Design and Synthesis of Angiotensin IV Peptidomimetics Targeting the Insulin-Regulated Aminopeptidase (IRAP)

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Abstract

Peptidomimetics derived from the bioactive hexapeptide angiotensin IV (Ang IV, Val\(^1\)-Tyr\(^2\)-Ile\(^3\)-His\(^4\)-Pro\(^5\)-Phe\(^6\)) have been designed and synthesized. These peptidomimetics are aimed at inhibiting the insulin-regulated amino peptidase (IRAP), also known as the AT\(_4\) receptor. This membrane-bound zinc-metallopeptidase is currently under investigation regarding its potential as a target for cognitive enhancers. The work presented herein was based on stepwise replacement of the amino acid residues in Ang IV by natural and unnatural amino acids, non-peptidic building blocks, and also on the introduction of conformational constraints. Initially, we focused on the introduction of secondary structure mimetics and backbone mimetics. The C-terminal tripeptide His-Pro-Phe was successfully replaced by a \(\gamma\)-turn mimetic scaffold, 2-(aminomethyl)phenylacetic acid (AMPA), which was coupled via an amide bond to the carboxyl terminus of Val-Tyr-Ile. Substitution of Val-Tyr-Ile, Val-Tyr, Tyr-Ile and Tyr, respectively, by 4-hydroxydiphenylmethane scaffolds comprising a 1,3,5-substituted benzene ring as a central moiety unfortunately rendered peptidomimetics that were less potent than Ang IV. The subsequent approach involved the introduction of conformational constraints into Val-Tyr-Ile-AMPA by replacing Val and Ile by amino acid residues appropriate for disulfide cyclization or ring-closing metathesis. Chemically diverse structures encompassing an N-terminal 13- or 14-membered macrocyclic tripeptide and a C-terminal non-peptidic moiety were developed. Tyr\(^2\) and AMPA were modified to acquire further knowledge about the structure-activity relationships and, in addition, to improve the metabolic stability and reduce the polarity. Several of the compounds displayed a high capacity to inhibit IRAP and exhibited \(K_i\) values in the low nanomolar range. Hence, the new compounds were more than ten times more potent than the parent peptide Ang IV. Enhanced selectivity over the closely related aminopeptidase N (AP-N) was achieved, as well as improved stability against proteolysis by metallopeptidases present in the assays. However, additional investigations are required to elucidate the bioactive conformation(s) of the relatively flexible N-terminal macrocycles. The compounds presented in this thesis have provided important information on structure-activity relationships regarding the interaction of Ang IV-related pseudopeptides and peptidomimetics with IRAP. The best compounds in the series constitute important starting points for further discovery of Ang IV peptidomimetics suitable as tools in the investigation of IRAP and other potential targets for Ang IV. The literature presents strong support for the hypothesis that drug-like IRAP inhibitors would serve as a new type of future cognitive enhancers with potential use in the treatment of cognitive disorders, e.g. Alzheimer’s disease.

Keywords: angiotensin IV, AT4, insulin-regulated aminopeptidase (IRAP), inhibitor, peptidomimetics, \(\gamma\)-turn mimetic, macrocycle

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Till min familj
This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aia</td>
<td>4-amino-1,2,4,5-tetrahydro-indolo[2,3-c]-azepin-3-one</td>
</tr>
<tr>
<td>Ala</td>
<td>alanine</td>
</tr>
<tr>
<td>Alg</td>
<td>allylglycine</td>
</tr>
<tr>
<td>AMBA</td>
<td>2-(aminomethyl)benzoic acid</td>
</tr>
<tr>
<td>AMPA</td>
<td>2-(aminomethyl)phenylacetic acid</td>
</tr>
<tr>
<td>Ang</td>
<td>angiotensin</td>
</tr>
<tr>
<td>AP-N</td>
<td>aminopeptidase N</td>
</tr>
<tr>
<td>Arg</td>
<td>arginine</td>
</tr>
<tr>
<td>Asn</td>
<td>asparagine</td>
</tr>
<tr>
<td>Asp</td>
<td>aspartic acid</td>
</tr>
<tr>
<td>BA</td>
<td>benzylamine</td>
</tr>
<tr>
<td>β^3^hTyr</td>
<td>β^3^-homotyrosine</td>
</tr>
<tr>
<td>CHO-K1</td>
<td>Chinese hamster ovary cell line</td>
</tr>
<tr>
<td>Cys</td>
<td>cysteine</td>
</tr>
<tr>
<td>DCE</td>
<td>1,2-dichloroethane</td>
</tr>
<tr>
<td>DIC</td>
<td>N,N'-diisopropylcarbodiimide</td>
</tr>
<tr>
<td>DIEA</td>
<td>N,N-diisopropylethylamine</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Fmoc</td>
<td>9-fluorenylmethoxycarbonyl</td>
</tr>
<tr>
<td>FMPB AM resin</td>
<td>4-(4-formyl-3-methoxyphenoxy)butyrylaminomethyl resin</td>
</tr>
<tr>
<td>Glu</td>
<td>glutamic acid</td>
</tr>
<tr>
<td>Gln</td>
<td>glutamine</td>
</tr>
<tr>
<td>GLUT4</td>
<td>glucose transporter type 4</td>
</tr>
<tr>
<td>Gly</td>
<td>glycine</td>
</tr>
<tr>
<td>HATU</td>
<td>O-(7-azabenzo triazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate</td>
</tr>
<tr>
<td>HBTU</td>
<td>O-(benzo triazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate</td>
</tr>
<tr>
<td>Hcy</td>
<td>homocysteine</td>
</tr>
<tr>
<td>HEK293</td>
<td>human embryonic kidney cell line</td>
</tr>
<tr>
<td>HGII</td>
<td>Hoveyda-Grubbs catalyst 2nd generation</td>
</tr>
<tr>
<td>His</td>
<td>histidine</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>K_i</td>
<td>equilibrium dissociation constant</td>
</tr>
</tbody>
</table>
Ile  isoleucine
IRAP  insulin-regulated aminopeptidase
Leu  leucine
LVV-H7  Leu-Val-Val-hemorphin-7
Met  methionine
MeTyr  N-methyltyrosine
Nle  norleucine
NMR  nuclear magnetic resonance
NOE  nuclear Overhauser effect
Phe  phenylalanine
Pro  proline
RAS  renin-angiotensin system
RCM  ring-closing metathesis
SAR  structure-activity relationship
SD  standard deviation
$t$Bu  tert-butyl
TES  triethylsilane
TFA  trifluoroacetic acid
THF  tetrahydrofuran
Thr  threonine
Trp  tryptophan
Trt  trityl
Tyr  tyrosine
Val  valine
Wang resin  $p$-benzyloxybenzyl alcohol resin
Xaa  unspecified amino acid
Xaa$^y$  amino acid residue at position y
1 Introduction

1.1 Peptides and Peptidomimetics

Peptides are involved in a wide range of physiological processes and most often exert their actions by interaction with proteins and other types of macromolecules. Consequently, peptides are highly interesting as starting-points in drug discovery. The biologically active hexapeptide angiotensin IV (Ang IV; Val\textsuperscript{1}-Tyr\textsuperscript{2}-Ile\textsuperscript{3}-His\textsuperscript{4}-Pro\textsuperscript{5}-Phe\textsuperscript{6}, Figure 1), which appears to play an important role in cognitive processes, was the focus of the research presented in this thesis.

\[\text{Figure 1.} \text{ Angiotensin IV is a bioactive peptide consisting of six amino acid residues.}\]

However, there are certain limitations on the practical use of peptides, both as pharmaceuticals and as pharmacological tools. These limitations typically include poor oral bioavailability, high metabolic instability and also generally low selectivity. By making structural modifications it is possible to obtain information on the parts of a peptide that are important for its biological activity, and how these parts should be arranged spatially. The peptide character of the starting compound can thus be decreased, ultimately leading to non-peptide compounds with retained biological activity and improved pharmacokinetic properties, i.e. peptidomimetics. General strategies for the development of peptidomimetics by rational design starting from a bioactive peptide have been used in the work presented here. These strategies are briefly described in the following section.
1.2 Development of Peptidomimetics

General strategies, combining rational design and nuclear magnetic resonance (NMR) spectroscopy, X-ray crystallography and computational methods, can be used in the transformation of biologically active peptides into peptidomimetics.\textsuperscript{4,10-13} In the initial phase of the transformation process, truncations and deletions are used to identify the minimum bioactive sequence. Amino acid scans are then used to obtain knowledge about the importance of the different side-chains. The individual residues are replaced one at a time by natural or unnatural amino acids, providing information on structure-activity relationships (SARs). Next, constraints can be introduced to derive a hypothesis of the conformation(s) of the peptide when interacting with the biological target, i.e. the bioactive conformation(s). Local restrictions can be induced via modified amino acids, short-range cyclizations and backbone modifications, while long-range cyclizations and secondary structure mimetics provide global constraints. Examples of amino acid modifications commonly used in the development of peptidomimetics are illustrated in Figure 2.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Illustration of amino acid modifications used in the development of peptidomimetics.}
\end{figure}

A three-dimensional pharmacophore model can be established by an iterative process involving the complementary biophysical methods described above. The information collected can be used to develop topographical mimetics with the pharmacophoric groups arranged in the correct orientation on a suitable non-peptide scaffold.

Compounds $A$ and $B$ in Figure 3 were derived from the octapeptide angiotensin II (Ang II; Asp\textsuperscript{1}-Arg\textsuperscript{2}-Val\textsuperscript{3}-Tyr\textsuperscript{4}-Ile\textsuperscript{5}-His\textsuperscript{6}-Pro\textsuperscript{7}-Phe\textsuperscript{8}), which is one of the main effector peptides in the renin-angiotensin system (RAS) and known to activate the AT\textsubscript{1} and AT\textsubscript{2} receptors. The truncated analogue $A$ encompasses a functionalized aromatic scaffold coupled to a modified C-terminal tripeptide sequence.\textsuperscript{14} Analogue $B$, which has a considerable peptide character, incorporates a benzodiazepine-based $\beta$-turn mimetic replacing the Val\textsuperscript{3}-Tyr\textsuperscript{4}-Ile\textsuperscript{5} residues in Ang II.\textsuperscript{15} Both compounds are high-affinity AT\textsubscript{2} receptor agonists, and display selectivity for the AT\textsubscript{2} receptor over the AT\textsubscript{1} receptor. Compound $C$ represents an AT\textsubscript{2} receptor selective
peptidomimetic with no peptide character,\textsuperscript{16} which has been proven to be bioactive in animal models.\textsuperscript{17-19}

![Figure 3. Compounds A and B are derived from the bioactive peptide Ang II. Both are potent AT\(_2\) receptor agonists and display selectivity for the AT\(_2\) receptor over the AT\(_1\) receptor. A consists of a tripeptide connected to a functionalized aromatic scaffold,\textsuperscript{14} while B incorporates a benzodiazepine-based \(\beta\)-turn mimetic replacing the Val\(^1\)-Tyr\(^4\)-Ile\(^5\) residues in Ang II.\textsuperscript{15} Compound C is a drug-like Ang II peptidomimetic and selective AT\(_2\) receptor agonist.\textsuperscript{16}](image)

1.2.1 Global Constraints

Rigid analogues often benefit from improved properties, such as enhanced bioavailability, proteolytic stability and selectivity for the specific target. A reduction in the loss of conformational entropy upon interaction with the target can also be achieved.\textsuperscript{20} There are several functional groups in a peptide that can be utilized for the introduction of global constraints via cyclization (Figure 4), i.e. the N- and C-terminals, side-chain functional groups and atoms of the backbone.\textsuperscript{10,11,20,21} Those groups that are not involved in recognition of the target are chosen as sites for cyclization. Disulfide, lactam and carbon-carbon bonds are common bridging units.

![Figure 4. Strategies for the introduction of global conformational constraints via cyclization.](image)
Secondary structures such as α-helices, β-sheets and reverse turns are characteristic elements in protein and peptide chains. Such conformational components appear to be central in peptide-protein interactions. For example, β-strands, β-turns and γ-turns are suggested to be adopted by a range of short, flexible bioactive peptides upon interaction with receptors and enzymes. Therefore, templates capable of reproducing a specific type of secondary structure, i.e. secondary structure mimetics, are attractive in the development of peptidomimetics.

A turn corresponds to a site on a peptide or protein where the chain reverses its overall direction. β-Turns consist of four amino acid residues while γ-turns involve three amino acid residues (Figure 5). γ-Turns are stabilized by an intramolecular hydrogen bond between CO$_{i}$ and NH$_{i+2}$ forming a pseudo-seven-membered ring. There are two categories of γ-turns, classical ($\phi_{i+1}$ 70 to 85, $\psi_{i+1}$ -60 to -70) and inverse ($\phi_{i+1}$ -70 to -85, $\psi_{i+1}$ 60 to 70). The latter is considerably more common. The side-chain of residue $i+1$ in an inverse turn is oriented in an equatorial position, while it assumes an axial orientation in a classical turn.

A seven-membered cyclic γ-turn mimetic previously incorporated into Ang II, to replace Val$_{3}$-Tyr$_{4}$-Ile$_{5}$, is depicted in Figure 5 (D). The β-turn template in structure B (Figure 3) was utilized as a tripeptide mimetic with a tyrosine side-chain in the $i+1$ position and an isoleucine side-chain in the $i+2$ position.

![Figure 5](image)

**Figure 5.** Illustration of a β-turn and a γ-turn with the corresponding Phi (ϕ) and Psi (ψ) backbone torsion angles. Structure D is an example of a γ-turn mimetic moiety previously incorporated into Ang II to replace the tripeptide fragment Val-Tyr-Ile.

### 1.3 Solid-Phase Synthesis

The concept of performing organic reactions on insoluble supports was originally introduced by Merrifield for the synthesis of peptides, and has developed into a widely applied methodology in organic synthesis.
Typically, the reactions are performed by mixing an immobilized intermediate with a solution of appropriate reactants and reagents, followed by filtration and washing with suitable solvents.

Polystyrene (PS) cross-linked with divinylbenzene (DVB, 1–2%) is one of the most common supports used in solid-phase synthesis. Functionalization with suitable linkers (Figure 6) is necessary for the reversible covalent attachment of the intermediates to the solid support. A suitable linker should enable straightforward attachment of the first moiety, withstand the reaction conditions to be used, and permit selective cleavage of the temporary covalent bond after completion of the target compound. Furthermore, simultaneous selective deprotection and cleavage is also a frequent requirement. In addition to the supports for solid-phase synthesis, immobilized reagents and scavengers also constitute valuable tools in solution-phase synthesis.

![Functionalized polystyrene resins](image)

**Figure 6.** Functionalized polystyrene resins used in the work presented in this thesis: 2-chlorotrityl chloride resin, 4-(4-formyl-3-methoxyphenoxy)butyrylaminomethyl resin (FMPB AM resin), p-benzzyloxybenzyl alcohol resin (Wang resin), and [3-((methyl-Fmoc-amino)-methyl)indol-1-yl]acetyl AM resin (Methyl Indole AM resin).

Transformations can be monitored using colorimetric methods, spectroscopic techniques and small-scale cleavage of the compounds from the solid support followed by solution-phase characterization. An important aspect to consider is that the intermediates cannot be separated from immobilized precursors or by-products. Hence, there is a risk of ending up with a complex mixture of compounds if consecutive reactions are not driven to completion. Since each synthetic step is followed by filtration and washing, it is possible to use reagents in large excess and repeat a reaction step, thereby enhancing the conversion. Another strategy employed is to prevent further reaction by blocking any residual functional groups with a suitable capping agent. A modified protection strategy or altered reaction conditions can also lead to improvements.
1.3.1 Solid-Phase Peptide Synthesis

The first stage in solid-phase peptide synthesis (SPPS) is generally the anchoring of the C-terminal amino acid residue to the linker of an appropriate resin via the carboxyl group.\(^1\) The sequence of desired amino acids is then assembled from the C-terminus to the N-terminus and is thereafter released from the resin. Reactive functional groups other than those involved in solid-phase attachment and peptide bond formation, i.e. side-chain and N\(^\alpha\)-amino groups are blocked to prevent side-reactions. A temporary protecting group that can be selectively removed after each step is used for the N\(^\alpha\)-amino groups. The side-chain protecting groups, on the other hand, are frequently not removed until after assembly of the complete sequence. One of the main protection schemes used in SPPS relies on 9-fluorenylmethoxycarbonyl (Fmoc)/\(t\)-butyl (tBu) protection.\(^1\) The temporary N\(^\alpha\)-Fmoc\(^32\) group is removed under mild basic conditions (e.g. 20% piperidine/\(N, N\)-dimethylformamide), while side-chain deprotection and generally also peptide-resin cleavage are performed under acidic conditions (e.g. 95% trifluoroacetic acid). Many variables must be considered when planning the synthesis of a peptide or a modified peptide. The overall efficiency is affected by the choice of resin, protection strategy, reagents and reaction conditions. Careful attention must be paid to preventing side-reactions and preserving the chiral integrity of the amino acid residues. A vast number of resins, coupling reagents and protected amino acids have been developed and are commercially available, providing a wide variety of possibilities.\(^1\)

1.3.2 Microwave-Assisted Solid-Phase Synthesis

In recent years, controlled microwave heating has been increasingly used in organic synthesis in solution as well as on solid-phase, including peptide synthesis.\(^33-38\) Compared with conventional methods, in which heat is transferred via the reaction vessel, a more uniform temperature profile is produced across the reaction mixture with microwave heating. Polar molecules and ions in the reaction mixture absorb microwave energy and convert it into heat, which results in efficient, rapid internal heating. Some of the advantages observed with microwave heating are reduced reaction times, higher yields and improved purity.

The stability of the polymer-bound linkers is one of the main concerns when applying microwave heating in solid-phase synthesis.\(^39\) Stability tests under the desired conditions are used to confirm resistance to degradation. The properties of the solid support, such as mechanical stability and tolerance to heat, are also important.\(^38-40\) Magnetic stirring, as well as elevated temperatures, might destroy the solid support. However, PS/DVB as well as other polymeric supports have been found to tolerate high temperatures (>160 °C) for short reaction times.
Since the discovery of renin by Tigerstedt and Bergman in the late 19th century, the renin-angiotensin system has been extensively studied. However, new components and functions are still being revealed. Its involvement in the regulation of cardio-vascular, body fluid and electrolyte homeostasis is widely recognized. Angiotensin II (Figure 7) is the main effector peptide, and is known to act via the G-protein coupled receptors (GPCRs) of the AT$_1$ and AT$_2$ subtypes. Ang II is produced from the precursor protein angiotensinogen by two successive proteolytic cleavages, the first by the aspartyl protease renin resulting in the inactive fragment Ang I, and the second by the zinc-metallopeptidase angiotensin converting enzyme (ACE).

**Figure 7.** Part of the expanded RAS. Ang II is generated from angiotensinogen through processing by renin and ACE. This octapeptide is then further degraded by ACE2 to Ang-(1-7) or by aminopeptidase A (AP-A) and aminopeptidase N (AP-N) to Ang III and Ang IV. Alternative proteolytic pathways exist.

Ang II was long considered to be the end product of the RAS but is now known to be further processed to generate Ang-(1-7), Ang III (Ang-(2-8)), Ang IV (Ang-(3-8)) and other metabolites. These fragments have been
shown to mediate effects to various degrees through the interaction with the *Mas* proto-oncogene (Mas), AT₁, AT₂ and AT₄ receptors.⁴³,⁴⁶-⁴⁸

The view of the renin-angiotensin system has changed over the years. In addition to the classical systemic RAS, it is now known that local renin-angiotensin systems with independent actions exist in many tissues, and even intracellular RAS have been suggested.⁴⁴ Furthermore, alternative proteolytic pathways have been discovered and are under investigation.⁴⁴,⁴⁵

In the initial studies of Ang IV, the peptide displayed poor affinity for the AT₁ and AT₂ receptors and was also unable to mediate classical angiotensin-independent effects. Hence, it was thought to be an inactive fragment in the RAS.⁴¹,⁴³ However, in 1988 Braszko et al. reported that intracerebroventricular injection of Ang IV in rats improved processes related to memory and learning.⁴⁹ In the following investigations, the peptide was also found to have other central as well as vascular and renal effects (e.g. blood flow regulation).⁴³,⁵⁰,⁵¹ Only a few years after angiotensin IV was found to be bioactive, a specific type of binding site was identified,⁵² as discussed below.

### 2.1 The AT₄ Receptor

A high affinity binding site for Ang IV that was distinct from the AT₁ and AT₂ receptors was discovered in 1992, and was later named the AT₄ receptor.⁵⁰,⁵²,⁵³ Ang II, Ang III and the AT₁ receptor antagonist [Sar¹, Ile⁶]Ang II displayed low affinity for the new binding site, which was found in various mammalian tissues, e.g. heart, kidney, adrenal gland and blood vessels.⁴¹,⁴³,⁵¹ In brain, the highest densities of the AT₄ receptor were located in areas associated with cognitive, sensory and motor functions.

Several studies were performed to establish the structure-binding characteristics of Ang IV at the AT₄ receptor. In the first study, involving a glycine and D-amino acid scan, truncations and extensions, the critical importance of the N-terminal tripeptide Val-Tyr-Ile was revealed.⁵⁴ Replacement of the individual amino acid residues produced marked reductions in affinity. The C-terminal tripeptide was found to be important, but not to play an essential role. Structural modifications were tolerated as well as extensions. Deletion of Phe⁶ was allowed, but further C-terminal truncations reduced the affinity significantly.

Additional information was derived from side-chain and backbone modifications of the N-terminal tripeptide.⁵⁵,⁵⁶ Again, the L-configuration was shown to be a prerequisite at all three positions. Methylation and acetylation of the N-terminal amine was not tolerated, but introduction of Pro at position one only resulted in a 10-fold decrease in affinity. Hydrophobic residues, in particular norleucine, were preferred at position one. The metabolic stability was increased and the affinity was retained when a reduced peptide bond (Ψ[CH₂NH]) was introduced between residues.
Aromatic amino acid residues were found to be superior at position two, while hydrophobic ones (e.g. Pro, Leu, Phe) were favoured at position three.

In addition to Ang IV, the decapeptide Leu-Val-Val-hemorphin-7 (LVV-H7, Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe) was identified as an endogenous AT4 receptor ligand. The two peptides exhibited equivalent binding affinity to sheep cerebellar membranes, as determined from the ability to compete with the binding of [125I]Ang IV, and displayed full cross-displacement.57,58 Later, both Ang IV and LVV-H7 were found to potentiate acetylcholine release in rat hippocampus. Thus, LVV-H7 was defined as an AT4 receptor agonist.59 Further studies demonstrated that the Tyr4 residue was crucial for activity, and showed that Val-Tyr-Pro-Trp-Thr was the shortest sequence required for high-affinity binding.60 Divalinal Ang IV (Val-$\Psi$[CH$_2$NH]-Tyr-Val-$\Psi$[CH$_2$NH]-His-Pro-Phe), on the other hand, was established as being an antagonist capable of blocking AT4 dependent actions.61 The modified amide bonds made the peptide more resistant to degradation in rat kidney homogenates than Ang IV. Norleual (Nle-Tyr-Leu-$\Psi$[CH$_2$NH]-His-Pro-Phe) was also identified as a high affinity antagonist.62

A series of tripeptide analogues, represented by Nle-Tyr-Ile-benzylamine, and an analogous series of compounds incorporating pseudopeptide bonds were patented by Kobori et al.63,64 The compounds were evaluated regarding their abilities to compete for [125I]Ang IV binding in guinea pig hippocampus. It was found in both series, that a wide range of aromatic moieties was accepted at the C-terminus.

In 2001, the AT4 receptor was purified from bovine adrenal membranes, and identified as the insulin-regulated aminopeptidase (IRAP).65 The IRAP/AT4 receptor has attracted considerable attention in recent years due to its potential application as a drug target for the treatment of cognitive disorders.66-70

### 2.1.1 The Insulin-Regulated Aminopeptidase

The insulin-regulated aminopeptidase (IRAP, Figure 8) is a single-spanning transmembrane zinc-metallopeptidase belonging to the M1 family of aminopeptidases. Cystinyl aminopeptidase (CAP, EC 3.4.11.3) and placental leucine aminopeptidase (P-LAP, soluble human homologue) are two of several alternative names. IRAP was initially identified in adipocytes where it was localized in vesicles containing the insulin-regulated glucose transporter GLUT4.71 Upon stimulation by insulin, these vesicles are redistributed to the cell surface and fused to the plasma membrane. The tissue distribution of IRAP was found to be wider than that of GLUT4, and it has consequently been speculated that IRAP is involved in processes unrelated to insulin action. The enzyme cleaves the N-terminal amino acid residue from several small bioactive peptides in vitro, including Met- and
Leu-enkephalin, dynorphin A, neurokinin A, somatostatin, cholecystokinin-8, oxytocin and vasopressin.  

Figure 8. The current hypotheses explaining the mechanisms by which Ang IV and its analogues mediate their effects, as illustrated by Vanderheyden.  

A) Through inhibition of IRAP resulting in the accumulation of neuropeptide substrates;  
B) through AT₄ receptor activation with IRAP acting as a membrane-bound receptor;  
C) through prolongation of IRAP and GLUT4 exposure at the cell surface resulting in an increase in glucose uptake; and  
D) through activation of the hepatocyte growth factor (HGF) receptor, also known as mesenchymal-epithelial transition factor (c-Met).
IRAP consists of three domains; an intracellular region of 110 amino acid residues, which is involved in intracellular localization and redistribution; a hydrophobic transmembrane segment consisting of 22 amino acid residues, and an extracellular region of 893 amino acid residues enclosing the catalytic site.

The M1 family of aminopeptidases shares a common His-Glu-Xaa-Xaa-His-(Xaa)15-Glu zinc-binding motif essential for enzymatic activity, and a Gly-Xaa-Met-Glu-Asn (Xaa = Ala in IRAP) exopeptidase motif. The zinc ion is coordinated to the His residues (His464 and His468 in IRAP), the second Glu residue (Glu487) and a water molecule, which is believed to be activated by the other Glu residue (Glu465) for peptide hydrolysis. The Gly-Ala-Met-Glu-Asn motif is involved in N-terminal recognition, and is important for binding and alignment of both substrates and competitive inhibitors, as demonstrated by mutational analyses.

Three hypotheses have been put forward, explaining the mechanisms by which Ang IV and its analogues mediate their effects via IRAP (Figure 8). First, they might act as inhibitors and extend the half-life of important bioactive neuropeptides such as vasopressin, somatostatin and cholecystokinin-8. Another suggestion is that IRAP also acts as a classical receptor and transfers extracellular information across the cell membrane upon interaction with suitable ligands. Finally, it has been suggested that Ang IV and its analogues extend the cell surface localization of IRAP as well as GLUT4, thereby modulating glucose uptake in neurons and other cells. Alternative targets of Ang IV, e.g. the hepatocyte growth factor receptor (HGFR/c-Met), will be discussed in Section 2.1.4.

2.1.2 Binding Studies versus IRAP Activity Measurements

When the AT4 receptor was identified as IRAP, known receptor ligands were evaluated regarding both their binding affinity and inhibitory activity. Inhibitory activities were assessed from the cleavage of synthetic substrates in the absence of metal chelators, while binding affinities were routinely obtained from radioligand competition binding experiments in the presence of chelators (i.e. ethylenediaminetetraacetic acid (EDTA) and 1,10-phenanthroline). Ang IV, LVV-H7, [Nle1]Ang IV and divalinal-Ang IV were all found to be potent inhibitors. However, a pronounced difference was observed between the enzyme activities and the binding affinities. Lower potencies and an altered rank order were observed in the enzyme assay. The lack of correspondence between the enzyme activities and the binding affinities was suggested to be due to the presence and absence of zinc in the catalytic site, respectively. The chelators were soon found to remove the zinc atom from the catalytic site, supporting this hypothesis. Distinct pharmacological profiles were revealed for the catalytically active and apo-forms of IRAP, and this was suggested as an explanation of the observed
differences. Thus, chelators must be omitted when examining ligand binding under physiologically relevant conditions, which in turn requires Ang IV analogues of high stability. Such studies are expected to provide a better comparison with results from enzyme and functional assays. Enzyme assays are frequently used to assess the structural requirements of Ang IV peptidomimetics for interaction with IRAP in a biologically relevant context.

2.1.3 IRAP Inhibitors

Recently, several potent peptidomimetic and non-peptidic IRAP inhibitors have been discovered. Lukaszuk et al. performed a $\beta$-homoamino acid scan of Ang IV. Replacement of Val$^1$ and Phe$^6$ by (R)$\beta^2$hVal and $\beta^5$hPhe, respectively, rendered a metabolically stable and potent IRAP inhibitor with a high selectivity for IRAP over aminopeptidase N (AP-N) and the AT$_1$ receptor (AL-11, Figure 9). Subsequently, His$^4$ or the His$^4$-Pro$^5$ dipeptide was replaced by different conformationally constrained residues. In this series, AL-40 incorporating (R)$\beta^2$hVal$^1$ and Aia$^3$-Gly$^5$, displayed the best results (Figure 9). The selectivity and stability were high and the inhibitory effect was enhanced compared with AL-11.

![Figure 9. Recently identified peptidomimetic (AL-11 and AL-40)$^{96,97}$ and non-peptidic (HFI-437) IRAP inhibitors.$^{98}$](image)

Interestingly, Albiston et al. have identified a series of drug-like non-peptide IRAP inhibitors of high potency using a virtual screening approach (e.g. HFI-437, Figure 9). A homology model of the catalytic domain of IRAP was generated based on the equivalent domain of the related M1 aminopeptidase leukotriene A4 hydrolase. In vivo efficacy was demonstrated for the 4-pyridine analogue of HFI-437. The performance in both spatial working and recognition memory paradigms was improved after
administration to the lateral ventricles. These compounds are currently being evaluated with regard to their therapeutic potential as cognitive enhancers targeting IRAP.

2.1.4 Alternative Targets of Ang IV

Not only IRAP has been suggested as a target of Ang IV. It has also been hypothesized that the AT\textsubscript{4} receptor corresponds to the tyrosine kinase receptor c-Met, also known as the HGF receptor (Figure 8).\textsuperscript{43,99} The AT\textsubscript{4} receptor antagonist norleual was recently found to be capable of inhibiting HGF mediated effects at picomolar concentrations and block binding of \textsuperscript{\textsuperscript{125}I}\textsuperscript{HGF} to c-Met. As in the case of IRAP, this receptor has attracted attention since it is believed to be involved in memory and learning consolidation. Of particular interest are also the findings that c-Met play a role in multiple cancers. It appears possible that both c-Met and IRAP are responsible for the observed effects of Ang IV and related compounds. Ongoing studies will hopefully provide answers to the questions currently being debated.

In addition to c-Met, the metallopeptidases in the same family as IRAP, such as aminopeptidase N (AP-N), constitute possible targets.\textsuperscript{100} Both Ang IV and LVV-H7 have been found to inhibit AP-N activity, albeit with a more than 10-fold lower potency than IRAP.\textsuperscript{93}
3 Aims of the Present Studies

The overall aim of the present studies was to convert the hexapeptide angiotensin IV into drug-like peptidomimetics using an iterative approach involving truncations and cyclizations. Potent, metabolically stable and selective Ang IV peptidomimetics would serve as attractive pharmacological tools for further characterization of IRAP, a potential target for the development of cognitive enhancers.

The specific objectives of the studies were:

- To obtain information about the bioactive conformation(s) of Ang IV by the introduction of conformational constraints.

- To truncate the peptide chain and reduce the peptide character of Ang IV by:
  - the introduction of turn mimetics
  - the introduction of unnatural amino acids
  - side-chain modifications
  - backbone modifications
  - the introduction of conformational constraints by cyclization

- To study structure-activity relationships of the new Ang IV analogues regarding their interaction with IRAP.
4 Incorporation of Secondary Structure and Backbone Mimetics (Papers I and II)

4.1 Design

As discussed in Section 2.1, the initial structure-binding studies showed that the L-amino acid residues at positions one to three were requisite for high binding affinity and that the structural variations in the side-chains were rather restricted. However, extensive changes were possible in the C-terminal region without having significant effects on the binding affinity.\textsuperscript{54-56} A disulfide scan performed on Ang IV demonstrated that cyclization between residues one and three as well as between four and six was well tolerated (Table 1).\textsuperscript{101} Analogue 1, with a disulfide bond between the side-chains of two cysteine (Cys) residues incorporated at positions four and six, was almost equipotent to Ang IV. Compound 2, with a disulfide bond between the side-chains of two homocysteine (Hcy) residues incorporated at positions one and three, displayed a 10-fold lower affinity compared to Ang IV.

Table 1. Binding affinities of Ang IV, disulfide analogues 1–3,\textsuperscript{101} and the C-terminally amidated analogue 4.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sequence</th>
<th>Binding affinity\textsuperscript{a} $K_i$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang IV</td>
<td>Val-Tyr-Ile-His-Pro-Phe-OH</td>
<td>4.6 (IC\textsubscript{50})</td>
</tr>
<tr>
<td>1</td>
<td>c[Cys\textsuperscript{4}, Cys\textsuperscript{6}]Ang IV</td>
<td>6.5</td>
</tr>
<tr>
<td>2</td>
<td>c[Hcy\textsuperscript{1}, Hcy\textsuperscript{3}]Ang IV</td>
<td>56.7</td>
</tr>
<tr>
<td>3</td>
<td>c[Cys\textsuperscript{1}, Cys\textsuperscript{3}]Ang IV</td>
<td>5000</td>
</tr>
<tr>
<td>4</td>
<td>Val-Tyr-Ile-His-Pro-Phe-NH\textsubscript{2}</td>
<td>10.0</td>
</tr>
</tbody>
</table>

\textsuperscript{a}[\textsuperscript{125}I]Ang IV competition binding in porcine frontal cortex membranes.

Previous conformational studies related to Ang II have demonstrated that cyclizations between terminal Hcy and/or Cys residues in tripeptide segments (e.g. Ac-Hcy/Cys\textsuperscript{i}-Ala/Tyr\textsuperscript{i+1}-Hcy/Cys\textsuperscript{i+2}-NHMe) provide ring structures with a propensity to adopt inverse \(\gamma\)-turns as opposed to classical \(\gamma\)-turn and \(\beta\)-turn conformations.\textsuperscript{28,102} The capacity to induce inverse \(\gamma\)-turns was shown to increase with decreasing ring size and was particularly pronounced in model peptides incorporating two Cys residues. Since c[Cys\textsuperscript{4}, Cys\textsuperscript{6}]Ang IV (1) was found to be almost equipotent to Ang IV, it was
hypothesized that Ang IV adopts an inverse $\gamma$-turn in the C-terminal part, centered on Pro$^5$. Another type of bioactive conformation appeared to be more probable in the N-terminal region as c[Cys$^1$, Cys$^3$]Ang IV (3) displayed a 1000-fold lower binding affinity than Ang IV. The higher potency of the less rigid analogue c[Hcy$^1$, Hcy$^3$]Ang IV (2) was also indicative of alternative conformations in the N-terminal part, e.g. a $\beta$-turn or a more extended conformation.

Next, the Cys-Pro-Cys moiety in 1 was replaced with various aromatic scaffolds. The C-terminally amidated analogue of Ang IV (4) was first evaluated and found to display a 2-fold lower binding affinity than the unmodified peptide (Table 1). Therefore, the carboxyl group was retained in future studies. Accordingly, structures III–VI (Figure 10) were selected and a conformational analysis was performed on model compounds represented by IIIm and IVm (Figure 11).

![Figure 10. A summary of the initial structural modifications of Ang IV. Val$^1$-Tyr$^2$ and Val$^1$-Tyr$^2$-Ile$^3$ were replaced by turn mimetic I. A closely related structure (II) was incorporated as a backbone mimetic at position two, and the C-terminal tripeptide was replaced by a set of $\gamma$-turn mimetics (III–VI).](image-url)
It was shown that all compounds could adopt conformations similar to the suggested bioactive conformation of 1, and two examples are shown in Figure 11. The four proposed γ-turn mimetics III–VI were introduced to replace the C-terminal tripeptide of Ang IV. In addition to the rigid turn mimetics, a highly flexible 7-aminoheptanoic acid was incorporated for comparison.

Structures I and II (Figure 10) were initially designed and incorporated into Ang II to mimic an anticipated γ-turn-like conformation in the Tyr4 region upon interaction with the AT1 and AT2 receptors.14,103,104 The possibility of employing I as a replacement of Val1-Tyr2-Ile3 or Val1-Tyr2 in Ang IV was investigated. Subsequently, attempts were made to introduce structure II as a backbone mimetic at position two, in combination with 2-(aminomethyl)phenylacetic acid (AMPA, III) or 2-(aminomethyl)benzoic acid (AMBA) in the C-terminal end. Internal deletions and truncations of these analogues were also performed in order to obtain further insights into the structure-activity relationships.

![Figure 11](image.png)

**Figure 11.** Two out of the four scaffolds which were examined as γ-turn mimetics. *Top:* The best fit of the model compounds of III and VI to Ac-Cys-Pro-Cys adopting an inverse γ-turn. *Bottom:* Model compounds IIIm and IVm used in the modelling studies.
4.2 Synthesis

Two series of Ang IV analogues were synthesized and the first of these incorporated a single turn mimetic moiety in the N-terminal (I) or in the C-terminal part (III–VI). In the second series, Tyr² was replaced by II, and the C-terminal tripeptide was substituted for AMPA (III) or AMBA. A solid-phase strategy was adopted, in which appropriately protected building blocks were coupled to the desired amino acids in a step-wise fashion.

4.2.1 Secondary Structure and Backbone Mimetics

Intermediates 9 and 10 (Scheme 1) were prepared from ethyl 2-methylbenzoate (5) and ethyl 2-methylphenylacetate (6) by benzylic bromination, followed by substitution with sodium azide, and hydrolysis under basic conditions. In the synthesis of building block 13, the benzylic bromination of 3-methylphenylacetic acid (11) was followed by substitution with ammonia, and finally Fmoc-protection. The corresponding para-substituted analogue 16 was obtained by electrophilic aromatic substitution of phenylacetic acid (14) using N-(hydroxymethyl)phthalamide and trifluoroacetic acid (TFA), hydrazinolysis of the resulting phthalamide liberated an amine which was Fmoc-protected. In addition to the building blocks described above, commercially available Fmoc-3-aminomethylbenzoic acid (17) and Fmoc-7-aminoheptanoic acid (18) served as starting materials in the solid-phase synthesis of 32 and 33, respectively.

Scheme 1.

Reagents and conditions: (a) N-bromosuccinimide, 2,2′-azobis(2-methylpropionitrile), MeCN, MW, 90 °C, 15 min; (b) NaN₃, N,N-dimethylformamide (DMF); (c) LiOH, tetrahydrofuran (THF)/MeOH/H₂O; (d) N-bromosuccinimide, (C₆H₅COO)₂, CCl₄, reflux; (e) NH₂/EtOH; (f) Fmoc-Cl, Na₂CO₃ (10% aq.), 1,4-dioxane; (g) N-(hydroxymethyl)-phthalamide, TFA, reflux; (h) N₂H₄ (aq.), reflux.
Compound 27 was synthesized essentially according to the route previously described by our group (Scheme 2).\textsuperscript{103} The first step was accomplished by a Negishi coupling,\textsuperscript{108} as an alternative to the original Friedel-Crafts acylation. Conversion of $p$-bromoanisole (19) to the corresponding Grignard reagent followed by transmetallation afforded the organozinc reagent 20, which was instantly coupled with the acid chloride of 21 using bis(triphenylphosphine)-palladium(II) chloride as the catalyst. Complete removal of palladium traces remaining after column chromatography was necessary to avoid side-reactions in the subsequent step. This was achieved using 3-(1-thioureido)propyl-functionalized silica gel as a suitable palladium scavenger. The yield of the Negishi coupling was comparable to that reported for Friedel-Crafts acylation. Reduction of the ketone by ionic hydrogenation followed by hydrolysis of the ester afforded intermediate 25. Catalytic hydrogenation of the resulting aniline, and cleavage of the aromatic methyl ether by boron trifluoride dimethyl sulfide complex generated the desired building block 27. The analogous building block Fmoc-3-aminomethyl-5-(4′-Fmoc-oxybenzyl)benzoic acid (28), corresponding to the Fmoc-protected precursor of 1 was available in house.\textsuperscript{104}

Scheme 2.

Reagents: (a) (i) Mg, MeI, THF, (ii) ZnCl$_2$; (b) SOCl$_2$, CH$_2$Cl$_2$; (c) (i) PdCl$_2$(PPh$_3$)$_2$, THF, (ii) 3-(1-thioureido)propyl-functionalized silica gel, THF; (d) Et$_3$SiH, CF$_3$SO$_2$H, TFA, CH$_2$Cl$_2$; (e) LiOH, THF/MeOH/H$_2$O; (f) (i) H$_2$, Pd/C, EtOH, (ii) Fmoc-Cl, Na$_2$CO$_3$ (aq.), 1,4-dioxane; (g) BF$_3$:S(CH$_3$)$_2$, CH$_2$Cl$_2$.  

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4.2.2 Solid-Phase Synthesis

The target compounds were synthesized by SPPS following the Fmoc/tBu protection strategy (Section 1.3.1) as outlined in Scheme 3. Manual synthesis of 29–33 and 37–50 was performed using 2-chlorotrityl chloride resin, while Wang resin preloaded with the appropriate amino acids was utilized in automated synthesis of 34–36. After attachment of the first building block, the desired product was obtained by stepwise coupling of the appropriate amino acids and building blocks using O-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HBTU) or (benzotriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate (PyBOP) in the presence of N,N-diisopropylethylamine (DIEA) or N-methylmorpholine (NMM) in DMF. Capping of unreacted sites after solid-phase attachment and couplings were performed using MeOH and acetic anhydride in DMF, respectively. Removal of the Fmoc group was performed with 20% piperidine/DMF. After completion of the linear sequence and Fmoc-deprotection the analogues were released from the resin by treatment with TFA in the presence of triethylsilane (TES). The products were isolated as the corresponding TFA salts by lyophilization after preparative reversed-phase high-performance liquid chromatography (RP-HPLC).

Scheme 3 Solid-phase synthesis of analogues 29–50.

Reagents: (a) (i) DIEA, CH₂Cl₂, (ii) MeOH, (iii) 20% piperidine/DMF (when needed); (b) (i) HBTU or PyBOP, DIEA, DMF, (ii) 20% piperidine/DMF; (c) TFA/TES/H₂O; (d) (i) HBTU, NMM, DMF, (ii) 20% acetic anhydride/DMF, (iii) 20% piperidine/DMF; (e) (i) N,N′-diisopropylcarbodiimide (DIC), 1-hydroxybenzotriazole (HOBt), CH₂Cl₂, (ii) PBu₃, (iii) 20% piperidine/DMF.

Building blocks 9 and 10, incorporating an azide functionality, were used instead of the corresponding building blocks incorporating an Fmoc-protected amine, since the latter are known to detach from the solid-phase via intramolecular cyclization in the deprotection step. The amide bond to the subsequent moiety was formed by a Staudinger/aza-Wittig reaction, involving transformation of the immobilized azide to the corresponding iminophosphorane in the presence of a suitable coupling partner.
4.3 Biological Evaluation

4.3.1 Binding Affinity Assays
The binding affinities of compounds 4 (Table 1) and 29–35 (Table 2) were assessed using porcine frontal cortex membranes,\textsuperscript{101} while membranes of Chinese hamster ovary (CHO)-K1 cells \textsuperscript{92,109} were used in the evaluation of 36–50 (Paper II). Both assays rely on $[^{125}\text{I}]$Ang IV competition binding after preincubation with different concentrations of compounds in the presence of chelators (i.e. EDTA in the former assay, and EDTA and 1,10-phenanthroline in the latter assay). The binding affinities are reported as equilibrium dissociation constants ($K_i$ values).

4.3.2 Binding Studies Focusing on Metabolic Stability
The stability of 29 to degradation by metalloproteases was determined in the presence of CHO-K1 cell membranes containing endogenous IRAP (Table 3).\textsuperscript{92,109} Preincubation with different concentrations of 29, either in the absence or presence of chelators, i.e. EDTA and 1,10-phenanthroline, was followed by further incubation with the radioligand $[^{125}\text{I}]$Ang IV in the presence of chelators. Binding affinities in the continuous presence of chelators refer to the IRAP apo-enzyme.\textsuperscript{92,93} When preincubation proceeds in the absence of chelators the competition curve will shift to the right if the compound is rapidly degraded by IRAP and/or other metalloproteases. Thus, the smaller the difference between the $K_i$ (or $pK_i$) values the more stable the compound.

4.3.3 Enzyme Assay
The ability of compounds 29 (Table 3) and 36–50 (Table 4) to inhibit the catalytic activity of IRAP and AP-N was tested in membranes of human embryonic kidney 293 (HEK293) cells transfected with either recombinant human IRAP or AP-N.\textsuperscript{93,109} The inhibitory activities were assessed from cleavage of the substrate L-leucine-$p$-nitroanilide (L-Leu-$p$NA) into L-leucine and $p$-nitroaniline (absorption maximum at 405 nM) and are presented as $K_i$ values.

4.3.4 Results
Compounds 29–33 (Table 2) in the initial series of C-terminally modified analogues displayed IRAP binding affinities ranging from 1.9 to 69.7 nM. The best result was demonstrated by 29 ($K_i = 1.9$ nM) incorporating an ortho-substituted phenylacetic acid and this analogue was slightly more potent than Ang IV (IC$_{50} = 4.6$ nM) and c[Cys$^4$, Cys$^6$]Ang IV (1, $K_i = 6.5$ nM). The binding affinity decreased 5 times when the carboxymethyl group
was moved to the \textit{meta}-position (30), and an additional 7 times when it was located in the \textit{para}-position (31). The \textit{meta}-substituted benzoic acid in 32 afforded a 4 times less potent analogue compared to the corresponding \textit{meta}-substituted phenylacetic acid in 30. Replacement of the aromatic scaffold in 31 by a flexible carbon chain rendered a ligand (33) with slightly higher affinity to IRAP.

\textbf{Table 2.} Binding affinity of C-terminally modified Ang IV analogues.

\begin{center}
\begin{tabular}{lll}
\hline
Compound & R & Binding affinity$^a$ \\
& & $K_i$ (nM) \\
\hline
29 & \includegraphics[width=0.2\textwidth]{compound29} & 1.9 \\
30 & \includegraphics[width=0.2\textwidth]{compound30} & 9.7 \\
31 & \includegraphics[width=0.2\textwidth]{compound31} & 69.7 \\
32 & \includegraphics[width=0.2\textwidth]{compound32} & 37.6 \\
33 & \includegraphics[width=0.2\textwidth]{compound33} & 42.0 \\
\hline
\end{tabular}
\end{center}

$^a$[\textsuperscript{125}I]Ang IV competition binding in porcine frontal cortex membranes.

The introduction of $\gamma$-turn mimetic I into the N-terminal part of Ang IV as replacement of either Val-Tyr-Ile or Val-Tyr, provided I-His-Pro-Phe (34) and I-Ile-His-Pro-Phe (35) (Paper I). Analogue 34 was inactive ($K_i > 10,000$ nM), while 35 displayed micromolar affinity to IRAP ($K_i = 2180$ nM), as in the case of $c[Cys^1, Cys^3]$Ang IV (3, $K_i = 5000$ nM). As discussed in Section 4.1, it was hypothesized that Ang IV does not adopt a $\gamma$-turn at the N-terminus upon interaction with IRAP. This was based on the fact that 3, which belongs to a group of macrocycles with strong $\gamma$-turn-inducing properties, displayed low affinity, while the more flexible $c[Hcy^1, Hcy^3]$Ang IV (2, $K_i = 56.7$ nM) exhibited a 100-fold stronger binding affinity, and only a 10-fold lower affinity than Ang IV. Thus, the poor affinity observed upon introduction of $\gamma$-turn mimetic I in the N-terminal region supports this hypothesis.

The most potent compound (29) in the series of C-terminally modified analogues was also evaluated regarding its ability to inhibit the catalytic
activity of IRAP and AP-N (Table 3). It was found to be an effective IRAP inhibitor with a potency comparable to that of Ang IV and disulfide 1. The difference between the IRAP and AP-N inhibitory activity was approximately 10 times greater for 29 than for Ang IV and 1. Thus, the selectivity for IRAP over AP-N was increased by the introduction of γ-turn mimetic III. Both 29 and 1 were more stable than Ang IV to degradation by metalloproteases in CHO-K1 cell membranes, as determined from the difference in binding affinity in the presence and absence of metal chelators.

Table 3. Inhibition of IRAP and AP-N activity of Ang IV, disulfide 1 and tripeptide 29, and results from stability experiments.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IRAP $K_i \pm SD$ (nM)</th>
<th>AP-N $K_i \pm SD$ (nM)</th>
<th>Binding affinity $pK_i \pm SEM$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang IV$^b$</td>
<td>62.4 ± 17.5</td>
<td>841 ± 38</td>
<td>8.10 ± 0.09</td>
</tr>
<tr>
<td>1$^c$</td>
<td>25.8 ± 3.2</td>
<td>222 ± 28</td>
<td>7.92 ± 0.16</td>
</tr>
<tr>
<td>29</td>
<td>43.6 ± 1.5</td>
<td>951 ± 85</td>
<td>7.94 ± 0.17</td>
</tr>
</tbody>
</table>

SD = standard deviation. SEM = standard error of the mean. $^a$[125I]Ang IV competition binding in CHO-K1 cell membranes after preincubation in the presence or absence of EDTA and 1,10-phenanthroline (chelators). $^b$Data from Demaegd et al.93 $^c$Data from Axén et al.101

Analogue 29 was used as a starting point to prepare a series of compounds incorporating a 4-hydroxydiphenylmethane scaffold (II) at position two (Table 4). Replacement of Tyr2 in Ang IV by II rendered a 20 times less potent IRAP inhibitor (36), with a 1000-fold decrease in binding affinity to IRAP in CHO-K1 cell membranes ($K_i = 0.008$ nM vs. $K_i = 6.8 \mu$M, Paper II). The lower potency implies that optimal interactions with IRAP were prevented by the introduction of scaffold II. When the C-terminal tripeptide in 36 was substituted by AMPA an equipotent IRAP inhibitor (38) with a similar binding affinity was obtained. Analogous results were observed when Ang IV was transformed in the same way (cf. Ang IV and 29). Replacement of AMPA by AMBA in the C-terminus of 38 did not change the potency (cf. 37 and 38).

Additional information on structure-activity relationships was obtained by varying the amino acid residues at positions one and three. Val1 was replaced by Nle, while Ile3 was substituted by Leu or Pro. These amino acids are known to produce high-affinity peptide analogues of Ang IV, when incorporated at the analogous positions.55,56,91 In summary, Val1 was found to be replaceable with Nle (cf. 37 and 38 vs. 39 and 40, Table 5), which in turn could be combined with Leu at position three (41 and 42). Introduction of Pro at position three, in combination with Nle or Val at position one produced inhibitors of a slightly lower potency (37–42 vs. 43–46). Similar or slightly lower potency was observed for the analogues with AMBA instead of AMPA in the C-terminus.
### Table 4. Inhibition of IRAP and AP-N activity by compounds incorporating II at position two in combination with His-Pro-Phe, AMBA or AMPA at the C-terminus.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>R</th>
<th>Enzyme inhibition $K_i \pm SD$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>36</td>
<td>-His-Pro-Phe</td>
<td>1.2 ± 0.6</td>
<td>5.0 ± 0.3</td>
</tr>
<tr>
<td>37</td>
<td></td>
<td>1.3 ± 0.5</td>
<td>20 ± 4.0</td>
</tr>
<tr>
<td>38</td>
<td></td>
<td>1.3 ± 0.5</td>
<td>21 ± 1.6</td>
</tr>
<tr>
<td>39</td>
<td></td>
<td>1.8 ± 1.8</td>
<td>17 ± 2.5</td>
</tr>
<tr>
<td>40</td>
<td></td>
<td>1.4 ± 0.6</td>
<td>13 ± 1.1</td>
</tr>
<tr>
<td>41</td>
<td></td>
<td>3.7 ± 1.1</td>
<td>10 ± 0.6</td>
</tr>
<tr>
<td>42</td>
<td></td>
<td>3.9 ± 2.5</td>
<td>16 ± 3.1</td>
</tr>
<tr>
<td>43</td>
<td></td>
<td>6.3 ± 0.0</td>
<td>17 ± 2.5</td>
</tr>
<tr>
<td>44</td>
<td></td>
<td>3.3 ± 0.9</td>
<td>11 ± 0.5</td>
</tr>
<tr>
<td>45</td>
<td></td>
<td>12 ± 3.9</td>
<td>55 ± 8.4</td>
</tr>
<tr>
<td>46</td>
<td></td>
<td>7.4 ± 0.0</td>
<td>49 ± 1.9</td>
</tr>
</tbody>
</table>
Attempts were also made to truncate 38 at the C-terminus (47) and substitute not only Tyr\(^2\) but also Tyr\(^2\)-Ile\(^3\) (48), Val\(^1\)-Tyr\(^2\) (49) or Val\(^1\)-Tyr\(^2\)-Ile\(^3\) (50) for I. However, all these modifications gave compounds with lower IRAP activity (Table 5). The best inhibitor was obtained by the deletion of Ile (48), and this analogue was 10 times less potent as an IRAP inhibitor than 38.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sequence</th>
<th>IRAP</th>
<th>AP-N</th>
</tr>
</thead>
<tbody>
<tr>
<td>38</td>
<td>Val-II-Ile-AMPA</td>
<td>1.3 ± 0.5</td>
<td>21 ± 1.6</td>
</tr>
<tr>
<td>47</td>
<td>Val-II-Ile</td>
<td>76 ± 7.0</td>
<td>69 ± 7.7</td>
</tr>
<tr>
<td>48</td>
<td>Val-II-AMPA</td>
<td>16 ± 0.3</td>
<td>33 ± 5.2</td>
</tr>
<tr>
<td>49</td>
<td>II-Ile-AMPA</td>
<td>97 ± 38</td>
<td>&gt;100</td>
</tr>
<tr>
<td>50</td>
<td>II-AMPA</td>
<td>35 ± 4.9</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

Potent IRAP inhibitors that display a low AP-N inhibitory activity, or vice versa, would serve as valuable tools for investigating the involvement of IRAP and AP-N in mediating the effects of Ang IV. Similar to Ang IV, compounds 36–50 as well as 29 exhibited low IRAP over AP-N selectivity, ranging from 1 to 20 (Tables 3–5).

The fact that compounds 36–46 exhibit the same or slightly higher potency in the IRAP inhibition assay than in the binding assay (Paper II) is difficult to explain. As discussed above (Section 2.1.2) differences have previously been observed in enzyme activities and binding affinities. However, lower potencies have then been found in the enzyme assay.

In conclusion, the C-terminal tripeptide of Ang IV was found to be interchangeable with small aromatic \(\gamma\)-turn mimetics, specifically AMPA (29). The introduction of scaffold II as replacement of Tyr in Ang IV or Val-Tyr-Ile-AMPA, was found to have a pronounced impact on the bioactivity, and confirmed the importance of the N-terminus in binding and inhibition. The analogues were 20 times less effective as IRAP inhibitors than Ang IV, and exhibited approximately 1000 times lower binding affinity. Inhibitors of similar activity were obtained by replacing Val by Nle, and/or Ile by Leu or Pro in Val-II-Ile-AMPA (38), while truncations and internal deletions further reduced the activity. It was also found that AMPA was replaceable with AMBA in the C-terminus. The successful truncation of the parent hexapeptide to a tripeptide incorporating a small aromatic moiety in the C-terminus (i.e. 29) represents an important step in the development of Ang IV peptidomimetics.
5 Design and Synthesis of Macrocyclic Ang IV Analogues (Papers III and IV)

5.1 Design

As discussed previously, Val-Tyr-Ile-AMPA (29, Figure 12) was identified as a potent IRAP inhibitor. Computational studies demonstrated that AMPA could mimic an inverse γ-turn. Thus, the successful incorporation of the AMPA moiety replacing His4-Pro5-Phe6 in Ang IV supported the hypothesis that the hexapeptide adopts an inverse γ-turn centred on Pro5 in its bioactive conformation. The next step towards developing non-peptide IRAP inhibitors was to introduce conformational constraints in the N-terminal part, while keeping AMPA in the C-terminus. As discussed in Section 1.2.1, macrocyclization of linear molecules is frequently used to obtain information on the bioactive conformation. In addition, the cyclic analogues can benefit from better metabolic stability, membrane permeability, target affinity and selectivity.20,110-112 Of the previously reported Ang IV analogues incorporating a cyclic moiety in the N-terminal part, compound 2 ($K_i = 56.7$ nM) displayed the best binding affinity.101 This macrocycle was therefore selected to be combined with AMPA. The resulting compound (51, $K_i = 22.5$ nM, Figure 12) was found to be a potent IRAP inhibitor, and was used as the starting point in the discovery of new Ang IV peptidomimetics. Thus, compound 51 was subjected to a number of structural modifications (Figure 12).

Both the macrocycle and AMPA were modified to obtain information on SARs and to reduce the peptide character. Two different cyclic structures were investigated: one in which the Tyr2 residue was replaced by $\beta^3$-homotyrosine ($\beta^3$hTyr), and another where the Hcy3 residue was also replaced by Cys. To assess the importance of AMPA and its carboxyl group, it was replaced by methylamine and benzylamine (BA), respectively.

The disulfide moiety was then replaced by a carbon-carbon double bond of $E$ or $Z$ configuration, or a saturated carbon-carbon bond. Such transformations can, for example, increase the metabolic stability, and alter the conformational properties, as well as the biological activity.111-117 Moreover, many substrates of IRAP contain disulfide bonds, e.g. vasopressin, somatostatin and oxytocin.72-74 The introduction of a carbon-carbon bond will render compounds structurally related to Ang IV and
similar IRAP inhibitors, for example, [Nle\textsuperscript{1}]Ang IV,\textsuperscript{91} incorporating aliphatic carbon chains at positions one and three. In the series of dicarba analogues, the impact of the ring-size, and the type and position of the carbon-carbon bond were investigated. The effects of replacing Tyr\textsuperscript{2} by $\beta^3$hTyr, Phe or N-methyltyrosine (MeTyr) were studied, and also the possibility of replacing AMPA by BA.

![Diagram](image)

**Figure 12.** IRAP inhibitory activity for Ang IV, 1, 2, 29 and 51. The combination of the macrocycle in 2, with AMPA in the C-terminus of 29, rendered a potent IRAP inhibitor (51). The rectangles represent the parts of 51 that were subjected to structural modifications.

### 5.2 Synthesis of Disulfides

The target compounds 51–60 were prepared as outlined in Scheme 4. Linear sequences were assembled by manual Fmoc/tBu SPPS (described in Sections 1.3.1 and 4.2.2), and then subjected to oxidative cyclization. Thiol containing side-chains were trityl-protected (Trt) during the SPPS process. Tyrosine and $\beta^3$-homotyrosine were generally coupled using $\textit{O}$-(7-azabenzotriazol-1-yl)-$\textit{N},N,N',N'$-tetramethyluronium hexafluorophosphate (HATU)
and DIEA in DMF. Cysteine is known to be prone to racemization under these conditions. Racemization can, however, be reduced by using a more hindered and/or weaker base, solvent combinations of lower polarity, and in situ activation.\textsuperscript{118} Accordingly, HATU, 2,4,6-trimethylpyridine (TMP), and DMF/CH$_2$Cl$_2$ were used for cysteine, and generally also for homocysteine incorporation. To prevent the thiol groups from reacting with $t$Bu cations during side-chain deprotection and cleavage, threo-1,4-dimercaptopbutane-2,3-diol (DTT) was used in the cleavage cocktail. After purification, the linear intermediates were subjected to disulfide cyclization in dimethylsulfoxide (DMSO)/TFA.\textsuperscript{119-121}

Scheme 4.

Reagents: (a) (i) DIEA, CH$_2$Cl$_2$, (ii) MeOH; (a') NaBH(OAc)$_3$, AcOH/DMF (1:99); (a'') 20% piperidine/DMF; (b) (i) DIC, HOBt, CH$_2$Cl$_2$, (ii) PBu$_3$, (iii) 20% piperidine/DMF; (c) (i) HATU, DIEA, DMF, (ii) 20% piperidine/DMF; (d) (i) HATU, TMP, CH$_2$Cl$_2$/DMF, (ii) 20% piperidine/DMF; (e) DTT, TFA/TES/H$_2$O; (f) 10% DMSO/TFA; (g) (i) AcOH (5% aq.), (NH$_4$)$_2$CO$_3$ (0.5 M aq.), pH 6, (ii) DMSO.

Analogues 51, 52, 55, 56, 59 and 60 were synthesized by initial attachment of 10 to the 2-chlorotrityl chloride resin. The subsequent coupling of cysteine or homocysteine was achieved by a Staudinger/aza-Wittig reaction (Section 4.2.2), while standard couplings were used for the second and third amino acids. For the synthesis of analogues 53, 57 and 58, the appropriate amine was attached to an FMPB AM resin by reductive amination. The reaction was performed under microwave heating at 60 °C for 20 min using sodium triacetoxyborohydride in 1% AcOH/DMF.\textsuperscript{122} Colorimetric tests were used to monitor the first and second steps. The presence of aldehyde groups was evaluated by the $p$-anisaldehyde test,\textsuperscript{123} while the chloranil test was used to
determine the presence of secondary amines. Standard coupling conditions were used to introduce the subsequent amino acids. The oxidative cyclization of the linear analogue of 58, was performed at pH 6 using DMSO in aqueous AcOH/(NH₄)₂CO₃. It would also have been interesting to apply this method to the linear intermediates of the other target compounds, especially those incorporating AMPA in the C-terminus. As will be discussed below, those compounds were epimerized during oxidative cyclization under acidic conditions using DMSO/TFA. The Methyl Indole AM resin allowed straightforward synthesis of the C-terminally modified analogue 54. The resin was Fmoc-deprotected before assembly of the appropriate amino acids, cleavage and oxidative cyclization.

Compound 51 was initially synthesized on a 100 μmol scale. When the reaction scale was increased 3 times extensive epimerization was observed in the cyclization step with DMSO/TFA. In fact, all compounds incorporating AMPA in the C-terminus were epimerized under these conditions. A combined spectroscopic and computational approach, described in the following section, was used to establish the absolute configuration of the diastereomeric pairs.

5.3 Configurational Analysis of Epimerized Disulphides

The synthesis of 51, 55, and 59 following the synthetic route outlined in Scheme 4 generated three sets of closely related product pairs. The compounds displayed comparable chemical properties and gave similar NMR spectra. Comparison of HPLC retention times (Paper IV), and NMR data (Figures 13 and 14) for the compounds of unknown chirality with those for which the chirality was known (i.e. 51, 53, 54 and 57, derived from an epimerization-free synthesis), suggested an L-configuration of the amino acid residues at positions 1–3 for compounds 51, 55, and 59. Furthermore, while one of the products in each set most likely had the chirality of the corresponding linear intermediates (i.e. LLL-configuration), stereomutation of one chiral centre of the other isolated compound (52, 56, and 60) appeared most plausible. Assignment of the absolute configuration of the three diastereomeric pairs, confirming the simplified preliminary examination described above, was established by a combined spectroscopic and computational analysis using the program NAMFIS (NMR analysis of molecular flexibility in solution).
Figure 13. A region of the 400 MHz $^1$H NMR spectrum of 51, 52 and 53 in CD$_3$OD/D$_2$O 9:1 (0.1% TFA). The non-epimerized compound 53 (LLL-configuration) is comparable to compound 51 (LLL-configuration), while compound 52 differs (epimer).

Figure 14. A region of the 400 MHz $^1$H NMR spectrum of 55–57, 59 and 60 in CD$_3$OD/D$_2$O 9:1 (0.1% TFA). The non-epimerized compound 57 (LLL-configuration) is comparable to compound 55 and 59 (LLL-configuration), while compounds 56 and 60 differ (epimers).
The feasible conformational populations of the possible LLL, DLL, LDL and LLD diastereomers of 52, 56, and 60 were predicted by restraint-free systematic Monte Carlo conformational searches. Examination of the complete set of molecular geometries resulting from the conformational search revealed a low flexibility in the central macrocycles (Figure 15). This rigidity allowed identification of the chirality of the diastereomers using interproton distances and dihedral angles derived from measurements of nuclear Overhauser effects (NOEs) and scalar couplings, respectively. NAMFIS analysis127 was used to derive a conformational ensemble from the conformational search best fitting the thermally averaged NMR data (NOEs and J’s). This was done by varying the mole fractions of the theoretical conformations, and comparing the sum of the probability-weighted parameters of the best fitting conformers of each investigated configuration with the observed NMR data. Best agreement with the experimental data was found for the LLL-configuration for compounds 51, 55, and 59, and for the LLD-configuration for compounds 52, 56, and 59. The fact that epimerization was only observed for the compounds containing AMPA in the C-terminal, implies that the carboxyl group might be involved in interactions promoting epimerization at the stereocenter of the neighbouring amino acid residue.

**Figure 15.** An overlay of the backbones of all (228) conformations of c[Hcy-$\beta^3$hTyr-Hcy]-AMPA (59), within 10 kcal/mol from the global minimum generated by restraint-free systematic Monte Carlo conformational search. A remarkable rigidity of the central cyclic core is indicated resulting in well-defined orientations of the CH$^\alpha$ and amide NH protons. Part of AMPA and the side-chain of $\beta^3$hTyr are omitted for clarity.
5.4 Synthesis of Macrocyclic Analogues by RCM

Ring-closing metathesis (RCM) was the key-step in the synthesis of the dicarba analogues. Two different strategies were adopted: cyclization of the Fmoc-protected linear sequence either in solution or attached to the solid-phase. The synthetic routes are outlined in Schemes 5 and 6. The linear intermediates, containing two amino acid residues with a terminal olefin on the side-chain, were prepared by manual Fmoc/tBu SPPS (described in Sections 1.3.1, 4.2.2 and 5.2). Coupling of 10 to Wang resin was mediated using DIC and a catalytic amount of 4-dimethylaminopyridine (DMAP).

The initial strategy was to perform RCM with the Fmoc-protected linear intermediate attached to the resin (Scheme 5). The Wang and FMPB AM resins were chosen as they are commonly employed in solid-phase organic synthesis, and have been shown to withstand a variety of conditions, including high temperatures. Cyclization was performed in 1,2-dichloroethane (DCE) under microwave heating for 5 min, using 15 mol% Hoveyda-Grubbs 2nd generation catalyst (HGII), repeated once. Complex product mixtures were obtained, as a result of double-bond migration and ring-contraction, leading to tedious separation and low yields.

Scheme 5.

Reagents and conditions: (a) (i) DIC, DMAP, DMF/CH₂Cl₂, (ii) acetic anhydride, pyridine, DMF; (a') NaBH(OAc)₃, AcOH/DMF (1:99); (b) (i) DIC, HOBt, CH₂Cl₂, (ii) PBu₃, (iii) 20% piperidine/DMF; (c) (i) HATU, DIEA, DMF, (ii) 20% piperidine/ DMF; (c') (i) HBTU, DIEA, DMF, (ii) 20% piperidine/DMF; (d) (i) HGII, DCE, MW, 140 °C or 150 °C, 5 min, repeated once, (ii) DMSO, CH₂Cl₂, (iii) 20% piperidine/DMF; (e) TFA/TES/H₂O (90:5:5); (f) H₂, Pd/C, EtOH.
Compound 61 contains a double bond, which has isomerized one step after RCM. The saturated analogues, 62 and 63, obtained by catalytic hydrogenation of the remaining products, contain a 14- and 13-membered macrocycle, respectively. Compound 64 includes a saturated 13-membered macrocycle connected to BA instead of AMPA.

In order to gain better control over the cyclization step, a solution-phase approach was adopted (Scheme 6). The Fmoc-protected linear intermediates (65–69) were released from the solid-phase and subjected to RCM after purification. Benzoquinone and electron-deficient analogues, e.g. 2,6-dichloro-1,4-benzoquinone, are known to prevent undesirable isomerization during RCM. The effect has been ascribed to prevention of the formation of metal hydrides via decomposition of the catalyst, or to scavenging of the metal hydrides. A pronounced difference was observed when the RCM was performed in the presence of 15 mol% of either of the two quinones, and 7 mol% of HGII in DCE for 5 min at 120 °C. The E and Z isomers of the desired products were obtained, and only minor amounts of by-products were observed. The final compounds (70–79) were obtained by purification followed by Fmoc-deprotection with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in MeCN/DMF/DMSO, in the presence of 3-mercaptopropyl-functionalized silica gel. DMF and/or DMSO were needed to dissolve the lipophilic compounds.

Scheme 6.

Reagents and conditions: (a) HGII, 1,4-benzoquinone or 2,6-dichloro-1,4-benzoquinone, DCE, MW, 120 °C or 140 °C, 5 min; (b) DBU, 3-mercaptopropyl functionalized silica gel, MeCN/DMF/DMSO, 30 min.
The double-bond configurations of the compounds were established by primitive exclusive correlation spectroscopy (P.E.COSY) and nuclear Overhauser effect spectroscopy (NOESY) experiments.\textsuperscript{139-141} Hence, coupling constants were determined from the $f_2$ 1D trace of the corresponding 2D P.E. COSY spectra ($^3J_E = 15.2–18.0$ Hz, $^3J_Z = 8.5–14.2$ Hz). Where extensive signal overlap of the olefinic protons of the $Z$ isomers made the direct extraction of active couplings difficult, they were extracted as passive couplings from the cross peaks to the neighbouring methylene groups. The configurational assignment of the $E$ isomers was further confirmed by the detection of NOEs between the methylene protons on either side of the double bond to the olefinic protons (Figure 16). In contrast, $Z$ configuration results in strong NOEs only between the neighbouring methylene and olefinic protons.

5.5 Biological Evaluation

5.5.1 Inhibition of IRAP and AP-N Activity

The 13- and 14-membered macrocyclic compounds were evaluated as described in 4.3.3. The $K_i$ values of 2 and 51–60, containing a disulfide bridge, are presented in Tables 6–8, while the results of the dicarba analogues 61–64, and 70–79 are presented in Tables 9–10. The selectivity for IRAP over AP-N is defined by the quotient of the AP-N activity ($K_i$) and IRAP activity ($K_i$). Of the 25 IRAP inhibitors, 21 inhibitors displayed $K_i$ values in the nanomolar range, and 11 inhibitors were more potent than Ang IV.

The combination of the 13-membered ring in 2 with AMPA resulted in an IRAP inhibitor (51) that was 3 times more potent than Ang IV, and 13 times more potent than 2 (Table 6). It was also more selective for IRAP over AP-
N. The corresponding epimer (52), with D-configuration at position three, was a weaker IRAP inhibitor almost equipotent to 2. When AMPA was replaced by benzylamine (53) and methylamine (54), the IRAP activity decreased 4 and 79 times, respectively. Thus, the carboxyl group was not essential for high potency, but substitution of the aromatic moiety for a methyl group was poorly tolerated.

Table 6. Inhibition of IRAP and AP-N activity by Ang IV and analogues incorporating a 13-membered c[Hcy-Tyr-Hcy]-type of macrocycle.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sequence/Structure</th>
<th>Enzyme inhibition (nM)</th>
<th>Kᵢ (AP-N)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IRAP ± SD</td>
<td>AP-N</td>
</tr>
<tr>
<td>Ang IV</td>
<td>Val-Tyr-Ile-His-Pro-Phe</td>
<td>62.4 ± 17.5</td>
<td>841 ± 38</td>
</tr>
<tr>
<td>2</td>
<td>c[Hcy-Tyr-Hcy]-His-Pro-Phe</td>
<td>303 ± 159</td>
<td>5974 ± 1450</td>
</tr>
<tr>
<td>51</td>
<td></td>
<td>22.5 ± 6.0</td>
<td>50 000 ± 36 000</td>
</tr>
<tr>
<td>52</td>
<td></td>
<td>282 ± 113</td>
<td>3588 ± 2293</td>
</tr>
<tr>
<td>53</td>
<td></td>
<td>95.7 ± 26.6</td>
<td>11 100 ± 1900</td>
</tr>
<tr>
<td>54</td>
<td></td>
<td>1752 ± 1257</td>
<td>16 800 ± 4800</td>
</tr>
</tbody>
</table>

aData from Demaegdt et al.93

Replacement of Tyr² by β³hTyr, and Hcy³ by Cys in 51, gave a 7 times more potent inhibitor with a Kᵢ value of 3.3 nM (55, Table 7). Similarly to 51, compound 55 displayed high selectivity for IRAP over AP-N, and a better potency than the corresponding LLD-epimer (56). Likewise, replacement of AMPA by BA resulted in a 6 times less potent inhibitor (57). The
introduction of a C-terminal 3-pyridyl functionality reduced the activity 28 times (58). Hence, among the two series of 13-membered macrocycles it was found that the second type (c[Hcy-β³hTyr-Cys]) delivered more potent inhibitors than the first type (c[Hcy-Tyr-Hcy]), and also that AMPA was slightly preferred over BA in the C-terminal.

Table 7. Inhibition of IRAP and AP-N activity by compounds incorporating a 13-membered c[Hcy-β³hTyr-Hcy]-type of macrocycle.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Enzyme inhibition</th>
<th>Kᵢ (AP-N)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Kᵢ ± SD (nM)</td>
<td>IRAP</td>
</tr>
<tr>
<td>55</td>
<td><img src="image" alt="Structure 55" /></td>
<td>3.3 ± 0.8</td>
<td>7245 ± 3897</td>
</tr>
<tr>
<td>56</td>
<td><img src="image" alt="Structure 56" /></td>
<td>242 ± 72</td>
<td>10 800 ± 2700</td>
</tr>
<tr>
<td>57</td>
<td><img src="image" alt="Structure 57" /></td>
<td>18.5 ± 4.8</td>
<td>48 900 ± 3700</td>
</tr>
<tr>
<td>58</td>
<td><img src="image" alt="Structure 58" /></td>
<td>93.7 ± 9.9</td>
<td>9217 ± 407</td>
</tr>
</tbody>
</table>

A third type of macrocycle was created by using β³hTyr in combination with two Hcy residues (Table 8). Inhibitors 59 and 60, containing a 14-membered ring-system, exhibited a potency similar to 55 and 56, respectively. Thus, for all three types of macrocyclic compounds the configuration at the third
position was found to be important. Compounds 52, 56 and 60 with LLD-configuration, displayed similar $K_i$ values, and were less efficient IRAP inhibitors than their corresponding LLL epimers 51, 55 and 59. The most potent inhibitors 55 and 59 also showed a high selectivity for IRAP.

Table 8. Inhibition of IRAP and AP-N activity by compounds incorporating a 14-membered c[Hcy-$\beta^3$hTyr-Hcy]-type of macrocycle.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Enzyme inhibition $K_i \pm SD$ (nM)</th>
<th>$K_i$ (AP-N)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IRAP</td>
<td>AP-N</td>
</tr>
<tr>
<td>59</td>
<td>5.2 ± 0.8</td>
<td>5436 ± 2621</td>
</tr>
<tr>
<td>60</td>
<td>230 ± 53</td>
<td>11 900 ± 5300</td>
</tr>
</tbody>
</table>

The following types of compounds contain double bonds of $E$ or $Z$ configuration, or more flexible saturated carbon-carbon bonds in the ring-structures instead of disulfide bonds (Table 9). The 13-membered macrocyclic inhibitors 63, 70 and 71, containing a saturated carbon bridge, an $E$-olefin and a $Z$-olefin, respectively, were all comparable to Ang IV and the corresponding disulfide (51, $K_i = 22.5$ nM). Hence, the structural modifications in the macrocycle did not change the IRAP activity significantly. Similarly to the disulfides, the replacement of AMPA by BA in 63 resulted in a 3 times less potent inhibitor (64). When the 13-membered macrocycle in 63 ($K_i = 64.2$ nM) was enlarged with one carbon, an approximately 3 times better inhibitor was obtained (62). Furthermore, the introduction of an olefin in the resulting 14-membered ring rendered a 6 times more potent IRAP inhibitor (61, $K_i = 4.1$ nM), with more than 1000 times selectivity for IRAP over AP-N inhibition. All the other dicarba analogues inhibited IRAP with less than 480 times selectivity over AP-N. Inhibitor 72 ($K_i = 1.8$ nM), containing a $\beta^3$hTyr residue in position two, showed a potency similar to the analogous disulfide 59 ($K_i = 5.2$ nM), while the $Z$ isomer of 72 was less active (73, $K_i = 30.4$ nM). In contrast, the corresponding 13-membered $E$ and $Z$ isomers, 70 and 71, were found to be
Table 9. Inhibition of IRAP and AP-N activity by Ang IV and dicarba analogues 61–64 and 70–75.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ring size</th>
<th>Sequence/Structure</th>
<th>Enzyme inhibition</th>
<th>$K_i$ (AP-N)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>$K_i \pm SD$ (nM)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IRAP</td>
<td>AP-N</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$K_i$ (IRAP)</td>
<td></td>
</tr>
<tr>
<td>Ang IV$^a$</td>
<td></td>
<td>Val-Tyr-Ile-His-Pro-Phe</td>
<td>62.4 ± 17.5</td>
<td>841 ± 38</td>
</tr>
<tr>
<td>61</td>
<td>14</td>
<td><img src="image1" alt="Image" /></td>
<td>4.1 ± 0.2</td>
<td>4955 ± 490</td>
</tr>
<tr>
<td>62</td>
<td>14</td>
<td><img src="image2" alt="Image" /></td>
<td>25.0 ± 4.0</td>
<td>12 000 ± 3500</td>
</tr>
<tr>
<td>63</td>
<td>13</td>
<td><img src="image3" alt="Image" /></td>
<td>64.2 ± 9.2</td>
<td>3283 ± 83</td>
</tr>
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<td><img src="image4" alt="Image" /></td>
<td>193 ± 50</td>
<td>8345 ± 239</td>
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<tr>
<td>70</td>
<td>13</td>
<td><img src="image5" alt="Image" /></td>
<td>50.0 ± 5.5</td>
<td>1074 ± 206</td>
</tr>
<tr>
<td>71</td>
<td>13</td>
<td><img src="image6" alt="Image" /></td>
<td>41.1 ± 9.7</td>
<td>5413 ± 1656</td>
</tr>
</tbody>
</table>

continued on next page
<table>
<thead>
<tr>
<th>Compound Ring size</th>
<th>Sequence/Structure</th>
<th>Enzyme inhibition $K_i \pm SD$ (nM)</th>
<th>$K_i$ (AP-N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>72 14</td>
<td><img src="image1" alt="Structure" /></td>
<td>1.8 ± 0.1 240 ± 28 133</td>
<td></td>
</tr>
<tr>
<td>73 14</td>
<td><img src="image2" alt="Structure" /></td>
<td>30.4 ± 0.8 1299 ± 260 43</td>
<td></td>
</tr>
<tr>
<td>74 13</td>
<td><img src="image3" alt="Structure" /></td>
<td>697 ± 20 1571 ± 163 2</td>
<td></td>
</tr>
<tr>
<td>75 13</td>
<td><img src="image4" alt="Structure" /></td>
<td>351 ± 51 12 000 ± 1400 34</td>
<td></td>
</tr>
</tbody>
</table>

aData from Demaegdt et al.93

almost equipotent. Compound 72 exhibited the best inhibitory activity among the Ang IV analogues in the series, but it was unfortunately also found to be the most potent AP-N inhibitor ($K_i = 240$ nM).

As discussed above, the inhibitors encompassing 13-membered rings exhibited similar activities. However, reduced activities were observed for compounds 74 and 75, most likely due to the Phe residue in position two, and the BA moiety at the C-terminus (cf. 70 and 71). Thus, the hydroxyl group of Tyr² is anticipated to be beneficial, but not a prerequisite for activity. Further studies are required to assess the importance of the hydroxyl group of Tyr².

The previous replacements of AMPA by BA were shown to reduce the IRAP activity only about 5-fold. The more drug-like BA analogue of inhibitor 1 ($K_i = 4.1$ nM) was therefore selected for $N$-methylation at the Tyr² residue (Table 10). These modifications rendered an inhibitor with
significantly decreased potency compared to the other evaluated macrocyclic analogues (76). Hindered coordination of the catalytic zinc ion by the amide nitrogen, and possibly also by the oxygen may explain the outcome of the N-methylation.\textsuperscript{82,85,142,143} However, the amide oxygen has previously been shown not to be essential for high potency. The Ang IV analogue divalinal-Ang IV ($K_i = 194$ nM), incorporating a reduced peptide bond between residues one and two, and between residues three and four, was found to be only 3 times less active than Ang IV.\textsuperscript{93} The configuration of the double bond did not change the activity (cf. 76 and 77), while a one step alteration of the double bond position resulted in a 2- to 3-fold decrease in activity (78 and 79).

<table>
<thead>
<tr>
<th>Compound Ring size</th>
<th>Sequence/Structure</th>
<th>Enzyme inhibition $K_i \pm SD$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>76 14</td>
<td><img src="image1" alt="Chemical Structure" /></td>
<td>5845 ± 1080 N.M.</td>
</tr>
<tr>
<td>77 14</td>
<td><img src="image2" alt="Chemical Structure" /></td>
<td>4011 ± 1513 N.M.</td>
</tr>
<tr>
<td>78 14</td>
<td><img src="image3" alt="Chemical Structure" /></td>
<td>12 000 ± 5400 N.M.</td>
</tr>
<tr>
<td>79 14</td>
<td><img src="image4" alt="Chemical Structure" /></td>
<td>10 800 ± 5000 N.M.</td>
</tr>
</tbody>
</table>

N.M. means not measurable (>100 μM).
5.5.2 Stability Experiments

Ang IV and the majority of macrocyclic compounds were evaluated as described in 4.3.2 using the radioligand [3H]AL-11. The \( K_i \) values of a selection of the most potent IRAP inhibitors are presented Table 11. A large difference between the \( K_i \) values obtained from the binding assay, with or without chelators, indicates that a compound is rapidly degraded in the CHO-K1 cell membranes. The chelators have the ability to block the proteolytic activity of IRAP, AP-N, and other metalloproteases.

**Table 11.** Results of stability experiments for Ang IV, 2 and selected potent IRAP inhibitors.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Binding affinity( ^a ) ( K_i \pm SD ) (nM)</th>
<th>( K_i ) (No chelators) / ( K_i ) (Chelators)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang IV</td>
<td>9.3 ± 2.9 / 1139 ± 1329</td>
<td>122</td>
</tr>
<tr>
<td>2</td>
<td>196 ± 17 / 1488 ± 89</td>
<td>8</td>
</tr>
<tr>
<td>51</td>
<td>25.3 ± 21.6 / 1501 ± 1167</td>
<td>59</td>
</tr>
<tr>
<td>55</td>
<td>35.1 ± 19.3 / 1265 ± 6</td>
<td>36</td>
</tr>
<tr>
<td>59</td>
<td>10.0 ± 5.6 / 357 ± 9</td>
<td>36</td>
</tr>
<tr>
<td>61</td>
<td>46.8 ± 35.3 / 133 ± 14</td>
<td>3</td>
</tr>
<tr>
<td>62</td>
<td>12.5 ± 0.5 / 517 ± 119</td>
<td>41</td>
</tr>
<tr>
<td>72</td>
<td>9.8 ± 0.6 / 74.2 ± 6.6</td>
<td>8</td>
</tr>
</tbody>
</table>

\( ^a \) [3H]AL-11 competition binding in CHO-K1 cell membranes.

Replacement of Val\(^1\) and Ile\(^3\) by Hcy residues followed by oxidative cyclization delivers an analogue with significantly increased stability compared to the parent peptide (cf. Ang IV and 2). Compound 29, containing AMPA in the C-terminus, exhibits a stability similar to 2 (Table 3). An inhibitor (51) with decreased stability was obtained by combining the N-terminal macrocycle of 2 with AMPA in 29, however, it is still more stable than Ang IV. The analogous compounds 55 and 59, incorporating different macrocycles (i.e. c[Hcy-\( \beta^3 \)hTyr-Cys] and c[Hcy-\( \beta^3 \)hTyr-Hcy]), displayed similar results. Hence, the introduction of a \( \beta \)-homoamino acid in position two did not increase the stability. The introduction of \( \beta^2 \)hVal\(^1\), on the other hand, has been shown to afford metabolically stable Ang IV analogues.\(^96\) By replacing the disulfides by carbon-carbon bonds the stability could be increased. The two best inhibitors among the dicarba analogues, 61 and 72, also exhibited the best stability. A less potent, selective and stable compound (62) was obtained when the olefin in 61 was reduced.
5.5.3 Summary

A wide variety of 13- and 14-membered macrocycles, incorporating a disulfide, olefin or saturated carbon-carbon bridge, could be introduced in the N-terminal part to deliver potent IRAP inhibitors. Replacement of the Tyr² residue with a β3hTyr rendered potent IRAP inhibitors, while N-methylation was found to be deleterious for activity, conceivably due to hindered coordination to the catalytic zinc ion. Removal of the carboxyl group in the C-terminal part afforded inhibitors of 5 times lower potency, and replacement of the aromatic moiety by a methyl group was highly unfavorable. L-Configuration was preferred over D-configuration in position three, suggesting that the spatial arrangement of the functional groups at the C-terminal end is important for activity. The highest selectivity over AP-N was displayed by the most potent IRAP inhibitors, and peptidomimetic 61 displayed the best stability. Together with 55, 59, and 72, this compound will serve as starting point for further optimization, and investigations to elucidate the bioactive conformations(s) of the N-terminal macrocycles. Proposed recognition elements important for IRAP interaction are presented in Figure 17.

Figure 17. The rectangles represent proposed recognition elements important for IRAP interaction of Ang IV and the Ang IV peptidomimetics as exemplified with 61. Alternatively, the amide oxygen is coordinated to the catalytic zinc ion.
6 Concluding Remarks

This thesis describes the design and synthesis of Ang IV peptidomimetics targeting IRAP. Stepwise replacement of the amino acid residues in Ang IV by natural and unnatural amino acids, non-peptidic building blocks, and the introduction of conformational constraints have led to the discovery of novel potent inhibitors and a better understanding of the SARs. The results are summarized below.

- The C-terminal tripeptide His$^{4}$-Pro$^{5}$-Phe$^{6}$ in Ang IV was successfully replaced by small aromatic γ-turn mimetics, specifically AMPA (29).

- Inhibitors in the low micromolar range (e.g. 38) were obtained by replacing one or more of the amino acid residues in the N-terminal Val$^{1}$-Tyr$^{2}$-Ile$^{3}$ sequence by 4-hydroxydiphenylmethane scaffolds. The importance of the N-terminal tripeptide for IRAP interaction was indicated by the decreased potency of these compounds.

- Compounds with low nanomolar inhibitory activity and high selectivity for IRAP over AP-N were obtained by combining the C-terminal aromatic moiety AMPA with different 13- and 14-membered macrocyclic disulfides replacing the N-terminal tripeptide Val$^{1}$-Tyr$^{2}$-Ile$^{3}$ (e.g. 55). The Tyr$^{2}$ residue could be replaced with β$^{3}$hTyr.

- Replacement of the disulfide bond by a carbon-carbon double bond or a saturated carbon-carbon bond rendered inhibitors with similar potency, and generally improved metabolic stability (e.g. 61).
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_Hanna, April 2010_
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