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A Frame Shifted Disulfide Bridged Analogue of Angiotensin II

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Abstract—*N*-(2-Mercaptoethyl)glycine [NMGly] was incorporated into the 3 and 5 positions of angiotensin II and oxidized to give the corresponding cyclized disulfide c[NMGly^{3,5}]Ang II. The binding affinity to the angiotensin II receptor (AT_1) of this conformationally constrained analogue, which is related to the potent Ang II agonist c[Hcy^{3,5}]Ang II, was examined. The analogue had no affinity to the AT_1 receptor. Theoretical conformational analysis was performed to compare the conformational characteristics of model compounds of c[Hcy^{3,5}]Ang II and the frame shifted analogue c[NMGly^{3,5}]Ang II in an attempt to explain the lack of affinity.

Introduction

The vast number of bioactive peptides has stimulated many research groups to devise strategies to identify their bioactive conformation in order to allow for development of non-peptidic compounds as potential drug candidates. In the approach that we have adopted, a key step is to stabilize a secondary structure of the peptide by introducing a cyclic constraint, while at the same time retain the pharmacological activity. If this can be achieved, conformationally relevant nonpeptidic scaffolds (e.g., γ- and β-turn mimetics) can be used to substitute the secondary structure. In order to induce these structures in a predictable manner, we are investigating different cyclization strategies.

Human angiotensin II (Ang II) is an important peptide in blood pressure regulation. The wealth of published conformational studies,[1–4] the known effects of amino acid alterations[5–7] and the availability of receptor assays[10] make Ang II a particularly attractive target to examine new methods for conformational control. Among a series of monocyclic Ang II analogues that have been synthesized and tested by Spear et al.,[9] c[Hcy^{3,5}]Ang II [**1**] was found to be an agonist equipotent to Ang II (Scheme 1). This disulfide cyclized Ang II analogue represents a successful example where agonist activity is retained and conformational freedom is reduced.

Cyclization by oxidation of two mercapto alkyl amino acids in a peptide, for example homocysteines, cysteines, penicillamines or combinations of these, constitutes one of the most common methods for introduction of conformational constraints. A disadvantage of using this approach is, that at least two of the amino acids of the native peptide have to be substituted by sulfur containing acids to enable cyclization (i.e., side chain-side to chain cyclization). An alternative approach has recently been exploited by Gilon et al.[10–14] and Undén et al.[15,16] using the backbone nitrogen atoms as the attachment points of the mercapto containing side chains to enable cyclization (backbone-to-backbone cyclization) and thereby keeping the original side chains as in the native peptide. We were interested to apply this methodology to the potent Ang II agonist **1** using the building block *N*-(2-mercaptoethyl)glycine [NMGly] and to synthesize the frame shifted analogue c[NMGly^{3,5}]Ang II **2** (Scheme 1). In addition, we wanted to synthesize the dicarba analogue **3** (Scheme 1) by a ring closing metathesis (RCM) reaction. In this study, we report our synthetic efforts to obtain **2** and our attempts to synthesize the

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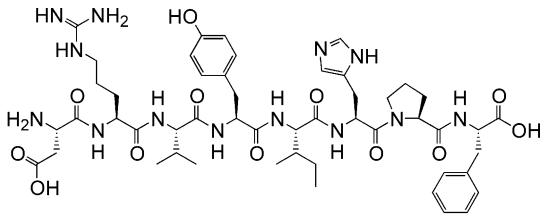
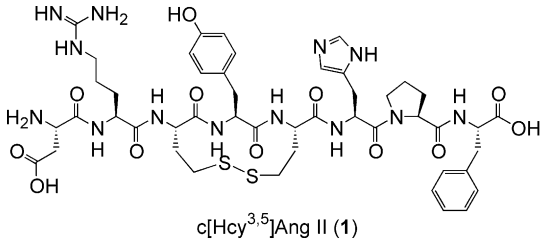
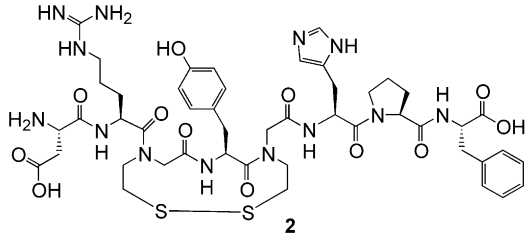


Angiotensin II (Ang II)



c[Hcy^{3,5}]Ang II (**1**)



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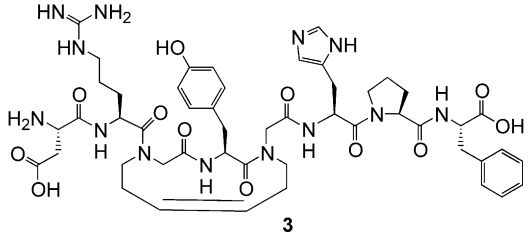


3

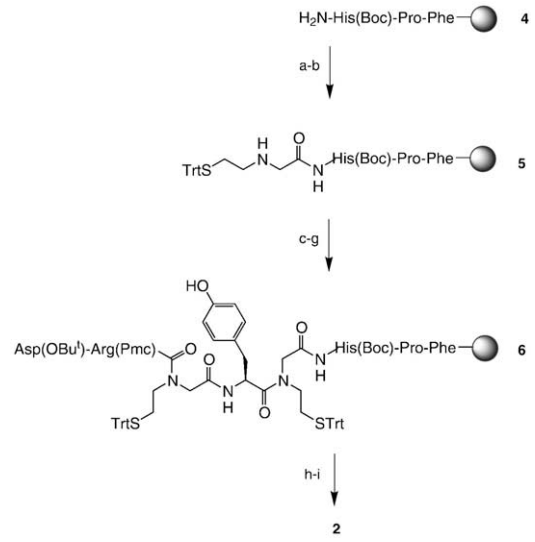
Scheme 1.

dicarba analogue **3**. Furthermore, we report the binding affinity of **2** for the Ang II AT_1 receptor as well as the conformational characteristics of a model compound of **2**.

Results and Discussion

Chemistry

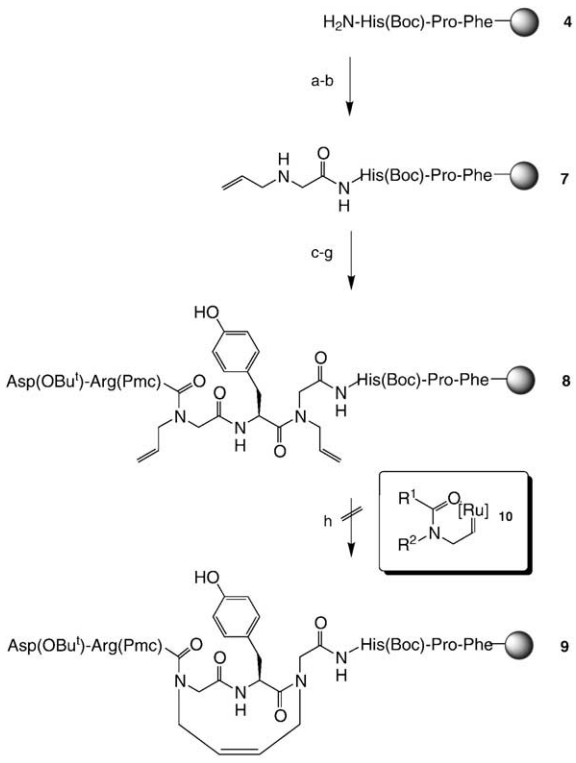
Compound **2** was assembled by Fmoc methodology on a Wang resin (Scheme 2). The *N*-alkylated glycines were obtained by acylation of the resin bound tripeptide (**4**) with bromoacetic acid[17] and diisopropylcarbodiimide. In order to avoid problems associated with the deprotection of the benzylthio derivatives,[12,13] we decided to use a more labile trityl protection on sulfur. Thus, the *N*-alkyl side chains were introduced by substitution with excess of *S*-Trityl-aminoethanethiol in DMF to afford **5**. The same sequence of reactions was used to introduce the *N*-alkyl derivative in position 3. The following elongation by tyrosine and arginine, respectively, crucially depends on the right agent to achieve condensation with the 2° amino termini. PyBroP® (bromotripyrrolidinophosphonium hexafluorophosphate), which gives good



Scheme 2. Reagents: $BrCH_2COOH$ (5 equiv), DIC, DMF; (b) $TrtSCH_2CH_2NH_2$ (15 equiv), DMF; (c) Fmoc-Tyr(^{t}Bu)–OH, PyBroP, DIEA, DMF; (d) $BrCH_2COOH$ (5 equiv), DIC, DMF; (e) $TrtSCH_2CH_2NH_2$ (15 equiv), DMF; (f) Fmoc-Arg(Pmc)–OH, PyBroP, DIEA, DMF; (g) Fmoc-Asp(O^{t}Bu)–OH, HBTU, DIEA, DMF; (h) cleavage/deprotection: TFA/H_2O/triethylsilane (90/5/5); (i) cyclization: 10% DMSO in TFA.

results[18] for *N*-methylated amino acids, turned out to be the reagent of choice. The oxidation of the resin bound peptide **6** by 10 equiv of iodine met with failure. Therefore, the octapeptide was cleaved from the resin and deprotected by TFA. The peptide was precipitated and washed with ether. Finally, oxidation of the free dithiol by 10% DMSO in TFA provided the crude cyclized peptide, which was purified by RP-HPLC to give pure **2** in 25% yield.

A new approach to constrained peptides was pioneered by R. Grubbs, who used ring closing metathesis (RCM) of 1,4-bis-*C*-allylated peptides[19–21] to synthesize β-turn analogues. This stimulated us to employ a similar approach to the angiotensin II analogue **3**, via the 1,3-bis-*N*-allylated peptide. For the solid-phase synthesis of **8** we modified the Zuckermann approach[17] to *N*-alkylated peptides (Scheme 3). Treatment of the resin-bound His-Pro-Phe tripeptide with chloroacetic acid anhydride in the presence of diisopropylethylamine (DIEA) resulted in the formation of a chloroacetamide, which was converted to the *N*-allylglycyl peptide by a large excess of allylamine. The further synthesis of the *N*-allylated peptide by SPPS was straightforward and without problems. The formation of the octapeptide was confirmed by PDMS on an aliquot prior to attempted ring closing metathesis. However, RCM of the resin bound peptide failed to give **9** for the two given catalysts in both DCM (CH_2Cl_2) and toluene. Neither for methyltrioxorhenium[22] nor with the Grubbs catalyst $[(PCy_3)_2Ru(CHPh)Cl_2]$ did we observe the cyclized peptide at or below 40 °C. This failure may be attributed either to chelation via a six-membered cyclic intermediate **10**, as it was postulated by Fürstner et al.,[23] which traps the catalyst, or to the considerable strain of the targeted 11-membered ring. Probably the build up of transannular interactions favours polymerization over cyclization. The formation of such 11-membered

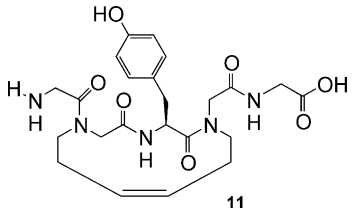


Scheme 3. Reagents: $(ClCH_2CO)_2O$ (5 equiv), DIEA, DMF; (b) allylamine (15 equiv), DMF; (c) Fmoc-Tyr(ᵗBu)–OH, PyBroP, DIEA, DMF; (d) $(ClCH_2CO)_2O$ (5 equiv), DIEA, DMF; (e) allylamine (15 equiv), DMF; (f) Fmoc-Arg(Pmc)–OH, PyBroP, DIEA, DMF; (g) Fmoc-Asp(OᵗBu)–OH, HBTU, DIEA, DMF; (h) $(PCy_3)_2Ru(CHPh)Cl_2$ or $MeReO_3$, CH_2Cl_2, rt.

rings by RCM has so far not been reported.[24] However, the precursor of the analogue 13-membered ring **11** (Scheme 4), which derived from replacement of allylamine by homoallylamine, cyclized readily under RCM conditions at 40 °C in DCM for 16 h. Similar observations of chain elongation in RCM were reported by B. Schmidt in 1998[35] and R. Liskamp in 1999.[25–27]

Conformational analysis

In a previous study using the MacroModel program[28] the conformational preferences of **11** (Scheme 4), a model compound of **1**, were investigated.[29] In that study 87 conformations within 20 kJ/mol of the lowest energy conformation were identified. Using a similar protocol in the present study (Amber* force field and the GB/SA solvation model for water), the conformational preferences of **12**, a model compound of **2** (Scheme 5), were investigated. Since this compound contains *N*-alkyl amino acid residues, we allowed for both *cis* and *trans* ω_1 and ω_3 amide bonds to be formed in the conformational search.[30,31] Thus, 469 conformations were identified within 20 kJ/mol of the lowest energy conformation of **12**. The large number of conformations found may be attributed to the considerable flexibility of glycine and that both *cis* and *trans* tertiary amide bonds (ω_1 and ω_3) were allowed in the search.



Scheme 4.

A classification of the identified conformations of **12** based on the propensity to form β-turns and γ-turns were performed in MacroModel. A β-turn is most often identified as any tetrapeptide sequence occuring in a nonhelical region in which the distance between Cα1 and Cα4 is less than or equal to 7 Å.[32–34] According to these criteria 122 out of the 469 conformations could be classified as β-turns when residues 1–4 were considered and 134 conformations when residues 2–5 were considered. To identify if any of the classical β-turn types were present (type I, I′, II, II′, III, III′, V, V′, and VIII)[35] we searched all the β-turn conformations and allowed a $\pm 30°$ deviation from the ideal Φ and Ψ values. Out of all the β-turn conformations only one type II β-turn conformation (with $\omega_3 = 0°$) fulfilled the above torsion angle criteria. No classic or inverse γ-turn conformations were identified for Φ_3 and Ψ_3 (allowed deviation $\Phi_3 = 75° \pm 20°$, $\Psi_3 = -65° \pm 20°$ and $\Phi_3 = -75° \pm 20°$, $\Psi_3 = 65° \pm 20°$). The rough similarity between the lowest energy conformation of **11** and **12** is shown in the superimposition in Figure 1.

Stereorepresentation of the best fit between the lowest energy conformation of compound **11** (light grey) and compound **12** (dark grey). The Cα atoms were used in the fitting procedure. Mean distance between fitted atoms = 1.13 Å.

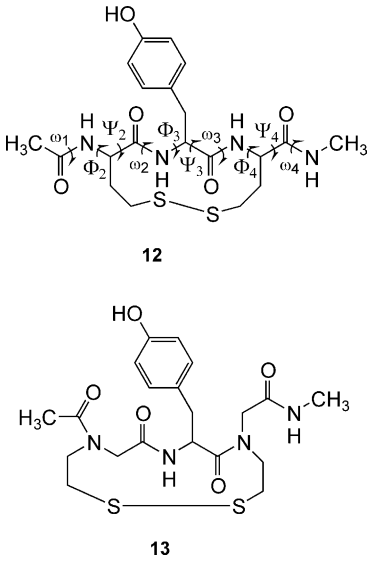
Pharmacology

Ang II, **1** and **2** were analyzed in a radioligand binding assay monitoring the displacement of [^{125}I]Ang II in the presence of BSA (0.2%) from chinese hamster ovary cells (CHO), transfected with the rat AT_1 receptor cDNA.[36] In previous studies it was shown that Ang II and **1** are equipotent ligands to the AT_1 receptor.[9,29] The high affinity of Ang II ($IC_{50} = 1.5$ nM) and **1** ($IC_{50} = 0.2$ nM) to AT_1 receptors was also confirmed in this study using CHO cells. In contrast, the frame shifted analogue **2** lacked affinity to the AT_1 receptor ($IC_{50} > 10^{-5}$ M).

Discussion

There may be at least two explanations of the lacking affinity of the frame shifted analogue **2** as compared to

Figure 1.



Scheme 5.

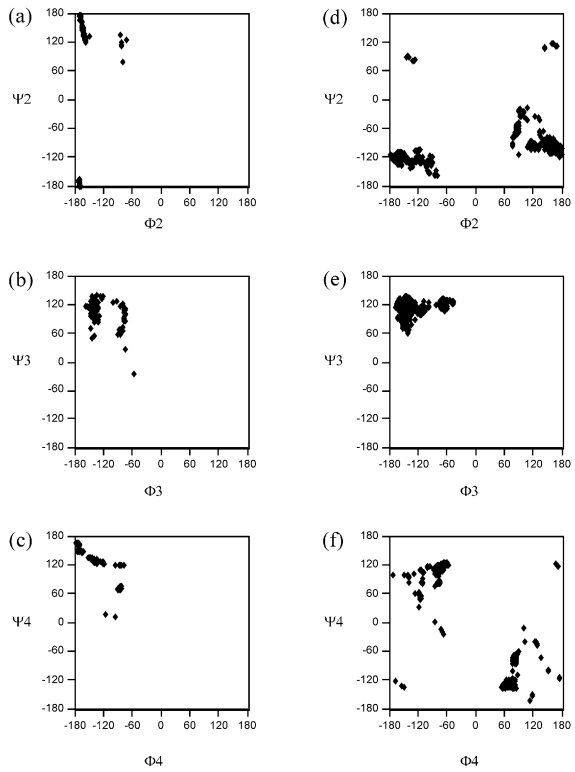


Figure 2. The Φ and Ψ backbone torsion angles of the conformations identified in the conformational search (ΔE <20 kJ/mol) of compound **12** (a–c) and **13** (d–f).

1: (a) the amide hydrogens of amino acids 3 and 5 in **1**, that are replaced by alkyl groups in **2**, are necessary for binding to the Ang II receptor and/or (b) the mode of cyclization using the amide nitrogens as the attachment points for the cyclic system may change the conformational preference of **2** as compared to **1**. *N*-Alkyl substitution is known to affect the conformation, because of the unability to form hydrogen bonds and/or steric effects, and therefore **2** may be unable to adopt the binding conformation of **1**.

The importance of the amide hydrogens for agonist activity can be deduced from *N*-alkyl substituted analogues of Ang II. In a recent study, the agonistic and antagonistic potency of Ang II analogues with constrained amino acids in position 3, 5 and 7 were investigated.[37] In this study [Sar^1,(N-Me)Ile^5]Ang II showed reduced agonist potency. This indicates that the N–H group of residue 5 is important for agonist activity. However, it cannot be concluded whether the lower potency of the synthesized Ang II analogues is due to the loss of hydrogen bonding capability to the receptor or due to conformational effects of the introduced *N*-Me group. Interestingly, the low activity of [Sar^5]Ang II has been rationalized in terms of conformational effects.[38]

To investigate the conformational similarities and/or dissimilarities between **1** and **2**, we used the model compounds **12** and **13**. The conformational profile of these compounds were analyzed by plotting the backbone Φ and Ψ torsion angles of the conformations identified in the conformational search with energies <20 kJ/mol above the lowest energy minimum (Fig. 2). These plots revealed that the available Φ/Ψ-space seems to be larger for **13** than for **12**. Inspection of the individual maps displays that the Φ_3/Ψ_3-angles have a similar distribution, except for one conformation of **12** having Φ_3-values around −60° and Ψ_3-values around −30°. In the Φ_2/Ψ_2 plot only compound **13** assumes conformations with Ψ_2-values around −120° and Φ_2-values around −120 or 120°. In contrast, compound **12** seems to adopt extended conformations preferentially with Φ_2 and Ψ_2 values around 180°. In the Φ_4/Ψ_4 plot, a unique conformational region having Φ_4-values between 60 and 180° and Ψ_4-values between −60 and −150° is present for **13**. Taken together, these results suggest that compound **12** can adopt some conformational families that cannot be adopted by **13**. However, it should be remembered that the present calculations were performed on model compounds of **1** and **2** and that the backbone torsion angles only were considered. Most certainly the side chains play a crucial role in binding to the receptor and therefore the whole peptide needs to be considered when trying to rationalize the biological activity.

Conclusion

We have synthesized a frame shifted disulfide bridged analogue of **1** which lacked affinity to the AT_1 receptor. Although the compound studied in the present work did not bind to the AT_1 receptor we believe that application of the backbone cyclization methodology is useful and provides a valuable complement to other methods in the search for the bioactive conformation of peptides. Attention should be drawn to the fact that the backbone-to-backbone cyclization in principle allows for incorporation of all types of amino acid side chains in the constrained region.

Experimental

Chemistry

General comments. Mass spectroscopy was done with an Applied Biosystems BIOION 20 plasma desorption mass spectrometer. PDMS-samples were applied on aluminized mylar foils, coated with electro-sprayed nitrocellulose, by drying from EtOH/H_2O/TFA mixtures and removal of excess liquid by nitrogen. Analytical and preparative HPLC was performed on an LDC chromatography system equipped with a variable wavelength detector using 218TP5415 VYDAC (10 μm, 4.6 × 150) or 218TP1010 VYDAC (10 μm, 10 × 250) columns. Amino acid analysis and peptide content determination was performed on 24 h hydrolysates with an LKB 4151 alpha plus analyser using ninhydrin detection.

Materials. SPPS resins, amino acid derivatives, for example Fmoc-His(Boc)–OH×CHA, Fmoc-Arg(Pmc)OH, Fmoc-Asp(O^{t}Bu)OH, Fmoc-Tyr(^{t}Bu)OH and condensation reagents (PyBroP®) were bought from Fluka Chemie (Switzerland), NovaBiochem (Switzerland), Millipore and Bachem (Switzerland). *S*-Trt-mercaptoethylamine was synthesized according to the literature.[39] DCM (HPLC grade, Rathburn, UK) and DMF (peptide synthesis grade, Millipore) were used without further purification. All chemicals and mediums for cell growing and receptor binding were purchased from Life Technologies, except 1,10-phenanthroline (Fluka), bacitracin and leupeptin (Sigma). Monoiodinated [^{125}I]-Ang II was made with the chloramine-T method by Euro-Diagnostica AB, Malmö, Sweden.

Solid-phase peptide synthesis (SPPS)

The peptides were synthesized by manual SPPS using Fmoc protected amino acids: Fmoc-His(Boc)–OH× CHA, Fmoc-Arg(Pmc)–OH, Fmoc-Asp(O^{t}Bu)–OH and Fmoc-Tyr(^{t}Bu)-OH. The starting polymer was preloaded Fmoc-Phe-Wang resin. The general procedure for amino acid coupling and deprotection was performed as published.[29]

3,5-*cyclo*-[Aspartyl-arginyl-(*N*-2-mercaptoethyl)glycyl-tyrosyl-(*N*-2-mercaptoethyl)glycyl-histidyl-prolyl-phenylalanine] (2). Fmoc-His(Boc)-Pro-Phe-Wang-resin (122 mg, 68.6 μmol) was suspended in DMF (2 mL), deprotected by 20% (v/v) piperidine–DMF solution (3×2 mL, 2×2 + 10 min) and rinsed by DMF (6×2 mL). The resin was suspended (3×) in a solution of bromoacetic acid (114 mg, 824 μmol) and diisopropylcarbodiimide (DIC, 113 mg, 832 μmol) in DMF (0.5 mL) and shaken for 30 min each. After rinsing the resin with DMF (6×2 mL), a solution of *S*-Trt-mercaptoethylamine (329 mg, 15 equiv) in DMF (0.8 mL) was added. The suspension was shaken for 16 h, washed with DMF (6×2 mL) and reacted with Fmoc-Tyr(^{t}Bu)–OH (158 mg, 343 μmol), PyBroP® (160 mg, 343 μmol) and DIEA (89 mg, 686 μmol) in DMF (1 mL) for 16 h. The resin was rinsed with DMF (3×2 mL), capped with 10% (v/v) Ac_2O–DMF solution and DIEA (53 mg, 412 μmol) during 15 min. Further elaboration to the full peptide was achieved by the general method except for a second bromoacetic acid/*S*-Trt-mercaptoethylamine/PyBroP sequence. Final cleavage from the resin and deprotection with TFA/H_2O/triethylsilane (90:5:5; 2 mL) over 2 h and precipitation (centrifuge) with cold Et_2O left the crude peptide, which was resuspended (3×) in Et_2O and centrifuged. Cyclization took place in 10% DMSO in TFA over 21 h, followed by precipitation and washing with ether. The dried peptide was purified on a 218TP1010 VYDAC column (10 μm, 1×25 cm, 0.1% TFA/H_2O, MeCN gradient 10–50%) and lyophilised to leave a colorless amorphous powder. PDMS: 1067.1 ($M+H^+$), amino acid analysis: Arg: 1.01, Asp: 1.00, His: 0.99, Pro: 1.09, Tyr: 0.89, peptide content: 72.4%.

Aspartyl-arginyl-(*N*-allyl)glycyl-tyrosyl-(*N*-allyl)glycyl-histidyl-prolyl-phenylalanine (8). This was obtained as **2** except for replacement of *S*-Trt-mercaptoethylamine by allylamine and bromoacetic acid by chloroacetic acid anhydride.

Cell growth

Chinese hamster ovary (CHO) cells, stably expressing rat AT_1-receptors were grown in RPMI 1640 medium with L-glutamine containing 10% fetal calf serum and 1% antibiotic/antimycotic solution. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. At 80% confluency, the medium was removed and the cells were detached from the flasks by incubating them with Hanks' Balanced Salt Solution (HBSS) containing 5 mM EDTA. After some gentle shaking the cells were harvested, centrifuged and used for radioligand binding experiments.

Radioligand binding assay

Receptor binding assays were performed on whole cells, resuspended in binding buffer (Minimum Essential Medium with Earl's salts, 25 mM HEPES, GlutaMax I, 0.2% BSA, 0.02% 1,10-phenanthroline, 0.5 mg/L leupeptin and 200 mg/L bacitracin) and distributed to a 96-well microtiter plate (50,000 cells/well). For competition binding experiments, cells were incubated for 1.5 h at ambient temperature with 50 μL binding buffer containing a fixed concentration of [^{125}I]-Ang II (50,000 cpm) and varying concentrations (0.03 nM–10 μM) of unlabelled peptide. After incubation, the cells were centrifuged, washed twice with ice-cold binding buffer (0.1 mL) and treated with 0.1 M NaOH (0.1 mL) at 37 °C for 30 min. The mixture was transferred to counting tubes and the radioactivity was determined. Nonspecific binding was defined as binding in presence of 1 μM unlabeled Ang II, and it was always less than 5%. All binding experiments were performed in duplicate and data analyzed by an iterative, non-linear curve fitting program (GraFit 3.0, SoftWindows).

Conformational energy calculations

Theoretical conformational analysis of **13** was performed using the Amber* all atom force field and the all

atom charge set as implemented in the program MacroModel version 5.5.[28] The General Born Solvent Accessible surface area (GB/SA) method for water developed by Still[40] was used in all calculations. The number of torsion angles allowed to vary simultaneously during each Monte Carlo step ranged from 2 to $n-1$ where n equals the total number of rotatable bonds ($n=14$ in **13**). All torsion angles, except the amide bonds ω_2 and ω_4 which were set in the trans configuration, were defined as rotatable. Conformational searches were conducted by use of the Monte Carlo Multiple Minimum (MCMM) method in the batchmin program. One 20,000-step run was performed, and those conformations within 50 kJ/mol of the global minimum were kept. The ring closure bond was defined as the bond between the sulfur atoms. Polak–Ribiere conjugate gradient (PRCG) minimization with a maximum of 500 iterations was used in the conformational search with the default derivate convergence set at a value of 0.05 (kJ/mol)/Å. In the subsequent minimization to fully converged structures a maximum of 5000 steps of PRCG minimization is followed by a maximum of 5000 steps of Full Matrix Newton Raphson (FMNR) minimization which is followed by a maximum of 5000 steps of Truncated Newton conjugated gradient (TNCG) with the convergence criteria being 0.001 (kJ/mol)/Å in all these runs.

Acknowledgements

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