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The Design and Synthesis of a Potent Angiotensin II Cyclic Analogue Confirms the Ring Cluster Receptor Conformation of the Hormone Angiotensin II

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Abstract—The novel amide linked Angiotensin II potent cyclic analogue, c-[Sar^1,Lys^3,Glu^5] ANG II **19** has been designed and synthesized in an attempt to test the aromatic ring clustering and the charge relay bioactive conformation we have recently suggested for ANG II. This constrained cyclic analogue was synthesized by connecting the Lys^3 amino and Glu^5 carboxyl side chain groups, and it was found to be potent in the rat uterus assay and in anesthetized rabbits. The central part of the molecule is fixed covalently in the conformation predicted according to the backbone bend conformational model proposed for Angiotensin II. The obtained results using a combination of 2D NMR, 1D NOE spectroscopy and molecular modeling revealed a similar Tyr^4-Ile^5-His^6 bend, a His^6-Pro^7 *trans* configuration and a side chain aromatic ring cluster of the key aminoacids Tyr^4, His^6, Phe^8 for c-[Sar^1,Lys^3,Glu^5] ANG II as it has been found for ANG II (Matsoukas, J. H.; Hondrelis, J.; Keramida, M.; Mavromoustakos, T.; Markriyannis, A.; Yamdagni, R.; Wu, Q.; Moore, G. J. *J. Biol. Chem.* **1994**, *269*, 5303). Previous study of the conformational properties of the Angiotensin II type I antagonist [Hser(γ-OMe)^8] ANG II (Matsoukas, J. M.; Agelis, G.; Wahhab, A.; Hondrelis, J.; Panagiotopoulos, D.; Yamdagni, R.; Wu, Q.; Mavromoustakos, T.; Maia, H.; Ganter, R.; Moore, G. J. *J. Med. Chem.* **1995**, *38*, 4660) using 1-D NOE spectroscopy coupled with the present study of the same type of lead antagonist Sarilesin revealed that the Tyr^4-Ile^5-His^6 bend, a conformational property found in Angiotensin II is not present in type I antagonists. The obtained results provide an important conformational difference between Angiotensin II agonists and type I antagonists. It appears that our synthetic attempt to further support our proposed model was successful and points out that the charge relay system and aromatic ring cluster are essential stereoelectronic features for Angiotensin II to exert its biological activity.

Introduction

Angiotensin II (ANG II) is a linear octapeptide (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) which is the active component of the Renin–Angiotensin system (RAS). Over the last decade a local RAS has been identified in various tissues including heart, brain, kidneys and vessel wall.[1] In particular, this hormone plays a pivotal role in cardiovascular homeostasis and the regulation of blood pressure.[2,3] Not only is it one of the most potent vasoconstrictive agents, but it was found to be also a growth factor implicated in cardiac, vascular hypertrophy and the ventricular remodeling following myocardial infarction.[4] As a result of its importance, Angiotensin II has been extensively studied since its discovery several years ago.[5] These studies have included theoretical,[6–10] physicochemical,[11–14] and spectroscopic investigations.[15–21]

In addition, a large number of linear and sterically restricted Angiotensin II analogues have been synthesized in order to help establish the roles of the ANG II

Keywords: angiotensin II; conformation; ring cluster; charge relay system; angiotensin II; cyclic analogues.
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residues and the relationship between biological activity and conformation. The structure–activity studies have illustrated the importance of the C-terminal aromatic residue phenylalanine (Phe[8]) for agonist activity. Replacement of Asp by Sar at position 1 results in the super agonist peptide analogue, [Sar[1]] ANG II, while additional replacement of the aromatic residue Phe at position 8 with an aliphatic one, as Ile, results in an antagonist, [Sar[1],Ile[8]] ANG II (Sarilesin). The structure–activity relationship work (SAR) has identified residues 2, 4, 6, and 8 to be critical for biological activity and 3, 5, and 7 for proper backbone orientation and has led to the proposal of several structural models for Angiotensin II, including conformations which contain an α-helix, a β-turn, a γ-turn, an ion–dipole interaction and a recently suggested charge relay system.[11,12,15,18,22,23]

All these models aimed to the design and synthesis of non-peptide receptor antagonists which can be utilized for the treatment of cardiovascular diseases. We have recently suggested a conformational model for Angiotensin II in which its active site is comprised by the three side chain aromatic rings of the residues Tyr[4], His[6], Phe[8], and the C-terminal carboxylate.[23] In particular, this model is characterized by a backbone bend at the sequence Tyr[4]-Ile[5]-His[6], a His[6]-Pro[7] *trans* configuration, a clustering of the Tyr[4], His[6], Phe[8] side chain aromatic rings and a charge relay system involving the triad Tyr[4] hydroxyl, His[6] imidazole and Phe[8] carboxylate. The clustering is the result of the backbone bend that brings the aromatic rings in spatial proximity. This derived model was based on NMR (COSY, TOCSY, ROESY, 1-D NOE), computational and fluorescence life time studies. The combination of NMR and computational analysis studies show the presence of the ring cluster while fluorescence life time experiments present evidence of a tyrosinate anion.[23] The proposed aromatic ring cluster which drives a charge relay system fits very well with the newly revealed non-peptide Angiotensin receptor antagonists as exemplified by Losartan and its potent analogue, 5-butyl-2-hydroxymethyl-1-[[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]imidazole, synthesized in a recent study. These superimposition studies were based on an optimization of their structural similarity with the C-terminal region of Angiotensin II.[19–21,24–32]

Developing an Angiotensin II receptor bound conformational model is not an easy task due to the fact that the peptide hormone is a linear molecule with high degree of flexibility. As a result of its linear structure, Angiotensin II can adopt in water solutions several conformations. However, the peptide hormone obviously adopts a predominant conformation as it approaches the receptor. The aim then is to determine which of the conformations observed in solution approximates best the receptor-bound conformation. There are three main ways of probing the biologically active conformation:

(1) Use of solvents to simulate biological environment

In particular, the use of receptor simulating solvents of intermediate or low polarity, which allow a more ordered peptide structure is an important issue in determining the possible bioactive conformations. The role of lipid-induced peptide folding to study peptide-receptor interactions is currently emphasized and the use of DMSO as solvent of lower dielectric constant (ε∼45) compared to H_2O (ε∼80) is justified.[33–37] Recent comparative studies in DMSO and H_2O have clearly shown these differences.[37]

(2) Synthesis of conformationally restricted analogues

Another way of investigating the Angiotensin II receptor-bound conformation is via conformational restriction. A limited number of conformationally restricted via cyclization Angiotensin II analogues have been reported by others. Cyclization was achieved either by the disulfide method using cysteine moieties at various locations of the peptide molecule[38–44] or by the amide-linkage method.[45–50] These studies have greatly assisted in the identification of the pharmacophoric groups for receptor activation and the development of a reliable receptor model accessible to non-peptide drugs.

(3) Synthesis of peptide mimetic analogues

A third important way of probing the Angiotensin II receptor-bound conformation can be through the design of a potent non-peptide compound to mimick the peptide hormone. The stereoelectronic characteristics of the non-peptide mimetic which resembles with pharmacophoric groups of peptide hormone when are properly superimposed may define their spatial arrangement towards the approach of the receptor.[29,51–53]

Results and Discussion

Our own interest in the Renin–Angiotensin system and the conformational model of Angiotensin II, which could be used as a basis for the synthesis of non-peptide receptor antagonists, prompted us to design and synthesize the novel cyclic amide linked Angiotensin II analogue c-[Sar[1],Lys[3],Glu[5]] ANG II **19** (Fig. 1) that potentially can pertain the Angiotensin II conformational characteristics (i.e. backbone bend and aromatic ring clustering). Cyclization was achieved by forming an amide-linkage between the -NH_2 and -COOH side chain

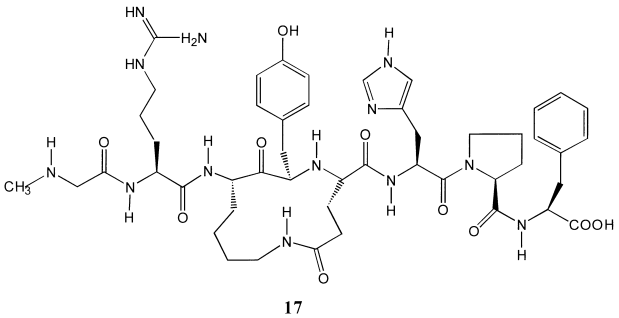

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Figure 1. Chemical structure of c-[Sar[1],Lys[3],Glu[5]] ANG II **19**.

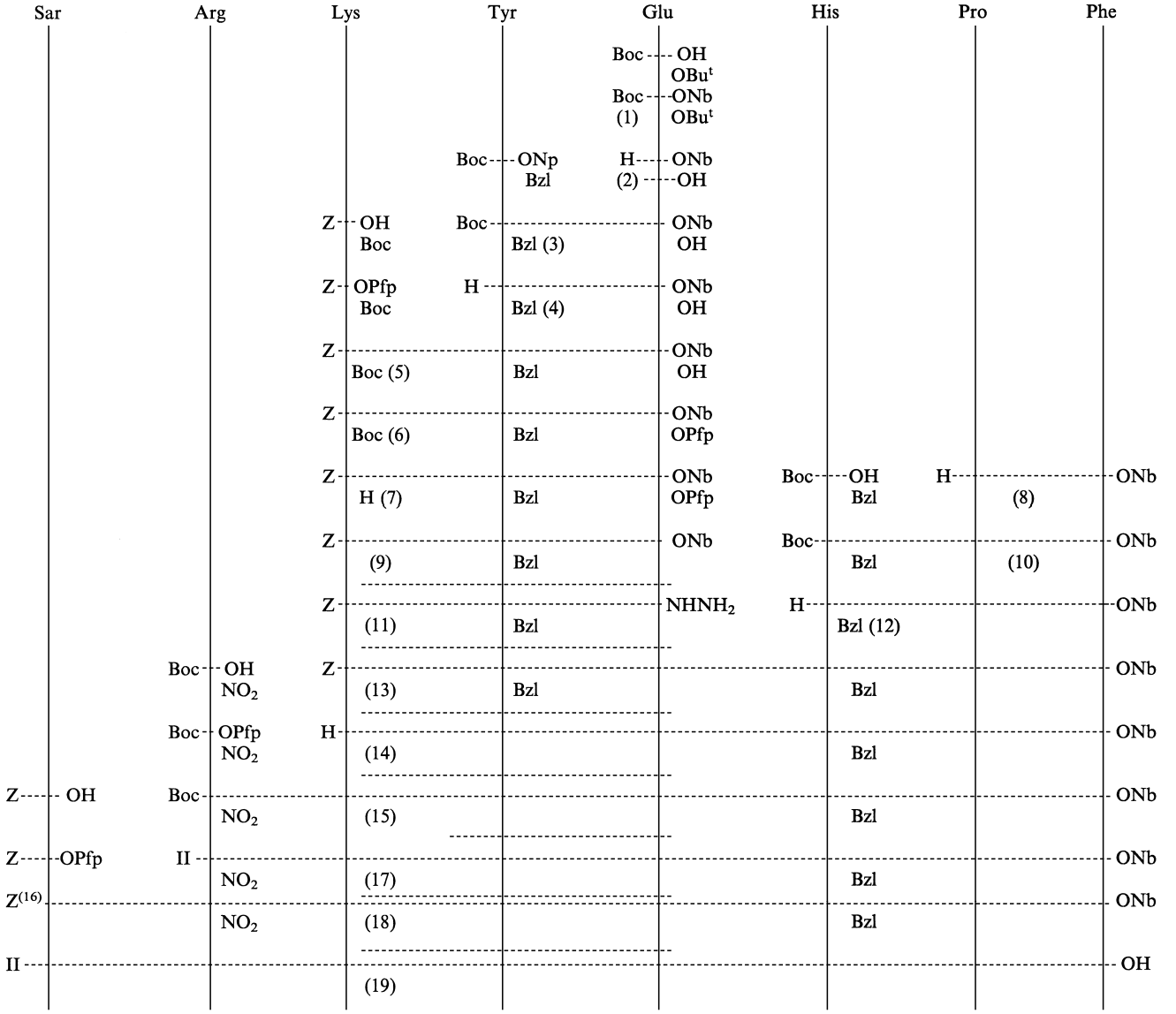
groups of Lys and Glu residues at positions 3 and 5, respectively, which are the least important for activity. With this study a confirmation of our Angiotensin II model was sought and further evidence to support the importance of the suggested ring cluster for receptor activation.

In addition, this cyclic peptide can also aid the investigation of the backbone differences between [Sar^1] ANG II and its type I antagonist Sarilesin. A comparison of the conformational properties of Angiotensin II with its type I antagonist Sarilesin would furthermore enhance our information about the side chain aromatic cluster formed by aromatic rings of the aminoacids Tyr^4-His^6-Phe^8 as an essential structural requirement for hormone activity.

Chemistry

The synthesis of the amide linked cyclic analogue of Angiotensin II, c-[Sar^1,Lys^3,Glu^5] ANG II **19** was carried out by solution methodology according to the synthetic route described in Table 1. Acquisition of cyclic structure at minimal peptide chain length, which was possible in the synthesis of this particular analogue, seemed preferable for reasons such as: (i) cyclization of short peptides proceeds with less problems related to the temporary protection and deprotection of groups that will be amide linked when compared to cyclization achieved at the last step of a synthesis and after a total synthesis of the linear molecule and (ii) the synthetic route as it is depicted in Table 1 offers a convenient way for the synthesis of other analogues with the same cyclic

Table 1. Synthetic route for cyclic [Sar^1,Lys^3,Glu^5] ANG II



core but with various modifications both in the N- and C-terminal parts of the molecule. The *p*-nitrobenzylester group was used to protect the carboxyl groups being in the central part of molecule and its C-end tripeptide. For temporary protection, the Bu^t, Boc and Z groups were used. Side chains of tyrosine and histidine were protected with the benzyl group. Coupling procedures were carried out with DCC/HOBT and active ester methology (using both *p*-nitrophenyl and pentafluorophenylesters) as well as by the azide method. The tripeptide of central part of the molecule was synthesized stepwise, with the γ-carboxyl group of Glu being not protected. Unprotection of the γ-carboxyl group did not cause any problems in the synthesis. Moreover, due to the reduced solubility of intermediates containing a free carboxyl group, isolation and purification procedures were simplified.[54]

Conformational analysis studies

NMR spectroscopy. Previous NMR studies on octapeptide Angiotensin II using 1-D NOE and 2-D ROESY techniques have shown a ring clustering and a strong Tyr^4-Ile^5-His^6 bend. In this study 2-D COSY, TOCSY, and 1-D NOE experiments on [Sar^1] ANG II, Sarilesin and c-[Sar^1,Lys^3,Glu^5] ANG II **19** have been conducted using DMSO-d_6 or DMSO-d_6:D_2O (9:1) as a solvent, as we have done previously for the conformational analysis of Angiotensin II.[23,51–53,55–58] While Angiotensin II and agonist peptides have been the subject of numerous conformational investigations, the conformation of Sarilesin was not explored. Therefore, it was of interest to investigate its backbone conformational features and compare them with those of [Sar^1] ANG II.

Figures 2–4 show reference and 1-D NOE difference spectra for c-[Sar^1,Lys^3,Glu^5]ANG II **19**, [Sar^1] ANG II and [Sar^1,Ile^8] ANG II. Assignment of aromatic proton resonances of peptide **19** was achieved by combining information from TOCSY spectra, NOE experiments and previous assignments of these resonances in Angiotensin II and its analogues.[23,51–58] The one-dimensional NMR spectrum of c-[Sar^1,Lys^3,Glu^5] ANG II was characterized by a well resolved down field region in which the Tyr^4, His^6 and Phe^8 aromatic resonances were distinguisable for 1-D NOE studies and distance information.

In the cyclic peptide analogue **19**, only the His^6 C_α proton resonance was distinct at $\delta = 4.67$ ppm, while all other C_α protons of the peptide analogue overlapped at $\delta =$ 4.3–4.4 ppm complicating backbone investigation. Enhancement of the resonance at $\delta = 4.27$ ppm overlapped with the water peak, upon saturation of the His^6 C_α proton, was tentatively assigned to the Tyr^4 C_α proton.

Tyr^4-Ile^5-His^6 bend. Saturation of His^6 protons in c-[Sar^1,Lys^3,Glu^5]ANG II **19**, [Sar^1]ANG II and Sarilesin gave valuable information concerning a Tyr^4-Ile^5-His^6 bend and a His^6-Pro^7 *trans* configuration.

Irradiation of the His^6 C_α proton at $\delta = 4.67$ ppm of c-[Sar^1,Lys^3,Glu^5] ANG II **19** resulted in enhancement

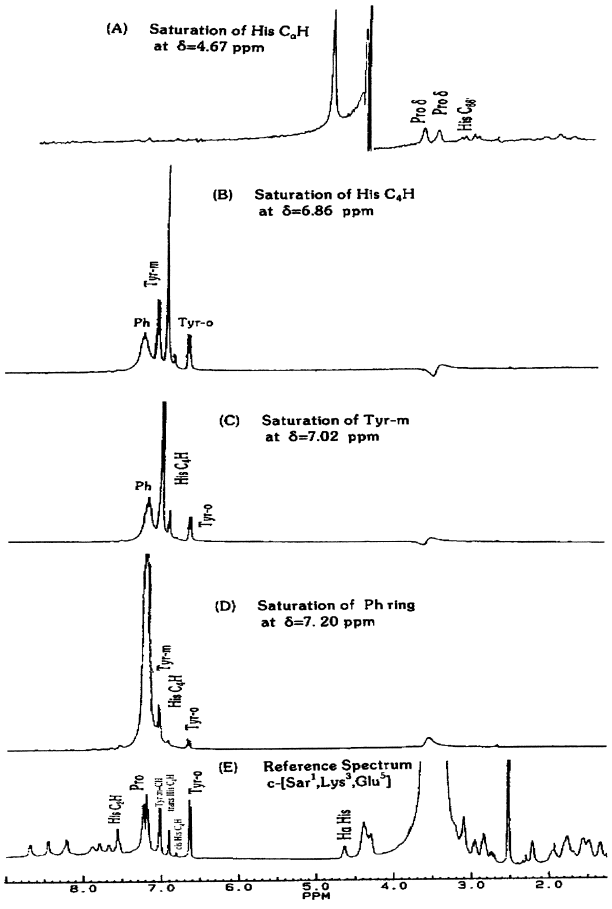


Figure 2. Reference spectrum (E) and NOE difference spectra for c-[Sar^1,Lys^3,Glu^5] ANG II **19** in dimethyl sulfoxide-d_6 obtained upon saturation of His^6 C_α (A) and His^6 C_4 (B) protons. Enhancement of Pro^7 δ and δ′ proton resonances in (A) indicates a *trans* His^6-Pro^7 amide bond. Enhancement of Phe^8, Tyr^4 *meta* and *ortho* aromatic proton resonances in (B) indicates ring clustering. NOE difference spectra for c-[Sar^1,Lys^3,Glu^5] ANG II in dimethyl sulfoxide-d_6 obtained upon saturation of Tyr^4 *meta* (C) and Phe^8 ring protons (D). Enhancements of Phe^8 and His^6 C_4 proton resonances in (C) and enhancements of Tyr^4 *meta* and *ortho* and His^6 C_4 proton resonances in (D) indicate ring clustering.

of the Tyr^4 C_α proton resonance at $\delta = 4.27$ ppm (17%) indicating a backbone bend (Fig. 2).

Similarly, irradiation of the His^6 C_α proton at $\delta =$ 4.68 ppm of [Sar^1] ANG II resulted in enhancement of the Tyr^4 C_α proton resonance at $\delta = 4.51$ ppm (22%) indicating a backbone bend (Fig. 3). The strong NOE between His^6 C_α and Tyr^4 C_α protons was found to be a reverse phenomenon. Thus, irradiation of Tyr^4 C_α proton produced enhancement of the His^6 C_α proton resonance (18%), indicating the presence of a Tyr^4-Ile^5-His^6 bend (Fig. 3).

For the antagonist peptide Sarilesin, no Tyr^4 or His^6 C_α proton resonance enhancements were observed upon saturation of the His^6 or Tyr^4 C_α protons at $\delta = 4.70$ ppm and $\delta = 4.42$ ppm, respectively (Fig. 4). Lack of such NOE effects indicated the absence of a Tyr^4-Ile^5-His^6 bend. Therefore, this study revealed that the Tyr^4-Ile^5-His^6 bend, a conformational property

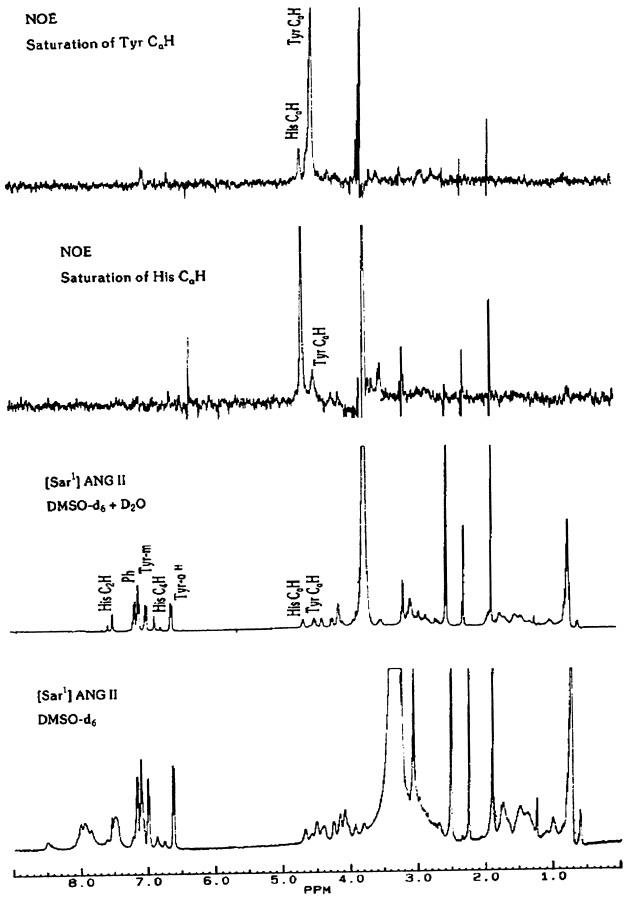


Figure 3. Reference spectrum and NOE difference spectra for [Sar^1] ANG II in DMSO-d_6:D_2O (9:1) obtained upon saturation of Tyr^4 C_α and His^6 C_α protons. Enhancement of the His^6 C_α proton resonance upon saturation of Tyr^4 C_α proton and vice versa, indicates a strong Tyr^4-Ile^5-His^6 backbone bend in the agonist peptide.

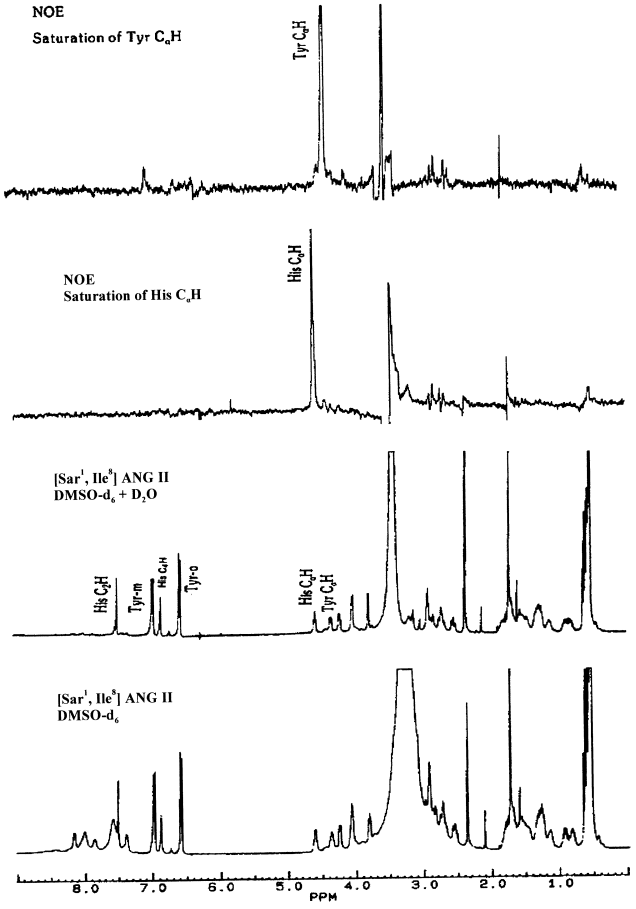


Figure 4. Reference spectrum and NOE difference spectra for [Sar^1, Ile^8] ANG II (Sarilesin) in DMSO-d_6:D_2O (9:1) obtained upon saturation of Tyr^4 C_α and His^6 C_α protons. Lack of enhancement of the His^6 C_α proton resonance upon saturation of the Tyr^4 C_α proton and vice versa indicates the absence of a Tyr^4-Ile^5-His^6 bend and a loose non-folded conformation in the antagonist peptide.

found in Angiotensin II and [Sar^1] ANG II is not present in [Sar^1,Ile^8] ANG II providing an important conformational difference between Angiotensin II and Sarilesin. As a result of the absence of such bend, Sarilesin assumes a loose non-folded backbone conformation.

His^6-Pro^7 *trans* configuration. For c-[Sar^1,Lys^3,Glu^5] ANG II **19** saturation of the His^6 C_α proton at $\delta = 4.67$, resulted in enhancements of the two δ Pro^7 proton resonances at $\delta_1 = 3.48$ ppm (6%) and $\delta_2 = 3.28$ ppm (5%) indicating a major His^6-Pro^7 *trans* conformer (Fig. 2). By integration of the two His C_2 or His C_4 peaks corresponding to *trans* and *cis* conformers a *trans:cis* ratio of 10:1 was calculated. The AB quartet revealed at $\delta = 2.88$ ppm (4%) was attributed to the enhancement of the vicinal His^6 $C_{\beta\beta'}$ proton resonances. This quartet was possible to be observed only after addition of D_2O, which resulted in the upfield shift of the water peak and allowed inspection of the region 2.50–3.50 ppm where the Pro^7 $C_{\delta\delta'}$ and His^6 $C_{\beta\beta'}$ protons resonate.

For [Sar^1] ANG II, enhancement of the Pro^7 C_δ proton resonances at $\delta = 3.15$ ppm (8%) and 3.48 ppm (13%) resulting from His^6 C_α proton saturation, indicated the presence of the His^6-Pro^7 *trans* conformer as the major one (not shown). In particular, His^6-Pro^7 *trans:cis* ratio was identified by examining the relative intensities of the doublets attributed to His^6 C_2 and C_4 protons. For Sar^1 ANG II, a His^6-Pro^7 *trans:cis* ratio of 4:1 was observed. Comparison of the obtained results with those of cyclic analogue **17** show that cyclization enhances the energy barrier between the *cis-trans* isomerization.

Strong enhancements of the Pro^7 C_δ proton resonances at $\delta = 3.30$ ppm and $\delta = 3.55$ ppm (overlapped with H_2O) upon saturation of the His^6 C_α proton in Sarilesin indicated a *trans* His^6-Pro^7 peptide bond configuration in the antagonist peptide (not shown).

Aromatic ring cluster. Spatial proximity of the three side chain aromatic rings of Tyr^4, His^6, Phe^8 in peptide **17** was indicated by NOE experiments involving irradiation of Tyr^4, His^6 and Phe^8 ring protons (Fig. 2).

Thus, NOE enhancements resulted in the His^6 C_4 ($\delta = 6.89$ ppm, 7%) and the Phe^8 aromatic ring proton resonances (7.20 ppm, 8%) upon saturation of the *m*-Tyr^4 proton at $\delta = 7.02$ ppm. The reverse NOE was also observed. Irradiation of the Phe^8 aromatic ring protons at $\delta = 7.20$ ppm resulted in enhancements of the *o*-Tyr^4

(6.63 ppm, 3%), *m*-Tyr^4 (7.02 ppm, 7%) and His^6 C_2 (7.57 ppm, 4%) and C_4 (6.89 ppm, 3%) proton resonances. This NOE establishes close proximity between the three key aminoacids Tyr^4, His^6 and Phe^8. As in the case of Angiotensin II and [Sar^1] ANG II (Fig. 3), the Phe^8 aromatic ring protons of the cyclic analogue appear as a broad multiplet at $\delta = 7.20$ ppm indicating restricted rotation (Fig. 2).[23,58]

For [Sar^1] ANG II saturation of the C_4 His^6 (6.87 ppm) proton resulted in enhancements of *o*-Tyr^4, (6.99 ppm, 3.2%), *m*-Tyr^4 (6.61 ppm and 2.4%) and the Phe^8 (7.15 ppm, 2.8%) ring proton resonances, and saturation of Phe^8 ring protons resulted also in enhancements of *o*-Tyr^4 (2.6%) and *m*-Tyr^4 (2.2%) proton resonances (Fig. 3).

Computational chemistry

Figures 5 and 6 show low energy structures for c-[Sar^1,Lys^3,Glu^5] ANG II **19** and Sarilesin generated using a combination of NOE data and molecular dynamics. The important stereoelectronic characteristics of the generated models are the following: The cyclic analogue **19** adopts a Tyr^4-Ile^5-His^6 bend, a *trans* amide His^6-Pro^7 configuration and a side chain aromatic cluster of the three key aminoacids Tyr^4, His^6 and Phe^8. Such stereoelectronic features are missing with Sarilesin. More detailed information about low energy conformers of c-[Sar^1,Lys^3,Glu^5] ANG II **19** will be given in a subsequent paper.

The difference in the magnitude of the biological activity between the cyclic analogue **19** and the parent compound ANG II is probably due to the different conformation that each molecule assumes as it approaches the receptor. Angiotensin II, like other linear peptide hormones, exists in several different structural forms in water, but as it approaches the receptor it adopts a predominant conformation with much reduced flexibility. However, cyclization restricts the number of possible conformations, thereby presumably preventing the peptide from assuming optimal conformation when it binds to the receptor.

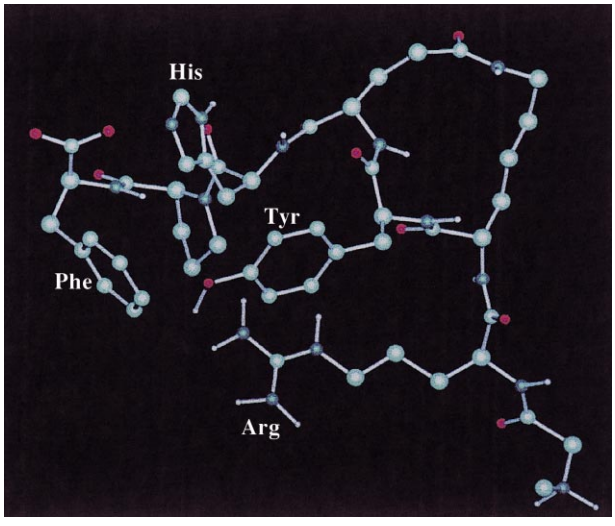


Figure 5. Proposed model of c-[Sar^1,Lys^3,Glu^5] ANG II generated by distance geometry and molecular dynamics. Distance constraints were obtained from 1D-NOE data. A cluster of the three aromatic rings of aminoacids (Tyr^4, His^6, Phe^8) and the Arg^2 guanidino group is favoured.

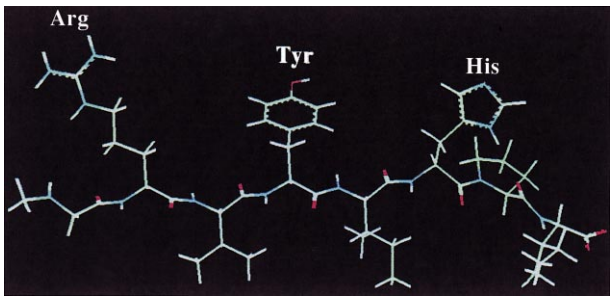


Figure 6. Proposed model of Sarilesin. The important molecular feature of this peptide which possess antagonist activity is the lack of the ring cluster.

Since the C-terminal carboxylate is essential for activity, the cyclic ANG II analogue **17** was designed to retain the C-terminal carboxyl moiety. The cyclization of Angiotensin II across residues 3 (Glu) and 5 (Lys) has resulted in the maintenance of potency revealing structural requirements necessary for activity. Obviously, the side chains of residues 3 and 5 though considered non-important for activity, as they are located at the opposite side of the functionally important aromatic side chains which represent the active site, seem to play an important role in controlling the agonist activity of ANG II. Taken together with our previous results, the current findings define a biologically active conformational model for Angiotensin II, characterized by the clustering of aromatic rings and a charge-relay system involving the triad Tyr^4 hydroxyl, His^6 imidazole and Phe^8 carboxylate. This system is analogous to that found in serine proteases (Fig. 7).[59]

Biological activity

The constrained amide linked cyclic Angiotensin II analogue **19** was found to be active in both *ex vivo* and *in vivo* experiments.

In the rat uterus assay, the agonist activity of the cyclic analogue **19** was determined by matching its response with the equivalent response to human Angiotensin II and it was found to be ~15% of that observed with the intact octapeptide.

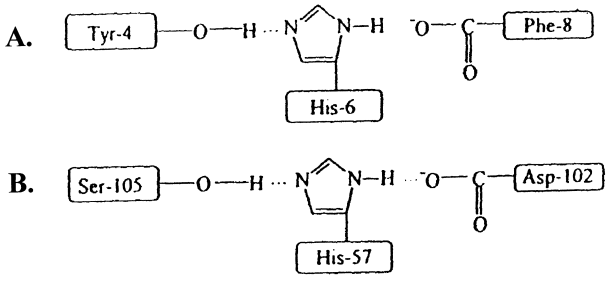


Figure 7. Charge relay system in Angiotensin II (A) and in Serine Proteases (B).

In anesthetized rabbits, previous testing had revealed that Angiotensin II infused at a rate of 2 mg/min exerts a significant and submaximal hypertensive response ($+105 \pm 13$ mmHg). Thus, in each animal a comparison was made between blood pressure changes produced by 2 μg/min of Angiotensin II with those produced by 10, 20, 40 and 80 μg/min of the cyclic analogue **19**. As shown in Figure 8, c-[Sar^1,Lys^3,Glu^5] ANG II is a potent agonist producing significant and dose-dependent blood pressure increases. The blood pressure started rising within approximately 1 min after the commencement of drug infusion, reached a plateau within 1–2 min and returned to preinjection levels within 2–4 min after discontinuation of the drug.

Conclusion

This research was aiming at establishing backbone differences between Angiotensin II agonist and antagonist peptides and at confirming the aromatic ring clustering conformational model for Angiotensin II which has been recently proposed on the basis of structure–activity relationships, NMR and fluoresence life time studies. A strong Tyr^4-Ile^5-His^6 bend exists in the superagonist [Sar^1] ANG II and Angiotensin II but not in [Sar^1,Ile^8] ANG II and type I antagonists with an aliphatic aminoacid at position 8. This backbone difference as revealed by 1-D-NOE studies, may constitute a major conformational feature responsible for the different activity of agonists and type I antagonists and may provide new avenues in designing non-peptide Angiotensin II antagonists. A constrained cyclic amide linked Angiotensin II analogue, c-[Sar^1,Lys^3,Glu^5] ANG II **19** designed to keep intact the clustering and backbone bend characteristics of the peptide hormone Angiotensin II has been synthesized and was found to be active in the rat uterus assay and in anesthetized rabbits. The molecule was designed with the hypothesis that residues 3 and 5 are not important for activity and exist on the other side of the molecule from the functionally important aromatic side chains and this structure can be accommodated in the charge relay conformation proposed for Angiotensin II. However, its lower potency points out that integrity of aminoacid residues 3 and 5 controls the agonist activity of ANG II. In the potent constrained analogue, the three rings are closely spaced at the same side of the cyclic ring and this is shown by NOE interactions and molecular modeling.

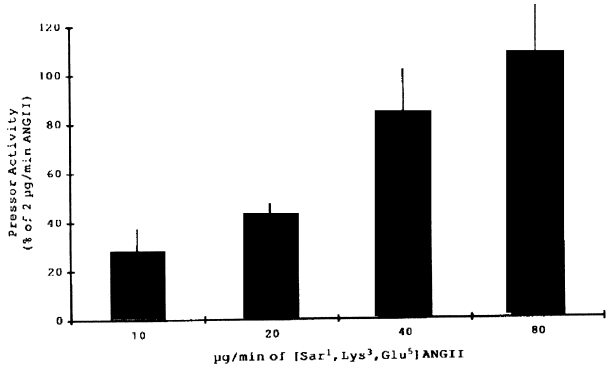


Figure 8. Dose dependency of the pressor activity produced by increasing doses of the cyclic analogue [Sar^1,Lys^3,Glu^5] ANG II. Results are expressed as percent of the hypertensive response produced by 2 μg/min of Angiotensin II and represent mean ± SEM of four experiments.

The obtained data confirm our hypothesis that the aromatic side chains together with the C-terminal carboxylate are the essential pharmacophoric groups for receptor activation. These data also emphasize the role of closely spaced residues 4, 6, and 8 to form a possible relay system.

We anticipate that further refinement of the charge relay conformational model will lead to the design and synthesis of improved ANG II receptor antagonists.

Experimental

The identity of the cyclic product **19** was established by FABMS and NMR spectroscorpy. FAB spectra were run on an AEI M29 mass spectrometer modified as described elsewhere.[60] FAB gun was run at 1 mA discharge current and at 8 kV. The FAB matrix used was a mixture of 6:1 dithiothreitol:dithioerythritol (Cleland Matrix). The purity of the final cyclic analogue **19** was checked by thin layer chromatography (TLC). TLC was carried out with precoated silica gel on glass (Merck Kieselgel 60 F254) TLC plates. Amino acid analysis of the cyclic product **19** was performed on a Beckman 6300 high-performance analyzer. Compositional analysis data were collected from 6 N HCl hydrosylates (100 °C, 18 h) with ninhydrin-based analysis.

Intermediate peptides and final product **19** were checked for purity on a Waters HPLC system equipped with a 600E system controller. Fractions were analysed by analytical reversed-phase column (Techsil C_{18}, 250×4.6 mm).[61,62]

The final cyclic product **19** was purified on a Waters HPLC system equipped with a 600E system controller.[61,62] The crude peptide material (18 mg) was dissolved in methanol (450 mL), clarified by centrifugation and the solution was injected through a Rheodyne 7125 injector with a 500 μL sample loop. A Lichrosorb RP-18 reversed-phase preparative column (250×10 mm) with 7 μm packing material was used. Separations were achieved with a linear gradient of acetonitrile in 0.1% aq TFA at a flow rate of 3 mL/min. In particular, the mobile phases used were 0.1% aq TFA (A) and 0.1% TFA in acetonitrile (B) and involved a linear gradient from 0 to 100% B over 60 min (3 mL/min). Fractions were manually collected at 0.5 min intervals, the eluent was monitored at 230 and 254 nm (Waters 996 Photodiode Array Detector) and the elution time of the major product was typically in the region of 25–30 min. The amino acid derivatives used in the synthetic procedures were purchased from Reanal (Hungary) and Nova Biochem.

NMR spectroscopy. NMR experiments were carried out using a Bruker 400-MHz NMR spectrometer. c-[Sar^1,

Lys[3],Glu[5]] ANG II **19**, [Sar[1]] ANG II and Sarilesin were studied by dissolving 5 mg of the compound in 0.5 mL of DMSO-d_6 or DMSO-d_6:D_2O (9:1). The chemical shifts were reported relative to the undeuterated fraction of the methyl group of DMSO-d_6 at 2.50 ppm with respect to TMS. One-dimensional spectra were recorded with a sweep width of 4.500 Hz and 32 K (zero filled to 64 K) data points and by methods previously described. The COSY/TOCSY experiment provided contour plots which were symmetrized with respect to the diagonal. One-dimensional NOE experiments were carried out in the difference mode using multiple irradiation. This procedure used a very low decoupler power setting (typically 10 dB lower than for a standard NOE experiment) so that it was possible to avoid partial saturation of resonances in close proximity. The selected lines were irradiated 20 times for 100 ms (total irradiation time 2.0 s). To monitor the NOE build-up, other irradiation times (0.2, 0.5, 1, and 5 s) were also employed in some experiments. Each line required a total of 1000 scans and the experimental time was 2 s. Time of acquisition for each transient was ≈3 s. Total experimental time was about 12–15 h for irradiating 10–15 groups of multiplets. NOE enhancements were measured as the point increase in signal size/proton after saturation of a distinct proton. The applied NOE experimental conditions (low power, different τ preirradiation times, saturation of control areas) aimed to minimize spin diffusion and partial saturation. The methods used for the COSY and NOE experiments were similar to those previously described.[23,63–73]

Molecular dynamics

In the molecular modeling, theoretical calculations were performed on a Silicon Graphics 02 work station using QUANTA package of Molecular Simulations and CHARMm force field. All calculations were run in DMSO environment ($\varepsilon = 45$). The model of the cyclic analogue c-[Sar[1],Lys[3],Glu[5]] ANG II **19** (Fig. 5) was obtained based on the proposed model of Angiotensin II.[23] Thus, Angiotensin II served as a matrix where the cyclization between Lys[3] and Glu[5] was achieved. It was then minimized using conjugate gradient and adopted-basis Newton Raphson algorithms.

In this obtained low energy conformation, molecular dynamics was performed at 300 K using 1 ps time frame for heating, equilibration and simulation steps to further relax the structure. One hundred structures from the simulated ones were minimized using 300 iteration steps and SD algorithm. Only structures that fit all the major NOE distance constraints were plotted. Details of the above techniques have been previously described.[23,68–72]

Rat uterus bioassay

Agonist activity was determined by matching the response to the cyclic-analogue **19** with an equivalent response to Angiotensin II (human).[50,73,74] Uterine horns from diethylstilbestrol-primed virgin Sprague–Dawley rats (150–250 g) were defatted and cut in half. Each tissue was suspended in 1 g of tension in a 3 mL tissue bath containing 150 mM NaCl, 5.6 mM KCl, 0.18 mM $NaHCO_3$ and 1.4 mM glucose at pH 7.0 gassed with oxygen. Contractions were monitored with Gould Metripak 753341-4202 isotonic transducers coupled to Gould 13-4615-50 transducer amplifiers housed in a Gould 2600s recorder.

In vivo cardiovascular experiments

Four adult male New Zealand white rabbits (3–3.5 kg) were anesthetized with pentobarbitone (40 mg/kg, iv), incubated through a tracheostomy and mechanically ventilated with 100% oxygen. Subsequently, two polyethylene catheters were inserted, one into the jugular vein for infusion of drug solutions and the other into the carotid artery for continuous blood pressure monitoring (Nihon-Kohden Recorder, Model 6000, Japan). The drug solutions were made by diluting ANG II (Hypertensin, CIBA) or c-[Sar[1],Lys[3],Glu[5]] ANG II **17** in 5% Dextrose at final concentrations of 5 and 50 mg/mL, respectively. Both drugs were then infused via a syringe pump (Harvard Apparatus Pump 22, Harvard Apparatus Inc., Natick, MA, USA) in random sequence, allowing at least 30 min between the infusions for blood pressure stabilization.

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