Conformational Stability!? 

Synthesis and Conformational Studies of Unnatural Backbone Modified Peptides

ANNA S. NORGREN
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Abstract

The beauty of the wide functionality of proteins and peptides in Nature is determined by their ability to adopt three-dimensional structures. This thesis describes artificial molecules developed to mimic secondary structures similar to those found crucial for biological activities.

In the first part of this thesis, we focused on post-translational modifications of a class of unnatural oligomers known as $\beta$-peptides. Through the design and synthesis of a glycosylated $\beta$-peptide, the first such hybrid conjugate was reported. In this first report, a rather unstable $\beta_1$-helical structure was found. Subsequently, a collection of six new glycosylated $\beta^3$-peptides was synthesized with the aim to optimize the helical stability in water.

The ability of natural proteins, i.e. lectins, to recognize the carbohydrate residue on these unnatural peptide backbones was investigated through a biomolecular recognition study.

The second part of this thesis concerns the design of conformationally homogeneous scaffolds, which could be of importance for biomedical applications. In paper V, four- and five-membered cyclic all-$\beta$-peptides were investigated for this purpose. In a subsequent paper, a completely different strategy was employed; herein, the ability of a single $\beta^3$-amino acid to restrict the conformational freedom of a cyclic $\alpha$-peptide was studied.

In the third part of this thesis, we synthesized and investigated the folding propensities of novel backbone modified oligomers, i.e. $\beta$-peptoids (N-substituted $\beta$-Ala) with $\alpha$-chiral side chains.

The collective results of these studies have established the procedures required for synthesis of glycosylated $\beta$-peptides and deepened our understanding of the factors governing folding among such oligomers. Moreover, it was established that $\beta$-amino acids can be a useful tool to increase conformational stability of cyclic peptides.

Keywords: Conformational investigations, $\beta$-peptides, glycosylation, biomolecular recognition, cyclic $\beta^3$-peptides, cyclic mixed $\alpha/\beta^3$-peptides, $\beta$-peptoids

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urn:nbn:se:uu:diva-7420 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-7420)
"If we knew what it was we were doing, it would not be called research, would it?".

Albert Einstein

Nobody puts Baby in a corner!

In dedication to my wonderful family and to the memory of those we have lost
List of Papers

This thesis is based on following papers, referred to in the text by their Roman number:

I Glycosylated Foldamers: Synthesis of Carbohydrate-modified \( \beta \)hSer and Incorporation into \( \beta \)-Peptides. Anna S. Norgren, Thomas Norberg, Per I. Arvidsson, *J. Pept. Sci.*, Accepted.


III Glycosylated \( \beta \)-Peptides: Relationship Between Peptide Sequence and 3\( \alpha \)-Helical Stability in Water. Anna S. Norgren, Per I. Arvidsson, *Submitted.*


Contribution Report

I  Planned and synthesized all compounds and peptides, performed the conformational studies, contributed significantly to the interpretation of the results and writing of the paper.

II  Synthesized the peptides, performed the conformational studies, contributed to the interpretation of the results and writing of the paper.

III  Contributed to the formulation of the research problem, synthesized all peptides, performed the conformational studies, performed the interpretation of the results. Contributed significantly to the writing of the paper.

IV  Contributed to the formulation of the research problem, synthesized all peptides, contributed partly to the interpretation of the results, contributed significantly to the writing of the paper. Dr. Matthis Geitmann performed the Biacore studies.

V  Performed the conformational studies, contributed significantly to the interpretation of the conformational results, contributed to the writing of the paper. Dr. Frank Büttner and Dr. Suode Zhang performed the synthetic work. Samran Pradpai and Prof. Palanpon Kongsaeeree performed the X-Ray crystallographic experiments.

VI  Performed the conformational studies and interpretation of the results, contributed significantly to the writing of the paper. Dr. Frank Büttner performed the synthetic work. Samran Pradpai and Prof. Palanpon Kongsaeeree performed the X-Ray crystallographic experiments.

VII  Purified all compounds, performed the conformational studies and the interpretation of the results, contributed significantly to the writing of the paper. Dr. Suode Zhang performed the synthetic work.
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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AgOTf</td>
<td>silver trifluoromethanesulfonate; silver triflate</td>
</tr>
<tr>
<td>Aoe</td>
<td>([(2\alpha,9\alpha)2\text{-amino-8-oxo-9,10-epoxy-decanoic acid}])</td>
</tr>
<tr>
<td>Boc</td>
<td>tert-butoxycarbonyl</td>
</tr>
<tr>
<td>BS-I</td>
<td>Bandeira Simplicifolia I</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>DBU</td>
<td>1,8-diazabicyclo[5.4.0]undec-7-ene</td>
</tr>
<tr>
<td>DIPEA</td>
<td>diisopropylethylamine</td>
</tr>
<tr>
<td>DMAP</td>
<td>4-(dimethylamino)pyridine</td>
</tr>
<tr>
<td>DMF</td>
<td>(N, N\text{-dimethylformamide})</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>Fmoc</td>
<td>9-fluorenylmethyloxycarbonyl</td>
</tr>
<tr>
<td>Fmoc-OSu</td>
<td>9-fluorenylmethyloxycarbonyloxy succinimide</td>
</tr>
<tr>
<td>GalNAc</td>
<td>(N\text{-acetyl galactosamine})</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>(N\text{-acetyl glucosamine})</td>
</tr>
<tr>
<td>GalN₃Br</td>
<td>3,4,6-tri-O-acetyl-2-azido-2-deoxy-(\alpha)-D-galactopyranosyl bromide</td>
</tr>
<tr>
<td>GMA</td>
<td>(Glycine\ \text{max\ agglutinin})</td>
</tr>
<tr>
<td>gHMBC</td>
<td>gradient-enhanced heteronuclear multiple bond correlation</td>
</tr>
<tr>
<td>HBTU</td>
<td>(N\text{-}[(1H\text{-benzotriazol-1-yl})(\text{dimethylamino})\text{methylen}]-N\text{-methylmethanaminium hexafluorophosphate N-oxide})</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylases</td>
</tr>
<tr>
<td>HOBT</td>
<td>1-hydroxybenzotriazole hydrate</td>
</tr>
<tr>
<td>HPA</td>
<td>(Helix\ \text{Pomatia\ agglutinin})</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>IBCF</td>
<td>(\text{iso-butylchloroformate})</td>
</tr>
<tr>
<td>NMM</td>
<td>(N\text{-methylmorpholine})</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NOE</td>
<td>nuclear Overhauser effect</td>
</tr>
</tbody>
</table>
NOESY
nuclear Overhauser effect spectroscopy
P.E.-COSY
primitive exclusive correlated spectroscopy
Pha
phenacyl ester
Phe
phenylalanine
PTM
post-translational modification
ROESY
rotating frame Overhauser effect spectroscopy
SPPS
solid phase peptide synthesis
TFA
trifluoroacetic acid
TIPS
triisopropylsilane
TOCSY
total correlation spectroscopy
Troc
trichloroethoxycarbonyl
TSA
trichostatin A
VVA B₄
Vicia Villosa agglutinin (Isolectin B₄)
WET
water suppression enhanced through T₁ effects

In the text that follows, the abbreviated nomenclature introduced by Seebach is adopted,¹ referring to β-amino acids as homologues of the natural α-amino acids bearing the same side chains by adding the letter “h” preceding the three-letter code of the natural amino acids; i.e. βhXxx.

Amino acids used in this thesis

Aspartic acid, Asp
Glutamine, Gin
Glutamic acid, Glu
Leucin, Lea
Lysine, Lys
Ornithine, Orn
Phenylalanine, Phe
Proline, Pro
Serine, Ser
Threonine, Thr
Valine, Val
1 Introduction

1.1 General Introduction

The use of peptides and proteins within medicinal chemistry is of great advantage as the peptide backbone sequence is naturally recognized by and interacts with for example enzymes and receptors. However, their therapeutic use is limited due to high degree of degradation and poor bioavailability. Therefore, the interest of finding artificial building blocks that retain the valuable characters of their natural analogues has lead to development of several new classes of non-natural compounds including small organic molecules, so-called peptidomimetica, as well as larger modified oligomers.2

The backbone of an amino acid is well characterized, consisting of a carboxylic acid and an amine separated by a methylene group. One widely used strategy to gain enhanced stability and bioavailability is to modify the amino acid’s backbone according to a replacement strategy. Examples of this is to replace the amide nitrogen by an oxygen (depsipeptide) or a sulphur (thioesterpeptide), replace the α-carbon by nitrogen (azapeptide) or an C-alkyl group (Cα-disubstituted amino acid), or having the carbonyl group exchanged by a methylene group (reduced amide bonds), see Figure 1.

![Figure 1. Selected examples of peptide backbone modifications developed and suggested to enhance stability and bioavailability.](image)
The replacement strategy has been widely used in the development of several new families of sequence-specific oligomers. Oligocarbamates and azatides (see Figure 2a) are typical examples of unstructured oligomer families, i.e. oligomers that so far have not been demonstrated to possess the ability to fold into secondary structures.

The folding of peptides and proteins is crucial for many of the known functions, such as molecular recognition, information storage, and catalysis. To be able to mimic the secondary structure, a variety of structure-inducing elements may be incorporated into the backbones of the α-amino acid residues (see Figure 2b). The chiral vinylogous aminosulfonic acid possesses an extended backbone with a strong negative charge meanwhile the oligopyrrolinone has its backbone structure rigidified by the presence of a 5-membered ring. The previous oligomers forms secondary structures predominant by hydrogen-bonding stabilization whereas the latter oligomer forms either intramolecular hydrogen bonds to mimic a β-strand or by intermolecular hydrogen bonds to promote β-sheets.

![Figure 2](image)

Figure 2. Examples of unstructured (a) and structured (b) unnatural oligomers.

N-Substituted glycines, so called peptoids, are structurally similar to peptides, but with side chains located on the backbone nitrogen atoms instead of on the carbon, see Figure 2 and Section 1.5.

Two rather similar families of foldamers (oligomers that adopt predictable and well-defined conformations) which have received a lot of interest recently for their ability to mimic natural peptides and proteins are the β- and the γ-peptides. β-Peptides represent the smallest step away from α-peptides in “backbone space”, having a methylene group incorporated into the backbone structure of the building blocks whereas γ-peptides has an ethylene group incorporated.
The majority of the papers included in this thesis (six of seven) are based on research on $\beta$-peptides. A more detailed introduction to the world of these unnatural oligomers is found in Section 1.2 below.

1.2 $\beta$-Peptides

1.2.1 General Introduction

The building blocks of $\beta$-peptides are called $\beta$-amino acids, see Figure 3. The additional carbon incorporated into the backbone results in unnatural oligomers containing side chains that can be recognized by side chain specific enzymes and receptors but hold a backbone structure which are significantly different from that found in natural $\alpha$-amino acids.

![Figure 3. Comparison of the backbone of the natural $\alpha$-amino acid (a) with the two most common $\beta$-amino acids; the $\beta$-amino acid (b) and the $\beta$-amino acid (c), respectively.](image)

The side chain of a $\beta$-amino acid can either be proteinogenic (homolog of the $\alpha$-amino acid) or synthetically modified, and because of the extra methylene group, the side chain can be positioned at either of the two backbone carbon atoms. A $\beta$-amino acid has the side chain on the $\beta$-carbon, whereas a $\beta$-amino acid has the side chain positioned on the $\alpha$-carbon. More advanced $\beta$-amino acids being di-, tri-, or even tetra-substituted have also been synthesized and incorporated into peptides.5

Extensive studies have proven $\beta$-peptides to be completely inert towards both metabolic and proteolytic degradation. Studies concerning the stability of $\beta$-peptides towards proteolytic enzymes (proteinases and peptidases) showed no degradation of these unnatural peptides regardless of stereochemistry, secondary structure, or whether the side chains were in $\beta$- or $\beta$- position. As the proteinases and peptidases are backbone specific enzymes and since the backbone-structure of $\alpha$-peptides and $\beta$-peptides differs significantly, this degrading stability may be expected, see Figure 4.

Proteolytic enzymes are known to be very specific towards the amino acid-sequence. Looking at the backbone structure of the $\alpha$-peptide, both the side chains and the amide bond are alternating compared to the $\beta$-peptide, where neither the side chains nor the amide bonds are alternating. The side chains can
be designed so that they are alternating, but nevertheless, the amide bonds will still point in the same direction and obviously lack the possibility to form a pleat structure with the enzyme.

![Figure 4](image)

Figure 4. Schematic picture of the active site of an enzyme (a) and a comparison between the backbone structures of α-peptides and β-peptides (b).

During solid phase synthesis of a peptide containing α-amino acids, there is always an overlaying risk of racemization of the amino acids. However, due to the incorporated methylene group between the carbon possessing the side chain and the carboxylic group, β-peptides do not racemize. However, for the β-amino acid, the problem with racemization is still present as the side chain is attached to the carbon positioned α to the carboxylic group.

1.2.2 Secondary Structure

An important feature of β-peptides is their ability to adopt many different secondary structures. Among these are a number of the major secondary structures found in natural peptides and proteins such as helices, parallel and antiparallel pleated sheets, and hairpins. However, there are also well-ordered structures that are not known for natural peptides, e.g. tubular stacking, ribbons, and alternative helices. Moreover, well-defined and remarkably stable secondary structures are found with β-peptides of much shorter chain length (4-6 amino acids) than a typical α-peptide.

1.2.2.1 Helical Secondary Structures

Peptides containing only glycine residues are known to have a higher flexibility than peptides consisting of amino acids with side chains other than hydrogen and for this reason β-peptides were generally expected to be more flexible than α peptides. However, whereas at least 15 residues are needed for α-peptide helices much shorter β-peptides can form secondary structures in protic solvents.

So far, five classes of helices have been found for β-peptides; the two major ones are the 3_14-helix and the 12/10-helix. Even though both will be described below, it is the 3_14-helix which has the major focus in this thesis.
The 12/10-helix is right-handed, and is formed by peptides composed of altering $\beta^{2}$- and $\beta^{3}$-residues. 12-Membered and 10-membered turns are alternated in the helix and arise as intramolecular hydrogen bonds are formed specifically between the amide groups surrounded by side chains and between the amide bonds surrounded by the methylene groups, respectively (see Figure 5a and b).

In the right-handed $\alpha$-helix there is a resulting macrodipole moment running from the N to the C–terminus (i.e. plus to minus). In the 12/10-helix, the amide C=O groups have alternating directions due to the alternation between $\beta^{2}$- and $\beta^{3}$-amino acids, resulting in a helical secondary structure with very small or even no macrodipole moment.

![Figure 5](image)

*Figure 5.* Nomenclature of the 310-helix and the 10/12-helix based on hydrogen-bonding pattern (a) and structures of the $\alpha$-helix, 310-helix and the 10/12-helix, respectively (b). The hydrogens are omitted for clarity, except the amide hydrogens (baby blue). Carbons are shown in grey, nitrogens in blue, and oxygens in red.

If the $\beta$-peptide is only composed of acyclic proteinogenic $\beta$-amino acid building blocks, the peptide is expected to fold into the 310-helical secondary structure (see Figure 5b), the so far most studied secondary structure of $\beta$-peptides. Depending on the stereochemistry of the building blocks, the $\beta$-
peptide can either form right- or left-handed helices, but the \( \beta \)-peptides containing \( \beta \)-amino acids derived from naturally occurring \( L \)-amino acids will adopt a left-handed helix. In contrast to the 12/10-helix, the 314-helix have a unique hydrogen bonding pattern which causes the helical macrodipole moment to orient in opposite direction relative the \( \alpha \)-helix, with a partial positive charge at the \( C \)-terminus and a partial negative charge at the \( N \)-terminus.

The 314-helix is stabilized by intramolecular hydrogen bonding between the amide protons in position \( i \) and the \( i+2 \) carbonyl, where each hydrogen bond closes a loop containing 14 atoms, see Figure 5a. Looking at the helical structure from above, three distinct sides of this helix can be seen, and therefore further stabilization can be gained if the sequence is designed so that the hydrophobic interactions are focused on one helical rim whereas residues able to form salt bridges are focused on at least one of the other rims.

Unfortunately, the 314-helical structure is known to be destabilized upon increase of solvent polarity, i.e. going from methanol to water. However, Schepartz et al. have developed a design-strategy to obtain enhanced stability of the secondary structure in aqueous solution.\(^1\)\(^8\) The approach suggests that residues with positively charged side chains (e.g. Orn, Lys) close to the \( N \)-terminus and residues possessing negatively charged side chains (e.g. Glu, Asp) close to the \( C \)-terminus (see Figure 6a) in combinations with free \( N \)- and \( C \)-termini minimizes the macro dipole moment. This minimization have shown to cause increased helical stability.\(^1\)\(^8\)

Since it also is of importance to use residues in the \( \beta \)-peptides that are not capable of salt bridge formation but nevertheless holds other important chemical properties, the group of Schepartz found an effect in stability depending on where these residues are positioned in the \( \beta \)-peptide sequence.\(^1\)\(^9\) The design strategy of Schepartz was utilized in one of our projects; see Section 4.3.2.2, Paper III. It should also be stated that \( \alpha \)-branched side chains, like Val, are better than e.g. \( \beta \)-branched Leu.

The group of Gellman also studied the loss of secondary structure in aqueous solution, whereupon they found that the helical stability can be increased if the hydrophobic residues are exchanged to \textit{trans}-2-aminocyclohexanecarboxylic acid (ACHC) instead of i.e. valine or leucine (see Figure 6b).\(^2\)\(^0\)
Figure 6. $\beta$-Peptide sequences designed to enhance secondary structure stability in aqueous solvent; Schepartz (a) and Gellman (b).

1.2.3 Applications
The ability of $\beta$-peptides to adopt stable secondary structures is useful in the design of foldamers with biological activities. Utilizing the ability to fold into helical structures, Seebach and co-workers demonstrated that short-chain $\beta$-peptides may inhibit small-intestinal cholesterol absorption.21 Further, the finding of potent and selective antimicrobial peptides is of great importance; Seebach and co-workers, Gellman and co-workers, and DeGrado and co-workers have all provided new $\beta$-peptides with potent antibacterial activity.22 Helical $\beta$-peptide analogues have also shown to be interesting in the search for cancer23 and HIV treatments.24 $\beta$-Peptides designed to mimic a $\beta$-turn secondary structure have also been described. Seebach and co-workers have reported mimics of the natural hormone somatostatin, which displayed biological activity and micromolar affinity for human receptors.25

1.3 Glycopeptides
The majority of proteins in living cells are post-translationally modified. Most common post-translational modifications (PTMs) includes phosphorylation, glycosylation and lipidation, but variations such as sulphation, bromination, racemization, and $\gamma$-carboxylation are frequently found.

The most abundant PTM is the protein glycosylation which introduces wide structural variety to proteins. Glycoproteins have an important role in the biological recognition processes, such as immuno-differentiation, cell adhesion, cell differentiation and regulation of cell growth26 and the role of biological selectivity is most often played by the carbohydrate residue.27 Further, carbohydrates help to stabilize the conformation of glycoproteins, and thereby
protect them towards denaturation and the associated loss of biological function. Unusual glycosylation is associated with autoimmune- and infectious diseases as well as cancer, to mention a few examples.

The naturally occurring glycosidic linkages can usually be classified into either N-glycosides or O-glycosides. The O-glycosides links to the side chains possessing a hydroxyl group, such as serine and threonine, and is the type of glycosylation of interest in this thesis.

The varieties of glycosides are very large, and they are not built from one common core. Instead, there are several core fragments known. Increased complexity lies in that the hydroxyl group attached to C-1 (the anomeric centre) can either end up in an axial (α) or an equatorial (β) position.

One of the most frequently observed core fragments is the α-D-GalNAc (N-acetyl galactosamine) residue attached to serine or threonine. This combination is a class of tumor-associated antigens which is found in mucins, proteins secreted from epithelial cells. The structures are dominated by a diverse number of tandem repeated sequences which all are rich in serine, threonine and proline residues. In normal tissue, the protein backbone carry complex oligosaccharides derived from structures with α-linked GalNAc as core fragment. In tumor cells, however, the expression of mucins is usually increased, and the carbohydrate side chains are altered due to incomplete glycosylation and premature sialylation.

Amongst the most important tumor-associated antigens are the T, Tn and sialyl-Tn antigens (see Figure 7a-c). The Tn and sialyl-Tn antigens are found in human colon cancer, ovarian cancer and breast cancer meanwhile the T antigen has been shown to be tumor specific in breast tissue.

Further, the Tn and the sialyl-Tn antigens have recently been discovered in a small amount (4-5 glycans) on the envelope glycoprotein gp120 of the human immunodeficiency virus (HIV). The gp120 has a carbohydrate content of about 50% and is one of the most heavily glycosylated proteins known today, having mostly N-linked glycans. While the N-glycosylation sites are quite similar to those of the host, and not generally immunogenic, the Tn and sialyl-Tn epitopes may be potential targets for immune intervention. This is due to their very limited occurrence, otherwise only produced in cancer cells as a result of abnormal glycosylation.
The N-acetyl glucosamine (GlcNAc) monosaccharide is another common glycosylic post-translational modification. The β-D-GlcNAc attached to either serine or threonine (see Figure 7d) occurs frequently in glycoproteins located in the nucleus and cytoplasm of nearly all eukaryotic cells, including filamentous fungi, plants, animals, and animal parasites, as well as viruses that infect eukaryotes.

The O-linkage of GlcNAc to protein appears to be as abundant and dynamic as protein phosphorylation. In fact, it has been found that the proteins that undergo O-GlcNAcylation also occur as phosphorylated proteins, where the modifications take place on either the same or adjacent hydroxyl moieties. Like phosphorylation, O-GlcNAcylation is responsive to the cell cycle, extracellular signals, glucose metabolism, and to the growth state of the cell. O-GlcNAcylation has also been suggested to play important roles in the regulation of transcription and translation.

1.4 Cyclic Peptides

Upon cyclization of small flexible peptides, there is a loss of conformational mobility. The increased backbone rigidity is valuable as it gives new and/or improved characteristic properties to the peptide such as increased biological activity and receptor specificity. Further, the biological response can be prolonged upon increased stability towards degrading enzymes.
There are numerous examples of cyclic peptide natural products with interesting biological properties. For instance, Chlamydocin (Figure 8a), HC-toxin, and trapoxin B, belong to a family of hydrophobic cyclic tetrapeptides that are potent inhibitors of histone deacetylases (HDAC). Another example is, Cyclosporine A (see Figure 8b), a cyclic undecapeptide which is being used clinically as an immunosuppressant in the treatment of autoimmune disorders and for preventing organ transplant rejection.38

Figure 8. Examples of cyclic peptides with interesting biological properties; chlamydocin (a), cyclosporine A (b), and somatostatin (c).

The application of cyclic peptides are numerous and to a certain extent dependent on the ring size. For instance, cyclic octapeptides can act as ion channel adapters,39 whereas cyclic hexapeptides are used e.g. as somatostatin agonists,40 δ-opioid receptor agonists41 and antifungal agents, acting through the inhibition of glycan synthesis.42

Extra conformational restrictions are often found in cyclic peptide backbones. Examples such as alternating use of D- and L-configured amino
acids, proline residues, N-alkylated amino acids, \( \alpha,\beta \)-dehydro amino acids and \( \alpha,\alpha \)-disubstituted amino acid residues are commonly found. Cyclic peptides which consists merely of \( \beta \)-amino acids residues, or where only one or two of the unnatural residues have been incorporated into the cyclic backbone, have also been reported (see Section 4.5.1 and 4.5.2, respectively).

1.5 \( \alpha \)- and \( \beta \)-Peptoids

\( \beta \)-Peptoids are yet another family of bioinspired oligomers awaiting to be explored, as compared to the \( \alpha \)-peptoids which have been extensively studied for more than ten years.

In the search for the ideal unnatural backbone modified peptide possessing a defined structure in solution, Zuckermann et al. presented in 1992 the oligomeric family of \( \alpha \)-peptoids, see Figure 9a.\(^{43} \) The \( \alpha \)-peptoids have an identical backbone sequence as compared to the \( \alpha \)-peptides but differs in the way that the side chains have been shifted to be positioned on backbone nitrogen atoms. The same resemblance and difference applies between the \( \beta \)-peptoids and the \( \beta \)-peptides, see Figure 9b.

Since the side chains are positioned on the nitrogen atoms, the backbone lacks both chiral centers and hydrogen bond donors. Despite this, \( \alpha \)-peptoids have shown to fold into stable secondary structures with a preferred handedness due to the inclusion of chiral side chains.\(^{44} \) Extensive circular dichroism (CD) studies of \( \alpha \)-chiral peptoids with varying side chains and chain length gives the indication that peptoids as short as pentamers adopt chiral secondary structures. Further, the CD spectra of \( \alpha \)-peptoids with aromatic \( \alpha \)-chiral side chains show spectral features similar to those arising from \( \alpha \)-peptides whereas the \( \alpha \)-peptoids with aliphatic side chains resembles the CD spectra given by polyproline type-I helices.\(^{45} \)
2 Aim of Present Studies

The molecules of biology are undoubtedly remarkable, considering that they can display such wide variety of structures and functions. Many of the interesting functions carried out by these macromolecules are dependent on stable well-defined three-dimensional structures. This factor is also important to consider in the development of new unnatural backbone modified peptides. As a result, we intended to extend the field of foldamers by: incorporating post-translational modifications, designing conformationally homogeneous scaffolds, and investigating novel classes of potential foldamers.

Therefore, this thesis is focused on the following specific objectives:

- Synthesis of O-glycosylated $\beta$-amino acids.
- Design and synthesis of glycosylated $\beta$-peptides, capable of adopting stable $3_{11}$-helical structures in both methanol and aqueous solution.
- Investigations concerning the ability of glycosylated $\beta$-peptides, with their unnatural backbone and natural carbohydrate part, to be recognized by macromolecules with relevant biological functions.
- Studies on how $\beta$-amino acids can be used for constraining cyclic peptide scaffolds in a) all-$\beta$-peptides and b) mixed $\alpha/\beta$-peptides.
- Investigate the folding propensities of $\beta$-peptoids with N-chiral side chains.
3 Applied Experimental & Theoretical Methods

3.1 Solid Phase Peptide Synthesis

3.1.1 General Introduction

In 1963, R.B. Merrifield published the first paper about solid phase peptide synthesis (SPPS). Only ten years later, in 1973, Meienhofer, in his important review about SPPS, were able to list more than 500 published solid phase syntheses. Solid phase peptide synthesis is today a very thoroughly developed and understood technique, with the possibility to use a large variety of protecting groups.

Synthesizing peptide sequences on a solid support instead of in solution offers many advantages. For instance, soluble reagents and solvents can efficiently be removed from the intermediate peptides by filtration. Further, the use of an excess of reagents will drive the reaction to completion whereas physical losses can be minimized as the peptide remains on the solid support.

This synthetic strategy opens for the possibility of automation, which saves both time and effort. However, one drawback with synthesis on solid support is that different byproducts that can be formed through side-reactions. Incomplete reactions or impure reagents will accumulate on the resin during the peptide synthesis and thereby contaminate the final product, and the purification can thus become a big challenge in itself. It is therefore not surprising that the success of SPPS came about the same time as developments in chromatographic techniques, i.e. HPLC.

The principles of SPPS are summarized in Figure 10.
The C-terminus of the first amino acid in the sequence is in the initial stage of the synthesis linked to the resin using a linker. The choice of linker is dependent on several aspects; whether a free or an amide protected C-terminus is wanted, what the reaction condition restrictions are for the cleavage from the resin, if the cleavage and removal of the side chain protecting groups should occur simultaneously or separately, etc. This thesis includes solid phase synthesis using four different linkers shown in Figure 11. The Wang (a) and the TentaGel S PHB (b) linkers do both form peptides with free C-terminus,
whereas the Rink amide linker (c) forms amide modified C-terminus. The 2-chlorotrityl linker (d) forms peptides with free C-terminus, but differs in cleavage conditions.49

Upon selecting the appropriate protecting groups in the synthesis of peptides, orthogonal protecting group strategy is important to consider.

![Molecular structures of Wang linker (a), TentaGel S PHB linker (b), Rink amide linker (c), and 2-chlorotrityl linker (d).](image)

**Figure 11.** Molecular structures of Wang linker (a), TentaGel S PHB linker (b), Rink amide linker (c), and 2-chlorotrityl linker (d).

### 3.1.2 Glycopeptide Solid Phase Synthesis

Glycopeptides can be synthesized according to two main strategies. Either the carbohydrate moiety can be conjugated to a full-length peptide, or it can be linked to the amino acid residue and then coupled in the same way as simple amino acids. The coupling of the carbohydrate group to the peptide has low stereoselectivity ($\alpha/\beta$-linkage), which is one reason why the latter strategy is preferred. Further, there are no observations indicating that the sterically large carbohydrate would interfere in the amino acid coupling reaction. As for any other peptide syntheses, the synthesis of a glycosylated peptide can be performed either in solution or by solid phase methodology. As already discussed, the latter is preferred over the former for longer sequences.

The use of Boc-protecting group as temporary masker of the amine group needs treatment of TFA for continuous cleavage, and very harsh acid hydrogen fluoride for cleavage of the peptide from the resin. This methodology is therefore not very suitable for glycopeptide chemistry, as the glycosylated amino acid is acid labile and would undergo loss of the carbohydrate moiety, see Figure 12a. Instead, the Fmoc/t-Bu chemistry is commonly employed.50 This protecting group strategy cleaves the peptide from the resin and simultaneous deprotects the amino acid side chains, using the moderately strong acid TFA. Even though the glycosidic bonds of most saccharides are stable towards TFA, the hydroxyl groups of the saccharide are usually protected
with acetyl or benzoyl groups to stabilize the glycosidic bonds during deprotection and cleavage.

\[ \text{Figure 12. All } O\text{-glycosides are cleaved by strong acid (a). Further on, the } O\text{-linked derivatives serine and threonine can undergo } \beta\text{-elimination under basic conditions (b).} \]

Amine-containing bases such as piperidine, morpholine or DBU are required to remove the temporary Fmoc backbone amino protecting group. This leads to a potential drawback, since the glycosides of serine and threonine can undergo \( \beta \)-elimination under basic conditions, see Figure 12b. For the same reason, the acetyl protection is preferred over benzoyl protection, since much milder conditions are required for deprotection.

According to Kihlberg et al., the stronger base piperidine is preferred over morpholine in the synthesis of glycopeptides despite the possible side-reactions that can arise during Fmoc-deprotection.\(^5\)

### 3.1.3 \( \beta \)-Peptoid Solid Phase Synthesis

In 1998 Hamper et al. published the first synthetic procedure of preparing \( \beta \)-peptoids.\(^3\) In this first report, the synthesis of trimeric \( \beta \)-peptoids with non-chiral side chains were described. Longer \( \beta \)-peptoid oligomers has latter been reported,\(^3\) but not where chiral side chains have been used. Our group (see Paper VII) developed the first synthetic methodology for preparing longer \( \beta \)-peptoids with \( \alpha \)-chiral N-substituents.
3.2 Conformational Analysis

3.2.1 Circular Dichroism

Circular dichroism (CD) spectroscopy is a chiroptical technique that gives useful information about the overall conformation of a molecule. It measures the wavelength dependence of how an optically active chromophore is able to differently absorb right and left circularly polarized light. In the case of peptides and proteins, the amide chromophore is usually investigated and for this reason, CD spectroscopy has become a powerful tool for determining the conformation of α-peptides in solution. The most common secondary structures of α-peptides are the right-handed α-helix, the β-sheet and the random-coil. The right-handed α-helix gives rise to CD minima at 208 and 222 nm and maximum at 190 nm. The β-sheet gives rise to a minimum at 218 nm and a maximum at 196 nm, whereas the CD spectrum of a random-coil has the minimum at 195 nm and the maximum at 212 nm, see Figure 13.

The CD spectrum of a left-handed 3_10-helix arising from a β-sheet-peptide in methanol is also well established and gives rise to a minimum, zero crossing, and maximum at 215, 208, and 198 nm, respectively (see Section 4.3).16

![Figure 13. Typical CD patterns seen from proteins and peptides adopting α-helices (α), β-hairpins (β) or random coils (r).](image)

In CD measurements of peptides and proteins, protic solvents such as water or methanol are most commonly used. A minor drawback with CD spectroscopy is that solvents that absorb in the ultraviolet spectral region, i.e. dichloromethane, cannot be used in the investigations. Further, CD spectroscopy is very dependent of the concentration and therefore intensity related errors can arise from the sample preparation. However, this risk can be
minimized by running two independent measurements, in combination with repeated lyophilization of the peptide.

CD spectroscopy is a valuable tool in the search for the folding propensities. However, it is important to keep in mind that the information retrieved in these experiments should always be considered as a complement to other analysis methods, such as NMR or X-Ray diffraction. This is especially true for unnatural oligomers with limited data available.

3.2.2 Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is one of the most prominent techniques for studying the three dimensional structure of molecules. Unfortunately, this technique has a relatively slow time scale and the gained parameters are therefore most often a population-weighted average of all conformations of a molecule in solution.

Some of the NMR parameters used for the determination of a conformation in solution are the chemical shift ($\delta$), the scalar coupling constant ($J$) and the nuclear Overhauser effect (NOE), parameters which are described in more detail below. The direct use of these parameters without a computation-based conformational analysis is, however, somewhat limited as this is valid only when the observed molecules assume a single conformation or when one conformation is so dominant at equilibrium that the contributions of all others are negligible.

3.2.2.1 Chemical Shifts

The chemical shift is a NMR parameter which is easily measured and gives a first indication of, e.g., whether amide protons are involved in hydrogen bonding interactions or not. As an example, an amide proton chemical shift ($\delta_{\text{NH}}$) in the range of 7-9 ppm indicates intramolecular hydrogen bonding whereas lower chemical shifts, in the range of 6-7 ppm, are typical for solvent exposed protons.

Amide proton chemical shift temperature coefficients ($\Delta\delta/\Delta T$) can give an additional indication of hydrogen bonding interactions. In polar aprotic solvents such as DMSO-$d_6$ used in this thesis, the $\Delta\delta/\Delta T$ values larger than 5 ppb/K are interpreted to identify solvent exposed amide protons, whereas values lower than 3 ppb/K imply that the amide protons are shielded from the solvent either due to intramolecular hydrogen bonding or by steric shielding.

The $H_\alpha$ chemical shift can also provide an indication regarding the secondary structures of proteins and peptides. By looking at the “secondary structure shift”, i.e. the difference between the observed chemical shift and the random coil value ($\delta_{\text{H}\alpha}^{\text{obs}} - \delta_{\text{H}\alpha}^{\text{rc}} = \Delta\delta_{\text{H}\alpha}$), it can be predicted whether the amino acid residue is part of an $\alpha$-helix or a $\beta$-sheet. If the $H_\alpha$ is shifted upfield, the residues are part of an $\alpha$-helix whereas if the $H_\alpha$ is shifted downfield, the residues are part of a $\beta$-sheet.
3.2.2.2 Coupling Constants

One factor influencing the vicinal coupling constants ($J_{\phi}$) between protons that is of particular interest for structural work is the dihedral angle ($\phi$). This dependence can be quantified by the Karplus relation, an empirical rule which is of great importance and used extensively in conformational analysis.60 Since a given value of vicinal coupling constant might result from four (or two) different dihedral angles, further parameters are required to determine the actual conformation.55 Furthermore, it should be emphasized that, in addition to the dihedral angle, other factors such as electronegativity of substituents, bond length, and steric strain modulate the size of vicinal coupling constants.

The $J_{3\text{HNCBH}}$ of $\beta$-amino acid residues are expected to have large values (7.5-9.5 Hz) both in linear peptides folded into a 314-helical secondary structure.

3.2.2.3 Nuclear Overhauser Effects (NOE)

Amongst the most important parameters for structure determination by NMR spectroscopy are those retrieved from NOE experiments, generating information regarding inter-atomic distances between nuclei close in space. Two spins, e.g. two protons $A$ and $S$, that are close in space but not necessarily linked through bonds relax each other through dipole-dipole relaxation. If one of the spins ($A$) is being perturbed by saturation (steady state NOE) or inversion (transient NOE), the signal of spin $S$ will have a change in intensity caused by a population change.61 Since this so-called NOE is dependent on this dipole-dipole relaxation, it is affected by both the distance between spins and molecular motion. Regarding the distance relationship, the NOE between spins $A$ and $S$ is proportional to $r^{-6}$, where $r$ is the distance between the two spins.

Concerning the dependency on molecular motion, the NOE is related to the motional frequencies of molecules which usually are described in terms of $\omega \tau_c$, where $\omega$ is the Larmor frequency of the observed spin and $\tau_c$ is the correlation time describing the movement of the molecules it resides in. For spins with a positive gyromagnetic ratio such as protons, if $\omega \tau_c$ is much smaller than 1 a positive NOE will be generated, corresponding to an increase of signal intensity. In a proton steady state NOE experiment the maximum increase of intensity is 50% whereas a smaller maximum value is observed (38.5%) for transient NOE. If $\omega \tau_c=1.12$, the NOE is zero, and if $\omega \tau_c$ has a value much larger than 1, a negative NOE is detected (minimum -100%), see Figure 14.

Small peptides with rapid molecular tumbling, corresponding to short correlation times, exhibit small positive NOEs, whereas larger peptides with slow tumbling give rise to large negative NOEs.
In such a situation of negative direct NOEs, the distance dependence is lost and so-called spin diffusion dominates. This means that a population disturbance, initially present only at one spin, spreads throughout the molecule by cross-relaxation from one spin to another until every spin in the molecule is more or less affected. Therefore, for larger molecules it is in some cases of greater advantage to use the rotating frame NOE (ROE) experiment instead. The ROE has a different dependence on molecular tumbling with the result that all spins behave as if they were in the positive NOE region.

Typically, a NOE or ROE can only be observed if the distance between spins is smaller than 3-4 Å.

The 2D NMR techniques, identified by their acronyms NOESY and ROESY, resemble 1D transient NOE experiments and the information extracted from these 2D experiments is often used in computational techniques to translate the observed parameters into a possible secondary structure of a peptide/protein by defining distance constraints.

### 3.2.2.4 Solvent Suppression

To be able to obtain 1H NMR signals and the corresponding important information from the amide region of peptides in solvents such as water and methanol, 90% non-deuterated solvent needs to be used. Due to this, solvent suppression techniques are required. One example is the WET technique, a $T_1$- and $B_1$-insensitive solvent-suppression method, which can be used in combination with various 1D and 2D NMR methods. It uses a series of variable-tip-angle solvent-selective RF (radio frequency) pulses, where each selective RF pulse is followed by a dephasing field-gradient pulse.
3.2.3 Computational Methods

As a good complement to the experimental conformational studies, a theoretical simulation can be made. In fact, the help of information retrieved from NMR experiments can propose a conformational profile in solution.

The studies in this thesis have been made using Monte Carlo simulation followed by PR Conjugate Gradient minimization in the molecular mechanics program MacroModel 7.0. In difference to quantum mechanic methods, molecular mechanics ignore the electronic motions and calculate the energy of a system as a function of the nuclear position only. This method is therefore used to make calculations on larger molecules, i.e. systems containing significant number of atoms and/or where multiple calculations are required.

Monte Carlo simulation generates low-energy conformations of a system by making random changes. A random search has the advantage that it can move from one region of the energy surface to a completely unconnected region in one single step. During each iteration, the conformation present is randomly changed and then energy minimized. If the minimized new conformation has not been found previously, it is stored. The conformational search proceeds until the number of iterations given is reached or if no new conformations are found. In the Monte Carlo search, the new conformation generated after energy minimization is used as starting point in the next cycle if it fulfills the requirement as such.

As distance restraints, parameters extracted from 2D NOESY and ROESY spectra were used and classified into three categories with upper bond distance limits: strong 2.5 ± 1 Å, medium 3.0 ± 1 Å and weak 4.0 ± 1 Å. The NH–C(ϕ)-H dihedral angle restraints, derived from the coupling constants, were used as restraints in the conformational search.

Even though this is a good complementary method, it needs to be noted that the reliability of the output is greatly dependent on the input.

3.2.4 X-Ray Diffraction

X-Ray crystallographic studies are very beneficial when it comes to determining the structure of a peptide in solid state. By beaming X-rays on grown crystal, the electrons will diffract the X-rays and cause diffraction patterns to arise. These patterns can be converted into electron-density maps by mathematical Fourier transformation which then can be fitted into atomic models by data processing.

A drawback with this method is that to be able to use this method at all, well-ordered crystals of the peptides have to be obtained. This is, however, not always possible. Even further, this method gives the conformation of a compound in the solid state, a conformation which is not always compatible with the secondary structure adopted in solution.
3.3 Biomolecular Recognition

3.3.1 Biacore 2000 Instrument

A Biacore instrument can measure the binding affinity between two molecules by attaching a biomolecule, a ligand, to a sensor surface and then detect the interaction between this ligand and a compound free in solution (analyte) (Section 4.4, Paper IV). The sensor surface is a glass medium coated with a thin layer of a conducting material (gold), and the detection can be made by a method based on surface plasmon resonance (SPR). This is an optical phenomenon that arises in thin conducting films at an interface between media with different refraction index. In this case, one of the media is the glass of the sensor chip whereas the second is the sample solution.

The ligand is attached to the surface of the sensor chip (immobilized) via a covalently bound matrix of carboxymethylated dextran and the binding can be made either covalently or through high affinity. After immobilization, the analyte is injected over the sensor surface and if the analyte binds to the ligand, a response arises that are directly proportional to the concentrations of the biomolecules on the surface. At the end of the injection, a buffer will replace the sample and the bound analyte can dissociate from the surface.

The binding response and the analyte dissociation are plotted in a so-called sensorgram, a plot of response against time, see Figure 15. From interaction studies by a Biacore instrument, $k_{on}$ and $k_{off}$ are retrieved, and by this, the association constant ($K_D$) can be determined ($K_D = k_{off} / k_{on}$).

![Figure 15. Schematic illustration of a sensorgram showing the progress of the interaction. The response is measured in response units (RU).](image-url)
4 Results and Discussion

4.1 Synthesis of $\beta$-Amino Acid Building Blocks

4.1.1 Synthesis of $\beta^3$-Amino Acids

$\beta^3$-Amino acids can efficiently prepared by Arndt-Eistert homologation of $\alpha$-amino acid derivatives, a method which proceeds stereospecifically and most often in high yields. The first step of the synthesis includes addition of freshly distilled diazomethane (CH$_2$N$_2$) in diethyl ether (Et$_2$O), forming a diazoketone which can be later left to undergo Wolff rearrangement. The last step has been shown to gain by ultrasonic promotion; and homologated $\beta^3$-amino acid can be obtained either as free carboxylic acid or as an ester-protected derivative, see Scheme 1.

All $\beta^3$-amino acids, but one, included in this thesis has been synthesized according to the Arndt-Eistert homologation. The only exception was the synthesis of $\beta^3$-Ser, which were prepared from the naturally occurring $\beta$-amino acid aspartic acid (1), see Scheme 2. By reducing the free carboxyl group of Fmoc- and side chain protected D-Asp (2) to an alcohol via activation of the carboxylic acid followed by mild reduction, the suitably protected $\beta^3$-Ser derivative (3) was obtained in 66%, overall yield.

---

Scheme 1. Reagents and Conditions: i) NMM, IBCF, THF, -15°C, 20 min; ii) CH$_2$N$_2$, Et$_2$O, 5 h; iii) 15% ROH or H$_2$O, THF; iv) Silver benzoate, NMM.

Scheme 2. Reagents and Conditions: i) Na$_2$CO$_3$, Fmoc-OSu, H$_2$O/THF (1:1), overnight, 83%; ii) NMM, IBCF, THF, -15°C, 1 min; iii) NaBH$_4$, H$_2$O, 80%.
4.1.2 Synthesis of $\beta^2$-Amino Acids

As described above, the synthetic pathway of $\beta^3$-amino acids includes few stereospecific high yielding steps. However, the $\beta^2$-amino acids are not accessible from $\alpha$-amino acids and despite numerous developments in this field,\textsuperscript{73} the synthesis of enantiomerically pure $\beta^2$-amino acids still require laborious multistep procedures.

The synthesis of the protected $\beta^2$-amino acids (Fmoc-(R)-$\beta^2$-Phe-OH and Fmoc-(S)-$\beta^2$-Phe-OH) used in Section 4.5.5 were performed by a procedure developed by Seebach,\textsuperscript{8a,74} utilizing Evans’ enolate chemistry.\textsuperscript{75}

4.2 Synthesis of $O$-Glycosylated Amino Acids (Paper I)

Using a synthetic route developed by Lemieux and Ratcliffe,\textsuperscript{76} and then latter improved by Kihlberg \textit{et al.},\textsuperscript{77} the bromide $4$ could be synthesized in an overall yield of 47% over four steps, Figure 16.

Chloride analogue $5$ (Figure 16) was synthesized with an overall yield of 43% according to the same procedures as 4 except for the last halogenation step.$^6$

\textit{Figure 16.} The glycosyl donors 3,4,6-tri-O-acetyl-2-azido-2-deoxy-$\alpha$-D-galactopyranosyl bromide (4) and chloride (5).

Only the $\alpha$-isomers of the C-1 halogenated monosaccharides $4$ and $5$ were formed due to the anomeric effect. This effect is a stabilizing interaction between C-1 (anomeric carbon) of the carbohydrate moiety and an electronegative substituent positioned in $\alpha$-configuration, which arises due to a delocalization of electrons.

4.2.1 Synthesis of $O$-Glycosylated Amino Acids

Mechanistically, the glycosidic bond can be formed through either $S_N1$- or $S_N2$-mechanisms. The ring oxygen facilitates the $S_N1$-mechanism since the lone-pairs of the oxygen stabilizes the carbocation intermediate that is formed as the leaving group departs, see Figure 17a. The departure of the leaving group is the rate-determining step of the $S_N1$-mechanism, which then is followed by an attack of a nucleophile. The attack can occur on either face of the carbocation, allowing the possible formation of $\alpha$ or $\beta$ products.

36
Figure 17. The two possible mechanisms of nucleophilic substitution at the anomeric position—SN1 (a) and SN2 (b). In figure c, the ether effect is demonstrated.

The ratio between the amount of α- and β-products formed upon glycosylation can to some extent be controlled by solvent and temperature. For instance, to assist the formation of α-product, the so-called ether effect can be utilized, see Figure 17c. An ethereal co-solvent such as Et₂O or THF will coordinate to the anomeric carbon in β-position and thereby leave the α-position as the preferred mode of nucleophilic attack.

Additionally, the α/β-ratio can be controlled via a neighboring participating effect from the group attached to carbon 2. The β-anomer can be formed exclusively due to a directing effect by the use of a glycosyl donor containing an acyl-group such as the 2-N-acetyl and 2-N-Troc (trichloroethoxycarbonyl) groups used in Section 4.2.1.2. However, having an azide in C-2 position (see Section 4.2.1.1), this group will not interfere with the entry of the nucleophile at all and will thereby increase the amount α-product being formed.

In certain cases, a competitive SN2-type process occurs. This gives a clean inversion of configuration at the anomeric carbon, forming the β-product exclusively, see Figure 17b.

4.2.1.1 Synthesis of αGalNAcβSer/Thr
The suitably protected β-hSer (3) could be linked to the glycosyl donor GalN3Br (4) at -40°C using AgOTf as promoting silver salt, see Scheme 3. Despite the low temperature and the use of an azide neighboring group, a mixture of the α- and the undesired β-anomer was obtained. The two
diastereomers had very similar Rf-values and their separation requires multiple purification by flash column chromatography.

$$\text{OAc}$$  $$\text{OAc}$$  $$\text{N}_3$$  $$\text{OAc}$$  $$\text{HO}$$  $$\text{O}$$  $$\text{OBn}$$  $$\text{Fmoc}$$

$$\text{OAc}$$  $$\text{OAc}$$  $$\text{N}_3$$  $$\text{OAc}$$  $$\text{HO}$$  $$\text{O}$$  $$\text{OBn}$$  $$\text{Fmoc}$$

$$\text{NH}$$  $$\text{O}$$  $$\text{OBn}$$  $$\text{Fmoc}$$

$$\text{NH}$$  $$\text{O}$$  $$\text{OBn}$$  $$\text{Fmoc}$$

$$\text{NH}$$  $$\text{O}$$  $$\text{OBn}$$  $$\text{Fmoc}$$

$$\text{Fmoc}$$  $$\text{Fmoc}$$  $$\text{Fmoc}$$  $$\text{Fmoc}$$

$$\text{NH}$$  $$\text{O}$$  $$\text{OBn}$$  $$\text{Fmoc}$$

$$\text{O}$$  $$\text{AcO}$$  $$\text{AcO}$$  $$\text{N}_3$$  $$\text{OAc}$$  $$\text{Br}$$

Scheme 3. Reagents and Conditions: i) CH$_2$Cl$_2$/THF (1:1), 4Å MS, -40°C, 1h. Then AgOTf in toluene, -40°C 6h, 36% ($\alpha$ anomer); ii) THF/Ac$_2$O/AcOH (3:2:1), activated Zn, CuSO$_4$ (sat), r.t. 2h, 84%; iii) 10% Pt/C, H$_2$, 1 atm, MeOH/H$_2$O (15:1), 5h, 92%.

In order to maximize the $\alpha$/$\beta$-ratio, the reaction conditions were optimized with respect to the choice of glycoside halogen (Br or Cl) and silver salt (AgOTf or AgClO$_4$), see Table 1. To minimize the margin of errors, two parallel reactions were performed for each method.

Table 1. Optimization of the coupling between $\beta$-Ser (3) and either monosaccharide 4 or 5. The silver salts were varied between AgOTf and AgClO$_4$, while the leaving group of the anomeric carbon was either a chloride or a bromide.

<table>
<thead>
<tr>
<th>Method</th>
<th>Yield (%)</th>
<th>$\alpha/\beta$ Ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I$^a$</td>
<td>71%</td>
<td>55:45</td>
</tr>
<tr>
<td>II$^b$</td>
<td>66%</td>
<td>65:35</td>
</tr>
<tr>
<td>III$^b$</td>
<td>65%</td>
<td>65:35</td>
</tr>
</tbody>
</table>

$^a$ AgOTf, CH$_2$Cl$_2$/Et$_2$O/Toluene, -10°C, 5 h.

$^b$ AgClO$_4$, CH$_2$Cl$_2$/Toluene, -40°C, 5 h.
From this investigation it could be concluded that there was a significant leaving group dependence, preferring the bromide (ratio: 65:35) over the chloride (ratio: 55:45). Further, it was observed that the $\alpha/\beta$-ratio was independent of what silver salt was used. Consequently, since AgOCl is known to be explosive, AgOTf was the silver salt of choice.

After the tedious separation to get the pure $\alpha$-anomer 6a, reductive acetylation was performed by treatment of 6a with Zn and saturated CuSO$_4$ in THF/Ac$_2$O/AcOH (3:2:1) and produced 7 rapidly in good yield (84%, Scheme 3). The final step of the synthesis was deprotection of the benzyl group using Pt/C in MeOH/H$_2$O under 1 atm of H$_2$, a procedure reported by the group of Danishefsky. Attempts to use Pd/C as the transition metal for the hydrogenation resulted in undesired backbone amine Fmoc-deprotection. By changing the transition metal from palladium to platinum, the benzyl group was selectively deprotected and the final product 8 was received in high yield (92%).

The same synthetic pathway as for glycosylated $\beta$-hSer was planned to be used for the threonine homolog. Therefore was the Fmoc-$\beta$-Thr(Ot-Bu)-OBn 9 synthesized by the Arndt-Eistert procedure, using Fmoc-L-Thr(Ot-Bu)-OH as starting material (overall yield: 43%).

Free hydroxyl group are required for glycosidic linkage. Unfortunately, upon removal of the $t$-Bu group of 9 by TFA and TIPS in CH$_2$Cl$_2$, rapid lactonization occurred of $\beta$-Thr forming a stable five-membered ring, see Scheme 4. The serine homolog did also undergo cyclization when exposed to acidic environment.

![Scheme 4. Lactonization of Fmoc-$\beta$-Thr(Ot-Bu)-OBn 9 upon treatment with TFA.]

Instead of using the $\beta$-amino acid as glycosyl acceptor, the suitably protected L-amino acid analogue could be linked to monosaccharide 4 and then use the sugar moiety as a protecting group during an Arndt-Eistert homologation. By this method, glycosylated $\beta$-hSer 8 was synthesized in 14% overall yields from the amino acid residue.

The overall yield was significantly lower in the latter strategy (26% vs. 14%) and was also less favorable from a safety point of view.

4.2.1.2 Synthesis of $\beta$GlcNAc$\beta$hSer/Thr

The N-acetylated glucose amine 11 was linked to the Fmoc-$\beta$-hSer-OBn 3 over two steps, see Scheme 5. In nature, this carbohydrate derivative is $\beta$-linked to either serine or threonine, and due to the directing effect of the 2-N-acetyl group, the $\beta$-linked anomer 12 was formed exclusively in 35% yield.
Deprotection of the carboxylic acid using the same conditions as for α-GalNAc-β-hSer-OH 8 gave the final product 13 in 29% yield, calculated from the β-hSer residue.

As discussed in previous section (Section 4.2.1.1), the synthesis of a β-hThr derivative suitable for glycosylation could not be performed. Coupling of the GlcNAc moiety to L-Ser followed by homologation gave 13 in 15% overall yield, with regard to the L-Ser derivative.
4.3 Glycosylated $\beta$-Peptides

4.3.1 Synthesis of Glycosylated $\beta$-Peptides

Seven glycosylated peptides 15-21, see Figure 18, were synthesized using standard Fmoc-chemistry on either Rink amide resin (15, 16, 18-20) or Wang resin (17, 21).

The syntheses started by leaving the resin to swell in DMF. For the peptides synthesized by Rink amide resin, the first step of the synthesis was removal of the Fmoc-protecting group, using a solution containing 2% piperidine and 2% DBU in DMF. After washing the resin with DMF, a mixture of $\beta$hVal, HBTU, HOBT, and DIPEA in DMF was added and left under N$_2$-atmosphere over night.

For the peptides synthesized using Wang resin, coupling of the first amino acid could be made directly after the swelling of the resin using N,N-diisopropylcarbodiimide and DMAP for preactivation.
Loading level of the resins was determined by measuring the UV-absorption of the fluorenyl-piperidine adducts (cleaved off by treating the resin with 20% piperidine in DMF for 30 min) at 290 nm.

Capping of unreacted amino groups by acetylation was performed after coupling of the first amino acid to the resin. This was made by treatment with a mixture of Ac₂O in CH₂Cl₂ (1:1 ratio) for 3 h.

As soon as the first amino acid was attached to the resin, the two different resins (Rink and Wang) had the same reaction cycle of peptide elongation and were composed of following steps; the terminal Fmoc protecting group was removed with 2% piperidine and 2% DBU in DMF and then treated for 5-24 h with activated β-amino acid. The activation was made using the mixture of HBTU, HOBT, and DIPEA in DMF and the coupling completion was controlled by TNBS (trinitrobenzenesulfonic acid)-test.79

After linkage of the glycosylated amino acid, a milder Fmoc cleaving mixture composed of 20% piperidine in DMF was used because of the base sensitivity of the glycoside (see Section 3.1.2).

The synthesis cycle was repeated until the desired length of the β-peptide was obtained. Between every step of the synthesis, the resin was washed at least five times with DMF to decrease the amount of by-products being formed.

After final Fmoc deprotection, the peptide was cleaved off from the resin using a standard TFA/H₂O/TIPS-mixture (95:2.5:2.5). Analysis of the crude material by reverse phased analytical HPLC on a C-18 column showed that the basic conditions used in the coupling of the last amino acid and the Fmoc-deprotection did also cause partial deprotection of the O-acetyl groups positioned on the carbohydrate moiety. Therefore, removal of the O-acetyl protecting groups was made by treating the crude peptides directly with saturated methanolic ammonia. Pure glycosylated β3-peptides were obtained after purification by reverse phase HPLC (C18 column).

In the synthesis of glycosylated β-peptides, it was found that N-capped peptide by-products were formed due to a small amount of acetic acid sometimes left from the purification of the Fmoc-βhSer(O-α-GalNAc)-OH by flash column chromatography. This could however be avoided by co-evaporation with toluene and repetitive lyophilization.

The unglycosylated analogue 14 was synthesized according to the same Rink amide strategy, except for the change of Fmoc cleavage mixture.

4.3.2 Conformational Studies of Glycosylated β-Peptides

4.3.2.1 Paper II

4.3.2.1.1 CD Spectroscopic Studies

Initial conformational studies by CD spectroscopic analysis was performed on the unglycosylated peptide 14 and the glycosylated analogue 15 in methanol at
25°C (c=0.1mM), see Figure 19. The characteristic features of the 3_14-helix (see Section 3.2.1) was observed for both peptides. Comparing the intensities of the minimum at 215 nm of the two peptides, one could conclude that the incorporation of a carbohydrate moiety has led to a destabilization of the 3_14-helix conformation in methanol.

![Figure 19. CD spectra of 14 (−) and 15 (−−) in methanol (high intensity curves) and water at pH 6.9 (low intensity curves)](image)

A dramatic decrease in the CD intensity was noted for both unglycosylated β-peptide 14 and the analogue 15 when running CD spectra of the β-peptides in aqueous buffer at pH 6.9 (c=0.1mM, 25°C), (Figure 19). Nevertheless, both peptides still gave rise to the characteristic signature of the 3_14-helix, suggesting that both peptides, at least partially, adopted this conformation also in aqueous solution.

4.3.2.1.2 NMR Studies

Detailed 1H and 2D NMR (TOCSY, P.E.-COSY, ROESY, gHMBC) studies for assignment of the NMR signals and further investigation of the secondary structure of the β-peptides were performed in CH_3OH/CD_3OD (9:1). Additional experiments were carried out in H_2O/D_2O (9:1) at pH=5.3. Solvent suppression by the WET technique (see Section 3.2.2.4) was utilized.

The 1H spectrum of the unglycosylated β-peptide 14 recorded in methanol showed good dispersion of the amide region, indicating the presence of a secondary structure. The J^\text{H} (NH–C(β)H) coupling constants were all large, i.e. in the range between 8.2-9.5 Hz, verifying the expected antiperiplanar orientation of these protons.

The β-amino acid residues could be differentiated and the protons were fully assigned by the use of the TOCSY spectrum. Further, the diastereotopic CH_2(α) protons were distinguished from each other by the use of P.E.-COSY data. In a helical structure, the β-amino acid residues would have one of the α-
protons arranged in an axial position, 180° relative the β-proton meanwhile the other α-proton would be equatorially positioned and in an angle to the β-proton closer to 90°. These dihedral angles would cause the axial proton to give rise to a signal with a large coupling whereas the equatorial α-proton would have a small coupling to H-C(β). In the P.E.-COSY, only one cross peak was seen from the α-protons, i.e. the axial proton.

Valuable information regarding the secondary structure of the β-peptide was obtained from the ROESY spectra. The assignment of axial and equatorial α-protons were shown to agree with the strength of the NOEs, which were larger between the H-C(β) and the equatorial H-C(α) protons than between H-C(β) and the axial H-C(α) protons. Further on, the ROESY spectra showed several non-sequential NOEs between NH→H-C(β)_{i+2} and NH→H-C(β)_{i+3}, confirming a 3_{11}-helical secondary structure, see Figure 20a.

![Figure 20. Backbone NOEs extracted from the ROESY spectrum of 14 (a) and 15 (b) recorded in methanol; C_\(i\) H(β)→C_{\(i+3\)} H(β) NOEs are in red and C_\(i\) H(α)→C_{\(i+3\)} H(α) NOEs are in blue.](image)

The amide region of 15 showed comparable dispersion as seen for 14 and all \(^3\)J(NH–C(β)H) coupling constants were large (8.7–9.5 Hz) and NOEs between the amino acid residues implied 3_{11}-helical conformation, see Figure 20b.

4.3.2.1.3 Computational Analysis

First peptide to be investigated by restrained Monte Carlo conformational search was the glycosylated peptide 15. The six H-N-C(β)-H torsional angles were fixed to 170 ± 20 according to the observed \(^3\)J NH–C(β)H using a modified Karplus curve. Conformational constraints were introduced using the upper bond distance limits categorized earlier in Section 3.2.3.
Figure 21 represents the superposition of ten low-energy structures resulting from this calculation. Two main conformational families of comparable energies were found and may be considered representative for the folded structure of 15 in MeOH solution. The structures of the two families differed mainly at the C-terminal side of the helix, most clearly seen in Figure 21c. The difference between the two families was caused by the interactions of $\beta^h$Orn in position 6 (position of the amino acid residue in the peptide sequence, relative the N-terminus); in one family (blue) this residue had the desired interaction with $\beta^h$Glu in position 3, while the other family (green) was characterized by an interaction between $\beta^h$Orn and the GalNAc moiety at position 2, leading to an “opening” of the helix at the C-terminus.

Since both conformational families of 15 possessed $\alpha_{14}$-helical structure, this result was in agreement with the information retrieved from the NMR experiments.

As in the calculations of 15, the six H-N-C($\beta$)-H torsional angles of 14 were fixed to 170° ± 20, according to the observed $J^1$ (NH–C($\beta$)-H). As expected from the CD measurements, only one conformational family was detected. The $\alpha_{14}$-helix was stabilized by hydrophobic interactions between the $\beta^h$Val residues positioned on one rim of the helical triangle, and a salt-bridge interaction between $\beta^h$Orn in position 6 and $\beta^h$Glu in position 3. Since the third rim of the helical structure consisted of $\beta^h$Thr in position 2 and $\beta^h$Glu in position 5, no ion-pair interaction was possible in this case. Instead, $\beta^h$Glu in position 5 interacted with the C-terminal amide nitrogen, an interaction which did not lead to destabilization of the $\alpha_{14}$-helical structure.
4.3.2.2 Paper III

4.3.2.2.1 CD Spectroscopic Studies

Glycosylated $\beta$-peptides 16-21 were designed and synthesized taking into account the results given in Paper II (Section 4.3.2.1) and Schepartz design strategy (see Section 1.2.2.1). CD spectroscopic studies were performed on all glycosylated $\beta$-peptides (15-21) in both methanol and water, see Figure 22.

The CD data of $\beta$-peptides have been extensively studied over the last ten years, and it is therefore rational to make the conclusion that changes in intensity of the $3_{14}$-helical pattern at 215 nm correlate to relative changes in overall mean $3_{14}$-helical population. Nevertheless, it is important to remember that the CD data of $\beta$-peptides always should be interpreted with care.

Figure 22. CD spectroscopic studies of glycosylated $\beta$-peptide 15-21 in methanol (a) and PBC-buffer (b) (1mM sodium phosphate/borate/citrate, pH 7) at 25°C and $\epsilon=0.1$mM.

In methanol ($\epsilon=0.1$mM at 25.0°C), the intensity of the absorption at 215 nm, suggested that all glycosylated $\beta$-peptides adopted $3_{14}$-helical structures with similar intensity, except the two glycosylated $\beta$-peptides 19 and 21, which had noticeably lower intensity than the others. This lowering in intensity corresponded to 37% and 34% loss of $3_{14}$-helical stability, respectively, using the helical intensity shown for the first synthesized glycosylated $\beta$-peptide 15 as reference.

In PBC buffer (pH 7), the CD spectroscopic results could be divided into three classes of intensity. The first class includes peptide 16, 17 and 20, being in the same intensity range and with a similar loss in overall mean $3_{14}$-helical population (53-56%, relative the $3_{14}$-helical intensity gained for respective $\beta$-peptide in methanol). The second family includes glycosylated $\beta$-peptides 15, 18, and 19. This class has only a low degree of its population folded into a $3_{14}$-helical structure, corresponding to 29%, 16% and 22% relative the helical intensity shown for peptide 15 in MeOH.

The third class includes peptide 21. This glycopeptide, with a free C-terminus and side chains sequenced to minimize the macrodipole moment,
gave rise to a CD pattern with low intensity in methanol. However, in water at pH 7, 21 showed a CD pattern with significantly much stronger intensity than any of the other structurally investigated glycosylated \( \beta \)-peptides, see Figure 22b. In fact, the overall helical content of peptide 21 has increased by 33\% upon change of solvent from methanol to water (pH 7) and possesses 87\% of the overall mean 3_14-helical population seen for glycosylated \( \beta \)-peptide 15 in methanol.

Studies concerning pH and temperature dependency of glycosylated \( \beta \)-peptides were performed on 15 and 16. While no temperature-dependence could be observed for any of the glycosylated \( \beta \)-peptides 15 and 16, (recorded in MeOH) they both showed an evident pH dependency. However, the results differed significantly between the two peptides (see Paper III).

Concentration dependence investigations were performed on 16, 17, 20 and 21 in both methanol and water in the range of 0.01 to 1 mM. According to Figure 23, it appears to be a possible tendency of aggregation at the highest concentration for peptides 16, 20 and 21. However, this was not observed in water at neutral pH.

The results from the concentration dependence study of 17 was unexpected, but follows the same trend in both methanol and water.

![Figure 23. Concentration dependence investigations of 16, 17, 20 and 21 in methanol (left) and water (right) in the range of 0.01 to 1 mM.](image)

### 4.3.2.2 NMR Studies

The glycosylated \( \beta \)-peptides 16-21 were all investigated by detailed \(^1\)H and 2D NMR (TOCSY, P.E.-COSY, ROESY,) studies in CH\(_3\)OH/CD\(_3\)OD (9:1). Solvent suppression was performed using the WET technique (see Section 3.2.2.4).

The \(^1\)H NMR spectra did all show good dispersion of the amide region, indicating the presence of secondary structures. The \( J \) (NH–C(\( \beta \)H)) coupling constants were large for all peptides, as seen for \( \beta \)-peptides 14 and 15.

Since the glycosylated \( \beta \)-peptide 21 gave rise to a CD pattern with much stronger intensity than any of the other peptides, but even more interestingly,
stronger intensity in water than in methanol, this peptide was additionally investigated in H₂O/D₂O (9:1) at pH 7.2. As expected, the amide protons gave rise to only one set of well-defined signals. The $J^3$ (NH–C(β)H) coupling constants were in the range of 8.3-9.7 Hz.

The NOEs were then used as distance limits restraints in the first computational analysis of a glycosylated β-peptide in water.

4.3.2.2.3 Computational Analysis

The NOE parameters derived from the ROESY spectra were used as conformational constraints using the upper bond distance limits categorized earlier in Section 3.2.3. The six H-N-C(β)-H torsional angles were fixed to $180°± 20$ according to the observed $J^3$ NH –C(β)H. The NOEs were then used as distance limits restraints in the first computational analysis of a glycosylated β-peptide in water.

![Figure 24. NMR structures of glycosylated β-peptide 21 in water (pH 7.2) as a bundle of six low-energy structures calculated from a restrained Monte Carlo conformational search shown from the side (a) and from the top (b). All hydrogens, except amide hydrogens, have been omitted for clarity. Carbons are shown in grey, nitrogens in blue, and oxygens in red. Figure (c) represents a structure (blue) where all side chains (except the carbohydrate moiety) and hydrogens (except the amide hydrogens) have been omitted for clarity.](image)

From the calculations, only one conformational family was found, see Figure 24, as expected from the CD measurements. The 3_14-helix was shown to be stabilized by a successful combination of a positively charged side chain close to the N-terminus, a negatively charged side chain close to the C-terminus and free C- and N-termini. Further, hydrophobic interactions between the β^3hVal residues, salt-bridge interaction between β^3hOrn in position 2 and β^3hGlu in position 5 and finally interactions between the N-acetyl group of the carbohydrate moiety in position 2 and the β^3hOrn residue in position 5 did also contribute to the enhanced 3_14-helical stabilization. Consequently, all elements of the β-design strategy were successfully combined to gain a glycosylated β-peptide with enhanced helical stability in aqueous solution.
4.4 Biomolecular Recognition of Glycosylated β³-
Peptides (Paper IV)

4.4.1 Lectins

Lectins (Latin legere, “to select”) are selective carbohydrate-binding proteins/glycoproteins present in most organisms, including viruses, bacteria, plants, fungi, invertebrates and vertebrates. They bind mono- and oligosaccharides reversibly through hydrogen bonds, hydrophobic- and van der Waals- interactions. Additionally, the contact between the protein and its ligands can also be mediated by water bridges.

The lectins were first discovered by Hermann Stollmark in 1888 but did not gain full attention until the late 1960’s when they were shown to play an important role as tools for structural and functional investigations of complex carbohydrates such as glycoproteins. Further, lectins can be used to examine changes in cell surface glycosylation pattern, occurring during physiological and pathological processes.

Dependently of their selectivity, the lectins are classified into five groups according to the monosaccharide for which they exhibit highest affinity: mannose, galactose/N-acetyl galactosamine, N-acetyl glucosamine, fucose, and N-acetyl neuraminic acid. In the bimolecular recognition studies included in this thesis, four lectins with varying, but still significant, N-acetyl galactosamine specificity were chosen: *Glycine max* (soybean) agglutinin (GMA), *Vicia villosa* (VVA), *Helix pomatia* agglutinin (HPA), and *Bandeiraea simplicifolia* lectin I (BS-I).

*Glycine max* agglutinin is a N-acetyl glucosamine and mannose containing glycoprotein made up of four nearly identical subunits. It recognizes galactosamine and galactose specifically. *Helix pomatia* agglutinin (HPA) is a lectin extracted from the albumen gland of roman snails. Its binding preferences has been established to be in decreasing order: Forssman antigen (αGalNAc1-3GalNAc-R)>blood group A substance (αGalNAc1-3[αFuc1-2]Gal)>Tn antigen (αGalNAc-Ser/Thr)>GalNAc>GlcNAc. The *Vicia villosa* agglutinin (VVA, isolectin B3) has specificity towards the monosaccharide N-acetyl-D-galactosamine linked to either serine or threonine (Tn antigen, see Section 1.3). This specificity seems possible by hydrogen bonding between the serine/threonine moiety of the Tn molecule and a hydroxyl group in the lectin.

The *Bandeiraea simplicifolia* BS-I lectin is a tetrameric lectin consisting of two types of subunits – A and B. These subunits are represented to various degrees in five existing BS-I isolectins, but it is the BSI-A1 isolectin that has the major affinity for terminal N-acetyl-α-D-galactosaminy1 residues.
4.4.2 Results

The ability of the four lectins described in Section 4.4.1 (GMA, VVA (B4), BS-I, and HPA) to recognize six carbohydrate-containing peptides (15, 16, 18, 22, 24, 25), and one monomeric αGalNAc-O-Thr residue (24) (see Figure 25) was investigated through a biomolecular recognition study.

The peptides to be investigated was two αGalNAc containing heptapeptides (15-16) and one analogue possessing a β-linked GalNAc moiety (18). Further, one αGalNAc containing mixed α/β-dipeptide (22) and a β-dipeptide including a βGlcNAc moiety (23) was investigated together with a glycosylated α-peptide analogue of 23 (25) and the monomer 24. The four lectin ligands were immobilized to the surface of a Biacore sensor chip and the carbohydrate possessing analytes dissolved in PBS (phosphate-buffered saline) buffer were added (see Section 3.3.1).

For each lectin, the binding and dissociation rate constants between every individual peptide/monomer were observed and summarized in interaction rate plots, see Figure 26. In this plot, the binding rate constant (on-rate, $k_{on}$) is plotted against the dissociation rate constant (off-rate, $k_{off}$). The diagonal lines represent the binding affinity with high affinity in the upper left corner and low affinity in the lower right corner.

From the binding study, it was observed that the lectins bound to the glycosylated β-peptides with different affinity and selectivity. In general, the monomer 24 had the highest binding affinity in all cases and the βGlcNAc possessing dipeptide 23 gave rise to affinities much lower than any of the GalNAc containing β- peptides. However, only the HPA lectin showed
significant difference in binding specificity between the $\alpha$GalNAc (16) and the $\beta$GalNAc (18) moieties.

![Figure 26.](image)

It could be concluded that glycosylated $\beta$-peptides can indeed be recognized by natural proteins. In this example it was shown that carbohydrate-binding lectins recognized the carbohydrate part of unnatural $\beta$-peptides. Furthermore, it could be observed that the binding properties of the carbohydrates to the lectins were influenced by variations in the $\beta$-peptide backbone. This first demonstration of such interactions represents an important step towards further designs of glycosylated $\beta$-peptides with more specific properties.
4.5 Cyclic Backbone Modified Peptides

4.5.1 Cyclic Tetra and Penta $\beta$-Peptides

The chemistry behind the secondary structure of acyclic $\beta$-peptides has been of interest for a long time, but it is not until recently that cyclic $\beta$-peptides have attracted attention.\textsuperscript{85,86} The first reported synthesis of a cyclic $\beta$-tripeptide was published almost 40 years ago (1968) and the attention was to mimic the bacterial Fe chelator enterobactin B.\textsuperscript{87} Since then, cyclic $\beta$-tripeptides have been synthesized using for example achiral or racemic $\beta$-amino acids,\textsuperscript{88} and enatiomerically pure $\beta$-amino acids.\textsuperscript{89} These structures have been conformationally studied in both solid-state,\textsuperscript{90} and in solution.\textsuperscript{89}

In solid-state, the cyclic $\beta$-tripeptides appeared to stack into tube-like hollow structures, so called nanotubes. The nanotubes are characterized by a polar hollow centre with apolar side chains. Since the C=O groups of the cyclic peptides are aligned in the same direction, this results in a macrodipole moment over the nanotube. Voltage gating and current rectification are two important new properties expected for such channel structures.\textsuperscript{91}

The group of Ghadiri observed formation of such nanotube stacking for cyclic D,L-$\alpha$-peptides in 1993.\textsuperscript{92} This group did also show that cyclic D,L-$\alpha$-peptides with suitable hydrophobic side chains can self-assemble in lipid bilayers to form highly efficient transmembrane ions and small molecule channels. With this in mind, they also synthesized cyclic $\beta$-tetrapeptides using homochiral $\beta$-amino acid residues, hoping that the side chains would occupy the equatorial positions on the exterior of the ring, while the interior and the axial positions remained unhindered so that the tubular stacking would be able to occur. Some of these cyclic $\beta$-tetrapeptides did not only stack into the nanotubes, but were also shown to be a new class of highly efficient artificial transmembrane ion channels.\textsuperscript{92}

The ability of $\beta$-peptides to mimic the natural $\alpha$-peptides in their recognition by (human) receptors was investigated by the group of Seebach as they synthesized cyclic $\beta$-peptides with the target to mimic the tetradecapeptide somatostatin (see Figure 8c, Section 1.4), which is e.g. a potent inhibitor of the pancreatic secretion of glucagons and insulin.\textsuperscript{93} They were first to show that a small $\beta$-peptide could mimic a natural peptide hormone, and while some proteins do not recognize the $\beta$-peptides, the somatostatin receptors did.\textsuperscript{94} The reason is that the receptors recognizes the hormone by side chains interaction and not from interaction with backbone.

Thus, it is of fundamental interest to find cyclic structures which possesses high degree of backbone rigidity, which then could be designed as suitable molecular scaffolds with pharmacophorically interesting groups as side chains. For this purpose, both cyclic $\alpha\beta$-$\beta$-peptides and $\alpha/\beta$-mixed peptides are of interest to investigate.
4.5.2 α/β²-Mixed Cyclic Tetrapeptides

Instead of preparing peptides consisting of solely β-amino acids, only one β-amino residue can be included into a α-peptide sequence. This incorporation has many advantages. As discussed earlier in Section 1.2, the unnatural β-amino acids have the great advantage of being completely stable towards enzymatic degradation. However, a wide use of β-amino acids in peptides are prevented by the high cost of either synthesizing or buying the β²- and β³-amino acid building blocks. Therefore, by designing cyclic peptides incorporating both α- and β-amino acids, these oligomers may keep the biological recognition properties of an α-peptide while the valuable biological stability of β-peptides are gained.95

The turn type of secondary fold is often an important element for biological interactions, and cyclic peptides do of necessity contain a turn in the sequence. In similarity to D-amino acids and proline,2a β-amino acids have proven to be turn-inducing residues with the potential of enhancing structural stability of short peptides.9,96 Stabilization of the overall structure can be gained by the use of these unnatural building blocks in cyclic peptides. Sewald et al. studied the incorporation of β³-amino acid residues into cyclic α-peptide tetra and pentamers.97 They found an enhanced stability of the overall secondary structure. More specifically, they found that the incorporated unnatural β-amino acid residue preferably occupied the central sequence position of a modified γ-turn conformation. Similar studies were published by Fairlie et al.,98 where they investigated how β³-amino acids influences the stability of cyclic tetrapeptides by incorporating a β³hPhe residue into the α-backbone sequence also containing the turn promoting element D-proline. They too observed an enhanced conformational stability of the resulting peptide, but only when the β³hPhe residue was positioned opposite and not adjacent to the D-proline residue.

4.5.2.1 Histone Deacetylase Inhibitors

The eukaryotic DNA is highly organized and tightly packed into the nucleus of the cell. This organization and packaging can be considered as a safety precaution by making all the information stored less accessible; this is achieved by forming a complex with proteins. This protein-DNA complex is called chromatin. The nucleosome, the fundamental subunit of chromatin, is composed of an octamer of four core histones that, as they are post-translationally modified, influence the entire conformational architecture of the chromatin. Examples of PTMs occurring are acetylation, methylation and phosphorylation. The best understood of these is the acetylation that occurs on the lysine residues located at the N-termini of the core histone subunits. The level of this modification is mainly related to transcription activity which is regulated and balanced by opposing activities of the two enzymes histone acetyltransferase and histone deacetylase (HDAC).99
Increasing levels of histone acetylation leads in general to local expansion of the chromatin and increased accessibility of regulatory proteins to DNA, whereas decreasing levels of the acetylation leads to chromatin condensation and repression of gene expression. Cancer cells manipulate these regulatory functions of HDAC which gives rise to an abnormal gene expression and therefore, HDAC inhibitors are exciting new anticancer agents that induce tumour cell death, differentiation and/or cell-cycle arrest. The search for a potent HDAC inhibitor has over the years led to the development of a large range of structurally different groups of substrates. One of the most potent found so far is TSA which belongs to a group of hydroxamic acids, see Figure 27a. Other examples of groups inhibiting HDAC are short-chain fatty acids, benzamides and cyclic tetrapeptides, see Figure 27b-d.

One family of cyclic tetrapeptide natural products extracted from fungi or bacteria have shown to exhibit high HDAC inhibition potential. This family is characterized by the complicated structure which includes one D-amino acid and one imino acid such as proline or piperolic acid in addition to the presence of the uncommon amino acid Aoe [(2S,9S)2-amino-8-oxo-9,10-epoxydecanoic acid].

4.5.3 Synthesis – A General Summary

Cyclic peptides are synthesized according to the same procedures described for linear peptides. However, in the end of the synthesis, the peptide oligomers is left to undergo cyclization. There are numerous strategies for cyclization, which can be performed either in solution-phase after the linear peptide has been cleaved off from the resin, or directly on the solid support. Since only the conformational studies, and not the synthesis, of the cyclic backbone modified peptides will be represented in this thesis, only a short summary of the synthesis will be described below. For further details, see paper V and VI.

The on-resin cyclization was utilized when synthesizing the cyclic tetra and penta-β-peptides (see Section 4.5.1 and 4.5.4). Two methods are possible; either the side-chain or the backbone is anchored to the solid phase. As the...
backbone-anchoring method allows syntheses of peptides without functionalized side chains, this is considered to be the more general pathway. However, if functionalized side chains are present, the side-chain anchoring strategy is the method of choice. For the synthesis of cyclic $\beta$-peptides according to this method, a three dimensional orthogonality of the protecting groups can be utilized, where Fmoc, Boc and Al groups are included.\(^{101}\)

The on-resin cyclization itself is promoted by the use of PyAOP and DIPEA in dry DMF, which is the final step before the freshly cyclized peptide is cleaved off from the resin and simultaneously side-chain deprotected under standard acidic conditions.

In-solution cyclization was used when synthesizing the mixed cyclic $\alpha/\beta^2$-peptides (see Section 4.5.2 and 4.5.5). Utilizing this method, the use of proper protecting groups are of great importance, as the $C$-terminus easily could be cyclized with a free amine side chain and the $N$-terminus easily with a free carboxylic acid side chain instead of with each other. Since the most commonly used resins require high concentrations of TFA upon cleavage of the peptide from the solid support, and as the carboxylic acids are most commonly $t$-Bu-protected and the amines Boc-protected, which are both quickly removed upon treatment with TFA, a resin where the peptide is cleaved off under milder conditions is of necessity. In Paper V, the 2-chlorotrityl chloride resin (Barlos resin, see Figure 9, Section 3.1.1) was used. This resin has a standard cleavage mixture consisting of CH$_2$Cl$_2$/trifluoroethanol/AcOH (3:1:1). Upon cyclization in solution, the linear peptide is preferably added over 20 h via a syringe pump to a well stirred reaction mixture consisting of PyBOP/DIPEA in dry DMF.

4.5.4 Conformational Studies of Cyclic Tetra and Penta $\beta^3$-Peptides (Paper V)

4.5.4.1 NMR Studies

Detailed 2D NMR (TOCSY, P.E.-COSY, ROESY, gHMBC) spectroscopic study was made to obtain high-resolution data on the conformations of cyclic $\beta^3$-peptides 26 and 27, see Figure 28. The studies were performed in methanol (CH$_3$OH/CD$_3$OD, 9:1) for both $\beta$-peptides, whereas only the conformation of pentapeptide 27 could be established in water (H$_2$O/D$_2$O, 9:1), due to solubility problems.

The sequence of 27 could be fully assigned by the use of gHMBC experiment, distinguishing $\beta^3$hLys4 and $\beta^3$hLys5 from each other.
The amide region of $^1$H NMR spectrum of 26 in methanol indicated the presence of one major conformer, but the wide range of the coupling constants of the NH-C$(\beta)$H (7.6-9.4 Hz) gave the indication of an unsymmetrical structure.

The two $\alpha$-protons could be distinguished from each other using the same approach as for the linear $\beta$-peptides (see Section 4.3.2.1.2) and they were classified into pro-($S$) and pro-($R$) instead of axial and equatorial, due to the indications of an unsymmetrical shape.

The existence of an unsymmetrical shape was further established by the ROESY spectra, which gave rise to different NOEs as compared with those expected from a symmetrical molecule. The structure of 26 will be further discussed in the section of theoretic conformational search (Section 4.5.4.2).

Cyclic $\beta$-peptide 27 gave rise to a $^1$H NMR spectrum with a broad shape of the $\beta$hLeu amide signal, indicating more than one major conformer in methanol. Large differences in size of the coupling constants for the amide protons also indicated that cyclic peptide 27 possessed an unsymmetrical shape.

In the ROESY spectra, weak NOE’s between amide protons and $\beta$-protons positioned on neighboring amino acid residues could be observed. This gave the indications of a flexible cyclic structure as these NOEs would not have arisen if the backbone of the cyclic $\beta$-peptide was more rigid.

The NMR spectra of 27 recorded in water concluded that the flexibility seen in methanol were present in water as well.

### 4.5.4.2 Computational Analysis

Regarding the computational search of the cyclic tetrapeptide 26, it could be concluded that even though 26 most probably fluctuated between oval and very symmetrical shapes, it preferably reached the boat-shaped oval structure, see Figure 29.
Cyclic \( \beta \)-peptide pentamer 27 was computationally investigated using both the information obtained in methanol and water, see Figure 30.

From the Monte Carlo search of the pentapeptide 27 in methanol, three different families of conformations were found. In between the families there were a high flexibility of the backbone structures observed, and the amide bonds did not have an unidirectional arrangement. Between the two amino acids \( \beta^h \text{Val} \) in position 1 and \( \beta^h \text{Lys} \) in position 5 was a turn similar to the \( \beta \)-
turn from a $\beta^\alpha/\beta^\alpha$-sequence observed. In water, however, the number of different conformational families was reduced to two families. These families had similar overall shape of the backbone conformations, but differed at one amine backbone dihedral angle.

4.5.5 $\alpha/\beta$-Mixed Cyclic Tetrapeptides (Paper VI)

As introduced in Section 4.5.2, previously published studies have shown how the incorporation of $\beta^\alpha$-amino acids into cyclic mixed $\alpha/\beta^\alpha$-tetrapeptides influenced the conformational homogeneity. In this study, the influence of a $\beta^\alpha$-substituted amino acid, i.e. $\beta^\alpha$hPhe, was investigated. The synthesis of these are summarized in Section 4.5.3.

![Structures of the mixed cyclic $\alpha/\beta^\alpha$-tetrapeptides](image)

Figure 31. Structures of the mixed cyclic $\alpha/\beta^\alpha$-tetrapeptides $29_{a,b}$ and $30_{a,b}$ and its $\alpha$-peptide analogue $28$.

The sequence chosen was cyclo(-Phe-D-Pro-Lys-Phe-) ($28$), where the $\beta^\alpha$hPhe residue with either (R) ($29_{a}$ and $30_{a}$) or (S) ($29_{b}$ and $30_{b}$) chirality were either incorporated at position $i-1$ ($29_{a,b}$) or $i+2$ ($30_{a,b}$) with respect to the D-proline. It should be noted that the (R)-isomer corresponded to the chirality of L-amino acids, see Figure 31.

4.5.5.1 NMR Studies

$^1$H NMR spectra of all five cyclic tetrapeptides ($28$, $29_{a,b}$-$30_{a,b}$) were first recorded in $\text{H}_2\text{O}/\text{D}_2\text{O}$ (9:1). The amide region of cyclic $\alpha$-tetramer $28$ gave rise to signals that were indicative of several conformers in slow interconversion. However, the amide regions of the spectra arising from the four mixed tetrapeptides $29_{a,b}$ and $30_{a,b}$ did all give the indication that by replacing one $\alpha$-amino acid residue with a $\beta^\alpha$-amino acid analogue, peptides with enhanced structural stability had been formed.
The amide proton temperature coefficients $\Delta \delta / \Delta T$ (ppb/K) of the cyclic $\beta^2$hPhe containing peptides $29a$, $29b$, $30a$, and $30b$ measured in DMSO-$d_6$ over the temperature range 25–85°C.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$\Delta \delta / \Delta T$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lys</td>
</tr>
<tr>
<td>$29a$</td>
<td>6.6</td>
</tr>
<tr>
<td>$29b$</td>
<td>4.7</td>
</tr>
<tr>
<td>$30a$</td>
<td>5.3</td>
</tr>
<tr>
<td>$30b$</td>
<td>4.9</td>
</tr>
</tbody>
</table>

Table 2. Amide proton temperature coefficients $\Delta \delta / \Delta T$ (ppb/K) of the cyclic $\beta^2$hPhe containing peptides $29a$, $29b$, $30a$, and $30b$ measured in DMSO-$d_6$ over the temperature range 25–85°C.

The amide proton temperature coefficients\(^{54,55}\) were measured for all peptides in DMSO-$d_6$, see Table 2. The measured values suggested that two amide protons were involved in intramolecular hydrogen bonding in peptide $29a$, while peptides $29b$ and $30b$ only had one amide proton involved in intramolecular hydrogen bonding in each peptide. The value of the temperature coefficient for the $\beta^2$hPhe amide proton in $30a$ in DMSO suggested that this proton was in-between solvent exposed and hydrogen bonded states.

Cyclic tetrapeptides $29a$, $29b$, and $30a$ were additionally evaluated by 2D NMR experiments (ROESY, TOCSY, P.E.-COSY, and gHMBC). These peptides were chosen as they gave rise to only one major conformer in H$_2$O/D$_2$O (9:1). From the 2D NMR experiments it could be concluded that all secondary amide bonds had the trans-configuration, whereas the amide bond of the compared D-Pro residue of $29b$ showed indication of being in a cis-conformation. This were in alignment with a shorter retention time on HPLC observed for $29b$, as compared to the other cyclopeptides. This behavior represents a remarkable change in overall hydrophobicity, a change which is possible to arise due to a cis-amide bond.

4.5.5.2 Computational Analysis

As a complement to the experimental conformational studies, theoretical conformational search was performed on the mixed cyclic peptides $29a$, $29b$, and $30a$.

Only one family of conformers was found formixed cyclic $\alpha/\beta^2$-tetrapeptide $29a$ (see Figure 32a). This structure was in almost perfect agreement with a structure found for the Boc-protected derivative by X-ray crystallography and corresponded perfectly to the prediction made from the temperature studies (data listed in Table 2).
Conformational study of the cyclic peptide possessing opposite chirality of \( \beta \)-hPhe residue (29b) showed the existence of two sets of conformers (see Figure 32b). The presence of a \( \psi \)-amide bond in the backbone prevents the formation of one of the stabilizing intramolecular hydrogen bonds, thus leaving one stabilizing \( i \) to \( i+2 \) hydrogen bond. As a consequence, the amide bond positioned opposite to the \( \psi \)-amide bond possesses an enhanced flexibility, which causes the amide bond to fluctuate in orientation at this position. Further, the amide proton of the \( \beta \)-hPhe residue in peptide 29b was predicted from the \( ^1 \)H NMR temperature studies to be involved in hydrogen bonding, a prediction which could be confirmed in this calculation.

Regarding 30a, only one conformational family was found (see Figure 32c). The position and chirality (\( R \)) of the \( \beta \)-hPhe residue in this peptide allowed the formation of one intramolecular \( i \) to \( i+2 \) hydrogen bond that involves both the stabilizing D-Pro residue and the opposing \( \beta \)-hPhe residue, thus again leading to a highly conformationally homogeneous peptide. Again, the temperature coefficient in DMSO suggested this, even if the value here was not as small as that observed for the other peptides (see Table 2).
4.5.5.3 Cyclic Tetrapeptides as HDAC Inhibitors

Cyclic tetrapeptide natural products can be, as introduced in Section 4.5.2.1, potent inhibitors of histone deacetylases (HDAC). Figure 27 shows examples of such cyclic peptides, containing the uncommon amino acid residue Aoe. However, the cyclic tetrapeptides also includes a family of chemically developed structures that are a combination of the cyclic tetrapeptides (Figure 27d) and hydroxamic acids (Figure 27b). This designed combination gives results in cyclic tetrapeptides with great inhibition potency and specificity. The hydroxamic acid residue, however, leads to enhanced stability in vivo, compared to the Aoe residue.

Incorporation of a $\beta$-hPhe residue was earlier in this thesis shown to increase the conformational homogeneity of cyclic tetrapeptides. Therefore, hydroxamic acid side chain containing analogues of 29a and 30a was synthesized (33 and 34, respectively) in order to investigate if the increased conformational restriction provided beneficial properties. In addition, the $\alpha$-peptide analogue 32 was synthesized for comparison. Further, a cyclic tetrapeptide containing two D-proline residues (35) was also synthesized, see Figure 33. These peptides were then used in a cell-based assay using cortical embryonic (E15) neural stem cells from rat. More specifically, it was the ability of cyclic tetrapeptides 32-35 to inhibit deacetylation of histone 3 lysine 9 (H3K9) (see Section 4.5.2.1), a lysine residue known to have low degree of acetylation in the studied cells. As a positive control, the potent class I and II HDAC inhibitor TSA was used (Figure 34a), whereas untreated cells (-FGF) were used as negative control.
Figure 34. The ability of cyclic tetrapeptides 32-35 to inhibit the histone deacetylase enzymes when tested on neural stem cells. The known inhibitor TSA were used as positive control (a). The results from the HDAC inhibition study can be seen in figure b, concluding that the synthesized peptides were potent HDAC inhibitors.

The results from this study can be seen in Figure 34b. The ability to inhibit HDAC, and thereby leave the H3K9 to undergo acetylation, could be detected by fluorescence. This study showed that all four synthesized cyclic tetrapeptides (32-35) have the ability to inhibit the HDAC enzyme.
4.5.6 Conformational Studies of $\beta$-Peptoids (Paper VII)

About ten years after the first report of $\alpha$-peptoids, Barron et al. succeeded to grow crystals of a peptoid pentamer with the aliphatic $\alpha$-chiral (R)-N-1-cyclohexylethyl side chain. The X-ray crystallographic analysis showed a left-handed helical conformation with repeating $\alpha\alpha\alpha$-amide bonds indicating a polyproline type-I helical shape, in line with the CD spectroscopic analysis. Continuing to consider the formation of secondary structures, the group of Hofmann presented in 2006 a systematic theoretical conformational study concerning the ability of both $\alpha$- and $\beta$-peptoids to adopt stable secondary structures. They could observe not only an ability of these oligomers to adopt several defined helical conformers with both trans- and cis- peptide bonds, but also that there was a close relationship between the $\alpha$-peptoid helical structure and those adopted by poly-glycines and poly-prolines. Even more interestingly, the possible secondary structures of the $\beta$-peptoids were suggested to resemble those adopted for $\beta$-peptides.

4.5.7 CD Spectroscopic Studies

The ability of linear $\beta$-peptoids with the $\alpha$-chiral side chain (S)-1-phenylethyl to adopt ordered secondary structures was studied by CD spectroscopy. Barron and co-workers have earlier shown that $\alpha$-peptoids with chiral aromatic side chains as short as five residues in length give rise to intense CD patterns. This suggests that a regularly repeating chiral secondary structure is present. 

![Figure 35. Structures of $\beta$-peptoid oligomers 36a-j and N-acetylated monomer 37.](image)

The folding propensities of the $\beta$-peptoids 36a-j (see Figure 35) were analyzed by CD spectroscopy in methanol at 25°C ($c=0.1$ mM), see Figure 36 a and c. All ten $\beta$-peptoid oligomers gave rise to CD spectra with patterns very similar those arising from $\alpha$-peptoids with same side chains and chirality but recorded in acetonitrile. The similarity regards both the shape and intensity.
Figure 36. CD spectra of $\beta$-peptoid oligomers 36a-j in methanol (a) and of 36 b, d, f, h, and j in acetonitrile (b). Included are also the normalized CD spectra in methanol (c) and acetonitrile (d), respectively, taking into account the number of chromophores.

The CD spectra were characterized by double minima near 204 and 218 nm, and when analyzing the folding propensities of 36 b, d, f, h and j in acetonitrile at 25°C ($c = 0.1$ mM), the same CD patterns arose in this solvent see Figure 36 b and d.

Barron$^{104}$ has reported an intensity increase proportional to increasing number of residues up to a certain chain length, after which no further change was observed. For the $\beta$-peptoids 36a-j, no such effect could be observed, which suggests that these oligomers do not adopt helical secondary structures, or, alternatively, that an ordered structure is present already in the trimer.

An acetylated monomer 37 were prepared and analyzed in both methanol and acetonitrile, giving rise to similar CD pattern as those originating from the oligomers. Based on this result, it appears most reasonable to conclude that $\beta$-peptoids with $\alpha$-chiral side chains lacks the high tendency of helical secondary structure formation characteristic of the analogous $\alpha$-peptoids, as the simple monomer would not be expected to adopt an ordered conformation.
5 Summary and Outlook

In summary, the projects included in this thesis stretch from the synthesis of glycosylated $\beta$-amino acid building blocks and solid phase peptide synthesis of glycosylated $\beta$-peptides to conformational investigations of not only glycosylated $\beta$-peptides but also cyclic $\alpha/\beta$-peptides, mixed cyclic $\alpha/\beta$-peptides and $\beta$-peptoids. The first biomolecular study with glycosylated $\beta$-peptides was also performed.

The conclusions and outlooks are as follows:

- O-Glycosylated $\beta$-amino acids are preferably synthesized by coupling the glycosyl donor to the homologated $\beta$hSer residue.

- The first reported synthesis of glycosylated $\beta$-peptides and fundamental studies of the $\beta$-peptide helical stability upon glycosylation were performed.

- An ideal glycosylated $\beta$-peptide with stable $3_{14}$-helical structure in water should include free $C$- and $N$-termini, a positively charged side chain close to the $N$-terminus and a negatively charged side chain close to the $C$-terminus. Further, a positively charged side chain should be sequenced for the possibility to interact with the $N$-acetyl group of the carbohydrate moiety. The carbohydrate moiety is preferably positioned closer to the $C$-terminus. This should be considered in the future when new and maybe even longer $\beta$-peptides are to be synthesized.

- Since both GalNAc and GlcNAc monosaccharides were successfully linked to the $\beta$hSer residue, $\beta$-peptides possessing larger oligosaccharides could potentially be prepared by the use of suitable sugar-attaching enzymes (glycosyltransferases). Such molecules could find applications as metabolically stable carriers for antigens, e.g. for vaccine development, and for glycosylation engineering purposes. Initial studies in this direction have shown promising results.
The biomolecular recognition study between glycosylated $\beta$-peptides and lectins showed that the carbohydrate moiety was still recognized when positioned on a $\beta$-peptide backbone. This opens for future studies including other proteins/biomolecules, e.g. antibodies or receptors.

The ability to fold is crucial for many, but not all, functions of peptides and proteins. In addition to the lectin study above, which apparently show little secondary-structure dependence, this thesis has also established the rules governing helical folding among such oligomers. This is of importance in cases where a helical interaction is involved, e.g. protein-protein interactions.

The design of the peptides could be changed to cause the peptide to fold into other secondary structures, i.e. a turn-like structure. This would open for interactions with turn-specific receptors, e.g. $\mu$- and $\delta$-opioid receptors.

Cyclic tetrapeptides including a (R)-$\beta$hPhe residue were shown to possess increased conformational homogeneity as compared to cyclic tetrapeptides containing only $\alpha$-amino acids. This could be of great advantage when it comes to receptor binding, e.g. in the search for more potent HDAC inhibitors; three (R)-$\beta$hPhe containing cyclic tetrapeptides have been tested on neural stem cells showing a inhibition of the acetylation process. To deeper investigate the HDAC inhibition potency and selectivity complementary in vitro studies needs to be made.

The conformation of $\beta$-peptoids with $\alpha$-chiral side chains was investigated. However, it could not be utterly established whether they did fold into secondary structures or not. By synthesizing $\beta$-peptoids with differently substituted side chains, the ability of these oligomers to fold into stable secondary structures could be more thoroughly investigated by high-resolution methods, such as NMR spectroscopy.
6 Acknowledgements

This thesis has described the chemistry I have done during my time as a Ph.D. student, but these years have also included so much more than that. I would like to express my sincere gratitude to the people who have traveled along with me on this journey, and made it so much better (and much more fun)!

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Stabila konformationer!? Syntes och konformationsstudier av onaturliga ryggradsmodifierade peptider.

Proteiner utgör en av huvudbeståndsdelarna i allt levande material och byggs upp av aminosyror, sammanlänkade i olika kombinationer. Då kedjan av aminosyror innehåller mindre än 50 byggstenar kallas dessa små proteiner för peptider.

Proteiner och peptider återfinns i alla levande celler och känns därmed naturligt igen av kroppens receptorer (budbärare) och enzym (katalysatorer) etc. I och med detta så skulle det optimala vara att använda sig av peptidernas/proteinernas egenskaper vid framställningen av läkemedel. Ett exempel på ett protein som redan används som läkemedel är insulin.

Tyvärr är det så att den stora fördelen med att de känns igen av kroppen även blir till deras största nackdel eftersom de på så sätt känns igen av bl.a. nedbrytande enzym. Detta är en av anledningarna till att läkemedel som utgörs av peptider och proteiner oftast har en låg upptagningsförmåga i kroppen. Idag finns det mycket forskning beträffande sökandet efter onaturliga peptidliknande byggstenar som fortfarande kan behålla de positiva egenskaperna hos en peptid eller ett protein, men som är så pass olik de naturliga byggstenarna (aminosyror) att de inte bryts ner av kroppen.

I denna avhandling behandlas två sådana peptidliknande byggstenar; \( \beta \)-aminosyror samt \( \beta \)-peptoider, se Figur I.

![Figur I. Jämförelse mellan den naturliga \( \alpha \)-aminosyran och ryggradsmodifierade \( \beta \)-aminosyror; hos en \( \beta \)-aminosyra sitter sidokedjan (R) på kolet närmast aminen (NH₂) medan hos en \( \beta \)-aminosyra sitter den på kolet närmast karboxylsyra-gruppen (O=C-OH). I figuren återfinns även en \( \beta \)-peptoid-byggesten vars sidokedja sitter på kvävet istället för på endera av de två kolen.](image-url)
Som tidigare nämnts byggs peptider och proteiner upp av aminosyror. Dessa byggstenar består av en kväveinnehållande (N) del, kallad amin, ett kol (C) där en sidokedja (R) är tillkopplad, samt en karboxylsyragrupp som utgörs av ett kol som binder dels till ett dubbelbundet syre (=O) och dels till en hydroxylgrupp (OH), se Figur I. Alla organsimer använder sig av samma 20 aminosyror som byggstenar vid bildandet av peptider och proteiner, och det är just i sidokedjan som variationen mellan dessa ligger.

Det som skiljer naturliga peptider och $\beta$-peptider åt är att de senare byggs upp av $\beta$-aminosyror, vilka innehåller ett extra kol i sin ryggrad. Eftersom det är kolot som har sidokedjan kopplad till sig, finns det hos $\beta$-aminosyror möjligheten att ha den placerad antingen på det kol som sitter närmast karboxylsyran, eller på kolot brevid aminen ($\text{NH}_2$). I det första fallet kallas aminosyran för en $\beta_2$-aminosyra och i det andra för en $\beta_3$-aminosyra (Figur I).

I och med att ett extra kol har inkluderats till aminosyrens ryggrad så har det visat sig att de peptider som är upphbyggda av $\beta$-aminosyror är helt stabila mot metabolisk nedbrytning. Den snabba nedbrytningen av vanliga peptider brukar anses som en stor begränsande faktor beträffande användningen av dessa som läkemedel.

Livsviktiga molekyler såsom DNA-strängar och proteiner bygger en stor del av sin enastående funktion på att de har förmågan att vika och vecka ihop sig till tredimensionella strukturer. Dessa ihopveckningar utgör ett proteins sekundära och tertiära struktur. $\beta$-Peptider har visat sig ha den värdefulla egenskapyen att bilda stabila sekundära strukturer liknande de som återfinns i $\alpha$-peptidevenvärlden och exempel på sådana är olika sorts helixar, flak och hårnål.

Den mest studerade sekundära strukturen hos $\beta$-peptider är $\beta_{14}$-helixen som uppkommer då $\beta$-peptiderna byggs upp av enbart $\beta^2$-aminosyror. Det är den helixstruktur som har studerats i denna avhandling.

Vanligt förekommande är att peptider och proteiner genomgår så kallad post-translationell modifiering, dvs att de på något sätt genomgår en förändring efter att de syntetiserats (tillverkats) i cellens ribosomer. Exempel på sådana förändringar är sammankoppling med lipider (fettkedjor) eller kolhydrater. En post-translationell modifiering innebär ofta både en strukturell och funktionell skillnad hos proteinets/peptidens funktion.

Vi har i delarbete I-IV undersökt hur en $\beta^3$-peptid, där förmågan att bilda $\beta_{14}$-helix är generellt påvisad, påverkas strukturellt av glykosylering, dvs när man kopplar ihop peptiden med en sockermolekyl.

I det första delarbetet har det framarbetats en metod till hur de glykosylerade $\beta^3$-aminosyrorerna kan syntetiseras. De glykosyleringar som är i fokus i denna avhandling är de O-glykosylerade, dvs då kolhydratdelen sammankopplas till aminosyran via en syrebrygg (O), vanligast i form av bindning till sidokedjan av antingen serin eller threonin, se Figur II. Då detta överfördes till $\beta^3$-aminosyror visade det sig att sockerdelens med störst fördel bör kopplas till $\beta$-serin ($\beta^3h\text{Ser}$).
Figur II. De två naturliga α-aminosyrorna serin (vänster) och threonin (mitten) sammanlänkade med en kolhydratsbyggesten (GalNAc) via sidokedjans syre. Till höger ses samma kolhydrat kopplad till β-serin.

Glykosylerade aminosyran αGalNAcβhSer (Figuur II, högra molekylen) användes som en byggnad vid syntes av glykosylerade β-peptider i delarbete II och III. Förutom att framarbeta en lämplig syntesväg ingick det även att i dessa två delarbeten studera till vilken grad de glykosylerade β-peptider bildar β3-helix. I det första delarbetet (Delarbete II) sattes en glykosylerad β-peptid i jämförelse med en oglykosylerad β-peptid analog. Det framkom då att den glykosylerade β-peptiden lyckligtvis antar en helixstruktur, men med något lägre förmåga än den oglykosylerade varianten.

I delarbete III var siktet inställt på att hitta en peptidsekvens som trots att den innehåller en kolhydratsdel fortfarande bibehåller sin sekundära struktur, och då helst i vatten eftersom detta är det ”biologiska” lösningsmedlet. Tack vare denna studie kunde de viktiga aspekternas för att få en glykosylerad β-peptid med stabil helix kartläggas.


Förutom studier som berör glykosylerade peptider utfördes även konformationsstudier på cykliska peptider och på β-peptoider. Att en peptid är cyklisk innebär i denna avhandling att peptidkedjornas två ändar har sammanlänkats med varandra.

I delarbete V undersöktes konformationen (dvs en molekyls form för ögonblicket p.g.a. dess rörlighet) av två cykliska β-peptider innehållandes fyra respektive fem β-aminosyror. Intresset för dessa ligger dels i att de har visat sig kunna ordna sig i rör-liknande ihåliga strukturer (så kallade nanorör) och i hopp
om att de ska komma att känna igen av kroppens enzymer och receptorer. Här säg man ett klart samband mellan antalet β-aminosyror och stabiliteten av ringen, med ökad flexibilitet med ökat antal byggnenar. Medan β-peptiden bestående av fem aminosyror var något flexibel skulle den ring innehållandes fyra byggnenar kunna användas som en rigid ram, där sidokedjorna kan bytas ut mot grupper som är av intresse inom läkemedelsforskning.

Effekten av att inkludera endast en β2-aminosyra i en cyklisk peptidsekvens som i övrigt bara består av naturliga aminosyror undersöcktes också (Delarbete VI). Här visade det sig att då en av fyra byggnenar utgjordes av β2-phenylalanin så minskade den cykliska strukturens flexibilitet, en egenskap som är värdefull vid kontakt med receptorer. Det kunde även kartläggas vilka parametrar som var viktiga för denna rigiditet. Denna slutsats skulle kunna vara till godo i forskning kring cykliska tetrapeptider och deras förmåga att hämma enzymet histonedeacetylas, ett enzym som påverkar gentranskriptionen.

β-Peptoider påminner i ryggradsstrukturen om β-peptider, men skiljer sig på så vis att de har sidokedjan kopplad till kvävet istället för till kolet, se Figur I. I delarbete VII genomförde vi en studie för att besvara om β-peptoider kan anta ordnade sekundära strukturer precis som β-peptidera. Trots att en rad olika mätningar gjordes gick det tyvärr ej att säkerställa till 100% om fallet var så eller ej.
8 References


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