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Synthesis, Receptor Binding Affinities and Conformational Properties of Cyclic Methylenedithioether Analogues of Angiotensin II

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Abstract—Cyclic 12-, 13- and 14-membered ring angiotensin II analogues related to disulfides but encompassing methylenedithioether bridges have been prepared. The affinity data from these derivatives were compared to those from the disulfides. The methylenedithioether analogues displayed good binding affinities to rat liver AT_1 receptors although in most cases somewhat lower than their disulfide counterparts. One of the methylenedithioethers with a 13-membered ring system demonstrated the highest binding affinity among the thioethers. Theoretical conformational analysis of model compounds of the two series were performed suggesting a similarity between the disulfide and the corresponding methylenedithioether analogues and also between the ring size homologues. This analysis also suggested that some of the model compounds were prone to adopt inverse γ-turn conformations, which was further supported by use of NMR spectroscopy of the 12-membered ring analogue in the series. The easily executed methylenedithioether cyclization should constitute a valuable complement to the common disulfide methodology for fine-tuning and for probing the bioactive conformation of peptides.

Introduction

During the last decade, numerous cyclization methods have been applied for the preparation of mono- and bicyclic peptides. In many cases, the objective was to probe the bioactive conformation(s) of a target peptide by reducing the conformational flexibility or to produce more metabolically stable compounds.[1,2] Disulfide and amide cyclizations are among the most commonly used monocyclization methods,[3] but other strategies have also been successfully employed including, for example, thioether cyclizations[4–12] and ring-closure metathesis reactions.[13–15]

Several cyclic analogues of the hypertensive octapeptide angiotensin II (Ang II) have been prepared and evaluated for affinity to the Ang II AT_1 receptor.[16–21] Among the monocyclic compounds synthesized, the 11-membered ring analogue c[$Cys^{3,5}$]Ang II (**1**), the 12-membered ring analogues c[$Cys^{3}Hcy^{5}$]Ang II (**2**) and c[$Hcy^{3}Cys^{5}$]Ang II (**3**) and the 13-membered ring analogue c[$Hcy^{3,5}$]Ang II (**4**) all showed high affinity for the AT_1 receptor (Chart 1). Interestingly, compound **4** was a full agonist, almost equipotent with Ang II, while the analogues **1**, **2** and **3** exerted less than 1% of the activity of Ang II.[16] This illustrates that disulfide cyclization can be used to produce Ang II analogues with retained affinity and that the AT_1 receptor is sensitive to small changes in the ring size of Ang II.

We wanted access to alternative monocyclization methods that would deliver ring systems with similar electronic and steric properties as disulfides and allow for fine-tuning of the structure–activity relationships (SARs) of Ang II. We became encouraged to further elucidate the effect of modifying the ring geometry of **1** to **4** above, via insertion of a methylene group between the sulfur atoms to form a thioacetal (S–CH_2–S). Only a few

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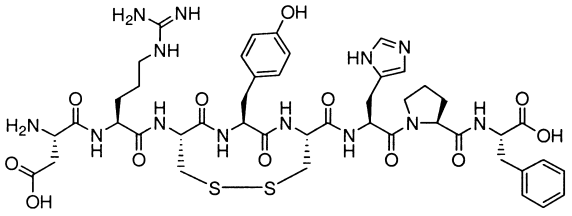
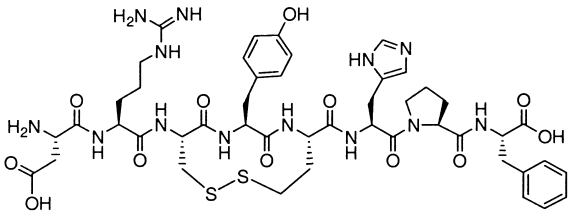
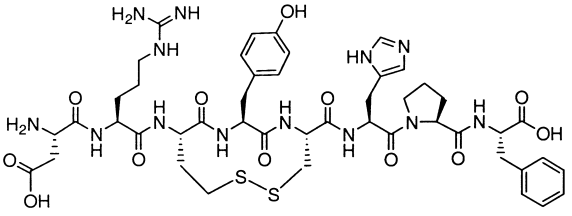


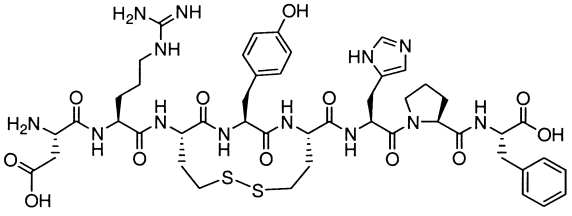
cyclo(S-S)[Cys^{3,5}]Ang II (**1**)



cyclo(S-S)[Cys^{3}Hcy^{5}]Ang II (**2**)



cyclo(S-S)[Hcy^{3}Cys^{5}]Ang II (**3**)

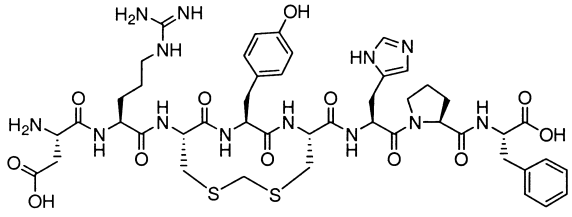


cyclo(S-S)[Hcy^{3,5}]Ang II (**4**)

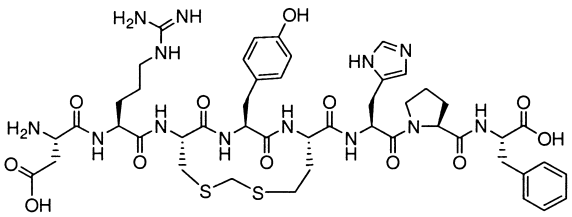
Chart 1.

examples have been reported where methylenedithioethers have been used for SAR studies and to our knowledge no comparative conformational analyses of *i* to *i*+2 cyclic disulfides and methylenedisulfides of the same ring size have yet been performed. From a pharmacokinetic viewpoint it would be of interest to study if the disulfide and methylenedithioether analogues had similar conformational and pharmacological properties since the methylenedithioether analogues most probably are more metabolically stable. The use of methylenedithioether bridges to form cyclic peptides was first described by Mosberg et al.[22] in the synthesis of enkephalin analogues.[22–24] More recently, Ueki et al. used a simplified procedure for the synthesis of both cyclic enkephalin[25] and vasopressin analogues[26] of this type.

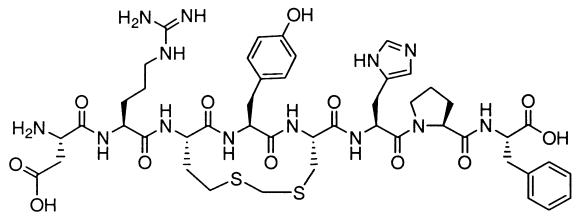
We have now prepared the methylenedithioether analogues **5**–**8** (Chart 2) of compounds **1**–**4** and evaluated their binding affinity to rat liver AT_1 receptors. Furthermore, the conformational properties of the cyclic moieties of the disulfides **1**–**4** and the methylenedithioethers **5**–**8** were compared using the model compounds **1m**–**8m** (Chart 3). In the conformational analysis we focused on the ability of the model compounds to adopt β- and γ-turns and also on comparison of key torsional angles between the two series.



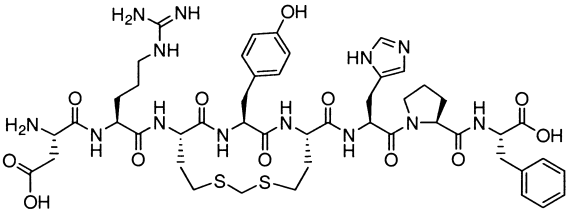
cyclo(S-CH$_2$-S)[Cys^{3,5}]Ang II (**5**)



cyclo(S-CH$_2$-S)[Cys^{3}Hcy^{5}]Ang II (**6**)

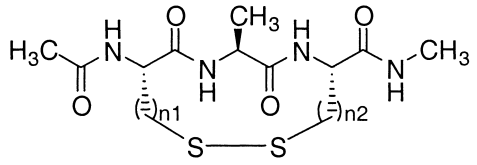


cyclo(S-CH$_2$-S)[Hcy^{3}Cys^{5}]Ang II (**7**)

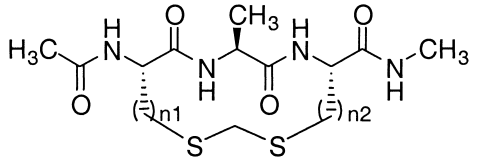


cyclo(S-CH$_2$-S)[Hcy^{3,5}]Ang II (**8**)

Chart 2.



1m: n1=1, n2=1
2m: n1=1, n2=2
3m: n1=2, n2=1
4m: n1=2, n2=2



5m: n1=1, n2=1
6m: n1=1, n2=2
7m: n1=2, n2=1
8m: n1=2, n2=2

Chart 3.

Results

Chemistry

The linear peptide precursors were synthesized by standard solid-phase techniques using Fmoc/*tert*-butyl protection. The methylenedithioether derivatives were prepared according to the method described by Ueki et al.[25] with minor modifications (Scheme 1). In short, the reduced peptide was treated with an excess of tetrabutylammonium fluoride (TBAF) under vigorous stirring in CH_2Cl_2/CH_3CN at room temperature. Acetonitrile was included in order to facilitate the solvation of the peptide. In the cyclization of **6** and **7**, 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 (Bu_3P),[27] to regenerate free thiol accessible to further thioacetalization, did not increase the yield of the desired products, not even when further addition of TBAF was employed. However, the reduction step was usually 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 **4** was prepared according to Maruyama et al.[28] using TBAF in the presence of carbontetrachloride (CCl_4).

In vitro binding affinity

Compounds **4**–**8** were evaluated in a radioligand-binding assay based on the displacement of [^{125}I]Ang II from AT_1 receptors in rat liver membranes[29] (Table 1). Ang II and **4** were used as reference substances. All compounds (**5**–**8**) were found to bind with high affinity to the AT_1 receptor. Compound **6** displayed the highest affinity ($K_i = 4$ nM) among the methylenedithioether analogues. The disulfide analogue **4** and Ang II exhibited almost the same K_i value. The IC_{50} values of compounds **1**–**4** using displacement of [^{125}I]Ang II have previously been reported by Spear et al.[30]

Table 1. In vitro AT_1 receptor binding affinities

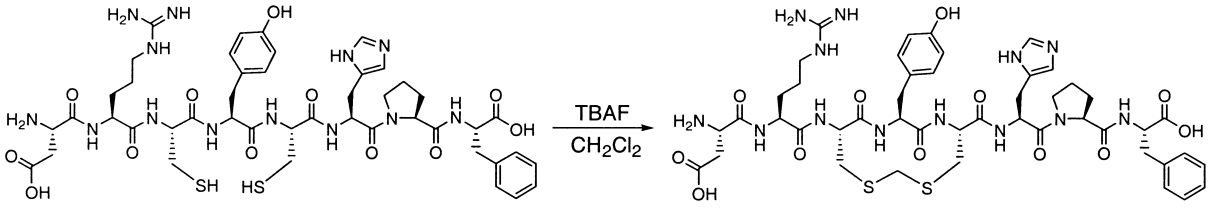
Compound	K_i (nM)±SEM
Ang II	0.31±0.08
DuP 753	25±5
4	0.23±0.14
5	44±1
6	4±1
7	24±2
8	23±1

NMR spectroscopy

The ^{1}H NMR spectrum (DMSO-d_6, 400 MHz) of peptide **5** displays a single set of signals over the monitored temperature range (25–55 °C). Signals were assigned from TOCSY[31] and ROESY[32] spectra as described previously.[20,33] NOEs were either sequential or intraresidual and did therefore not give any conformational information. NMR temperature coefficients of the NH chemical shifts of compound **5** are given in $\Delta\delta/\Delta T$ (ppb/K) and were Arg-2 (3.0), Cys-3 (5.7), Tyr-4 (3.7), Cys-5 (1.0), His-6 (5.0), Phe-8 (5.4). The low temperature coefficient of residue 5 indicates that this proton is either intramolecularly hydrogen bonded or unexposed to solvent.[34]

Conformational characterization of 1m–9m

Conformational analysis was performed on blocked tripeptide model compounds of **1**–**8**. The model compounds have the general formula Ac-X-Ala-Y-NHMe where X and Y represent Cys or Hcy (Chart 3). The conformational properties of model compounds **1m**–**4m**



5

Scheme 1.

have been reported previously.[35] We reanalyzed **1m**–**4m** to allow for a direct comparison with **5m**–**8m**, and the blocked tripeptide Ac-Ala-Ala-Ala-NHMe (**9m**) was included in the analysis as a linear reference compound. The Amber* force field and the GB/SA water solvation model[36] within Macromodel (version 6.5)[37] 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 global energy minimum found for model compounds **1m**, **2m**, **3m** and **4m** was 9, 13, 9 and 52, respectively, and for **5m**, **6m**, **7m** and **8m** it was 9, 36, 21 and 162, respectively. For the reference compound **9m,** 20 conformations were identified. The conformational flexibility of the methylenedithioether model compounds was in general higher than for the disulfide model compounds.

The conformational preferences of the peptides, and their potential to induce the β- and γ-turn geometries[38] were analyzed. In a β-turn, by definition, the distance between $C_\alpha(i)$ to $C_\alpha(i+3)$ must be shorter than 7 Å and the tetrapeptide should not be part of an α-helical region.[39] For the model compounds two locations of the β-turn are conceivable, either between residues 1 and 4 or between residues 2 and 5 (see Fig. 1 for a description of the parameters used to characterize the model compounds). The results from this analysis can be seen in Figure 2(a). Not all of the compounds could adopt the β-turn geometry. In both **1m** and **5m**, four out of the nine conformations adopted the β-turn as defined by $C_\alpha2$–$C_\alpha5$. In **2m** one conformation out of 13, and in **8m** five conformations out of 162, adopted a β-turn geometry. Thus, based on the computational protocol used it seems that, except for **1m** and **5m**, i to $i+2$ cyclization does not induce the β-turn geometry.

A γ-turn consists of a seven-membered ring involving three amino acid residues. Two types of γ-turn exist, the classic with Φ_{i+1} and Ψ_{i+1} values corresponding to 70° to 85° and −60° to −70° and the inverse γ-turn with Φ_{i+1} and Ψ_{i+1} values corresponding to −70° to −85° and 60° to 70°.[40] We allowed a further ±30° deviation from the limits defined above in identifying γ-turn-like conformations.[40] Several previous studies have demonstrated that i to $i+2$ disulfide cyclization induces the inverse γ-turn geometry.[17,35,41,42] This can also be seen in the Φ_3, Ψ_3 plots of the model compounds (Fig. 3(a)). When all the compounds are plotted in the same Φ_3, Ψ_3 plot and only the region around the inverse γ-turn is considered it is seen that all model compounds (**1m**–**9m**) are able to adopt the inverse γ-turn conformation (Fig. 2(b)). In contrast to the classic γ-turn conformation which is only found in **8m** and **9m**, conformations similar to the inverse γ-turn are strongly preferred in compounds **1m** (7 out of 9) and **5m** (7 out of 9). All the other compounds can also adopt the inverse γ-turn geometry with various propensities.

To determine if the two series of analogues adopt different backbone torsion angles, the Φ_3, Ψ_3, Φ_4 and Ψ_2 angles, which lie within the cyclic moiety, were compared. In

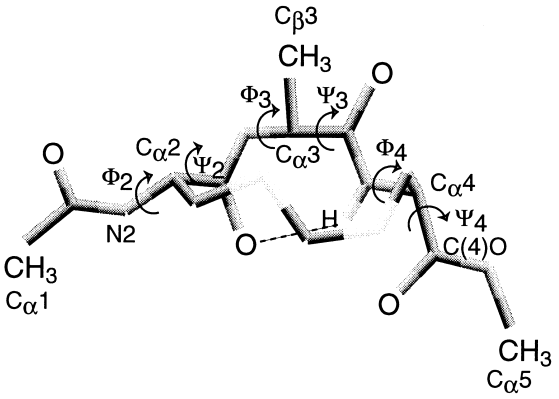


Figure 1. Parameters used to characterize the model compounds. The lowest energy conformation of model compound **5m** is shown.

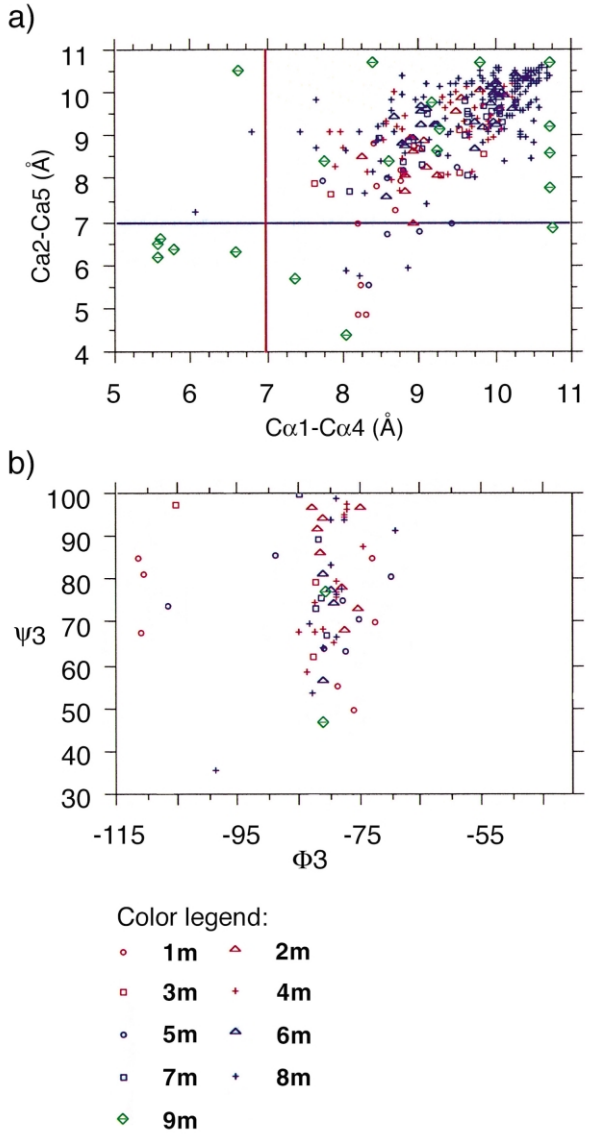


Figure 2. (a) Scatter plot of $C_\alpha1$–$C_\alpha4$ and $C_\alpha2$–$C_\alpha5$ distances for all conformations below 5 kcal/mol of the lowest energy conformation for model compounds **1m**–**9m**. Conformations to the left of the red line have $C_\alpha1$–$C_\alpha4$ distances below 7 Å and conformations beneath the blue line have $C_\alpha2$–$C_\alpha5$ distances below 7 Å. (b) Scatter plot for backbone torsion angles Φ_3 and Ψ_3 for all conformations below 5 kcal/mol of the lowest energy conformation for model compounds **1m**–**9m**. Only Φ_3 values between −40° and −115° and Ψ_3 values between 30° and 100° are shown.

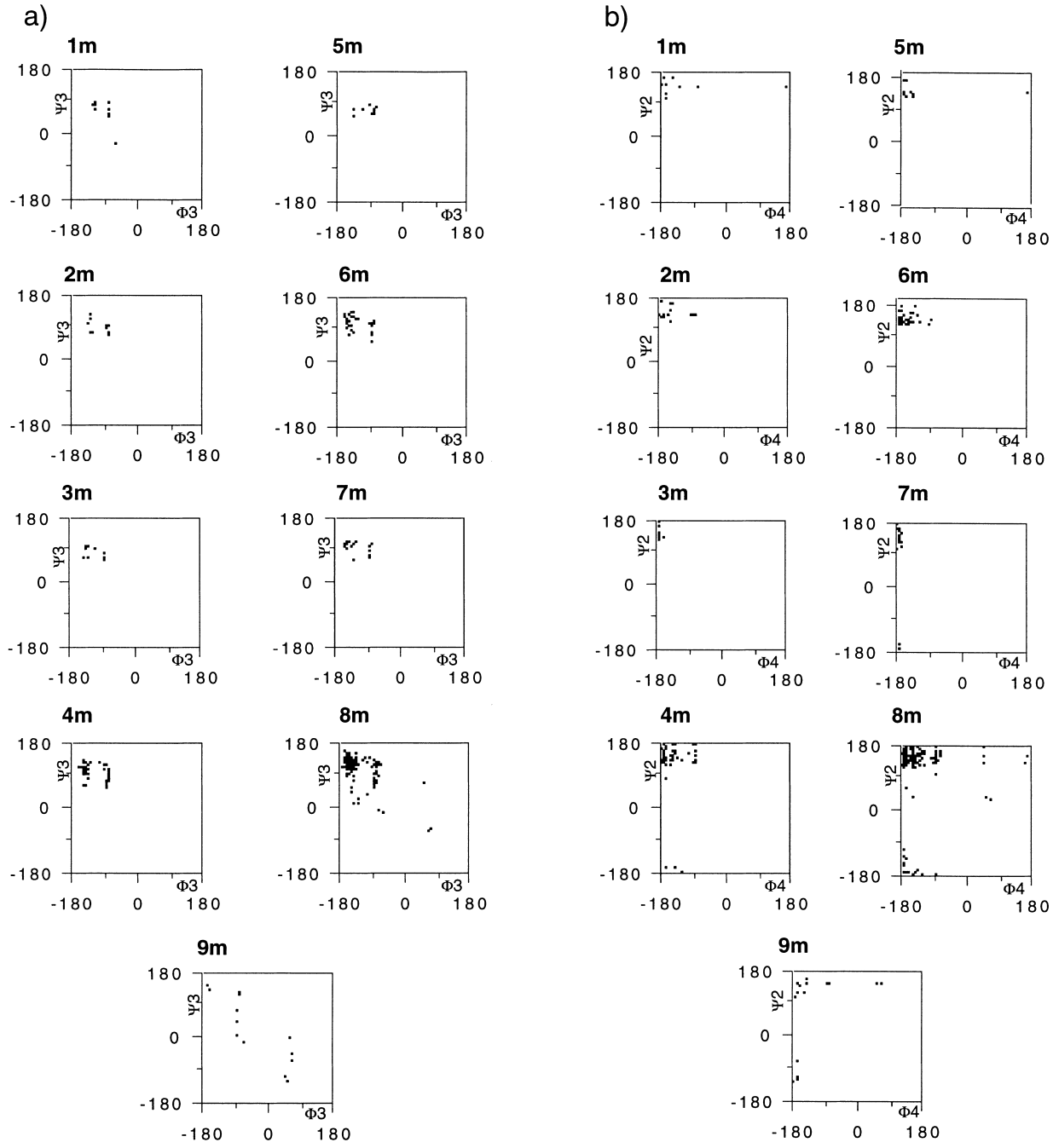


Figure 3. Scatter plots of torsion angles for all conformations below 5 kcal/mol of **1m**–**9m**: (a) Φ_3 versus Ψ_3; (b) Φ_4 versus Ψ_2.

Figure 3(a) and (b), respectively, the Φ_3, Ψ_3 and the Φ_4, Ψ_2 plots of the two series are shown. Qualitatively, both the plots in Figure 3(a) and (b) display similar characteristics. The exception to this is that the larger rings display greater flexibility. As expected the 12-membered ring homologues **2m**, **3m** and **5m** and the 13-membered ring homologues **4m**, **6m** and **7m**, respectively, adopt similar backbone torsion angles (Fig. 3(a) and (b)). A pairwise comparison of the disulfide and methylenedithioether analogues (**1m** to **5m**, **2m** to **6m**, etc.) also shows a similarity in allowed torsion angles. The 14-membered ring model compound **8m** was the least constrained peptide and adopted backbone torsion angles comparable to the linear reference compound **9m**.

The impact of cyclization was further monitored by analyzing the virtual torsion angles X1 = (N2-C_α2-C_α3-C_β3), X2 = (C_β3-C_α3-C_α4-C(4)O), and X3 = (N2-C_α2-C_α4-C(4)O) (Fig. 4(a) and (b)). These torsion angles describe the directions of the incoming backbone, the side chain of R_2 and the outgoing backbone (see Fig. 1). In these plots in Figure 4(a) the X2 angle of **1m**, **3m**, **5m** and **7m** seems to be somewhat restricted. This may be due to the Cys residue in position 3 which seems to restrict this angle. The X3 angle in the plots in Figure 4(b) of **2m**, **4m** and **6m**–**8m** has much greater flexibility. As was seen for the backbone torsion angles (Fig. 3), the 12-membered ring homologues and the 13-membered ring homologues adopt similar X1, X2 and X3 torsion angles. In

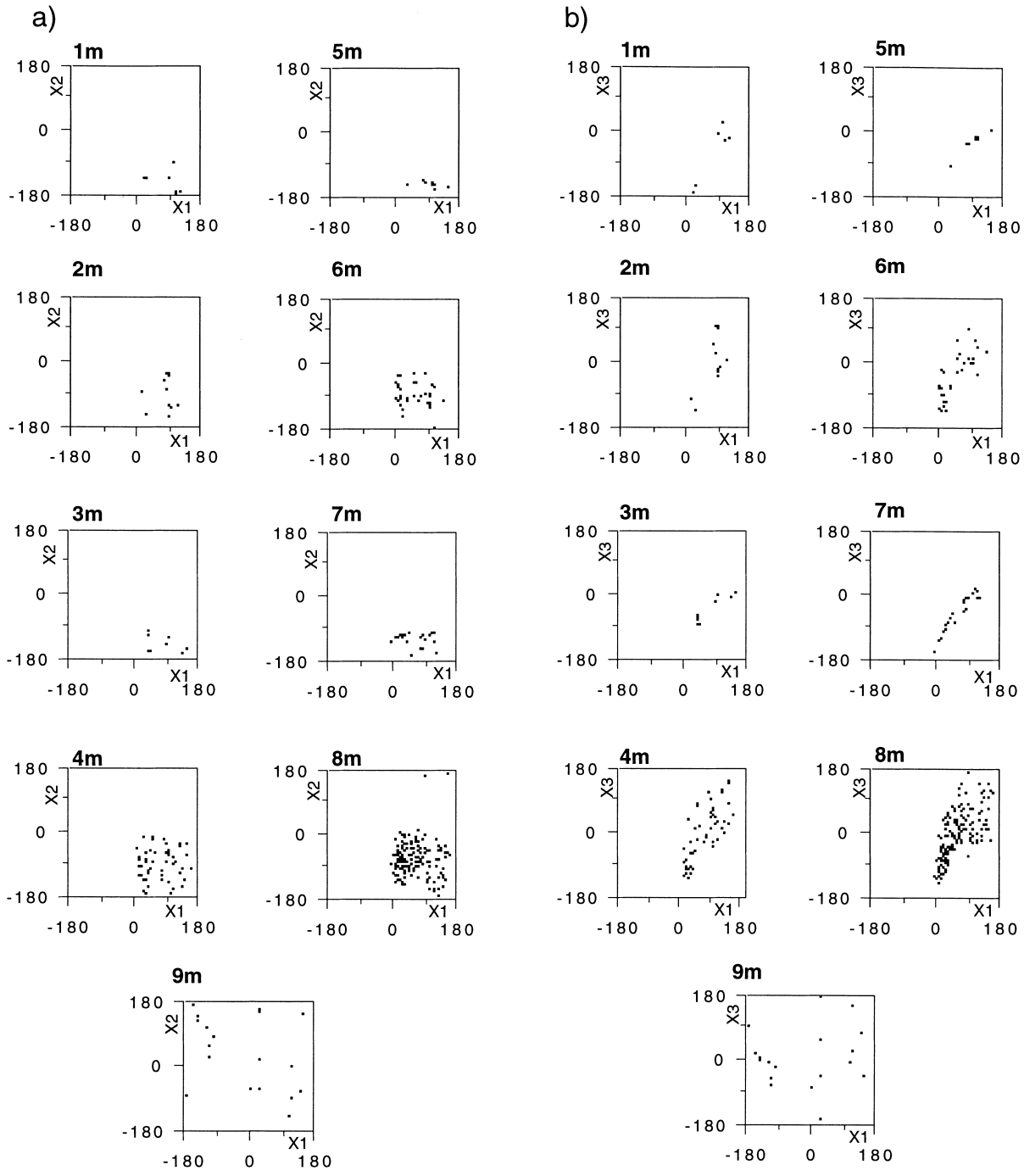


Figure 4. Scatter plots of torsion angles for all conformations below 5 kcal/mol of **1m**–**9m**: (a) X1 versus X2; (b) X1 versus X3.

the pairwise comparison of the disulfide and methylenedithioether analogues an even better similarity is seen than in the comparison of backbone torsion angles. The linear compound **9m** seems to be able to adopt conformations as described by X1, X2 and X3 that the other model compounds cannot adopt.

Discussion

Displacement of Val-3 and Ile-5 in Ang II by Hcy or Cys to give compounds **1**–**4** have previously been demonstrated to afford compounds with high affinity to Ang II receptors.[16] We have now introduced the methylenedithioether bridge in the analogues **1**–**4** to form **5**–**8**. The effect of ring size, and thus conformation, on the binding affinity of **1**–**8** has been assessed. We hypothesized that the 13-membered ring analogues **6** and **7** should exert similar binding affinity to the AT_1 receptor and exhibit similar conformational properties as **4**. As can be seen in Table 1, compound **6** is 17-fold less potent in binding than compound **4,** and compound **7** is about 100-fold less potent than **4**. The 12-membered ring analogue **5** should have similar properties as **2** and **3**. A comparison between **5** and the disulfides **2** and **3**[16] reveals that these compounds have roughly the same

affinity. The 14-membered ring analogue **8** had a binding affinity similar to **7**.

The conformational effects of introducing a methylene group between the sulfur atoms in disulfides **1m**–**4m** to give the methylenedithioethers **5m**–**8m** were studied by conformational analysis. An analysis of the backbone torsion angles and virtual torsion angles X1, X2 and X3 indicates that a change in ring size does not have a dramatic effect on the conformational properties of these analogues. Introduction of an extra methylene group between the sulfur atoms in the disulfides modified the conformational behavior of the corresponding disulfide only slightly. However, there is a qualitative resemblance in the plots between the disulfide and corresponding methylenedithioether analogues and also between the ring size homologues. This is also reflected in the comparable binding affinities of these compounds. The 13-membered ring size homologues **4** and **6** had the highest affinity to the AT_1 receptors in this study. Notably, when the conformational plots of these compounds are compared, there is a strong similarity between them, which may explain the high affinity of **6**. Compound **8** can also adopt the same conformations as **4** but the extra steric bulk of the larger ring may reduce the affinity.

Piling evidence suggests that Ang II adopts a turn conformation centered on residues 3–5 when interacting with the AT_1 receptor.[16–18,21,43–47] For example, replacing amino acid residues 3–5 with a seven-membered γ-turn mimetic delivered an AT_1 receptor agonist.[17,47] It is notable that the methylenedithioether model compound **5m** seems to be conformationally similar to **1m** and is as likely as the disulfide to adopt an inverse γ-turn geometry as suggested by theoretical calculations. Furthermore, the low temperature coefficient (1.0) of the Cys-5 residue amide proton in **5** indicates that this proton is intramolecularly hydrogen bonded. There are several possible hydrogen bonding arrangements for Cys-5 in **5** including the γ-turn around Tyr-4. In general, all ring formations that induce stabilization of secondary structures such as γ- and subtypes of β-turns in target peptides constitute valuable research tools. The easily executed methylenedithioether cyclization should therefore be a valuable complement to the common disulfide methodology for fine-tuning and for probing the bioactive conformation of peptides.

Conclusion

In summary we have prepared cyclic Ang II analogues with 12-, 13- and 14-membered rings encompassing methylenedithioether bridges. These analogues displayed good binding affinities to rat liver AT_1 receptors although somewhat lower than their disulfide counterparts. A comparison of the conformational properties of the two series of model compounds suggested that there was a conformational similarity not only between the ring size homologues but also between the disulfide and corresponding methylenedithioether analogues, which was reflected in the similar AT_1 receptor affinity.

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 with 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. The purity of compounds **5**–**8** was also established by LC-MS on a Gilson-Finnigan ThermoQuest AQA system, ESI mode, using two different chromatographic conditions: (1) Zorbax Stable Bond C8, 5μ, 4.6×50 mm with a 7 min gradient of 0–40% B at a flow rate of 2.0 ml/min and with detection at 214 nm (A: 0.05% HCOOH, B: 0.05% HCOOH in CH_3CN) (2) Zorbax Extend C18, 3.5μ, 4.6×50 mm using a 7 min gradient of 0–40% B at a flow rate of 1.2 mL/min and with detection at 214 nm (A: 5 mM NH_4OH, B: 5 mM NH_4OH in 95% CH_3CN). Plasma desorption mass spectroscopy was carried out on an Applied Biosystems (Uppsala, Sweden) BIOION 20 instrument. Amino acid analyses 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. Peptide contents were determined based on these amino acid analyses. ^1H NMR spectra were recorded on a Varian Unity 400 spectrometer at 400 MHz. Chemical shifts are indirectly referenced to the residual solvent signal at 2.49 ppm.

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) 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), Tyr(^{t}Bu), Cys(Trt), Hcy(Trt) and His(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 Fmoc-Hcy(Trt) which was 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/CH_3CN 1:1 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 the formed disulfide.[27] 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)[$Cys^{3,5}$]Ang II (5). Fmoc-Phe-Wang resin (161 mg, 100 μmol) was reacted as described above (SPPS) to yield the partially protected peptide resin (334 mg). A portion of the resin (158 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 60.4 mg of crude peptide. A portion of the crude peptide (17.5 mg, 16.8 μmol) was cyclized according the general procedure without the addition of Bu_3P to yield 5.0 mg (15%) of **5**. Amino acid analysis: Asp 1.02, Arg 1.00, Tyr 0.94, His 1.03, Pro 1.01, Phe 0.99 (53% peptide); PDMS (MW 1052.2): 1052.9 $(M+H)^+$, 1075.3 $(M+Na)^+$; LC–MS, cond (1): 526.7 $(M+2H)^{2+}$, 1052.5 $(M+H)^+$; cond (2): 526.8 $(M+2H)^{2+}$, 1052.5 $(M+H)^+$. ^1H NMR (DMSO-d_6, 25 °C, 400 MHz) δ 1.45 (m, 2H, Hβ′-Arg, Hγ′/Hγ-Arg), 1.50 (m, 1H, Hγ/Hγ′-Arg), 1.62 (m, 1H, Hβ-Arg), 1.78 (m, 1H, Hβ′-Pro), 2.01 (m, 1H, Hβ-Pro), 2.61 (m, 1H, Hβ′-Tyr), 2.65 (m, 1H, Hβ′-Asp), 2.73 (m, 1H, Hβ′-Cys^5), 2.79 (m, 1H, Hβ-Asp), 2.88 (m, 2H, Hβ-Cys^5, Hβ′-His), 2.92 (m, 1H, Hβ′-Phe), 2.93 (m, 1H, Hβ′-Cys^3), 2.99 (m, 1H, Hβ-Cys^3), 3.00 (m, 2H, Hβ-His, Hβ-Phe), 3.05 (m, 1H, Hβ-Tyr), 3.06 (m, 2H, Hδ-Arg, Hδ′-Arg), 3.33 (m, 2H, Hγ-Pro, Hγ′-Pro), 3.45 (m, 1H, Hδ′/Hδ-Pro), 3.56 (m, 1H, Hδ/Hδ′-Pro), 3.63 (d, $J=13.6$ Hz, 1H, S–CH_2–S), 3.74 (d, $J=13.6$ Hz, 1H, S–CH_2–S), 4.10 (m, 1H, Hα-Asp), 4.37 (m, 1H, Hα-Arg, Hα-Pro), 4.39 (m, 1H, Hα-Cys^5), 4.42 (m, 1H, Hα-Phe), 4.48 (m, 1H, Hα-Cys^3), 4.53 (m, 1H, Hα-Tyr), 4.75 (m, 1H, Hα-His), 6.62 (m, 2H, *meta*-Tyr), 7.02 (m, 2H, *ortho*-Tyr), 7.16- 7.29 (m, 5H, Phe), 7.34 (m, 1H, H4-His), 7.41 (d, $J=7.7$ Hz, 1H, NH-Cys^5), 7.56 (dd, $J=5.4$, 5.4 Hz, 1H, NHε-Arg), 8.05 (d, $J=7.3$ Hz, 1H, NH-Cys^3), 8.33 (d, $J=7.8$ Hz, 1H, NH-Phe), 8.42 (d, $J=8.0$ Hz, 1H, NH-His), 8.60 (d, $J=7.9$ Hz, 1H, NH-Arg), 8.80 (m, 1H, H2-His), 8.94 (d, $J=8.8$ Hz, 1H, NH-Tyr).

Cyclo(S–CH_2–S)[Cys^3,Hcy^5]Ang II (6). The peptide was synthesized according to the procedure used for **5**. The partially protected peptide resin (137 mg) was cleaved as described above to yield 49.1 mg of crude peptide. A portion of the crude peptide (11.5 mg, 10.9 μmol) was cyclized according the general procedure. The final yield of **6** was 3.2 mg (15%). Amino acid analysis: Asp 1.06, Arg 1.01, Tyr 0.91, His 1.10, Pro 0.90, Phe 1.03 (55% peptide); PDMS (MW 1066.2): 1067.3 $(M+H)^+$, 1089.0 $(M+Na)^+$; LC–MS, cond (1): 533.7 $(M+2H)^{2+}$, 1066.9 $(M+H)^+$; cond. (2): 533.7 $(M+2H)^{2+}$, 1066.4 $(M+H)^+$.

Cyclo(S–CH_2–S)[Hcy^3,Cys^5]Ang II (7). The peptide was synthesized according to the procedure used for **5**. The partially protected peptide resin (145 mg) was cleaved as described above to yield 54.4 mg of crude peptide. A portion of the crude peptide (11.1 mg, 10.5 μmol) was cyclized according the general procedure. The final yield of **7** was 2.3 mg (12%). Amino acid analysis: Asp 1.04, Arg 0.98, Tyr 0.88, His 1.04, Pro 1.06, Phe 1.01 (58% peptide); PDMS (MW 1066.2): 1067.2 $(M+H)^+$, 1089.9 $(M+Na)^+$; LC–MS, cond (1): 533.7 $(M+2H)^{2+}$, 1066.6 $(M+H)^+$; cond (2): 533.8 $(M+2H)^{2+}$, 1066.5 $(M+H)^+$.

Cyclo(S–CH_2–S)[$Hcy^{3,5}$]Ang II (8). The peptide was synthesized according to the procedure used for **5**. The partially protected peptide resin (150 mg) was cleaved as described above to yield 52.1 mg of crude peptide. A portion of the crude peptide (13.7 mg, 12.8 μmol) was cyclized according the general procedure. The final yield of **8** was 2.0 mg (9%). Amino acid analysis: Asp 1.03, Arg 1.01, Tyr 0.88, His 1.05, Pro 1.03, Phe 1.00 (63% peptide); PDMS (MW 1080.2): 1082.2 $(M+H)^+$, 1103.8 $(M+Na)^+$; LC–MS, cond (1): 540.8 $(M+2H)^{2+}$, 1080.6 $(M+H)^+$; cond (2): 540.7 $(M+2H)^{2+}$, 1080.5 $(M+H)^+$.

Cyclo(S–S)[$Hcy^{3,5}$]Ang II (4).[16] 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 became clear almost immediately and the reaction was quenched after 20 min by addition of glacial acetic acid (100 μL). The final yield of **4** was 4.9 mg (13%). Amino acid analysis: Asp 1.00, Arg 0.98, Tyr 1.01, His 1.00, Pro 0.99, Phe 1.01 (72% peptide); PDMS (MW 1066.2): 1067.5 $(M+H)^+$, 1089.6 $(M+Na)^+$.

Rat liver membrane AT_1 receptor binding assay. Rat liver membranes were prepared according to the method of Dudley et al.[29] Binding of [^{125}I]-Ang II to membranes was conducted in a final volume of 0.5 mL of 50 mM Tris–HCl (pH 7.4) containing 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.025% bacitracin, 0.2% BSA, liver homogenate corresponding to 5 mg of the original tissue weight, [^{125}I]-Ang II (0.036 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. Non-specific binding was determined in the presence of 1 μM Ang II. All experiments were performed in triplicate except for Ang II and **4** which were performed in quadruplicate. K_i values were calculated using the Cheng–Prusoff equation ($K_d = 1.7 \pm 0.1$ nM, [L] = 0.057 nM)

Conformational energy calculations. The calculations of **1m**–**9m** were performed using the Amber* all atom force field as implemented in the program Macromodel 6.5.[37] The General Born Solvent Accessible (GB/SA) surface area method for water developed by Still[36] 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 (SUMM) search method[48] in the batchmin program (command SPMC). 25000-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 3. Torsional memory and geometrical preoptimization were used. Truncated Newton conjugated gradient (TNCG) minimization (**1m**–**4m**) or PR Conjugate Gradient (PRCG) minimization (**5m**–**9m**) 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 (**1m**–**4m**) or PRCG (**5m**–**9m**) was used with the convergence criteria set to 0.001 (kJ/mol)/Å.

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