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Design, Synthesis and Biological Evaluation of Cyclic Angiotensin II Analogues with 3,5 Side-Chain Bridges: Role of C-Terminal Aromatic Residue and Ring Cluster for Activity and Implications in the Drug Design of AT1 Non-peptide Antagonists

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Abstract—The novel amide linked Angiotensin II (ANG II) cyclic analogues: γ,ε-cyclo(3, 5)-[Sar[1]-Glu[3]-Lys[5]-Ile[8]] ANG II (I) and γ, ε-cyclo(3, 5)-[Sar[1]-Glu[3]-Lys[5]-Phe[8]] ANG II (II) have been designed, synthesized and bioassayed in anesthetized rabbits in order to unravel structural ring cluster characteristics important for receptor activation. Analogue I with Ile at position 8 was an inhibitor of Angiotensin II while analogue II with Phe at position 8 was found to be an agonist. Similar results were reported for cyclic compounds that have reversed the linking between positions 3 and 5. The overall results show that positions 3 and 5 do not govern the biological activity of the synthetic analogues. It also appears that the aromatic ring cluster (Tyr-His-Phe) in agonist peptides is an essential stereo-electronic feature for Angiotensin II to exert its biological activity. A non-peptide mimetic of ANG II, 1-[2′-[(*N*-benzyl)tetrazol-5-yl]biphenyl-4-yl]methyl]-2-hydroxymethylbenzimidazole (BZI8) has been designed and synthesized. This molecule is more rigid and much less active than AT_1 non-peptide mimetic losartan probably because it lacks to mimic the orientation of tetrazole and the pharmacophore segments of butyl chain and imidazole ring. © 2002 Published by Elsevier Science Ltd.

Introduction

The octapeptide Angiotensin II (ANG II, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) is the main pressor component of the Renin-Angiotensin System (RAS).[1] Accumulated experimental evidence for Angiotensin 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 agonist activity.[4,5] Thus, conformational analysis using modern 2D NMR techniques in receptor-simulating environments has shown proximity of the three key aminoacids sidechains and the formation of tyrosinate has been demonstrated by nanosecond time resolved fluorescence studies.[6,7] Comparative nuclear magnetic resonance studies of the backbone structure between peptide agonists and antagonists have shown that only agonists display ring clustering and form a charge relay

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system. In addition, the proposed conformation of peptide antagonists overlay the recently discovered non-peptide ANG II receptor antagonist losartan and analogues when molecular modeling techniques and superimposition studies are applied.[8] 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 anesthesized rabbits.[5] This potent cyclic analogue was designed to have as a major molecular feature the integrity of the ring cluster. Other structure–activity studies have illustrated the importance of the C-terminal aromatic residue 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).[1,2]

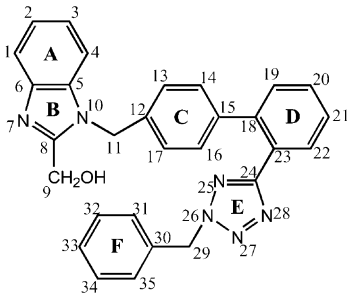
In this work, two cyclic ANG II analogues γ,ε-cyclo(3,5)-[Sar^1-Glu^3-Lys^5-Ile^8] ANG II (I) and γ,ε-cyclo(3,5) -[Sar^1-Glu^3-Lys^5-Phe^8] ANG II (II) have been synthesized with Glu, Lys residues at positions 3 and 5 and with Ile or Phe respectively at position 8 (Fig. 1). The aim of this work was to investigate furthermore the role of the 3,5-positions and the ring cluster receptor conformation in agonist activity and shed light to stereoelectronic differences in activity and conformation upon replacement of aromatic residue Phe with aliphatic Ile. While replacement of Phe with Ile at position 8 produces an antagonist, the reverse of the Lys^3-Glu^5 of the earlier reported cyclic peptides to Glu^3-Lys^5 has a minimal effect in the agonist or antagonist activity of cyclic ANG II analogues. A 15-membered ring in the central core X-Ôyr-Y allows a ring clustering and activity regardless of the Glu-Tyr-Lys or Lys-Tyr-Glu sequence in the agonist cyclic peptide. Based on these structure activity relationships which demand the presence of Phe, Tyr and His residues in ANG II as well as in linear and constrained ANG II analogues to possess biological activity, it can be inferred that the ability to form a ring cluster and consequently a charge relay system may be the key stereoelectronic molecular features of ANG II for exerting biological activity.

Based on this information and modeling comparisons between ring cluster and pharmacophoric groups of AT_1 antagonists we have synthesized an AT_1 non-peptide antagonist 1-[2′-[(*N*-benzyl)tetrazol-5-yl]biphenyl-4-yl]methyl]-2-hydroxymethylbenzimidazole. Detailed synthetic procedure for this analogue will be reported elsewhere. This molecule was found to be much less active inhibitor of ANG II than losartan in anesthetized rabbits. For this reason we sought to compare its superimposition ability with losartan (Fig. 1).

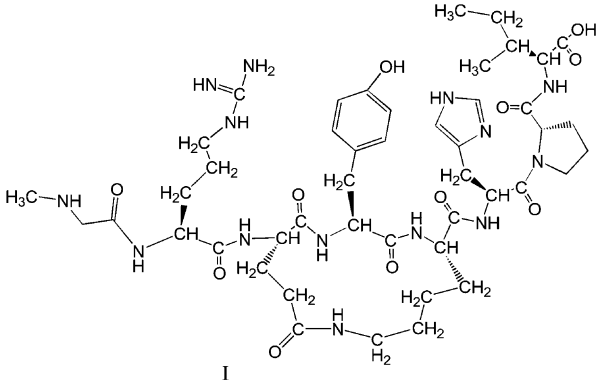
Rationale for the Synthesis of Cyclic Peptide Analogues

The limited stability of peptides often severely restricts their medical and industrial application. Therefore, the engineering of stable proteins is of great technological and economical 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. Furthermore, cyclic analogues are important intermediates in the design and synthesis of non-peptide mimetics with the potential to be used as drugs.[9–11] With this aim, our group has been involved for several years in the design and synthesis of cyclic analogues of important peptides such as, Angiotensin II, Thrombin Receptor Peptides and Myelin Basic Protein[12–15] involved respectively in hypertension, cancer and multiple sclerosis.

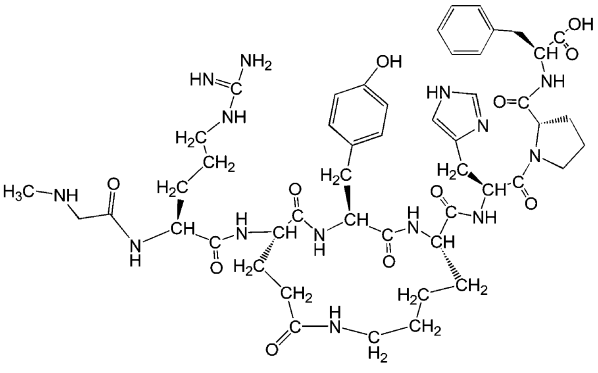
So far, a limited number of conformationally restricted Angiotensin II analogues via cyclization have been reported. In these studies cyclization was achieved either by the disulfide method using cysteine moieties at various locations of the peptide molecule or by the amide-linkage method.[16–27]



BZI 8

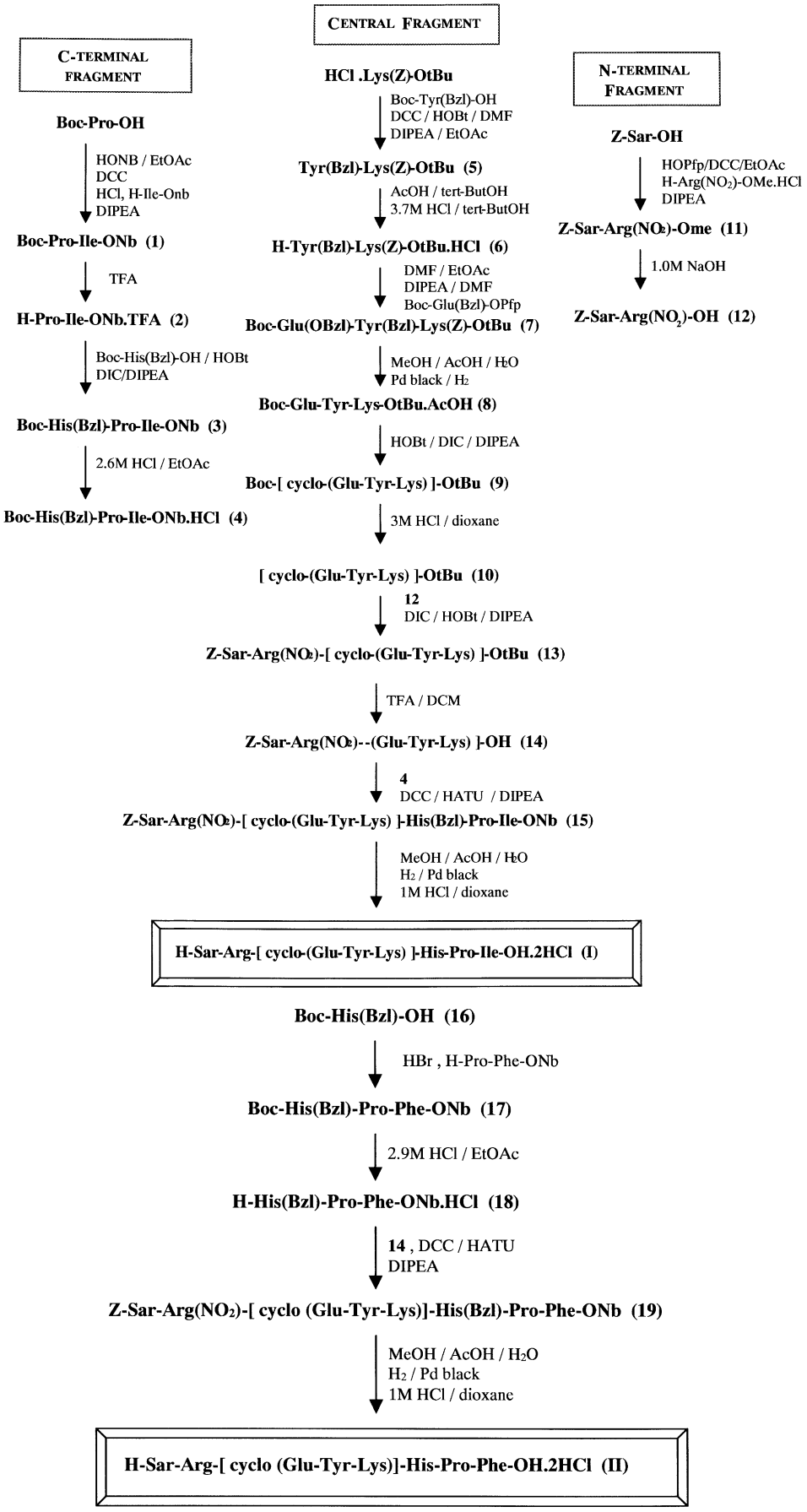


I



II

Figure 1. Chemical structures of: BZI8 (top), Sar^1-Arg^2-[-cyclo(Glu^3-Tyr^4-Lys^5)]-His^6-Pro^7-Ile^8 (middle), and Sar^1-Arg^2-[-cyclo(Glu^3-Tyr^4-Lys^5)]-His^6-Pro^7-Phe^8 (bottom).



Scheme 1. Synthetic routes for the peptides **I** and **II**.

Our interest in 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 two novel cyclic amide linked Angiotensin II analogues, cyclo(3,5)-[Sar^1-Glu^3-Lys^5-Ile^8] ANG II (I) and cyclo(3,5)-[Sar^1-Glu^3-Lys^5-Phe^8] ANG II (II) (Fig. 1) that differ only in residue at position 8. Analogues I and II as it was stated above differ from cyclo(3,5)-[Sar^1-Lys^3-Glu^5-Phe^8] ANG II and cyclo(3,5)-[Sar^1-Lys^3-Glu^5-Ile^8] ANG II recently reported[5] only in the positioning of the Lys-Glu bridging residues.

Strategy of the synthesis of two cyclic analogues. Cyclization was achieved by forming an amide-linkage between the $-NH_2$ and –COOH side chain groups of Glu and Lys residues at positions 3 and 5, respectively, which are the least important for activity.

Scheme 1 shows the synthesis of the new cyclic analogues I and II.

Biological data. 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. The tidal volume used 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 and the other one in the jugular vein for the administrations of solutions made by diluting ANG II and its cyclic analogues γ,ε-cyclo(3,5) [Sar^1-Glu^3-Lys^5-Phe^8] ANG II and γ,ε-cyclo(3,5) [Sar^1-Glu^3-Lys^5-Ile^8] ANG II in 5% dextrose at final concentration of 5 and 50 μg/mL, respectively. Each solution and dose was given in random sequence after a wash-out period of 30 min.

The hypertensive responses to angiotensin II infusion at 1, 2 or 3 μg/min and to γ,ε-cyclo(3,5) ANG II analogues at 10, 20 or 30 μg/min were recorded continuously. Angiotensin II-dependent hypertension was then induced and maintained by a constant infusion of angiotensin II via a syringe pump at a rate of 0.2 mL/min (1 μg/min). Five min after the establishment of hypertension, boluses of different doses of losartan (0.05, 0.1 and 0.2 mg), BZ18 (1 and 2 mg) and the γ,ε-cyclo(3,5) ANG II analogues (75, 150 and 300 mg) were given via an ear vein in random sequence and the drop in mean blood pressure was recorded.

The results presented in Figure 2 represent the mean of three experiments. The cyclic ANGII analogue [Sar^1, Glu^3, Lys^5] ANG-II was capable to induce a dose-related hypertensive response, which was of less magnitude compared with that elicited by an isovolemic and equimolar solution of angiotensin II (ANG II). In contrast, the cyclic analogue [Sar^1, Glu^3, Lys^5, Ile^8] ANG II, in which an isoleucin was substituted for phenylalanine at position 8, did not preserve any hypertensive action and in fact, it was transformed in a potent angiotensin II antagonist reducing the angiotensin II-dependent hypertension in a dose-related manner.

As expected, losartan produced a significant dose-dependent hypotensive response, when it was given as

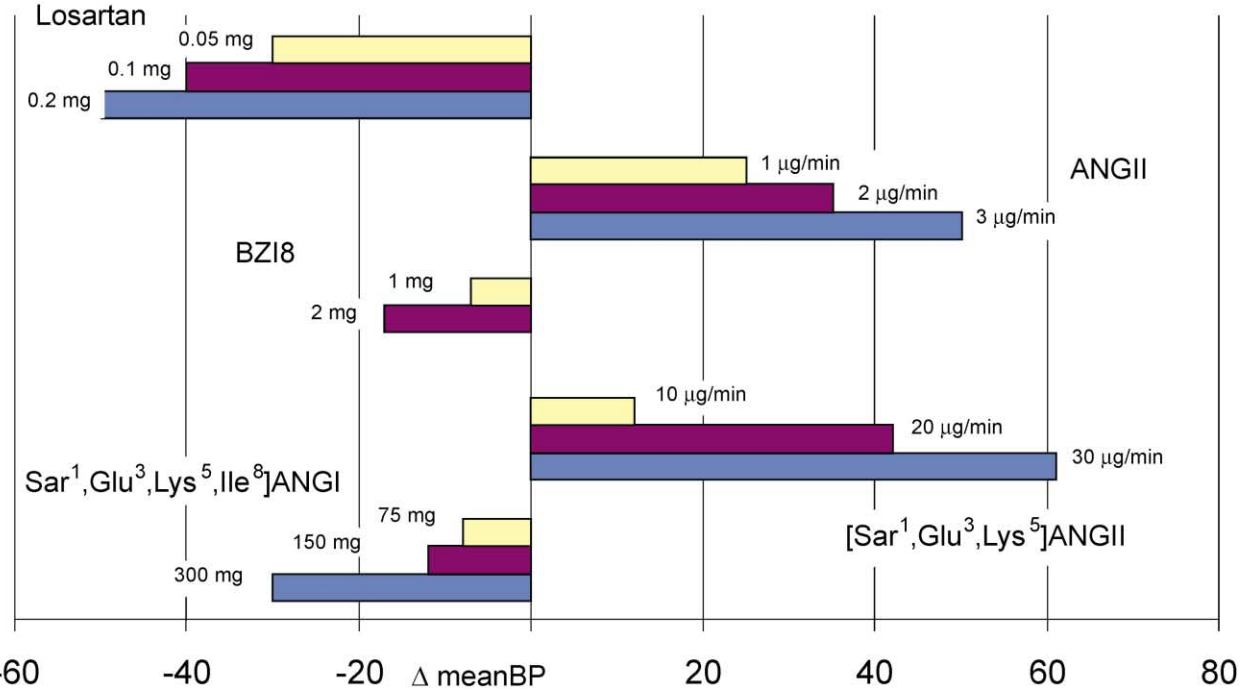


Figure 2. Dose-dependent hypertensive responses were observed when solutions of ANG II or its cyclic analogue [Sar^1, Glu^3, Lys^5] ANG II were infused in anesthetized rabbits. In contrast, dose-related reduction of the mean blood pressure was seen when boluses of [Sar^1, Glu^3, Lys^5, Ile^8] ANG II, losartan or BZ18 were given in anesthetized rabbits with angiotensin-II-induced hypertension.

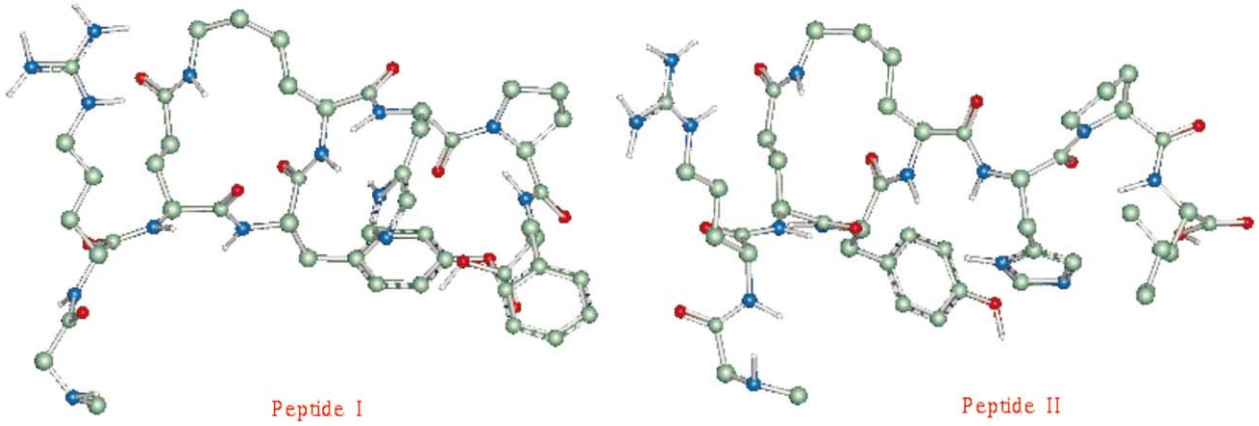


Figure 3. Low energy conformers of cyclic agonist I and antagonist II peptides derived using conformational search methods.

a bolus in our model of ANG II-dependent hypertension in anesthetized rabbits. A similar but less potent hypotensive response was observed when boluses of the novel non-peptide ANG II receptor antagonist BZI8 were given in the same experimental model.

Conformational analysis of cyclic analogues I and II. The new cyclic analogues were considered as mutated structures of previously reported synthetic cyclic analogues[5] differing only in the order of aminoacids 3 and 5. Figure 3 shows low energy structures for c-[Sar^1-Lys^3-Glu^5] ANG II and c-[Sar^1-Lys^3-Glu^5-Ile^8] ANG II generated using a combination of minimization algorithms and molecular dynamics. The important stereoelectronic characteristics of the generated models are the following: the cyclic analogue c-[Sar^1-Lys^3-Glu^5-Phe^8] ANG II 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 ring cluster stereoelectronic feature is missing.

The cyclic agonist and cyclic antagonist peptides adopt the same conformation at the central part of the molecule in which the three residue (Lys-Tyr-Glu) bridge is the same for both agonist and antagonist peptides. Therefore the difference in activity originates exclusively from the C-terminal segment. The difference in the magnitude of the biological activity between the cyclic agonist analogue and the parent ANG II peptide hormone is probably due to the different conformations 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. Cyclization of Angiotensin II restricts the number of possible conformations, thereby presumably preventing the cyclic peptide from assuming optimal conformation when it binds to the receptor.

Structure elucidation and conformational analysis of BZI8. BZI8 was structurally elucidated using 2D DQF COSY, ROESY and literature reported data on losartan and eprosartan.[28] ROESY and NOESY experiments gave valuable information on the spatial proximity between protons of the flexible part of the molecule. The most critical observed NOE was between H4 and H29. This suggests a clustering between the phenyl rings A and F. The two rings that constitute the biphenyl system are not linear to each other as it is also observed with Losartan and Eprosartan.[8,28] Absence of NOE between –OH and H11 suggests that –OH group is oriented away from the two protons of H-11. Based on this information distance constraints were used along with dynamics experiments and minimization procedures to generate low energy conformers that are consistent with NMR data. A representative low energy conformer for BZI8 is shown in Figure 4. Computational details about the methods used for the conformational analysis are reported in our previous publication.[8] Superimposition

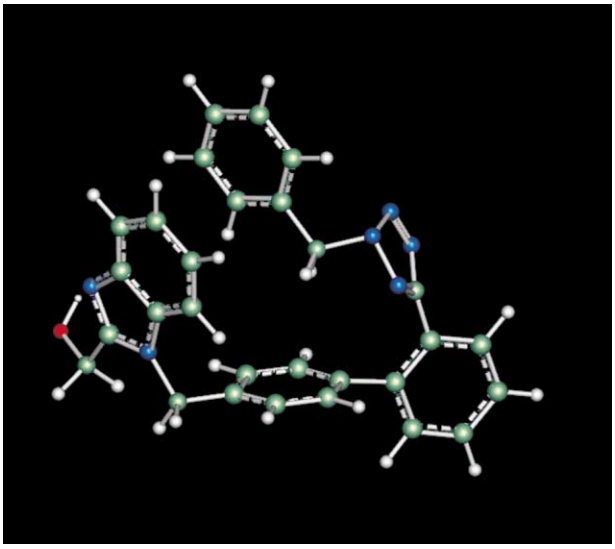


Figure 4. A representative low energy conformer of BZI8 derived using a combination of 2D NMR spectroscopy and computational analysis.

between losartan and BZI8 is shown in Figure 5. As it can be seen the two tetrazole rings are oriented in the opposite site. The biphenyl system of both antagonists occupy the same space as well as the imidazole rings. The butyl chain of losartan has not a satisfactory counterpart even the phenyl ring attached to the tetrazole is located towards it. Superimposition between II and BZI8 is shown in Figure 6. As it can be observed benzyl tetrazole is in a close vicinity with C-terminal carboxylate of Ile[8] and its alkyl chain. However, imidazole of His[6] and phenolic hydroxyl group of Tyr[4] are not in a spatial vicinity with 2-hydroxymethylbenzimidazole.

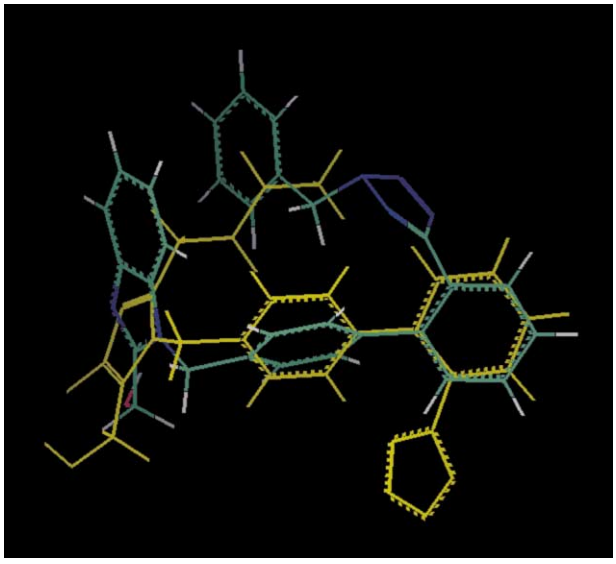


Figure 5. Superimposition of low energy conformer of BZI8 with Losartan.

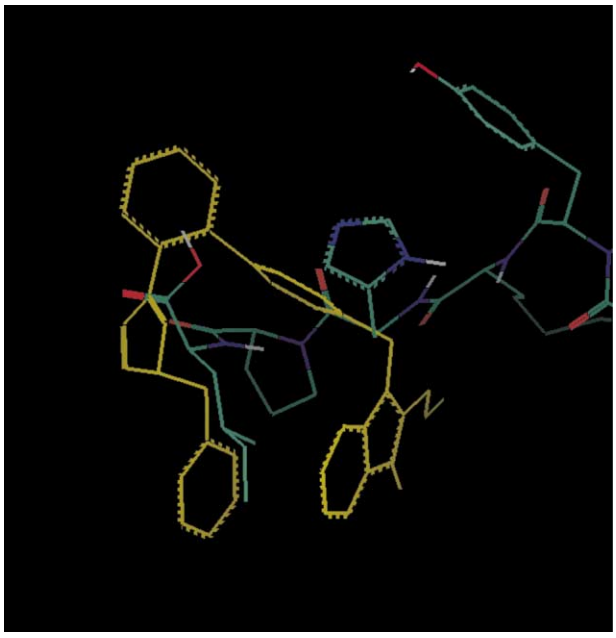


Figure 6. Superimposition of low energy conformers of BZI8 with peptide antagonist I.

Conclusion

This research was aiming at establishing 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 fluorescence life time studies. A strong Tyr[4]-Ile[5]-His[6] bend exists in cyclic agonist and antagonist cyclic analogues which both bear the same central Glu-Tyr-Lys or (Lys-Tyr-Glu as it is reported in earlier study) ring moiety, indicating that the difference in activity is resulting mainly from the nature of the C-terminal residue. An aromatic residue such as Phe allowing the formation of a ring cluster is necessary for exertion of agonist activity in ANG II. The constrained cyclic amide linked ANG II analogue c-[Sar[1]-Glu[3]-Lys[5]-Phe[8]] and earlier reported c-[Sar[1]-Lys[3]-Glu[5]] ANG II were designed to keep intact the clustering and backbone bend characteristics of the peptide hormone ANG II. On the contrary, the cyclic antagonists [Sar[1]-Glu[3]-Lys[5]-Ile[8]] ANG II and [Sar[1]-Lys[3]-Glu[5]-Ile[8]] ANG II without an aromatic residue at position 8, lack the ring cluster conformation. The molecules were designed with the hypothesis that residues 3 and 5 do not govern the biological 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. The lower potency of both agonist and antagonist cyclic analogues compared to ANG II and Sarilesin points out that the achieved conformation by cyclic analogues is not the optimal in inducing or suppressing ANG II caused hypertension. This supports the hypothesis and SAR studies that positions 3 and 5 are not responsible for agonist or antagonist activity even though they contribute to the optimum activity. In the potent constrained agonist analogues the three rings are indeed closely spaced at the same side of the cyclic ring as it has been shown by NOE interactions and molecular modeling. Activity is similar regardless of Lys-Tyr-Glu or Glu-Tyr-Lys order in cyclic agonist or antagonist peptides.

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 the closely spaced residues 4, 6 and 8 to form a possible relay system, which is not possible in Sarilesin and cyclic ANG II antagonists, lacking a C-terminal aromatic residue.

Superimposition studies between the weak antagonist BZI8 and losartan showed not a good mimicking between their pharmacophore segments. For example, their tetrazole moieties were located in the opposite site. However, their biphenyl rings showed a good matching. The lipophilic butyl chain of losartan was mimicked, although not perfectly, by two rings. These structural similarities and differences may give a plausible explanation of the weak antagonist activity of BZI8. It is of special interest that while the orientation of tetrazole in

the two molecules is in the opposite site the hydrophobic parts are located in spatial proximity.

Acknowledgements

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