05-6.15 (m, 1H, H5’); 6.39 (d, J = 7.7, 1H, H2); 6.47-6.51 (m, 1H, H3’); 7.28-7.32 (m, 1H, H4’); 7.62 (dd, J1 = 1.6, J2 = 7.1, 1H, H6’).

13C-NMR (75 MHz, CDCl3): -5.52, -5.51, -5.14, -5.05 (4 CH3-Si); 17.77, 18.18 (2 (CH3)3C-Si); 25.58, 25.88 (2 (CH3)3C-Si); 27.97 ((CH3)3C-O); 40.93 (C3); 61.07 (CH2O); 70.05 (C5); 70.33 (C2); 73.78 (C4); 80.91 ((CH3)3C-O); 104.61 (C5’); 119.80 (C3’); 134.02 (C6’); 138.95 (C4’); 152.74 (C=O); 162.48 (C2’).
Experimental Part

MS (El⁺): 538 (8, M⁺), 444 (17), 344 (100), 330 (25), 286 (27), 154 (46), 89 (32).

\[ \text{Integral (ppm)} \]

\[
\begin{align*}
0.0 & \quad 0.5 & \quad 1.0 & \quad 1.5 & \quad 2.0 & \quad 2.5 & \quad 3.0 & \quad 3.5 & \quad 4.0 & \quad 4.5 & \quad 5.0 & \quad 5.5 & \quad 6.0 & \quad 6.5 & \quad 7.0 & \quad 7.5 & \quad 8.0 \\
1H-NMR-spectrul of 38
\end{align*}
\]
23. (4S,5R)-4-[(tert-butyldimethylsilyl)oxy-5-[(tert-butyldimethylsilyl)oxymethyl-aza-cyclopent-1-ene (39)

\[
\begin{align*}
\text{C}_{17}H_{39}NO_2Si_2 & \quad \text{Mol. Wt.: 345.67} \\
\text{C}_{17}H_{37}NO_2Si_2 & \quad \text{Mol. Wt.: 343.65}
\end{align*}
\]

To a soln. of 27 (1.0 g, 2.89 mmol) in hexane (40 ml) was added N-chlorosuccinimide (500 mg, 3.74 mmol). After 1 h, the suspension was filtered over Celite and evaporated. The residue was dissolved in dry THF (50 ml) and cooled to –78°C. A soln. of tetramethylpiperidine (1.18 ml, 6.97 mmol) in THF (30 ml), previously treated with BuLi (1.6 M in hexane, 4.06 ml, 6.50 mmol) at 0°C and stirred for 30 min at 0°C, was added over 1 h to the soln. at –78°C until no N-chloro compound was detected by TLC. After 1 h, the mixture was warmed to rt, most of the THF was evaporated and the residue distributed between tert-butylmethylene and H₂O. The org. layer was dried (MgSO₄) and evaporated. FC (hexane/AcOEt 5:1 to 4:1) gave 730 mg (74 %, 2.12 mmol) of 39 as a yellow oil.

TLC (hexane/AcOEt 4:1): Rₜ 0.34

\(^1\)H-NMR (300 MHz, CDCl₃): 0.02, 0.04, 0.08 (3 s, 12 H, 4 CH₃-Si); 0.87, 0.88 (2 s, 18 H, 2 (CH₃)₃C-Si); 2.44 (d, 1 H, J = 18.4, H-C(3)); 2.75 (dd, 1 H, J = 6.2, 18.4, H-C(3)); 3.56 (dd, 1 H, J = 4.8, 10.3, CH₂-OTBDMS); 3.86 (dd, 1 H, J = 3.3, 10.3, CH₂-OTBDMS); 4.06 (m, 1 H, H-C(4)); 4.37 (d, 1 H, J = 6.6, H-C(5)); 7.61 (s, 1 H, H-C(2)).

\(^13\)C-NMR (75 MHz, CDCl₃): -5.55, -5.44, -4.72, -4.64 (4 q, 4 CH₃-Si); 18.01, 18.25 (2 s, 2 (CH₃)₃C-Si); 25.79, 25.85 (2 q, 2 (CH₃)₃C-Si); 47.39 (t, C(3)); 63.08 (t, CH₂-OTBDMS); 71.12 (d, C(4)); 83.47 (d, C(5)); 165.95 (d, C(2)).

MS (FAB\(^+\)): 344 (100, [M + H]\(^+\)), 154 (10), 115 (17).
$^1$H-NMR-spectrum of 39
24. 2-(2-Benzyloxy-pyridin-5-yl)-4-(tert-butyl-dimethyl-silyloxy)-5-(tert-butyl-dimethyl-silyloxy)methyl)-pyrrolidine (41)

To a stirred solution of 34 (1.90 g, 7.2 mmol) in dry THF (33 ml) at -78°C was added dropwise BuLi (1.6 M in hexane, 4.49 ml, 7.2 mmol). After 1 h, a solution of 39 (823 mg, 2.4 mmol) in dry THF (12 ml) was slowly added and the mixture was stirred for 2 h. The reaction was quenched at -78°C with 40 ml of water and the layers were separated. The aqueous phase was extracted with 40 ml of ether. The combined org. phases were dried (MgSO₄) and evaporated to yield yellow oil. FC (AcOEt/hexane 1:8 to 1:5) afforded 809 mg of α/β mixture of 41 as a yellow oil which will be used for the next step without further purification. A pure fraction of the β anomer was isolated for analytical characterization.

TLC (AcOEt/hexane 1:7): Rf 0.27

¹H-NMR (300 MHz, CDCl₃): 0.06, 0.07, 0.08 (3s, 12H, 4 CH₃-Si); 0.90, 0.91 (2s, 18H, 2 (CH₃)₃C); 1.73-1.90 (m, 2H, H3β, NH); 1.97-2.04 (m, 1H, H3α); 3.14-3.18 (m, 1H, H5); 3.55-3.69 (m, 2H, CH₂O); 4.23-4.27 (m, 1H, H4); 4.42 (dd, J₁ = 6.4, J₂ = 9.7, 1H, H2); 5.36 (s, 2H, CH₂ Bn); 6.77 (d, J = 8.8, 1H, H3’); 7.30-7.47 (m, 5H, Bn); 7.62 (dd, J₁ = 2.6, J₂ = 8.8, 1H, H4’); 8.12 (d, J = 2.6, 1H, H6’).

¹³C-NMR (75 MHz, CDCl₃): -5.39, -4.69, -4.57 (4 CH₃-Si); 18.05, 18.33 (2 (CH₃)₃Si); 25.86, 25.94 (2 (CH₃)₃C-Si); 43.78 (C3); 57.64 (C2); 64.81 (CH₂O); 67.57 (CH₂ Bn); 68.86 (C5); 74.26 (C4); 111.01 (C3’); 127.74, 127.91, 128.42 (5 CH Bn); 132.66 (C5’); 137.40 (C4’); 137.45 (C Bn); 144.97 (C6’); 162.93 (C2’).
Difference-NOE (500 MHz, CDCl₃): 1.73-1.90 (H3β) → 1.97-2.04 (6.5 %, H3α), 3.55-3.69 (2.6 %, CH₂O), 4.23-4.27 (6.8 %; H4), 7.62 (3.5 %, H4’), 8.12 (2.6 %, H6’); 1.97-2.04 (H3α) → 1.73-1.90 (19.1 %, H3β), 3.55-3.69 (2.1 %, CH₂O), 4.23-4.27 (3.1 %, H4), 4.42 (5.1 %, H2), 7.62 (2.0 %, H4’), 8.12 (2.1 %, H6’); 3.14-3.18 (H5) → 3.55-3.69 (5.1 %, CH₂O), 4.23-4.27 (2.8 %, H4), 4.42 (3.4 %, H2); 3.55-3.69 (CH₂O) → 3.14-3.18 (5.1 %, H5), 4.23-4.27 (5.7 %, H4); 4.23-4.27 (H4) → 1.73-1.90 (6.0 %, H3β), 1.97-2.04 (1.4 %, H3α), 3.14-3.18 (2.4 %, H5), 3.55-3.69 (2.8 %, CH₂O); 4.42 (H2) → 1.97-2.04 (5.7 %, H3α), 3.14-3.18 (3.1 %, H5), 7.62 (2.2 %, H4’), 8.12 (7.9 %, H6’); 6.77 (H3’) → 5.36 (1.1 %, CH₂ Bn), 7.62 (10.1 %, H4’); 7.62 (H4’) → 6.77 (14.2 %, H3’); 8.12 (H6’) → 4.42 (5.3 %, H2), 5.36 (1.0 %, CH₂ Bn).

MS (ESI⁺): 1058 (34), 530 (100, [M + H]⁺), 344 (8).

HR-MS (ESI⁺, [M + H]⁺): 529.3275 (C₂₉H₄₉N₂O₃Si₂ requires: 529.3281).

\[ ^1H-NMR-spectrum \text{ of } 41 \]
Experimental Part

25. 4-(tert-Butyl-dimethyl-silanyloxy)-5-(tert-butyl-dimethyl-silanyloxymethyl)-2-(2-oxo-pyridin-5-yl)-pyrrolidine (43)

To a solution of 41 (α/β mixture, 809 mg, 1.5 mmol) in MeOH (50 mL) under argon was added 10% Pd/C (10% w/w, 81 mg) and the black suspension was stirred under an atmosphere of hydrogen. The reaction’s evolution was followed by TLC in AcOEt/hexane 1:4 stained with ninhydrin. As soon as no starting material was detected anymore, typically after 30 to 45 min, the palladium catalyst was filtered off over celite, washed with MeOH and the filtrate was evaporated and coevaporated with AcOEt to yield greenish foam. F.C. (AcOEt/THF 1:0 to 4:1) afforded 352 mg of 43 (35 % over two steps) as yellowish foam.

TLC (CH₂Cl₂/MeOH 9:1): Rf 0.43

¹H-NMR (300 MHz, CDCl₃): 0.05, 0.06, 0.07, 0.07 (4s, 12H, 4 CH₃-Si); 0.89 (2s, 18H, 2 (CH₃)₃C); 1.62-1.69 (m, 1H, H3β); 1.93 (ddd, J₁ = 1.7, J₂ = 4.7, J₃ = 9.6, 1H, H3α); 3.13-3.16 (m, 1H, H5); 3.47 (dd, J₁ = 4.9, J₂ = 7.6, 1H, CH₂O); 3.59 (dd, J₁ = 3.9, J₂ = 7.6, 1H, CH₂O); 4.18-4.21 (m, 1H, H2); 6.55 (d, J = 7.1, 1H, H3’); 7.37 (d, J = 1.9, 1H, H6’); 7.51(dd, J₁ = 1.9, J₂ = 7.1, 1H, H4’).

¹³C-NMR (75 MHz, CDCl₃): -5.39, -5.37, -4.69, -4.61 (4 CH₃-Si); 18.02, 18.34 (2 (CH₃)₃C-Si); 25.83, 25.95 (2 (CH₃)₃C-Si); 42.88 (C3); 56.91 (C2); 65.38 (CH₂O); 68.53 (C5); 74.19 (C4); 120.10 (C3’); 123.22 (C5’); 131.49 (C6’); 141.36 (C4’); 165.05 (C2’).

Difference-NOE (500 MHz, CDCl₃): 1.62-1.69 (H3β) → 1.93 (7.3 %, H3α), 3.47 (1.5 %, CH₂O (a)), 4.18-4.21 (5.6 %, H4), 4.26 (1.8 %, H2), 7.37 (3.0 %, H6’), 7.51 (3.6 %, H4’); 1.93 (H3α) → 1.62-1.69 (7.6 %, H3β), 4.18-4.21 (1.7 %, H4), 4.26 (3.8 %, H2), 7.37 (2.9 %, H6’), 7.51 (1.0 %, H4’); 3.13-3.16 (H5) → 3.47 & 3.59 (5.7 %, CH₂O), 4.18-4.21 (2.9 %, H4), 4.26 (2.5 %, H2); 3.47
(CH₂O(a)) → 3.59 (19.9 %, CH₂O (b)), 4.18-4.21 (2.6 %, H4); 3.59 (CH₂O (b)) → 3.13-3.16 (4.1 %, H5), 3.47 (10.7 %, CH₂O (a)), 4.18-4.21 (2.3 %, H4); 4.18-4.21 (H4) → 1.62-1.69 (3.1 %, H3β), 1.93 (1.6 %, H3α), 3.13-3.16 (2.3 %, H5), 3.47 & 3.59 (2.4 %, CH₂O); 4.26 (H2) → 1.93 (2.8 %, H3α), 3.13-3.16 (2.0 %, H5), 7.37 (3.1 %, H6'), 7.51 (2.8 %, H4'); 6.55 (H3’) → 7.51 (4.5 %, H4'); 7.37 (H6') → 1.62-1.69 (1.2 %, H3β), 4.26 (3.3 %, H2); 7.51 (H4’) → 1.62-1.69 (2.5 %, H3β), 4.26 (2.6 %, H2), 6.55 (9.1 %, H3').

MS (ESI⁺): 878 (6), 439 (100, [M + H]⁺), 346 (5).


₁H-NMR-spectrum of 43
26. 4-(tert-Butyl-dimethyl-silyloxy)-5-(tert-butyl-dimethyl-silyloxy)methyl)-2-(2-oxo-pyridin-5-yl)-pyrrolidine-1-carboxylic acid 9H-fluoren-9-ylmethyl ester (44)

A solution of Fmoc-OSu (541 mg, 1.6 mmol) in THF (8.5 mL) was added to a suspension of 43 (352 mg, 0.8 mmol) in dioxane (8.5 mL) and 1M aq. NaHCO₃ (8.5 mL). When no starting material was detected anymore by TLC (1.5 to 2h), 60 mL aq. sat. NaCl was added and the product was extracted with 3x30 mL AcOEt. The organics were dried (MgSO₄) and evaporated to yield yellow foam. The product will be used for the next step without further purification. A pure sample of 44 was obtained as white foam after F.C. (AcOEt) for characterization.

TLC (THF): Rf 0.63

MS (ESI⁺): 1322 (20), 661 (100, [M + H]⁺), 509 (4).

HR-MS (ESI⁺, [M + H]⁺): 661.3488 (C₃₇H₅₃N₂O₅Si₂ requires: 661.3493).
27. 4-Hydroxy-5-hydroxymethyl-2-(2-oxo-pyridin-5-yl)-pyrrolidine-1-carboxylic acid 9H-fluoren-9-ylmethyl ester (45)

To a solution of crude 44 (530 mg, 0.8 mmol) in MeOH (11 mL) at 0°C was added 1 M aq HCl (1.1 mL). After the addition, the ice-water bath was removed and the solution was stirred for 20 h. The violet solution was then evaporated and the violet solid purified by FC (CH₂Cl₂/MeOH 8:1 to 6:1) to give 306 mg (88 % over 2 steps) of 45 as brownish foam.

TLC (THF): Rf 0.15

MS (ESI⁺): 865 (28), 455 (48, [M + Na]⁺), 433 (100, [M + H]⁺), 239 (9).

28. 5-[Bis-(4-methoxy-phenyl)-phenyl-methoxymethyl]-4-hydroxy-2-(2-oxo-pyridin-5-yl)-pyrrolidin-1-carboxylic acid 9H-fluoren-9-ylmethyl ester (46)

\[
\begin{align*}
45 & \quad \text{C}_{25}H_{24}N_2O_5 \\
\text{Mol. Wt.:} & \quad 432.47
\end{align*}
\]

\[
\begin{align*}
46 & \quad \text{C}_{46}H_{42}N_2O_7 \\
\text{Mol. Wt.:} & \quad 734.83
\end{align*}
\]

To a solution of 45 (150 mg, 0.4 mmol) in dry pyridine (2 mL) was added DMT-Cl (141 mg, 0.4 mmol) in 3 portions (47 mg each) every 30 min. If the reaction was not complete according to TLC, another portion (47 mg) was added after 1h30. As soon as all the starting material has reacted, typically after 5 h, the solution was diluted with AcOEt (15 mL) and washed with water (20 mL). The aqueous phase was again extracted with AcOEt (10 mL) and the combined organic phases were dried (MgSO₄) and evaporated. The residual yellow foam was purified by FC (CH₂Cl₂/EtOH 15:1) to yield 204 mg (80 %) of 46 as white foam. The FC column was conditioned with elution solvent + 1 % Et₃N.

TLC (THF + 1 % Et₃N): Rf 0.50

MS (ESI⁺): 735 (10, [M + H]⁺), 303 (100).

Experimental Part

29. 5-[Bis-(4-methoxy-phenyl)-phenyl-methoxymethyl]-4-[(2-cyano-ethoxy)-
diisopropylamino-phosphanyloxy]-2-(2-oxo-pyridin-5-yl)-pyrrolidine-1-carboxylic acid

9H-fluoren-9-ylmethyl ester (13)

\[
\begin{align*}
\text{To a solution of 46 (332 mg, 0.5 mmol) in dry THF (10 mL) was added iPr}_2\text{Net (236 } \mu\text{L, 1.4 mmol) followed by CEP-Cl (151 } \mu\text{L, 0.7 mmol). After 2 h, a solution of 1 M aq NaHCO}_3 (10 mL) was added and the product was extracted with 2x10 mL AcOEt. The organic layer was dried (MgSO}_4 \text{ and evaporated resulting in colourless oil. FC (CH}_2\text{Cl}_2/\text{EtOH 50:1) yielded 359 mg (85 \%) of 13 as white foam. The FC column was conditioned with elution solvent + 1 \% Et}_3\text{N.}}
\end{align*}
\]

TLC (CH\textsubscript{2}Cl\textsubscript{2}/EtOH 15:1 + 1 \% Et3N): Rf 0.47

\[\text{\textsuperscript{31}P-NMR (161.9 MHz, CDCl}_3\text{): 143.13, 148.01}\]

MS (ESI\textsuperscript{+}): 935 (28, [M + H\textsuperscript{+}]), 459 (100), 353 (66), 303 (63).
Experimental Part

30. 4-(tert-Butyl-dimethyl-silyloxy)-5-(tert-butyl-dimethyl-silanyloxymethyl)-2-{2-[2-(4-nitro-phenyl)-ethoxy]-pyridin-5-yl}-pyrrolidine-1-carboxylic acid 9H-fluoren-9-ylmethyl ester (47)

\[
\text{TBDMSO} \quad \text{TBDMSO} \quad \text{TBDMSO} \quad \text{TBDMSO}
\]

\[
\text{N} \quad \text{Fmoc} \quad \text{ONPE}
\]

\[
\begin{align*}
\text{To a solution of 44 (278 mg, 0.4 mmol) in dry dioxane (4 mL) under argon was added Ph}_3\text{P (408 mg, 1.6 mmol) and p-nitro-phenyl ethanol (260 mg, 1.6 mmol). DIAD (302 \mu L, 1.6 mmol) was then added dropwise to the stirred solution. After 8h, the volatiles were removed by rotary evaporation and the yellow oily residue was purified by FC (AcOEt/hexane 2:3 to 1:1) to yield 118 mg (35 \%) of 47 as yellow foam.}
\end{align*}
\]

TLC (AcOEt): Rf 0.41

MS (ESI\(^+\)): 1621 (18), 1089 (59), 810 (87, [M + H]\(^+\)), 658 (100), 279 (81).

HR-MS (ESI\(^+\), [M + H]\(^+\)): 810.3963 (C\(_{45}\)H\(_{59}\)N\(_3\)O\(_7\)Si\(_2\) requires: 810.3969).
31. 4-Hydroxy-5-hydroxymethyl-2-\{2-[2-(4-nitro-phenyl)-ethoxy]-pyridin-5-yl\}-pyrrolidine-1-carboxylic acid 9H-fluoren-9-ylmethyl ester (48)

To a solution of 47 (151 mg, 0.2 mmol) in MeOH (2.56 mL) was added a solution of 1 M aq HCl (256 µL). The solution was stirred at rt for 24-27 h until no starting material was detected anymore on TLC and then evaporated to give a violet solid. FC (CH$_2$Cl$_2$/MeOH 19:1) afforded 73 mg (67 %) of 48 as a slightly pink solid.

TLC (AcOEt): Rf 0.10

MS (ESI$^+$): 1163 (10), 582 (100, [M + H]$^+$), 360 (12).

HR-MS (ESI$^+$, [M + H]$^+$): 582.2223 (C$_{33}$H$_{32}$N$_3$O$_7$ requires: 582.2240).
32. 5-[Bis-(4-methoxy-phenyl)-phenyl-methoxymethyl]-4-hydroxy-2-[2-(4-nitro-phenyl)-ethoxy]-pyridin-5-yl]-pyrrolidine-1-carboxylic acid 9H-fluoren-9-ylmethyl ester (49)

To a solution of 48 (50 mg, 0.1 mmol) in dry pyridine (0.5 mL) was added DMT-Cl (35 mg, 0.1 mmol) in 3 portions (16.3 mg) every 30 min. Additional portions were added every hour till no starting material was detectable by TLC. The solution was diluted with AcOEt (5 mL), washed with water (10 mL) and the aqueous phase extracted with AcOEt (5 ml). The combined organics were dried (MgSO₄) and evaporated to give yellow oil. The product was subjected to FC (AcOEt/THF 1:0 to 4:1) to yield 56 mg (74 %) of 49 as white foam. The FC column was conditioned with elution solvent + 1 % Et₃N.

TLC (AcOEt/THF 4:1): Rf 0.38

MS (ESI⁺): 906 (40, [M + Na]⁺), 273 (78), 215 (100).

33. 5-[Bis-(4-methoxy-phenyl)-phenyl-methoxymethyl]-4-[(2-cyano-ethoxy)-
diisopropylamino-phosphanyloxy]-2-{2-[2-(4-nitro-phenyl)-ethoxy]-pyridin-5-yl}-pyrrolidine-
1-carboxylic acid 9H-fluoren-9-ylmethyl ester (50)

To a solution of 49 (56 mg, 0.06 mmol) in dry THF (1.4 mL) was added iPr2NEt (55 µL, 0.3 mmol) followed by CEP-Cl (35 µL, 0.2 mmol). After 2h, a solution of 1 M aq NaHCO₃ (5 mL) was added and the product was extracted with AcOEt (2x5 mL). The organic phases were dried (MgSO₄) and evaporated. The resulting yellow foam was purified by FC (AcOEt/hexane 9:1) to afford 56 mg (82 %) of 50 as white foam. The FC column was conditioned with elution solvent + 1 % Et₃N.

TLC (AcOEt/hexane 9:1): Rf 0.46, 0.52

³¹P-NMR (161.9 MHz, CDCl₃): 147.84, 147.90, 148.23
To a solution of 36 (2.15 g, 5.2 mmol) in dry THF (23 mL) at -78°C was added dropwise BuLi (1.6 M in hexane, 3.25 mL, 5.2 mmol). After one hour a solution of 39 (595.9 mg, 1.7 mmol) in dry THF (8 mL) was added dropwise and the reaction mixture was stirred at -78°C. After 2 h, 25 mL of water was added and the solution was allowed to warm up to rt. The layers were separated and the aqueous phase extracted with 25 mL of Et₂O. The combined organic phases were dried (MgSO₄) and evaporated to yield yellow oil. FC in AcOEt/hexane 1:6 to 1:5 gave 304 mg (26%) of pure β anomer 51 as yellow oil.

TLC (AcOEt/hexane 1:99): R_f 0.20

¹H-NMR (300 MHz, CDCl₃): 0.05, 0.06, 0.07, 0.08 (4s, 12H, 4 CH₃- Si); 0.89, 0.90 (2s, 18H, 2 (CH₃)₃C); 1.77-1.87 (m, 2H, H3, NH); 1.93-2.01 (m, 1H, H3); 3.10-3.14 (m, 1H, H5); 3.57-3.69 (m, 2H, CH₂O); 3.78 (s, 6H, 2 OCH₃); 4.22-4.26 (m, 1H, H4); 4.33 (dd, J₁ = 6.2, J₂ = 9.5, 1H, H2); 4.68 (s, 4H, 2 CH₂); 6.44 (d, J = 8.8, 1H, H3’); 6.80-6.85 (m, 4H, CH PMB); 7.12-7.16 (m, 4H, CH PMB); 7.39 (dd, J₁ = 2.6, J₂ = 8.8, 1H, H4’); 8.15 (d, J = 2.6, 1H, H6’).

¹³C-NMR (75 MHz, CDCl₃): -5.40, -4.69, -4.55 (4 CH₃-Si); 18.06, 18.31 (2 (CH₃)₃C-Si); 25.86, 25.95 (2 (CH₃)₃C-Si); 43.41 (C3); 50.15 (CH2); 55.26 (OCH₃); 57.84 (C2); 64.38 (CH₂O); 68.84 (C5); 74.26 (C4); 105.86 (C3’); 113.91 (CH PMB); 127.08 (C5’); 128.34 (CH PMB); 130.53 (C PMB); 136.08 (C4’); 146.50 (C6’); 158.10 (C2’); 158.61 (C PMB).

MS (ESI⁺): 678 (17, [M + H⁺]), 558 (90), 450 (61), 426 (22), 340 (17), 121 (100).

HR-MS (ESI⁺, [M + H⁺]): 678.4126 (C₃₈H₆₉N₅O₄Si₂ requires: 678.4122).
\( ^1 \text{H-NMR-spectrum of 51} \)
35. 2-\{2-[Bis-(4-methoxy-benzyl)-amino]-pyridin-5-yl\}-4-(tert-butyl-dimethyl-silanyloxy)-5-(tert-butyl-dimethyl-silanyloxymethyl)-pyrrolidine-1-carboxylic acid 9H-fluoren-9-ylmethyl ester (52)

A solution of Fmoc-OSu (214 mg, 0.6 mmol) in THF (3.3 mL) was added to a suspension of 51 (215 mg, 0.3 mmol) in dioxane (3.3 mL) and aq 1 M NaHCO₃ (3.3 mL). After 4 h, brine (30 mL) was added and the mixture was extracted with AcOEt (3x10 mL). Drying and evaporation of the organic phase resulted in yellow oil. 184 mg (65 %) of 52 was obtained as slightly yellow foam after FC (AcOEt/hexane 1:7).

TLC (AcOEt/hexane 1:4): Rf 0.52

MS (ESI⁺): 900 (100, [M + H]⁺).

36. 5-Bromo-2-(2,2,5,5-tetramethyl-[1,2,5]azadisilolidin-1-yl)-pyridine (53)

A solution of 2-amino-5-bromopyridine (20, 5.00 g, 28.9 mmol) in dry THF (80 mL) was treated at -78°C with nBuLi (1.6 M in hexane, 18.1 mL, 28.9 mmol). After 1h at -78°C a solution of 1,2-bis(chlorodimethylsilyl)ethane (6.22 g, 28.9 mmol) in THF (15 mL) was added dropwise. After another 90 min at -78°C nBuLi (1.6 M in hexane, 18.1 mL, 28.9 mmol) was added, the mixture was allowed to reach r.t. and then stirred for additional 2 h. Ice cold brine (50 mL) was added and the mixture quickly extracted with Et2O (2 x 200 mL). The combined organic phases were dried (MgSO4) and concentrated. Kugelrohr distillation (110-120°C/0.1 mbar) gives 7.20 g (79%) of the stabase adduct 53 as a white solid.

TLC (AcOEt/hexane 1:99): Rf 0.8

1H-NMR (300 MHz, CDCl3): 0.30 (s, 12H, 4 CH3-Si); 0.82 (s, 4H, 2 CH2-Si); 6.46 (dd, J1 = 0.8, J2 = 8.9, 1H, H3); 7.46 (dd, J1 = 2.6, J2 = 8.9, 1H, H4); 8.14 (dd, J1 = 0.8, J2 = 2.6, 1H, H6).

13C-NMR (75 MHz, CDCl3): -0.58 (4 CH3-Si); 8.54 (2 CH2-Si); 108.52 (C5); 113.26 (C3); 139.29 (C4); 148.67 (C6); 159.43 (C2).
$^1$H-NMR-spectrum of 53
37. 2-(2-Amino-pyridin-5-yl)-4-(tert-butyl-dimethyl-silanyloxy)-5-(tert-butyl-dimethyl-silanyloxymethyl)-pyrrolidine (54)

To a solution of 53 (4.30 g, 13.6 mmol) in dry THF (60 mL) at -78°C was added dropwise BuLi (1.6M in hexane, 8.51 mL, 13.6 mmol). After 1 h a solution of 39 (1.56g, 4.5 mmol) in dry THF (20 mL) was added slowly to the first solution and the mixture was stirred 2h at -78°C. The reaction was quenched with 80 mL of water, the temperature was allowed to reach room temperature and the mixture was stirred another hour. The layers were then separated and the aqueous layer was extracted with 80 mL ether. The combined organics were dried (MgSO4) and evaporated to give yellow oil. FC with first AcOEt/hexane 1:4 allowed to recover 1.19g of remaining 39 (76%) and then AcOEt/THF 1:0 to 4:1 gave 378 mg (19%) of the desired product 54 in the right stereochemistry.

TLC (AcOEt): Rf 0.19

$^1$H-NMR (300 MHz, CDCl3): 0.05, 0.06, 0.07, 0.07 (4s, 12H, 4 CH3-Si); 0.89, 0.90 (2s, 18H, 2 (CH3)3C); 1.71-1.81 (m, 1H, H3β); 1.93-2.01 (m, 2H, H3α, NH); 3.10-3.15 (m, 1H, H5); 3.57-3.69 (m, 2H, CH2O); 4.21-4.25 (m, 1H, H4); 4.30-4.37 (m, 3H, H2, NH2); 6.47 (d, J = 8.5, 1H, H3’); 7.46 (dd, J1 = 2.3, J2 = 8.5, 1H, H4’); 8.02 (d, J = 2.3, 1H, H6’).

$^{13}$C-NMR (75 MHz, CDCl3): -5.43, -4.73, -4.60 (4 CH3-Si); 18.01, 18.28 (2 (CH3)3C-Si); 25.82, 25.90 (2 (CH3)3C-Si); 43.68 (C3); 57.82 (C2); 64.47 (CH2O); 68.85 (C5); 74.26 (C4); 108.55 (C3’); 129.39 (C5’); 136.52 (C4’); 146.26 (C6’); 157.58 (C2’).

Difference NOE (500 MHz, CDCl3): 3.10-3.15 (H5) → 3.57-3.69 (4.8 %, CH2O), 4.21-4.25 (2.8 %, H4), 4.30-4.37 (2.6 %, H2); 3.57-3.69 (CH2O) →3.10-3.15 (4.9 %, H5), 4.21-4.25 (3.4 %, H4);
6.47 (H3’) → 7.46 (9.6 %, H4’); 7.46 (H4’) → 1.71-1.81 (1.6 %, H3β), 4.30-4.37 (1.5 %, H2), 6.47 (10.4 %, H3’); 8.02 (H6’) → 1.71-1.81 (0.9 %, H3β), 4.30-4.37 (4.3 %, H2).

MS (ESI⁺): 438 (100, [M + H]⁺), 324 (26), 255 (13), 192 (20).


1H-NMR-spectrum of 54
38. 2-(2-Amino-pyridin-5-yl)-4-(tert-butyl-dimethyl-silanyloxy)-5-(tert-butyl-dimethyl-silanyloxymethyl)-pyrrolidine-1-carboxylic acid 9H-fluoren-9-ylmethyl ester (55)

![Chemical structure of compounds 54 and 55]

A solution of Fmoc-OSu (583 mg, 1.7 mmol) in THF (9 mL) was added to a suspension of 54 (378 mg, 0.9 mmol) in dioxane (9 mL) and 1 M aq NaHCO₃ (9 mL). After 30 min, sat aq NaCl (75 mL) was added and the product was extracted with 3x40 mL AcOEt. The organic phase was dried (MgSO₄) and evaporated. The obtained yellow foam was purified by FC (AcOEt/hexane 6:4) to yield 425 mg (75 %) of 55 as white foam.

TLC (AcOEt): Rf 0.55

MS (ESI⁺): 1321 (15), 660 (100, [M + H]⁺), 433 (30).

39. 4-(tert-Butyl-dimethyl-silanyloxy)-5-(tert-butyl-dimethyl-silanyloxymethyl)-2-[2-(2-phenoxy-acetylamino)-pyridin-5-yl]-pyrrolidine-1-carboxylic acid 9H-fluoren-9-ylmethyl ester (56)

To a solution of 55 (425 mg, 0.6 mmol) in dry pyridine (6.4 mL) was added Pac₂O (664 mg, 2.3 mmol). After 2 h, the reaction was quenched with water (10 mL) and the mixture was concentrated. The residue was dissolved in AcOEt (120 mL), washed with 0.1 M aq NaOH (2x16 mL), dried (MgSO₄) and evaporated to yield yellow oil. FC (AcOEt/hexane 1:4) afforded 506 mg (99 %) of 56 as white foam.

TLC (AcOEt/hexane 3:7): Rf 0.53

MS (ESI⁺): 794 ([M + H]⁺), 739 (91), 381 (100), 319 (16).

40. 4-Hydroxy-5-hydroxymethyl-2-[2-(2-phenoxy-acetylamino)-pyridin-5-yl]-pyrrolidine-1-carboxylic acid 9H-fluoren-9-ylmethyl ester (57)

To a solution of 56 (126 mg, 0.2 mmol) in MeOH (2.2 mL) was added 1 M aq HCl (220 µL) and the solution was stirred at rt. As soon as all the starting material has reacted according to TLC (20 to 24 h) the pink solution was evaporated and the resulting pink foam was purified by FC (CH₂Cl₂/MeOH 19:1 to 9:1) to yield 89 mg (99 %) of 57 as slightly yellow foam.

TLC (AcOEt/THF 9:1): Rf 0.28

MS (ESI⁺): 566 (100, [M + H]⁺), 149 (34), 121 (34).

41. 5-[Bis-(4-methoxy-phenyl)-phenyl-methoxymethyl]-4-hydroxy-2-[2-(2-phenoxo-acetylamino)-pyridin-5-yl]-pyrrolidine-1-carboxylic acid 9H-fluoren-9-ylmethyl ester (58)

To a solution of 57 (277 mg, 0.5 mmol) in dry pyridine (2.7 mL) was added portionwise DMT-Cl (199 mg, 0.6 mmol) over 1 h. 1 h after the last addition, the solution was diluted with AcOEt (20 mL), washed with water (45 mL) and the aqueous phase was extracted with AcOEt (20 mL). The combined organic layers were treated with brine (30 mL), dried (MgSO₄) and evaporated. FC (AcOEt/hexane 7:3 to 8:2) of the yellow foam afforded 372 mg (88 %) of 58 as white foam. The FC column was conditioned with elution solvent + 1 % Et₃N.

TLC (AcOEt): Rf 0.62

MS (ESI⁺): 868 (7, [M + H]⁺), 609 (100).

42. **5-[Bis-(4-methoxy-phenyl)-phenyl-methoxymethyl]-4-[2-(2-cyano-ethoxy)-diisopropylamino-phosphanyloxy]-2-[2-(2-phenoxy-acetylamino)-pyridin-5-yl]-pyrrolidine-1-carboxylic acid 9H-fluoren-9-ylmethyl ester (59)**

To a solution of 58 (372 mg, 0.4 mmol) in dry THF (9.5 mL) was added iPr2NEt (373 µL, 2.1 mmol) followed by CEP-Cl (239 µL, 1.1 mmol). After 2h, a solution of 1 M aq NaHCO3 (30 mL) was added and the product was extracted with AcOEt (2x30 mL). The organic phases were dried (MgSO4) and evaporated to give colourless oil. FC (AcOEt/hexane 1:1) afforded 412 mg (90 %) of 59 as white foam. The FC column was conditioned with elution solvent + 1 % Et3N.

TLC (AcOEt/hexane 1:1): Rf 0.33, 0.42

31P-NMR (161.9 MHz, CDCl3): 147.95, 148.08, 148.30

MS (ESI+): 1068 (100, [M + H]+), 359 (41), 341 (56), 313 (33), 253 (32), 200 (25).

43. 2-(2-Amino-pyridin-5-yl)-4-hydroxy-5-hydroxymethyl-pyrrolidine-1-carboxylic acid 9H-fluoren-9-ylmethyl ester (60)

\[
\text{C}_{25}\text{H}_{25}\text{N}_3\text{O}_4 \quad \text{Mol. Wt.: 431.48}
\]

To a solution of 56 (511 mg, 0.6 mmol) in MeOH (9 mL) was added 1 M aq HCl (900 µL) and the solution was stirred at rt. After 30 h, the pink solution was evaporated and the resulting pink foam was purified by FC (CH₂Cl₂/MeOH 19:1 to 9:1) to yield 277 mg (76 %) of 57 (described earlier) together with 64 mg (23 %) of 60 as white solid.

TLC (CH₂Cl₂/MeOH 19:1): Rf 0.04

MS (ESI⁺): 432 (100, [M + H]⁺), 254 (21), 210 (10).
44. 2-(2-Amino-pyridin-5-yl)-4-hydroxy-5-hydroxymethyl-pyrrolidine (61)

To a suspension of 60 (61 mg, 0.1 mmol) in DMF (650 µL) was added piperidine (94 µL, 1.0 mmol) and the mixture was stirred at rt overnight. The next morning the volatiles were removed by rotary evaporation to yield a brown solid. FC (AcOEt/MeOH 1:0 to 1:1) afforded 21 mg (71 %) of 61 as a slightly yellow solid.

TLC (CH2Cl2/MeOH 9:1): Rf 0.04

1H-NMR (300 MHz, CDCl3): 2.17-2.25 (m, 1H, H3α); 2.39-2.50 (m, 1H, H3β); 3.55-3.60 (m, 1H, H5); 3.85 (d, J = 4.9, 2H, CH2O); 4.42-4.45 (m, 1H, H4); 4.76 (dd, J1 = 6.0, J2 = 12.1, 1H, H2); 6.69 (d, J = 8.8, 1H; H3'); 7.68 (dd, J1 = 2.4, J2 = 8.8, 1H, H4'); 8.03 (d, J = 2.3, 1H, H6').

13C-NMR (75 MHz, CDCl3): 41.24 (C3); 61.01 (C2); 61.59 (CH2O); 70.82 (C5); 73.80 (C4); 110.71 (C3'); 121.23 (C5'); 139.09 (C4'); 148.15 (C6'); 161.66 (C2').

Difference NOE (500 MHz, MeOD): 2.17-2.25 (H3α) → 2.39-2.50 (15.6 %, H3β), 4.42-4.45 (2.2 %, H4), 4.76 (6.5 %, H2); 2.39-2.50 (H3β) → 2.17-2.25 (19.5 %, H3α), 4.42-4.45 (9.3 %, H4), 4.76 (2.1 %, H2); 7.68 (6.0 %, H4'), 8.03 (3.2 %, H6'); 3.55-3.60 (H5) → 3.85 (4.0 %, CH2O), 4.42-4.45 (2.6 %, H4), 4.76 (4.0 %, H2); 3.85 (CH2O) → 3.55-3.60 (4.8 %, H5), 4.42-4.45 (3.0 %, H4); 4.42-4.45 (H4) → 2.17-2.25 (1.4 %, H3α), 2.39-2.50 (4.8 %, H3β), 3.55-3.60 (2.5 %, H5), 3.85 (2.8 %, CH2O); 4.76 (H2) → 2.17-2.25 (4.7 %, H3α), 3.55-3.60 (3.5 %, H5), 7.68 (2.6 %, H4'), 8.03 (5.8 %, H6'); 6.69 (H3') → 7.68 (7.6 %, H4'); 7.68 (H4') → 2.39-2.50 (2.9 %, H3β), 4.76 (3.4 %, H2), 6.69 (9.8 %, H3'); 8.03 (H6') → 2.39-2.50 (2.1 %, H3β), 4.76 (7.0 %, H2).

MS (ESI⁺): 210 (100, [M + H⁺]), 192 (88), 122 (21).
Experimental Part


\[
\begin{array}{cccccc}
0.8753 & 1.0270 & 1.0258 & 1.5317 & 1.1161 & 2.0225 \\
0.9991 & 1.2015 &
\end{array}
\]

\[
\text{Integral (ppm)}
\]

1H-NMR-spectrum of 61
5.3 Oligonucleotide synthesis and analysis

5.3.1 Synthesis, purification and characterization

The modified oligonucleotides as well as the natural targets or references were generated using standard solid-phase phosphoramidite chemistry (see Annexe 1). The synthesis was performed either on the 1.3 µmol scale on a Pharmacia LKB Gene Assembler Special DNA-synthesizer or on the 1.0 µmol scale on a PerSeptive Biosystems Expedite Nucleic Acid Synthesis System. The natural phosphoramidites and the (deoxy)nucleoside-CPG (1 µmol) were purchased from Glen Research. The modified phosphoramidite 13, 50 and 59 were prepared as previously described. The concentration used for all phosphoramidites was 0.1 M. The solvents and reagents used for the synthesis were prepared according to the manufacturer's indications. Minor modifications to the synthesis cycle were introduced for the incorporation of the non-natural building blocks. More precisely, the coupling time was extended from 1.5 to 6 min and the standard activator tetrazole was replaced by the more powerful (S-benzylthio)-1H-tetrazole. The following solutions have been used:

**Deblocking:** 3 ml dichloroacetic acid
97 ml dichloroethane

**Activator 0.25M:** 1 g Ethyl thiotetrazole
31 ml CH₃CN

**Capping A:** 6.15 g DMAP
100 ml CH₃CN

**Capping B:** 25 ml CH₃CN
10 ml Ac₂O
15 ml sym-collidine

**Oxidizing:** 104 ml CH₃CN
412 mg I₂
9.6 ml sym-collidine
48 ml H₂O

For the preparation of the oligonucleotides dp14-17 containing dp2AP, the classical acetyl capping group were replaced by phenoxyacetyl (Pac). The capping A solution was identical and the capping B solution used was:

**Capping B:** 40 ml CH₃CN
7.5 g Phenoxyacetic anhydride
10 ml sym-collidine.
## Experimental Part

**Table 5.1:** Summary of the properties of the synthesized oligonucleotides.

<table>
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<tr>
<th>N°</th>
<th>Sequence</th>
<th>Modif.</th>
<th>$\varepsilon_{260}^a$</th>
<th>ESI-MSb m/z exp m/z calc</th>
<th>yieldc OD$_{260}$</th>
<th>Deprot. methodd</th>
<th>Purif. Method</th>
<th>IE$^e$ (tR)</th>
<th>RP* (tR)</th>
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<tr>
<td>T1-pu</td>
<td>5'-d(GCTA$_6$(GA)$_5$TCG)</td>
<td>/</td>
<td>230100</td>
<td>6560.8</td>
<td>29.2</td>
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<td>IE1 (24.6)</td>
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<td>/</td>
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<td>6288.9</td>
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<td>D1</td>
<td>IE2 (24.4)</td>
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<td>/</td>
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<tr>
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<td></td>
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<td>4285.0</td>
<td>7.9</td>
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<td>IE7 (28.8)</td>
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<td>dpψiC</td>
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<td>D2</td>
<td>RP3 (22.2)</td>
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</tbody>
</table>

---

*a The extinction coefficients of the natural oligonucleotides were determined using the Schepartz Lab Biopolymer Calculator. For the modified oligonucleotides the following values were added: $\varepsilon$(dpψU) = 7920; $\varepsilon$(dpψiC) = $\varepsilon$(drψiC) = 5995; $\varepsilon$(dp2P) = negligible, almost no absorption at 260 nm [2]; $\varepsilon$(dp2AP) = 650.

*b MS were measured by ESI-MS (negative mode). The samples were dissolved in a 1:1 mixture of MeCN/H$_2$O containing 1 % Et$_3$N.

c after isolation by HPLC.

d D1: 33 % ammonia at 55 °C for 16h. D2: a) ethanolic ammonium hydroxide (EtOH/NH$_4$OH 1:3) at 55°C for 12h; b) 0.5 M TBAF (1 M solution in THF) at rt for 6h. D3: 33 % ammonia at rt for 40h. D4: a) 1 M TBD in MeCN at 60°C


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*Ref* [3]
Experimental Part

for 8h; b) 33 % ammonia at 55°C for 16h. D5: 33 % ammonia at rt for 18h.

IE1: MonoQ, buffer 1, 20 to 60 % B in 30 min; IE2: MonoQ, buffer 1, 20 to 50 % B in 30 min; IE3 MonoQ, buffer 1, 20 to 80 % B in 30 min; IE4: MonoQ, buffer 1, 0 to 35 % B in 30 min; IE5: Nucleogen, buffer 1, 27 to 50 % B in 30 min; IE6: Nucleogen, buffer 1, 15 to 40 % B in 30 min; IE7: MonoQ, buffer 2, 20 to 80 % B in 30 min; IE8: MonoQ, buffer 1, 0 to 50 % B in 30 min.

RP1: Pep RPC, buffer 3, 0 to 30 % B in 30 min; RP2: Aquapore, buffer 3, 5 to 15 % B in 30 min; RP3: Aquapore, buffer 3, 0 to 15 % B in 30 min; RP4: Pep RPC, buffer 3, 0 to 50 % B in 30 min; RP5: Aquapore, buffer 3, 5 to 25 % B in 30 min; RP6: Aquapore, buffer 3, 0 to 20 % B in 30 min.

Coupling efficiencies for the modified units were typically > 97%, according to trityl assay. After chain elongation and final detritylation, the natural deoxyoligonucleotides were cleaved from the resin and deprotected (see Table 5.1) and filtered through (Titan HPLC-filters, teflon, 0.45 µm, Infochroma AG). The crude oligonucleotides were purified by ion exchange HPLC (DEAE-HPLC) (ET 125/4 NUCLEOGEN DEAE 60-7 column, 125 × 4.0 mm, Macherey Nagel) or FPLC (Mono Q HR 5/5 column, Pharmacia biotech) and then were desalted over Sep-Pack C18 cartridges (Waters) according to the manufacturer’s protocol. Purified oligonucleotides were controlled for purity by reversed-phase HPLC (Aquapore RP-300, 7 µm, 220 × 4.6 mm, Brownlee or NUCLEOSIL 100-5, C18, 220x5 mm, Macherey-Nagel) or FPLC (Pep RPC HR 5/5 column, Pharmacia biotech). The following buffers have been used:

Buffer 1 (DEAE-HPLC and FPLC)

A: 20 mM NaH₂PO₄, in 4:1 H₂O/CH₃CN, pH 6.0.
B: 20 mM NaH₂PO₄, 1M NaCl in 4:1 H₂O/CH₃CN, pH 6.0.

Buffer 2 (FPLC)

A: 0.01 M NaOH in H₂O, pH 12.0.
B: 0.01 M NaOH, 1M NaCl in H₂O, pH 12.0.

Buffer 3 (RP-HPLC and FPLC)

A: 0.1 M Triethylammonium acetate in H₂O, pH 7.0.
B: 0.1 M Triethylammonium acetate in 1:4 H₂O/CH₃CN, pH 7.0.

The integrity of all oligonucleotides was confirmed by ESI-MS (Table 5.1).

5.3.2 Analysis

UV spectroscopy

ODs of oligonucleotides were measured at 260 nm with a NanoDrop ND-1000 spectrophotometer. UV melting experiments were performed on a Varian Cary 100 BIO UV-Vis spectrophotometer equipped with a temperature controller. Data were collected with a generic Pentium II™ PC running with the Cary WinUV thermal software. All measurement were conducted in a buffer that approximates the intracellular cationic environment as closely as possible (140 mM KCl, 7 mM NaH₂PO₄, 0.5 mM MgCl₂) at a oligonucleotide concentrations of 1.2 µM; the pHs were
Experimental Part

measured directly in the sample. Melting curves were recorded at 260 nm in a consecutive heating-cooling-heating cycle (0-90°C) with a temperature gradient of 0.5°C/min. For temperatures < 20°C, the spectrophotometer was flushed with nitrogen. To avoid evaporation of the solutions, 6-8 drops of dimethylpolysiloxane were added on top of the samples. \( T_m \) values were determined from the first derivative of the melting curve with the software package Origin\textsuperscript{TM} V5.0.

Circular Dichroism

A Jasco J-715 spectropolarimeter equipped with a Jasco PFO-350S temperature controller was used. The temperature was measured directly in the sample. The strand concentration was 1.2 \( \mu \)M in 140 mM KCl, 7 mM NaH\(_2\)PO\(_4\), 0.5 mM MgCl\(_2\) at pH 6. The buffer was used as blank. The samples were scanned at a speed of 50 nm/min, band width of 1 nm, response of 1 sec and in a 210-320 nm range at constant temperature. Each spectrum was taken as an average of 3 scans using a 10 mm cell. Subsequently, the graphs were smoothed with a noise filter. For the temperature scan, the spectra were measured at fixed wavelength between 10 and 80°C with a resolution step of 0.1°C and a slope of 50°C/h. were used.

Speed Vac

Oligonucleotide samples were dried on a Savant Speed-Vac SC 110 during 4-5h.

Non-denaturing polyacrylamide gel electrophoresis

Pharmacia Biotech, Power supply EPS 3500 XL, Life technologies, Vertical Gel electrophoresis and 180 × 180 × 0.4 mm glass plates were used. The plates were previously coated with SIGMA cot, a siliconizing reagent. Non-denaturing gel: 20% [150 ml 40% acrylamide-bis (19:1) solution, SERVA; 30 ml TBM 10\( \times \) (Tris-Borate 90 mM, MgCl\(_2\) 10 mM) and 120 ml mQ H\(_2\)O, pH 7.0]. To 50 ml of this solution, 50 \( \mu \)l of ammonium peroxide disulfate (10% in mQ H\(_2\)O) and 50 \( \mu \)l of N, N, N', N'-tetramethylendiamine (TEMED) were added. The gel was poured and polymerized for at least 1h. TBM 1\( \times \) buffer was used as electrophoresis buffer.

The concentration of the loaded samples was between 0.7 and 0.9 nmol for each single strand. The oligonucleotides were mixed in a ratio 1:1 (duplex) or 1:1:1 (triplex), dried at the speed vac and taken up in 10 \( \mu \)l of loading buffer (8% saccharose in TBM buffer). The samples were heated to 90 °C for 2 min, cooled slowly to rt and then to 0 °C for another 5 min. Bromophenol blue and Xylencyanol FF were used as markers: 1 \( \mu \)g of each was dissolved in 1ml formamide; 2.5 \( \mu \)l of this solution was then added to 10 \( \mu \)l loading buffer.
The gel was equilibrated at a constant voltage of 120 V for 1h. After loading of the samples, electrophoresis was performed at 120 V for 18h at 4°C. Bands were detected by UV shadowing on a TLC plate at 254 nm.
Annexes

Annexe I: Solid phase oligonucleotide synthesis

All oligonucleotides were synthesized on an automated DNA synthesizer either on a 1.0 µmol scale (*Expedite Nucleic Acid Synthesis System, PerSeptive Biosystems*) or on a 1.3 µm scale (*Pharmacia LKB Gene Assembler Special DNA synthesizer*) using standard solid phase phosphoramidite chemistry (Figure A.1).

![Solid phase oligonucleotide synthesis cycle with standard phosphoramidite chemistry.](image)

The phosphoramidite method for oligonucleotide synthesis is the methodology of choice for most laboratories because of efficient and rapid coupling and the stability of the starting materials.
The first 5’-DMT protected nucleoside is linked to the CPG (controlled pore glass) solid support at its 3’-end. The first step of the cycle consists in the removal of the DMT group with 3% Cl₂CHCOOH in dichloroethane (DCE); in step 2 the incoming 5’-DMT protected nucleoside (1.0 µM in CH₃CN) is first activated by tetrazole (0.45 M in CH₃CN), which protonates the nitrogen atoms of the phosphoramidite making the phosphorus more reactive to nucleophilic attack. Then the free 5’-hydroxyl group of the CPG bound nucleoside can react with the phosphoramidite to form a 3’-5’ internucleotide linkage. The coupling reactions are performed for 1.5 min under completely anhydrous conditions, by means of anhydrous solvents and addition of 3 Å molecular sieves, to avoid competition reactions with H₂O. Coupling efficiencies are only qualitatively estimated from detritylation assays, in which the UV absorption of the trityl cation containing solution released during deprotection is monitored. The next step (step 3, capping) terminates every chain which did not undergo addition by simple acetylation of the free hydroxyl groups (Sol A: 6% DMAP in CH₃CN; sol B: 20% Ac₂O in CH₃CN/Collidine). The internucleotide linkage is then oxidized from phosphate (+ III) to the more stable phosphortriester (+ V) using iodine as the oxidizing agent and water as the oxygen donor (step 4, oxidation, 0.01M Iodine in Collidine/H₂O/CH₃CN). After each individual step the solid support is washed with CH₃CN to remove excess reactants and reaction byproducts. The cycle starts all over again with the cleavage of the DMT group and is repeated until chain elongation is complete.

Natural DNA oligonucleotides are then completely deprotected and cleaved from the solid support by using concentrated aq. NH₃ (33%) at 55°C for 16 to 18 hours. The crude oligomers are purified by ion exchange HPLC (DEAE-HPLC) and desalted over Sep-Pak Cartridges. The purity of the oligomers is confirmed by RP-HPLC and their integrity is proven by negative electrospray mass spectrometry analysis. The mass are typically found to be within 0.05% of the expected mass.

All the nucleoside phosphoramidite building blocks, including the natural DNA, the 2’-deoxy-5-methylcytidine and the nucleosides bound to the CPG solid support, have been purchased from Glen Research. Minor modifications to the standard synthesis cycle presented above were introduced for the incorporation of the non-natural building blocks. More precisely, the coupling time was extended from 1.5 to 6 min and the standard activator tetrazole was replaced by the more powerful (S-benzylthio)-1H-tetrazole (0.25 M in CH₃CN).

A complete list of the synthesized oligonucleotides and more details concerning their purification and characterization can be found in the experimental part.
Annexe II: UV-melting curve experiment

DNA absorbs UV light, result from the combination of the strong UV absorption of the purine and pyrimidine bases, with a major peak at approximately 260 nm (Figure A.2).

Figure A.2: UV spectrum of a DNA strand.

The intensity and exact position of this maximum, $\lambda_{\text{max}}$, is function of different parameters like base composition, salt concentration, pH and base pairing interaction. This last point plays an essential role in UV-melting curves. When nucleic acids strands come together and form a complex (duplex, triplex...) the base stacking interactions become much stronger and a part of the UV absorbance is quenched. For the same reason, when the strands denature, an increase of 20 to 40% in UV absorption, known as hyperchromic shift or hyperchromicity, is observed. The temperature at which the strands are half denaturated is called the melting temperature, Tm. The denaturation process is highly co-operative: the strands hold fast until the Tm and than rapidly separate resulting in a jump in the absorbance. Thus, the melting of a complex can be monitored using the temperature dependent changes in UV absorbance, and the resulting sigmoidal plot is known as a melting curve. As example, the typical melting curve of a triplex is depicted in Figure A.3 (left). It displays two transitions: the one at lower temperature represents the dissociation of the third strand from the duplex; the one at higher temperature corresponds to the duplex to single strands melting. The first derivative of the melting curves gives more precisely the Tm values (Figure A.3 right).
**Figure A.3:** Absorbance versus temperature melting curve (left) and first derivative of the curve (right).
Annexe III: CD spectroscopy and gel shift experiments in triplex study

Under certain conditions the two transitions usually obtained for triple helical system in UV-melting curves can be very close and sometimes even overlap making the presence of triplex difficult to detect. Additional techniques including CD-spectroscopy or gel electrophoresis can help to confirm the formation of a triple helical structure.

Circular dichroism (CD) measurements are used to determine the conformation of nucleic acids in solution. This technique is based on the difference in absorbance of left and right circularly polarized light as a result of the chirality of the molecule under investigation. In many cases, the CD spectra of triplexes differ from the arithmetical sum of the spectra of the constituent double and single stranded nucleic acids. This observation added to the appearance of an intense and characteristic negative short-wavelength (210-230 nm) band in the CD-spectra strongly accounts for the presence of the triple-helical structures (Figure A.4).

![Figure A.4: Typical triplex CD spectrum (in black), which differs from the calculated CD spectrum obtained from the sum (in magenta) of the CD spectra of the target duplex (red) and third strand (blue). Note also the negative band around 220 nm.](image)

The measurement of CD spectra at different temperatures results upon heating in the transformation of the minimum at around 220 nm to a maximum at about the same wavelength reflecting the thermally induced release of the third strand from the triplex (Figure A.5 left). At this point, when the triplex is not formed anymore, the curve exhibit a shape similar to the one obtained from the sum of the duplex and the third strand curves. A temperature scan at a fixed wavelength around 220 nm leads to a sigmoidal CD-melting curve (Figure A.5 right) and the first derivative reveals the triplex melting temperature.
Another technique used to validate triplex formation is the non-denaturing gel electrophoresis method. Polyacrylamide gels allow the separation of nucleic acids on the basis of size, electric charge, shape of the molecule and other physical properties. Usually triplex formation results in the appearance of a novel band in the gel, which migrates slower than an unbound oligonucleotide strand or a DNA duplex band (Figure A.6). The bands can be visualized via UV-shadowing or with the help of staining solutions.

Figure A.6: Typical gel shift assay obtained during investigation of triplex formation.
Curriculum Vitae

1978    Born on the 20th of January in Bitche (France)

1981-1984    Nursery school in Saint Louis-lès-Bitche (France)

1984-1989    Elementary school in Saint Louis-lès-Bitche (France)

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1996-1999    Undergraduate studies in Chemistry at the University of Metz
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1999-2001    Undergraduate studies in Organic Chemistry at the University
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