Synthesis of Aldehyde-Functionalized Building Blocks and Their Use for the Cyclization of Peptides

Applications to Angiotensin II

BY

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ABSTRACT


This study addresses the issue of how to convert peptides into drug-like non-peptides with retained biological activities at peptide receptors. Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe, Ang II) was used as a model peptide.

Knowledge of the bioactive conformations of endogenous peptides is invaluable for the conversion of peptides into less peptidic analogues. Effectively constrained cyclic analogues, with retained pharmacological activities, may provide valuable information about the bioactive conformations of the peptide in question.

This thesis describes the development of synthesis for a number of protected, aldehyde-functionalized building blocks for standard solid phase peptide synthesis, and their use for the preparation of cyclic peptide analogues. The effect of variations in the side-chain lengths of the building blocks, on the outcome of the cyclizations was studied. Incorporation of a building block derived from L-aspartic acid afforded bicyclization towards the C-terminal end of the peptide, while for the corresponding L-glutamic acid derived building block, N-terminal directed bicyclization was achieved. A building block derived from L-2-aminoadipic acid was exploited for monocyclization furnishing *cis-* and *trans-* vinyl sulfide bridged peptide analogues.

The described cyclization methods have been applied to the synthesis of a number of conformationally constrained Ang II analogues, for which the pharmacological properties have been evaluated. Two of the Ang II analogues synthesized displayed high affinities and full agonist activities at the AT1 angiotensin receptor, and have proven to be useful tools in the search for the bioactive conformation of Ang II.

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Till minne av farfar
Som hade varit stolt...
PAPERS DISCUSSED

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


IV. Petra Johannesson, Gunnar Lindeberg, Máté Erdéyi, Per-Anders Frändberg, Anders Karlén, and Anders Hallberg. Synthesis of a Tyrosine Derivative α-Substituted with a Masked Aldehyde – A Building Block for Bicyclic Dipeptide Mimetics. Incorporation into Angiotensin II. *Manuscript.*
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>ACE</td>
<td>angiotensin converting enzyme</td>
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<td>Ang II</td>
<td>angiotensin II</td>
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<tr>
<td>Boc</td>
<td>tert-butoxycarbonyl</td>
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<td>BTD</td>
<td>bicyclic turned dipeptide, or $\beta$-turn dipeptide</td>
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<tr>
<td>CHO cells</td>
<td>Chinese hamster ovary cells</td>
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<tr>
<td>DIBAL</td>
<td>diisobutylaluminium hydride</td>
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<tr>
<td>DIEA</td>
<td>diisopropylethylamine</td>
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<td>DMF</td>
<td>dimethylformamide</td>
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<td>Fmoc</td>
<td>9-fluorenylmethyloxycarbonyl</td>
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<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
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<tr>
<td>HBTU</td>
<td>2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate</td>
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<tr>
<td>Hcy</td>
<td>homocysteine</td>
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<td>HOBt</td>
<td>1-hydroxybenzotriazole</td>
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<tr>
<td>KHMDS</td>
<td>potassium hexamethyldisilylamide</td>
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<tr>
<td>LC-MS</td>
<td>liquid chromatography-mass spectrometry</td>
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<tr>
<td>Mpc</td>
<td>cis-4-mercaptoproline</td>
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<tr>
<td>Mpt</td>
<td>trans-4-mercaptoproline</td>
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<tr>
<td>NMM</td>
<td>$N$-methylmorpholine</td>
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<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<tr>
<td>NOE</td>
<td>nuclear Overhauser enhancement</td>
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<tr>
<td>PbF</td>
<td>2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl</td>
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<tr>
<td>PCC</td>
<td>pyridinium chlorochromate</td>
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<tr>
<td>Pen</td>
<td>penicillamine</td>
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<td>Pmc</td>
<td>2,2,5,7,8-pentamethylchromane-6-sulfonyl</td>
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<tr>
<td>PyBOP</td>
<td>(benzotriazol-1-yl)tripyrrolidinophosphonium hexafluorophosphate</td>
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<tr>
<td>RAS</td>
<td>renin-angiotensin system</td>
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<td>rms</td>
<td>root mean square</td>
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<td>RP-HPLC</td>
<td>reversed phase - high performance liquid chromatography</td>
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<tr>
<td>SAR</td>
<td>structure-activity relationship</td>
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<tr>
<td>Sar</td>
<td>sarcosine, $N$-methyl glycine</td>
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<tr>
<td>SPPS</td>
<td>solid phase peptide synthesis</td>
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<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
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<td>Trt</td>
<td>trityl</td>
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<tr>
<td>TsOH</td>
<td>$p$-toluenesulfonic acid</td>
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<tr>
<td>Z</td>
<td>benzyloxy carbonyl, or carbenzyloxy</td>
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1 INTRODUCTION

A century ago, in 1902, Emil Fischer* and Franz Hofmeister made the discovery that peptides and proteins are made up of amino acids linked by peptide bonds. Fifty-one years later, in 1953, James D. Watson and Francis Crick proposed the double helical structure of DNA, and postulated that the nucleotide sequence in DNA carries encoded genetic information. Within a decade, this discovery led to an understanding of the fact that it is the genetic code that determines the amino acid sequences of peptides and proteins.

Ever since then, a large number of naturally-occurring and bioactive peptides has been discovered and characterized. Peptides are known to influence almost all vital physiological processes, and peptide receptors often have been identified as targets for drug discovery. The sequencing of the human genome, completed in 2001 will probably add thousands more ligands and targets to be explored.

The inherent properties of peptides, such as poor bioavailability, short biological half-lives and low receptor selectivity, have however limited their use as drugs. Rather than being used as drugs directly, biologically active peptides are therefore most useful as starting points in the search for small “drug-like” organic molecules that mimic bioactive peptides, i.e. peptide mimetics, or peptidomimetics.

1.1 GENERAL STRATEGIES FOR THE DEVELOPMENT OF PEPTIDE MIMETICS

Most endogenous peptides exert their effects through binding and activation of G protein-coupled receptors (GPCRs). X-ray structures of enzyme-inhibitor complexes fruitfully have allowed the direct and structure-based design of peptidomimetic enzyme inhibitors. Successful examples include the development of e.g. HIV-protease inhibitors. The design of peptidomimetics for peptides acting on receptors would, in a similar manner, greatly benefit from 3D structural data of complexes between the peptides and their G-protein coupled receptor targets. The first X-ray of a GPCR at high resolution (2.8 Å), that of bovine rhodopsin, was reported in 2000, and hopefully such 3D

* In the same year (1902) Emil Fischer was awarded the Nobel prize in chemistry for his work on sugars and purines.
structures also will be available for other GPCRs within the near future. Until then, more indirect, ligand-based design processes will have to be applied.

Peptides are inherently very flexible molecules. In solution they are present in a number of different conformations, and this is sensitive to the conditions of the experimental environment, such as the solvent. Furthermore, receptors often select a minor conformer and assist in transforming this conformation into the one that binds and activates the receptor, i.e. through induced fit. The question therefore arises, whether studies of conformations of linear peptides in solution will have any relevance, since preferred solution conformations do not necessarily correspond to those adopted when activating the receptor.

In the search for bioactive conformations, the key strategy, therefore is instead to use effectively constrained peptide analogues with retained pharmacological activities. Such analogues may serve as valuable tools in the elucidation of the relative 3D arrangement of the pharmacophoric groups displayed in the biologically active conformation.

Several groups have outlined rational strategies for the design of peptidomimetics, similar to the one depicted in Figure 1.1. The different steps in Figure 1.1 will be described briefly:

1. After identifying the primary sequence of a biologically active peptide, the structure-activity relationships (SAR) of the individual amino acids should be explored. Systematic exchange of the different amino acids, for example by alanine (alanine scan), or by D-amino acids, and reduction of the peptide length, will lead to identification of a minimal fragment needed for recognition and activation of the receptor.

2. The next step will be to synthesize conformationally-constrained peptide analogues in order to limit the possible relative orientations of the key residues identified as required for recognition and activation. The effects of the introduced constraints should be carefully analyzed using biophysical methods, such as NMR and molecular modeling. The different ways of introducing conformational constraints will be further discussed in section 1.2. This second step hopefully will provide information about the receptor-bound and/or biologically active conformation, and may optimally lead to a suggested 3D pharmacophore model.

3. In the third step the topographic information obtained from 3D pharmacophore models should be used to construct a non-peptide mimetic where the important pharmacophores have been mounted onto a carefully
selected non-peptidic scaffold or template, such as a small ring or ring system, of defined stereochemistry.

**Figure 1.1.** Rational strategy for development of peptidomimetics.

If discussing strategies for rational *discovery* (not only rational *design*) of peptide mimetics, the entries of random screening definitely should be added.\textsuperscript{18-20} This includes screening of either natural products, in-house synthetic collections, or libraries from combinatorial chemistry. In fact, most of
the compounds that are known today to act as peptidomimetics have evolved by optimization of lead structures found in such screening programs.

It should also be pointed out that the entries depicted in Figure 1.1 should be used in an iterative manner, and that the access to reliable biological testing methods is a prerequisite. Still, the design of “drug like” non-peptides from peptides remains the ultimate challenge, since it requires comprehensive understanding of the structural requirements for recognition and activation. The field of peptidomimetic design and development has been reviewed thoroughly.11-13,15-17,21-34

1.2 CONSTRAINED PEPTIDE ANALOGUES

The different ways of introducing conformational restriction into peptides broadly can be divided into global constraints and local constraints, but clearly there is an overlap between them. The incorporation of secondary structure mimetics may either have global or more local effects and deserves to be discussed separately.

An observed loss in activity after the introduction of a conformational constraint could have two possible explanations. Either it can be caused by steric hindrance between the ligand and the receptor, due to the added constraining atoms, or by the inability of the ligand to adopt the proper conformation. If the activity, on the other hand, is retained in the constrained analogue, the analogue is able to adopt the proper conformation, and binding is allowed in spite of the added restricting atoms.

Global constraints: The usual way of achieving global constraints is through long-range cyclization, and the idea is to lock-in the bioactive conformation. Ideally this should result in an increased affinity, since there is relatively little conformational entropy loss upon binding to the receptor.35 The different methods for cyclization of peptides will be discussed in section 1.3. Cyclization of peptides also often reduces the susceptibility of the peptides to peptidases.36

Local Constraints: Methods to achieve local constraints include modification of the amide bond,37,38 incorporation of modified amino acids,13,17,28,32 and short-range cyclization39 either within a single, or between adjacent amino acid
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residues (Figure 1.2). The latter example will produce constrained dipeptide mimetics.

\[ \text{Figure 1.2. Possibilities for short-range cyclization between two consecutive amino acids, leading to dipeptide mimetics. (Modified from ref.17)} \]

\[ \text{Secondary Structure Mimetics: There are three main classes of regular secondary structures present in peptides and proteins, } \alpha \text{-helices, } \beta \text{-sheets and reverse turns. Reverse turns often have been implicated as recognition elements for peptide receptor interactions.}^{40} \text{ The peptide backbone in a reverse turn serves as a scaffold, and presents the side-chains in a highly accessible 3D arrangement around a compact folded backbone.}^{35} \text{ The reverse turns can be divided into } \beta \text{-turns and } \gamma \text{-turns. A } \beta \text{-turn is most often defined as any tetrapeptide sequence occurring in a non-helical region, in which the distance between } C_\alpha \text{ of the first } (i) \text{ residue and the fourth } (i + 3) \text{ residue is less than 7Å.}^{41} \text{ A } \beta \text{-turn is often stabilized by a hydrogen bond between the carbonyl function of residue } i \text{ and the NH-group of residue } i + 3, \text{ to give a ten-membered ring (Figure 1.3.). A } \gamma \text{-turn, which is a more rare turn conformation, is similarly made up by a hydrogen bond between residues } i \text{ and } i + 2, \text{ to form a seven-membered ring (Figure 1.3). Much work has been devoted to the synthesis of structures that mimic or induce reverse turns.}^{26,42-49} \text{ } \beta \text{-Turn mimetics will be discussed further in sections 1.4 and 1.5.} \]
1.3 METHODS FOR CYCLIZATION OF PEPTIDES

The possibilities depicted in Figure 1.2 for short-range cyclizations are also valid for more long-range cyclizations, i.e. by covalent connections end to end (head to tail), side-chain to side-chain, N-backbone to N-backbone, end to N-backbone, end to side-chain or side-chain to N-backbone. Examples of different methods used for cyclization of peptides are shown in Figure 1.4. Formation of disulfide (1-3,) and amide bonds (4) are those most commonly used.

For methods involving the side-chains it is critical that the side-chains used for cyclization are not the same as those identified as crucial for the biological activity. One way to circumvent this problem is to use chimeric, or hybrid, amino acids such as penicillamine (Pen) (used in 2) or trans-4-mercaptoproline (Mpt), in which an extra functional group has been added, facilitating cyclization. Mpt has been utilized by Marshall's group to achieve effectively constrained bicyclic structures (3). For connections N-backbone to N-backbone however, no side-chains have to be modified. The concept of N-backbone cyclization has been explored by the group of Gilon, using connection through amide bonds (5) and more recently by the group of Liskamp, using ring-closing metathesis (6). The chemistry of ring-closing metathesis has also been successfully utilized for connections end to end, side-chain to N-backbone and side-chain to side-chain (7). Other successful connection strategies include formation of thioether- (8), dithioether- (9), ureido- and saturated aliphatic bridges.
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Figure 1.4. Methods for cyclization of peptides.

An elegant example of a side-chain to side-chain and N-terminal end cyclization was reported by Tam and coworkers. By this approach an N-terminal cysteine is reacted with an N\textsuperscript{ε}-lysyl glyoxaldehyde, to afford a thiazolidine fused to a large ring, consisting of 10 amino acid residues (Figure 1.5).

Figure 1.5. Cyclization reported by Tam et al.

Even though a large number of methods for the cyclization of peptides have been reported, there is still a need for new, simple and complementary cyclization methods. These methods should preferably be used prior to the initiation of synthesis of more complex organic scaffolds.
1.4 β-TURN MIMETICS

The β-turns, which comprise a rather diverse group of structures, have been divided into the classical, well-defined turn types (I, I’, II, II’, III, III’, VIa and VIb) based on the φ and ψ backbone torsion angles of residues $i+1$ and $i+2$ in the turn (Figure 1.6).⁸¹ Types I, II and III are the most commonly occurring turn types, and about 40% of the β-turns found in proteins can not be classified into any of the classical turn types.⁸² However, it has been argued that the most important features of the β-turn are the relative positions of the side-chains of residues $i+1$ and $i+2$, in addition to the angle at which the peptide chain enters and leaves the turn, and that these features are only indirectly and not clearly defined by the classification based on the peptide backbone.¹⁷,⁴²,⁸³ This led Ball et al. to introduce an alternative description of the β-turn, based on a single dihedral angle $C_1-O-C\alpha_2-C\alpha_3-N_4$ (Figure 1.6), defined as $\beta$.⁸³ The $\beta$-angle gives a more complete description of the spatial relationship between the entry- and exit-bonds of the turn, and the orientations of the $i+1$ and $i+2$ side-chains.

![Figure 1.6. A β-turn with the φ and ψ backbone torsion angles of residues $i+1$ and $i+2$ depicted (left), and the dihedral angle $C_1-O-C\alpha_2-C\alpha_3-N_4$, defined by Ball et al.⁸ as $\beta$ (right).](image)

β-Turn mimetics may either be of the external- or of the internal type.⁴² For external β-turn mimetics, the skeleton lies outside the induced 10-membered ring, while internal β-turn mimetics have its framework inside the turn, often with the stabilizing hydrogen bond of the β-turn replaced covalently.

A large number of compounds intended to mimic or induce β-turns have been reported (Figure 1.7).²⁶,⁴²-⁴⁹ The majority of these are dipeptide mimic
replacements for the $i + 1$ and $i + 2$ residues in the corners of the turn. The first generation of $\beta$-turn mimetics often suffered from the lack of side-chains, but during recent years many side-chain functionalized $\beta$-turn mimetics also have been reported.

The incorporation of $\beta$-turn mimetics into bioactive peptides has however frequently met with limited success,$^{13,45,47}$ often due to the large difficulty in both exactly predicting and reproducing the conformational features of the turn. During the last years several groups have turned towards the synthesis and screening of $\beta$-turn mimetic libraries that include many possible combinations of side-chains and relative orientations thereof.$^{19,24,47,48}$ These recent $\beta$-turn mimetics, which often lack $N$- and $C$-terminal ends, are not intended to be incorporated into peptides, but to be screened as receptor ligands per se. Some examples of $\beta$-turn mimetics are given in Figure 1.7.

Figure 1.7. Examples of well characterized $\beta$-turn mimetics; $^{10,84} 11^{85}, 12^{86}, 13^{87}, 14^{88}, 15^{89}, 16^{90}, 17^{91}$. 
1.5 THIAZABICYCLOALKANES AS β-TURN MIMETICS

One example of structures that have been used successfully to induce β-turns is constituted by the family of thiazabicycloalkane dipeptide mimetics, with the general structure 18 (Figure 1.8). The first reported β-turn mimetic of this structure was BTD (Bicyclic Turned Dipeptide, or β-Turn Dipeptide) 19. BTD was shown to be a type II’ β-turn mimetic of the external type, and lacks side-chains at the $i+1$ and $i+2$ positions. BTD has been incorporated into a number of different biologically active peptides.

Several similar structures, of which 20-23 are shown in Figure 1.8, have been synthesized and studied by the group of Johnson. Dipeptidomimetic 20 was designed as a type II β-turn mimetic, and more recently the corresponding side-chain functionalized compounds 21a-c, shown by X-ray also to induce type II β-turns, have been reported. In the spiro-bicyclic type II β-turn mimetic 22, three of the torsion angles of the type II β-turn are restricted. Compound 23 was designed as a type II’ β-turn mimetic and is functionalized with two side-chains. The side-chain functionalized reverse turn mimetics 24-26, prepared by a five-step synthesis, were reported recently by Hruby and coworkers.

Further examples also have been reported (27, 28). Besides being used as turn mimetics, thiazabicycloalkane dipeptide mimetics also have been synthesized and used as γ-lactam analogues of β-lactam antibiotics, and for incorporation into inhibitors of metalloproteases, illustrated by compounds 29 and 30 respectively.

BTD was synthesized utilizing the synthetic route to penicillin as a model scheme. With some recent exceptions, most thiazabicycloalkanes are still prepared by the same approach. That is, the syntheses mostly involve condensation of an ω-formyl α-amino acid with a cysteine residue, forming a thioaminal, followed by intramolecular N-acylation as the last step (Figure 1.9).
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Figure 1.8. Examples of thiazabicycloalkane dipeptide mimetics.

Figure 1.9. The most commonly used strategy for synthesis of thiazabicycloalkane dipeptide mimetics.

While numerous investigations have been devoted to thiazabicycloalkane dipeptide mimetics, studies of the corresponding tripeptides are rare.
2 AIMS OF THE PRESENT STUDY

This study is part of an ongoing project with the overall aim of gaining knowledge of how to convert peptides into non-peptidic small molecules with retained biological activity.

The hypertensive octapeptide angiotensin II (Ang II) (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) was selected as the model peptide for four main reasons; (a) it is a well studied peptide, with a well established structure-activity relationship, (b) it comprises a diverse set of amino acids, (c) the role of the AT₁-receptor is well understood, and (d) we had access to in vitro models for receptor binding and functional assays.

The specific objectives of this study were:

- To synthesize a number of protected aldehyde-functionalized building blocks with different side-chain lengths that are compatible with standard solid phase peptide synthesis (SPPS) using the Fmoc/tert-butyl protection strategy.

- To explore the utility of the synthesized building blocks for the cyclization of peptides, with special emphasis on cyclization methods that produce systems to stabilize secondary structures.

- To synthesize effectively constrained analogues of Ang II, preferably with retained biological activities. It was decided to focus our major efforts around the Tyr⁴ residue, crucial for agonism, and where a turn had previously been postulated to be adopted. We also wanted to perform cyclizations in the His⁶-region of Ang II, which had not been explored as extensively.

- To study the structure activity relationships of Ang II and its cyclic analogues, with the aim of obtaining information about the bioactive conformation.

Before the results are presented and discussed, a brief background on the selected model peptide angiotensin II is given in section 3.
3 ANGIOTENSIN II

3.1 PHARMACOLOGY

Angiotensin II (Ang II) is a linear octapeptide with the sequence Asp$^1$-Arg$^2$-Val$^3$-Tyr$^4$-Ile$^5$-His$^6$-Pro$^7$-Phe$^8$ (Figure 3.4). Ang II is the main effector of the renin-angiotensin system (RAS) (Figure 3.1), and plays a major role in regulating blood pressure and cardiovascular homeostasis.$^{117-119}$ Sequential cleavage of angiotensinogen, by the enzymes renin and angiotensin converting enzyme (ACE), produce Ang II via the inactive decapeptide Angiotensin I. In addition to being a circulating endocrine system, local RAS are also found in many tissues, including the vasculature, heart, kidney and brain, which are also capable of producing Ang II. Ang II is broken down into peptide fragments of which angiotensin (1-7),$^{119,120}$ angiotensin (3-8), referred to as Ang IV,$^{117,119,121}$ and angiotensin (2-8), Ang III,$^{117}$ also show biological activities.

![Figure 3.1](image.png)

*Figure 3.1. The renin-angiotensin system, RAS.*

The actions of Ang II are mediated by at least two receptors, designated as AT$_1$ and AT$_2$.$^{119,121}$ Most of the well-known actions of Ang II are mediated through the AT$_1$-receptor. Less is known about the AT$_2$-receptor, even though it has received increasing attention lately.$^{119,121-124}$ Other angiotensin receptors, such as AT$_3$ and AT$_4$ also have been proposed. Ang IV shows high affinity and selectivity for the AT$_4$-receptor.$^{119}$

Over-activity of RAS has been implicated in the development of
cardiovascular diseases such as hypertension, congestive heart failure, coronary ischaemia and renal insufficiency. Drugs that interfere with the RAS, such as the ACE-inhibitors and the non-peptide AT₁-receptor antagonists (section 3.2, Figure 3.3) have therefore been of great therapeutic and commercial success.

3.2 THE AT₁-RECEPTOR

The AT₁-receptor (Figure 3.2) is a seven trans-membrane G protein-coupled receptor (GPCR) belonging to the rhodopsin subclass.\textsuperscript{125} It has been cloned and characterized in human as well as in several other mammals, and is composed of 359 amino acids. The extracellular part contains two disulfide bridges that are critical for the tertiary structure of the receptor. The human AT₁-receptors are primarily found in the brain, adrenal glands, heart, vasculature and kidney.\textsuperscript{118,119,121}

In rodent, two isoforms, termed AT₁A and AT₁B have been found.\textsuperscript{118,119,121} These isoforms are 95% identical, and are also similar in terms of ligand binding and activation. The AT₁A-receptor is primarily found in the kidney, lung, liver and vascular smooth muscle, whereas AT₁B mainly is distributed in the adrenal and pituitary glands.

There are five classical transduction mechanisms for the AT₁-receptor.\textsuperscript{119} Stimulation of phospholipase C, which causes a rise in 1,4,5 inositol triphosphate (Ins(1,4,5)P₃) and diacylglycerol (DAG) levels is the most well described signaling pathway.\textsuperscript{118,119,121} Ins(1,4,5)P₃ mediates the release of Ca²⁺, and DAG activates protein kinase C. Both these events lead to vasoconstriction.

![Figure 3.2. Schematic picture of the AT₁-receptor.](image)
The trans-membrane domain and the extracellular loops are important for the binding of Ang II. Several homology based 3D models of the AT$_1$-receptor and its complex with Ang II have been suggested (see Nikiforovich and Marshall\textsuperscript{126} and references therein). The non-peptide AT$_1$-receptor antagonists interact with the transmembrane domain of the receptor. Even though the binding site of the AT$_1$-receptor antagonists is different from that of Ang II, they still appear to share parts of the same binding pocket between the helices.\textsuperscript{119}

The non-peptide AT$_1$-receptor antagonists\textsuperscript{127-130} originate from lead compounds found by screening at Takeda Chemical Industries. The first compound released on the market was DuP 753 or Losartan (Cozaar\textsuperscript{®}). Currently there are six approved orally active AT$_1$ antagonists on the Swedish market that are used to treat hypertension\textsuperscript{131} (Figure 3.3). Losartan is also being used for treatment of congestive heart failure.

\textbf{Figure 3.3.} Orally active AT$_1$-receptor antagonists, originating from lead compounds found by screening.\textsuperscript{131}
3.3 Structure-Activity Relationships of Ang II and its Cyclic Analogues

The structure-activity relationships (SAR) of the amino acid residues of Ang II are summarized in Figure 3.4.132 The aromatic side-chains of the Tyr\(^4\), His\(^6\) and Phe\(^8\), in addition to the C-terminal carboxylate have been identified as the pharmacophores. Substitution of Asp\(^1\) with Sar\(^1\) (sarcosine\(^1\), N-methyl glycine\(^1\)) is known to give 3-10 times enhanced binding,\(^{133,134}\) as well as enhanced potency and resistance to degradation.

Figure 3.4. SAR of the amino acid residues in Ang II. Modified from ref.\(^{132}\)

Several cyclic analogues of Ang II also have been prepared,\(^{51,76,134-142}\) in order to study the conformational requirements for binding and agonist activity. The Val\(^3\), Ile\(^5\) and Pro\(^7\) residues of Ang II mainly have conformational stabilizing roles,\(^{132}\) and these positions may therefore be used advantageously for cyclization.

In 1990 Spear et al.\(^{137}\) reported a series of disulfide \(i\) to \(i + 2\) cyclized Ang II analogues, of which 31a-d and 32 are depicted in Figure 3.5. Compounds 31a-d all showed high affinity to the AT\(_1\)-receptor. While the 11- and 12-membered
Angiotensin II

ring analogues 31a-c showed less than two percent of the agonist activity of Ang II, the 13-membered c[Hcy3,5]Ang II, 31d was shown to be a full agonist only two times less potent than Ang II itself ($pD_2 = 8.48$ versus 8.81 for Ang II). c[Pen3,5]Ang II, 32 also had high binding affinity to AT1-receptors and was found by Spear et al. to exhibit partial agonist-like properties. Analogue 32 was however re-tested by Schmidt et al. and was found to display full agonist activity in their assay. Conformational analyses of tripeptide model compounds of analogues 31a and 32, performed by the same group, suggested that the cyclic parts of these compounds adopted inverse $\gamma$-turn conformations. Based on this finding, four Ang II analogues were designed, incorporating $\gamma$-turn mimetics into positions 3-5. Indeed, two of these $\gamma$-turn containing compounds, the epimers of 33 displayed high AT1-receptor binding, and one of the epimers was found to be an agonist, 300-fold less potent than Ang II.

The highly constrained bicyclic agonist c[Sar1, Hcy3, Mpt5]Ang II, 34 was prepared by Marshall and coworkers, and allowed Nikiforovich and Marshall to postulate a 3D model for the bioactive conformation of Ang II (see section 3.4). Several 3-5 amide-cyclized Ang II analogues 35a-c and 36 also have been reported. Of these the 14-membered ring analogue 35b was found to be an agonist, as well as Ang II analogue 36, with the ring size of 15 atoms. Lindman et al. reported the dithioether-bridged Ang II analogues 37a-d, with ring sizes of 12-14 atoms. All of these compounds displayed high AT1-receptor binding affinities and the 13-membered ring analogue 37b was recently shown to be an agonist also, only 20 times less potent than Ang II.

Interestingly, for the 3-5 cyclized Ang II analogues so far reported, the 13-membered ring size seems to be the most favorable for producing agonists, except for analogues made by amide cyclization, where the 14-membered ring analogues have been the most successful. Indeed, it has been reported by Kataoka et al., that the rigidity of a 13-membered disulfide-cyclized peptide corresponds to that of a 14-membered amide-cyclized analogue.

While a large number of 3-5 cyclized Ang II analogues have been made, the 5-7 region, has not been explored as extensively. In this region, the Pro7 residue of Ang II has been postulated to participate in a second backbone turn. With the exception of the bicyclic c[Sar1, Hcy5, Mpc7]Ang II, 38 which displayed partial agonist activity and had 10 times lower binding affinity than Ang II, cyclization between residues five and seven has to date only afforded inactive compounds.
Figure 3.5. Previously reported biologically active cyclic analogues of angiotensin II.
3.4 **Bioactive Conformation**

The study of Ang II and its analogues has enabled the postulation of several 3D models for the biologically active conformation of Ang II. Nikiforovich and Marshall in 1993/1994 proposed two possible conformers of Ang II as being the bioactive ones.\textsuperscript{143,144} One of these, (designated as number II) was supported recently by modeling of a series of potent Ang II agonists.\textsuperscript{146} This proposed bioactive conformation of Ang II is shown in Figure 3.6.

*Figure 3.6. Bioactive conformation of Ang II, as proposed by Nikiforovich and Marshall.\textsuperscript{143,144,146} Residues 3-8 are depicted.*

Models of the bioactive conformation of Ang II also have been proposed by several other groups.\textsuperscript{141,142,147-151} The model suggested by Moore and coworkers involves a charge relay system that requires clustering of the aromatic rings of the Tyr\textsuperscript{4}, His\textsuperscript{6} and Phe\textsuperscript{8} side-chains.\textsuperscript{141}
4 SYNTHESIS OF ALDEHYDE-FUNCTIONALIZED BUILDING BLOCKS

As described above (section 2) our focus was on exploring aldehyde-mediated cyclizations. In contrast to the common method for the synthesis of thiazabicycloalkanes, we wanted to incorporate protected ω-formyl-α-amino acids into the peptides by standard SPPS, and thereafter effect the cyclizations by connection of side-chains and backbone in a final step. We decided to synthesize ω-formyl-α-amino acids with side-chain lengths varying between two and four carbon atoms, and to explore the effect of chain length on the outcome of the cyclizations. The most commonly used aldehyde protecting group, the dimethyl acetal, was selected for protection of the formyl group of the building blocks, in order to prove the concept.

Our general strategy for synthesis of the aldehyde functionalized building blocks has been to start from a commercially available amino acid derivative and: 1) introduce necessary protecting groups, 2) introduce, or transform the original side-chain into a dimethyl acetal protected aldehyde, and 3) exchange the protecting groups into ones that are standard for SPPS using Fmoc/tert-butyl protection.

4.1 THE ASPARTIC ACID DERIVED DIMETHYL ACETAL (Paper I)

Scheme 1
The synthesis of the aldehyde-functionalized building block with a chain length of two carbon atoms was started from commercially available Boc-L-Asp-O'Bu (39) (Scheme 1). The starting material (39) was converted to the mixed carbonic anhydride 40, in order to enable the selective reduction of the side-chain carboxylic acid into an alcohol in presence of the tert-butyl ester. The alcohol derivative formed 41 was subsequently oxidized with PCC to give the aldehyde 42.

![Scheme 2]

The aldehyde 42 was protected as the dimethyl acetal 43, using catalytic amounts of p-toluenesulfonic acid in methanol. The Boc and tert-butyl protecting groups were thereafter cleaved with HCl in methanol. The removal of these groups was accompanied with formation of considerable amounts of α-methyl ester, and subsequent treatment with potassium hydroxide was necessary to liberate the zwitterion. The zwitterion was not isolated, but was treated directly with Fmoc-Cl and sodium carbonate in water/dioxane to give the Fmoc protected building block 44.

Alternatively, the aldehyde 42 could be transformed directly into the Fmoc derivative 44 in approximately the same yield as for the two-step procedure via compound 43. The synthetic route via compound 43 was still preferred, since it was found be more reliable.

Selective cleavage of the Boc and tert-butyl protecting groups was attempted using trimethylsilyl iodide, but proved to be unsuccessful due to concomitant
deprotection of the aldehyde function.\textsuperscript{156} The Fmoc protecting group was introduced at the end of the reaction sequence, since it was found to be unstable to the conditions used for the oxidation step using PCC.

### 4.2 The Glutamic Acid Derived Hemi-aminal (Paper II)

Attempts to follow the same synthetic procedure as in Scheme 1 and 2 for the synthesis of the corresponding aldehyde-functionalized building block with a chain length of three carbon atoms proved unsuccessful. Upon its formation from alcohol derivative 45 (Scheme 3), the corresponding aldehyde immediately cyclized to the nitrogen with the resulting formation of the five-membered hemi-aminal 46.\textsuperscript{157} This hemi-aminal should be in equilibrium with the open-chain aldehyde, although the equilibrium is shifted towards the cyclic form. Hemi-aminal 46 can therefore be regarded as an equivalent to the open-chain aldehyde and it should be possible to use it as a building block for aldehyde mediated peptide cyclizations. Under the oxidative conditions used however, hemi-aminal 46 was further oxidized immediately to form the undesired pyroglutamic acid derivative 47.\textsuperscript{157}

The hemi-aminal derivative 46 was prepared by alternative means under reductive conditions (Scheme 4), by following the procedure described by Wernic et al.\textsuperscript{153} for the synthesis of the corresponding aspartic acid derived aldehyde. The commercially available Boc-L-Glu-O\textsuperscript{t}Bu (48) was transformed into Weinreb\textsuperscript{158} amide 49.\textsuperscript{159} Reduction with diisobutylaluminium hydride (DIBAL) gave the aldehyde, which immediately cyclized to give the hemi-aminal derivative 46. This hemi-aminal (46) was formed as a pair of
diastereomers. No attempts to separate these diastereomers were made, since under acidic conditions they are in equilibrium with the open-chain form of the aldehyde.

NMR spectra recorded at 20 °C (DMSO-\(d_6\)) showed each diastereomer to exist as a mixture of two rotamers. The existence of rotamers is caused by the relatively high rotation barrier of the nitrogen-carbon bond of the carbamate function.

Variable temperature NMR experiments confirmed the existence of a rotamer mixture.

The Boc and tert-butyl protecting groups were removed using trimethylsilyl iodide (TMS-I), and the resulting zwitterion was directly converted to the Fmoc protected building block 50. For this compound also (50), each diastereomer was shown by NMR to exist as a mixture of rotamers.

![Scheme 4](image)

**Scheme 4**

### 4.3 The Glutamic- and 2-AminoAdipic Acid Derived Dimethyl Acetals (Papers II and III)

Even though the 5-membered hemi-aminal 50 can be regarded as an equivalent to the open-chain form of the L-glutamic acid derived aldehyde, we still wanted access also to the linear, dimethyl acetal-protected building block 58 (Scheme 6). In order to avoid the spontaneous cyclization of the nitrogen in compound 54 onto the aldehyde function, the nitrogen needs to be diprotected. The synthesis of building block 58 therefore was started from the commercially available
benzyloxycarbonyl (Z) protected L-glutamic acid 51, which was protected also as an oxazolidinone moiety. The latter group was used for the simultaneous protection of the nitrogen and the α-carboxyl group. The resulting compound 52 was then ready for transformation of the side-chain carboxyl function into a formyl group. We chose to do this conversion according the procedure used by Holcomb et al., by reduction to the alcohol 53 with borane dimethyl sulfide and subsequent oxidation with PCC. The resulting aldehyde 54 was then protected as the dimethyl acetal 55.

Scheme 5

The dimethyl acetal 55 was transformed into the free amine 57 following the procedure used by Fukuyama et al., i.e. through cleavage of the oxazolidinone with sodium methoxide in methanol to give the methyl ester 56, followed by removal of the benzyloxycarbonyl group by catalytic hydrogenation. The cleavage of similar oxazolidinones have recently been reported also to be achievable under conditions using sodium hydrogen carbonate under reflux.
The methyl ester of compound 57 was hydrolyzed with potassium hydroxide and the formed zwitterion was directly converted to the Fmoc protected building block 58.

![Scheme 6]

We also wished to prepare the next homologue in the series of the dimethyl acetal protected building blocks, that with a chain length of four carbon atoms (67). The aldehyde with the four-carbon side-chain has been reported to be prone to cyclize onto the nitrogen also, with formation of a 6-membered ring. Diprotection of the nitrogen atom was therefore used for the synthesis of building block 67. The starting material for this synthesis (Scheme 5) was the commercially available L-2-aminoadipic acid, 59. The benzyloxycarbonyl-protecting group was introduced at the first step, and for subsequent steps the same synthetic route that had been developed for the L-glutamic acid derived building block was successfully applied (Schemes 5 and 6).

**4.4 THE \(\alpha,\alpha\)-DISUBSTITUTED BUILDING BLOCK (Paper IV)**

The \(\alpha,\alpha\)-disubstituted amino acid derivative 75 (Scheme 8) was designed as a building block for the preparation of tyrosine side-chain functionalized thiazabicycloalkane dipeptide mimetics, which would introduce a local constraint around the important Tyr\(^4\) residue of Ang II. Building block 75 can be regarded
as a chimeric amino acid derivative that contains one side-chain to be used for cyclization and one for recognition by the receptor. Since we wanted access to both enantiomers of compound 75, a racemic synthesis was chosen. The synthesis to the intermediate \( p \)-\( \text{tert} \)-butoxyoxybenzyl substituted aldehyde derivative 71 (Scheme 7) was based on a strategy outlined by Baldwin et al.\(^{113} \) for the synthesis of the corresponding phenylalanine side-chain functionalized thiazabicycloalkanes.

Commercially available \( L \)-Z-Tyr(\( \text{O}^\text{tBu} \))-OH (68) (Scheme 7) was used as starting material. In this case acidic catalysis with \( p \)-toluenesulfonic acid could not be used for the formation of oxazolidinone 69, due to the presence of the acid labile \( \text{t} \)-butyl protecting group. The conversion to oxazolidinone 69 by reaction with paraformaldehyde, employing azeotropic removal of water was hard to put to completion, resulting in the low yield of 38\%. The starting material could however be recovered, and based on the consumed starting material the yield was 95\%.

Scheme 7
Oxazolidinone 69 was deprotonated with potassium hexamethyldisilylamide (KHMDS) and the enolate formed was alkylated with allyl iodide to form the racemic quartenary substituted amino acid derivative 70. Oxidative cleavage of the double bond with sodium periodate and a catalytic amount of osmium tetroxide\textsuperscript{172} afforded aldehyde 71, which was directly protected as the dimethyl acetal to give compound 72.

\begin{equation}
\begin{array}{c}
\text{MeO} \\
\text{MeO} \\
\text{MeO} \\
\text{N} \\
\text{O} \\
\text{Z} \\
\end{array}
\xrightarrow{\text{NaOMe, MeOH, reflux}}
\begin{array}{c}
\text{MeO} \\
\text{MeO} \\
\text{MeO} \\
\text{NH} \\
\end{array}
\xrightarrow{\text{H₂, Pd-C, EtOH, 65 °C}}
\begin{array}{c}
\text{MeO} \\
\text{MeO} \\
\text{MeO} \\
\text{OH} \\
\end{array}
\end{equation}

\textbf{Scheme 8}

The cleavage of the oxazolidinone- and benzyloxy carbonyl groups from compounds 72 and 73 (Scheme 8) required more elevated temperatures than used for the deprotection of the corresponding mono-substituted compounds (Scheme 6). Reflux conditions were also required to effect hydrolysis of the methyl ester 74. Finally the primary amine was protected to give the \(\alpha,\alpha\)-disubstituted building block 75. NMR spectra of compounds 69-72 were recorded at elevated temperatures, due to the existence of rotameric mixtures at lower temperatures. For compound 72 it was however necessary to report the NMR data as for a mixture of rotamers.

In summary, five aldehyde-functionalized building blocks with chain lengths varying between two and four carbon atoms, have been synthesized. The preparation of the different building blocks required different protection schemes.
and/or modified conditions for protection and deprotection. Reductive and/or several oxidative strategies were utilized.

The final protected aldehyde-functionalized building blocks were all isolated and purified through extensive extractions. They were not stable to flash chromatography on silica, and some of them were found to decompose slowly when stored at room temperature. 2D NMR experiments were commonly required in order to allow structural confirmation and assignments for the final building blocks and their synthetic intermediates.
5 NOVEL METHODS FOR THE CYCLIZATION OF PEPTIDES

5.1 BICYCLIZATION DELIVERING THIAZABICYCLOALKANE TRIPLEPTIDE MIMETICS (Papers I and II)

The building blocks 44 and 58, with chain lengths of two and three carbon atoms respectively, were subjected to SPPS and incorporated into position 5 of the dimercapto peptide precursors 76 and 77 respectively (Scheme 9). These initial experiments were designed to assess the propensity of the formyl group to cyclization towards the N-terminal or C-terminal end of the peptide.

Upon deprotection and cleavage from the solid phase with TFA, the formyl groups were found to cyclize to one of the neighboring backbone nitrogens, in addition to the sulfur atom of one of the two incorporated cysteine residues. The thiazabicycloalkane tripeptide containing peptides 78 and 79a-b were thus formed (Scheme 9). The aspartic acid derived building block 44 cyclized towards the C-terminal end of the peptide, while for the glutamic acid derived building block 58, N-terminal directed cyclization was observed. Thus, it seemed that the regioselectivity of the bicyclization could be directed either towards the C-terminal or N-terminal end of the peptide, simply by altering the chain length of the incorporated ω-formyl-α-amino acid. The ready formation of a five-membered ring, in preference to other ring sizes was anticipated as providing the driving force for the observed regioselectivity.

The C-terminal directed bicyclization occurred stereoselectively, with formation of the diastereoisomer with the ring-junction hydrogen on the opposite side of the incoming and outgoing backbone of the bicyclic ring system. In contrast, the N-terminal directed cyclization delivered both the epimers 79a and 79b. The thiazabicycloalkane tripeptide unit produced by the C-terminal directed cyclization can be regarded as a five-membered “Freidinger-type” lactam84 (see structure 10, Figure 1.7), fused to an eight-membered ring. For N-terminal directed cyclization on the other hand, the formed tripeptidic thiazabicycloalkane ring system is a proline derivative fused to a nine-membered ring.
Scheme 9. Reagents: (a) (i) His(Trt)-Cys(Trt)-Phe-Wang resin, HBTU, NMM, DMF, (ii) piperidine, DMF; (b) (i) Fmoc-Tyr(tBu), HBTU, NMM, DMF, (ii) piperidine, DMF; (c) (i) Fmoc-Cys(Trt), HBTU, NMM, DMF, (ii) piperidine, DMF; (d) (i) Fmoc-Arg(Pmc), HBTU, NMM, DMF, (ii) piperidine, DMF; (e) (i) Fmoc-Asp(OtBu), HBTU, NMM, DMF, (ii) piperidine, DMF.
The utility of the alternative glutamic acid derived building block, hemi-alaminal 50, was also examined by incorporating it into the dimercapto precursor peptide 80 (Scheme 10). Treatment with TFA delivered the same epimeric octapeptides 79a and 79b, produced by N-terminal directed cyclization, as when the linear building block 58 was used (Scheme 9). The linear building block 58 was however found to deliver more easily purified bicyclized peptides than the hemi-alaminal 50, and therefore this route was selected when further bicyclized peptides containing this ring system were to be synthesized.

**Scheme 10.** Reagents: (a) (i) His(Trt)-Cys(Trt)-Phe-Wang resin, PyBOP, HOBt, DIEA, DMF; (ii) piperidine, DMF; (b) (i) Fmoc-Tyr(tBu), PyBOP, HOBt, DIEA, DMF; (ii) piperidine, DMF; (c) (i) Fmoc-Cys(Trt), HBTU, NMM, DMF; (ii) piperidine, DMF; (d) (i) Fmoc-Arg(Pmc), HBTU, NMM, DMF; (ii) piperidine, DMF; (e) (i) Fmoc-Asp(OtBu), HBTU, NMM, DMF; (ii) piperidine, DMF.

The simplicity and the obvious potential of the bicyclization strategy encouraged us to explore this method for the preparation of a series of constrained Ang II analogues. The Ang II analogues prepared by the C-terminal directed cyclization procedure is depicted in Scheme 11, and the Ang II analogues, produced from the N-terminal directed cyclization, in Scheme 12. Previous mono- and bicyclizations in the Tyr^4^ and His^6^- region of Ang II provided bioactive compounds (Figure 3.5), and we were interested in investigating both regions. The building block 44 was therefore incorporated both into positions 3 and 5, in order to furnish 3-5 and 5-7 bicyclized Ang II analogues (Scheme 11). Similarly, the glutamic acid derived building block 58 was incorporated into positions 5 and 7 in order to give both Ang II analogues constrained around the Tyr^4^ and His^6^- region (Scheme 12). In addition to using cysteine residues in positions 3, 5 or 7, homocysteine residues were also used for the preparation of Ang II analogues 83-84 and 87, containing bicyclic systems with the size of the larger ring increased by one atom.
Scheme 11. C-terminal directed bicyclization, using the aspartic acid derived building block 44 was utilized for preparation of Ang II analogues 81-84. (Reagents used in the SPPS, were essentially the same as those given in Scheme 9.)

Ang II analogues 81-87 were produced as the predominant components of the reaction mixtures. According to LC-MS analyses, none of the major side-products (>5% yields, as estimated from peak areas) had the same molecular weights as the target peptides, suggesting that the synthesis of the building blocks was accompanied by negligible racemization, and that the bicyclizations
occurred with high regioselectivity and/or stereoselectivity. Ang II analogues 81-87 were isolated in 15-40% yields, after purification by RP-HPLC.

Scheme 12. N-terminal directed bicyclization, using the glutamic acid derived building block 58, was utilized for preparation of Ang II analogues 85-87. (Reagents used in the SPPS, were essentially the same as those given in Scheme 9.)

The structural assignment of Ang II analogues 81-87 was made from P.E. COSY\(^{173}\), TOCSY\(^{174}\) and NOESY\(^{175,176}\) or ROESY\(^{177}\) NMR experiments.
For Ang II analogues 81-84, selected carbon-proton connectivity information regarding the bicyclic portions of the molecules was also obtained from HMQC\(^{178}\) and HSBC\(^{179}\) NMR experiments.\(^{180}\) The stereochemistry of the bicyclic ring systems was determined from NOE information. Conformational analyses of the bicyclic tripeptide segments of Ang II analogues 81-87 will be discussed in section 7.

The results from the receptor binding assays (see section 6) showed Ang II analogues 81-87 to have little or no affinity to the AT\(_1\)-receptor. Apparently they cannot adopt a suitable conformation that allows binding to the receptor. Therefore, it was decided to progress the project in two different directions: (a) to increase the flexibility of the \(i\) to \(i+2\) cyclized segments by using monocyclic systems instead of bicyclic ones (section 5.2): (b) to continue the bicyclization strategy, but to concentrate the conformational constriction to more localized regions of Ang II, by performing \(i\) to \(i+1\) bicyclizations instead of \(i\) to \(i+2\) bicyclizations (section 5.3). We wanted to introduce the local constraints close to the important Tyr\(^4\) residue where the incorporation of a 7-membered \(\gamma\)-turn mimetic was shown previously to deliver an active agonist.\(^{140}\)

5.2 Monocyclization through Formation of Vinyl Sulfide Bridges (Paper III)

Initial experiments which incorporated a cysteine residue into position \(i\), and the building block 67 into position \(i+2\) of a suitable peptide (Scheme 13) showed that the expected cyclization to nitrogen and sulfur, forming a 6,9-fused thiazabicycloalkane ring system (89) did not occur. Instead, the formyl function was found to cyclize to the sulfur only, with formation of monocyclic cis- and trans- vinyl sulfide bridged ring systems such as 90. We postulate that the reaction occurs via the formation of hemi-acetal like compounds, with subsequent elimination of water.

However, we envisioned that the 13-membered vinyl sulfide cyclized ring systems could be exploited as bioisosteric segments of the 13-membered c[Hcy \(^{3,5}\)] ring system (cf. compound 31d, Figure 3.5) produced by disulfide cyclization of two \(i\) and \(i+2\) positioned homocysteine residues. That is, the disulfide CH\(_2\)-S-S segment is replaced by a bioisosteric S-CH=CH segment. We also expected that the vinyl sulfide ring systems would restrict the
conformational freedom to a greater extent than the corresponding disulfide based systems, due to the planarity of the double bond introduced. The vinyl sulfide ring systems therefore should exhibit interesting complementary properties and have the potential to be exploited for conformational fine-tuning of SARs of cyclic peptide analogues. Furthermore it would be of interest from a pharmacokinetic viewpoint to examine whether the vinyl sulfide ring systems display higher metabolic stability than the corresponding disulfides.

Scheme 13. Initial experiments using the building block 67, with the chain length of four carbon atoms, did not give the expected 9,6-fused ring system 89. Instead the monocyclic cis- and trans- vinyl sulfide ring systems 90 were formed.

Encouraged by the high affinity and agonist properties of c[Hcy3,5]Ang II137 (compound 31d in Figure 3.5), the preparation of Ang II analogues 91-92 (Scheme 14), containing vinyl sulfide bridges between positions 3 and 5, was undertaken. As an additional example of the vinyl sulfide cyclization, Ang II analogues 93-94, bridged between positions 5 and 7, were also prepared.
Incorporation of the building block 67 was achieved by standard SPPS. Upon acidic deprotection and cleavage from the resin, cyclization occurred spontaneously, with formation of both the cis- and trans- vinyl sulfide bridged segments. For the 3-5 cyclized Ang II analogues 91 and 92, the cis/trans ratio was 1:2, and the total yield after purification by RP-HPLC was 10%.

Scheme 14. Vinyl sulfide cyclized Ang II analogues 91-94, prepared by using building block 67, with the chain length of four carbon atoms. (Reagents used in the SPPS, were essentially the same as those given in Scheme 9.)

For the 5-7 cyclized peptides 93 and 94, we were not able to separate the cis- and trans- isomers by RP-HPLC, but the ratio of cis/trans was determined from
NMR to be approximately 3:2. The total isolated yield of the Ang II analogues 93 and 94, was 8%.

In relation to the yields of the vinyl sulfide cyclized Ang II analogues, which were only modest, it should be noted that the formation of 13-membered rings in particular is a difficult task by other cyclization methods also.50,58,90,181

Structural determinations of the 3-5 cyclized Ang II analogues 91-92 were made by assignment of the proton NMR signals from P.E.COSY, TOCSY and ROESY spectra. The connectivity between the CH2 and S-CH=CH segments of the vinyl sulfide bridges was established by NOE from Hε of the residue derived from building block 67 to Hβ of cysteine. The cis- or trans- geometry of the vinyl sulfide double bond (Ang II analogues 91 and 92) was established by the magnitude of the coupling constants. P.E.COSY, TOCSY and ROESY spectra were also recorded on the mixture of the 5-7 cyclized Ang II analogues 93 and 94. Assignment of certain key signals together with comparison of the spectra from compounds 91 and 92, allowed the identification of analogues 93 and 94.

The conformational- and the pharmacological properties of the vinyl sulfide bridged Ang II analogues will be discussed further in sections 6 and 7.

5.3 **BICYCLIZATION DELIVERING TYROSINE SIDE-CHAIN FUNCTIONALIZED THIAZABICYCLOALKANE DIPEPTIDE MIMETICS**

(Paper IV)

As discussed in section 5.1 we decided to continue the bicyclization strategy, by concentrating the conformational restriction to more localized regions of Ang II, by performing i to i + 1 cyclizations instead of the i to i + 2 cyclizations described in section 5.1. We wanted to focus around the Tyr4 residue of Ang II, where a turn has been postulated, and where the incorporation of a 7-membered γ-turn mimetic has been proved to be very successful.140

The chimeric amino acid building block 75 was designed therefore to be incorporated into position 4 of the precursor peptide 95 (Scheme 15), to provide Ang II analogues encompassing thiazabicycloalkane dipeptide mimetics such as 96a-d. Building block 75 is incorporated into the peptides as the racemate, and an additional asymmetric centre of unknown configuration is created at the ring junction of the bicyclic system. Ang II analogues encompassing all four diastereomeric core structures 96a-d are therefore possible, but may not be formed necessarily.
Scheme 15. The building block 75 was designed to be incorporated into position 4 of the precursor peptide 95, for the delivery of Ang II analogues encompassing thiazabicycalkane dipeptide mimetics such as 96a-d.

The SPPS of peptides containing the building block 75 did, however, turn out to be more complicated than expected. The incorporation of other quartenary substituted building blocks and similar cyclizations have also been reported to be difficult.\textsuperscript{100,106} The coupling of the building block 75 to the C-terminal tetrapeptide Cys-His-Pro-Phe on Wang resin (Scheme 15) was challenging, but manageable. The subsequent coupling of Fmoc-Val was even more problematic. After several unsuccessful attempts with a number of different coupling reagents treatment with 2% TFA in dichloromethane, followed by the addition of 1% triethylsilane, finally allowed both the cyclization and the following coupling of Fmoc-Val-F to be performed successfully. This acidic treatment was however accompanied by the undesired cleavage of the peptide from the resin. This
caused us to start looking for alternative resins, with linkers of greater acid stability than possessed by the Wang resin.

Scheme 16. Reagents: (a) Cys(Trt)-His(Trt)-Pro-Phe-P-linker-2-TentaGel, PyBOP, DIEA, DMF, over night; (b) 95% TFA (aq.); (c) piperidine, DMF; (d)(i) Fmoc-Val-F, DIEA, DMF, (ii) piperidine, DMF; (e) (i) Fmoc-Arg(Pbf), PyBOP, DIEA, DMF, (ii) piperidine, DMF; (f) (i) Fmoc-Asp(Ot Bu), PyBOP, DIEA, DMF, (ii) piperidine, DMF; (g) 95% TFA (aq.), (h) hν (350 nm).

After unsuccessful attempts with the palladium(0) labile HYCRAM® resin,182,183 the SPPS incorporation and on-resin cyclization was finally successfully completed using the photolabile P-linker-2184,185 on TentaGel, as outlined in Scheme 16. The synthesis afforded two main products with the
expected mass and amino acid analysis values, tentatively assigned as diastereomers of Ang II analogues 99. These two Ang II analogues, referred to as 99(I) and 99(II) were each isolated in 5% yield after purification by RP-HPLC.

The structural assignment of 99(I), and determination of the stereochemistry of the bicyclic part of the molecule was determined using data obtained from phase sensitive DQF-COSY\textsuperscript{186-188} and gNOESY\textsuperscript{189,190} NMR experiments. The structure of Ang II analogue 99(I) was found to encompass the thiazabicyclo-alkane dipeptide mimetic 96d (Scheme15), and is depicted in Figure 5.1.

![Figure 5.1. The structure of Ang II analogue 99(I).](image)

Determination of the absolute configuration, and confirmation of the structure of Ang II analogue 99(II) will be undertaken within the near future.
6 **Pharmacology**

The Ang II analogues **81-87** were first screened in a radioligand binding assay based on displacement of $[^{125}]$Ang II from rat AT$_1$-receptors expressed by Chinese Hamster Ovary (CHO) cells$^{191,192}$ (Table 6.1). Compounds exhibiting affinity (i.e. Ang II analogue **87**) were further evaluated in a radioligand binding assay on AT$_1$-receptors in rat liver membranes$^{51,193}$ (Table 6.1 and 6.2). Ang II analogues **91-94, 99(I)** and **99(II)** were directly tested in the rat liver membrane assay.

The $i$ to $i + 2$ bicyclic Ang II analogues **81-87** were all shown to lack affinity to the AT$_1$-receptor, except for analogue **87**, which showed a moderate binding affinity of 750 nM in the rat liver assay (Table 6.1).

<table>
<thead>
<tr>
<th>compound</th>
<th>binding on rat AT$_1$ receptors expressed by CHO cells</th>
<th>binding on AT$_1$ receptors in rat liver membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC$_{50}$ (nM)</td>
<td>$K_i$ (nM)</td>
</tr>
<tr>
<td>Ang II</td>
<td>1.5</td>
<td>0.53</td>
</tr>
<tr>
<td>c[Hcy$^{3,5}$]Ang II</td>
<td>0.2</td>
<td>0.19</td>
</tr>
<tr>
<td>DuP 753</td>
<td>40</td>
<td>15</td>
</tr>
<tr>
<td><strong>81</strong></td>
<td>$&gt;10000$</td>
<td>$&gt;10000$</td>
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<tr>
<td><strong>84</strong></td>
<td>$&gt;10000$</td>
<td>$&gt;10000$</td>
</tr>
<tr>
<td><strong>85a</strong></td>
<td>$&gt;10000$</td>
<td>-</td>
</tr>
<tr>
<td><strong>85b</strong></td>
<td>$&gt;10000$</td>
<td>-</td>
</tr>
<tr>
<td><strong>86</strong></td>
<td>$&gt;10000$</td>
<td>-</td>
</tr>
<tr>
<td><strong>87</strong></td>
<td>800</td>
<td>750</td>
</tr>
</tbody>
</table>

The results for the vinyl sulfide cyclized Ang II analogues (Table 6.2) were however more encouraging. Both of the 3-5 position, *cis*- and *trans*- vinyl sulfide cyclized analogues **91** and **92** were found to bind with high affinity to the AT$_1$-receptor ($K_i = 1.7$ nM). These two compounds therefore were also evaluated for possible agonist activity in a vascular contractility study using rabbit aorta (Table 6.2 and Figure 6.1).
**Table 6.2.** Vinyl sulfide cyclized Ang II analogues (paper III); in vitro rat liver AT1-receptor binding affinities and agonistic activities in vascular contractility studies on rabbit aorta.

<table>
<thead>
<tr>
<th>compound</th>
<th>rat AT1 receptor binding affinities $K_i$ (nM) ± SEM</th>
<th>agonistic activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang II</td>
<td>0.31 ± 0.08</td>
<td>1.40 ± 0.28</td>
</tr>
<tr>
<td>c[Hcy$^{3,5}$]Ang II</td>
<td>0.23 ± 0.14</td>
<td>-</td>
</tr>
<tr>
<td>DuP 753</td>
<td>25 ± 4.7</td>
<td>-</td>
</tr>
<tr>
<td><strong>91</strong></td>
<td>1.7 ± 0.26</td>
<td>26.0 ± 6.79</td>
</tr>
<tr>
<td><strong>92</strong></td>
<td>1.7 ± 0.32</td>
<td>16.1 ± 3.52</td>
</tr>
<tr>
<td><strong>93</strong></td>
<td>&gt; 1 000$^a$</td>
<td>-</td>
</tr>
<tr>
<td><strong>94</strong></td>
<td>&gt; 1 000$^a$</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$ Compounds **93** and **94** were tested for binding as a mixture (approximate ratio 3:2).

**Figure 6.1.** Ang II analogues **91** and **92** displayed full agonist properties in the vascular contractility study using rabbit aorta.

Both Ang II analogues **91** and **92** were found to behave as full agonists, and were only 20 and 10 times less potent respectively, than Ang II itself. The agonistic properties of analogues **91** and **92** were also shown to be completely blocked upon addition of the non-peptide AT1 antagonist DuP 753 (Losartan, Cozaar®, Figure 3.3). The position 5-7 vinyl sulfide cyclized compounds **93** and
94 on the other hand, lacked affinity to the AT₁-receptor, and were thus not tested in the contractility study.

The two $i$ to $i + 1$ bicyclized Ang II analogues 99(I) and 99(II) (paper IV) were both shown to lack affinity to the AT₁-receptor. No IC₅₀ values below 10µM were found.

The agonistic c[Hcy³⁵]Ang II¹³⁷ (compound 31d, Figure 3.5) and the non-peptide AT₁-receptor antagonist DuP 753 were used as reference compounds in the binding assays.
7 CONFORMATIONAL ANALYSIS

7.1 CALCULATIONS ON MODEL TRIPEPTIDES (Papers I, II, and III)

The mono- and bicyclic peptide core structures of the synthesized Ang II analogues were modeled as the blocked tripeptide model compounds 1m-7m depicted in Figure 7.1. Calculation at the tripeptide level was chosen not only for the reason of simplicity, but also in order to provide a general conformational description, that could be useful when incorporating the tripeptidic core structures into other target peptides.

Figure 7.1. The blocked tripeptide model compounds 1m-10m.

Conformational analyses were carried out by performing Monte Carlo conformational searches, using the Amber* force field and the GB/SA water solvation model\textsuperscript{194} within Macromodel (version 5.5 in papers I and II, and version 6.5 in paper III).\textsuperscript{195} A summary of the number of low-energy conformations found for the model compounds, within 5 kcal/mol of the lowest energy conformation identified, is presented in Table 7.1 and 7.2. Model compound 8m was included in the analyses, as a linear reference peptide.
Model compounds 1m and 2m, derived from C-terminal i to i + 2 bicyclization (paper I): These model compounds were found to be the most rigid of those investigated (Table 7.1). The highly similar geometries of the low-energy conformations of 1m (a) and 2m (b), within 5 kcal/mol are shown in Figure 7.2. As can be seen from this figure, model compounds 1m and 2m have reverse turn inducing properties.

![Figure 7.2](image)

---

**Table 7.1. Summary of the number of low-energy conformations found within 5 kcal/mol of the lowest energy minimum, for each of the model compounds 1m-5m and 8m-9m. Macromodel version 5.5**

<table>
<thead>
<tr>
<th>compound</th>
<th>conformations &lt;5kcal/mol</th>
<th>compound</th>
<th>conformations &lt;5kcal/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1m</td>
<td>4</td>
<td>5m</td>
<td>44</td>
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<tr>
<td>2m</td>
<td>10</td>
<td>8m</td>
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<td>3m</td>
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<td>9m</td>
<td>58</td>
</tr>
<tr>
<td>4m</td>
<td>21</td>
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</tbody>
</table>

---

**Figure 7.2. Stereo image of the conformations within 5 kcal/mol of the lowest energy minimum found for 1m (a) and 2m (b).**
Some, but not all, of the low-energy conformations were shown to adopt β-turns, as defined by the distance of $C_\alpha i$ to $C_\alpha i + 3$ being less than 7Å. The β-turn conformations could not, however, be classified into any of the classical turn types (I, I’, II, II’, III, III’ VIa or VIb). Analysis of NMR temperature coefficients of the NH chemical shifts of the octapeptides corresponding to 1m (81-82) and 2m (83-84), together with analysis of the calculated low-energy conformations of 1m and 2m, indicated a hydrogen bond between the NH proton on the N-terminal side of the five membered ring (residue $i + 1$) and the carbonyl of the $i + 4$ residue. This hydrogen bond creates an 11-membered ring, that is shifted from the hydrogen bond between the carbonyl function of residue $i$ and the NH-group of residue $i + 3$ making up the 10-membered ring that is normally seen in β-turn conformations (Figure 1.3).

Considering the constrained nature of the bicyclic scaffolds of model compounds 1m and 2m, it is reasonable to assume that octapeptides which encompass these scaffolds would also adopt reverse turn conformations in the corresponding regions. The fact that about 40% of the β-turns found in proteins do not belong to any of the classical turn types, implies that there is also an incentive for developing turn mimetics for non-classical reverse turns.

Model compounds 3m, 4m and 5m, derived from N-terminal i to i + 2 bicyclization (paper II): Model compounds 3m, 4m and 5m were found to be more flexible (Table 1) and more conformationally diverse than those described above. Some of the low-energy conformations were found to adopt β-turns, whereas some were found to be more extended, as those shown in Figure 7.3.

![Figure 7.3](image_url)
Among the β-turn conformations found for 4m and 5m, some were found to be classical type I β-turns, like the one depicted for model compound 4m in figure 7.4.

**Figure 7.4.** Type I β-turn conformation of model compound 4m, $\Delta E = 1.0$ kcal/mol.

Besides analyzing the backbone torsion angles of model compounds 3m, 4m and 5m, we also monitored the $X_1= (N2-C_\alpha 2- C_\alpha 3-C_\beta 3)$, $X_2= (C_\beta 3- C_\alpha 3- C_\alpha 4-C(4)O)$ and $X_3= (N2-C_\alpha 2- C_\alpha 4-C(4)O)$ torsion angles (see Figure 7.4 for notation of the atoms). $X_1$, $X_2$ and $X_3$ describe the relative orientations of the side chain connected to $C_\alpha 3$, and the incoming- and outgoing backbone with respect to each other. The $X_1$, $X_2$ and $X_3$ angles should be more relevant for an analysis of the relative topographical arrangement of the pharmacophoric side-chain connected to $C_\alpha 3$, in relation to other pharmacophoric groups positioned outside the tripeptidic core structures.

A comparative study, based on both the backbone torsion angles and on the $X_1$, $X_2$ and $X_3$ angles, was performed, comparing model compounds 5m and 9m (Figure 7.1). This was done in order to try to rationalize the difference in binding between Ang II analogue 87 (Scheme 12) ($K_i = 750$ nM) and Ang II analogue 34 in Figure 3.5 ($IC_{50} = 1.3$ nM), reported by Marshall and coworkers. These Ang II analogues differ with respect to the position of the sulfur atom, the stereochemistry at the ring junction of the bicyclic ring system, and also with
respect to position 1, where a Sar$^1$ residue is present in the analogue reported by Marshall and coworkers. Such a Sar residue is known to enhance the binding 3-10 times, but cannot account for the large difference in binding between the two Ang II analogues. Our conformational analysis however showed model compound 5m and 9m to possess conformations with overall similar topography, as is illustrated in Figure 7.5, and did not enable rationalization of the difference in binding affinity.

![Figure 7.5. Stereo image of the rms best fit of the lowest energy conformations of 5m (green) and 9m (black). N2, C$_{\alpha}$2, C$_{\alpha}$3, C$_{\beta}$3, C$_{\alpha}$4, and C(4)O were included in the fitting procedure.](image)

It has been suggested recently by Nikiforovich et al. that the whole hexapeptide fragment Val$_3$-Phe$_8$ of Ang II, has to be taken into account when attempting to distinguish analogues that are potent binders (e.g. 34) from those that have low binding affinity (e.g. 87).

Vinyl sulfide cyclized model compounds 6m and 7m (Paper III): The number of low-energy conformations found for 6m and 7m are summarized in Table 7.2. Model compounds 6m and 7m were expected to display slightly different, yet broadly similar conformational properties compared to the disulfide based ring system in model compound 10m (Figure 7.1) (cf. Ang II analogue 31d, Figure 3.5). Analyses of the backbone torsion angles as well as of the X$_1$, X$_2$ and X$_3$ angles showed that model compounds 6m, 7m, and 10m displayed very similar conformational properties. This can be seen in Figure 7.6, were the lowest energy conformations of 6m, 7m, and 10m are superimposed.
Table 7.2. Number of low-energy conformations found within 5 kcal/mol of the lowest energy minimum for model compounds 6m-7m, 8m and 10m. Macromodel version 6.5.

<table>
<thead>
<tr>
<th>compound</th>
<th>conformations &lt;5kcal/mol</th>
<th>compound</th>
<th>conformations &lt;5kcal/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>6m</td>
<td>36</td>
<td>8m</td>
<td>20</td>
</tr>
<tr>
<td>7m</td>
<td>22</td>
<td>10m</td>
<td>50</td>
</tr>
</tbody>
</table>

As expected, due to the planarity of the double bond, the vinyl sulfide containing compounds were found to be more rigid than the disulfide 10m, in the context of fewer low-energy conformations being identified (Table 7.2). Analysis of the identified low-energy conformations however revealed that the conformations found for 6m and 7m were as conformationally diverse as those found for the more flexible disulfide ring system 10m. Due to the relatively high flexibility of the monocyclic tripeptides 6m, 7m, and 10m overall, it was not possible to detect the small conformational differences imposed by the cis- and trans- vinyl sulfide bonds, which we aimed to exploit for conformational fine tuning.

7.2 Calculations on Model Dipeptides (Paper IV)

The four possible bicyclic core structures of Ang II analogue 99 were modeled as the blocked dipeptide model compounds 11m(a)-11m(d) (Figure 7.7), as described for the calculations in section 7.1, and using Macromodel (version
6.5). Preliminary results showed 11m(a) to possess β-turn inducing properties, and 11m(b), 11m(c) and 11m(d) to adopt more extended conformations.

\[ 
\text{Figure 7.7. The blocked dipeptide model compounds 11m(a)-11m(d).} 
\]

7.3 MODELING OF VINYL SULFIDE CYCLIZED OCTAPEPTIDES (Paper III)

As part of a collaboration, the agonistic vinyl sulfide cyclized Ang II analogues 91 and 92 were also modeled on the octapeptide level by Gregory Nikiforovich, Washington University. The low-energy conformers of Ang II analogues 91 and 92 were fitted to the bioactive conformation of Ang II (number II), earlier proposed by Nikiforovich and Marshall.\textsuperscript{143} When the C\textsubscript{α} and C\textsubscript{β} atoms of Val\textsuperscript{3}-Phe\textsuperscript{8}, as well as the C-terminal carboxyl group were used in the fitting procedure, a good overlap was obtained. The conformers of analogue 91 (green) and 92 (magenta) with the best fit to Ang II (white) are displayed in Figure 7.8.

The general spatial positions of the pharmacophore side-chains of Tyr\textsuperscript{4}, His\textsuperscript{6} and Phe\textsuperscript{8}, and the C-terminal carboxyl group overlap well. The main difference can be seen in the C\textsubscript{α}-C\textsubscript{β} bond vector of the Tyr\textsuperscript{4} residue. It should be pointed out that the build up procedure employed in the modeling procedure does not specify low-energy conformations at the accuracy required to distinguish side-chain rotamers. However the particular conformations in Figure 7.8 are of low energy.

The agonistic vinyl sulfide cyclized Ang II analogues 91 and 92 have furthermore been used as valuable tools for computational modeling that has resulted recently in a refined model of the bioactive conformation of Ang II.\textsuperscript{146}
Figure 7.8. Low-energy conformers (relative energy less than 10 kcal/mol) of Ang II analogues 91 (green) and 92 (magenta), with the best fit to the proposed bioactive conformation of Ang II (white). The rms values were 1.1 Å. and 1.0 Å. for 91 and 92, respectively. Residues 3-8 are depicted.
8 CONCLUDING REMARKS

In this thesis I have described the synthesis of a number of protected aldehyde-functionalized building blocks and their utility for the cyclization of peptides. The results are summarized below:

- Five aldehyde-functionalized amino acid derived building blocks have been synthesized that are compatible with SPPS.

- The preparation of the building blocks required different protecting schemes, and several reductive and oxidative methodologies were utilized.

- The building blocks were incorporated into positions 3, 5 and/or 7 of angiotensin II (Ang II). Liberation of the aldehydes delivered bicyclic or monocyclic peptides. The effects of varying the side-chain lengths of the building blocks on the outcome of the cyclizations are summarized below:

  - The building block derived from L-aspartic acid, with a chain length of two carbon atoms, reacts by C-terminal directed bicyclization.

  - The building blocks derived from L-glutamic acid, with a chain length of three carbon atoms, reacts by N-terminal directed bicyclization.

  - The building block derived from L-2-aminoadipic acid, with the chain length of four carbon atoms reacts by monocyclization affording vinyl sulfide bridged systems.

  - The α,α-disubstituted tyrosine-derived building block, with a chain length of two carbon atoms reacts by C-terminal directed bicyclization.

We believe that the cyclization methods described should be sufficiently general in nature to be readily applicable to the synthesis of other target peptides.
• The cyclization methods described herein have been utilized for the synthesis of a number of constrained analogues of Ang II.

• Two of the synthesized Ang II analogues displayed high affinities and full agonist activities at the AT₁-receptor, and have proven to be useful tools in the search for information on the bioactive conformation of Ang II.

In conclusion, I think that this thesis has illustrated both the scope and limitations of using constrained peptide analogues to obtain information about bioactive conformations.

The position 3-5 cis- and trans- vinyl sulfide cyclized Ang II analogues displayed high affinities and full agonist properties. Active analogues constrained even further could be even more informative for the elucidation of the bioactive conformation of Ang II. This might be achieved by the synthesis of analogues similar to the vinyl sulfide cyclized derivatives, but containing tyrosine mimetics with conformationally constricted side-chains in position 4.32,196

The rigid i to i + 1 and i to i + 2 bicyclized Ang II analogues, of which one is very similar to the agonistic mercaptoproline containing analogue reported by Marshall,51 showed little or no affinity to the AT₁-receptor. This illustrates the fact that the topography of the pharmacophore groups must be mimicked in a rather exact manner, and that subtle differences in geometry may have strong impact on the affinity to the receptor.

Figure 8.1. Graphical summary.
ACKNOWLEDGEMENTS

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I would like to express my sincere gratitude to all present and former colleagues at the department, for making my time there very enjoyable. Special thanks to:

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----------

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10 References

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(192) CHO cells expressing the AT1-receptor were generously provided by Professor Thue Schwartz, University Department of Clinical Biochemistry, Rigshospitalet, Copenhagen, Denmark.
Errata

To

Synthesis of Aldehyde-Functionalized Building Blocks and Their Use for the Cyclization of Peptides

*Applications to Angiotensin II*

BY

PETRA JOHANNESSON

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