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Effect of 3–5 Monocyclizations of Angiotensin II and 4-AminoPhe^6-Ang II on AT_2 Receptor Affinity

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Abstract—The endogenous angiotensin II (Ang II) and the synthetic AT_2 selective agonist 4-aminoPhe^6-Ang II respond very differently to identical cyclizations. Cyclizations of Ang II by thioacetalization, involving the 3 and 5 amino acid residue side chains, provided ligands with almost equipotent binding affinities to Ang II at the AT_2 receptor. In contrast, the same cyclization procedures applied on the AT_2 selective 4-aminoPhe^6-Ang II delivered significantly less potent AT_2 receptor ligands, although the AT_2/AT_1 selectivity was still very high. The fact that different structure–activity relationships are observed after imposing conformational restrictions on Ang II and 4-aminoPhe^6-Ang II, respectively, suggests that the peptides, despite large similarities might adopt quite different backbone conformations when binding to the AT_2 receptor.

Introduction

The angiotensin II AT_2 receptor has attracted interest in recent years, partly since it constitutes a new potential therapeutic target. This receptor plays a role during fetal development and is expressed with high density in fetus.[1,2] In adult tissues, it is less abundant but importantly it is up-regulated in certain pathological conditions.[3] Activation of the AT_2 receptor can induce apoptosis and counteract several of the growth responses initiated by the AT_1 and growth factor receptors. Furthermore, it contributes to the regulation of blood pressure and renal function and is also reported to affect neuronal cell differentiation and nerve regeneration.[2] The AT_2 receptor is a G-protein coupled receptor with 34% sequence homology with the AT_1 receptor.[4] The homology is mainly localized to the transmembrane hydrophobic domains. The amino acid residues essential for binding of the endogenous octapeptide angiotensin II, **1** (Ang II; Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) to the AT_1 receptor seem well preserved in the AT_2 receptor. Eschers group applied photoaffinity labeling and site-directed mutagenesis to determine contact points between Ang II and the AT_2 receptor.[5,6] These studies and modeling of the AT_2 receptor to the rhodopsin scaffold[7] suggested that Ang II binds parallel to the transmembrane region and interestingly, in an extended form.[5]

Considerable research efforts have been devoted to structure–activity relationships (SARs) studies of angiotensin II analogues, but unfortunately these investigations to a large extent predate the discovery of the subtypes of the Ang II receptor. Several models of bioactive conformations of Ang II, when interacting with the AT_1 receptor have been proposed.[8–14] However, the understanding of molecular recognition phenomena when the peptide is interacting with the AT_2 receptor subtype is more limited.[5,15–20] When comparing the AT_1 receptor with the AT_2 receptor, regarding binding affinity of Ang II analogues, it has been demonstrated that: (a) Asp-1 in Ang II is not needed for affinity,[21] (b) aromatic amino acids in position 7 in combination with hydrophobic amino acids in position 8 seem to enhance the AT_2 selectivity,[21,22] (c) Ang II, when binding to both the AT_1 and AT_2 receptor seems to adopt a turn centred at positions 3–5, as deduced from studies of cyclized [Sar^1]Ang II analogues[23] and

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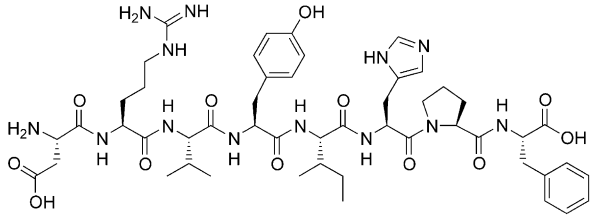
(d) modifications of Ang II at most of the positions are reasonably well tolerated by the AT_2 receptor,[24] while the same modifications were detrimental to AT_1 receptor binding affinity. This latter feature is nicely reflected in the fact that displacement of His by 4-aminoPhe in position 6 gives a peptide **2** (4-aminoPhe^6-Ang II) with almost the same AT_2 receptor affinity as Ang II, while the affinity to the AT_1 receptor subtype is negligible.[25] Peptide **2** and the more structurally divers N^{α}-nicotinoyl-Tyr-(N^{α}-Cbz-Arg)Lys-His-Pro-Ile-OH (CGP 42112A) represent the only examples of agonists with high selectivity for the AT_2 receptor and have consequently been extensively exploited as research tools for functional studies of the AT_2 receptor[26] (Chart 1).

Marshall's group has reported that disulfide monocyclizations in the 3–5 region of [Sar^1]Ang II resulted in retained binding affinity to AT_1 but a more than 4-fold reduction of the affinity to the AT_2 receptor.[23] Bicyclizations in the same part of the peptide, on the other hand, gave high affinities for the AT_2 receptor, but in general very low affinities for the AT_1 receptor.[23] These exciting findings encouraged us to evaluate methylenedithioether monocyclized Ang II analogues[27] and their binding affinities to the AT_2 receptor but including Asp in position 1, to copy the native peptide. Furthermore, the effects of cyclizations applied on the commonly employed highly AT_2 selective agonist analogue **2** were assessed to enable a direct comparison and since, to the best of our knowledge, such data are not available.

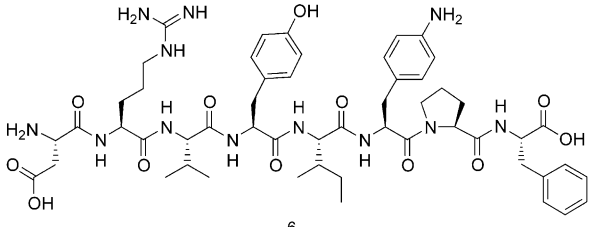
We herein report that cyclizations of Ang II by thioacetalization, an alternative to disulfide cyclization, give ligands (**3**–**6**, Chart 2) with high AT_2 binding preferences and with K_i values in the low nanomolar range. In contrast, the same cyclization procedures applied on **2** provide significantly less potent AT_2 receptor ligands (**7**–**11**, Chart 2), although the AT_2/AT_1 selectivity is still very high.

Chemistry

The linear peptide precursors were synthesized by standard solid-phase techniques using Fmoc/*tert*-butyl protection. The synthetic thioacetalization procedure developed by Ueki et al.[28] for monocyclizations of peptides was applied with some minor modifications for the synthesis of compounds **7**–**11**. In short, the reduced peptide was treated with an excess of tetrabutylammonium fluoride (TBAF) under vigorous stirring in methylenedichloride at room temperature. In the

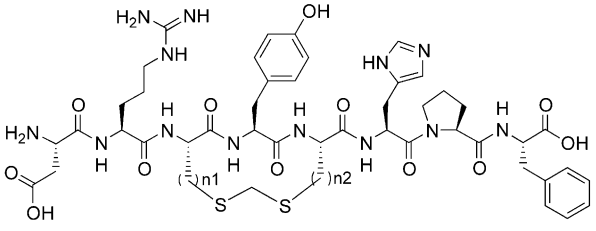


Angiotensin II (Ang II) (**1**)

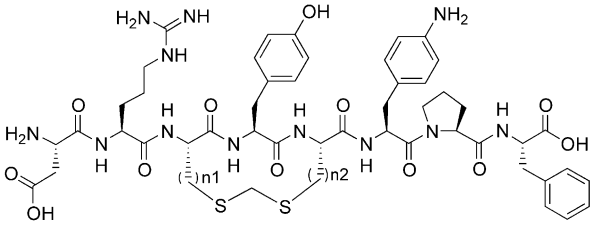


4-AminoPhe^6-Ang II (**2**)

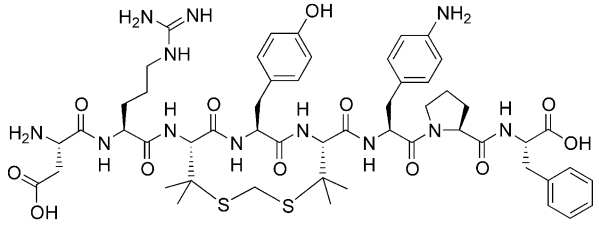
Chart 1.



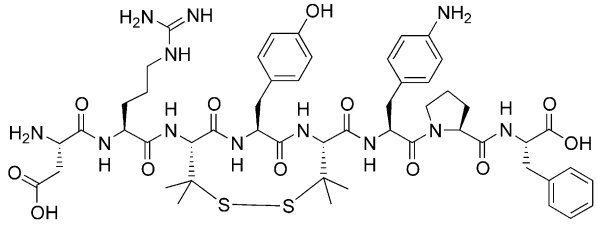
3: n1=1, n2=1
4: n1=1, n2=2
5: n1=2, n2=1
6: n1=2, n2=2



7: n1=1, n2=1
8: n1=1, n2=2
9: n1=2, n2=1
10: n1=2, n2=2



11



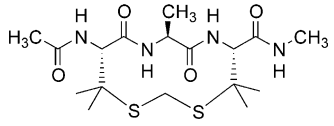
12

Chart 2.

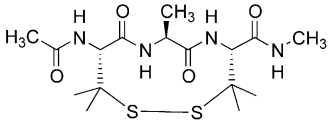
cyclization of **8** and **9**, the corresponding disulfides were formed to a significant extent despite precautions to exclude oxygen from the reaction mixture. Reduction of the disulfides by addition of tributylphosphine,[29,30] to regenerate free thiol accessible to further thioacetalization, did not increase the yield of the desired products, not even when more TBAF was added. However, the reduction step was included at the end of the cyclizations since the reduced peptides were more easily separated from the target compound than were the disulfides. The disulfide analogue **12** was prepared according to Maruyama et al.[31] using TBAF in the presence of carbontetrachloride.

In Vitro Binding Affinity

Compounds **1**–**12** were evaluated in radioligand-binding assays based on displacement of [^{125}I]Ang II from AT_1 receptors in rat liver membranes and from AT_2 receptors in pig uterus membranes (Table 1). Ang II (**1**), losartan, c[Hcy^{3,5}]Ang II and c[Pen^{3,5}]Ang II were used as reference substances. Compounds **3**–**6** were found to bind with high affinity to the AT_1 receptor but even better to the AT_2 receptor. Compound **4** displayed the highest affinity (K_i = 4 nM) to the AT_1 receptor among the methylenedithioether analogues, while compound **3** displayed the highest affinity to the AT_2 receptor. The methylenedithioether analogues **7**–**11** were AT_2-selective compounds but with lower affinity than **3**–**6**. The disulfide analogue **12** displayed the lowest K_i-value among the compounds with 4-aminoPhe in position 6.



11m



12m

Chart 3.

Conformational characterization of 11m and 12m

We have recently reported the conformational preferences of the cyclic methylenedithioether moieties of **3**–**10** and their disulfide counterparts.[27] Conformational analysis has now been performed on the blocked tripeptide model compounds **11m** and **12m** of the penicillamine derivatives **11** and **12**, respectively (Chart 3). The Amber* force field and GB/SA water solvation model[32] within Macromodel (version 6.5)[33] were used in the calculations, and all conformations within 5 kcal/mol of the lowest-energy minimum were characterized.

The number of conformations within 5 kcal/mol of the lowest energy minimum found for **11m** and **12m** were 10 and 7, respectively. The backbone torsion angles and key distances were recorded and the propensity of each of the conformations to adopt γ- and β-turns[34,35] were studied (see Fig. 1 for a description of the parameters used to characterize the model compounds.) We allowed ±30° deviation from the limits defined for the different turns in identifying γ-turn like and β-turn conformations.[36] Using these criteria, five out of the 10 conformations of **11m** and five out of the 7 conformations of **12m** were found to adopt inverse γ-turn like conformations centered on residue 3. In the case of β-turns, four out of the conformations of **11m** and 3 of the conformations of **12m** adopt $C_\alpha 2$–$C_\alpha 5$ distances shorter than 7 Å. In **11m**, two of the conformations were identified as type I β-turns and in **12m** only one as a type I β-turn.

To determine whether **11m** and **12m** adopt different backbone torsion angles, the angles Φ_3, Ψ_3, Φ_4 and Ψ_2, located within the cyclic moiety, were compared (Fig. 2). The angles Φ_3, Ψ_3 as well as Φ_4, Ψ_2 display similar characteristics. This seems also to be the case when the impact of cyclization was further monitored by the analyzing the virtual torsion angles X1 = [N2-Cα2-Cα3-Cβ3], X2 = [Cβ3-Cα3-Cα4-C(4)O], and X3 = [N2-Cα2-

Table 1. In vitro binding affinities of monocyclic Ang II analogues

Compd	AT_1 (rat liver membranes) K_i (nM) ±SEM	AT_2 (pig uterus myometrium) K_i (nM) ±SEM	AT_2 selectivity (AT_1/AT_2)
Ang II (**1**)	0.31±0.08	0.63±0.16	0.5
4-AminoPhe^6-Ang II (**2**)	3296±154	1.97±0.02	1670
Losartan	25±5	—	
c[Hcy^{3,5}]-Ang II	0.23±0.14	4.0±0.4	0.06
c[Pen^{3,5}]-Ang II	253±52	6.2±0.2	41
3	44±1	0.62±0.04	73
4	4±1	1.6±0.4	2.5
5	24±2	2.4±0.1	10
6	23±1	3.3±0.7	7
7	>10,000	224±7	>45
8	>10,000	262±16	>38
9	>10,000	83±49	>120
10	>10,000	107±25	>93
11	>10,000	38±14	>263
12	>10,000	31±11	>322

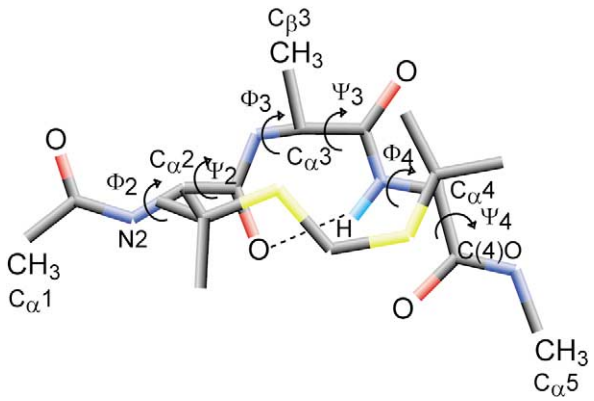


Figure 1. Parameters used to characterize the model compounds. The second lowest energy conformation of the model compound **11m** is shown.

Cα4-C(4)O] (Fig. 2). These torsion angles describe the directions of the incoming backbone, the side chain of residue 3 and the outgoing backbone with respect to each other. Thus, methylenedithioether cyclization does not change the conformational preferences of **11m** substantially, as compared to the disulfide cyclized **12m**.

Results and Discussion

The affinities to the pig uterus AT_2 receptor of the 3-5 monocyclized thioacetals **3–6** derived from **1**, and **7–11** derived from **2**, are shown in Table 1. The affinities of **1**, **2** and the disulfides c[$Hcy^{3,5}$]Ang II, c[$Pen^{3,5}$]Ang II and **12** are also included for comparison.

It has been proposed that the amino acid residues Val-3, Ile-5 and Pro-7 are engaged in interactions stabilizing the bioactive conformation of Ang II.[8] Based on this hypothesis Spear et al.[37] synthesized 3–5 monocyclic compounds with high affinity to Ang II receptors. Following this finding, the binding affinities to Ang II receptors of several 3–5 and 5–7 cyclized analogues have been reported.[23,38–43] With respect to the AT_2 receptor subtype (rabbit uterus), Marshall et al.[23] found that neither c[$Sar^1Hcy^{3,5}$]Ang II (IC_{50}; 80 nM), c[$Sar^1Cys^3Hcy^5$]Ang II (IC_{50}; 28 nM), nor c[$Sar^1Cys^{3,5}$]Ang II (IC_{50}; 27 nM) with 13-, 12- and 11-membered rings, respectively, exhibited good binding affinities. The same trend as noted above, that a smaller ring size gives an increased affinity for the AT_2 receptor is also observed when the Ang II analogues **3–6** are compared. It is interesting to note that the thioacetals, as opposed to the disulfides, exert a pronounced affinity to the AT_2 receptor (Table 1). The smallest ring analogue **3**, that is the most potent in the group, binds as tightly to the AT_2 receptor as Ang II itself and exhibits a 70-fold AT_2 selectivity, suggesting it to be a candidate for further investigations. In general, while the disulfides reported by Marshall were very potent binders to the AT_1 receptor, the thioacetals with the 13-membered ring, compound **4**, as an exception, were comparably weak AT_1 receptor ligands with K_i values of 23–44 nM.

The monocyclized analogues (**7–11**) of **2** lost all affinity to the AT_1 receptor as a result of displacement of His-6, an amino acid residue important for efficient binding to the AT_1-receptor, for the 4-aminoPhe residue. Interestingly, while methylenedithioether cyclization of Ang II afforded ligands that were almost equipotent to Ang II at the AT_2-receptor, the same conformational restriction imposed on **2** gave approximately 100-fold less efficient AT_2 receptor ligands. In the 4-aminoPhe[6] series (**7–10**) large rings seemed to promote high affinity, whereas in the Ang II series the opposite seemed to be the case (cf **10**; $K_i = 107$ nM, **7** and $K_i = 224$ nM vs **6**; $K_i = 3.3$ nM and **3**; $K_i = 0.6$ nM, respectively). Even though the binding affinities of **7–11** to the AT_2 receptor were low, we found the high AT_2/AT_1 selectivity obtained very attractive.

Marshall et al.[23] imposed conformational constraints on c[$Sar^1Hcy^{3,5}$]Ang II and identified two ligands, c[$Sar^1Mpt^3Hcy^5$]Ang II and c[$Sar^1Hcy^3Mpt^5$]Ang II, that exhibited a significant improvement of binding affinity to the AT_2 receptor and with preference for the same receptor. We introduced steric and conformational constraints by the introduction of four methyl groups in **7** to afford **11**. Compound **11** had 6 times higher affinity to AT_2-receptors than **7** but still no measurable AT_1 receptor affinity. This 12-membered ring analogue, smoothly prepared by thioacetalization of the penicillamine precursor, was found to be slightly less potent than the disulfide **12** derived from oxidation of the same starting material. The constrained disulfide **12** containing an 11-membered ring scaffold that seems to adopt an inverse γ-turn conformation centered at Tyr-4[40] was the most potent analogue in the 4-aminoPhe[6] series.

We recently reported on the conformational preferences of the N- and C-terminally blocked cyclic tripeptide model compounds of **3–10**.[27] That study suggested that the cyclic moieties in **3** and **7** induce inverse γ-turn like conformations around Tyr. This seems to be the case also for the model compounds **11m** and **12m**. However,

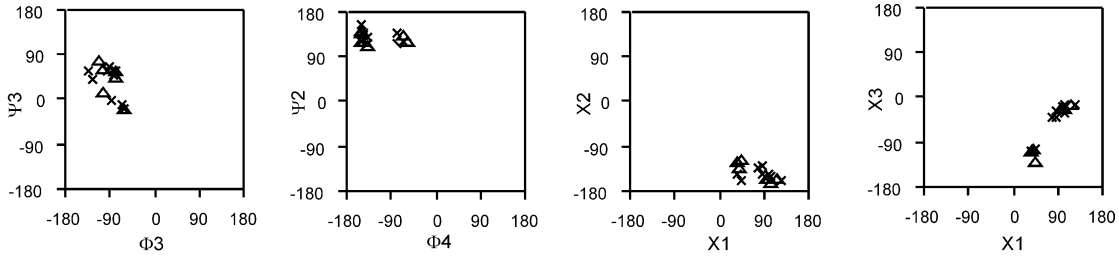


Figure 2. Scatter plots of torsion angles for all conformations below 5 kcal/mol of **11m** (×) and **12m** (△).

the surprising finding that **3** but not **7** displays high affinity to the AT_2-receptor suggests different binding modes of the two peptides when interacting with the AT_2 receptor. Potent cyclized analogues of **2** are still not identified but it appears that also this peptide adopts a turn conformation centered at Tyr-4, and it is also most likely that the ring conformations differ when cyclic analogues of Ang II and of **2** are binding to the receptor. A substitution of histidine for 4-aminophenylalanine strongly affects the conformation of a substantial part of the peptide.

With this in mind and considering the fact that different SARs are observed after imposing conformational restrictions on **1** and **2**, respectively, we suggest that the peptides despite large similarities might adopt quite different backbone conformations when binding to the AT_2 receptor. Thus, diverse cyclized scaffolds are probably required to induce optimal side chain receptor recognitions in the two peptides Ang II (**1**) and 4-aminoPhe^6-Ang II (**2**).

Conclusion

Endogenous Ang II (**1**) and the synthetic AT_2 selective agonist **2** respond very differently to identical cyclizations. Cyclizations of Ang II (**1**) by thioacetalization, involving the 3 and 5 amino acid residue side chains, provided ligands almost equipotent to Ang II at the AT_2 receptor. In contrast, the same cyclization procedures applied on the AT_2 selective 4-aminoPhe^6-Ang II (**2**) gave significantly less potent AT_2 receptor ligands, although the AT_2/AT_1 selectivity was still very high. A cyclized penicillamine analogue of **2**, the thioacetal **11**, exhibited an AT_2/AT_1 selectivity exceeding 200. This compound was also one of the most potent AT_2-receptor ligands among the 4-aminoPhe^6-Ang II analogues synthesized in this series ($K_i = 38$ nM). We believe that the thioacetalization protocol employed herein offers a useful complement to disulfide cyclization when probing a peptide's bioactive conformation(s).

Experimental

Chemistry. General comments

Preparative RP-HPLC was performed on a Vydac 10-μm C18 column (2.2×25 cm) using a 80 min gradient of 5–45% CH_3CN in 0.1% aqeuous TFA at a flow rate of 3 mL/min and detection at 230 nm. Analytical RP-HPLC was performed on a Vydac 10-μm C18 column (0.46×15 cm) using a 25-min gradient of 10–35% CH_3CN in 0.1% aqeuous TFA at a flow rate of 1.5 mL/min and detection at 220 nm). Mass spectroscopy was carried out on an Applied Biosystems (Uppsala, Sweden) BIOION 20 plasma desorption mass spectrometer. Amino acid analyses and peptide content determinations were performed at the Department of Biochemistry, Uppsala University, Sweden, on oxidized samples after 24 h hydrolysis using an LKB 4151 alpha plus analyzer with ninhydrin detection.

Materials

Fmoc-Phe-Wang resin and amino acid derivatives were obtained from Bachem (Bubendorf, Switzerland), Calbiochem-Novabiochem (Läufelfingen, Switzerland), or Alexis Corporation (Läufelfingen, Switzerland). DMF (peptide synthesis grade) was obtained from Perseptive Biosystems (Hamburg, Germany) and was used without further purification. 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt) were purchased from Richelieu Biotechnologies (St-Hyacinthe, QC, Canada). Tetrabutylammonium fluoride (TBAF, 1.0 M solution in THF) was purchased from Aldrich and tributylphosphine (Bu_3P, 85%) was obtained from Fluka. All other reagents were obtained from commercial sources and used as received.

Solid-phase peptide synthesis (SPPS)

The peptides were synthesized on a 100-μmol scale with a Symphony instrument (Protein Technologies Inc., Tucson, AZ, USA) using Fmoc/*tert*-butyl protection. The starting polymer was Fmoc-Phe-Wang resin (0.62 mmol/g), and for the Fmoc amino acids the side chain protecting groups were as follows: Asp(O^{t}Bu), Arg(Pbf), 4-AminoPhe(Boc), Tyr(^{t}Bu), Cys(Trt), Hcy(Trt) and Pen(Trt). Removal of the Fmoc group was achieved by reaction with 20% piperidine in DMF for 5 + 10 min. Coupling of the amino acids (125 μmol) was performed in DMF (2.50 mL) using HBTU (125 μmol) in the presence of NMM (500 μmol). Double couplings (2×30 min) were used for all amino acids, except for 4-AminoPhe(Boc), Fmoc-Hcy(Trt) and Fmoc-Pen(Trt) which were coupled once for 60 min. After the introduction of each amino acid, remaining amino groups were capped by addition of 20% acetic anhydride in DMF (1.25 mL) to the coupling mixture and allowing the reaction to proceed for 5 min. After completion of the synthesis, the Fmoc group was removed and the partially protected peptide resin was washed with several portions of DMF and CH_2Cl_2 and dried in a stream of nitrogen and in vacuo.

General procedure for S–CH_2–S cyclization

The crude peptide (1 equiv) was suspended in helium-purged CH_2Cl_2 at a concentration of 0.5 mM. TBAF (25 equiv) was added and the reaction mixture was stirred for 20 min, before addition of Bu_3P (2 equiv) for reduction of eventually formed disulfide.[29,30] After 5 min the reaction was quenched with glacial acetic acid (100 μL) and the solvent was removed in vacuo. The residue was dissolved in 0.1% aq TFA (6 mL) and purified by preparative RP-HPLC. Selected fractions where analyzed by analytical RP-HPLC and by PDMS. Yields for the purified Ang II analogues were corrected for peptide content.

Cyclo(S–CH_2–S)[Cys^{3,5}, 4-aminoPhe^6]Ang II (7). Fmoc-Phe-Wang resin (161 mg, 100 μmol) was reacted as described above (SPPS) to yield the partially protected peptide resin (323 mg). A portion of the resin (153 mg)

was treated with TFA/H_2O/triethylsilane (90:5:5; 1 mL) for 1.5 h. The mixture was filtered through a small plug of glass wool in a Pasteur pipet, and the resin was washed with TFA (3×0.3 mL). The product was precipitated by the addition of cold, anhydrous ether (12 mL). The precipitate was collected by centrifugation, washed with ether (4×6 mL) and dried to furnish 69.1 mg of crude peptide. The peptide (31.2 mg, 29.3 μmol) was cyclized according the general procedure above without the addition of Bu_3P to yield 12.5 mg (27%) of **7**. Amino acid analysis: Asp 1.00, Arg 1.01, Tyr 0.98, Pro 1.00, Phe 1.01, 4-aminoPhe 0.93 (69% peptide); PDMS (M_r 1077.2): 1078.6 ($M+H^+$), 1100.4 ($M+Na^+$).

Cyclo(S–CH_2–S)[Cys^3,Hcy^5, 4-aminoPhe^6]Ang II (8). The peptide was synthesized according to the procedure used for **7**. The partially protected peptide resin (151 mg) was cleaved as described above to yield 66.4 mg of crude peptide. The peptide (26.0 mg, 24.1 μmol) was cyclized according the general procedure above. The final yield of **8** was 6.0 mg (16%). Amino acid analysis: Asp 1.01, Arg 0.99, Tyr 0.92, Pro 1.00, Phe 1.01, 4-aminoPhe 0.94 (70% peptide); PDMS (M_r 1091.2): 1092.7 ($M+H^+$), 1114.9 ($M+Na^+$).

Cyclo(S–CH_2–S)[Hcy^3,Cys^5, 4-aminoPhe^6]Ang II (9). The peptide was synthesized according to the procedure used for **7**. The partially protected peptide resin (156 mg) was cleaved as described above to yield 74.9 mg of crude peptide. The peptide (34.4 mg, 31.9 μmol) was cyclized according the general procedure above. The final yield of **9** was 5.5 mg (11%). Amino acid analysis: Asp 1.01, Arg 0.98, Tyr 0.90, Pro 1.01, Phe 1.00, 4-aminoPhe 0.92 (67% peptide); PDMS (M_r 1091.2): 1092.7 ($M+H^+$).

Cyclo(S–CH_2–S)[Hcy^{3,5}, 4-aminoPhe^6]Ang II (10). The peptide was synthesized according to the procedure used for **7**. The partially protected peptide resin (146 mg) was cleaved as described above to yield 64.2 mg of crude peptide. The peptide (42.9 mg, 39.2 μmol) was cyclized according the general procedure above. The final yield of **10** was 6.7 mg (12%). Amino acid analysis: Asp 1.00, Arg 0.99, Tyr 0.83, Pro 1.00, Phe 1.01, 4-aminoPhe 0.90 (77% peptide); PDMS (M_r 1105.2): 1106.8 ($M+H^+$).

Cyclo(S–CH_2–S)[Pen^{3,5}, 4-aminoPhe^6]Ang II (11). The peptide was synthesized according to the procedure used for **7**. The partially protected peptide resin (148 mg) was cleaved as described above to yield 66.3 mg of crude peptide. The peptide (43.2 mg, 38.5 μmol) was cyclized according the general procedure above. The final yield of **11** was 19.7 mg (32%). Amino acid analysis: Asp 1.01, Arg 0.99, Tyr 0.93, Pro 1.01, Phe 0.99, 4-aminoPhe 0.84 (70% peptide); PDMS (M_r 1133.3): 1134.7 ($M+H^+$).

Cyclo(S–S)[Pen^{3,5}, 4-aminoPhe^6]Ang II (12). The crude peptide (27.9 mg) was suspended in CH_2Cl_2 (25 mL) and CCl_4 (5 mL) followed by addition of TBAF, 1 M in THF, (20 equiv, 525 μL). The solution got clear almost immediately and the reaction was quenched after 20 min by addition of glacial acetic acid (100 μL). The final yield of **12** was 8.1 mg (22%). Amino acid analysis: Asp 1.00, Arg 0.99, Tyr 0.94, Pro 0.99, Phe 1.02, 4-aminoPhe 0.78 (76% peptide); PDMS (M_r 1119.3): 1121.2 ($M+H^+$).

Rat liver membrane AT_1 receptor binding assay

Rat liver membranes were prepared according to the method of Dudley et al.[44] Binding of [^{125}I]Ang II to membranes was conducted in a final volume of 0.5 mL containing 50 mM Tris–HCl (pH 7.4), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.025% bacitracin, 0.2% BSA (bovine serum albumin), liver homogenate corresponding to 5 mg of the original tissue weight, [^{125}I]Ang II (70 000 cpm, 0.03 nM) and variable concentrations of test substance. Samples were incubated at 25 °C for 1 h, and binding was terminated by filtration through Whatman GF/B glass-fiber filter sheets using a Brandel cell harvester. The filters were washed with 4×2 mL of Tris–HCl (pH 7.4) and transferred to tubes. The radioactivity was measured in a gamma counter. The characteristics of the Ang II binding AT_1 receptor was determinated by using six different concentrations (0.03–5 nmol/L) of the labelled [^{125}I]AngII. Non-specific binding was determined in the presence of 1 μM Ang II. The specific binding was determined by subtracting the non-specific binding from the total bound [^{125}I]AngII. The dissociation constant ($K_d=1.7\pm0.1$ nM, [L] = 0.057 nM) were determined by Scatchard analysis of data obtained with Ang II by using GraFit (Erithacus Software, UK). The binding data were best fitted with a one-site fit. All experiments were performed in triplicate except for Ang II and c[Hcy^{3,5}]Ang II, which were performed in quadruplicate.

Porcine (pig) myometrial membrane AT_2 receptor binding assay

Myometrial membranes were prepared from porcine uteri according to the method by Nielsen et al.[45] A presumable interference by binding to AT_1 receptors was blocked by addition of 1 μM losartan. Binding of [^{125}I]Ang II to membranes was conducted in a final volume of 0.5 mL containing 50 mM Tris–HCl (pH 7.4), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.025% bacitracin, 0.2% BSA, homogenate corresponding to 10 mg of the original tissue weight, [^{125}I]Ang II (70,000 cpm, 0.03 nM) and variable concentrations of test substance. Samples were incubated at 25 °C for 1 h, and binding was terminated by filtration through Whatman GF/B glass-fiber filter sheets using a Brandel cell harvester. The filters were washed with 3×3 mL of Tris–HCl (pH 7.4) and transferred to tubes. The radioactivity was measured in a gamma counter. The characteristics of the Ang II binding AT_2 receptor was determinated by using six different concentrations (0.03–5 nmol/L) of the labelled [^{125}I]Ang II. Non-specific binding was determined in the presence of 1 μM Ang II. The specific binding was determined by subtracting the nonspecific binding from the total bound [^{125}I]Ang II. The dissociation constant ($K_d=0.7\pm0.1$ nM, [L] = 0.057 nM) were determined by Scatchard analysis of data obtained with Ang II by using GraFit

(Erithacus Software, UK). The binding data were best fitted with a one-site fit. All experiments were performed in triplicate except for Ang II, **11** and **12**, which were performed in quadruplicate.

Conformational energy calculations

The calculations of **11m** and **12m** were performed using the Amber* all atom force field as implemented in the program Macromodel 6.5.[33] The General Born Solvent Accessible surface area (GB/SA) method for water developed by Still[32] was used in all calculations. The number of torsion angles allowed to vary simultaneously during each Monte Carlo step ranged from 1 to $n-1$ where n equals the total number of rotatable bonds. Amide bonds were fixed in the *trans* configuration. Conformational searches were conducted by use of the Systematic Unbound Multiple Minimum search (SUMM) method[46] in the batchmin program (command SPMC). 20000-step runs were 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 C_β and C_γ atoms of the side chain of residue three. Torsional memory and geometrical pre-optimization were used. Truncated Newton conjugated gradient (TNCG) minimization (**11m**) or PR Conjugate Gradient (PRCG) minimization (**12m**) with a maximum of 5000 iterations was used in the conformational search with derivative convergence set to 0.05 (kJ/mol)/Å. In the subsequent minimization to fully converged structures, a maximum of 5000 steps of TNCG (**11m**) or PRCG (**12m**) was used with the convergence criteria set to 0.001 (kJ/mol)/Å.

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substituent is pseudo equatorially oriented. The characteristic torsion angles for $\Phi i+1$, $\Psi i+1$ in classic γ-turns are: 70 to 85°, −60 to −70° and in inverse γ-turn: −70 to −85°, 60 to 70°. The characteristic torsion angles for the $\Phi i+1$, $\Psi i+1$ and $\Phi i+2$ $\Psi i+2$ angles of β-turns are as follows: type I, −60, −30, −90, 0°, type II, −60, 120, 80, 0°, type III, −60, −30, −60, −30°, type I′, 60, 30, 90, 0° etc. See, for example: Richardsson, J. S. *Adv. Protein Chem.* **1981**, *34*, 167 and Smith, J. A.; Pease, L. G. *CRC Crit. Rev. Biochem.* **1980**, *8*, 315.

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