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DEVELOPMENT OF A NEW PNA ANALOGUE AS A POTENTIAL ANTISENSE DRUG AND TOOL FOR LIFE-SCIENCE STUDIES

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ABSTRACT

The work described in this thesis focuses on applying synthetic organic chemistry methods (supported by modern synthesizers and analytical techniques) for the preparation of peptides and modified nucleic acids in order to affect certain properties in these biomolecules, which can lead to their application as drugs and/or tools for life sciences studies.

The first part of the thesis reports the design and synthesis of a novel pyrrolidine-based peptide nucleic acid (PNA). Two enantiomers of the chiral pyrrolidine-containing unit were chemically synthesized and further incorporated into PNA fragments using modern automated solid-phase assembly methods. By comparison of their binding affinities to both DNA and RNA targets, it was found that such PNAs, due to their enhanced rigidity are able to recognize and bind to the complementary RNA strands with significantly larger affinity than to the complementary DNA. These findings may be utilized in the development of RNA-specific molecular probes, binding assays for cell extracts that contain mixtures of RNA and DNA, or targeting of viral RNAs, i.e. act as basis for the development of antisense drugs.

With the rapid advancement of biochemistry and related sciences, there is an increasing demand for synthetic peptides. The development and application of a new type of peptide-coupling agent is described. This coupling agent is superior in some ways to the most commonly used commercial agents, since peptides obtained by its action are generally of higher enantiopurity, i.e. a significant reduction of racemization of the amino acid during coupling is obtained. The potential use of this agent in linking peptide segments has been studied and discussed. So called 'segment coupling' becomes economically valuable when either large synthetic peptides are to be made, or when a number of peptides, consisting mainly of highly conserved parts with differences in only a few amino acid residues, are desired. Alternatively, this coupling agent could be used for solid phase synthesis of PNA sequences containing racemization-prone units.

As a spin-off from the above project, new sulfilimine derivatives of natural nucleosides have been successfully synthesized, isolated, characterized and studied. The synthesis of deoxycytidine and deoxyadenosine sulfilimine dimethyl and diphenyl sulfilimines is reported and the results of tests of their stability under a variety of conditions – mostly those relevant to oligonucleotide chemistry are discussed. The results of these stability studies lead to the conclusion that the sulfilimine group can serve as UV-detectable labels for short oligonucleotides. This label can be either transient or permanent (dimethyl or diphenyl sulfilimine, respectively) and can simplify the detection of tagged oligonucleotides during their purification and in assays, which can otherwise be a complicated task.

In the final part of this thesis it is demonstrated how a sequence-specific PNA clamp is used to suppress a dsDNA-digesting enzyme Exonuclease III. The rate of the enzymatic degradation has been studied in different environments and added-PNA concentrations. It was found that PNA indeed specifically suppresses the action of the enzyme, in addition to non-specific inhibition. This, previously non-documented finding, helps to better understand the mechanism of action of these enzymatic processes, as well as having potential applications within both DNA sequencing and gene therapy.

LIST OF PUBLICATIONS

I Andis Slaitas, Esther Yeheskiely. Synthesis and Hybridization of Novel Chiral Pyrrolidine Based PNA Analogue. *Nucleosides, Nucleotides & Nucleic Acids* **2001**, *20*, 1377–1379.

II Andis Slaitas, Esther Yeheskiely. A Novel N-(Pyrrolidinyl-2-methyl)glycine-Based PNA with a Strong Preference for RNA over DNA. *Eur. J. Org. Chem.* **2002**, 2391-2399.

III Fast and Efficient Peptide Bond Formation Using *bis*-[*α,α-bis*(trifluoromethyl)-benzyloxy]diphenylsulfur. Part I. *J. Peptide Res.* **2002**, *60*, 283-291.

IV Synthesis and Reactivity of Nucleoside Sulfilimines. *Phosphorus, Sulfur, and Silicon* **2004**, *179*, 153–171.

V Andis Slaitas, Charlotte Ander, Zeno Földes-Papp, Rudolf Rigler, Esther Yeheskiely. Suppression of Exonucleolytic Degradation of Double-Stranded DNA and Inhibition of Exonuclease III by PNA. *Nucleosides, Nucleotides & Nucleic Acids* **2003**, *22*, 1603-1605.

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LIST OF ABBREVIATIONS

Ac	Acetyl
Ade	Adenine (6-aminopurine)
Aeg	*N*-(2-Aminoethyl)glycine
All	Allyl
Alloc	Allyloxycarbonyl
Base	Heterocyclic nucleobase (where appropriate)
Bhoc	Benzhydryloxycarbonyl (diphenylmethyloxycarbonyl)
Boc	*t*-Butyloxycarbonyl
BTBDS	[α,α-*bis*(trifluoromethyl)benzyloxy]diphenylsulfur
Bz	Benzoyl
Bzl	Benzyl
Cbz	Benzyloxycarbonyl
Cyt	Cytosine (4-aminopyrimidin-2one)
DCA	Dichloroacetic acid
DCE	1,2-Dichloroethane
DCM	Dichloromethane
DIEA	Diisopropylethylamine (Hünig's base)
DMF	N,N-Dimethylformamide
DMT	Dimethoxytrityl [*bis*(4-methoxyphenyl)(phenyl)methyl]
DNA	Deoxyribonucleic acid
ds	Double-stranded
Gua	Guanine (2-aminopurin-6-one)
Fmoc	9-Fluorenylmethyloxycarbonyl
HATU	*O*-(7-Azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate
HBTU	*O*-(Benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate
HMBA	4-Hydroxymethylbenzoic acid
HOBt	1-Hydroxybenztriazole
LNA	'Locked' nucleic acid
MMT	Monomethoxytrityl [(4-methoxyphenyl)(diphenyl)methyl]
Ms	Mesyl (methanesulfonyl)
NCS	N-Chlorosuccinimide
PG	Protecting group
Pmg	Pyrrolidin-2-yl methyl glycine
PNA	Peptide (polyamide) nucleic acid
PS	Polystyrene (as in 'PS solid support')
PyBroP®	Bromotripyrrolidinophosphonium hexafluorophosphate
RNA	Ribonucleic acid
RP	Reversed-phase
ss	Single-stranded
TEA	Triethylamine
TEAB	Triethylammonium bicarbonate
Tf	Trifluoromethanesulfonyl (triflyl)
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
Thy	Thymine (5-methylpyrimidin-2,4-dione)
Trt	Trityl (triphenylmethyl)
Ts	4-Methylbenzenesulfonyl (tosyl)

1. GENERAL INTRODUCTION

Nucleic acids

Native nucleic acids - DNA and RNA

DNA and RNA – carriers of the genetic information, have fascinated scientists for now nearly a century.

Nucleic acids are built from repeating units – nucleotides, which consist of a phosphorylated sugars (ribose or deoxyribose) attached to a heterocycle – adenine, cytosine, guanine and thymine or uracil. Nucleotides are linked as phosphate diesters to form the chains of DNA or RNA (Figure 1.1).

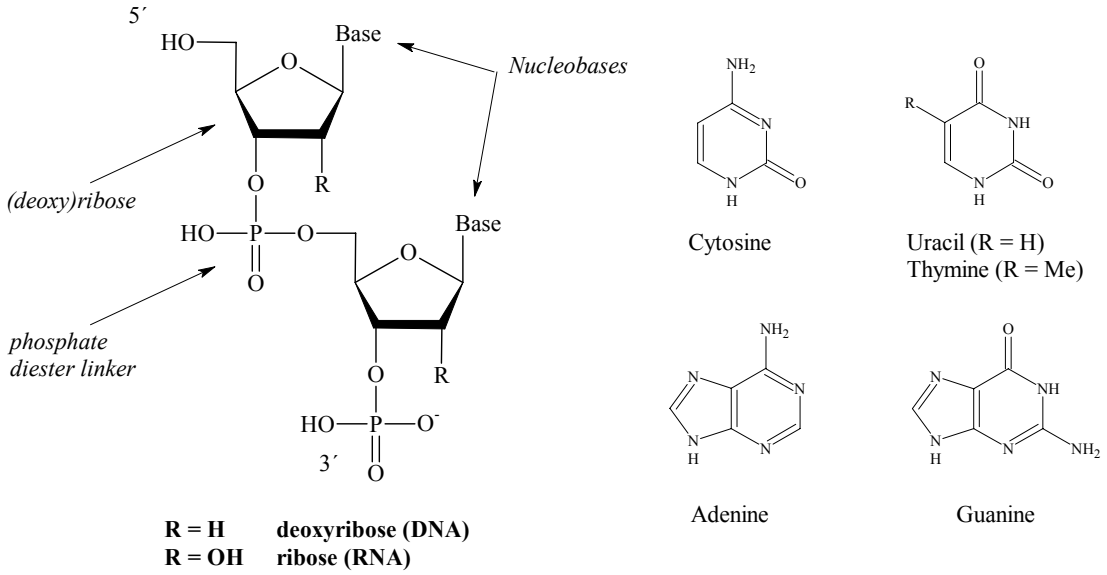


Figure 1.1. Chemical structure of DNA (RNA).

The chemical structure alone cannot be credited for the unique properties of nucleic acids. In the first half of the 20th century it was found that the amount of adenine in DNA is equal to the amount of thymine, and the amount of cytosine to that of guanine. This important finding made perfect sense after the proposed DNA structure by Watson and Crick in 1953. By their model the DNA structure is a double helix, formed from two nucleic acid strands with the heterocyclic nucleobases positioned inside the helices and sugar phosphates on the outside. The structure is held together

by hydrogen bond interactions of the heterocyclic bases. These interactions are the strongest between the adenine-thymine pair and the cytosine-guanine pair (
Figure **1.2**).
This ability of nucleic acids to form duplexes with their complementary counterparts is the key to the storage and replication of the genetic information.

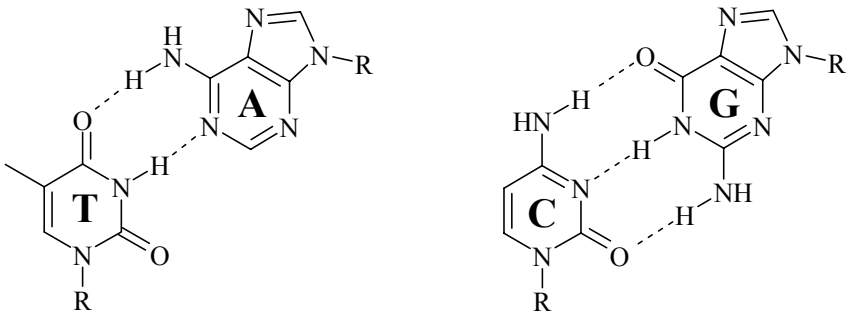


Figure 1.2. H-bonding in nucleobases leading to Watson-Crick complementarity. R = ribose or deoxyribose

PNA – an artificial nucleic acid

Peptide nucleic acids were first described by P. E. Nielsen et al. in 1991.[1] PNA was designed by a computer-assisted modelling of triple helices. The third strand (also known as the Hoogsteen strand) was stripped of its deoxyribose backbone and a new – pseudopeptidic *N*-(2-aminoethyl)glycine (Aeg) backbone was constructed, thus obtaining a DNA mimic, which is not charged and achiral. The nucleobases are attached to the Aeg backbone via a methylene carbonyl linkage (Figure 1.3).

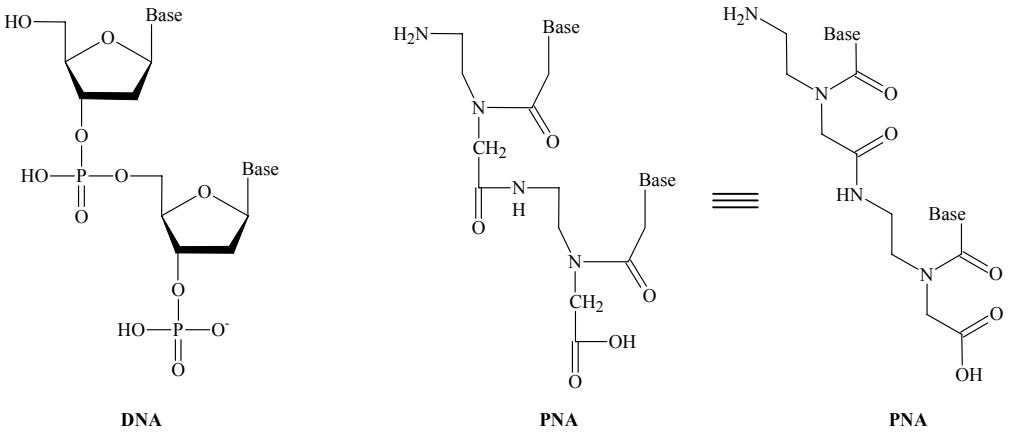


Figure 1.3. The chemical structure of PNA and its similarity to DNA.

PNA Synthesis

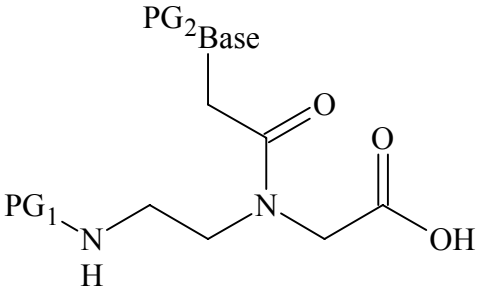


Figure 1.4. General structure of a PNA monomer

A PNA monomer consists of N-protected (2-aminoethyl)glycine (PG_1) to which a protected nucleobase (PG_2) is attached. These two protecting groups have to be orthogonal i.e. PG_2 must be stable to the conditions used to remove PG_1 (Figure 1.4). There are several combinations of protecting groups reported for the PNA synthesis, and the most commonly used are summarized in Table 1.1.

Table 1.1. Commonly used protecting group strategies for PNA synthesis.

Entry	PG_1	PG_2	Removal of PG_1	Removal of PG_2	References
1	Boc	Cbz	50% TFA	HF or TfOH	2, 3, 4, 5
2	Fmoc	Bhoc	20% piperidine	95% TFA	6
4	MMT	Acyl[a]	2% DCA[b]	NH_3[c]	7, 8, 9, 10
5	Fmoc	MMT	20% piperidine	2% DCA[b]	11

[a] e.g. benzoyl for Ade and Cyt, isobutyryl for Gua
[b] could be replaced by 1% TFA
[c] saturated aqueous or methanolic

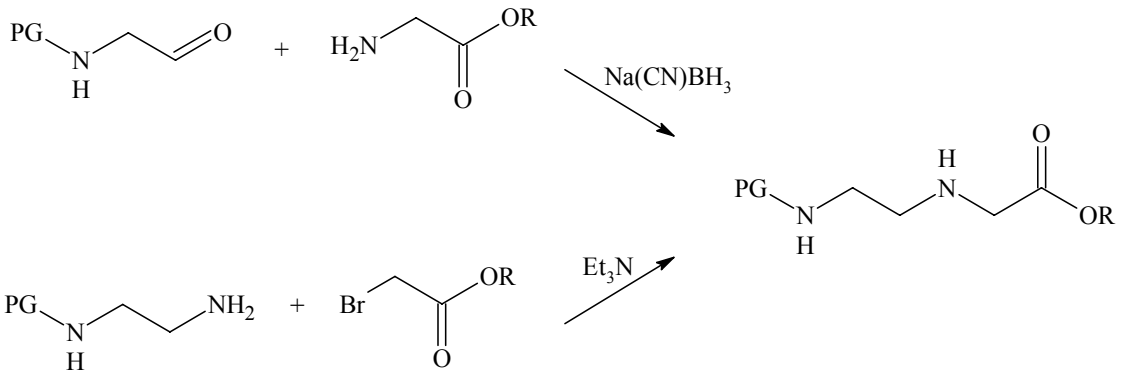
Boc/Cbz and Fmoc/Bhoc PNA monomers are commercially available and are used for routine PNA synthesis with the possibility of using commercial peptide synthesizers.

A new strategy – MMT/Acyl was adopted for PNA synthesis and used in conjunction with assembly PNA-DNA chimera,[7-10] since the removal conditions of CBz or Bhoc groups are not compatible with DNA, which is acid-sensitive. The removal of the MMT group is affected by treatment with low concentrations of dichloro- or trifluoroacetic acid.

Synthesis of PNA monomers

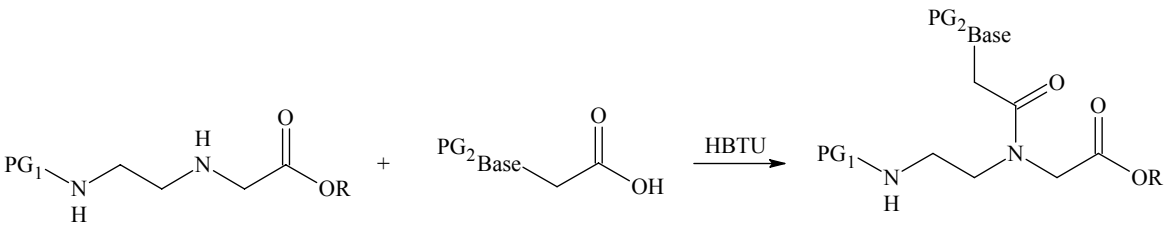
Although PNA monomers are commercially available, they are also relatively easy to synthesize from inexpensive starting materials (Scheme 1.1).

As previously mentioned, PNA consists of repeating N-(2-aminoethyl)glycine units with an attached nucleobase. The synthesis of the backbone unit can be achieved either via reductive amination of a glycine ester with an *N*-protected aminoacetaldehyde,[12-14] or via an alkylation of mono-protected ethylenediamine with a bromoacetic acid ester.[9,15-17] Either route gives high yields of the desired intermediate.



Scheme 1.1. Synthesis of protected (2-aminoethyl)glycine

In the next step protected nucleobases are attached via an amide bond using a coupling agent, e.g. DCC[18] or HBTU.[19] (Scheme 1.2).

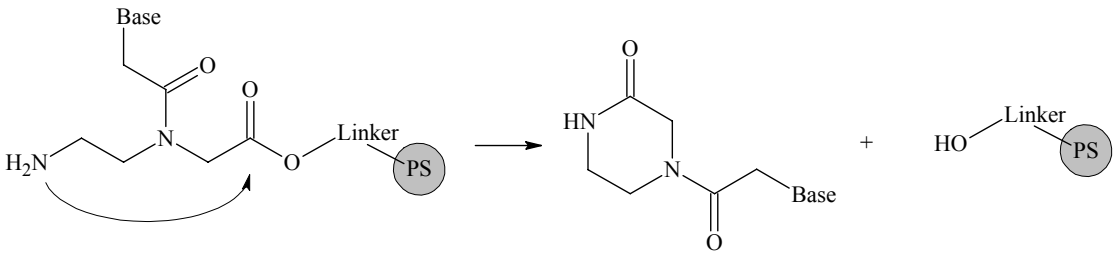


Scheme 1.2. Synthesis of Aeg-PNA monomers

Solid phase PNA synthesis

The assembly of PNA, using either of the above-described monomers, is usually performed on a solid support, preferably via automated synthesis.

The solid support is usually functionalised polystyrene to which an *N*-protected amino acid (e.g. glycine) is attached via its C-terminus to a cleavable linker. It is not advisable to link the PNA monomers directly onto the linker since, due to the potential formation of a ketopiperazine during the first deprotection step, the support may get de-functionalised.[20]



Scheme 1.3. De-functinalization of support due to cyclization.

The choice of the linker is very important and depends on the protecting group strategy used. For instance, a Wang linker [4-(hydroxylmethyl)phenol)] is used for Fmoc/Bhoc chemistry and the product is cleaved from the resin by acid treatment, for instance using TFA,[21] while a more acid stable HMBA [4-(hydroxymethyl)benzoic acid] linker is used for MMT/Acyl chemistry.[9,22]
The loading of the solid support can range from 30 μM/g to >1 mM/g. Low loading resins are usually used in the synthesis of PNA-DNA chimera.[9]

The PNA synthesis cycle consists of the following operations:

- Chain elongation (coupling step)
- Wash
- Removal of the transient protecting group
- Wash

The chain elongation is the critical step in the PNA assembly. The efficiency of the elongation step, and thus the choice of the coupling agent, is crucial for the overall yield and purity of the desired PNA, especially when somewhat longer fragments are to be assembled.

HATU is probably the most commonly used coupling agent for PNA synthesis. It is reported to give better than 99% coupling yields in a relatively short time (15-30 min).[23]
In our work we used the less expensive and more available coupling agent HBTU and still managed to maintain the same efficiency.
The intermediate wash steps are necessary for the removal of unreacted monomers, coupling agent and other non-resin bound material from the polystyrene resin. The wash procedure is usually performed by alternating resin-swelling and non-swelling

solvents e.g. DMF and acetonitrile. Insufficient washing can have a devastating effect on both the quality and the quantity of the desired end product.

Normally the size of the PNA fragments that are constructed on a solid-support do not exceed 16 bases. There are two main reasons behind this: first, a 16-mer PNA has usually already high affinity to both DNA and RNA, greatly surpassing the affinities of the native nucleic acids; second, being rather lipophilic, PNA has a tendency to self-aggregate both on the solid support during synthesis, leading to low yields of the desired product as well as in solution[24] leading to precipitation and non sequence-specific interactions.[25,26]

After the synthesis the PNA is usually purified by reversed-phase HPLC, using conditions similar to those utilized in the purification of small peptides i.e. a low pH buffer.[8]

Properties of PNA

What made the discovery of PNA invaluable is that, although being an artificial nucleic acid, PNA was binding DNA and especially RNA with an affinity greater than that of native nucleic acids. For instance a pentadecamer H-tgt acg tca caa cta-NH_2 was forming a duplex with complementary anti-parallel DNA (i.e. the amino terminus of PNA is facing the 3´ end of the DNA) with a T_m[†] of 69.5 °C, while the corresponding DNA-DNA duplex has a T_m of 53.3 °C.[27] The duplex with a complementary anti-parallel RNA was even more stable with a measured T_m of 72.3°C, compared to the DNA-RNA duplex with the T_m of 50.6 °C. [27] Interestingly, PNA is also able to bind to a parallel DNA targets (i.e. the amino terminus of PNA facing the 5´-end of the DNA), although with lower affinity (56.1 °C). Kinetic binding studies have shown that the binding of PNA to anti-parallel DNA is much faster (< 30 s) than compared to parallel targets.[28]

The influence of single-point mismatches is more pronounced in PNA•DNA than DNA•DNA duplexes. For example, a single Cyt to Gua mismatch in the middle of a DNA pentadecamer showed a T_m depression of 9 °C when hybridized to DNA, but 16 °C in the case of PNA.[27]

[†] T_m denotes thermal melting, i.e. the temperature at which 50% of the duplex is dissociated, as measured by an increase in the UV absorption. A higher T_m value means higher duplex stability and vice versa.

The increased stability of PNA•DNA and PNA•RNA duplexes in comparison to DNA•DNA(RNA) duplexes is mainly attributed to the lack of electrostatic repulsion between the two strands. This is supported by experiments showing that the thermal melting stability of DNA-DNA duplexes increases with increasing ionic strength of the medium and becomes equal to that of a PNA•DNA duplex at ionic strength above 1 M NaCl. At the same time the change in ionic strength has little effect on the stability of PNA•DNA duplexes.[27]

Homopyrimidine PNAs or PNAs with a high pyrimidine:purine ratio bind to complementary DNA via the formation of $(PNA)_2$•DNA triplexes.[1,29] These complexes are very stable and are dependent on the length of the oligomers, an average increase of 10 °C per base pair is observed.[29] Triplex formation involving Cyt is pH dependent, in accordance to the Hoogsteen binding model, i.e. cytosine needs to be protonated at N^3 in order to form a hydrogen bond to the N^7 of guanine.

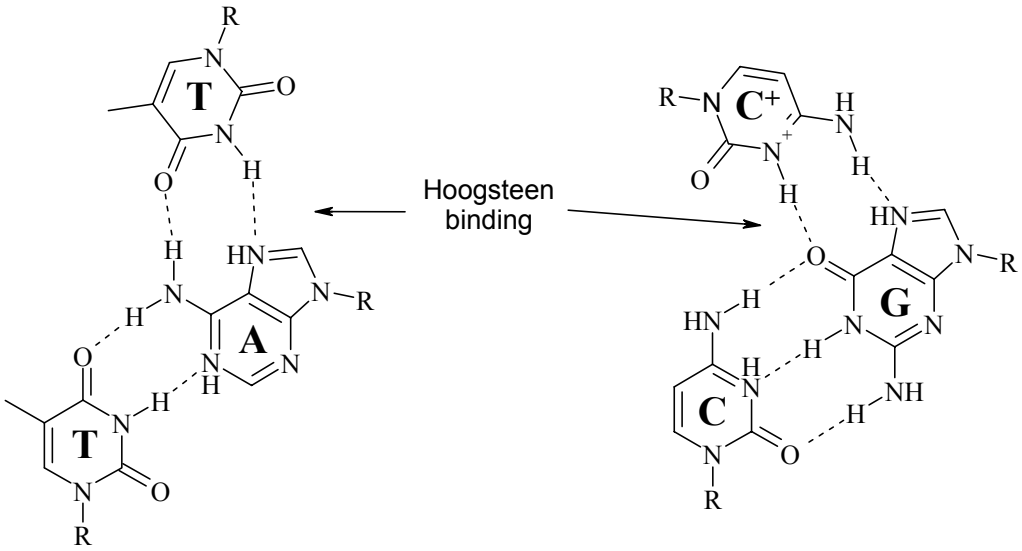


Figure 1.5. Hoogsteen binding model (triple strand formation), R = ribose or deoxyribose

Thermal melting of $(PNA)_2$•DNA hybrids exhibits very pronounced hysteresis, i.e. the difference between the melting (higher) and annealing temperatures (lower), indicating that the rate of formation of the triplex is very slow.[30,31]

Strand invasion

Homopyrimidine PNA oligomers, when targeting dsDNA, displaces the pyrimidine strand of the dsDNA and binds to the purine strand forming a $(PNA)_2$•DNA triplex and a looped out ssDNA – so called P-loop.[1,32] This process is unique for PNA and takes place only in a low ionic strength buffer (< 50 mM). However, once pre-formed in low salt buffer, the P-loop structures are stable in salt concentrations as high as 500 mM.[33,34]

The strand invasion concept has been proved by several techniques. For example, when a dsDNA target was incubated with a homothymine PNA 10-mer, a footprinting experiment showed a protection of a $d(A)_{10}$ target, while a $d(T)_{10}$ region of the same target was cleaved by *Staphylococcus* nuclease as well as S1 nuclease. Both these nucleases are known to prefer ssDNA. In addition the displaced $d(T)_{10}$ strand was probed with $KMnO_4$, which oxidizes the C^5-to-C^6 double bonds in thymine residues not involved in the base-pairing.[1]

Advantages and disadvantages of PNA compared to native nucleic acids

PNA has many advantages over oligodeoxy- and oligoribonucleotides.

- PNA is generally more stable than DNA or RNA fragaments. Being a polyamide-based molecule, PNA is very stable under acidic and moderately stably under basic conditions, as well elevated temperatures.
- PNA is not a substrate for proteases, peptidases or nucleases. All these characteristics facilitate synthesis, purification, storage and application of PNA fragments.
- The lack of charge in the PNA backbone, results in the lack of charge repulsion between the strands, and thus a greater affinity towards its targets. Actually, introduction of a positive charge in the PNA strand can be beneficial for the formation and stability of triple helices.[35]
- PNA is a more specific binder, single-point mismatches are better pronounced in PNA than DNA or RNA.[27]
- PNA forms stable triplexes with DNA, while the $(DNA)_3$ hybrids are rather unstable.
- PNA binding to its complementary target is rather unaffected by the ionic strength of the medium.[27,36]

PNA has also some shortcomings:

- PNA has a lower solubility compared to both DNA and RNA due to the lack of charge in the molecule.[2]
- PNA has very low cellular permeability, thus limiting its applications for antigene or antisense therapies (*vide infra*).[37]
- PNA is rather non-selective binding DNA vs. RNA.[27]
- In biological systems, the triplex formation is limited only to guanine-poor targets, since the physiological pH does not affect the protonation of cytidine residues.[38]

In order to overcome these limitations of PNA, and to add more positive features, further modifications are being developed. For instance, the solubility issue has been addressed by adding hydrophilic amino acids or short peptides at the end of PNA fragments. These modifications greatly enhance the solubility of the PNA, but usually have no negative effect on its binding ability.[1,39] In order to achieve hybridization to the nuclear DNA, The cell permeability has been increased by conjugating the PNA with peptides.[40,41]

PNA modifications

Being of a relatively simple structure, PNA monomers are good targets for chemical modifications. By PNA modifications one usually understands changes in the (2-aminoethyl)glycine backbone and the methylene carbonyl linkage from the backbone to the nucleobase.

The scientific literature has an ample range of examples reporting PNA modifications. The aim of these modifications has been mostly to further improve the properties of PNA, such as binding affinity and solubility, and to synthesize new DNA mimics in order to get a better understanding of the structural and biological features of the native nucleic acids.

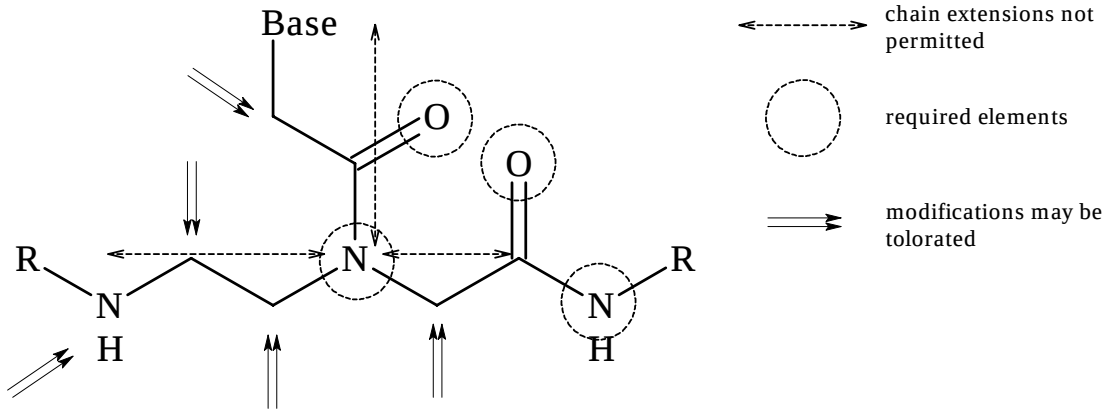
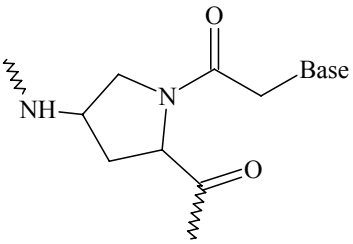
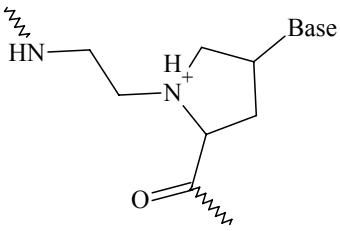
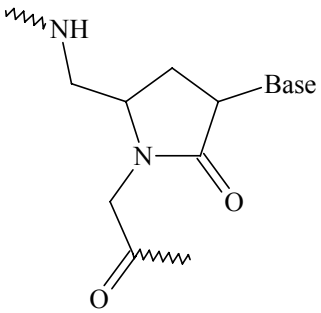


Figure 1.6. Towards the SAR of an acyclic backbone-based PNA.

Modifications to the aminoethylglycine backbone are not very well tolerated in terms of effect on the hybridization properties of PNA (Figure 1.6). Changes in the distances between the N-atoms, as well as the distance from the tertiary amide nitrogen to the secondary amide (glycine fragment) or to the nucleobase are generally not favoured.[42,43] Increasing the linkage length by one carbon and incorporation in the middle (shown bold) of Aeg-PNA decamer 'H-gta ga**t** cac t-NH_2' resulted a T_m depression of 8-20 °C.[43] The tertiary amide nitrogen is a required structural element, since the reduction to the tertiary amine resulted in a T_m depression of 22 °C.[44] In these, so-called 1st generation modifications only the α-position of the glycine tolerates substitution. For example, introducing a D or L-alanine instead of the glycine, resulted in slight depression of T_m in the binding of DNA (0.7 and 1.8 °C, respectively).[42] Introduction of D-lysine resulted in a slight increase of the binding affinity towards DNA (ΔT_m = +1.0 °C), while the L-isomer had the opposite effect – a 1 °C destabilization.[42]

In more recent years much work has been done on investigating various cyclic backbone-based PNAs.[43-49] By linking various parts of the PNA backbone, the influence of conformational restriction and chirality on the binding affinity of PNA can be evaluated. Table 1.2 shows the most successful modifications made as well as commenting on the actual advantages.

Table 1.2. Cyclic backbone-based PNAs.
(A review by V. A. Kumar gives an in-depth analysis of cyclic backbone PNAs.[45])

Entry	Structure	Features	Reference
1	Aminoprolyl PNA 	Single modification inserted at the N-terminus of Aeg PNA shows discrimination of anti-parallel vs. parallel binding to the target DNA. Fully modified sequence does not bind target. Alternating Aeg and aminoprolyl PNA shows higher binding affinity than pure Aeg-PNA	46, 47, 48, 49
2	Aminoethylprolyl PNA (Aep-PNA) 	Backbone protonated at physiological pH. The oligomers containing 4*S*, 2-*S/R* Aep-thymine units showed favourable binding properties towards target sequences without affecting the specificity. The stereochemistry at C^2 did not have any effect on the binding abilities.	50
3	Pyrrolidine PNA (PyrPNA) 	All four diastereomers of adenine-9-yl pyr-PNA have been synthesized. The oligomers incorporating (3*S*,5*R*) isomer were shown to have the highest affinity towards RNA in comparison with DNA. The fully modified decamer was binding rU_{10} with a small decrease in efficiency compared to Aeg-PNA.	51

Applications of PNA

The unique properties of PNA make it an attractive tool for a variety of applications. In theory, PNA could be used wherever there is a demand for sequence-specific recognition of nucleic acid (DNA or RNA) fragments, and especially in applications where DNA or RNA would have limited use e.g. cell extracts containing nucleases.

The high affinity of PNA has been utilized in designing hybridization assays, for example, the presence of *Mycobacterium tuberculosis* was detected by the hybridization of the microorganisms *m*RNA to a fluorescently labelled PNA.[52]

Another interesting application is PCR-clamping, where a PNA fragment binds the PCR primer site, thus effectively blocking the PCR product, while a sequence with a single mutation binds the site and is PCR-amplified.[53] Following a similar strategy, point mutations were detected in the K-*ras* gene in the presence of excess amounts of non-mutant DNA.[54]

The so-called biosensor approach entails monitoring the hybridization of DNA targets to an immobilized PNA probe by means of measurable signals produced by the probe.[55-57]

Perhaps even more interesting is the use of PNA for the inhibition of transcription (antigene) or translation (antisense) of genetic material.

A site-specific termination of reverse transcription as well as *in vitro* translation at the point of formation of a PNA-RNA duplex has been reported.[58]

More recently it has been shown that an 11-mer PNA directed toward the initiation codon, dose-dependently inhibited the expression of the neurotrophin receptor p75NTR (associated with both neurodevelopment and neurodegenerative disorders).[59] In comparison a 19-mer phosphorothioate sequence failed to show any activity against the target.

Numerous other examples of inhibition of expression,[60-62] reverse transcription[63-65] or replication[66,67] have been reported.

2. N-(PYRROLIDINYL-2-METHYL)GLYCINE-BASED PNA (PAPERS I AND II)

PNAs, which are based on amino acids other than glycine, can form stable complexes with complementary DNA and RNA.[68-72] Reports on these compounds demonstrate that the chirality of the backbone is an important factor determining the stability of the complexes. In addition, recent studies indicate that introduction of conformational restriction into oligonucleotides,[73,74] PNA-DNA[75,76] chimera and modified PNAs[77,78] has a beneficial effect on their hybridization properties. It is believed that conformational restriction in the nucleic acid backbone should result in a better positioning relative to the target strand, thus giving higher affinity. It would be interesting to investigate the hybridization properties of a non-charged PNA modification that will combine conformation restriction with chirality. Therefore, a new chiral PNA analogue, the backbone of which contains both isomers of *N*-(2-pyrrolidine-methyl)glycine (Pmg) **I** (Figure 2.1), was designed and prepared.

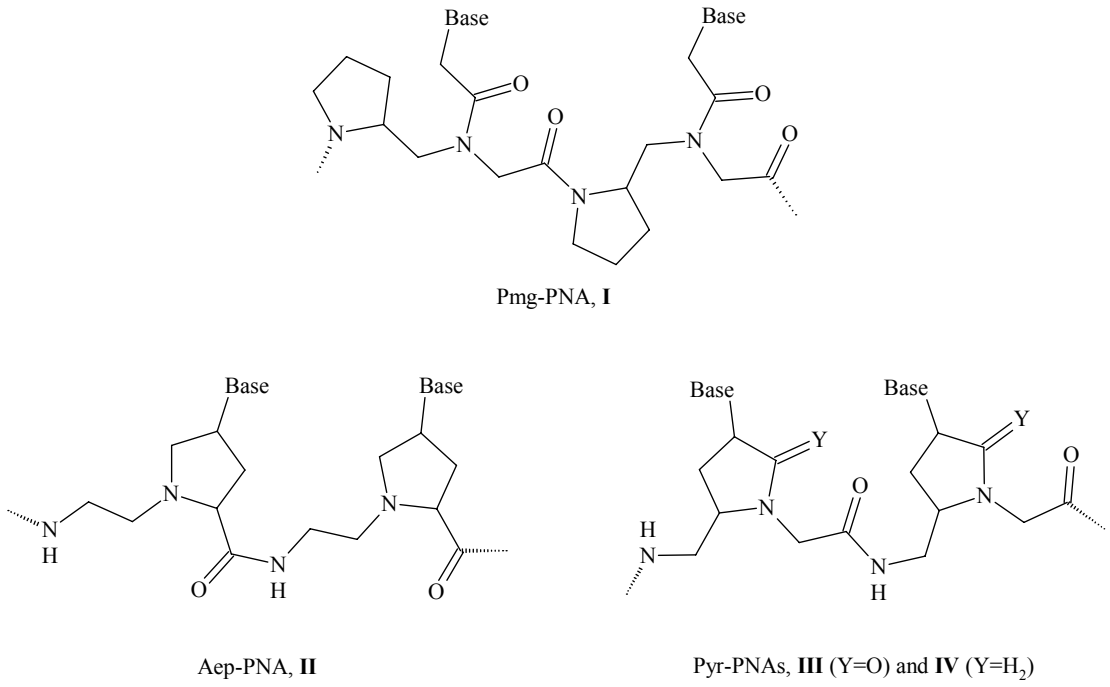


Figure 2.1. Structures of Pmg-PNA, aminoethylprolyl PNA, and pyrrolidine PNAs

It was reasoned that the pyrrolidine moiety will introduce restraints and chirality, while the backbone would still contain the requisite six covalent bonds in the monomeric unit as the Aeg-PNA. Fragments would be constructed by introduction of an amide bond between the carboxyl and the pyrrolidine amino functions of two monomers. The second amino group in the Pmg monomeric unit would be the attachment point (carbonyl methylene) of the nucleobase. Unlike other pyrrolidine-based PNAs, e.g. aminoethylprolyl PNA[79] (Aep-PNA) **II** (Figure 2.1) and pyrrolidine PNA[80] (**IV**), which are positively charged, the Pmg backbone will remain non-charged at physiological pH. Pyrrolidine PNA **III** is also non-charged at neutral pH, but the insertion of a single modification caused a Tm drop of 3.5 °C, while a fully modified decamer showed a Tm depression of 1 °C per modification.[81]

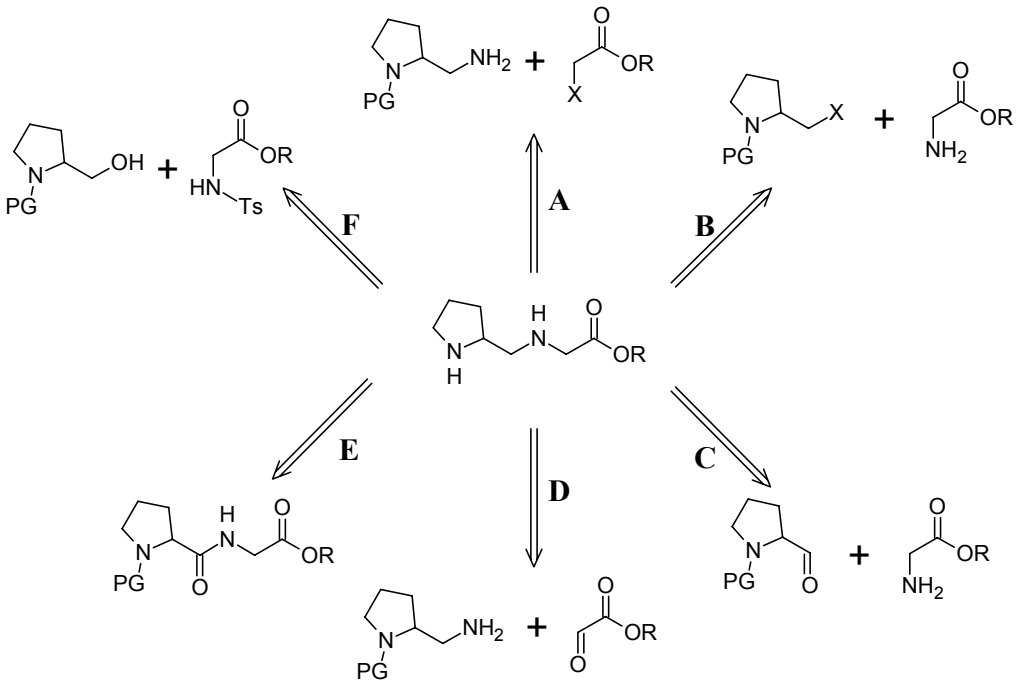
Poly-cationic species are generally known to form stable complexes with DNA or RNA due to charge attraction; however, non-specific binding may be a consequence.[82]

Studying the hybridization of Pmg-PNA will permit the determination of the conformational restrictions and chirality effects on duplex formation in the absence of positive charges. This may provide useful information for the development of new PNA analogues.

Synthesis of monomers

A first step, prior to the assembly of Pmg-containing PNA fragments on a solid support, is the preparation of properly protected Pmg building blocks.

Disconnection of the Pmg fragment leads to a number of valid synthetic routes (Scheme 2.1). It would reasonable to introduce the 2-methylene-pyrrolidine fragment from a proline derivative, since there are many L and D-proline derivatives commercially available as pure enantiomers.



Scheme 2.1. Disconnections of Pmg fragment.

In analogy to the synthesis of the Aeg backbone unit (See Chapter 1), the Pmg fragment could be obtained through alkylation of the selectively protected diamine, i.e. 2-aminomethylpyrrolidine (Pathway A).

Alternatively, a glycine ester could be alkylated with an *N*-protected (2-halomethyl)pyrrolidine (Pathway B).

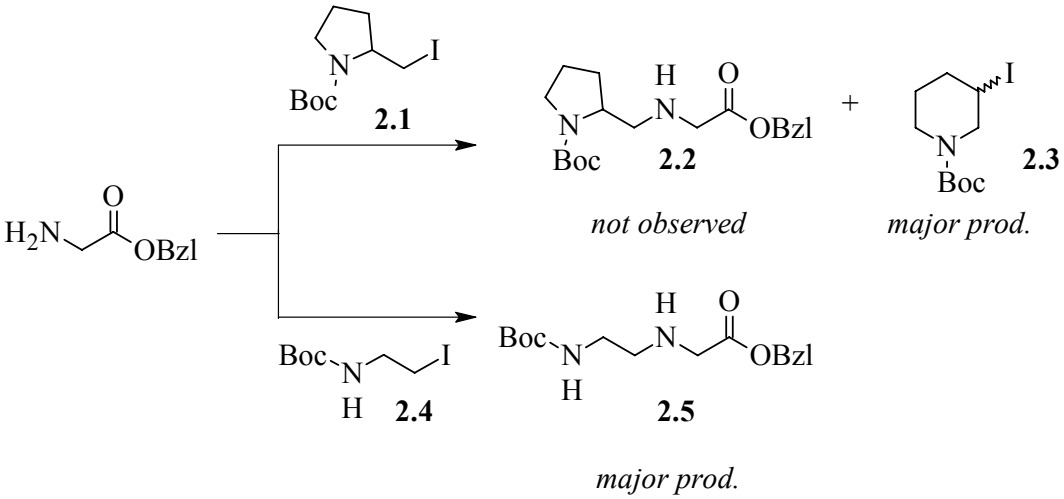
Another approach would be to introduce the secondary exocyclic amino function via a reductive amination, either using *N*-protected prolinal (pyrrolidine 2-carboxy-aldehyde) and a glycine ester, or 2-aminomethyl-pyrrolidine and a glyoxalic ester. (Pathways C and D).

Considering the structural similarity of Pmg to a dipeptide, one could attempt to perform a selective reduction of the amide bond of the properly protected Pro-Gly dipeptide (Pathway E).[83]

During the course of this study another suitable route was published, i.e. an alkylation of *N*-tosyl or *p*-nitrobenzenesulfonyl derivatized glycine with prolinol under Mitsunobu conditions (Pathway F).[84]

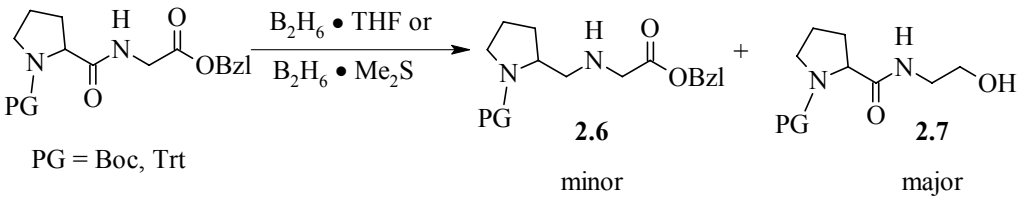
Of the above mentioned routes several were tested in the synthesis of a protected Pmg backbone. Alkylation of glycine benzyl ester (Pathway B) with 2-iodomethyl-*N*-Boc-pyrrolidine (**2.1**) did not yield the expected product **2.2**, however a new product was

observed, which was later, according to its ^{1}H NMR spectrum, identified as N-Boc-2-iodopiperidine (**2.3**). Such ring expansion via a strained aziridine intermediate has been previously reported.[85] In comparison, the alkylation of glycine ester with Boc protected 2-iodoethylamine **2.4** proceeded smoothly, giving the expected *N*-(2-aminoethyl)glycine **2.5**.



Scheme 2.2. Alkylations of glycine ester.

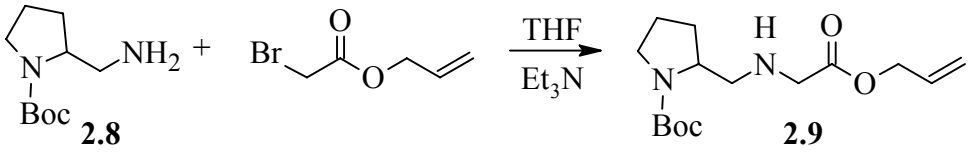
Reduction of the amide bond of a dipeptide, Boc-Pro-Gly-OEt, was attempted (Pathway E) with THF or Me_2S-complexed diborane. Performing the reduction at low temperatures gave a poor yield of the desired product Boc-Pmg-OEt (**2.6**), and increasing the temperature gave side products, mostly the reduction of the ester to an alcohol **2.7**. Although borane is generally more reactive towards amides than esters, it was reported that amino acid esters can be reduced by borane.[86]



Scheme 2.3. Dipeptide reduction with borane.

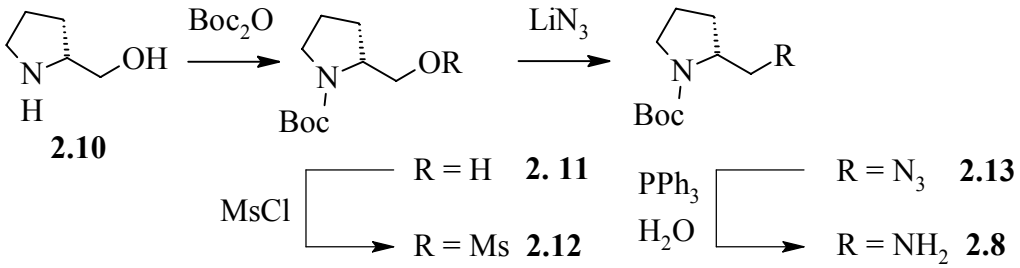
Reductive amination of prolinal (Pathway C) was not attempted since the reaction could lead to a certain degree of racemization at C^2 of pyrrolidine.[87]

The route that gave the best results was alkylation of *N*-Boc-(2-aminomethyl)pyrrolidine (**2.8**) with allyl bromoacetate (Pathway A). Following this route a protected *S*-Pmg backbone unit **2.9** was obtained in 65% isolated yield using THF as a solvent and Et_3N as base (Scheme 2.4).



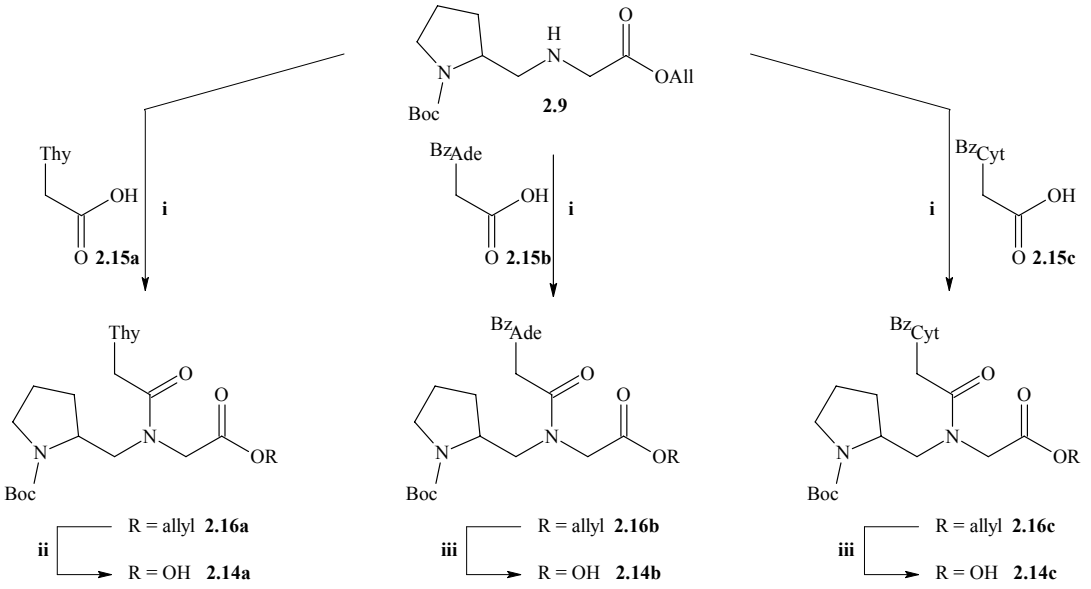
Scheme 2.4. Alkylation of 2-(aminomethyl)pyrrolidine.

Both isomers of *N*-Boc-(2-aminomethyl)pyrrolidine are commercially available,[88] however, due to their relatively high cost and long delivery time, they were synthesized from the more available prolinol (2-hydroxymethyl-pyrrolidine, **2.10**). The key step was a nucleophilic displacement of prolinol mesylate **2.12** with lithium azide, furnishing 2-azidomethylpyrrolidine (**2.13**), which was subsequently reduced to the corresponding amine **2.8** through action of triphenylphosphine in the presence of water (Scheme 2.5). These reaction conditions are mild, allowing the use of other N-protecting groups than Boc for N^1 of pyrrolidine, such as Fmoc, Trt, Alloc.[89]



Scheme 2.5. Synthesis of 2-(aminomethyl)pyrrolidine.

Having made the *R*-Pmg backbone, the *R*-Pmg-PNA monomers **2.14a-c** were synthesized by acylation of the free secondary amino function of compound **2.9** with protected nucleobase acetic acids **2.15a-c** in conjunction with HBTU, followed by cleavage of the the allyl esters **2.16a-c** (See Scheme 2.6). Although there are numerous methods available for making an amide bond, HBTU was selected because of its rapid action and easy availability.



Scheme 2.6. Synthesis of Pmg-PNA monomers. (i) HBTU, DIEA, DMF; (ii) NaOH, dioxane; (iii) Bu_3SnH, $[Pd(PPh_3)_2]Cl_2$, AcOH, DCM.

Allyl esters have an advantage over the more commonly used methyl/ethyl or benzyl esters, since the allyl esters can be cleaved both under basic (base hydrolysis) or neutral (catalytic hydrostannylation) conditions.[90] The latter comes in handy when there are other base-sensitive functionalities present in the molecule, like the benzoyl groups on the exocyclic amines of adenine and cytosine. Besides the above-mentioned advantages, the monomers, after hydrostannolytic cleavage and workup, are obtained as triethylammonium salts, which allows their purification via standard flash silica chromatography. An alternative method, specially used in the synthesis of MMT-protected Aeg-PNA monomers, is cleavage of the allyl esters by aqueous tetrabutylammonium hydroxide in methanol (producing Bu_4N^+-salts), which can be contaminated with an excess of $Bu_4N^+OH^-$ and are purified by laborious multiple extractions with water.[91]

PNA synthesis

The PNA sequences that we wished to synthesize, and test, were 10-mers with one or two Pmg monomers included in the middle of the Aeg-PNA strand. The assembly was performed on an automated DNA synthesizer (Gene Assembler) using a highly crosslinked polystyrene functionalized with Fmoc-glycine via a 4-(hydroxymethyl)benzoic acid (HMBA) linker.

Since the MMT protected Aeg-PNA monomers were already available (Paper V), the assembly of the Pmg-containing PNA would utilize three different protecting groups, i.e. Fmoc, MMT and Boc. Thus, the synthesizer was programmed to execute the following steps:

1. Removal of Fmoc group (deprotection of support-bound glycine)
2. Coupling
3. Removal of MMT group (deprotection of Aeg-PNA units)
4. Removal of Boc group (deprotection of Pmg-PNA unit(s))
5. Intermediate washes

Assembly of PNA decamers including Pmg

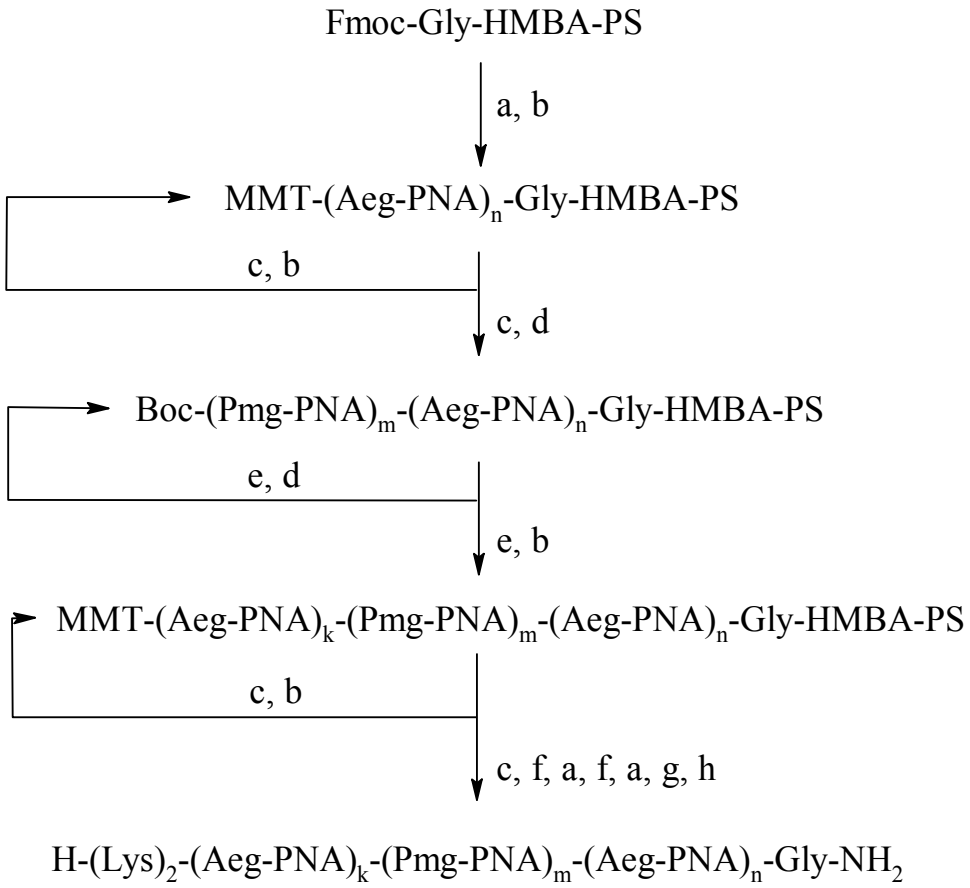
Four PNA decamers **VI-IX** were constructed containing one or two, *S*- or *R*-Pmg units in the middle of the PNA strand. All PNA decamers were terminated with two consecutive lysines, in order to enhance the solubility of the assembled PNAs in water at pH 7.[92]

Table 2.1. Steps involved in the solid phase synthesis using a mixed protection group strategy.

Step	Function	Solvents and reagents	Time, min.
1	Fmoc deprotection of resin-bound glycine and terminal lysines	22 % piperidine in NMP	2×3.5
2	Single coupling	MMT Aeg-PNA monomers[b], HBTU[c], DIEA[c]	17.5
3	MMT deprotection during chain elongation	1 % TFA/DCE	3
4	Double coupling	Boc Pmg-PNA monomer[c], HBTU[b], DIEA[c]	2×17.5
5	Boc deprotection	50 % TFA/DCM	2×15
6	Lysine termination	Fmoc-*L*-Lys(Boc)-OH[b], HBTU[b], DIEA[c]	2×17.5

[a] Synthesis was performed on 1 μmol scale; [b] 0.3 M solution in NMP; [c] 0.4 M solution in NMP.

The post-synthetic procedures included removal of the N^{ε}-Boc groups from the terminal lysines, and the release of the synthesized PNA fragments from the solid support by ammonolysis. This provided crude PNAs with glycine amide at their C-termini (Scheme 2.7).



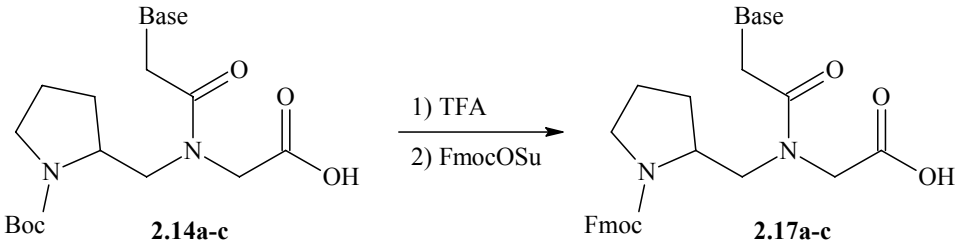
Scheme 2.7. Solid phase synthesis of Pmg-PNA using a mixed protecting group strategy; reagents: (a) 22% piperidine/NMP; (b) MMT-Aeg-PNA monomer, DIEA, HBTU; (c) 1% TFA/DCE; (d) Boc-Pmg-PNA monomer, DIEA, HBTU; (e) 50% TFA/DCM; (f) Fmoc-*L*-Lys(Boc)-OH, DIEA, HBTU; (g) 95% aq. TFA; (h) NH_3/MeOH.

In addition to the four PNA fragments, it was also decided to assemble a fully modified PNA decamer, i.e. having a uniform Pmg-backbone.

The preliminary thermal melting data indicated that the PNA fragments containing the *R*-Pmg modification show greater binding affinity to their complementary targets in comparison to *S*-Pmg containing PNAs. At this point it was decided to prepare only the fully modified *R*-Pmg PNA.

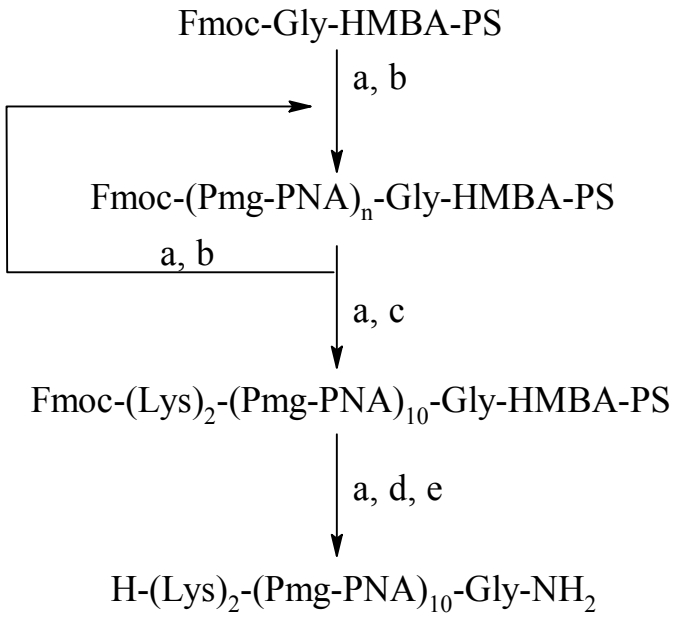
Further, it was understood that having Boc protection is inconvenient since now all the deprotection steps would have to be done manually. Therefore, the Boc group was

replaced with Fmoc, which is removed under basic conditions usually using about 20% piperidine solution. Thus, the existing Boc-Pmg monomers were first treated with 50% TFA in DCM resulting in cleavage of the Boc group and then treated with FmocOSu (Scheme 2.8) and purified by crystallization giving the desired thymine, N^6-benzoyladenine, and N^4-benzoylcytosine building blocks **17a-c** in excellent yields.



Scheme 2.8. Synthesis of Fmoc *R*-Pmg monomers; Reagents:
(a) TFA/DCM/MeOH/H_2O 16:16:1:1; (b) FmocOSu, DIEA, THF.

Once the Fmoc *R*-Pmg monomers were prepared an automated solid phase synthesis of the fully modified *R*-Pmg-PNA decamer **X** was performed (Scheme 2.9).



Scheme 2.9. Synthesis of all-*R*-Pmg PNA decamer **X**; reagents: (a) 22% piperidine/NMP; (b) Fmoc-Pmg-PNA monomer, DIEA, HBTU; (c) Fmoc-*L*-Lys(Boc)-OH, DIEA, HBTU; (d) 95% aq. TFA; (e) NH_3/MeOH.

Table 2.2. Steps involved in solid phase synthesis of PNA **X**[a]

Step	Function	Solvents and reagents	Time, min.
1	Fmoc deprotection	22 % piperidine in NMP	2×3.5
2	Coupling	Fmoc Pmg-PNA monomer[b], HBTU[b], DIEA[c]	2×17.5
3	Lysine termination	Fmoc-*L*-Lys(Boc)-OH[b], HBTU[b], DIEA[c]	2×17.5

[a] Synthesis was performed on 1 μmol scale; [b] 0.3 M solution in NMP; [c] 0.4 M solution in NMP.

Purification

The purifications of the PNAs were performed by RP-HPLC using a C_{18} column running a gradient of acetonitrile in a low pH buffer (0.1% TFA). Major peaks were collected and analyzed by MS and the product-containing fractions were lyophilized to give pure PNAs.

UV thermal melting experiments

Thermal melting values of the corresponding DNA•DNA and DNA•RNA duplexes were recorded and used as references.

The ability of the Pmg containing PNA fragments to form duplexes with complementary DNA and RNA was evaluated by UV thermal melting experiments. The data obtained from these experiments are collected in Table 2.3.

Table 2.3. UV thermal melting data (λ = 260 nm).

Entry	PNA #	Sequences	T_m, °C	ΔT_m(RNA vs. DNA), °C	ΔT_m/mod., °C
1		d(TCACTTCCAT):DNA	35.0		
2		d(TCACTTCCAT):RNA	38.0	3.0	
3	**V**	tcacttccat : DNA	40.0		
4	**V**	tcacttccat : RNA	53.0	13.0	
5	**VI**	tcact**T**^{S}ccat : DNA	21.0		– 19.0[a]
6	**VI**	tcact**T**^{S}ccat : RNA	39.0	18.0	– 14.0[b]
7	**VIII**	tcact**T**^{R}ccat : DNA	24.0		– 16.0[a]
8	**VIII**	tcact**T**^{R}ccat : RNA	44.0	20.0	– 9.0[b]
9	**VII**	tcac**T**^{S}**T**^{S}ccat : DNA	– [c]		– [c]
10	**VII**	tcac**T**^{S}**T**^{S}ccat : RNA	27.0	–	– 13.0[b]
11	**IX**	tcac**T**^{R}**T**^{R}ccat : DNA	5.0		– 17.5[a]
12	**IX**	tcac**T**^{R}**T**^{R}ccat : RNA	36.0	31.0	– 8.5[b]
13	**X**	(**TCACTTCCAT**)^{R} : DNA	– [c]		– [c]
14	**X**	(**TCACTTCCAT**)^{R} : RNA	– [c]	–	– [c]

All PNA sequences are of the following structure (N → C): H-$(Lys)_2$-$(PNA)_{10}$-Gly-NH_2. Lowercase letters in PNA sequences denote Aeg-PNA (a, c, t) and bold uppercase denotes Pmg-PNA ($\mathbf{T}^{X}$) where the superscript letter indicates the isomer.
[a] Compared to the T_m of PNA:DNA duplex; [b] compared to the T_m of PNA:RNA duplex; [c] no sigmoidal transition was observed.

Summary

In summary, very clear trends can be observed: a) *R*-Pmg containing PNAs form more stable duplexes both with DNA and RNA, compared to *S*-Pmg containing PNAs, but less stable than those obtained with non-modified Aeg-PNA; b) both the *R*- and the *S*- Pmg containing PNAs form tighter duplexes with complementary RNA than with DNA; c) both the *S*- and the *R*- Pmg PNA exhibit substantially stronger discrimination between RNA and DNA compared to all-Aeg-PNA and native DNA; d) fully modified 'all-Pmg' PNA does not form tight duplexes with either DNA or RNA.

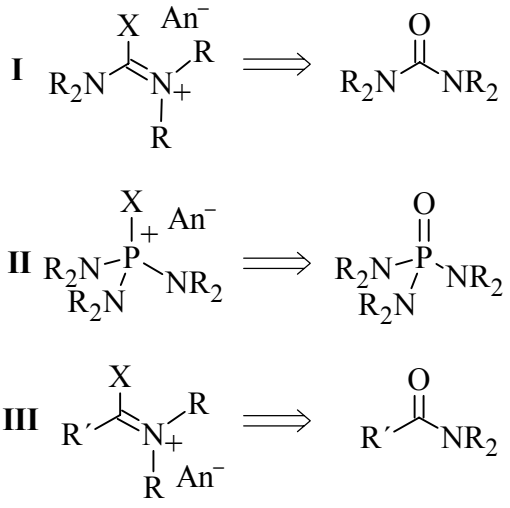
This enhanced selectivity, especially of the *R*-Pmg-containing fragments towards RNA, can be utilized in several possible ways. For instance, by increasing the Aeg-PNA part around *R*-Pmg based units, a more stable RNA-preferring nucleic acid analogue can be created. This analogue could be used to label or identify specific RNA in cell extracts, gels and may have other applications in studies where discrimination between RNA and DNA is desired.

3. SULFURANE-MEDIATED PEPTIDE BOND FORMATION (PAPER III)

Peptide coupling agents

Amide bond formation plays a central role in the synthesis of peptides and numerous other biologically active molecules.[93,94] The introduction of amide bonds is not only crucial for the construction of peptide backbones, but it is also an essential step in the protection of both amino and carboxylic acid functions[95-98] both of which can be rendered relatively non-reactive by converting them into amides. Over the past decades a large number of reagents have been developed for the introduction of amide (peptide) linkages.[99-106] A useful coupling agent should fulfil the following criteria: rapid action, high coupling yield and low racemization (in the condensation of chiral components), as well as shelf-stability and ease to handle.

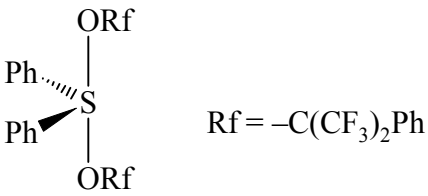
A large number of the commonly used coupling agents are uronium (I), phosphonium (II) or immonium (III) salts, which can be derived from *N,N*-dialkylated ureas,[101,102] *tris*-(*N,N*-dialkylamino) phosphanes (phosphamides)[103,104] or *N,N*-dialkyl carboxyamides [105,106] respectively (Scheme 3.1).



Scheme 3.1. Commonly used peptide coupling agents and their synthetic precursors.

Racemization during peptide coupling

It is well established within peptide chemistry that racemization occurs mainly during activation of the carboxyl function due to base-mediated deprotonation of an azalactone intermediate.[107,108] It has also been reported that using mild bases such as *N*-methylmorpholine or 2,4,6-trimethylpyridine instead of the commonly applied *N,N*-diisopropylethylamine (DIEA) results in reduced racemization, although the yields and time of couplings are somewhat compromised.[109-113] Not many procedures are reported in which the external base is completely excluded.[114,115] From previous studies directed towards the applications and mechanism of action of BTBDS[116-127] it seemed plausible that the sulfurane functionality could be used for the construction of an amide bond without an external base. In this work the possibility of using BTBDS for amide bond formation was investigated. The extent of racemization of BTBDS-mediated coupling was also studied and compared to that of two of the most commonly used coupling agents - HATU and HBTU.



3.1

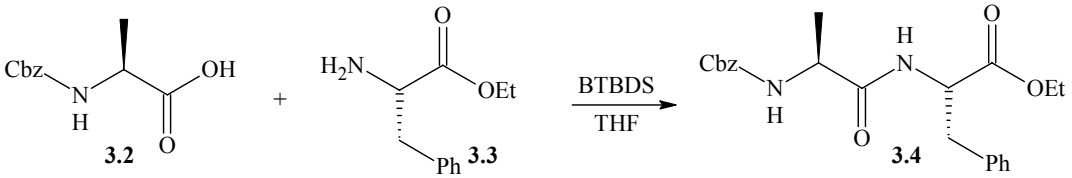
Figure 3.1. Structure of *bis*-[α,α-*bis*(trifluoromethyl)benzyloxy]diphenylsulfur (BTBDS)

BTBDS - Martin Sulfurane Dehydrating agent

BTBDS is a commercially available reagent, sold by Aldrich Chemical Co. under the name 'Martin Sulfurane Dehydrating agent'. As the name suggests, BTBDS is used for dehydrations of a wide variety of substrates, including but not limited to secondary and tertiary alcohols producing alkenes,[117,118] formation of cyclic ethers from diols (epoxides from 1,2-diols)[119] and preparation of nitriles from aminomethyl aryls.

In the current study BTBDS was used for activation of an *N*-protected amino acid in order to promote a reaction with another amino acid, thus forming a peptide bond.

The first successful synthesis of a dipeptide using BTBDS was the condensation of Z-L-Ala-OH and H-L-Phe-OEt in THF using 2 equivalents of BTBDS. The desired dipeptide was identified first by TLC (comparing the reaction mixture with a standard) and later also by MS.



Scheme 3.2. Formation of protected dipeptide Ala-Phe.

Yield optimisation of BTBDS couplings

After establishing that BTBDS can indeed promote condensation of two amino acids to give a dipetide, a set of reactions were performed over a period of 20 min using components **3.2**, **3.3** and BTBDS in DCM under various conditions (Table 3.1). All these reactions resulted in the formation of dipeptide Cbz-Ala-Phe-OEt (**3.4**), which was isolated, quantified and analyzed by ESI-TOF mass spectrometry and NMR spectroscopy.

It was found that using an excess of sulfurane **3.1** gives poor yields of dipeptide **3.4** (Table 3.1, entry 1), partially due to the formation of a side-product which, according to ESI-TOF MS could be sulfilimine **3.5** (Scheme 3.3). This type of compounds, which has also been reported in the literature,[120] was not detected in the experiments presented in entries 2-6. In addition, from the data in Table 3.1, it is clearly beneficial if the carboxyl component **3.2** is added in excess to both BTBDS and H-L-Phe-OEt.

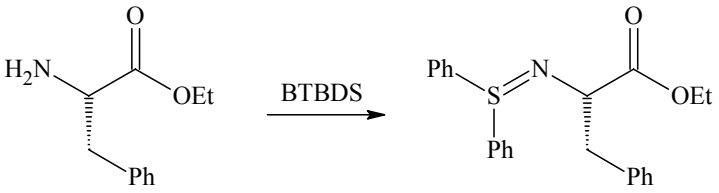
Table 3.1. Coupling of 1 equivalent H-L-Phe-OEt with various amounts of Cbz-L-Ala-OH and BTBDS.

Entry	Component **3.2**, equivalents[a]	BTBDS, equivalents[a]	Isolated Yield[b] %
1	1	2	< 20
2	1	1	43.7
3	2	1	76.3
4	3	2	95.1
5	4	2	98.4
6	4	2	95.1[c]
7	4	2[d] (HATU)	95.6

[a] Equivalents relative to **3.3**.
[b] Reaction time 20 min.
[c] Component **3.3** was used as HCl salt followed by addition of 1 eq. of DIEA.
[d] 2 eq. of HATU instead of BTBDS and 5 eq. DIEA in DMF.

The best and virtually quantitative yield of Cbz-Ala-Phe-OEt was obtained using 4 equivalents of Cbz-Ala-OH and 2 equivalents of BTBDS Table 3.1, entry 5). Similar yields were obtained using the HCl salt of H-Phe-OEt together with one equivalent of DIEA (entry 6) and when BTBDS or HATU in a ratio of 3:2 and 4:2, respectively (entries 4 and 7) were used.

It is important to note that in a BTBDS-assisted condensation, the yields are dependent on the order of the addition of reactants. When BTBDS was added to H-L-Phe-OEt prior to the treatment with carboxyl component **3.2**, the major product was sulfilimine **3.5**.



3.3 **3.5**

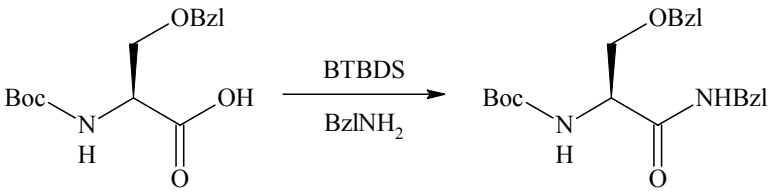
Scheme 3.3. Sulfilimine formation from amine and BTBDS.

The highest yields of the dipeptide **3.4** were obtained by pre-activating the carboxylic residue **3.2** with BTBDS for at least 30 seconds,s followed by condensation with H-L-Phe-OEt.

Racemisation in BTBDS-mediated coupling

In the next stage, after optimization of the reaction conditions, it was important to determine the extent of racemization caused by BTBDS activation of Cbz-L-alanine. For this purpose ^{1}H and ^{13}C NMR spectra of dipeptide **3.4** obtained by BTBDS-mediated formation were recorded. The spectra indicated the presence of only one compound, which was identical to the Cbz-L-Ala-L-Phe-OEt prepared by HATU-assisted condensation. The detection limit of this method was found to be about 3%. This led to the conclusion that racemization in the BTBDS-assisted formation of compound **3.4** did not exceed the level of racemization observed in a HATU-mediated coupling (Table 3.1, entries 5, 6 and 7, respectively).

The results obtained from NMR spectroscopy stimulated us to investigate the racemization in more detail. It was performed by analyzing the coupling products of racemization-sensitive amino acids, i.e. serine [110,121] and cysteine,[109,122] with benzylamine using HPLC analysis with a chiral-phase column.



3.6 **3.7**

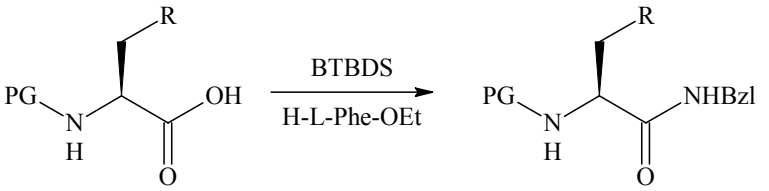
Scheme 3.4. Formation of the protected serine benzylamide

At first, condensation of Boc-L-Ser(Bzl)-OH with benzyl amine was investigated (Scheme 3.4). Treatment of the above amino acid with HBTU, HATU or BTBDS in DCM followed by addition of benzyl amine gave the protected serine benzyl amide **3.7**.[123] DCM was selected as solvent, since it is known that the rate of racemization is substantially lower in DCM compared to polar solvents, such as DMF.[109] In addition, our previous experiments showed that DCM is a suitable solvent for the BTBDS mediated couplings (See Table 1.) The reactions were allowed to proceed for 20 min, after which an aliquot was withdrawn and quenched in the HPLC mobile phase. The crude mixture was then analyzed by HPLC.

HBTU- and HATU-assisted condensations were performed by reacting the carboxyl component, amino nucleophile, coupling agent and DIEA in a ratio of 1:1:1:2,

respectively. These conditions are often used to effect fast and efficient amide bond formation utilizing the above-mentioned uronium salts. The reactions involving BTBDS were performed under the conditions specified in Table 3.1, entry 5. It was observed that after 20 min HBTU, HATU and BTBDS couplings gave 5.5 %, 2.2 % and 1.8 % D-isomer of amide **3.7** and that the total yield of both isomers was 93 %, 100 % and 99 %, respectively. An additional experiment was executed using BTBDS, showing that the extent of racemization is concentration dependent, i.e. higher concentrations of the components resulted in increased racemization.

Experiments were then conducted to determine the degree of epimerization during the condensation of Boc-L-Ser(Bzl)-OH and Boc-L-Cys(Trt)-OH with the less basic amino component **3.3**. The resulting diastereomeric compositions of dipeptides Boc-Ser(Bzl)-Phe-OEt and Boc-Cys(Trt)-Phe-OEt were analyzed using chiral-phase HPLC as described above for the synthesis and analysis of compound **3.7**. The coupling conditions and their respective outcomes are summarized in Table 3.2 and Table 3.3.



PG = Boc, R = OBzl **3.6**
PG = Boc, R = STrt **3.8**
PG = Bz, R = Ph **3.11**

PG = Boc, R = OBzl **3.6**
PG = Boc, R = STrt **3.10**
PG = Bz, R = Ph **3.12**

Scheme 3.5. Formation of dipeptides Boc-AA-Phe-OEt.

Table 3.2. Coupling of Boc-L-Ser(Bzl)-OH with H-L-Phe-OEt.

Entry	Coupling agent	Equivalents C	N	CP	DIEA	C_C, M	Preact. time, min.	Racem. % D,L	Relative Yield, %
1.	HBTU	1	1	1	2	0.13	15	8.3	99
2.	HATU	1	1	1	2	0.13	4	3.7	100
3.	BTBDS	4	1	2	0	0.14	4	- [a]	> 99
4.	BTBDS	4	1	2	5	0.14	4	2.1	> 99

C, N and CP in the table denote carboxyl component, amino component and coupling agent, respectively; C_C is the concentration of the carboxyl component.
[a] Below the detection limit.

The results depicted in Table 3.2 and Table 3.3 indicate that BTBDS-mediated couplings of Boc-L-Ser(Bzl)-OH and Boc-L-Cys(Trt)-OH to H-L-Phe-OEt proceeded in high yields and with undetectable or low racemization.

Table 3.3. Coupling of Boc-L-Cys(Trt)-OH with H-L-Phe-OEt.

Entry	Coupling agent	Equivalents				$C_{C,\ M}$	Preact. time, min.	Racem. % D,L	Relative Yield, %
		C	N	CP	DIEA				
1.	HBTU	1	1	1	2	0.07	15	6.1	96
2.	HATU	1	1	1	2	0.07	4	4.1	100
3.	BTBDS	4	1	2	0	0.14	4	0.9	92
4.	BTBDS	4	1	2	0	0.07	4	-	75

They also show that in the BTBDS-mediated formation of dipeptide **3.10**, the concentration affects not only the extent of racemization, but also the yield. The results given in Table 3.3 suggest that, in comparison to the HBTU- and HATU-mediated formation of dipeptide **3.10**, the BTBDS reaction requires higher concentrations or longer reaction time.

The low or virtually non-existent racemization in BTBDS-assisted formation of dipeptide **3.9**, versus the 1.8 % D-isomer found in serine benzylamide **3.7** can be attributed to two factors. The first is the difference in basicity of these amines. Benzylamine has a pK_a value of 9.36[124] while that of H-L-Phe-OEt is 2 units lower (7.23).[125] This pK_a difference makes the environment of the Boc-L-Ser(Bzl)-OH/benzylamine mediated condensation more basic than that of the Boc-L-Ser(Bzl)-OH/H-L-Phe-OEt (given in Table 3.2, entry 3), with the consequence that racemization increased to 1.8%. The second factor that influences racemization is the pre-activation time. The higher level of racemization can be the outcome of the longer pre-activation time, i.e. 15 vs. 4 min for the formation of compounds **3.7** and **3.9**, respectively. As expected, racemization also increased when DIEA was added in excess to the C-component to the BTBDS coupling mixture, as indicated in Table 3.2, entry 4. The absence of racemization in the condensation of urethane-protected amino acids prompted us to extend our investigations to a system in which the α-amino function has been converted to an amide.

It is well established that urethane-type protecting groups like Boc, Fmoc and Cbz suppress the formation of azalactones (main factor in racemization) of activated amino acids.[126] However, it is not always possible to use urethane-linked amino acids.

In segment condensations, the amino function adjacent to the activated carboxyl moiety is part of the peptide backbone i.e. an *N*-acyl (amide) component. Activation of such C-terminal amino acids mostly leads to substantial racemization.[127] Stimulated by the performance of BTBDS we decided to apply it in the coupling of Bz-L-Phe-OH (**3.11**), which could be considered as a model for a carboxylic function in a peptide fragment. Compound **3.11** was coupled to H-L-Phe-OEt as described below in Table 3.4.

Table 3.4. Coupling of Bz-L-Phe-OH with H-L-Phe-OEt.

Entry	Coupling agent	Equivalents C	N	CP	DIEA	C_C M	Preact. time, min.	Racem. % D,L	Relative Yield, %
1	HBTU	1	1	1	2	0.13	15	47.4	75[a]
2	HBTU	1	1	1	2	0.14	15	47.8	92[b]
3	HATU	1	1	1	2	0.14	4	36.7	91[a]
4	HATU	1	1	1	2	0.14	4	40.7	100[b]
5	BTBDS	4	1	2	0	0.14	4	13.2	64[a]
6	BTBDS	4	1	2	0	0.14	4	19.2	94[b]

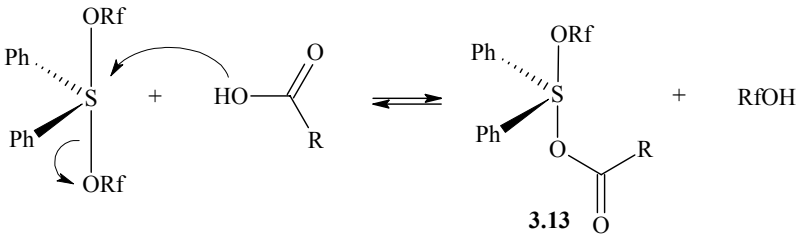
[a] 20 min reaction time.
[b] 100 min reaction time.

HBTU- and HATU-mediated amide bond formations resulted in almost complete racemization of Bz-Phe in dipeptide **3.12** (48% and 41% of D,L-diastereomer, respectively, Table 3.4, entries 2 and 4), while BTBDS gave 19 % of the D,L-diastereomer (entry 6). In all cases a longer time (100 min) was required to drive the reactions to completion.

As mentioned earlier, the coupling of Bz-Phe-OH with an amino nucleophile can be considered to mimic segment condensation. The promising results shown in Table 3.4 suggest that sulfurane-based coupling of properly protected peptide fragments is worth further investigation.

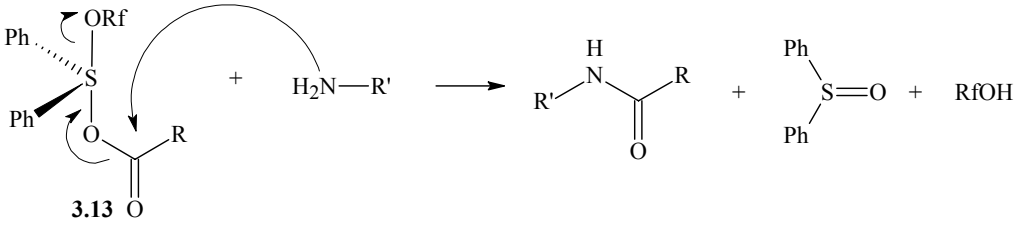
Proposed mechanism of action of BTBDS

Formation of an amide bond between a carboxylic acid and an amine generally requires activation of the acid. Taking into account that BTBDS has two easily exchangeable ligands - *α,α-bis*(trifluoromethyl)benzyloxy groups - the first step of the reaction could be the following:



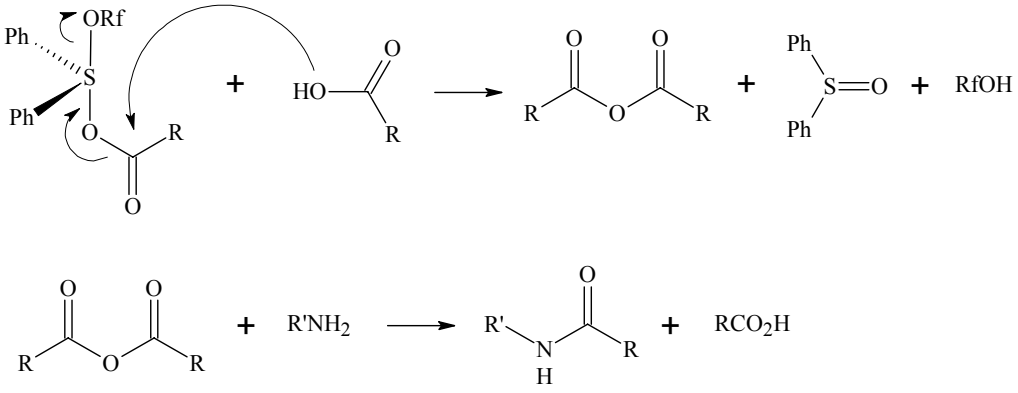
Scheme 3.6. Reaction of a carboxylic acid with BTBDS.

Now the intermediate **3.13** can react with an amine yielding an amide:



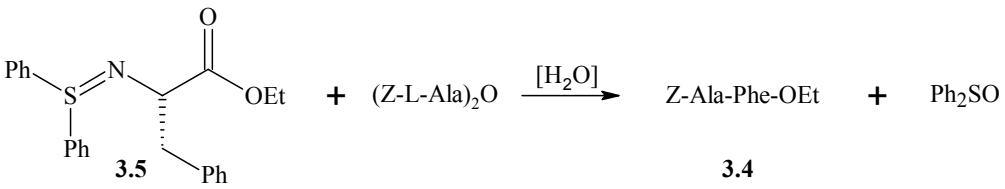
Scheme 3.7. Reaction of an activated carboxylic acid with an amine.

The active intermediate could also react with another molecule of carboxylic acid yielding a symmetric anhydride, which, once formed, can react with the amine giving an amide:



Scheme 3.8. Formation of amide via an anhydride.

In addition, it was observed that amino acid diphenylsulfilimines, formed in the reaction of an amine with BTBDS (Scheme 3.3) can be acylated with acid anhydrides, yielding amides and diphenylsulfoxide:



Scheme 3.9. Acylation of an amino acid sulfilimine with acid anhydride.

In order to get more information on the possible mechanistic pathways, an additional experiment was performed: phenylalanine ethyl ester was acylated with 2 equivalents of Z-alanine anhydride under conditions maximally similar to those used in BTBDS reactions, i.e. solvent, component concentration, reaction time. This reaction would be similar to the one described in Table 3.1, entry 5, if the condensation reaction proceeds via the symmetric anhydride.

The yield of the fully protected dipeptide was 84%, compared to 98% in the case of BTBDS-mediated coupling. This means that it is feasible that the BTBDS-mediated coupling proceeds through the symmetric anhydride. It does, moreover, not exclude that the reaction could proceed wholly or at least partially through the active intermediate **3.13** (Scheme 3.7). The level of racemization in the above-described sym-anhydride reaction did not exceed the ones observed for BTBDS-mediated coupling.

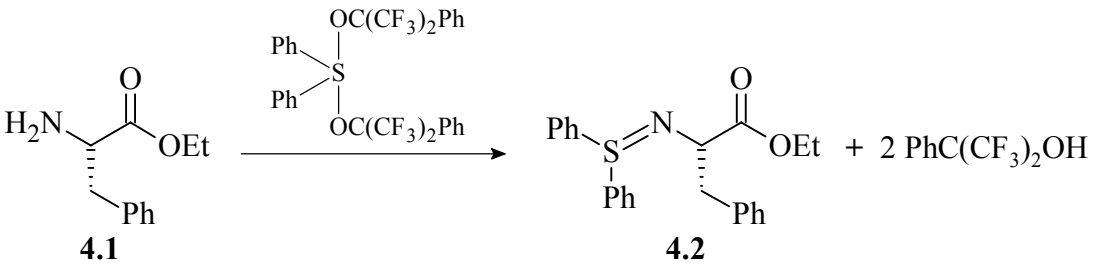
Summary

In summary, this initial investigation concerning the development of sulfurane-based coupling agents shows that *bis*-[α,α-*bis*(trifluoromethyl)benzyloxy]diphenylsulfur can be used to effect fast and efficient condensations of urethane protected amino acids with remarkably low racemization. The yields and racemization levels were compared to two of the most commonly used uronium salt (i.e. HBTU and HATU) and it is shown that in all cases BTBDS-mediated coupling in the absence of a tertiary external base gives better results. These data, obtained from the condensation experiments executed on relatively racemization-sensitive amino acids, i.e. serine and cysteine, suggest that similar outcomes may be achieved for other properly protected amino acids. Also the coupling studies of Bz-L-Phe-OH indicate that, after additional improvement, sulfuranes could become useful for segment condensation. In conclusion, the sulfurane concept is worth further exploration and extension in

several directions, such as testing of other sulfuranes for their ability to serve as peptide coupling agents, studies of sulfurane mediated peptide segment condensation and sulfurane-based amide bond formation of hindered amino acids as well as in the synthesis of chiral PNAs.

4. NUCLEOSIDE SULFILIMINES (PAPER IV)

While investigating the potential of a novel, sulfurane-based coupling agent (Chapter 3), a side-product of the coupling reaction was isolated, later identified as an amino acid-derived sulfilimine. This compound was formed in the reaction of an amine with the coupling agent BTBDS.



Scheme 4.1. Formation of a sulfilimine from an amine and BTBDS.

This finding promoted our interest in this class of substances. We noticed that while there is a limited number of reports dealing with amino acid-derived sulfilimines,[128,129] no reports at all were found of nucleoside-derived sulfilimines. Driven by pure scientific curiosity we synthesized, characterized and explored the chemical stability and proposed some potential applications of these novel compounds. The results of these studies are presented in this chapter.

Sulfilimines

Sulfilimines (sulfimides, iminosulfuranes) are a class of λ^4 sulfur compounds. The first reports of sulfilimines date back to the beginning of the 20th century. Raper reported the formation of a crystalline compound in a reaction of chloramine-T and mustard gas.[130] A few years later Nicolet and Willard obtained a crystalline product from the reaction of diethylsulfide with chloramine-T.[131] A large number of sulfilimines have been prepared from sulfides and *N*-chloroarenesulfonamides. A review from Gilchrist and Moody gives an excellent overview.[132]

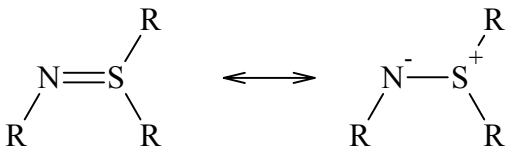
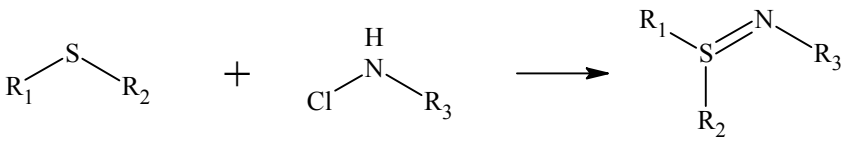


Figure 4.1. Structure of sulfilimines.

The data on sulfilimines, including X-ray structural analysis lead to description of sulfilimines as resonance hybrids. The substituents have very little effect on the bonding and all examined sulfilimines show similar features.[133,134]

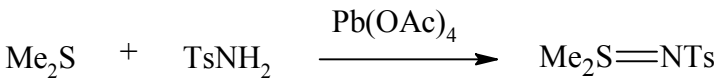
Synthesis of sulfilimines

Sulfilimines can be obtained in a variety of ways. The first method is a reaction of sulfides with N-halo compounds. A variety of N-halo compounds have been utilized, for example, N-chloroarenesulfonamides,[135,136] N-chloroamides,[137,138] and N-chloroanilines.[139,140]



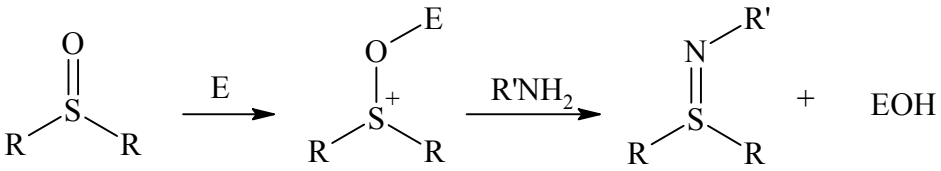
Scheme 4.2. Formation of sulfilimines in a reaction between sulfide and N-haloamine.

Sulfilimines are also formed in reactions of sulfides, amines and reagents such as lead (IV) acetate,[141] NCS[142] or sulfuryl chloride.[139]



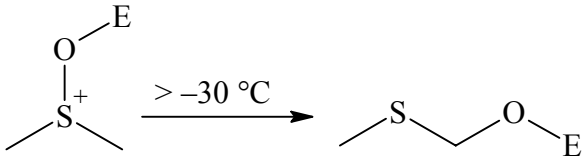
Scheme 4.3. Formation of tosyl-dimethlsulfilimine.

Swern et al.[143,144] have published a number of reports on the synthesis of sulfilimines utilizing sulfoxide as the source of sulfur. Intially, the sulfoxide is converted into a oxysulfonium species, which is further allowed to react with an amine.



Scheme 4.4. Formation of oxysulfonium intermediate and reaction with an amine (E – electrophile).

Phosphorus(V)oxide,[143] sulfur(VI)oxide,[143] boron(III)fluoride,[143] acetic and trifluroacetic anhydrides,[144] have been used as electrophiles to activate sulfoxide. It is worth mentioning that, while P_2O_5 and BF_3 complexes with DMSO can be isolated as very hygroscopic solids and the reaction with amines performed at ambient temperatures,[143] trifluroacetic anhydride reacts violently with DMSO, thus the reaction has to be performed at greatly reduced temperature (-60 °C) in the presence of moderating solvent (DCM).[144] Another reason this reaction has to be properly cooled, is to prevent Pummerer rearrangement occurring at temperatures exceeding -30 °C.[145]



Scheme 4.5. Pummerer rearrangement.

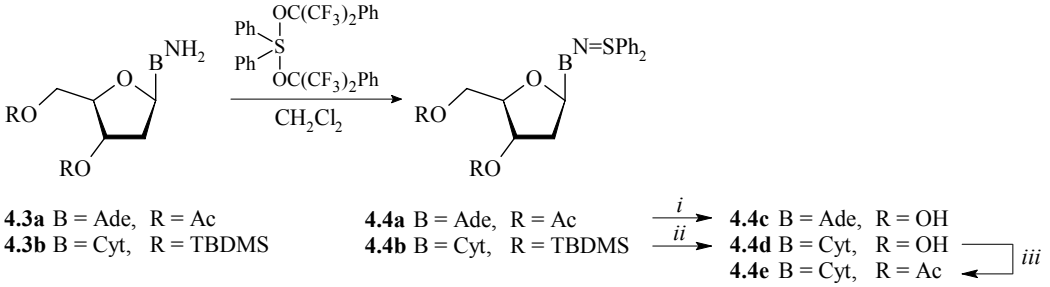
At ambient temperature acetic anhydride and DMSO does not form the active oxysulfonium intermediate, it is, however, produced upon heating.[143,145]

Martin et al. have reported formation of diphenylsulfilimines not only from aliphatic and aromatic amines, but also from primary and secondary amides, and sulfonamides.[146,147,148]

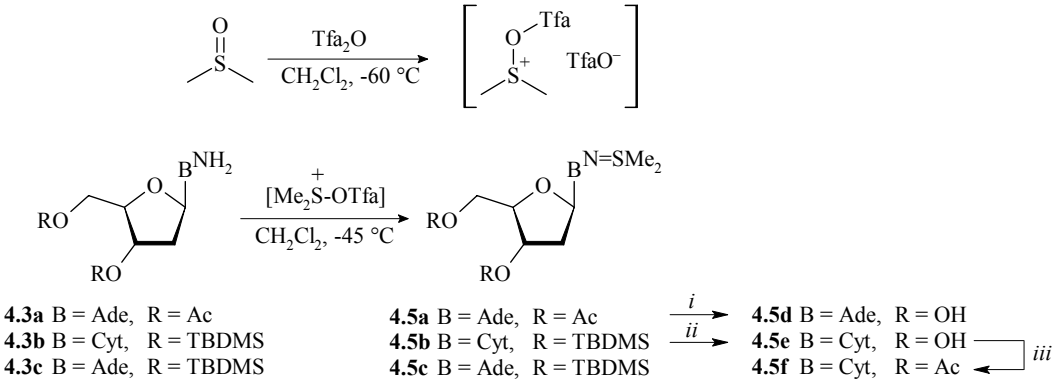
Synthesis of nucleoside sulfilimines

In order to explore the properties of aromatic as well as aliphatic nucleoside sulfilimines, we embarked on the preparation of the diphenyl- and the dimethylsulfilimine derivatives of suitably-protected deoxycytidine and

deoxyadenosine. Our intention is to use the information obtained from investigating these compounds as the basis for a broader study of the properties of nucleoside sulfilimines as potential protecting groups for nucleobases. For the synthesis of the nucleoside diphenylsulfilimine, BTBDS was the reagent of choice, while for making the nucleoside dimethylsulfilimines a protocol employing dimethylsulfoxide/ trifluoroacetic anhydride was followed.[144] This method relies on the activation of dimethylsulfoxide (DMSO) followed by addition of an amine. For the synthesis of dimethylsulfilimines **4.4a** and **4.4b**, Tfa_2O was chosen, since it was reported to perform better than P_2O_5, BF_3 and SO_3.[13]



Scheme 4.6. Synthesis of nucleoside diphenylsulfilimines. (i) NH_3/MeOH; (ii) $Et_3N \times 3$ HF/THF; (iii) Ac_2O/Py.



Scheme 4.7. Synthesis of nucleoside dimethylsulfilimines. (i) NH_3/MeOH; (ii) $Et_3N \times 3$ HF/THF; (iii) Ac_2O/Py.

The treatment of 3′,5′-*O*-protected deoxynucleosides **4.3a**[149] and **4.3b**[150] with *bis*-[*α,α-bis*(trifluoromethyl)benzyloxy]diphenylsulfur in DCM resulted in the formation of diphenylsulfilimines **4.4a** and **4.4b** in 82% and 88% yield, respectively (Scheme 4.7). It should be mentioned, that protection of the free hydroxyl functions of the nucleosides during the BTBDS and the DMSO/trifluoroacetic anhydride reactions is

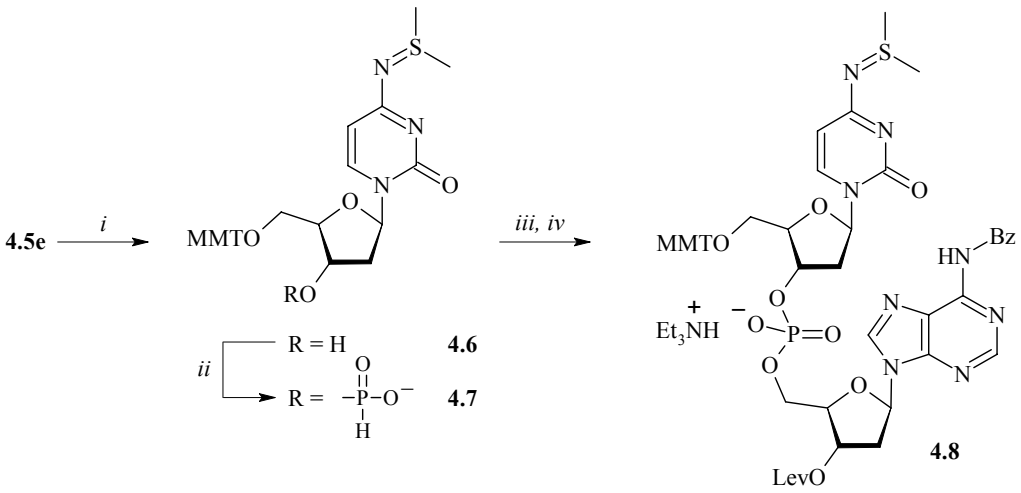
mandatory, since BTBDS can cause elimination of alcohols to produce alkenes,[151,152] and the DMSO/trifluoroacetic anhydride mixture can bring about their oxidation.[153,154] Next, compound **4.4c** was obtained by ammonolysis of diacetylated **4.4a** in anhydrous NH_3/MeOH. Fluoride-ion assisted cleavage of the *t*-butyldimethylsilyl (TBDMS) groups from **4.4b** resulted in desilylated compound **4.4d**, which was further acetylated with acetic anhydride in pyridine to give, after work-up and purification, **4.4e** (92%).

Nucleoside dimethylsulfilimines **4.5a**, **4.5b**, and **4.5c** were prepared from **4.3a**, **4.3b**, and **4.3c**[150] in 70%, 62% and 63% yield, respectively (Scheme 4.7) by the reactions of the properly *bis*-*O*-protected nucleosides with DMSO/ trifluoroacetic anhydride in CH_2Cl_2. Dimethylsulfilimine derivative **4.5a** was further ammonolyzed as mentioned above for *bis*-acetylated **4.4a,** while **4.5f** was prepared by removal of the TBDMS group from **4.5b** with $Et_3N \times 3$ HF for 18 h (70% isolated yield), followed by acetylation with acetic anhydride in pyridine, which proceeded in quantitative yield. The dimethylsulfilimine group was completely intact during the desilylation and the acetylation reactions.

While engaged in the synthesis of **4.4a-e** and **4.5a-f**, we found that the sulfilimines have characteristic UV absorbance maxima at 284-296 nm. Within this wavelength range, the nucleobases have an absorbance minimum.[155] This UV absorbance simplified purification and analysis of the above-mentioned compounds, since it enabled a fast and clear identification of sulfilimine-containing compounds in the reaction mixtures.

Synthesis of a sulfilimine-containing dinucleotide

In the next stage after the preparation of the completely protected nucleoside sulfilimines, we focused our attention on testing of their stability during oligonucleotide synthetic conditions for the H-phosphonate approach. In the course of their preparation, it became apparent that dimethylsulfilimines **4.5a-f** are less stable than their diphenyl counterparts **4.4a-e.** Consequently, a successful application of the former derivatives in the assembly of dimer **4.8** will indicate that diphenylsulfilimine will also survive this procedure. Therefore, deoxycytidine dimethylsulfilimine **4.5e** was selected as starting material in the preparation of dinucleotide **4.8** as outlined in Scheme 4.8.



Scheme 4.8. Synthesis of a dinucleotide containing dC dimethylsulfilimine (i) MMT-Cl/Py (ii) H_3PO_3/Py/PivCl (iii) 3′-O-Lev-dA^{Bz}, PivCl/Py/MeCN or OXP (iv) I_2/Py/H_2O.

Tritylation of compound **4.5e** with monomethoxytrityl chloride (MMT-Cl) in pyridine afforded compound **4.6** (49%). Then, phosphonylation in pyridine using phosphorous acid and pivaloyl chloride (PivCl) led, after hydrolysis, to the formation of H-phosphonate monoester **4.7** (56% isolated yield). Next, condensation of monomer **4.7** with 6-*N*-benzoyl-2′-deoxy-3′-*O*-levulinoyl-adenosine (3′-*O*-Lev-dA^{Bz})[156] under the mediation of *bis*-(2-oxo-3-oxazolidinyl)phosphoryl chloride (OXP)[157] afforded, after oxidation with iodine/water and purification by column chromatography, dimer **4.8** (87%). In addition, dinucleotide **4.8** was also synthesized by treating phosphonate **7** and 3′-*O*-Lev-dA^{Bz} in acetonitrile and pyridine with pivaloyl chloride, which is the most common coupling agent in oligonucleotide synthesis by the H-phosphonate method.[158] After 15 min, oxidation with iodine in pyridine/water for 5 min afforded phosphate diester-containing dimer **4.8**. There was a complete conversion of starting material **4.7** into **4.8** as judged by ^{31}P NMR spectroscopy and HPLC analysis of crude compound **4.8** (using dimer **4.8** previously prepared from the OXP reaction as a reference).

Stability of nucleoside sulfilimines

Stimulated by the outcome of the phosphonylation and oxidation steps, which are two of the most crucial parts in solid-phase oligonucleotide synthesis, we then explored the stability of the dimethylsulfilimine group under conditions for another essential

step, i.e. detritylation.[158] This is a repeated mild acidolysis of the di- or monomethoxytrityl groups that serve as temporary protection of the 5´-hydroxy functions of the nucleosides (at the extension point of the oligonucleotide chain). In addition, the iodine oxidation[158] was further investigated in more detail. Moreover, although a capping[159] (acetylation) protocol is not regularly used in the H-phosphonate methodology, acetylation is often performed in order to protect the hydroxyl functions of the nucleosides and is also standard in the amidite approach. It was therefore included in the stability studies. The results of these tests are summarized in Table 4.1.

Table 4.1. Stability of adenosine and cytidine dimethylsulfilimines.[a]

Time,	2% DCA/DCE		0.01 M Iodine[b]		Capping mixture[c]	
min	dA **4.5a**	dC **4.5f**	dA **4.5c**	dC **4.5b**	dA **4.5c**	dC **4.5b**
5	99	~100	~100	~100	~100	90
15	99	97	~100	90	~100	90
30	92	96	~100	88	~100	79
60	90	94	99	81	~100	76
120	83	91	99	77	~100	72

[a] The values given in the table correspond to the percentage of the intact sulfilimine as determined by straight-phase HPLC (mean error ± 3%).
[b] Solution in collidine/water/MeCN.
[c] Ac_2O/collidine/MeCN/DMAP.

The data in Table 4.1 show that the dimethylsulfilimine function of nucleosides **4.5a** and **4.5f** was not stable in 2% dichloroacetic acid (DCA) in 1,2-dichloroethane (DCE). Some degradation was observed already after 30 min for both compounds **4.5a** and **4.5f**. This implies that the dimethylsulfilimine group on deoxyadenosine and deoxycytidine may not survive the repeated detritylation steps. Nonetheless, it may be stable during the several detritylations necessary for the preparation of a short oligonucleotide fragment. In contrast to the observed lability under acidic conditions of compound **4.5a,** the dimethylsulfilimine of derivative **4.5c** was not degraded when exposed to the conditions required for the oxidation of the phosphonate diester function into a phosphate diester[158,160] or to the capping solution (Ac_2O/*sym*-collidine/DMAP/MeCN) commonly used in some solid-phase nucleic acid synthesis protocols.[161] On the other hand, when cytidine dimethylsulfilimine **4.5b** was dissolved in the above mentioned iodine solution, it was intact for the first 5 min.

However, slightly longer treatment led to cleavage of the sulfilimine (10% within the 15 min) as indicated inTable 4.1. Moreover, exposure of **4.5b** to the capping mixture proved to have a deleterious effect on the sulfilimine integrity. In this case, 10% of nucleoside **4.5b** was affected within 5 min. The information summarized in Table 4.1 suggests that only properly protected dimethylsulfilimine derivatives of deoxyadenosine may endure oligonucleotide synthesis protocols that require repeated oxidation steps or oxidation times longer than 5 min. This is also correct for methods that endorse repeated capping step.

Next, the diphenylsulfilimino group of compounds **4.4a**, **4.4b**, and **4.4e** was subjected to the same conditions as described in Table 4.1 for the dimethylsulfilimine function. The values obtained from these stability tests are given in Table 4.2, and they indicate that, in contrast to their dimethyl counterparts, compounds **4.4a** and **4.4b** were stable during treatment with 2% DCA in DCE for 120 min. Prolonged exposure (24 h) to this mixture showed that the deoxycytidine derivative **4.4d** remained unaffected, while $Ac_2dA{=}SPh_2$ (**4.4a**) was less stable and was partially degraded (9%).

Table 4.2. Stability of adenosine and cytidine diphenylsulfilimines.

	2% DCA/DCE		0.01 M Iodine		Capping mixture	
Time, min	Ac_2dA-SPh_2 **4.4a**	Ac_2dC-SPh_2 **4.4e**	Ac_2dA-SPh_2 **4.4a**	Si_2dC-SPh_2 **4.4b**	Ac_2dA-SPh_2 **4.4a**	Si_2dC-SPh_2 **4.4b**
5	~100	~100	~100	~100	~100	~100
15	~100	~100	~100	~100	~100	~100
30	~100	~100	~100	~100	~100	~100
60	~100	~100	99	~100	~100	~100
120	~100	99	94	~100	~100	~100

The diphenylsulfilimine moiety was also found to be much more stable in the oxidation mixture of I_2/collidine in aqueous acetonitrile. After 2 h, the deoxycytidine derivative **4.4b** was still completely intact in contrast to **4.4a** which was partially cleaved (6%) as shown in Table 4.2 Furthermore, no cleavage of the diphenylsulfilimine function in **4.4a** and **4.4b** was observed in a mixture of Ac_2O/collidine/DMAP in MeCN for at least 2 h.

The stability of the diphenylsulifilimino group towards the conditions listed in Table 4.2 suggests that it will endure the assembly of longer nucleic acid fragments on a solid support using the H-phosphonate approach. A successful application of nucleoside diphenylsulfilimines in the phosphoroamidite chemistry is also very likely, provided they would survive the repeated phosphonylation step.

In the following stage, the stability of dA=SMe_2, dC=SMe_2, dA=SPh_2 and dC=SPh_2 (**4.5d**, **4.5e, 4.4c** and **4.4d**, respectively) in an aqueous environment and during ammonolysis, which is the final step in oligonucleotide synthesis,[158] employed to remove the protecting groups from the nucleobases and detach the oligonucleotide fragment from the solid-support, was examined.

Exposure to 33% aq. NH_3 for 16 h at 21 °C showed that **4.4c**, **4.4d**, and **4.5d** were un-degraded while **4.5e** lost 12% of the dimethylsulfilimine group. Upon treatment with 33% aq. NH_3 at elevated temperature, the diphenylsulfilimine derivatives **4c** and **4d** remained unaffected, but compounds **4.5d** and **4.5e** were almost completely converted into the corresponding deoxyadenosine and deoxycytidine, respectively. In anhydrous NH_3 in methanol, at 21 °C, all four nucleoside sulfilimines **4.5d**, **4.5e**, **4.4c**, and **4.4d** were found to be stable for at least 2 h.

The increased stability of the diphenylsulfilimines **4.4c** and **4.4d**, compared to the dimethyl- **4.5d** and **4.5e**, is also evident in water and 50 mM TRIS buffer (pH 9.0).

The diphenylsulfilimines were stable in aqueous solutions at 21 ºC for 16 hours, while the dimethyl analogues were substantially degraded under the same conditions. In H_2O at elevated temperature however, only dA=SPh_2 (**4.4c**) was stable, while compound dC=SPh_2 (**4.4d**) lost 37% of the sulfilimino group, and derivatives **4.5d** and **4.5e** were only detected in trace amounts.

The outcome of the stability tests in aqueous solutions is consistent with the results of the tests reported above, which show the increased stability of the diphenylsulfilimine function compared to the dimethylsulfilimine. This outcome leads to the conclusion that the diphenyl-sulfilimine group would not be cleaved at the final stage of nucleic acids synthesis, i.e. the deprotection of the nucleobases and detachment from the solid-support which is brought about by ammonolysis either at ambient temperature or at 60 °C.

This stability to ammonolysis and to the above-mentioned conditions depicted in Table 4.2 permits retaining the diphenylsulfilimine group during and after oligonucleotide synthesis, a fact that can make them useful for the introduction of

different functionalities into the nucleic acid fragment which can be accomplished by substitution and/or extension of the sulfilimine aromatic ring(s) accordingly.

Finally, since it was reported that 2-mercaptoethanol (β-ME) could cause cleavage of sulfilimines in the presence of acid,[162] we were curious to determine the effect of β-ME solution on **4.4a, 4.4b**, **4.5b**, and **4.5c**. It was found that the dimethylsulfilimines **4.5b** and **4.5c** underwent a rapid conversion (within 5 min) into their corresponding starting materials **4.3b** and **4.3c**, while the diphenylsulfilimines were much more stable. After 2 h, the deoxycytidine derivative **4.4b** was still completely intact in contrast to **4.4a**, which was partially cleaved (10%).

Table 4.3. Stability of 3′,5′-unprotected nucleoside sulfilimines in aqueous solutions.[a]

No.	Compound	50 mM TRIS (pH 9.0), 21 °C, 16 h	H_2O, 21 °C 16 h	H_2O, 60 °C, 16 h	33% aq. NH_3, 21 °C, 16 h	33% aq. NH_3, 60 °C, 16 h
1.	dA=SMe_2 **4.5d**	90	96	0	98	1
2.	dC=SMe_2 **4.5e**	59	77	3	88	7
3.	dA=SPh_2 **4.4c**	~100	~100	98	~100	~100
4.	dC=SPh_2 **4.4d**	99	99	64	~100	98

[a] The values given in the table correspond to the percentage of the intact sulfilimine as determined by reversed- phase HPLC (mean error ± 3%).

Summary

Nucleoside diphenyl- and dimethylsulfilimines were synthesized in good yields. The stability tests clearly indicated that the nucleoside diphenylsulfilimines are significantly more stable than those with the dimethylsulfilimine group. These tests suggest that nucleoside dimethylsulfilimines are not suitable for the solid-phase synthesis of long oligonucleotide fragments by the H-phosphonate approach. However, they may be used in the synthesis of short fragments, such as dimers, and possibly trimers and tetramers, either in solution or on a solid-support with reasonably good yields.

Unlike the dimethylsulfilimine nucleosides, it should be possible to use the diphenylsulfilimine derivatives in the solid-support assembly of long oligonucleotides. Incorporating one or several nucleoside diphenylsulfilimines can be

advantageous due to the fact that they are unaffected by ammonolysis and that the sulfilimine moiety absorbs at 290-300 nm. Thus, they could be used as UV tags for oligonucleotides via pinpointing of their location in the presence of unlabeled fragments. Another possible advantage is that by modifying one or both of the phenyl rings accordingly, diarylsulfilimine-containing nucleotides could be used to introduce functionalities, such as, for example an intercalator, at a predetermined position in a nucleic acid fragment.

Changing the substituents on the sulfur, for example, by synthesizing methyl-phenyl sulfilimines, could tune the stability of the nucleoside sulfilimines. The aim of these modifications would be to obtain enhanced stability towards the oligosynthesis conditions, while still preserving the ease of removal. The use of sulfilimines as potential UV tags and/or protecting groups is not limited to DNA synthesis, but extendable to RNA, PNA and PNA-DNA chimera.

5. SUPPRESSION OF EXONUCLEOLYTIC DEGRADATION OF DOUBLE-STRANDED DNA AND INHIBITION OF EXONUCLEASE III BY PNA (PAPER V)

A new method of single-molecule DNA sequencing, is based upon the detection and identification of single fluorescently labelled mononucleotides released by the exonucleolytic degradation of single-stranded DNAs. [163] The key element in this novel DNA sequencing approach is the suppression of the degradation of double-stranded DNA. As a part of a collaboration project, we investigated the ability of PNA to suppress the degradation of dsDNA by Exonuclease III.

First, it was necessary to ascertain that PNA can indeed suppress the degradation of a double-stranded DNA in a sequence-specific manner as well as to determine the efficacy of the suppression.

Thus, two shorter fragments of the master DNA (from the sequencing project), a $30^{mer}/45^{mer}$ were chemically synthesized.[♣] The formation of a duplex from these fragments will create a protruding 3′-end that is adjacent to the anti-parallel (-) DNA strand, which is not to be cleaved by 3′→5′ exonucleases that are double strand specific (See Figure 5.1). This will ensure that the exonucleolytic degradation will be only mono-directional i.e. 3′→5′, thus simplifying the quantification of the degradation. The quantification of the nucleotides released by the degradation in the presence and in the absence of PNA will provide the answers to the questions about the binding stability of such PNA complexes.

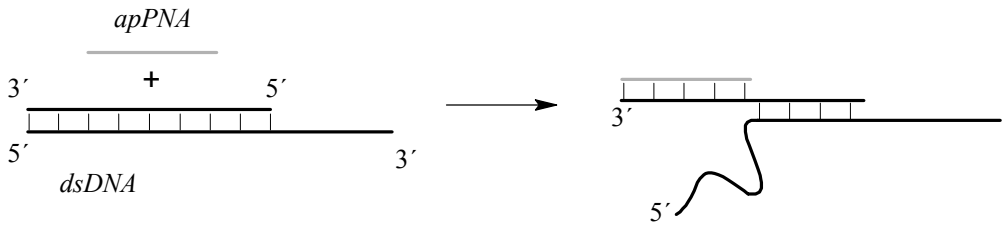


Figure 5.1. System design. dsDNA consists of 30- and 45-mer[*] fragments (3′-overhang); apPNA is a 16-mer anti-parallel PNA.

[♣] Purchased from TAG Copenhagen.

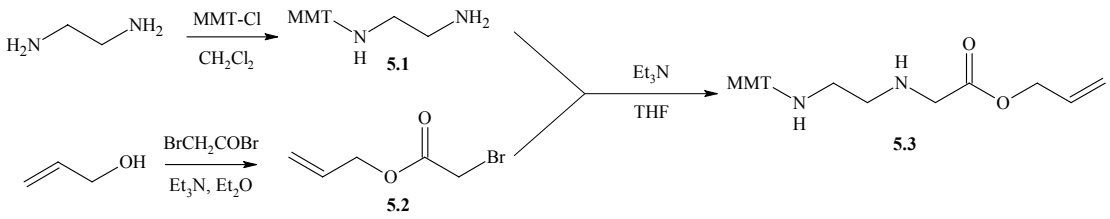
[*] 5′-TCT-TCA-CCT-CTC-TCT-CTT-TCT-GTC-TCT-CTC-TTT-CTT-TGT-CCT-CTT-3′

Synthesis of PNA

The length of the PNA has to be carefully chosen so that the thermal stability of the PNA•DNA duplex would be higher than that of the parent DNA•DNA duplex; otherwise the stand invasion (i.e. the displacement of one of the DNA strands of the duplex by the PNA) would not be thermodynamically favoured. On the other hand, it is known that long PNA fragments are prone to self-aggregation, thus leading to poor solubility in physiological media. Taking this in to account and using empirical calculations of the thermal stability, it was decided that a 16-mer PNA fragment would fulfil the criteria.

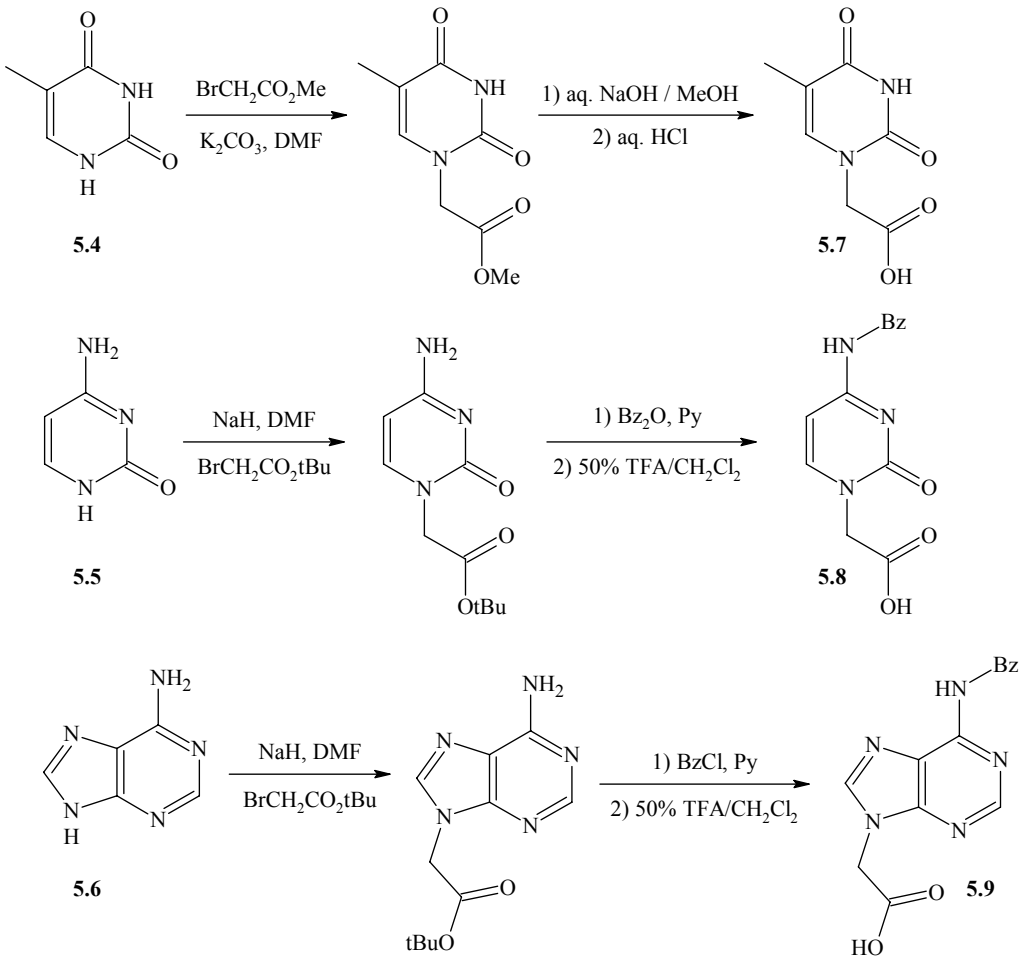
The assembly of the target PNA commenced with the syntheses of the properly protected monomers, which were performed in accordance to the previously published procedures[164] and consisted of three parts:

First, the synthesis of protected *N*-(2-aminoehtyl)glycine unit (**5.3**) comprised of *N*-alkylation of MMT-protected ethylene diamine **5.1** with allyl bromoacetate.



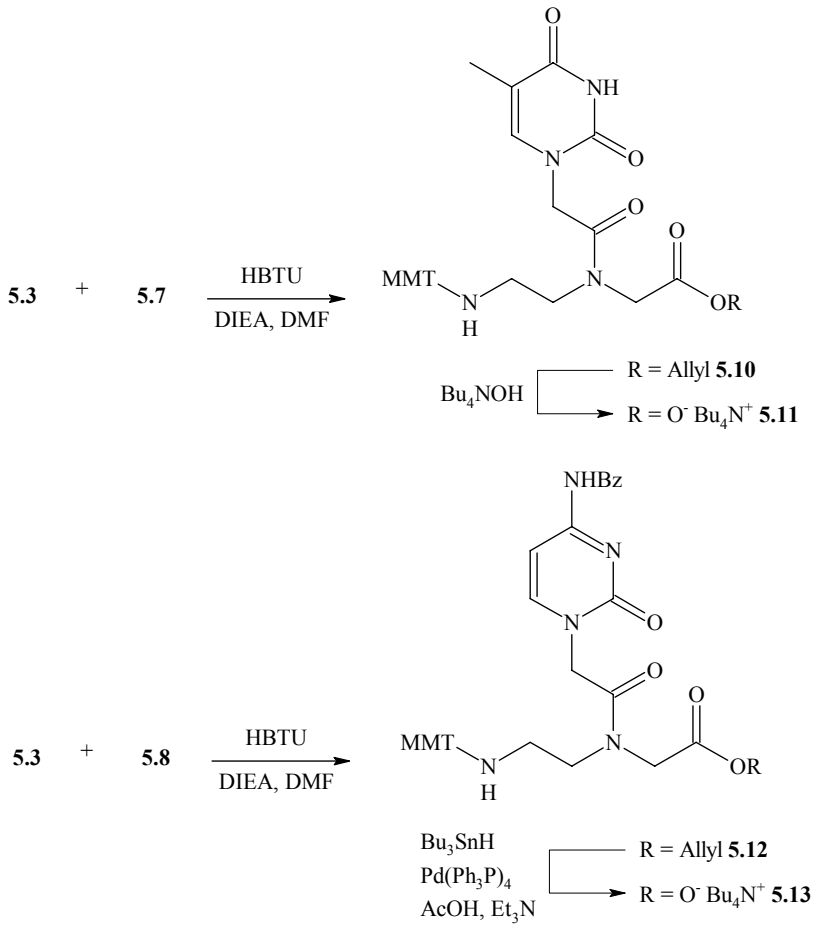
Scheme 5.1. Synthesis of protected *N*-(2-aminoehtyl)glycine unit.

Second, the nucleobase acetic acids **5.7**, **5.8** and **5.9** were made by alkylation of the corresponding heterocycles - thymine, cytosine and adenine (**5.4**, **5.5**, **5.6**) with alkyl bromoacetates.



Scheme 5.2. Synthesis of protected nucleobase acetic acids.

Finally, the nucleobase acetic acids were coupled to the Aeg backbone unit yielding fully protected Aeg-PNA monomers, which were further subjected to ester hydrolysis in order to liberate free carboxylates.



Scheme 5.3. Synthesis of MMT-protected Aeg-PNA monomers.

The assembly of the 16-mer PNA was performed using automated solid-phase synthesis on a low loading (33μM/g) highly cross linked polystyrene support, functionalized with Fmoc-glycine linked via a 4-(hydroxymethyl)benzoic acid (HMBA) linker. The synthesis steps are summarized in Table 5.1.

Table 5.1. Solid phase synthesis of PNA.

Step	Function	Solvents and reagents	Time, min.
	Fmoc deprotection of resin-bound glycine and terminal lysines	22 % piperidine in NMP	2×3.5
	Coupling	MMT Aeg-PNA monomers[a], HBTU[a], DIEA[c]	17.5
	MMT deprotection during chain elongation	1 % TFA/DCE	3
	Boc deprotection	50 % TFA/DCM	2×15
	Lysine termination	Fmoc-L-Lys(Boc)-OH[a], HBTU[a], DIEA[b]	2×17.5

[a] As a 0.3 M solution in NMP
[b] As a 0.4 M solution in NMP

A pure PNA 16-mer was obtained by reverse-phase HPLC purification of the crude PNA after detachment of the PNA from the resin.

UV thermal melting experiments

Prior to the enzymatic degradation experiments, the ability of a 16-mer PNA fragment, complementary and anti-parallel to the 30-mer, to hybridize to ssDNA and dsDNA was evaluated (Table 5.2).

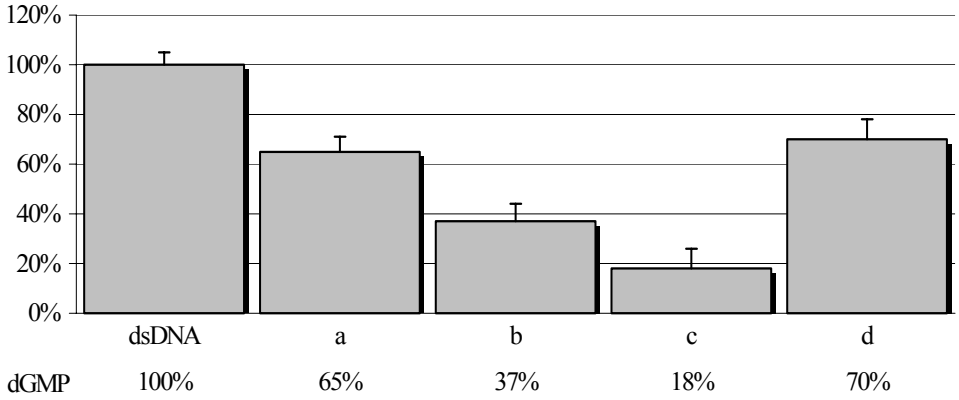
Table 5.2. UV thermal melting data of the nucleic acid components.

No.	Components[a]	T_m, °C[b]
1.	DNA (45) : DNA (30)	57
2.	DNA (30) : apPNA	74
3.	dsDNA : apPNA	37, 74

As expected, it was found that the PNA•DNA duplex was more stable than the DNA•DNA duplex (ΔT_m = 17 °C), which should allow the strand invasion to occur.

Enzymatic degradation studies

The following experiments were set up – 1 nmol of dsDNA in a TRIS buffer (66 mM, pH 8.0), containing 0.66 mM Mg^{2+} and 2-mercaptoethanol (1 mM) were mixed with various amounts of apPNA (0, 1, 5, and 7 nmol), briefly heated up to 90 °C and cooled to ambient temperature over a period of 90 min. Exonuclease III (2 units[†]) was added and the assay was incubated at 37 °C. Aliquots were withdrawn at fixed time intervals (30 and 60 min) and analyzed for the amounts of the formed mononucleotides by reverse-phase HPLC. The amounts of the mononucleotides released from the degradation of dsDNA with no apPNA added were assigned to 100% and used as a reference point.



a) dsDNA + 10 nM apPNA; b) dsDNA + 50 nM apPNA; c) dsDNA + 70 nM apPNA; d) dsDNA + 70 nM non-complementary PNA 10-mer.

Figure 5.2. Relative amount of degradation of dsDNA by Exonuclease III after 60 min (by the amount of the released dGMP).

By plotting the amounts of the released mononucleotides versus the concentration of apPNA, it was apparent that increasing the concentration of apPNA from 10 to 70 nM resulted in increased suppression of degradation (Figure 5.2, entries a-c).

Further, it was important to determine whether the observed suppression of degradation is specific due to the base pairing of PNA with the dsDNA (strand invasion) or non-specific, due to the interactions of PNA with the DNA duplex or the

[†] One unit is defined as the amount of enzyme required to produce 1nmol of acid-soluble nucleotides from double-stranded DNA in 30 minutes at 37°C.

enzyme. Thus, when a non-complementary PNA 10-mer was used instead of the apPNA, the enzymatic activity was observed to be 70% (Figure 5.2, entry ‘d’).

Summary

It can be concluded that anti-parallel PNA does indeed suppress the activity of Exonuclease III in a concentration-dependant and sequence-specific manner, in addition to some degree of non-specific inhibition, which is less pronounced than the specific suppression. The extent of the suppression could be further increased by optimizing the binding affinity of the PNA. Factors such as length of PNA, guanine-cytosine content, ionic strength of the media, incubation temperature and time, concentration of Mg^{2+}, use of various additives could be changed in order to achieve maximum specific suppression values, while keeping the non-specific inhibition low.

CONNECTIONS AND CONCLUSIONS

The work described in the above five chapters deals with various aspects of the development of new potential molecular tools for biochemistry and related disciplines.

A new PNA modification has been introduced, which has an enhanced ability in its binding to discriminate between complementary DNA and RNA. The assembly of backbone-modified PNAs can be problematic due to potentially lower reactivity of the monomers in which novel structural elements are included. To deal with such problems, the use of a new type of amide bond-forming reagent has been proposed and investigated.

The right choice of protecting groups for the chemical synthesis of nucleic acid fragments is one of the keys for success. Often the problems are caused by premature removal of protecting groups or on the contrary – partial loss of product because of incomplete deprotection. A new type of potential protecting group and/or UV label has been introduced and their stability towards a variety of conditions have been tested and reported.

Further, a PNA fragment has been successfully applied as a means to enhance the stability of a DNA strand against enzymatic degradation, a finding that may have an application in a novel sequencing approach.

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