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Synthesis and Study of a Cyclic Angiotensin II Antagonist Analogue Reveals the Role of π*–π* Interactions in the C-terminal Aromatic Residue for Agonist Activity and Its Structure Resemblance with AT_1 Non-peptide Antagonists

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Abstract—The novel amide linked Angiotensin II (ANG II) cyclic analogue cyclo(3, 5) -[Sar[1]-Lys[3]-Glu[5]-Ile[8]] ANG II (**18**) has been designed, synthesized and bioassayed in anesthetized rabbits. The constrained cyclic analogue with a lactam amide bridge linking a Lys-Glu pair at positions 3 and 5 and possessing Ile at position 8, was synthesized by solution procedure using the maximum protection strategy. This analogue was found to be inhibitor of Angiotensin II. NMR spectroscopy coupled with computational analysis showed clustering between the side chains of the key aminoacids Tyr[4]-His[6]-Ile[8] similar to that observed with ANG II. The obtained data show that only π*–π* interactions observed in ANG II or its superagonist Sar[1] [ANG II] are missing. Therefore, it can be concluded that these interactions are essential for agonist activity. Conformational analysis comparisons between AT_1 antagonists losartan, eprosartan and irbesartan with C-terminal segment of cyclic compound **18** revealed structural similarities.

Introduction

The octapeptide Angiotensin II (ANG II, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) is the main pressure component of the Renin-Angiotensin System (RAS).[1,2] Accumulated experimental evidence for ANG II supports a bioactive conformation characterized by a charge relay system between Tyr hydroxyl, His imidazole and Phe carboxylate, analogous to that found in serine proteases,[3] as well as a ring cluster of the triad key aminoacids Tyr[4]-His[6]-Phe[8] which appears to be responsible for activity. Thus, conformational analysis of ANG II in phospholipid membrane bilayers showed that ANG II adopts a preferred conformation.[4] Conformational analysis using modern 2D NMR techniques in receptor-simulating environments has shown proximity of the three key aminoacid sidechains and the formation of tyrosinate has been demonstrated by nanosecond time resolved tyrosinate fluorescence studies.[5–11] In addition, the proposed conformation for Sarmesin, an antagonist of Angiotensin II overlays the recently discovered non-peptide ANG II receptor antagonist losartan when molecular modeling techniques and superimposition studies are applied.[10] Furthermore, the ring cluster conformation was recently supported by the design and synthesis of a novel constrained ANG II cyclic analogue, [Sar[1]-Lys[3]-Glu[5]] ANG II, which possesses biological activity when tested in the rat uterus assay and in anesthetized rabbits. This potent cyclic analogue was designed to have as a major molecular feature the integrity of the ring cluster.[7]

Other structure–activity studies have illustrated the importance of the C-terminal aromatic residue

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phenylalanine (Phe) for agonist activity. Replacement of residue Phe at position 8 with an aliphatic one, as Ile, results in an antagonist [Sar[1]-Ile[8]] ANG II (Sarilesin).[12,13] Mutation studies have also shown that the aromaticity of Phe[8] position is important for receptor activation.[14]

In this work, the cyclic [Sar[1]-Lys[3]-Glu[5]-Ile[8]] ANG II with a Lys-Glu pair at positions 3 and 5 was synthesized (Fig. 1). The aim of this work was to investigate furthermore the role of a ring cluster receptor conformation in agonist activity and shed light to intriguing differences in activity and conformation upon replacement of aromatic residue Phe with aliphatic Ile. Replacement of Phe with Ile at position 8 produces an antagonist, and the cyclization between 3 and 5 positions does not affect the antagonist activity. Such a cyclic analogue is more suitable to study the cluster system between the key aminoacids Tyr[4]-His[6]-Phe[8] (or Ile[8]) in comparison with the linear ones which adopt more low energy conformers and prevent the establishment of definite conclusions. A cluster between Tyr[4]-His[6]-Ile[8] provides strong evidence that π*–π* interactions between Tyr[4]-Phe[8] must be a key stereoelectronic element for agonist activity.

NMR spectroscopy coupled with computational analysis was performed in order to establish the significance of π*–π* interactions. In addition, an existing cluster between Tyr[4]-His[6]-Ile[8] initiated a research activity which involves the conformational analysis of AT_1 antagonists in an attempt to study their stereoelectronic similarities with such a cluster. Conformational analysis of losartan, eprosartan and irbesartan (Fig. 1) is performed in order to examine the possibility of their structural similarity with C-terminal segment of cyclic antagonist.

Results and Discussion

Rationale for the synthesis of cyclic peptide analogues

The limited stability of peptides often severely restricts their medical and industrial application. Therefore, the engineering of stable proteins is of great technological and economic importance. If designed carefully without causing drastic changes in the conformation of active peptides, the rigid geometry of the cyclic peptides enhances the binding affinity towards a selected target molecule compared to their linear counterparts..[14–16] Furthermore, cyclic analogues are important intermediates in the design and synthesis of non-peptide mimetics with the potential to be used as drugs.[17,18]

So far, a limited number of conformationally restricted via cyclization Angiotensin II analogues have been

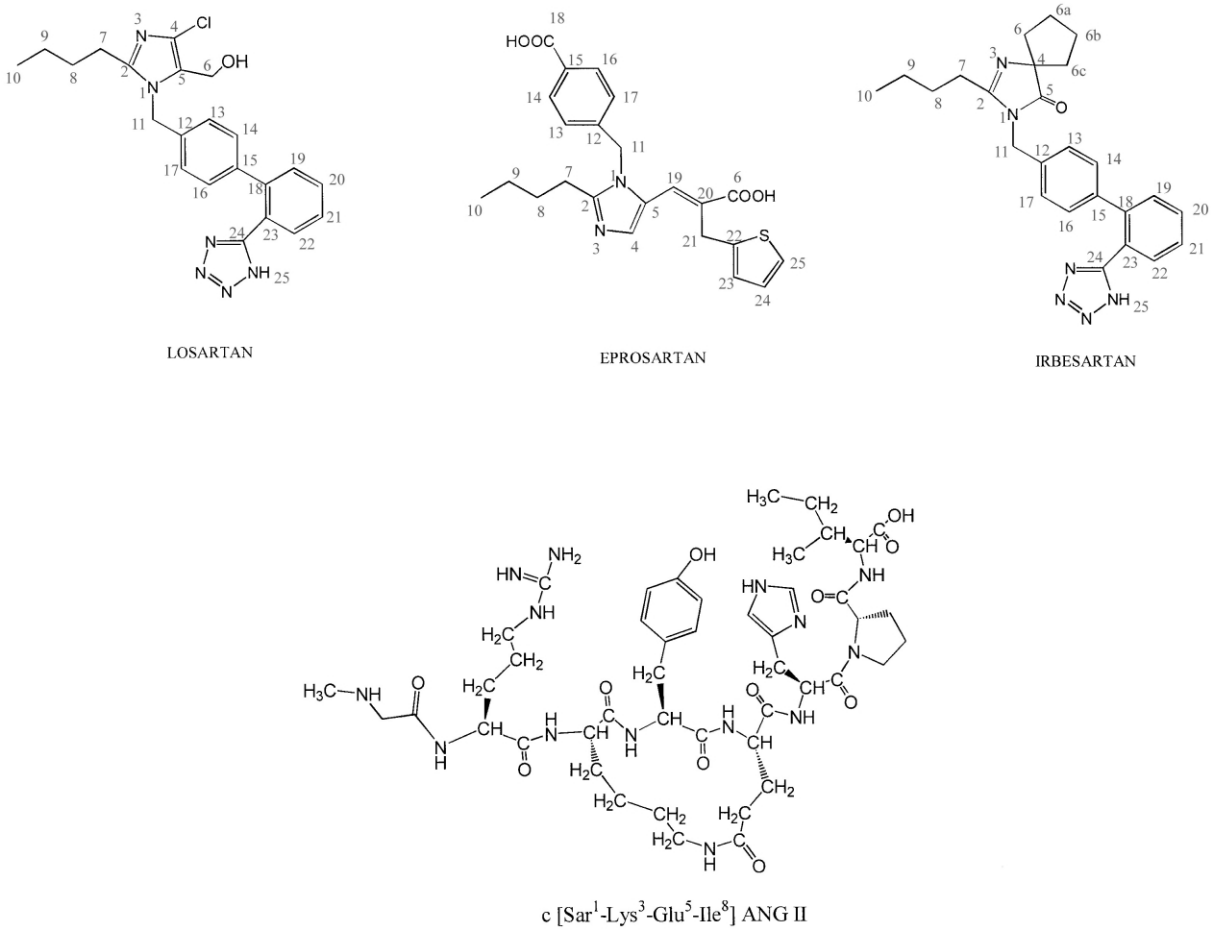


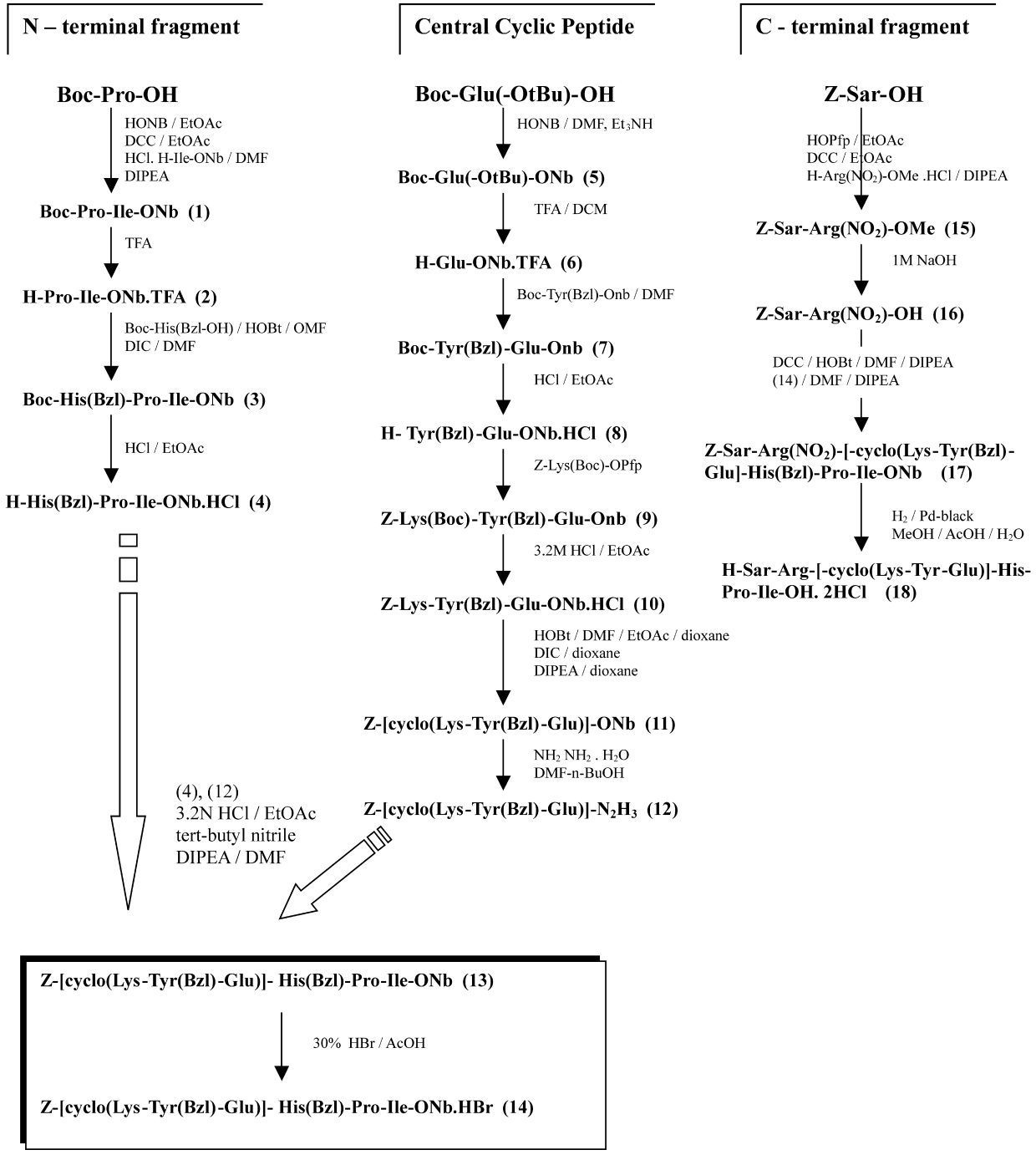
Figure 1. Chemical structures of cyclic compound **18** and AT_1 antagonists losartan, eprosartan and irbesartan.

reported by us and others. In these studies cyclization was achieved either by the disulfide method using cysteine moieties at various locations of the peptide molecule or by the amide-linkage method.[15–22]

Our interest in the conformational model of ANG 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 ANG II analogue c-[Sar^1-Lys^3-Glu^5-Ile^8] ANG II (Fig. 1). This allows to further explore the role of the aromatic residue at position 8 for agonist activity. Cyclization was achieved by forming an amide-linkage between the -NH_2 and -COOH side chain groups of Lys and Glu at positions 3 and 5, respectively, which are the least important for biological activity.

Strategy of the synthesis of c-[Sar^1-Lys^3-Glu^5-Ile^8] ANG II

The 3,5-cyclic analogue of ANG II with lactam amide bridges linking a Lys^3-Glu^5 pair was synthesized by the solution procedure in combination with the maximum protection strategy as shown in Scheme 1. This analogue was constructed from three major fragments: the linear N- and C-terminal fragments and the central



Scheme 1.

cyclic tripeptide part to produce the fully protected octapeptide. The final unprotected cyclic analogue, cyclo (3,5)-Sar[1]-Lys[3]-Glu[5]-Ile[8]-ANG II was obtained after catalytic hydrogenolysis.

The key step in the preparation of this analogue was the cyclization performed at the tripeptide stage by connecting the side chains of lysine and glutamic acid residues separated by tyrosine residue in the linear sequences Lys[3]-Tyr[4]-Glu[5] resulting in the formation of a cyclic amide (lactam) bond. The fully protected linear tripeptide **9** was synthesized stepwise using Boc-Glu(OtBu)-ONb (**5**) as starting material. The protected cyclic tripeptide Z-[-cyclo(Lys-Tyr(Bzl)-Glu)]-]-Onb (**11**) was prepared after deprotection of the side chain of the bridgehead residue **9** and by coupling with the linear precursor **10** via its *N*-hydroxybenzotriazole active ester in situ using DIC/HOBt/DIPEA. Cyclic dimer (~10%) formed as by-product during the cyclization, was removed by HPLC chromatography in the last stage of purification.

The N- and C-terminal fragments were synthesized step by step from their respective *C*-amino acid ester. *Z*-Sar-Arg(NO_2)-OH (**16**) was obtained by saponification from methyl ester **15**. The protected tripeptide Boc-His(Bz)-Pro-Ile-ONb (**3**) was synthesized with *p*-nitrobenzyl ester protection for the C-terminal and Boc group for the α-amino function. After Boc acidolytic cleavage by HCl/EtOAc, hydrochloride of C-terminal tripeptide **4** was used for preparation of the protected hexapeptide **13**. The hexapeptide **13** was prepared by the azide method: this method includes hydrazinolysis of the protected cyclotripeptide *p*-nitrobenzyl ester **11** leading to hydrazide **12** and treatment of **12** with *tert*-butyl nitrite, and condensation of the obtained azide with H-His(Bzl)-Pro-Ile-ONb.HCl (**4**) in the presence of DIPEA. Further selective cleavage of the *Z*-protecting group from **13** was achieved using 30% HBr/AcOH to afford **14**. This compound was then condensed with *Z*-Sar-Arg(NO_2)-OH (**16**) by activating the carboxyl function with DCC in the presence of HOBt resulting finally in the protected peptide **17**. Simultaneous catalytic hydrogenolysis of all protective groups from **17**, further purification by reversed-phase HPLC, conversion of the hygroscopic trifluoroacetate salts to the hydrochlorides and crystallization from *iso*-propanol provided the

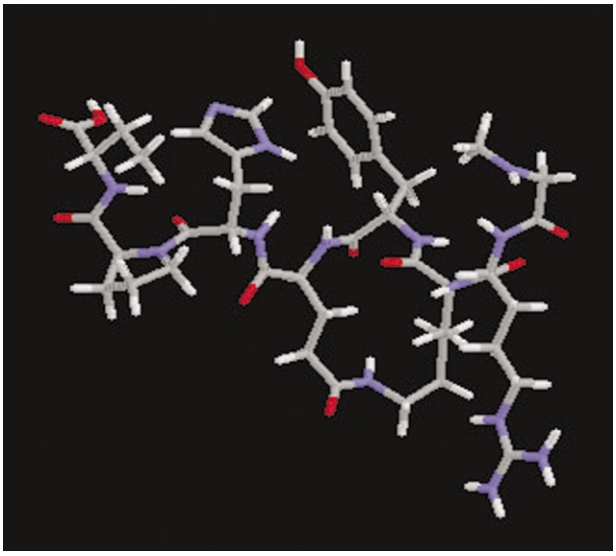


Figure 2. Low energy conformer of c(3,5)-[Sar[1]-Lys[3]-Glu[5]-Ile[8]] ANG II.

Table 1. Major NOEs that define conformers of antagonist c-[Sar[1]-Lys[3]-Glu[5]-Ile[8]] ANG II (2)[a]

Sample	NOEs	Intensity
Antagonist c-[Sar[1]-Lys[3]-Glu[5]-Ile[8]] ANG II	NH Arg(2)-αHHis(6)	m
	NH Ile(8)-αHArg(2)	s
	NH His(6)-3,5HTyr(4)	s
	4H His(6)-3,5HTyr(4)	s
	4H His(6)-δIle(8)	m

[a]s = strong and m = medium.

Table 2. Major NOEs that define conformers of AT_1 antagonists eprosartan, irbesartan and losartan

Sample	NOEs	Intensity
Eprosartan	H 11–H 28	m[a]
	H 19–H 24	s
	H 4–H 24	m
	H 19–H 11	s
Irbesartan	H 6a,6b,6c—H 13,17,14,16	s
	H 6a,6b,6c—H 10	m
	H 10–H 13,17,14,16	l
Losartan	H 7–H 11	m
	H 6–H 11	m
	H 7–H 13, 17	m

[a]s = strong, m = medium, l = light.

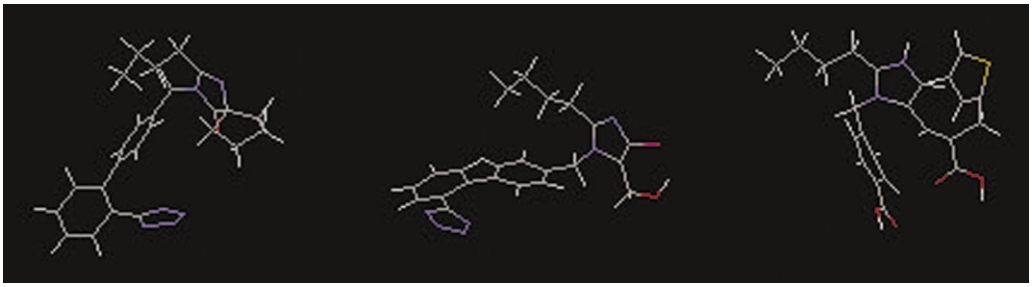


Figure 3. Low energy conformers of the three AT_1 antagonists losartan, eprosartan and irbesartan derived using a combination of NMR spectroscopy and computational analysis.

desired final-cyclo(lysyl-tyrosyl-glutamyl)-containing octapeptide (**18**).

NMR spectroscopy and computational chemistry

NMR spectroscopy and computational analysis was performed for the antagonist c-[Sar^1-Lys^3-Glu^5-Ile^8] ANG II. Figure 2 shows a low energy structure for c-[Sar^1-Lys^3-Glu^5-Ile^8] ANG II generated using a combination of NOE data and minimization procedures. The most important NOEs that define the conformation of the cyclic analogue **18** are shown in Table 1.

NOEs in this molecule show a spatial proximity between Sar^1-Tyr^4. It was found in most agonists and antagonists studied that as a general rule Sar^1 or Arg^2 protrude to the cluster system, due to energetically favor rotation through Cα of Arg^2. NOEs between Tyr^4-His^6 and His^6-Ile^8 were also observed. These results evident a cluster system between the key amino acids Tyr^4-His^6-Ile^8.

The important stereoelectronic characteristics of the generated model are the following. The cyclic antagonist 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. In cyclic antagonist peptide c-[Sar^1-Lys^3-Glu^5-Ile^8] ANG II with Ile at position 8, the π*–π* interactions are missing since this molecule contains the non aromatic aminoacid Ile^8 instead of the aromatic Phe^8. In recent studies the importance for such interactions is highlighted.[15]

To compare the C-terminal region of cyclic antagonist with AT_1 antagonists the conformational analysis of the well known antihypertensive AT_1 antagonists losartan, irbesartan and eprosartan were studied using a combination of computational analysis and NMR spectroscopy.[23–28] The major NOES observed for these three analogues and served as NOE constraints are shown in Table 2.

Low energy conformers for the three AT_1 antagonists under study consistent with NOE constraints are shown in Figure 3. Detailed conformational analysis of these molecules will be sought in a subsequent study.

Superimposition of the three AT_1 antagonists with the cyclic analogue were achieved in an attempt to reveal the similarities of the C-terminal segment with specific structural features of AT_1 antagonists.

Such superimpositions are very important because (a) mutation studies point out that C-terminal region of ANG II and ANG II peptide antagonists act on the same region of AT_1 receptor located in the transmembrane region.[29–35] In particular, the N-terminal region of peptides seem to dock on the extracellular part of AT_1 receptor while the C-terminal part as the AT_1 nonpeptide antagonists are located in the transmembrane part of the receptor and especially between TMIII-TMVII helices; (b) Potent AT_1 antagonists such as losartan were designed based on the C-terminal part of AII;[36–42] (c) Superimposition studies may explain the different kind of activities between antagonists especially because their receptor active site is not yet established due to not conclusive results obtained by the mutation studies; (d) Superimposition studies may discover the structural requirements for agonist and antagonist activity. These requirements can be tested with the synthesis of novel synthetic compounds.

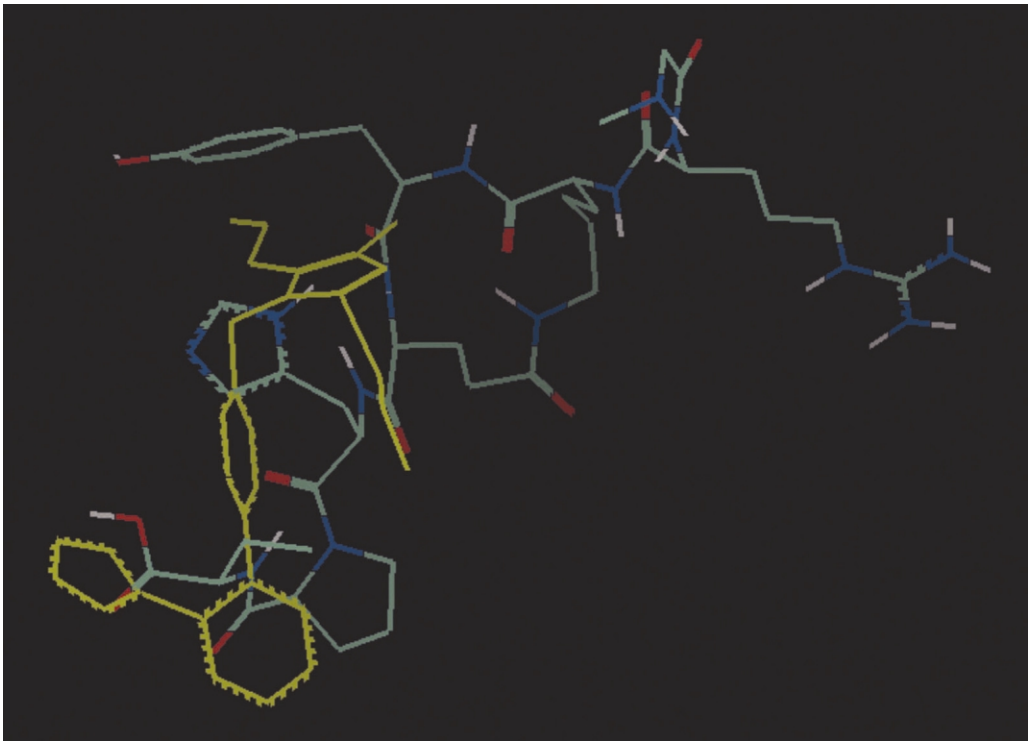


Figure 4. Superimposition of losartan with cyclic analogue **18**.

Superimposition of losartan with c-[Sar^1-Lys^3-Glu^5-Ile^8] ANG II

From the different possible overlays, the best superimposition between the two molecules was achieved when the following groups were matched: (i) losartan's hydroxymethylimidazole with c-[Sar^1-Lys^3-Glu^5-Ile^8] ANG II His^6; (ii) losartan's tetrazole with c-[Sar^1-Lys^3-Glu^5-Ile^8] ANG II isosteric carboxylate of Phe^8; (iii) losartan's spacer phenyl ring with c-[Sar^1-Lys^3-Glu^5-Ile^8] ANG II pyrrolidine group of Pro^7. Interestingly, losartan mimics the γ-turn formed around Pro^7 in c-[Sar^1-Lys^3-Glu^5-Ile^8] ANG II (iv) losartan's hydroxymethyl group with phenolic hydroxyl group of Tyr^4 (Fig. 4).

Superimposition of eprosartan with c-[Sar^1-Lys^3-Glu^5-Ile^8] ANG II

From the possible overlays, the best superimposition between the two molecules was achieved when the following groups were matched: (i) eprosartan's carbox-

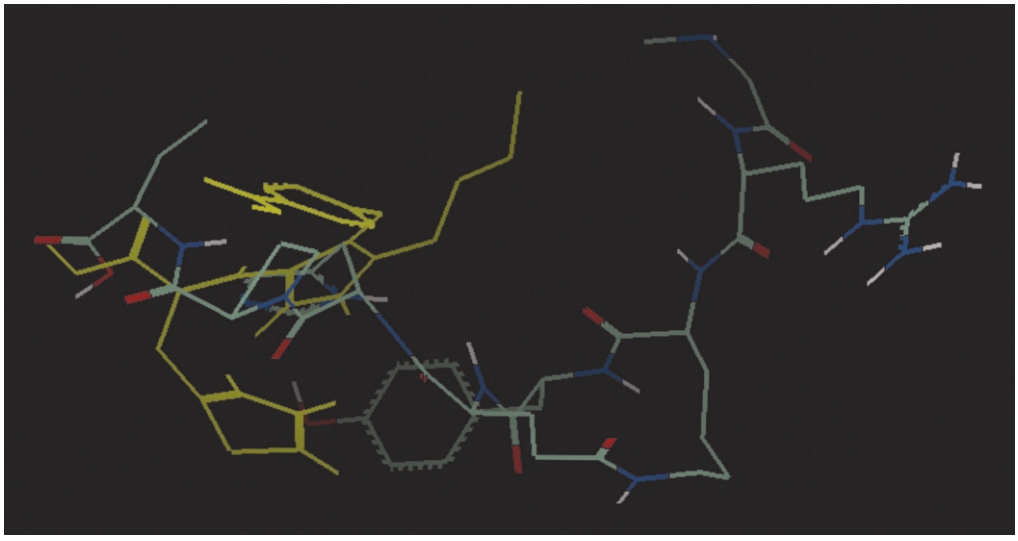


Figure 5. Superimposition of eprosartan with cyclic analogue **18**.

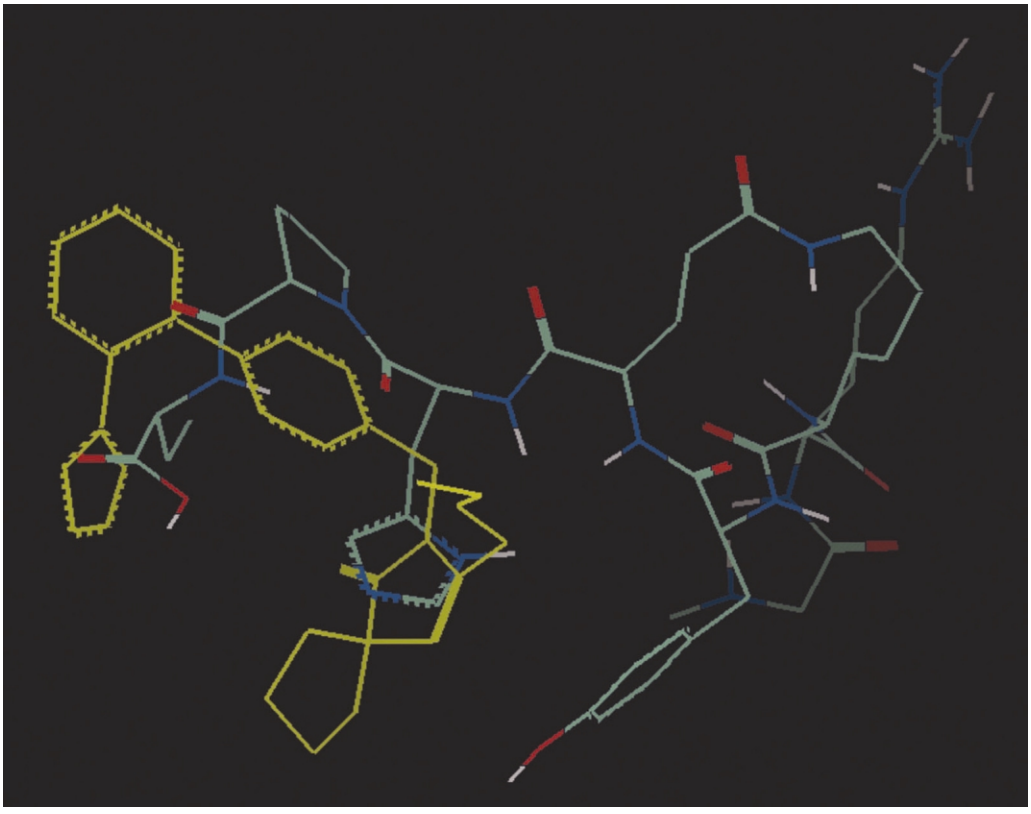


Figure 6. Superimposition of irbesartan with cyclic analogue **18**.

ylate with c-[Sar^1-Lys^3-Glu^5-Ile^8] ANG II carboxylate of Phe^8; (ii) eprosartan's spacer phenyl ring with c-[Sar^1-Lys^3-Glu^5-Ile^8] ANG II pyrrolidine group of Pro^7. Interestingly, eprosartan as losartan mimics the γ-turn formed around Pro^7 in c-[Sar^1-Lys^3-Glu^5-Ile^8] ANG II; (iii) eprosartan's imidazole with c-[Sar^1-Lys^3-Glu^5-Ile^8] ANG II His^6 (Fig. 5).

Superimposition of irbesartan with c-[Sar^1-Lys^3-Glu^5-Ile^8] ANG II

Similarly, from the possible overlays, the best superimposition between the two molecules was achieved when the following groups were matched: (a) irbesartan's tetrazole with c-[Sar^1-Lys^3-Glu^5-Ile^8] ANG II isosteric carboxylate of Phe^8; (b) irbesartan's spacer phenyl ring with c-[Sar^1-Lys^3-Glu^5-Ile^8] ANG II pyrrolidine group of Pro^7. Interestingly, irbesartan like losartan and eprosartan mimics the γ-turn formed around Pro^7 in c-[Sar^1-Lys^3-Glu^5-Ile^8] ANG II; (c) irbesartan's substituent 1,3-diazaspiro[4,4]non-1-3en-4-one with c-[Sar^1-Lys^3-Glu^5-Ile^8] ANG II His^6 (Fig. 6).

The similarities in the superimposition of the three AT_1 antagonists suggest a similar way of interacting with AT_1 receptor. Superimposition studies between losartan with sarmesin[10] or c-[Sar^1-Lys^3-Glu^5-Ile^8] ANG II His^6 reveal identical manner of superimposition. Losartan-sarmesin superimposition has additional matching between the butyl chain of losartan with sarmesin's Ile^5 alkyl chain. Due to the lack of Ile^5 aminoacid in c-[Sar^1-Lys^3-Glu^5-Ile^8] ANG II such a lipophilic matching is missing. This result points out that lipophilic segments of AT_1 non-peptide mimetics may not be equivalent with that of alkyl group of Ile^5.

Assess of biological activity of [Sar^1-Lys^3-Glu^5-Ile^8] ANG II

As opposed to the c-[Sar^1-Lys^3-Glu^5-Phe^8] ANG-II, which was found capable to induce a dose-related hypertensive response after infusion into anesthetized rabbits[43] the cyclic analogue [Sar^1, Lys^3, Glu^5, Ile^8] ANG-II, where an isoleucine was substituted for phenylalanine at position 8 did not preserve any hypertensive action. In contrast, [Sar^1-Lys^3-Glu^5-Ile^8] ANG-II was transformed to a potent ANG- II antagonist and reduced ANG II-dependent hypertension in a dose-related manner (Fig. 7).

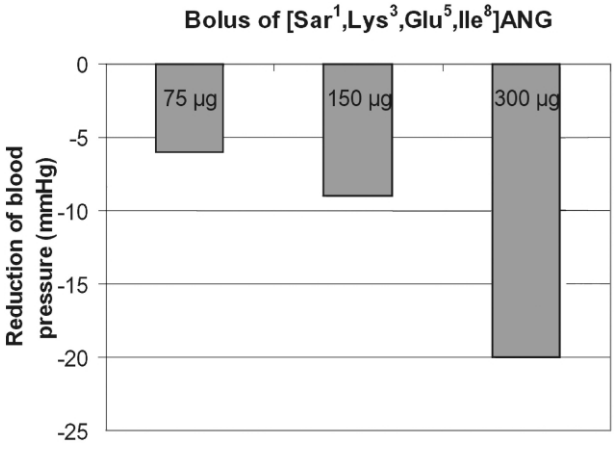


Figure 7. [Sar^1-Lys^3-Glu^5-Ile^8] ANG II causes dose-related reduction of ANG II induced hypertension in anesthetized rabbits.

Experimental

Chemistry

The identity of the cyclic product **18** was established by FAB/MS and NMR spectroscorpy. FAB spectra were run on an AEI M29 mass spectrometer modified as described elsewhere.[44] 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 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 **18** 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 **18** were checked for purity on a Waters HPLC system equipped with a 600E system controller. Fractions were analyzed by analytical reversed-phase column (Techsil C_{18}, 250×4.6 mm).

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% aqueous TFA at a flow rate of 3 mL/min. In particular, the mobile phases used were 0.1% aqueous 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 Bruker 300 MHz and Varian 600 MHz NMR spectrometers. c-[Sar^1,Lys^3,Glu^5] Ile^8 ANG II **18**, [Sar^1] ANG II and the AT_1 antagonists losartan, eprosartan and irbesartan were studied by dissolving 5 mg of the compounds in 0.5 mL of DMSO. The chemical shifts were reported relative to the non-deuterated fraction of the methyl group of DMSO-d_6 at 2.50 ppm with respect to TMS. One-dimensional spectra, and two-dimensional spectra

COSY/TOCSY and NOESY experiments aided in the assignments of the cyclic peptide, and the AT_1 antagonists as well as their conformational analysis. Standard parameters were used for the experiments provided in the software and installed with the instrument.

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 lower energy conformer of the cyclic analogue c-[Sar^1-Lys^3-Glu^5-Ile^8] ANG II was obtained based on the proposed model of ANG II. Thus, ANG 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 under NOE constraints. The conformational analysis of losartan is described elsewhere.[10] Irbesartan and eprosartan were manipulated in an identical way. Thus, they were built and minimized under NOE constraints. Details of the above techniques have been previously described.[45]

In vivo cardiovascular experiments

Four adult normotensive male New Zealand White rabbits weighing between 2.5 and 3.3 kg were used in the study. All animals were anesthetized by pentobarbitone (30 mg/kg), intubated and mechanically ventilated with 100% oxygen using a respirator for small animals (MD Industries, Mobile, AL, USA). The tidal volume was 15 mL and the rate was adjusted to keep blood gases within normal range. Two polyethylene catheters were inserted, one in the carotid artery for continuous blood pressure monitoring via a transducer attached to a multichannel recorder (Nihon-Kohden, Model 6000, Japan) and the other one in the jugular vein for angiotensin II administration.

Based on previous testing angiotensin II-dependent hypertension was induced by infusing angiotensin via a syringe pump (Harvard Apparatus Pump 22, Harvard Apparatus, Natick, MA, USA) at a constant rate of 0.6 mL/min (3 μg/min). Angiotensin solution was prepared by diluting angiotensin II (Hypertensin, CIBA) in 5% dextrose at a final concentration of 5 μg/mL. Five min after the establishment of hypertension, boluses of 75, 150 or 300 μg of the novel cyclic angiotensin II antagonist [Sar^1, Lys^3, Glu^5, Ile^8] ANG-II were given via an ear vein in random sequence and the changes of blood pressure were recorded.

Acknowledgements

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