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Computational Modeling of the AT_2 Receptor and AT_2 Receptor Ligands

Investigating Ligand Binding, Structure–Activity Relationships, and Receptor-Bound Models

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Abstract
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Rational conversion of biologically active peptides to nonpeptide compounds with retained activity is an appealing approach in drug development. One important objective of the work presented in this thesis was to use computational modeling to aid in such a conversion of the peptide angiotensin II (Ang II, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe). An equally important objective was to gain an understanding of the requirements for ligand binding to the Ang II receptors, with a focus on interactions with the AT_2 receptor.

The bioactive conformation of a peptide can provide important guidance in peptidomimetic design. By designing and introducing well-defined secondary structure mimetics into Ang II the bioactive conformation can be addressed. In this work, both γ- and β-turn mimetic scaffolds have been designed and characterized for incorporation into Ang II. Using conformational analysis and the pharmacophore recognition method DISCO, a model was derived of the binding mode of the pseudopeptide Ang II analogues. This model indicated that the positioning of the Arg side chain was important for AT_2 receptor binding, which was also supported when the structure–activity relationship of Ang II was investigated by performing a glycine scan.

To further examine ligand binding, a 3D model of the AT_2 receptor was constructed employing homology modeling. Using this receptor model in a docking study of the ligands, binding modes were identified that were in agreement with data from point-mutation studies of the AT_2 receptor.

By investigating truncated Ang II analogues, small pseudopeptides were developed that were structurally similar to nonpeptide AT_2 receptor ligands. For further guidance in ligand design of nonpeptide compounds, three-dimensional quantitative structure–activity relationship models for AT_1 and AT_2 receptor affinity as well as selectivity were derived.

Keywords: Angiotensin II, AT1, AT2, SAR, bioactive conformation, turn mimetic, peptidomimetic, DISCO, homology model, 3D-QSAR, CoMFA

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“1-2-3-4!”

Douglas Colvin

List of Papers

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

I. Rosenström, U.; Sköld, C.; Lindeberg, G.; Botros, M.; Nyberg, F.; Karlén, A.; Hallberg, A. A selective AT_2 receptor ligand with a γ-turn-like mimetic replacing the amino acid residues 4–5 of angiotensin II. *J. Med. Chem.*, **2004**, 47, 859–870.

II. Rosenström, U.; Sköld, C.; Lindeberg, G.; Botros, M.; Nyberg, F.; Hallberg, A.; Karlén, A. Synthesis and AT_2 receptor-binding properties of angiotensin II analogues. *J. Peptide Res.*, **2004**, 64, 194–201.

III. Rosenström, U.; Sköld, C.; Plouffe, B.; Beaudry, H.; Lindeberg, G.; Botros, M.; Nyberg, F.; Wolf, G.; Karlén, A.; Gallo-Payet, N.; Hallberg, A. New selective AT_2 receptor ligands encompassing a γ-turn mimetic replacing the amino acid residues 4–5 of angiotensin II act as agonists. *J. Med. Chem.*, **2005**, 48, 4009–4024.

IV. Georgsson, J.; Sköld, C.; Plouffe, B.; Lindeberg, G.; Botros, M.; Larhed, M.; Nyberg, F.; Gallo-Payet, N.; Gogoll, A.; Karlén, A.; Hallberg, A. Angiotensin II pseudopeptides containing 1,3,5-trisubstituted benzene scaffolds with high AT_2 receptor affinity. *J. Med. Chem.*, **2005**, 48, 6620–6631.

V. Rosenström, U.; Sköld, C.; Lindeberg, G.; Botros, M.; Nyberg, F.; Karlén, A.; Hallberg, A. Design, synthesis, and incorporation of a β-turn mimetic in angiotensin II forming novel pseudopeptides with affinity for AT_1 and AT_2 receptors. *J. Med. Chem.*, **2006**, 49, 6133–6137.

VI. Sköld, C.; Nikiforovich, G.; Karlén, A. Modeling binding modes of angiotensin II and pseudopeptide analogues to the AT_2 receptor. *Manuscript*.

VII. Georgsson, J.; Sköld, C.; Botros, M.; Lindeberg, G.; Nyberg, F.; Karlén, A.; Hallberg, A.; Larhed, M. Synthesis of a new class of druglike angiotensin II C-terminal mimics with affinity for the AT_2 receptor. *J. Med. Chem.* **2007**, 50, 1711–1715.

VIII. Sköld, C.; Karlén, A. Development of CoMFA models of affinity and selectivity to angiotensin II type-1 and type-2 receptors. *J. Mol. Graph. Model.*, **2006**, doi:10.1016/j.jmgm.2006.10.004.

Contents

Abbreviations

3D	Three-dimensional
Ang	Angiotensin
Ac	Acetyl
ACE	Angiotensin-converting enzyme
Ala	Alanine
Arg *or* R	Arginine
Asp *or* D	Aspartic acid
Bpa	*p*-Benzoylphenylalanine
CoMFA	Comparative molecular field analysis
Cys	Cysteine
DISCO	Distance comparison
DTT	Dithiothreitol
ECL	Extracellular loop
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
GPCR	G protein-coupled receptor
His *or* H	Histidine
Ile *or* I	Isoleucine
Lys *or* K	Lysine
MCMM	Monte Carlo multiple-minimum
Met *or* M	Methionine
NMR	Nuclear magnetic resonance
PDB	Protein data bank
Phe	Phenylalanine
PLS	Partial least squares projection to latent structures
Pro	Proline
QSAR	Quantitative structure–activity relationship
RAS	Renin–angiotensin system
Sar	Sarcosine
SAR	Structure–activity relationship
SUMM	Systematic unbound multiple minimum
TM	Transmembrane
Tyr	Tyrosine
Val	Valine

1 Introduction

1.1 Peptides in Drug Discovery

Many physiological effects are mediated by endogenous peptides acting as hormones or neurotransmitters, which makes them interesting in drug development.[1,2] Unfortunately, peptides are not optimal as drugs because of their low oral absorption and rapid degradation in the body.[3] However, there are examples of nonpeptide compounds that can mimic the interactions and effects of a peptide and that also have more favorable pharmacokinetic properties.[1,4-6] Such compounds are referred to as peptide mimetics, or peptidomimetics.[7] One of the most well-known peptidomimetics is the opiate morphine (Figure 1). Although morphine has been used in medicine for centuries, endogenous peptide ligands to the opiate receptors were not discovered until 1975 (the enkephalins, Figure 1) and 1997 (the endomorphins, Figure 1).[8,9] In contrast to the opiate system, often only the endogenous peptide is known. Thus, one of the goals in medicinal chemistry is to convert such peptides into peptidomimetics.

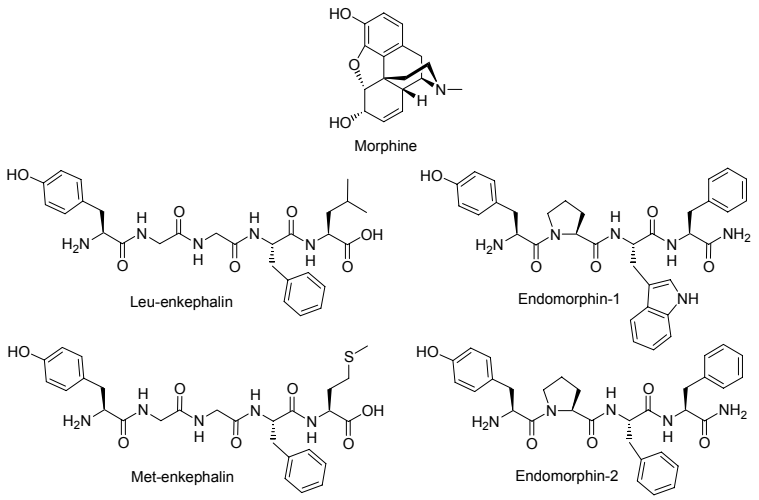


Figure 1. The peptidomimetic compound morphine and endogenous opiate receptor peptides.

1.2 Peptidomimetic Design Strategy

Figure 2 describes a general strategy for designing ligands to peptide receptors. Two main methods are used: rational step-wise conversion of the peptide ligand and screening of compounds already lacking peptide character. These methods can be integrated to gain information from both routes.

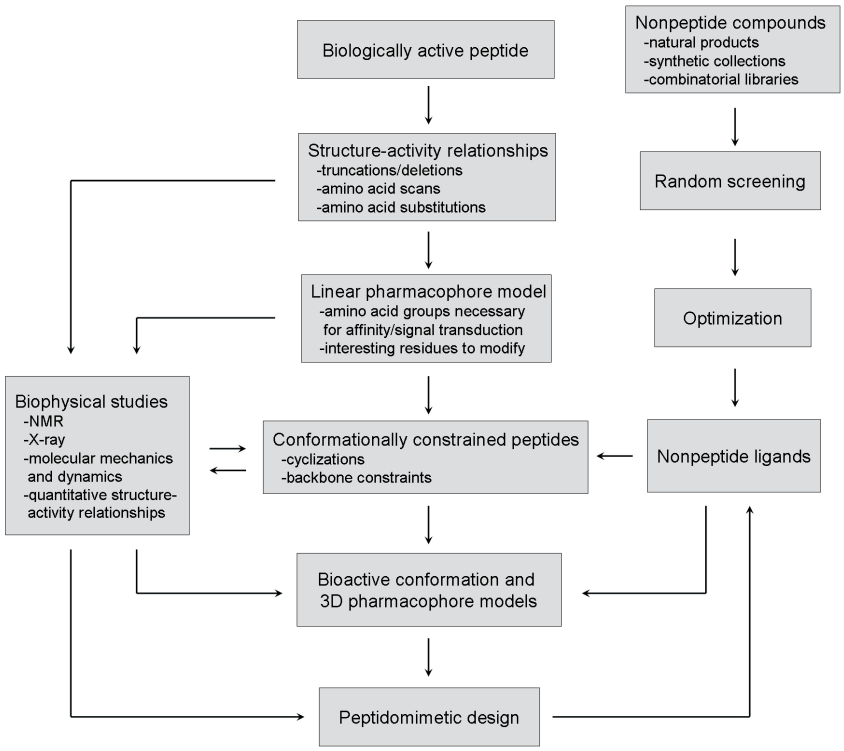


Figure 2. A general strategy for ligand design targeting peptide receptors, as described by Hruby.[2]

1.2.1 Bioactive Conformation

One of the central steps in peptidomimetic design is to elucidate the bioactive conformation of the peptide, i.e. the conformation adopted when it is bound to the receptor or enzyme. Many high-quality 3D structures of ligands, when bound to their macromolecular target, have been obtained using X-ray crystallography, but this technique is not readily applicable to all types of systems. This is, for example, the case for G protein-coupled receptors (GPCRs), to which many of the endogenous peptides bind. Since direct modeling studies of ligand binding are not possible for these receptors

they present a challenging target for drug design. The only GPCR with a known 3D structure at high resolution is bovine rhodopsin, first reported in 2000.[10] This 3D structure has been used for building models of other related GPCRs using homology modeling, as will be discussed in Chapter 2. With a model of the receptor, potential receptor-bound conformations can be explored in the receptor environment for further guidance in ligand development.

To model the receptor-bound conformation when the 3D structure of the target is unknown, experimental or theoretical studies of only the ligand are often performed. It should be noted, however, that the conformations adopted by flexible linear peptides are numerous, and are strongly influenced by interactions with the environment.[11] Therefore, to limit the conformational flexibility, conformational constraints can be introduced into the peptide to provide information about the bioactive conformation. In many cases the constrained part of the peptide is not completely rigid (e.g., using side chain cyclizations as constraints) and therefore several differently constrained analogues of the ligand may be needed to derive a putative receptor-bound conformation. When peptides bind to their receptors they become an integral part of the protein structure and thus can be predicted to adopt secondary structure motifs as part of the bioactive conformation.[2] Therefore, conformational constraints that induce or mimic secondary structures can give valuable information when searching for the bioactive conformation.

1.2.2 Secondary Structure Mimetics

The main secondary structures found in proteins and peptides are the α-helix, β-sheet and turns. For peptide ligands, secondary structures correspond to local rigidified structure motifs with a specific arrangement of the residue side chains, which could provide important recognition elements during receptor binding and activation.[11] Thus, mimicking such structural elements of the peptide with organic scaffolds is a rational approach in the development of peptidomimetic compounds. In addition, such an approach may also give compounds with increased metabolic stability and higher receptor specificity, and provide a rational basis for exploring the conformational effect on activity.[12] There are indications that turn structures are present in several peptide ligands when bound to their receptors, and this makes it appealing to mimic turns.[11] The major types of turns present in proteins and peptides are the β-turn and the γ-turn, depicted in Figure 3, where turn mimetic examples also can be found.

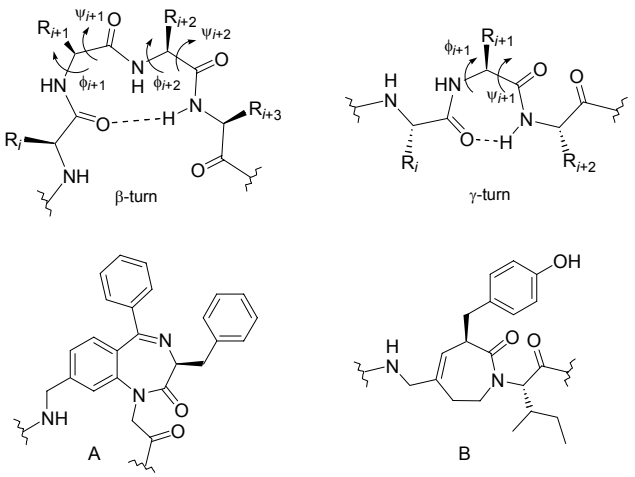


Figure 3. A β- and γ-turn conformation of a peptide chain and examples of a β-turn mimetic (A)[13] and a γ-turn mimetic (B)[14]. The backbone torsion angles phi (ϕ) and psi (ψ) are indicated for the relevant residues in the peptide turns.

Venkatachalam first suggested the presence of β-turns in proteins based on modeling studies in 1968.[15] The β-turn causes reversal of the peptide backbone and is defined as a sequence of four amino acid residues in a non-helical segment with a distance of less than 7 Å between $C_{\alpha,i}$ and $C_{\alpha,i+3}$.[16] The β-turns are often, but not necessarily, stabilized by a CO_i–NH_{i+3} hydrogen bond. The β-turn has been divided into several types depending on the values of the backbone torsion angles of residues *i*+1 and *i*+2. The most common β-turns found in proteins are types I, I′, II, II′, and VIII (Table 1).[15,17-19] In addition to these, other classes such as III, III′, VIa, VIb and VII have been proposed.[15,16] The miscellaneous category IV has been introduced to accommodate β-turns that do not fit any specific type.

Table 1. Idealized backbone torsion angles for the most common β-turn types[15,18] and the backbone torsion angles for the classic and inverse γ-turn.[20,21]

	Residue i+1		Residue i+2	
β-Turn	Phi (ϕ)	Psi (ψ)	Phi (ϕ)	Psi (ψ)
I	−60	−30	−90	0
I′	60	30	90	0
II	−60	120	80	0
II′	60	−120	−80	0
VIII	−60	−30	−120	120
γ-Turn (classic)	70 to 85	−60 to −70		
γ-Turn (inverse)	−70 to −85	60 to 70		

The γ-turn is not as common as the β-turn but has been found to be adopted by peptides as short as tripeptides.[22] A γ-turn spans over three amino acid residues with a hydrogen bond between CO_i and NH_{i+2}, resulting in the shape of a seven-membered ring. There are two types of γ-turns: the classic γ-turn with the *i*+1 residue side chain in an axial geometry and the inverse γ-turn with the side chain in an equatorial geometry (Table 1).[20,21] Although the classic γ-turn was proposed first, it has been shown to be very rare and the inverse γ-turn is the significantly more common of the two.[23] The classic γ-turn conformation causes reversal of the backbone direction and is mainly found in β-hairpin structures, as a tight turn, forming an antiparallel β-sheet structure. The inverse γ-turn can be found as backbone kinks and rarely causes reversal of the backbone direction.[23]

2 Computational Chemistry

Computational chemistry is commonly used in the drug discovery process with the intention of extracting information, predicting properties, and increasing our understanding of ligand–target interaction, which can be used for decisions regarding the design of potential drugs.[24] In drug discovery projects a multidisciplinary team is assembled and computational chemistry is integrated into many of the steps in the discovery process.[25] This thesis relies heavily on results obtained by computational methods, and a brief introduction to the methods used will be given in this chapter.

2.1 Molecular Mechanics

Using molecular mechanics, geometries and conformational energies can be readily calculated for molecular structures.[26] Unlike more precise quantum chemistry methods, in which the motion of the electrons is considered, the energy calculated by molecular mechanics is approximated to be dependent only on the position of the atomic nuclei. The advantage of this approximation is faster calculations but the downside is lower accuracy. However, the results obtained are often accurate enough for most studies and molecular mechanics can be applied to large molecular systems that are not suitable for quantum mechanical calculations.

In molecular mechanics the potential energy function of a molecular system is described by a force field. The force field contains terms for bond stretching, angle bending, torsional rotation, and non-bonded interactions, presented in their general forms in Equations 1–4, respectively. These equations are dependent on the distance (r), angle (θ), and torsion angle (ϕ) between atoms. The other parameters in the equations are adjusted to reproduce results obtained, for example, by experimental methods or quantum mechanical calculations. Several sets of potential energy functions with different fitted parameters are available, forming different force fields such as AMBER, MMFF94, and OPLS-AA, which can be used in molecular mechanic calculations.[27-29]

$$E_{\text{bond}} = \sum_{\text{bonds}} K_r (r - r_{\text{eq}})^2 \tag{1}$$

$$E_{\text{angle}} = \sum_{\text{angles}} K_\theta (\theta - \theta_{\text{eq}})^2 \tag{2}$$

$$E_{\text{torsion}} = \sum_{\text{torsions}} K_\phi \left[1 + \cos(n\phi - \delta)\right] \tag{3}$$

$$E_{\text{nb}} = \sum_{\text{nonbonded}} \left(\frac{q_i q_j}{4\pi\varepsilon_0 r_{ij}} + \left[\left(\frac{A_{ij}}{r_{ij}^{12}} \right) - \left(\frac{B_{ij}}{r_{ij}^{6}} \right) \right] \right) \tag{4}$$

2.2 Conformational Analysis

Every molecule has one, or most often, several stable conformations which are different minima on the potential energy surface described by the force field used. Starting from 3D structures of a molecule and applying an energy minimization algorithm to the energy function, the geometry and energy of the molecule in these low-energy conformations can be obtained.[30] The minimization algorithms move downhill on the energy surface and thus only find the energy minimum closest to the starting geometry. To thoroughly explore the potential energy surface of a molecule, a number of different approaches can be used, such as molecular dynamics simulation, random or systematic searching, and distance geometry.[31]

In this work the conformational sampling was performed using random or systematic searches. In these methods different starting geometries are generated on the potential energy surface followed by an energy-minimization step. The Monte Carlo Multiple-Minimum (MCMM)[32] method is a random search method, in which starting geometries are generated by randomly altering selected torsion angles of the molecule. In a systematic search the starting geometries are instead generated by systematic alterations of each investigated torsional angle of the molecule. This method can be applied in different ways. In the Systematic Unbound Multiple Minimum (SUMM)[33] method the torsion angles are first searched at a low resolution to cover the energy surface quickly. When all the starting geometries have been generated with a low resolution, new starting geometries are sampled using a higher resolution for the torsion angles.

In each search step, the starting geometry obtained is energy-minimized to a predefined convergence criterion and saved in the list of conformations, if it is considered unique and within a specified energy window compared to the conformation with the lowest energy in the list. The test for unique con-

formations is usually based on the atom-pair distances of superimposed conformations, where a threshold distance defines whether the conformation is unique. The number of conformations obtained in a conformational analysis can be very high, especially if the molecules are flexible and have many rotatable bonds. This can make the analysis of the output quite complex. To make this analysis easier, the values of the energy window, number of atoms compared and the atom-pair distance threshold can all be modified to reduce the number of conformations identified. The convergence criterion in the minimization step can also be used in this process, since two quite similar conformations can minimize to the same conformation when an increased convergence criterion is used.

2.3 Ligand-Based Modeling

When the structure of the macromolecular target of a ligand is unknown, modeling of the ligand–target interactions has to be performed based solely on the properties of the ligands. This is referred to as ligand-based modeling.

2.3.1 Pharmacophores

Different ligands that bind to the same target probably have some necessary structural features in common that are important for the ligand–target interaction. These could, for example, be hydrogen bond acceptor/donor atoms, hydrophobic regions, or charged groups. Such features are central in the pharmacophore concept, attributed to Paul Ehrlich.[34] Peter Gund later defined a pharmacophore as: "a collection of atoms spatially disposed in a manner that elicits a biological response".[35]

A number of pharmacophore modeling methods and programs are available to search for possible pharmacophores in a set of active ligands.[36] In this work the DISCO (DIStance COmparison) method,[37] implemented as DISCOtech in the molecular modeling program SYBYL,[38] was used in several studies. In addition to the derivation of pharmacophores, DISCOtech is useful as a tool for structural alignment based on superimposition of pharmacophore groups. The input to DISCOtech consists of a set of conformations derived for each active compound. In the analysis a network of distances between the structural features representing possible pharmacophore groups is calculated for all conformations of the compounds. These distance networks are compared, and if a sub-graph of the distance network can be matched within a specified tolerance to all the compounds included, this distance network is reported as a possible pharmacophore. A simplified example of the method is depicted in Figure 4.

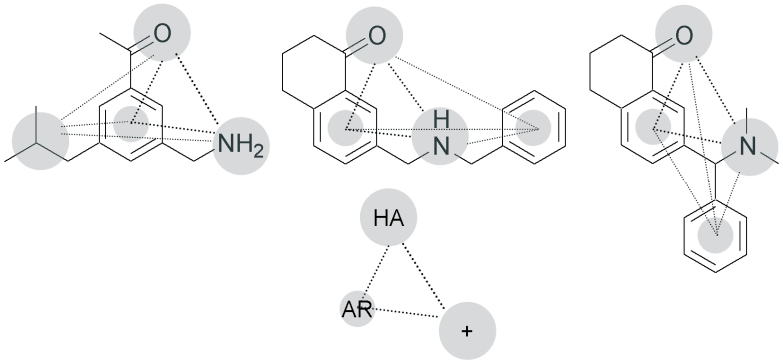


Figure 4. Distance network between structural features in three compounds with the possible pharmacophore depicted (HA, hydrogen bond acceptor; AR, aromatic center; +, potential positive charge).

DISCOtech has some limitations as a fully automated approach to pharmacophore recognition.[39] For example, as only the distances in the ligands are analyzed, the overall structural alignment (e.g., those parts of the structures that are not included in the pharmacophore model) is not considered in the deduction of the models. This can result in models in which the structures are not always intuitively well-aligned and thus DISCOtech analyses usually require a post-processing step. Also, since all conformations of each compound are compared, DISCOtech is limited to include a maximum of 300 conformations per compound. This can lead to difficulties when large, flexible compounds with many conformations are analyzed. In addition, large compounds usually contain many possible pharmacophore features, resulting in rather complex distance networks. Thus, such compounds take a long time to analyze and may produce many proposed pharmacophores if all possible pharmacophore features are included in the analysis.

2.3.2 Quantitative Structure–Activity Relationship

One important task for medicinal chemists is to derive general structure–activity relationships (SARs) and quantitative SARs (QSARs) by designing, synthesizing, and modeling series of compounds. This generates information that can be used for the development of new compounds with, for example, improved activity, selectivity, or pharmacokinetic properties. QSAR models describing the correlation between the responses and the descriptors of ligands are often obtained by using partial least squares projection to latent structures (PLS).[40] In short, PLS is a method of relating two data matrices, such as a descriptor table and a response table, to form a linear multivariate model.

In this work comparative molecular field analysis (CoMFA)[41] was used to derive QSAR models. CoMFA is a QSAR method belonging to the 3D-QSAR class, in which the 3D arrangement of atoms in the ligands is also considered. The descriptors in CoMFA consist of the interaction energies between the molecules in the series and a probe atom positioned in a grid surrounding the molecules (Figure 5). By aligning common parts of the molecules, for example, a central scaffold, different interaction energies in different areas of the ligands are obtained depending on their substituents. Using PLS these interaction energies are correlated with a response, most often affinity, and a 3D model of how the substituent pattern affects the response is obtained. Many of the steps in the analysis can affect the outcome of the CoMFA modeling, such as the selected conformations of the ligands, the method used for calculating partial charges, alignment, and CoMFA settings.[42,43] Optimally, the conformations and alignment should reflect the receptor-bound conformation of the ligands, especially if comparison are to be made of the CoMFA model obtained and the receptor structure.

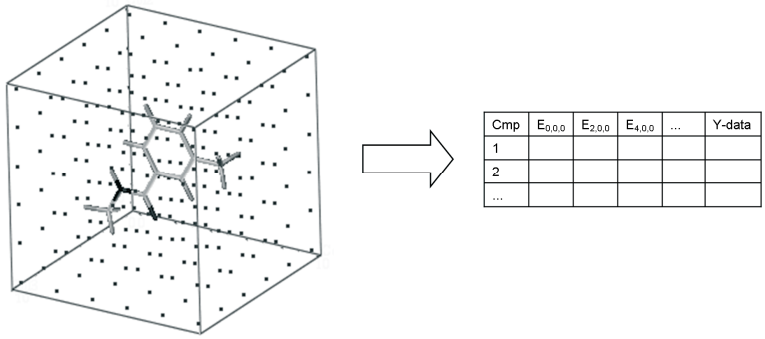


Figure 5. Visualization of the CoMFA grid points for calculation of ligand–probe interaction energies at each point. These energies are collected in a table for subsequent correlation with a measured response to obtain the CoMFA model.

2.4 Structure-Based Modeling

The 3D structure of a macromolecular target, such as an enzyme or receptor, can provide important information on how the ligand interacts with the target, especially when obtained with a bound ligand. This information can be used to modify the ligand to improve the interaction with the target. When the 3D structure of the target is included in the modeling of the ligand–target interaction the modeling is referred to as structure-based modeling.

2.4.1 Homology Modeling

Unfortunately, high-quality 3D structures of all targets are not readily available. This is especially the case for GPCRs where the receptors are incorporated into the membrane of the cells, which makes 3D structures of these receptors very difficult to derive. Nevertheless, the 3D structure of one GPCR, bovine rhodopsin, has been determined by X-ray crystallography[10] and can therefore be used in homology modeling. Homology modeling is based on the idea that homologous proteins have a common 3D structure and that the known 3D structure of one protein can be used as a template to create a model of a protein with unknown 3D structure. Thus, by using the 3D structure of bovine rhodopsin, a receptor model of related GPCRs can be obtained. The general steps involved in homology modeling are listed below.[44]

1. Fold assignment and template selection
2. Sequence alignment of the target and template
3. Modeling of structurally conserved regions
4. Modeling of structurally variable regions
5. Refinement of the generated 3D model
6. Validation of the 3D structure model

When modeling structures with an amino acid sequence identity greater than 30% with the template, homology modeling is likely to produce high-quality model structures.[45] In the case of GPCRs, the amino acid identity is usually not that high. However, due to the presence of conserved motifs in the transmembrane region of GPCRs even a low sequence identity can be expected to give more accurate models than homology models of other types of proteins based on low sequence identity.[46] The extramembrane domains of GPCRs are not expected to be modeled accurately using the 3D structure of bovine rhodopsin as template. This is because there is very low or no sequence identity in these structural regions, and the template may be influenced by crystal packing forces.[44,47] Although homology modeling using the rhodopsin 3D structure is currently the most successful approach to obtaining 3D structures of GPCRs, a simple and general homology modeling protocol will probably not be sufficient to obtain reliable receptor models.[48,49] Also, the 3D structure of rhodopsin was obtained in the inactivated state, and this may be rather different from the activated receptor state,[50] which could have implications in modeling ligand binding of agonists and antagonists.

2.4.2 Molecular Docking

When a 3D structure of a target has been obtained, direct modeling of ligand-binding is possible: this is referred to as docking. Depending on the

objective of the study, different methods can be used. For example, to screen for a suitable starting compound in a drug discovery project several hundred thousand compounds can be quickly evaluated regarding their fit to the target using a fast docking protocol. The results from such a study can be used to increase the probability of finding promising compounds in later experimental screening.[51] When only a few compounds are to be investigated more precise and more computationally demanding docking methods, such as molecular dynamic simulations or conformational analysis of the ligand in the target binding site, can be applied.[52,53]

3 Aims of the Present Study

This study is part of a research project aiming to convert peptides to nonpeptidic drug-like compounds using an iterative process. As the model peptide, angiotensin II was selected and the main focus of the project was its interaction with the AT_2 receptor.

The specific objectives of this study were:

- to design and characterize γ- and β-turn mimetics for incorporation into angiotensin II;
- to investigate the structure–activity relationships of angiotensin II and angiotensin II analogues when interacting with the AT_2 receptor;
- to investigate the binding mode of angiotensin II using conformationally constrained angiotensin II analogues;
- to derive a 3D model of the AT_2 receptor and investigate the binding mode of angiotensin II and angiotensin II analogues with the AT_2 receptor;
- to derive 3D-QSAR models based on nonpeptide ligands for the AT_1 and AT_2 receptors.

4 Angiotensin II

Angiotensin II (Ang II, Asp[1]-Arg[2]-Val[3]-Tyr[4]-Ile[5]-His[6]-Pro[7]-Phe[8]) is the central ligand in the renin–angiotensin system (RAS). It is formed through stepwise cleavage starting from the protein angiotensinogen, from which the enzyme renin cleaves off the decapeptide Ang I. Ang I is further cleaved to the active octapeptide Ang II via angiotensin-converting enzyme (ACE).[54] Ang II acts on two receptors in the RAS, the AT_1 and AT_2 receptors, which both belong to the seven-transmembrane domain GPCR superfamily.[55] A picture of the path to receptor activation is presented in Figure 6. The metabolites of Ang II are also important effectors in the RAS: Ang III (Ang 2–8) acts as an agonist on the Ang II receptors while Ang 1–7 and Ang IV (Ang 3–8) act on other receptors.[56,57] A major function of the RAS is the regulation of blood pressure, and this effect has been of great interest to the pharmaceutical industry for several decades in attempts to produce antihypertensive drugs. The successful approaches have been to inhibit the hypertensive actions of Ang II by either inhibit the formation of Ang II using ACE inhibitors or to block its binding to the AT_1 receptor using antagonists.[58] Renin inhibitors have also been investigated for a considerable time, but only recently have suitable compounds emerged.[59]

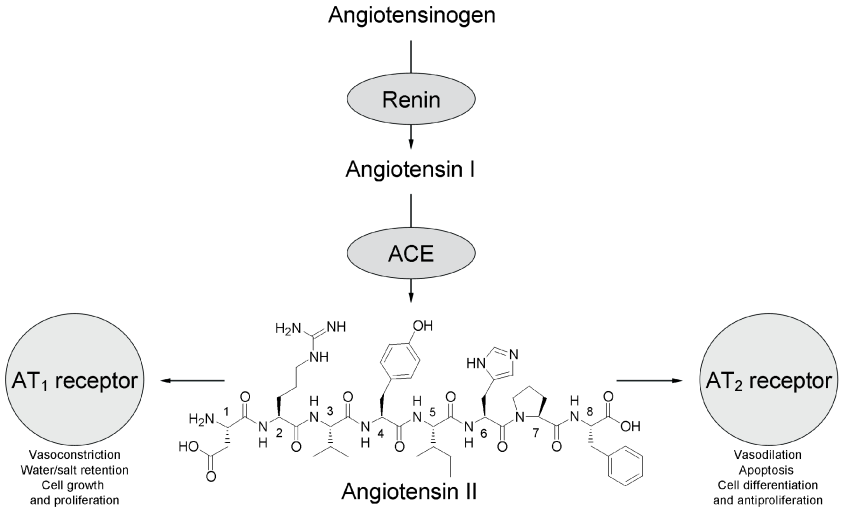


Figure 6. Formation of Ang II and some of the AT_1 and AT_2 receptor effects.

4.1 The AT_1 Receptor

The AT_1 receptor belongs to the rhodopsin-like GPCR class A (IUPHAR receptor code 2.1:ANG:1:AT1).[55,60] The AT_1 receptor mediates virtually all well-known physiological actions of Ang II, such as vasoconstriction, aldosterone release, water and salt retention, sympathetic transmission, cell growth and proliferation.[61,62] AT_1 receptors are primarily found in the brain, adrenal gland, vasculature, heart, and kidney.[63]

4.1.1 Ang II SAR for the AT_1 Receptor

By studying Ang II analogues, the importance of each amino acid for affinity and functional response has been thoroughly investigated and reviewed[64-69] and a short summary will be given below.

Position one in Ang II (Asp) is the least important position for both affinity and functional response. Many modifications, including deletion and elongation, of this position are possible without severely disrupting the biological activity. An important modification of this position that should be mention is when sarcosine (Sar, N-methylglycine) is introduced. [Sar^1]Ang II shows enhanced *in vitro* and *in vivo* activities and increased metabolic stability.[70,71] Thus, [Sar^1]Ang II analogues are often used in studies involving Ang II.

Position two (Arg) is important for affinity, which is attributed to the positive charge of the guanidino group.

Position three (Val) is considered to have a conformational role since Val can be replaced by Ala or Pro and cyclization between position three and five is well-tolerated by the AT_1 receptor.

Tyrosine in *position four* plays a critical role for the agonistic function. The phenolic hydroxyl group is crucial since methylation or deletion produces antagonists.

Position five (Ile) is, similar to position three, considered to mainly have a conformational role. However, β-branched amino acids are important in this position. [Val^5]Ang II, the endogenous Ang II peptide in bovine species, is interchangeably used in studies involving Ang II in non-bovine species.

In *position six*, His has a critical role in retaining both functional response and binding. [4-NH_2-Phe^6]Ang II lacks affinity for the AT_1 receptor, but is an agonist to the AT_2 receptor.[72]

Position seven (Pro) is considered to have a conformational role. Secondary amino acids are necessary for high-affinity binding.

Position eight (Phe) is the primary determinant for the agonistic or antagonistic activity of Ang II. The C-terminal carboxyl group is required for both agonism and binding affinity. Aliphatic residues such as Ile or Ala in this position give AT_1 receptor antagonists. The AT_1 receptor antagonists

[Sar[1], Ile[8]]Ang II and [Sar[1], Val[5], Ala[8]]Ang II (saralasin) have been commonly used in studies of the AT_1 receptor.

4.1.2 AT_1 Receptor-Bound Conformation of Ang II

Many studies suggest that Ang II adopts a turn structure in the 3–5 region, around Tyr, when binding to the AT_1 receptor.[14,73-81] Also, cyclization in the 5–7 region of Ang II has produced ligands with good affinity to the AT_1 receptor, implying a turn structure also in this region.[82] However, an extended receptor-bound conformation has also been suggested by the results of photoaffinity labeling and homology modeling studies.[83,84] Based on the numerous suggestions regarding the receptor-bound conformation and data from diverse cyclized analogues with high AT_1 receptor affinity, Marshal and coworkers suggested that no specific secondary structure was necessary for binding to the receptor as long as important ligand–receptor interactions take place.[77]

4.1.3 Nonpeptide AT_1 Receptor Ligands

A major research area in the field concerning the AT_1 receptor has been to develop AT_1 receptor antagonists for the treatment of hypertension. Guided by lead compounds, such as S-8307 (Figure 7), originating from Takeda Chemical Industries, losartan was developed by DuPont-Merck as the first oral, nonpeptide, AT_1 receptor antagonist.[85] Losartan reached the market in 1994 and was followed by several similar compounds to form a new class of drugs known as the "sartans" (some examples are shown in Figure 7).

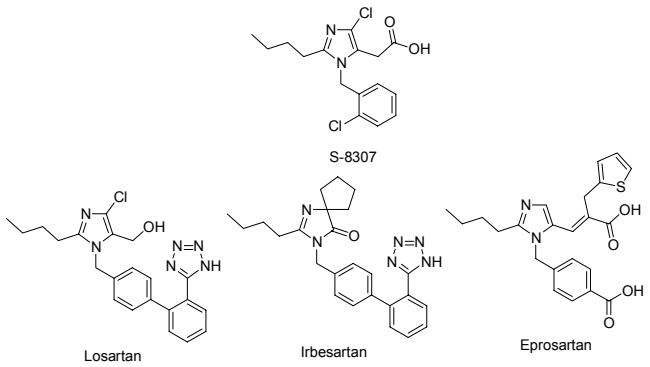


Figure 7. The antihypertensive lead compound S-8307 that was developed into clinically used antihypertensive drugs such as losartan, irbesartan and eprosartan.

4.1.4 The AT_1 Receptor Binding Site

Point-mutation studies of the AT_1 receptor have provided valuable information on potential ligand–receptor contacts during AT_1 receptor binding. For Ang II analogues, mutation of Lys^{102}, Arg^{167}, Lys^{199}, Trp^{253}, Phe^{259}, Asp^{263} or Asp^{281} results in receptors with reduced ligand affinity.[86-88] His^{256} has been suggested to play an important role in signal transduction by interaction with the aromatic side chain of Phe^{8} in Ang II.[89,90] Two disulfide bridges important for binding have been suggested in the AT_1 receptor, Cys^{101}–Cys^{180} and Cys^{18}–Cys^{274}, where the former is conserved within the receptor family. Point mutation of these cysteine residues or reduction of the disulfide bridges with dithiothreitol (DTT) lowers both Ang II and nonpeptide ligand affinity.[91]

It has been indicated that the nonpeptide AT_1 receptor ligands, such as losartan, have an overlapping binding pocket with the C-terminal part of Ang II, forming interactions with Lys^{199} and His^{256}.[87,89] This binding site may reside deeper in the TM region as mutations in the exterior domain have shown little effect on nonpeptide binding.[92] Thus, such nonpeptide compounds may be regarded as mimics of the C-terminal part of Ang II, which was also one of the original design ideas for these compounds.[93] Homology modeling has been employed in several studies to derive 3D models of the AT_1 receptor.[84,94-98] These receptor models have been used to investigate ligand–receptor binding and have mainly focused on nonpeptide binding in the Lys^{199}/His^{256} region.

4.2 The AT_2 Receptor

The selective AT_1 receptor antagonists, together with DTT and selective synthetic peptide ligands such as [4-NH_2-Phe^{6}]Ang II and CGP-42112A (Figure 8), were recognized as key compounds in the discovery and characterization of the angiotensin II receptor heterogeneity in the late 1980s.[72,99-101]

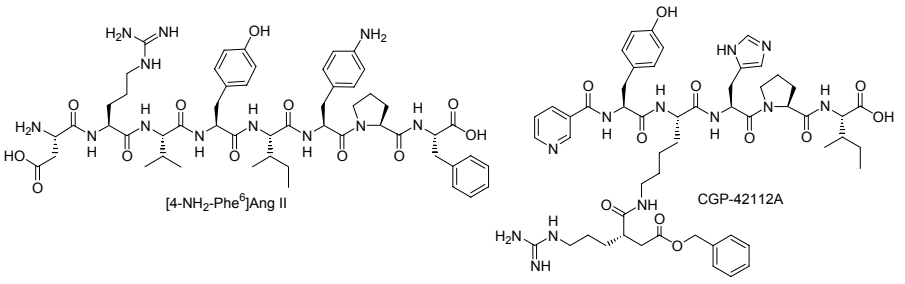


Figure 8. The selective AT_2 receptor ligands [4-NH_2-Phe^{6}]Ang II and CGP-42112A.

Analogous to the AT_1 receptor, the AT_2 receptor belongs to the rhodopsin-like GPCR class A (IUPHAR receptor code 2.1:ANG:2:AT2).[55,60] Although both AT_1 and AT_2 receptors are activated by the same ligand, they share only 32–34% sequence identity.[102,103] The AT_2 receptor has been shown to mediate apoptosis, cell differentiation, alkaline secretion in the duodenal mucosa and some anti-AT_1 receptor effects, such as antiproliferation and vasodilatation.[61,62,104,105] The AT_2 receptor is believed to play an important role in fetal development since this receptor is the predominant Ang II receptor prior to birth. The expression of the AT_2 receptor in adults is low, and can mainly be found in specific tissues such as the heart, uterus, ovary, vascular endothelium, adrenal gland, and distinct brain areas.[61,106] However, the AT_2 receptor is up-regulated during certain pathological conditions such as heart and kidney failure, vascular, skin and axonal injury, wound healing, and myocardial infarction,[63,105] indicating a role in these conditions. Recently it has been shown that treatment with a selective AT_2 receptor agonist improves the heart function after myocardial infarction in rats.[107] Furthermore, there are indications that some of the beneficial effects of the AT_1 receptor antagonists could be attributed to indirectly increased stimulation of the AT_2 receptor,[108,109] but no drug has yet reached the market intentionally targeting the AT_2 receptor.

Functional assays for the AT_2 receptor are complex and not generally available, and thus the functional properties of only a few ligands have been identified. Two commonly used compounds for functional studies of the AT_2 receptor are CGP-42112A and PD-123,319,[110] an AT_2 receptor agonist and antagonist, respectively.[111]

4.2.1 Ang II SAR for the AT_2 Receptor

In contrast to the manifold investigations of peptide SAR for AT_1 receptor ligands, only a few systematic SAR studies have been reported for AT_2 receptor binding of Ang II and Ang II analogues.[69,112-114] The lack of an easily accessible functional assay has resulted in SAR being confined to investigation of binding affinity only. An amino acid scan of [Sar^1]Ang II has been reported by Miura et al.,[69] where Ala was introduced in six positions and Gln was used to study the importance of Arg in position two. In general, no single amino acid was shown to be crucial for AT_2 receptor affinity. The greatest effect was observed when Arg^2 was replaced by Gln, resulting in a 20-fold decrease in affinity. Other significant reductions in AT_2 receptor affinity were seen when either Tyr^4 or His^6 was replaced by Ala. Also, amidation of the C-terminal carboxyl group of Phe gave significantly reduced affinity.

Position one in Ang II has been thoroughly investigated[114] and, as in the case of affinity to the AT_1 receptor, the presence of the Asp in this position seems to be of minor importance. Many modifications of this position, such as methylation of the N-terminal amine, and even deletion of the whole resi-

due, gave analogues with improved affinity. Position eight was also investigated in the same study. An Ile or Met residue in position eight gave analogues with increased AT_2 receptor affinity, and substituents on the Phe aromatic ring gave increased affinity in many cases. Amino acids as bulky as Bpa (*p*-benzoylphenylalanine) in position eight were also found to be well tolerated by the AT_2 receptor.

4.2.2 AT_2 Receptor-Bound Conformation of Ang II

In contrast to the many hypotheses concerning AT_1 receptor binding, significantly less has been reported on the preferred conformation of Ang II when binding to the AT_2 receptor. The AT_2 receptor was unknown when many of the receptor-bound conformational studies of Ang II were performed. Therefore less data have been obtained for the AT_2 receptor. Also, the lack of an easy functional assay may have contributed to the limited number of studies. However, incorporation of secondary structure mimetics and side chain cyclization in Ang II have provided support for a turn structure around the Tyr residue, as also suggested for Ang II binding to the AT_1 receptor.[77,115] Also similar to the case of AT_1 receptor binding, photoaffinity labeling data have been used in combination with a homology model of the AT_2 receptor to propose an extended receptor-bound conformation of Ang II when bound to the AT_2 receptor.[84]

4.2.3 Nonpeptide AT_2 Receptor Ligands

In vivo studies of selective AT_1 receptor antagonists have shown that these drugs increased Ang II plasma concentration, with unknown long-term effects due to the potential increase in AT_2 receptor activation.[116] In a study aimed at developing antagonists that would have a safer pharmaceutical profile, a large series of nonselective AT_1/AT_2 receptor ligands was produced.[117,118] In that project, compounds were also identified that led to the discovery of the first nonselective, nonpeptide AT_1 receptor agonist L-162,313 (Figure 9).[119,120] This compound was later shown to also posses agonistic properties to the AT_2 receptor.[121] Using structural modifications of L-162,313, the first selective, nonpeptide AT_2 receptor agonist M024 (Figure 9) was developed in 2004.[122]

The selective AT_2 receptor antagonist PD-123,319 (Figure 9) was discovered in a project aimed at producing Ang II receptor ligands with antihypertensive effects. Although the compounds in the project did bind to Ang II receptors, they showed no or little antihypertensive effect because of the AT_2 receptor selectivity.[110] Even though the original aim was not achieved, PD-123,319 has been of great use as a reference compound in studies of AT_2 receptor effects. Recently M132 (Figure 9), a compound closely related to M024, has been reported to act as a selective AT_2 receptor antagonist.[123]

Thus, the structurally similar M024 and M132 could prove to be valuable research tools in the evaluation and elucidation of AT_2 receptor effects and requirements for the functional response of nonpeptide ligands.

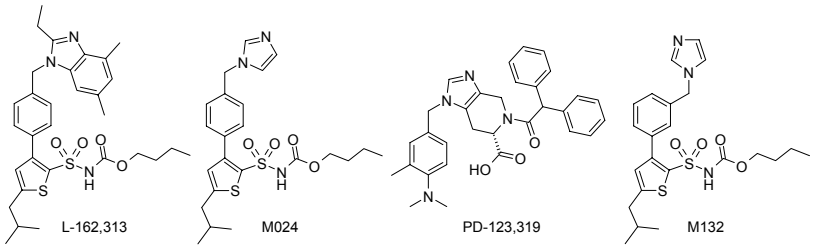


Figure 9. The nonpeptide AT_2 receptor ligands L-162,313, M024, PD-123,319, and M132.

4.2.4 The AT_2 Receptor Binding Site

As shown by point-mutation studies, a number of AT_2 receptor residues are important for affinity of Ang II and Ang II analogues. The point-mutation studies of the AT_2 receptor have been focused on conserved or similar receptor areas/residues shown to be important for ligand binding to the more well-studied AT_1 receptor. Point-mutation of Arg^{182}, Lys^{215}, His^{273}, Asp^{279}, or Asp^{297} in the AT_2 receptor results in receptors with lower affinity for Ang II, indicating that these residues are potential ligand–receptor contact points.[124-130] In photoaffinity labeling studies, the C-terminal in Ang II has been suggested to be in the proximity of Met^{128} and Met^{138}, and the N-terminal segment of Ang II has been suggested to be in the proximity of the N-terminal tail of the AT_2 receptor during binding.[131,132] Similarly to the AT_1 receptor, two disulfide bridges have been proposed in the extracellular domain of the AT_2 receptor, Cys^{117}–Cys^{195} and Cys^{35}–Cys^{290}, both of which have implications for ligand binding.[133,134] The Cys^{117}–Cys^{195} bridge is conserved in the receptor family and, as for the AT_1 receptor, disruption of this bridge produces unstable AT_2 receptors which are unable to bind Ang II. The second disulfide bridge is proposed to connect Cys^{35} in the N-terminal segment with Cys^{290} in the third extracellular loop. However, mutation of Cys^{35} or Cys^{290}, which eliminates this disulfide bridge, produces AT_2 receptors with slightly increased affinity for Ang II. Also, an intermolecular disulfide bridge has been suggested between the Cys^{35} residue in one AT_2 receptor and the Cys^{290} residue in another AT_2 receptor, forming a homo-oligomer with induced cell signaling.[135] These studies of the disulfide bridges make the role, occurrence and importance of the intramolecular Cys^{35}–Cys^{290} disulfide bridge somewhat unclear for the AT_2 receptor.

4.3 Assays

The biological assays for Ang II receptor binding and functionality that were used to evaluate the compounds described in this thesis are briefly presented below.

4.3.1 Binding Affinity Assays

The binding affinity data were obtained using radioligand-binding assays relying on displacement of [^{125}I]Ang II from AT_1 receptors in rat liver membrane[136] or AT_2 receptors in pig uterus myometrium.[137] The natural ligand Ang II and the selective AT_2 receptor ligand [4-NH_2-Phe^6]Ang II were used as reference compounds in the assays. All experiments were performed in triplicate.

4.3.2 Functional Assays

Functional properties were obtained for some of the compounds. The classic contractile rabbit aorta strip assay was used for the evaluation of compound functionality on the AT_1 receptor.[138] Two functional assays were used for the evaluation of compound functionality on the AT_2 receptor, based on induced neurite outgrowth in NG108-15 cells[139,140] or suppression of proliferation in PC12 cells.[141] In the neurite outgrowth assay, cells with at least one neurite longer than a cell body were counted as positive for neurite outgrowth (Figure 10).

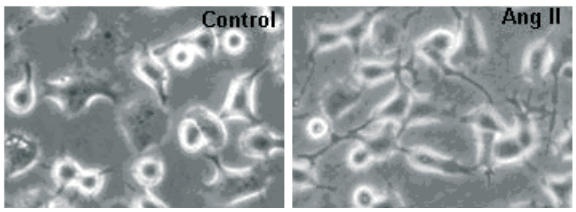


Figure 10. Unaffected NG108-15 cells (left) and cells with induced neurite outgrowth when treated with Ang II as an AT_2 receptor agonist (right).

5 Ligand-Based Modeling of AT_2 Receptor Ligands (Papers I–V)

A compilation of the structures of the investigated compounds described in this and the following chapter, including their biological data, can be found in Table A1 in the Appendix.

5.1 Background

Cyclization of Ang II in the Val^3-Tyr^4-Ile^5 region has proven to be a successful approach for producing high-affinity AT_2 receptor ligands. Plucinska et al. have published a series of disulfide-based bicyclizations of [Sar^1]Ang II in the 3–5 region, which in many cases produced Ang II analogues with high affinity to the AT_2 receptor.[77] Our group has shown that 3–5 monocyclized Ang II analogues also had high AT_2 receptor affinity, where the most active compound **1** (K_i = 0.62 nM) contained a methylenedithioether ring structure (Figure 11).[115] Based on conformational analysis, this ring structure was shown to preferentially adopt an inverse γ-turn conformation around the central Tyr residue.[80] These results indicated that a rational approach to less peptide-like Ang II analogues with AT_2 receptor affinity would be to incorporate rigidified γ-turn mimetics replacing the Val^3-Tyr^4-Ile^5 segment in Ang II. An isoquinolinone-based bicyclic scaffold has previously been used as a γ-turn mimicking moiety with the intention of producing a pseudopeptide with AT_1 receptor affinity, although incorporation of this scaffold in Ang II gave two diastereomers **2a** and **2b** (Figure 11), which both lacked affinity to the AT_1 receptor.[14]

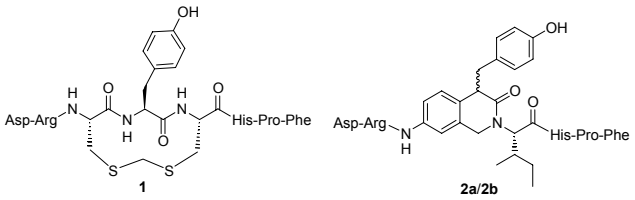


Figure 11. The constrained Ang II analogues **1** and **2a**/**2b**.

5.2 Benzodiazepine-Based γ-Turn-Like Mimetics (Paper I)

When the previously synthesized diastereomers, **2a** and **2b**, were evaluated for AT_2 receptor affinity, one of them was shown to possess fairly good affinity (K_i = 61 nM). Insertion of NH into the γ-turn mimetic moiety of **2** gives the benzodiazepine core structure that has previously been recognized as a turn mimetic.[142] Thus, to further explore bicyclic scaffolds as γ-turn mimetics, the benzodiazepine-based scaffold A (Figure 12) was evaluated.

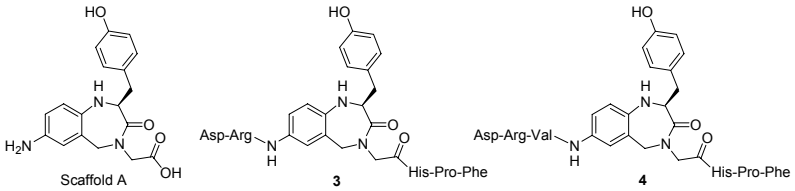


Figure 12. Scaffold A and pseudopeptides **3** and **4** comprising scaffold A.

First, the geometry of scaffold A was compared with the cyclic scaffolds in **1** and **2**, using conformational analysis and scaffold alignment. The alignment of the structures was focused on the *i*+1 and *i*+2 γ-turn residues since the peptide backbone in the γ-turn region that corresponds to the *i* residue is only present in **1**. When the turn-mimicking moieties were superimposed it was evident that the geometries of the residues entering the γ-turn region were different (Figure 13). Because of the geometric differences of the *i* residue, both compound **3** and **4** (Figure 12) were evaluated as Ang II analogues, where the latter had a Val residue also present, analogous to Val^3 in Ang II.

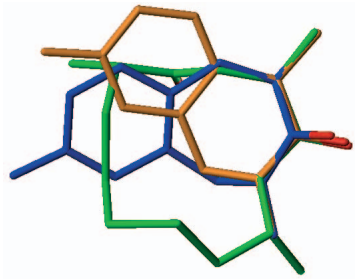


Figure 13. Scaffold A (blue) with the turn-mimicking moieties in **1** (green) and **2** (orange) superimposed. Only atoms in residues *i*+1 and *i*+2 were superimposed.

Both **3** and **4** lacked AT_1 receptor affinity, but interestingly they exhibited different affinities to the AT_2 receptor in that compound **3** lacked AT_2 receptor affinity (K_i > 10,000 nM) while **4** had high AT_2 receptor affinity (K_i = 3.0 nM). Since the structural difference in the N-terminal end of **3** and **4** resulted

in such a drastic difference in affinity we could start to formulate hypotheses concerning which structural properties are important for AT_2 receptor affinity. One hypothesis explaining the observed difference in affinity was that the Val residue in **4** could act as a spacer, enabling the N-terminal residues Asp and Arg to reach an area in the receptor favorable for interactions. This receptor area should also be accessible to Asp and Arg in **1**, but not in **3**.

To test this hypothesis, conformational analysis was performed on model structures of **1**, **3** and **4**, where the Asp residue was replaced by an acetyl capping group, the Tyr residue replaced by an Ala residue, and His-Pro-Phe replaced by an NHMe capping group. When the cyclic moieties in the different conformations for each compound were superimposed, it was found that the guanidino group of Arg and the N-terminal end occupied different regions in space in **3** and **4**. When the conformations of **1** were included in the analysis it was shown that areas could be located that were only accessible to the guanidino group and the N-terminal end of the active ligands (Figure 14, in which **3** and **4** are shown). This supported the hypothesis that Val enabled **4** to make similar, favorable contacts with the AT_2 receptor as **1**, which are not accessible to **3**. To investigate the importance of the Arg residue, compound **5** (Table A1, Appendix), in which Arg in **4** was replaced by Ala, was tested regarding AT_2 receptor affinity. This compound lacked AT_2 receptor affinity, which supported the importance of the Arg residue in **4**.

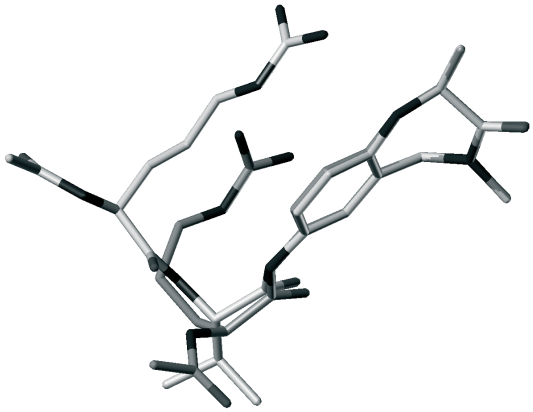


Figure 14. Scaffold superimposition of **3** (dark gray) and **4** (white), showing that Arg and the N-terminal end of **3** cannot reach areas accessible to **4**. The Arg guanidino group and Asp residue of **1** can also reach the respective positions of these groups in **4** when the scaffolds are superimposed.

5.3 Ang II Structure–Activity Relationship (Paper II)

Based on the systematic positional scan of Ang II analogues by Miura et al. it has been shown that no single amino acid was essential for AT_2 receptor affinity.[69] To complete, complement and confirm these results using our assay setup, a glycine scan of Ang II was performed (compounds **6**–**13**, Table 2). It should be noted that an alanine scan is preferable since the conformational preference of the backbone is more preserved than in a glycine scan. However, the glycine scan also gave an opportunity to investigate the influence of the small alanine side chain in the Ang II analogues. In addition to the glycine scan, Ang II analogues truncated or prolonged in the N-terminal side between Arg and Tyr were tested with regard to AT_1 and AT_2 receptor affinity (compounds **14**–**19**, Table 2) to further explore the results obtained for pseudopeptides **3** and **4**.

Table 2. Peptide Ang II analogues.

Compound no	Compound	K_i (± SEM) AT_1	K_i (± SEM) AT_2
Ang II	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe	1	0.6
6	Gly-Arg-Val-Tyr-Ile-His-Pro-Phe	6.1 ± 0.2	4.0 ± 0.6
7	Asp-Gly-Val-Tyr-Ile-His-Pro-Phe	> 10,000	55 ± 5
8	Asp-Arg-Gly-Tyr-Ile-His-Pro-Phe	1.6 ± 0.1	1.2 ± 0.2
9	Asp-Arg-Val-Gly-Ile-His-Pro-Phe	48 ± 2	5.2 ± 0.8
10	Asp-Arg-Val-Tyr-Gly-His-Pro-Phe	1.6 ± 0.1	2.3 ± 0.1
11	Asp-Arg-Val-Tyr-Ile-Gly-Pro-Phe	14.3 ± 0.06	7.6 ± 1.0
12	Asp-Arg-Val-Tyr-Ile-His-Gly-Phe	146 ± 7	4.3 ± 0.6
13	Asp-Arg-Val-Tyr-Ile-His-Pro-Gly	> 10,000	1.1 ± 0.1
14	Arg-Val-Tyr-Ile-His-Pro-Phe	10.5 ± 0.3	2.2 ± 0.2
15	Gly-Val-Tyr-Ile-His-Pro-Phe	> 10,000	5.4 ± 0.4
16	Ac-Gly-Val-Tyr-Ile-His-Pro-Phe	17.3 ± 0.2	2.8 ± 0.3
17	Asp-Arg-Gly-Val-Tyr-Ile-His-Pro-Phe	> 10,000	2.9 ± 0.3
18	Asp-Arg-Val-Gly-Tyr-Ile-His-Pro-Phe	> 10,000	255 ± 12
19	Asp-Arg-Tyr-Ile-His-Pro-Phe	204 ± 10	238 ± 7

Similar to the scan performed by Miura et al., the results of the glycine scan showed that the AT_2 receptor was very tolerant with respect to the removal of side chains in Ang II. The most pronounced effect was seen when replacing Arg by Gly, resulting in an almost 100-fold decrease in affinity. In the case of the AT_1 receptor, all affinity was lost when replacing either Arg or Phe with Gly. It was thus confirmed that the Arg side chain was important for both AT_1 and AT_2 receptor affinity. Surprisingly, this was not the case for Ang III (**14**), where only a 2-fold decrease in AT_2 receptor affinity was

seen when Arg was replaced by Gly (**15**). To rule out the possibility that the positive charge of the N-terminus of Ang III interacts at the Arg binding site, the acetylated Gly analogue **16** was tested. However, **16** had even higher AT_2 receptor affinity, showing that a positive charge on the N-terminal end of Ang III was not required for high-affinity AT_2 receptor binding. These results suggest that, compared to Ang II, the slightly shorter Ang III does not need the Arg side chain to ensure good interaction with the AT_2 receptor. However, for binding to the AT_1 receptor, the Arg side chain in Ang III is very important, since **15** lacks affinity to the AT_1 receptor, although, by acetylating the N-terminus of **15**, much of the affinity can be recovered (**16**).

As previously indicated from the study of the relatively long pseudopeptide **4**, the Ang II analogue **17** showed that elongation between Arg and Tyr was tolerated by the AT_2 receptor. However, the location of the inserted Gly was important since **18** showed a considerable decrease in AT_2 receptor affinity. When Ang II was shortened between Arg and Tyr (**19**), loss in both AT_1 and AT_2 receptor affinity was observed.

5.4 Improved Benzodiazepine-Based γ-Turn Mimetics (Paper III)

Based on the superimposition shown in Figure 13, it can be speculated that the positioning of the N-terminal handle on scaffold A may be more favorable in the 9-position than in the 7-position. Comparison of the geometries of these scaffolds showed that better correspondence with an inverse γ-turn was in fact obtained for this new scaffold, B (Figure 15).

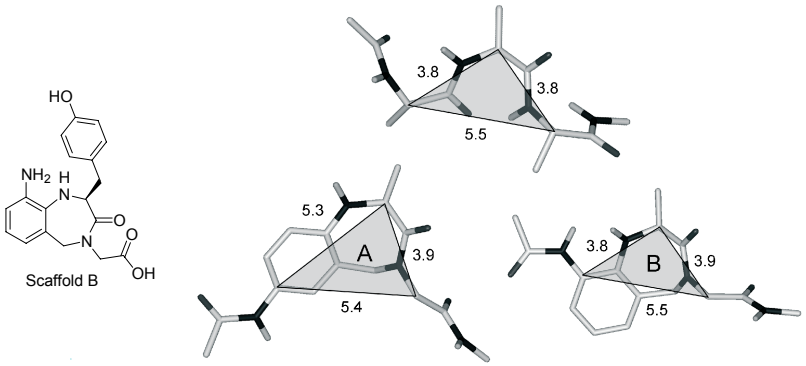


Figure 15. Scaffold B and geometrical comparison (C_α distances) of the γ-turn mimetic scaffolds A and B with an inverse γ-turn (upper triangle).

Scaffold B was also compared to inverse γ-turns extracted from a diverse set of crystallized proteins obtained from the Protein Data Bank (PDB)[143-145] and this comparison further supported the γ-turn-mimicking properties of scaffold B (Figure 16).

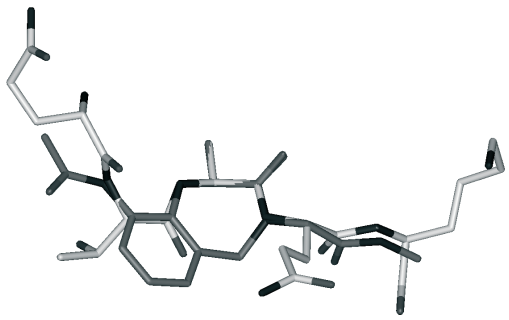


Figure 16. Superimposition of scaffold B (dark gray) on an inverse γ-turn (residue *i*+1 superimposed) found in a crystallized protein (PDB code 1NNF, chain A, Ala[83] as central turn residue).

Scaffold B was incorporated into Ang II, replacing Val[3]-Tyr[4]-Ile[5], giving pseudopeptide **20** (Figure 17). Interestingly, **20** had high AT_2 receptor affinity (K_i = 2.8 nM), despite the fact that it lacked the Val residue, which was a necessary structural feature for binding when scaffold A was used. Pseudopeptide **21** (Figure 17), comprising a Val residue, was also evaluated and was shown to have an even higher AT_2 receptor affinity. However, although the affinity increased, a Val residue was no longer crucial for affinity to the AT_2 receptor when scaffold B was used. Also, the SAR of the Arg residue in compound **20** and **21** was more in line with the SAR of Ang II since when Arg was replaced by Ala in these compounds, giving **22** and **23** (Table A1, Appendix), only about a 10-fold decrease in AT_2 receptor affinity was seen. This supports the hypothesis that the Val residue in **4** mainly functions as a spacer to correct for the non-optimal geometry of scaffold A.

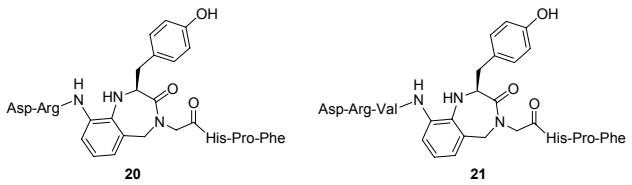


Figure 17. Pseudopeptides comprising scaffold B, in which the N-terminal handle is positioned in the 9-position of the scaffold.

To further develop scaffold B an Ile side chain was incorporated at the C-terminal handle in an attempt to mimic Ile[5] in Ang II. Two diastereomers of

unassigned stereochemistry were obtained when this moiety was incorporated into Ang II replacing either Val-Tyr-Ile (**24a** and **24b**, Figure 18) or Tyr-Ile (**25a** and **25b**, Figure 18).

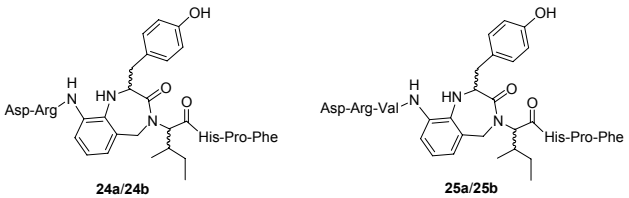


Figure 18. Pseudopeptides comprising an Ile side chain in the turn mimetic scaffold.

When the diastereomers **24a** and **24b** were evaluated regarding AT_2 receptor affinity, one was found to have decreased affinity (K_i = 117.4 nM) while the other showed similar affinity (K_i = 2.6 nM) to the corresponding pseudopeptide without the Ile side chain, **20**. Interestingly, when the diastereomers **25a** and **25b** were evaluated regarding AT_2 receptor affinity, both displayed higher affinity than the corresponding pseudopeptides lacking the Ile side chains, **21**. One showed slightly better affinity (K_i = 0.3 nM), while the other showed a 10-fold increase in affinity (K_i = 0.08 nM). Compared to Ang II this was a 3-fold increase in affinity, making this pseudopeptide the first in the series with higher AT_2 receptor affinity than the endogenous peptide.

The three pseudopeptides **4**, **20**, and **21** were shown to be AT_2 receptor agonists in the neurite outgrowth assay, and **4** was also found to act as an agonist in the proliferation assay. These compounds also had high affinity to and selectivity for the AT_2 receptor, despite having geometrically different fragments between Arg and Tyr. A study was therefore initiated to investigate whether these three pseudopeptides were able to interact with the AT_2 receptor in a similar fashion. For this study the pharmacophore searching program DISCOtech was used, with the aim of generating an unbiased alignment of potentially important structural elements in the three pseudopeptides. To reduce the size of the long flexible pseudopeptides, smaller model structures (Figure 19) were built before performing conformational analysis.

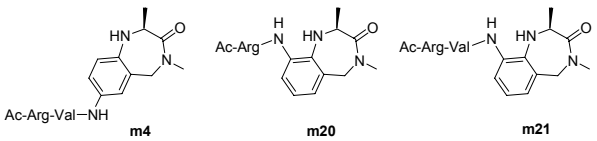


Figure 19. Model structures of **4**, **20**, and **21**, used in a DISCOtech analysis to derive binding mode models.

It was assumed that the three amino acids His-Pro-Phe found in all compounds were positioned in the same area of the receptor. Therefore, these amino acids were removed for the model structures, retaining only part of the C-terminal handle of the scaffolds. Similarly, only the C_β atom of the Tyr side chain was included in the model structures, and the N-terminal Asp residue was replaced by an acetyl capping group. For the DISCOtech analysis, pharmacophore features were added to represent the position of the truncated groups, and only pharmacophore features originating from these groups and the Arg guanidino group were included in the analysis.

DISCOtech found 82 different models but many had a very low structural overlap in those areas of the structures not included in the analysis. To identify relevant models, their structural volume was calculated and used as an indication for the structural overlap. The model with the lowest volume corresponded to a plausible binding mode for the pseudopeptides and is presented in Figure 20. In this model, the N-terminal acetyl group (corresponding to the position of Asp), the Arg guanidino group, the C-terminal handle and the Tyr C_β of the scaffolds are positioned in approximately the same areas. To challenge this model, a new DISCOtech analysis was performed including the corresponding model structure of the inactive pseudopeptide **3**. In this analysis, no models were found that had a convincing alignment. Furthermore, none of the conformations of the active compounds in the previous model (Figure 20) was present in any of these new models, in which **3** was included. This supported the idea that the active pseudopeptides **4**, **20**, and **21** may have a common binding mode not accessible to the inactive compound **3**.

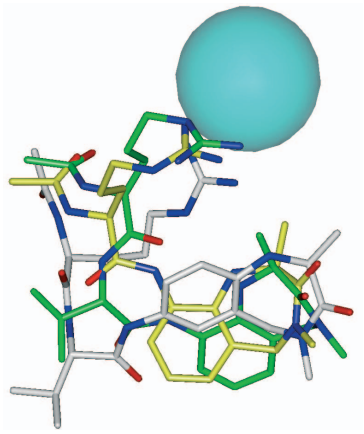


Figure 20. A common binding mode of the model compounds of **4** (white), **20** (yellow) and **21** (green) found by DISCOtech. The cyan sphere corresponds to the receptor area interacting with the Arg guanidino group, as suggested by DISCOtech.

5.5 1,3,5-Trisubstituted Benzene-Based γ-Turn Mimetics (Paper IV)

Although several studies have suggested that Ang II adopts a turn around the Tyr residue when binding to the AT_1 receptor, the pseudopeptides comprising the benzodiazepine-based scaffolds A or B did not show any AT_1 receptor affinity. Also, both diastereomers **2a** and **2b** comprising the isoquinolinone-based scaffold lacked AT_1 receptor affinity, while one possessed AT_2 receptor affinity. It therefore appears that the requirement of the correct geometry of the turn mimetic moiety in the 3–5 region of Ang II is more important to obtain AT_1 receptor affinity than AT_2 receptor affinity. In pseudopeptides targeting the AT_1 receptor, the synthetically easily accessible 1,3,5-trisubstituted benzene scaffold C (Figure 21) has previously been investigated as a γ-turn-mimicking scaffold by our group. Incorporation of this scaffold into the 3–5 region of Ang II gave a pseudopeptide that lacked AT_1 receptor affinity.[80] However, because of the differences between the AT_1 and AT_2 receptor regarding the sensitivity of the turn mimetic region, we wanted to investigate whether incorporation of scaffold C, and perhaps the even more compact, novel scaffold D (Figure 21), could render ligands with high AT_2 receptor affinity.

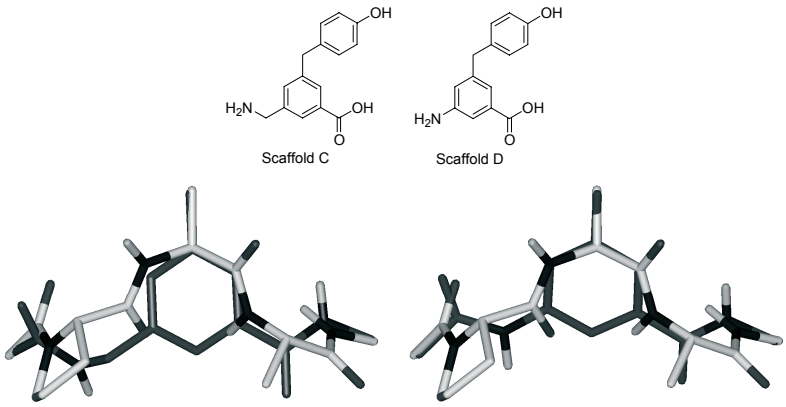


Figure 21. The 1,3,5-trisubstituted benzene scaffolds C and D and superimposition of scaffold C (left, dark gray) and scaffold D (right, dark gray) on an inverse γ-turn (white) found in a crystallized protein (PDB code 1EUV, chain A, Leu^{597} as central turn residue). Only backbone and C_β atoms are shown.

Scaffolds C and D were compared with γ-turns in crystallized proteins and both were shown to be well-suited as inverse γ-turn mimetics (Figure 21), albeit not with as good backbone alignment to residues *i* and *i*+2 as scaffold B. Nevertheless, incorporation of scaffolds C and D in Ang II gave the pseudopeptides **26**–**30** (Table A1), which all had affinity the AT_2 receptor. Compound **28** also showed AT_1 receptor affinity (K_i = 30.3 nM) and in functional

studies **28** was shown to act as an AT_2 receptor agonist but no agonistic effect was seen at the AT_1 receptor.
For the AT_2 receptor there were now a number of rather different scaffolds which, when incorporated into the 3–5 region in Ang II, all gave pseudopeptides with a K_i value of 10 nM or lower (**4**, **20**, **21**, **26**, **27**, and **28**). As previously done for **4**, **20**, and **21**, we now wanted to investigate whether this extended set of pseudopeptides comprising the different γ-turn-mimicking moieties could also adopt a common binding mode. Therefore a DISCOtech analysis was performed on model compounds of these six pseudopeptides (Figure 22).

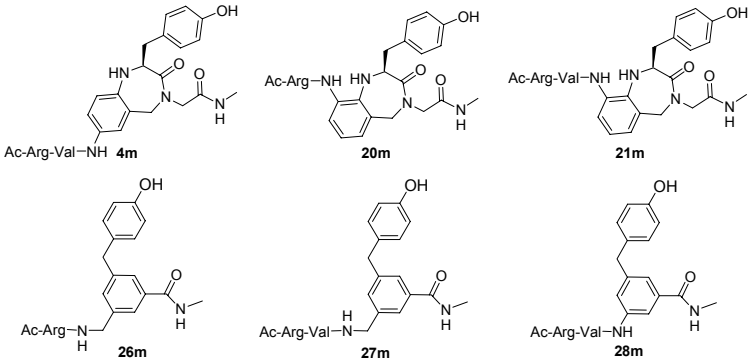


Figure 22. Model structures of **4**, **20**, **21**, **26**, **27**, and **28**, used in the DISCOtech analysis.

Compared to the previous DISCOtech analysis, larger parts of the compounds were used in the analysis, including the Tyr side chains and the region up to the C_α of the His residue (represented by an NHMe capping group). Because of the increased size and flexibility of the new model structures, the number of conformations in the conformational analysis preceding the DISCOtech analysis was rather high. To include the most relevant conformations, i.e. those that would have a high chance of being included in the DISCOtech models, sets of conformations for the DISCOtech analysis were selected based on comparison with each other. Only those conformations of each compound capable of giving approximate alignment with the conformations of the other compounds were included in the final sets. When the conformations were selected, unique DISCOtech features were created for the N- and C-terminal cap, the phenol oxygen atom of Tyr, and the receptor sites for Arg interactions. All other potential pharmacophore groups in the structures were removed to facilitate the analysis. To allow for the possibility that not all the compounds would align well, DISCOtech was set to find models where one compound could be excluded.

In the DISCOtech analysis 316 models were found. A few models including all six structures were identified; one is shown in Figure 23. This model

suggests that all six compounds can interact similarly with the AT_2 receptor, despite their geometrical differences. Also, this model was similar to the DISCOtech model obtained when only **4**, **20**, and **21** were included (Figure 20). Notably, the Val residues present in many of the compounds were fairly well aligned, which may suggest that a specific receptor interaction with Val could be obtained for these compounds. In summary, we now had developed a binding mode model of the region from C_α of Asp to C_α of His for these pseudopeptides, which may reflect how Ang II binds to the AT_2 receptor.

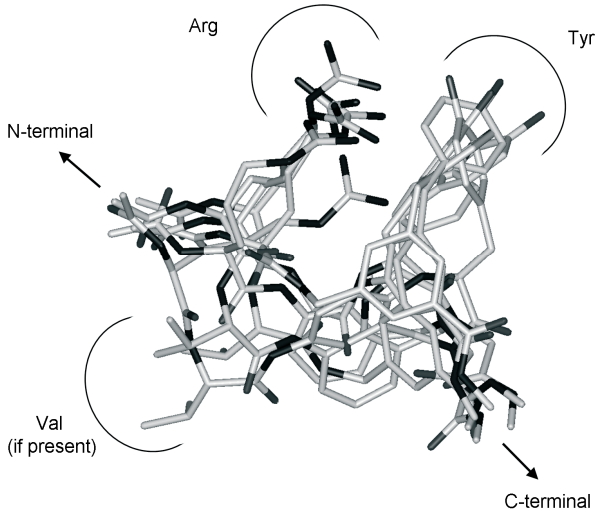


Figure 23. A DISCOtech model obtained from model structures of **4**, **20**, **21**, **26**, **27**, and **28** with aligned regions highlighted.

5.6 Benzodiazepine-Based β-Turn Mimetics (Paper V)

The β-turn mimetic scaffold E was developed by introducing one more side chain into scaffold A. By comparing scaffold E with β-turns found in crystallized proteins it was found that the new scaffold best mimicked a type II β-turn (Figure 24).

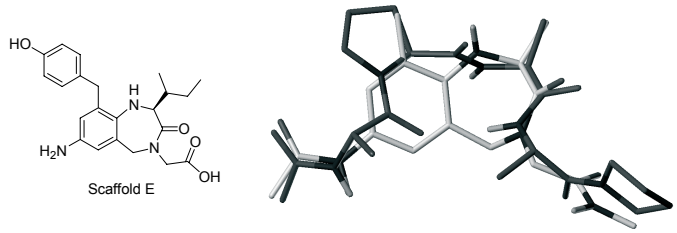


Figure 24. Scaffold E and the scaffold (dark gray) superimposed on a type II β-turn (PDB code 1H2C, chain A, Pro^{143} and Asp^{144} as central turn residues). Only backbone and C_β atoms are shown.

This scaffold was incorporated into Ang II to replace Val-Tyr-Ile, Tyr-Ile or Val-Tyr-Ile-His (**31**–**33**, respectively, Table A1). Compounds **31** and **32** showed high AT_2 receptor affinity, with K_i values of 4.7 nM and 1.8 nM, respectively, comparable to the pseudopeptides comprising γ-turn mimetic moieties. This was interesting since we had now developed pseudopeptides with high AT_2 receptor affinity that both comprised both γ-turn- and β-turn-mimicking scaffolds. We hypothesized that although the scaffolds were different from each other, they could position the N- and C-terminal residues in the same areas in the receptor and still render the same interactions with the Arg and Tyr side chains. To test this hypothesis, conformational analysis of model structures of **31** and **32** (analogous to the model structures in Figure 22, but with scaffold E) was performed, and the conformations obtained were superimposed on the previously obtained DISCOtech model presented in Figure 23. This superimposition suggested that the β-turn mimetic moiety was also able to position the N- and C-terminal parts and the Tyr side chain in the AT_2 receptor in similar positions to those in the γ-turn mimetics (Figure 25). However, the Ile residues in **31** and **32** did not have any correspondence in the γ-turn mimetics when superimposed as in Figure 25, which may provide extra receptor interactions for the β-turn mimetics.

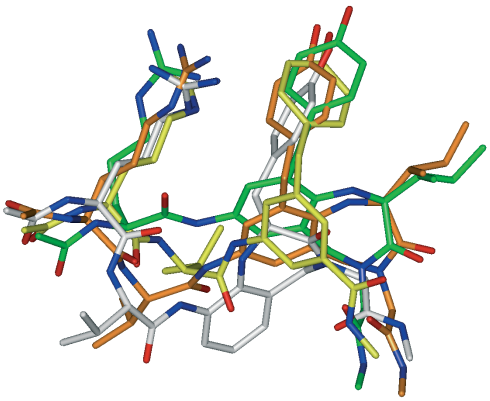


Figure 25. Model structures of **31** (green) and **32** (orange), comprising β-turn mimetic moieties, superimposed on the DISCOtech model structures of **21** (white) and **27** (yellow), comprising γ-turn mimetic moieties.

6 Structure-Based Modeling of AT_2 Receptor Ligands (Paper VI)

To further explore the binding mode of Ang II and the pseudopeptide Ang II analogues, a model of the AT_2 receptor was constructed. The transmembrane (TM) domain was modeled employing homology modeling using the 3D structure of the bovine rhodopsin receptor as template. To account for the flexibility of the extracellular loops (ECLs), the three ECLs were modeled using step-wise elongation of each loop, followed by energy minimization into families of loop conformations. The N-terminal tail and the intracellular domain of the receptor were excluded in the model. As an initial AT_2 receptor model, the receptor structure with the most open set of ECLs was chosen for further consideration. However, localization of the putative binding pocket for Ang II, based on important receptor residues reported in the literature, showed that ECL2 had folded down in the region most likely to be occupied by Ang II during binding. Therefore ECL2 was temporarily removed from the receptor model, and the peptide Arg-Val-Tyr-Val-His-Pro-Phe, serving as a model peptide of Ang II, was docked in the located binding pocket. The backbone of the ligand was constrained in a plausible bioactive conformation, and two ligand–receptor distance constraints were introduced (Arg guanidino group to Asp^{297} and Phe C-terminus to Lys^{215}) to constrain the ligand to the putative binding pocket. When the ten ligand–receptor complexes with the lowest energy were examined it was clear that the major part of the removed ECL2 was tolerated by the ligand poses. However, to fully accommodate the ligand, the central residues 188–199 of ECL2 needed to be remodeled, which resulted in a final receptor model suitable for further docking studies. A flexible docking study was performed of the Ang II model peptide in the AT_2 receptor model. The side chain conformations of the receptor residues Lys^{215}, Asp^{279}, and Asp^{298}, situated in the binding pocket, were also investigated in the docking study.

Based on the number of ligand contacts with receptor residues indicated to be important or in the proximity during binding, one ligand pose was found to fulfill most of the contacts (Figure 26), albeit in a high-energy ligand–receptor complex. In general, the conformation of the ligand was extended, with a γ-turn adopted around the Tyr residue. Interestingly, the conformation of the Ang II model peptide was also similar to a crystal structure of Ang II, obtained in complex with an antibody.[146] Figure 27 shows the docked pose superimposed on the Ang II crystal structure (root mean square distance of 0.76 Å when C_α atoms are superimposed).

For the receptor–ligand complex, ligand contacts or close proximities to the ligand can be found for Met^{128}, Lys^{215}, Asp^{279},and Asp^{297}, and to some extent also Arg^{182} and His^{273}, which are receptor residues that have been suggested to be involved in the binding of Ang II to the AT_2 receptor. However, all the suggested contact points could not be accounted for in the docking results. Phe in Ang II has been suggested to be in the proximity of Met^{138} during binding but this residue is too far down in the TM region of the AT_2 receptor model to be reached with this ligand binding mode.

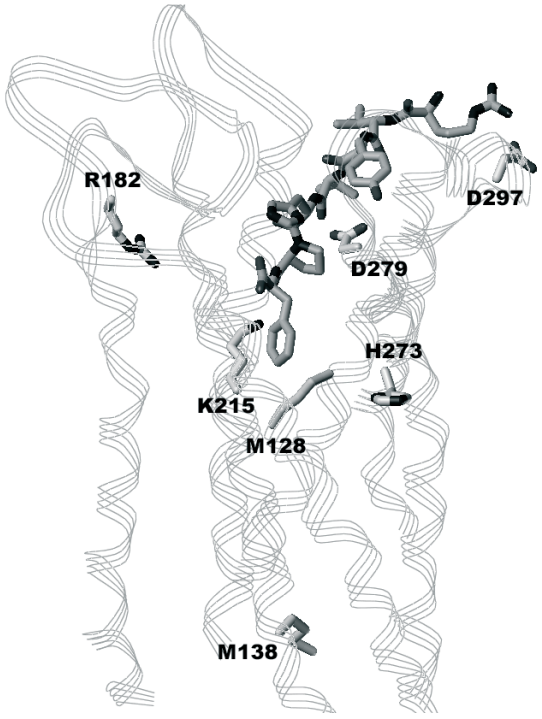


Figure 26. One of the plausible binding models found for the Ang II model peptide when docked in the AT_2 receptor model. The receptor residues shown are reported to affect binding affinity (Arg^{182}, Lys^{215}, His^{273}, Asp^{279}, and Asp^{297}) or to be in the proximity of the ligand during binding (Met^{128} and Met^{138}).

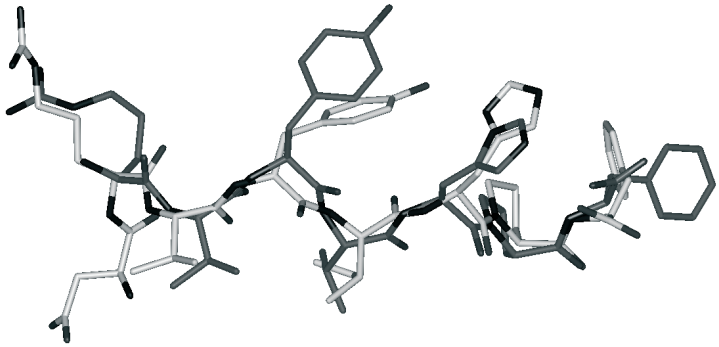


Figure 27. Docked pose of the Ang II model peptide (dark gray) superimposed on a crystal structure of Ang II (white).

Although this ligand–receptor complex had high energy, a low-energy complex with a similar ligand pose was also identified. The main difference between the two ligand poses was in the local conformation of the C-terminal Phe residue (Figure 28). This part of the ligand was located in a region of the receptor with tightly packed residues in both complexes. Since the receptor structure was frozen during the docking analysis, more favorable ligand–receptor packing might have been obtained if the receptor had been allowed to move. Thus, both these ligand configurations can be regarded as plausible binding models for the binding of Ang II to the AT_2 receptor.

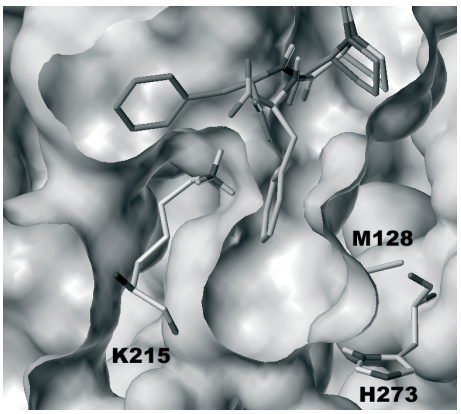


Figure 28. The different orientations of Phe in the plausible binding models found, shown in dark gray (low-energy complex) and light gray (high-energy complex). The AT_2 receptor surface, and the receptor residues Lys^{215}, His^{273}, and Met^{128}, situated in this region, are also shown.

Using the result obtained from the docking of the Ang II model peptide, the pseudopeptide ligands **4**, **20**, **21**, **26**, **27**, **28**, **31** and **32** were docked in the

binding pocket. To try to elucidate the inactivity of **3**, this pseudopeptide was also included in the analysis. To reduce the number of degrees of freedom, both the receptor and the His-Pro-Phe segment of the pseudopeptides were kept rigid during docking. Furthermore, the Asp residue of the pseudopeptides was replaced by an acetyl capping group.

The docking results suggested that **4**, **20**, **21** and **27** could adopt a binding mode similar to the Ang II model peptide (Figure 29), with an Arg–Asp^{297} interaction and with Tyr in the same receptor region. The docking result for **4** supported the hypothesis that the extra Val residue inserted into this compound acted as a spacer to compensate for the non-optimal scaffold and to enable contact with Asp^{297}, as seen in Figure 30. In addition, the docking results of the pseudopeptides also supported the previously derived hypothesis that the incorporated Val residues also contributed to the affinities also by a direct receptor interaction, as indicated in Figure 30, with the Val residue of **4** in proximity to Ile^{47}.

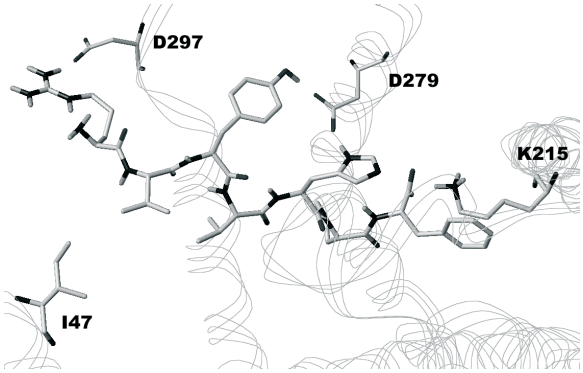


Figure 29. Docked pose of the Ang II model peptide in the AT_2 receptor model. The receptor residues Ile^{47}, Lys^{215}, Asp^{279}, and Asp^{297} are also shown.

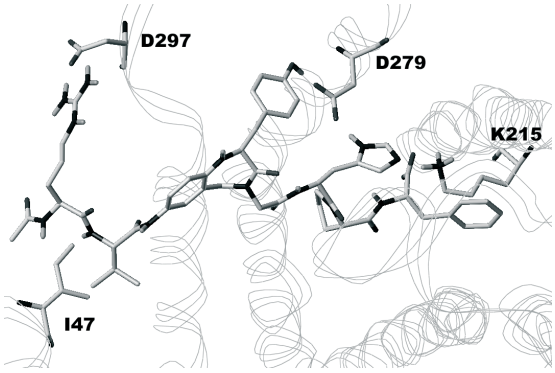


Figure 30. Docked pose of **4** in the AT_2 receptor model. The receptor residues Ile^{47}, Lys^{215}, Asp^{279}, and Asp^{297} are also shown.

The docked poses of **26**, **28**, **31**, and **32** differed from the binding mode shown in Figure 30. For these pseudopeptides the Tyr side chain was oriented very differently to allow the pseudopeptides to reach the Arg contact with Asp^{297}, as seen in Figure 31 for **28**. The inactive compound **3** also adopted a similar binding mode, which makes it difficult to rationalize the inactivity of this compound based on this binding mode. For the pseudopeptides comprising the benzodiazepine-based β-turn-mimicking scaffold (**31** and **32**), the energetically less favorable classic γ-turn conformation of the scaffolds was found in these docked poses.

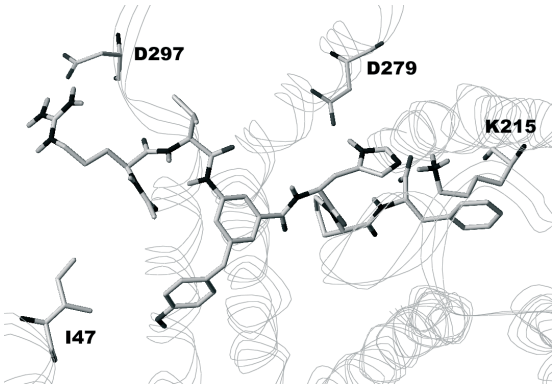


Figure 31. Docked pose of **28** in the AT_2 receptor model. The receptor residues Ile^{47}, Lys^{215}, Asp^{279}, and Asp^{297} are also shown.

When the docked poses of **4**, **20**, **21** and **27** were compared with the previously derived ligand-based model, obtained using DISCOtech, they were found to be in good agreement. The binding modes of **21** obtained from the two different approaches are shown in Figure 32, where it can be seen that the main difference lies in the orientation of the outgoing C-terminal segment from the scaffold.

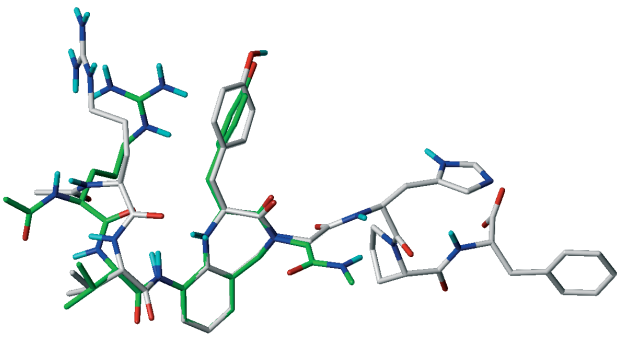
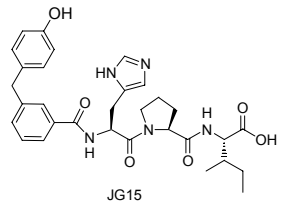


Figure 32. The conformation of **21** obtained from the ligand-based DISCOtech model (green) presented in Figure 23, superimposed on the structure-based model (AT_2 receptor docked pose) of **21** (white).

The docking of **26**, **28**, **31** and **32** did not result in the binding mode derived using ligand-based modeling regarding the positioning of the Tyr side chain. Thus, it would be interesting to investigate whether this side chain has any specific importance for AT_2 receptor binding. This is especially interesting for the pseudopeptides that adopted the classic γ-turn conformation of the benzodiazepine-based scaffold during docking, which indicates that a Gly or D-amino acids in the ring may be more energetically favorable, since an axial substituent would not be present.

7 Modeling of Ang II C-Terminal Mimetics (Papers VII and VIII)

In 1991 de Gasparo et al. reported that the truncated and acylated Ang II analogues Ac-Tyr-Val-His-Pro-Phe and Ac-Tyr-Val-His-Pro-Ile had affinity and selectivity to the AT_2 receptor.[147] This finding was further investigated by our group, showing that the closely related Ac-Tyr-Ile-His-Pro-Phe and Ac-Tyr-Ile-His-Pro-Ile had K_i values of 38 nM and 3 nM, respectively, to the AT_2 receptor.[148] In the same study, scaffolds A–D were incorporated into these pentapeptides, replacing the Tyr-Ile residue fragment. Several of the resulting short pseudopeptides were found to have high affinity to the AT_2 receptor and a few also showed AT_1 receptor affinity. Compound JG15 (Figure 33) bound selectively to the AT_2 receptor and had the highest affinity in the series, with a K_i of 0.5 nM. This compound was also shown to act as an AT_2 receptor agonist, making it a suitable lead structure for further development towards smaller, fully nonpeptide AT_2 receptor ligands.



JG15

Figure 33. The truncated pseudopeptide Ang II analogue JG15, acting as a selective AT_2 receptor agonist.

7.1 His-Pro-Phe-Based Ang II Analogues (Paper VII)

As previously described, losartan and structurally similar compounds have been proposed to mimic the interactions of the C-terminal residues of Ang II when binding to the AT_1 receptor. Because of their structural similarity, this may also apply to the selective AT_2 receptor agonist M024 when binding to the AT_2 receptor. Structural comparison between M024, Ang II and JG15 shows that no apparent structural motif in M024 corresponds to the Tyr side chain in Ang II and JG15. Therefore, we hypothesized that Tyr could be removed from JG15 to produce analogues without great loss of AT_2 receptor affinity. To test this hypothesis, four tripeptides (**34**–**37**, Table A1) and eight

pseudopeptides (**38**–**45**, Table A1), corresponding to the His-Pro-Phe C-terminal segment of Ang II, were evaluated with regard to AT_1 and AT_2 receptor affinity. The pseudopeptides comprised aromatic scaffolds with either a His side chain, as in Ang II, or an N-imidazolemethyl side chain, as in M024.

In the series of compounds evaluated, **45** (Figure 34) bound selectively and with good affinity (K_i = 16.6 nM) to the AT_2 receptor. From structural comparison of **45** and M024 it can be seen that the structures have several groups in common (e.g., the imidazole rings, phenyl rings, acidic moieties and alkyl chains), which may be interact with the same receptor areas during binding. However, when comparing the 2D structures it is not evident to which group in M024 the Ile side chain in **45** corresponds.

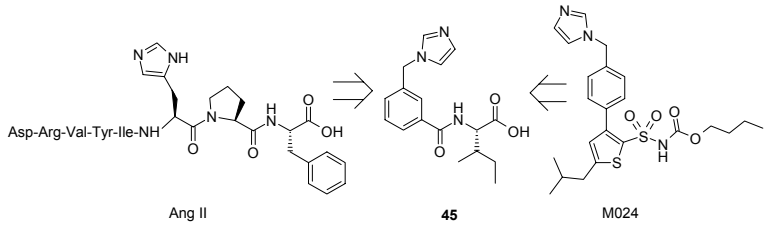


Figure 34. Compound **45** developed from both the endogenous peptide Ang II and the nonpeptide agonist M024.

To explore the possibilities, pharmacophore superimposition was performed of **45** and M024 using DISCOtech. Among the suggested pharmacophore models, two were found that presented plausible superimpositions of the compounds (Figure 35). Both these models had the imidazole and the acidic moiety well-aligned, but the Ile side chain in **45** is oriented towards different parts of the M024 structure.

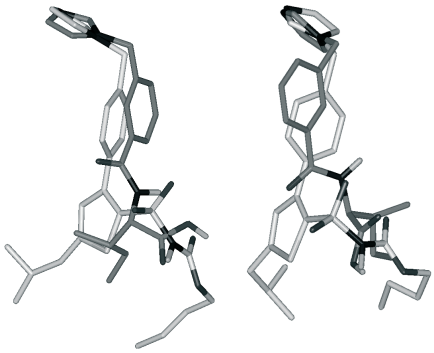


Figure 35. Superimposed structures from DISCOtech models based on **45** (dark gray) and M024 (white). The Ile side chain of **45** is positioned towards the thiophene ring and isobutyl side chain of M024 (left), or the *n*-butyl side chain of M024 (right).

Both these models seemed reasonable, and compound **45** thus represents a compound that was derived using a rational approach, starting from the endogenous peptide and which shows potentially similar interactions with the AT_2 receptor as the nonpeptide ligand M024. However, M024 will have an additional receptor interaction with the "extra" side chain, which may be one of the reasons why M024 has a higher AT_2 receptor affinity than **45**. In the docking results described earlier for the C-terminal part of the full-length Ang II analogues, the Phe side chain was found in two different positions (Figure 28). Thus, it could be speculated that **45** can occupy one of these Phe-positions while M024 could occupy both, to obtain a more optimal receptor interaction.

7.2 Nonpeptide Ligand 3D-QSAR Models (Paper VIII)

Since we now had approached structures that were similar to the fully nonpeptide Ang II receptor ligands such as M024 and the "sartan" class of compounds, we were interested in investigating such compounds in more detail. A data set of 244 nonpeptide angiotensin II receptor ligands was therefore compiled from the literature.[149-156] These compounds had been evaluated in a common binding assay and had reported AT_1 and AT_2 receptor affinity. This made it possible to derive models for AT_1 and AT_2 receptor affinity as well as selectivity towards these receptors. The general SAR for this data set has been reported previously,[117,118] but we now used the data set to obtain quantitative models for future affinity and selectivity predictions of new ligands.

To obtain these models the data set was analyzed using CoMFA. First, a conformational analysis was performed on two of the compounds in the data set, MK1 and MK2 (Figure 36), representing the two different structural classes in the data set. To reduce the number of conformations of MK1 and MK2, the constrained conformational properties of the macrocyclic analogue MK3 (Figure 36) were used as a filter.

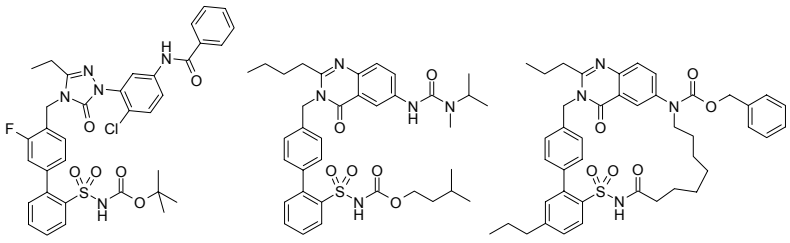


Figure 36. Structures used to create the data set alignment, using the macrocyclic compound as a conformational filter.

The alignment of MK1 and MK2 was obtained by superimposing possible pharmacophore groups found in the two compounds using the DISCOtech program. This resulted in the alignment presented in Figure 37. From this alignment the rest of the compounds in the data set were built in SYBYL. Semi-empirical AM1 charges[157] were calculated for the structures, followed by calculation of the CoMFA interaction energies. A diverse training set consisting of half of the total data set was selected using hierarchical clustering based on the CoMFA fields, AT_1 and AT_2 receptor affinity, and AT_1/AT_2 receptor selectivity. The remaining compounds were used as an external test set to asses the predictive ability of the CoMFA models.

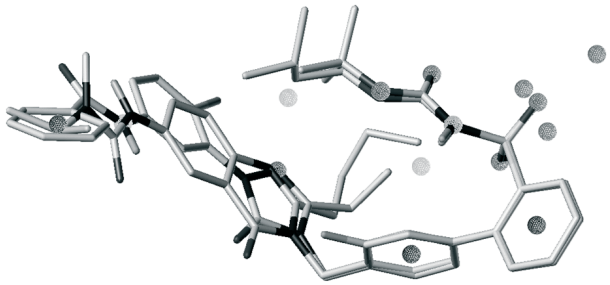


Figure 37. Alignment of MK1 and MK2 using DISCOtech. The spheres correspond to the DISCOtech features included in the model.

The CoMFA interaction energies of the compounds in the training set were correlated with the responses using PLS to obtain models for AT_1 receptor affinity, AT_2 receptor affinity and AT_1/AT_2 receptor selectivity. The three models obtained had both good internal and external predictive ability, as can be seen in Table 3. The R^2_{pred} values were derived from the predictions of the test set using Equation 5.

Table 3. Statistical results of the CoMFA models for each response.

Response	c^a	R^2	Q^{2b}	R^2_{pred}
pAT1	4	0.78	0.54	0.69
pAT2	3	0.82	0.69	0.86
pSel	5	0.89	0.69	0.80

[a]Number of components
[b]Leave-20%-out cross-validation

$$R^2_{pred} = 1 - \frac{\sum (y_{pred} - y_{obs})^2}{\sum (y_{obs} - \bar{y}_{obs})^2} \quad (5)$$

The CoMFA contour plots of each response can be seen in Figure 38. The CoMFA contours are in accordance with the qualitative SAR previously reported for this data set in several areas. The most easily interpreted contour

is the one originating from the alkyl side chain of the heterocyclic rings. A long chain favors AT_1 receptor affinity and selectivity, shown by the green contour in this area. A long chain is correlated with lower AT_2 receptor affinity, shown by the yellow contour. The partial negative charge of the *o*-fluoro substituent in the biphenyl group is correlated with higher AT_2 receptor affinity, but is not significant for AT_1 receptor affinity in the contour level shown. However, the AT_1/AT_2 receptor selectivity is affected: a positive charge increases the selectivity.

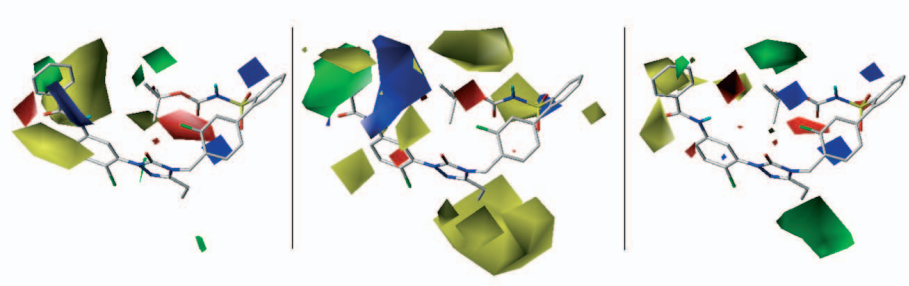


Figure 38. The CoMFA contour plots of AT_1 receptor affinity (left), AT_2 receptor affinity (middle) and AT_1/AT_2 receptor selectivity (right), at upper 85^{th} and lower 15^{th} percentiles. Red and blue contours correspond to areas favorable for negative and positive charge, respectively. Green and yellow contours correspond to sterically favorable and unfavorable areas, respectively.

The information from these QSAR models and the ability to predict affinities and selectivity can now be used as guidance for future development of new nonpeptide compounds, both those originating from Ang II and in series of M024 analogues.

8 Conclusions

In the present work, new insight has been gained into the binding mode of Ang II and Ang II analogues when binding to the AT_2 receptor.

- Benzodiazepine-based and 1,3,5-trisubstituted benzene scaffolds were designed, characterized and evaluated as γ-turn mimetic moieties.

- A benzodiazepine-based scaffold was designed, characterized and evaluated as a β-turn mimetic moiety.

- Computational modeling of the Ang II analogues comprising the turn mimetic moieties revealed a binding mode that all active compounds could adopt.

- A homology-based model of the AT_2 receptor was constructed. Using this receptor model in a docking study, enabled binding modes of Ang II and pseudopeptide Ang II analogues to be identified that were in agreement with contact points suggested for the binding of Ang II to the AT_2 receptor. The identified binding modes were in agreement with a turn structure around Tyr, while still adopting an overall extended binding mode, and were similar to the binding mode identified using the ligand-based approach.

- Using step-wise modification of Ang II, a low-molecular-weight compound was developed that could potentially provide interactions with the AT_2 receptor similar to the nonpeptide agonist M024.

- CoMFA models were derived for affinity and selectivity to the AT_1 and AT_2 receptors for nonpeptide ligands. These models were in general agreement with the SAR of the compound series and showed high quantitative predictive ability.

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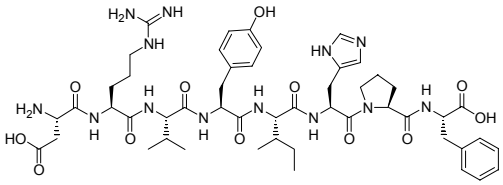
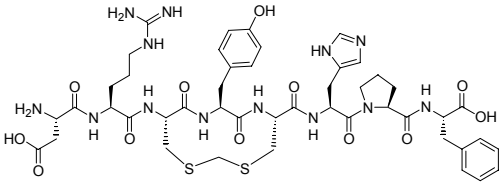
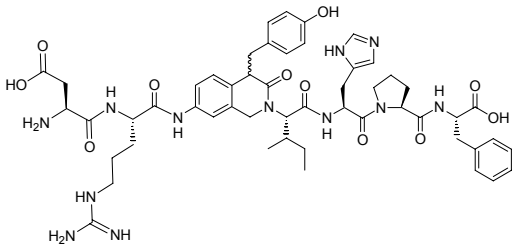
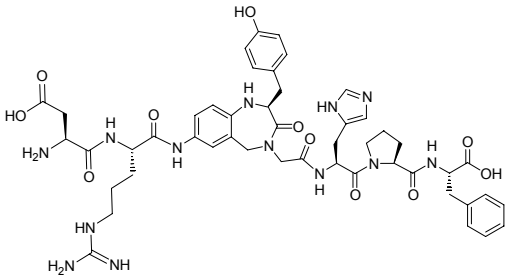
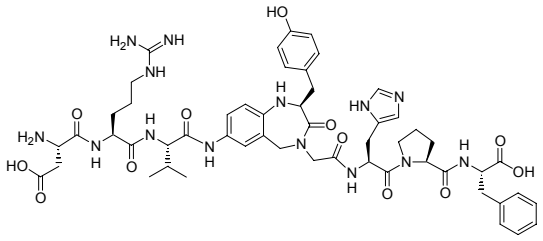
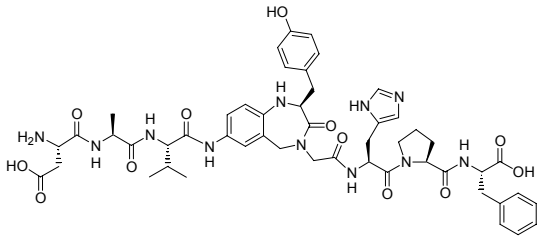
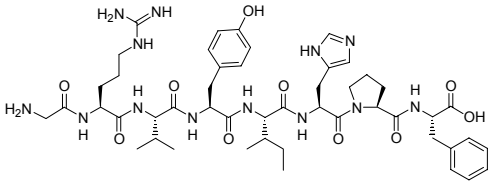
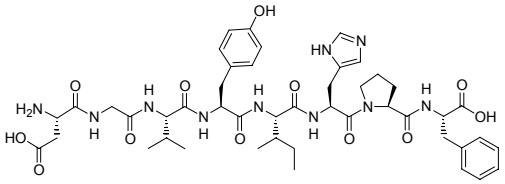
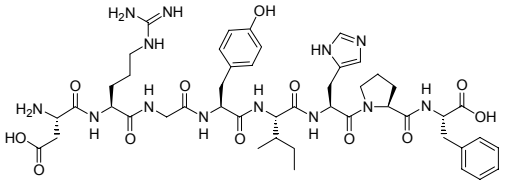
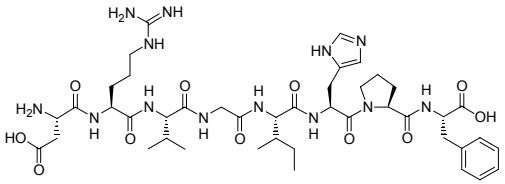
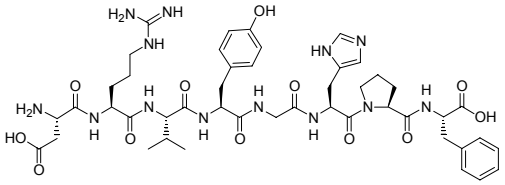
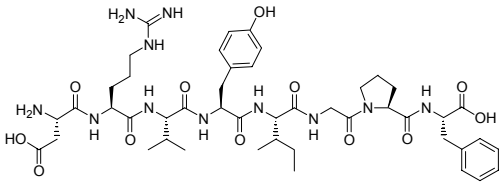
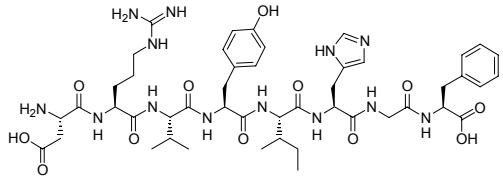
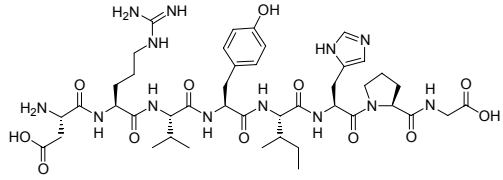
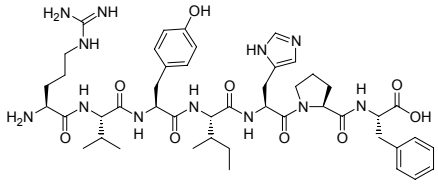
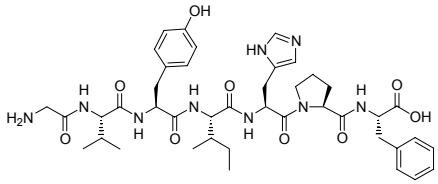
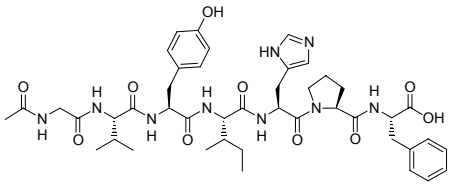
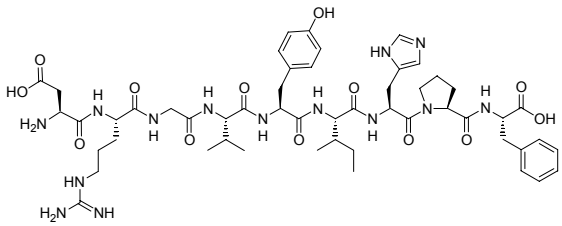
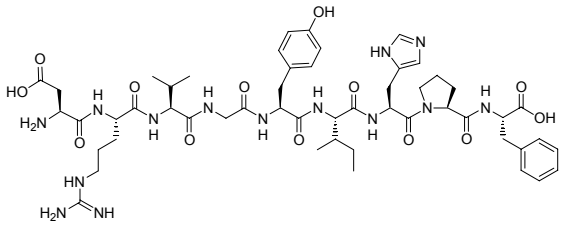
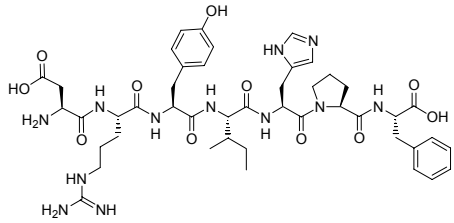
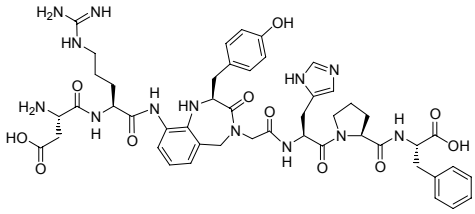
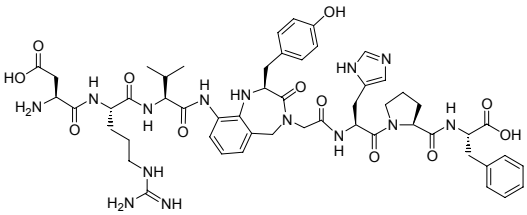
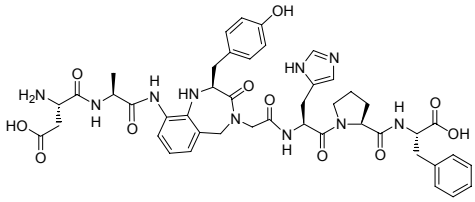
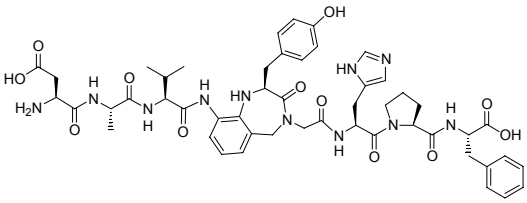
11 Appendix

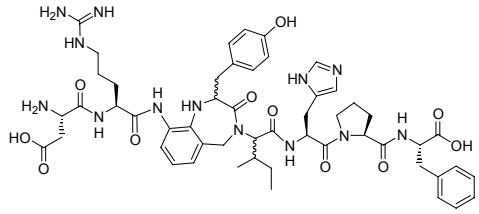
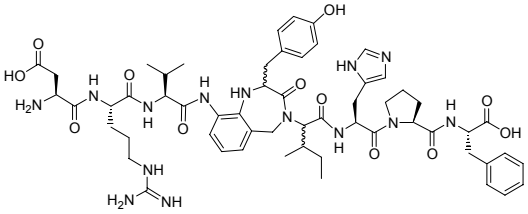
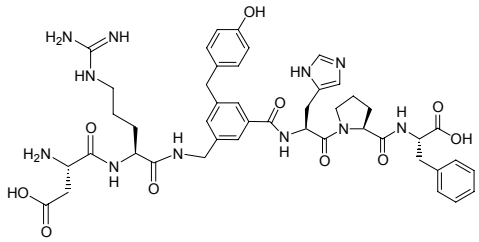
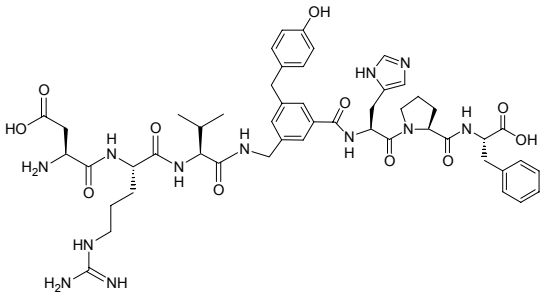
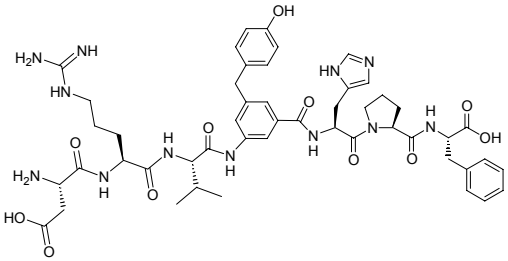
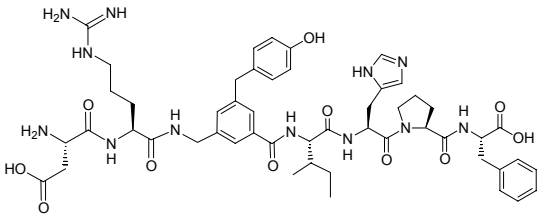
Table A1. Compilation of structures and AT_1 and AT_2 receptor affinities for peptides and pseudopeptides in paper I-VII. Functionality is assigned if tested.[a]

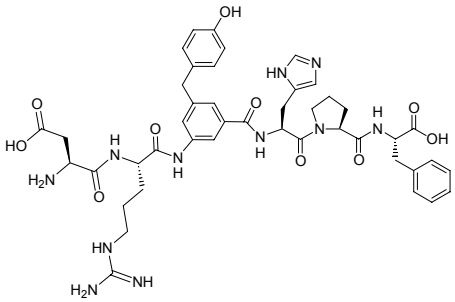
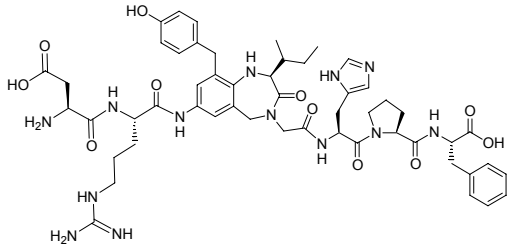
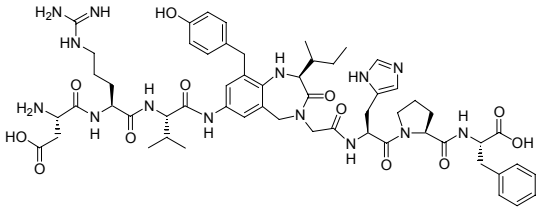
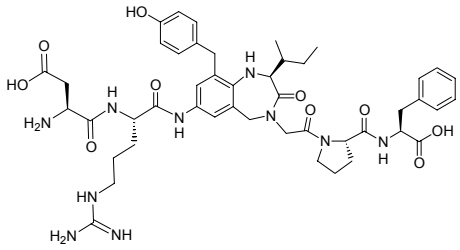
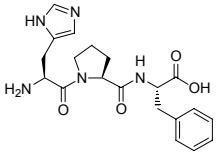
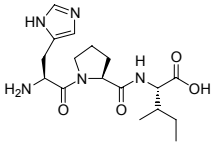
No.	Structure	AT_1[b] K_i (nM) ± SEM	AT_2[c] K_i (nM) ± SEM
Ang II		0.24 ± 0.07 agonist	0.23 ± 0.01 agonist
1		44 ± 1	0.62 ± 0.04
2a[d]		> 10000	61 ± 2
2b[d]		> 10000	> 10000
3		> 10000	> 10000

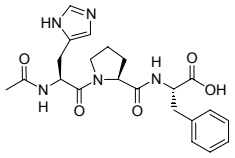
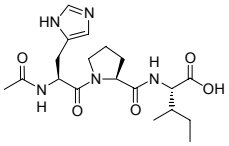
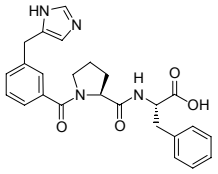
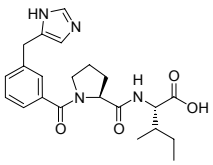
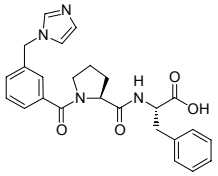
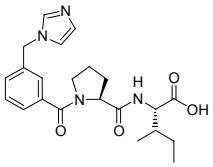
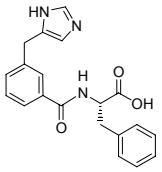
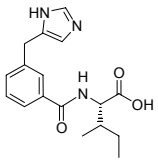
4		> 10000	3.0 ± 1.1 agonist
5		> 10000	> 10000
6		6.1 ± 0.2	4.0 ± 0.6
7		> 10000	55 ± 5
8		1.6 ± 0.1	1.2 ± 0.2
9		48 ± 2	5.2 ± 0.8
10		1.6 ± 0.1	2.3 ± 0.1

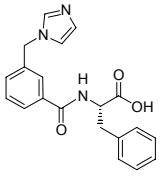
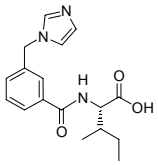
11		14.3 ± 0.06	7.6 ± 1.0
12		146 ± 7	4.3 ± 0.6
13		> 10000	1.1 ± 0.1
14		10.5 ± 0.3	2.2 ± 0.2
15		> 10000	5.4 ± 0.4
16		17.3 ± 0.2	2.8 ± 0.3
17		> 10000	2.9 ± 0.3

18		> 10000	255 ± 12
19		204 ± 10	238 ± 7
20		> 10000	2.8 ± 0.2 agonist
21		> 10000	0.8 ± 0.1 agonist
22		> 10000	37.4 ± 2.1
23		> 10000	9.3 ± 1.4

24a[d]		> 10000	2.6 ± 0.2
24b[d]		> 10000	117.4 ± 7.7
25a[d]		> 10000	0.3 ± 0.01
25b[d]		> 10000	0.08 ± 0.003
26		> 10000	10.1 ± 0.6
27		> 10000	1.85 ± 0.1
28		30.3 ± 1.5	9.8 ± 0.3 agonist
29		> 10000	463.7 ± 10.9

30		> 10000	41.3 ± 0.8
31		1668 ± 20	4.7 ± 0.3
32		14.9 ± 0.4	1.8 ± 0.04
33		2108 ± 33	53.1 ± 1.7
34		1598 ± 66	> 10000
35		> 10000	> 10000

36		> 10000	> 10000
37		> 10000	1110 ± 17
38		> 10000	> 10000
39		> 10000	> 10000
40		> 10000	> 10000
41		> 10000	> 10000
42		> 10000	40.6 ± 1
43		> 10000	> 10000

44		> 10000	37.5 ± 0.6
45		> 10000	16.6 ± 0.5

[a] rabbit aorta strip assay for the AT_1 receptor, neurite outgrowth assay for the AT_2 receptor
[b] rat liver membranes
[c] pig uterus myometrium
[d] a and b denote single diastereomers of unassigned absolute stereochemistry

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