Multiple Binding Modes for the Receptor-Bound Conformations of Cyclic AII Agonists†,‡

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Angiotensin II, Asp-Arg-Val-Tyr-His-Pro-Phe, binds its receptor with a postulated turn centered at residue four. Analogs of angiotensin II which contain a disulfide bridge between the side chains of residues 3 and 5 retain significant activity consistent with this hypothesis. Incorporation of 4-mercaptoproline residues, a hybrid, or chimeric amino acid which combines the properties of proline and homocysteine, into either of these positions with analogous disulfide bridges allows retention of high affinity for the receptor. These more highly constrained bicyclic systems give new insight into the details of molecular recognition of residues 3–5 of angiotensin by the receptor. Retention of activity by the antiparallel dimer of [Sar^1,Cys^{3,5}]-AII in which the peptide backbone is held in an extended conformation was unexpected. Analysis of the conformational constraints imposed in these active analogs suggests that AII agonists bind to their receptor with different backbone conformations in the region of the central tyrosine residue.

Introduction

The receptor-bound conformation of angiotensin II (AII, Asp^1-Arg^2-Val^3-Tyr^4-Val^5-His^6-Pro^7-Phe^8) has been suggested[1-3] to possess a turn centered on residues 3–5 based on the activity of [MeF^4]-AII, [Pro^3]-AII, and [Pro^5]-AII. Previous models based on physical studies of AII in solution have suggested,[2] among others, an inverse γ-turn[4] centered on residue 4. Cyclization by side chain disulfide formation between Cys or Hcy residues incorporated at positions 3 and 5 in AII led to potent agonists and antagonists,[5,6] further supporting some sort of a central turn for the active conformation. Conformational studies by Kataoka et al.[7] have shown that many backbone conformations are still compatible with position 1–3 side chain cyclization of Cys or Hcy residues. In order to further define the receptor-bound conformation, a series of cyclic analogs of AII have been prepared based on mercaptoproline analogs (4-*trans*, MPt, and 4-*cis*, MPc, Figure 1) in positions 3 and 5 in order to constrain the peptide backbone even further.

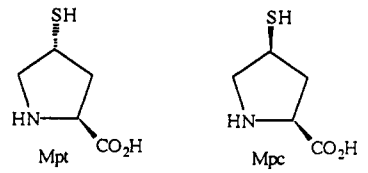

Figure 1. Structures of Mpt and Mpc.

Several other analogs in which cyclization between residues 3 and 5 utilized an amide bond instead of the disulfide were also prepared. Other disulfide cycles between residues 3 and 8, 5 and 8, and 3 and 7 using pairs of cysteine residues were also tested as controls.

Results and Discussion

Synthesis of Cyclic AII Monomers and Dimers. Procedures for the synthesis of the cyclic monomer c[Sar^1,Cys^{3,5}]-AII have been described by Spear *et al.*[5] We found that oxidation with potassium ferricyanide, even under relatively dilute conditions, leads mainly to the mixture of cyclic, antiparallel and parallel dimers and higher oligomers as determined by HPLC and characterization of individual peaks. The ratio of the two dimers differs depending on the pH value (pH = 7–8.5) of the reaction mixture; the antiparallel dimer was always formed with much higher yield, but the ratio of antiparallel to parallel dimer was decreased with lower pH. As a rule, the peptides were carefully oxidized after HF cleavage with a large excess of I_2 in HOAc/H_2O/MeOH to obtain monomers, or by more standard oxidation conditions to obtain the cyclic antiparallel dimer, 3–5′,5–3′,bis[Sar^1,Cys^{3,5}]-AII, and traces of the parallel dimer. The optimum yield was obtained at pH 8.5 and these procedures are described in the Experimental Section. These results demonstrate the differences in stability of the two dimers (see Zhang and Snyder[8] for further discussion of the inherent stability of antiparallel homodimers). The compound which is energetically more stable, the antiparallel dimer 3–5′,5–3′,-bis[Sar^1,Cys^{3,5}]-AII, is formed under thermodynamically controlled conditions, i.e., disulfide–sulfide exchange

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‡ Abbreviations: The abbreviations for natural amino acids and nomenclature for peptide structures are those recommended (*J. Biol. Chem.* 1971, *247*, 977). AII, angiotensin II; Hcy, homocysteine; MPc, *cis*-4-mercaptoproline; MPt, *trans*-4-mercaptoproline; Dtc, 5,5-dimethylthiazolidine-4-carboxylic acid; MeF, α-methylphenylalanine; MeY; α-methyltyrosine; Acm, acetamidomethyl; DIC, *N,N′*-diisopropylcarbodiimide; DCC, *N,N′*-dicyclohexylcarbodiimide; Dup 753, 2-*n*-butyl-4-chloro-5-(hydroxymethyl)-1-[[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-imidazole; Fm, 9-fluorenylmethyl; Fmoc, 9-fluorenylmethoxycarbonyl; TBTU, 1,1,3,3-bis(tetramethylene)chlorouronium hexafluorophosphate; HBTU, *O*-benzotrizolyl-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate; BOP, benzotriazolyloxytris(dimethylamino)phosphonium hexafluorophosphate; DIPEA, diisopropylethylamine; TFA, trifluoroacetic acid, DCM, dichloromethane; HOBt, hydroxybenzotriazole.

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(reaction at pH higher than 7). This specific case of n to $n + 2$ oxidation contradicts the generally accepted view that intramolecular bridging across the nearest neighbor positions is not only possible but energetically preferable to intermolecular dimerization due to entropic considerations. Thus, the synthesis of the cyclic, n to $n + 2$ monomer has to be performed under reaction conditions where disulfide exchange is kept at the lowest possible rate as the desired monomer is relatively unstable. Iodine oxidation under kinetic control (large excess of I_2) under acidic conditions represents such conditions.

Determination of molecular ions by FAB-MS can be difficult to interpret as the disulfide bonds of these dimers are readily fragmented, and the monomer fragment ions are much more intense than the ion of the parent dimers; this was particularly true for the less stable parallel heterodimers. Thus, the molecular ion of the dimer might be misinterpreted as a peak characterizing molecular association seen frequently with FAB-MS. Electrospray MS, which does not cause the fragmentation of the disulfide heterodimer, was used to confirm the molecular weight of synthetic disulfide heterodimers (unpublished data).

Enzymatic hydrolysis is an excellent supplementary tool to determine the type of dimer formed during the oxidation of disulfide-bridged peptides, if a susceptible bond for enzymatic hydrolysis is appropriately located in the cyclic disulfide structure. The relative arrangement of the peptide chains in the angiotensin dimers was confirmed by enzymatic digestion with chymotrypsin at the susceptible Tyr^4-X^5 bond, yielding two cleavage products in the case of parallel dimers and one cleavage product in the case of the antiparallel dimer (see Experimental Section). A synthetic route to produce the parallel dimer selectively by differential (but not orthogonal) disulfide protection gave an authentic sample for comparison (see Experimental Section for the synthesis of the parallel, cyclic dimer, 3-3′,5-5′,bis[Sar^1,Mpc^3,Cys^5]-AII (7)).

Boc-Pro(*cis*-4-S-MBzl) and Boc-Pro(*trans*-4-S-MBzl) were incorporated into position 3 and/or 5 of angiotensin II analogs utilizing normal solid-phase synthesis protocols. Yields of highly constrained analogs were generally lower than those of less constrained peptides and would convert to dimers during purification under nonalkaline conditions. The yields of the cyclic monomer consistently correlated with the estimated ring strain as determined by molecular mechanics calculations in accord with the results of Burns and Whitesides.[9] For example, the propensity for cyclization was much higher in the "Cys-Tyr-Mpt" tripeptide than in the "Cys-Tyr-Mpc" analog according to theoretical calculations.[7] Increasing the iodine concentration during the cyclization of the *trans*-mercaptoproline analog, i.e., [Sar^1,Cys(Acm)^3,Mpt^5]-AII (3) resulted in cyclic monomer formation, while for the *cis*-analog [Sar^1,Cys(Acm)^3,Mpc^5]-AII (4) the main product was a linear dimer. This combination of Cys(Acm) and Cys(H) had been found previously by Kamber *et al.*[10] to increase the efficiency of cyclization in unconstrained systems. In one case (3), it was not as efficient as the use of two unprotected cysteines and led to intermolecular reactions; in another case (4), it allowed the isolation of the cyclic monomeric product prepared in a modified solvent system.

Three cyclic lactam analogs in which position 3 contained the carboxyl-bearing residue (Asp or Glu) and position 5 contained the amine-bearing side chain (Orn or Lys) have been prepared using a solid-phase protocol which allowed resin-bound cyclization. Use of base-labile protecting groups (Fm and Fmoc) on the side chains of the carboxyl residues and the amine component allowed deprotection on the resin with 20% piperidine, followed by lactam formation overnight using DCC/HOBt activation.

Table I. Cyclic Angiotensin II Analogs and Their Receptor Affinities and Agonist Activity

compound	binding affinity[a] (IC_{50}, nM) AT1	AT2	rabbit aorta (EC_{50}, nM)
c[Sar^1,$Hcy^{3,5}$]	1.3	80	3.2
c[Sar^1,Cys^3,Hcy^5]	2.6	28	NT[b]
c[Sar^1,$Cys^{3,5}$]	7.6	27	NT
bis-c[Sar^1,$Cys^{3,5}$] (antiparallel, 0)	28	65	NT
c[Sar^1,Hcy^3,MPt^5] (1)	1.3	0.65	13
c[Sar^1,Hcy^3,MPc^5] (2)	110	9.1	690
c[Sar^1,Cys^3,MPt^5] (3)	18	0.82	160
c[Sar^1,Cys^3,MPc^5] (4)	62	7.4	NA[c]
c[Sar^1,MPc^3,Cys^5] (5)	4700	12	NA
bis-c[Sar^1,MPc^3,Cys^5]	110	25	870
(antiparallel, 6, parallel, 7)	190	23	460
c[Sar^1,MPc^3,Hcy^5] (8)	510	4.8	440
c[Sar^1,MPt^3,Cys^5] (9)	87	6.1	NT
c[Sar^1,MPt^3,Hcy^5] (10)	29	0.47	700
c[Sar^1,Cys^3,Cys^8] (11)	520	NT	NT
c[Sar^1,Cys^5,Cys^8] (12)	1200	NT	NT
c[Sar^1,Cys^3,Cys^7] (13)	1230	NT	NT
c[Sar^1,Asp^3,Lys^5] (14)	12	86	53
c[Sar^1,Glu^3,Lys^5] (15)	89	NT	NT
c[Sar^1,Asp^3,Orn^5] (16)	683	NT	NT
AI, decapeptide	300	116	>10000
AII	1.02	2.5	2.5
AIII, heptapeptide	5.57	1.74	32
saralasin, [Sar^1,Ala^8]-AII	1.69	1.32	NT

[a] AT1 = rat liver; AT2 = rabbit uterus. [b] NT = not tested. [c] NA = not active.

Biological Activity of Cyclically Constrained AII Analogs. Binding assays were performed with both rat liver (AT1) and rabbit uterus (AT2) tissues in order to measure the affinity for the two types of angiotensin receptors.[11] Contractile responses on rabbit aorta (AT1) were used to characterize the agonistic activity of selected analogs. Most, if not all, of the normal biological activities associated with AII correlate with occupancy of the AT1 receptor. The physiological role of AT2 receptor has not yet been fully elucidated. The results presented in Table I indicate that some of the cyclically constrained, mercaptoproline-containing analogs of AII bind more tightly to the AT2 receptor than does AII itself, suggesting that their conformation may closely mimic that of the receptor-bound conformation. One constrained analog, c[Sar^1,-Hcy^3,MPt^5]-AII, binds almost as tightly to the AT1 receptor as AII itself, suggesting it as a candidate for further study.

If the cyclic constraint locked angiotensin II into a conformation truly mimicking the receptor-bound conformation, then one would expect to see a dramatic increase in affinity. This would be due to the decrease in entropy loss on binding the peptide to the receptor. It has been estimated[12-14] that binding which freezes out torsional degrees of freedom requires approximately 0.8 kcal/mol per degree of rotational freedom. A cyclic constraint in chemistry eliminates at least four degrees of freedom (consider hexane with five torsional degrees of freedom compared with cyclohexane with one). In the case of most of the peptides in the literature[15,16] in which cyclization has been used to constrain the system, additional degrees of torsional freedom have been introduced through the

Table II. Possible Biologically Relevant Conformations for Tyrosine-4 for AII Analogs

conformation	analogs	
	compatible	noncompatible
inverse γ-turn	c[Cys^{3,5}]	[MeF^4], [MeY^4], [Pro^5], c[Cys^3,MPt^5], dimers
Φ_4 = −70° to −85°		
Ψ_4 = 60° to 70°		
β-turns		
type III′ ($Y^4 = i + 2$)	c[Cys^{3,5}]	c[MPc^3,Hcy^5], dimers
type I ($Y^4 = i + 1$)	c[MPc^3,Hcy^5]	c[Cys^3,MPt^5], dimers
type III ($Y^4 = i + 1$)	c[MPc^3,Hcy^5]	c[Cys^3,MPt^5], dimers
Tyr^4 constraints (Φ, Ψ)		
right-handed helix	[MeF^4], [MeY^4], [Pro^5]	c[Cys^3,MPt^5], dimers
left-handed helix	[MeF^4], [MeY^4], [Pro^5] c[Cys^3,MPt^5]	dimers
"open turn"		
(Φ_4 = −130°, Ψ_4 = 65°)	c[Cys^3,MPt^5]	dimers
β-sheet		
parallel (Φ_4 = −119°, Ψ_4 = 113°)	3-3′,5-5′,bis[Sar^1,Cys^{3,5}]	[MeF^4], [MeY^4]
antiparallel (Φ_4 = −139°, Ψ_4 = 135°)	3-5′,5-3′,bis[Sar^1,Cys^{3,5}]	[MeF^4], [MeY^4]

use of longer side chains, such as Glu or Lys. The major advantage which proline affords as a scaffold for the introduction of functional groups for subsequent cyclization is the relative rigidity of the pyrrolidine ring.[7] The 4-mercaptoproline analogs used in this study can be considered as rigidified Hcy analogs, hybrid or chimeric amino acids, combining the properties of the two side chains, Pro and Hcy. Analogs of bradykinin containing 4-mercaptoproline[17] and 3-mercaptoproline, a chimeric amino acid combining the side chains of proline with that of cysteine (Kaczamarek, Skeean, and Marshall, unpublished results), have been prepared.

The fact that the affinity of these rigidified angiotensin analogs for the AT1 and AT2 receptors is not significantly increased over that of the control probably implies that the true receptor-bound conformation is not identical with that stabilized by these modifications. One must remember that the receptor is not required to optimally bind its peptide ligand in a conformation which can be mimicked by a cyclic compound with the appropriate bond distance forming the cyclic constraint. There is no guarantee that a cyclic analog could be constructed using the limited repertory of organic chemistry which would mimic exactly that of a linear peptide bound to its receptor. Alternatively, a modification might shift the conformational equilibrium unfavorably by introducing favorable intermolecular interactions stabilizing a conformation not recognized, rather than simply restricting the system. An example where the potential increase in affinity has been observed is the cyclic analog of α-melanotropin, c[Cys^{4,10}]-MSH, reported by Sawyer *et al.*[18] Further support of the concept that one has stabilized a conformation similar, but not identical, to the ideal comes from comparison of binding affinities with agonist activities. Decreasing the ring size of **2**, c[Sar^1,Hcy^3,MPc^5]-AII, by one methylene by replacing Hcy-3 with Cys-3 to give c[Sar^1,Cys^3,MPc^5]-AII (**4**) enhances the affinity for the AT1 receptor while converting the compound from a weak agonist to a presumed antagonist (Table I) as the lack of contractile activity on the rabbit aorta reflects lack of activation of the AT1 receptor. Similarly, the binding affinity for c[Sar^1,Hcy^3,MPt^5]-AII (**1**) is nearly identical to that for AII, but its contractile efficacy on the rabbit aorta has been reduced. The underlying assumption that compounds of similar chemical structure bind to the receptor in a common conformation and a common binding mode is, of course, subject to question in the light of these observations.

Implications for the Receptor-Bound Conformation of Angiotensin II. A main motivation behind the introduction of steric and cyclic conformational constraints is the determination of the receptor-bound conformation for angiotensin II (AII: Asp^1-Arg^2-Val^3-Tyr^4-Val^5-His^6-Pro^7-Phe^8) itself. For an overview of these efforts on angiotensin agonists and antagonists, the recent work of Samanen *et al.*[19] is recommended. Several simplifying assumptions are usually implicitly made in the analyses of structure–activity data which bear scrutiny. Certainly, a common binding mode in which the backbone torsional angles as well as side chain orientations are nearly identical is a common simplifying assumption. By focusing on agonists which are both recognized by the receptor with high affinity and which are capable of stimulating activation and second messenger production, it is often assumed that conformational constraints acceptable in one compound are applicable to others in the series; i.e., a common binding mode is present. The set of analogs of AII available in the literature and in this study provide an opportunity to test this assumption due to their different conformational constraints.

Spear *et al.*[5] have recently reported cyclic analogs of AII having relatively high contractile activity. These analogs (c[Hcy^{3,5}]-AII, c[Hcy^3,Cys^5]-AII, c[Cys^3,Hcy^5]-AII, and c[Cys^{3,5}]-AII) all contain cyclic tripeptides at residues 3, 4, and 5 of AII. Similar analogs in which the amide bond was used for cyclization of the side chains of residues 3 and 5 are reported in Table I. Optimal activity for the cyclic amide analogs was found for c[Asp^3,Lys^5]-AII (**14**) with reduced activity when the ring size was reduced in c[Asp^3,Orn^5]-AII (**16**). Results from the study of Kataoka *et al.*[7] on the conformational constraints introduced by side chain cyclization in tripeptides are applicable to these cyclic analogs of AII. In these studies, the amide cyclization was more restrictive than that of the disulfide, probably due to be the shortened bond length as well as the higher torsional barrier to rotation. This help rationalize the reduction seen with the amide-containing ring in c[Asp^3,Orn^5]-AII (**16**) when the disulfide analog with a comparable ring size, c[Hcy^{3,5}]-AII, binds almost as well as AII itself (Table I).

Let us examine the ability of the set of active AII analogs to assume a common backbone conformation in the vicinity of the Tyr-4 residue (Table II).

γ-Turn. The inverse γ-turn centered at tyrosine-4 has previously been proposed by Printz *et al.*[4] for the receptor-bound conformation of AII. For the inverse γ-turn, Φ =

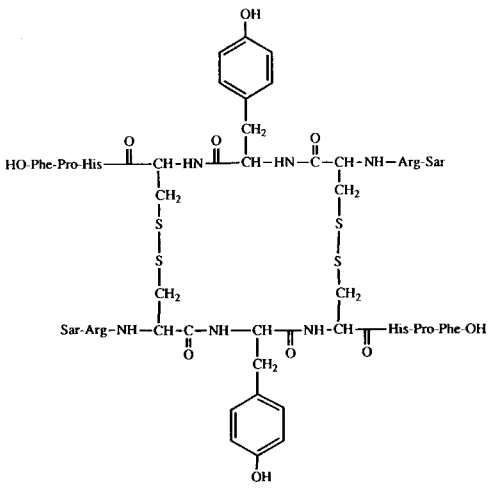
−70 to −85 and Ψ = 60–70°, the Φ_2,Ψ_2 plots calculated by Kataoka *et al.*[7] indicates that the 4 cyclic analogs of Spear *et al.*[5] and c[Sar^1,MPc^3,Hcy^5]-AII are consistent with this hypothesis in that each plot shows an allowed angle range consistent with the γ-turn. NMR studies of c[Sar^1,Cys^{3,5}]-AII showed (Plucinska, Kao, Nikiforovich, and Marshall, unpublished) a small negative shift of the Cys-5 NH upon changing the solvent from chloroform to DMSO, similar to that seen[20] with c[Boc-Cys-Ala-Cys-NHCH$_3$], a model γ-turn peptide. In addition, the temperature dependence of the amide chemical shifts point unequivocally to a strong hydrogen bond at the amide of Cys-5 (unpublished data), again implicating the γ-turn with Tyr-4 as the central residue. Both the NMR and CD data indicate a dynamic equilibrium, however, with interconversion between the M and P configurations of the chiral disulfide bond (Plucinska, Kao, Nikiforovich, and Marshall, unpublished). The high levels of activity of analogs containing α-methyltyrosine, MeY,[21] and α-methylphenylalanine, MeF,[22] at position four (92.6% and 19%, respectively) coupled with the evidence correlating the presence of α,α-dialkylamino acid residues with torsional values (Φ,ψ) associated with helical conformations[1,2,23] make, however, a common receptor-bound conformation of the backbone featuring a γ-turn centered on residue 4 less likely. In addition, the activity of [Pro^5]-AII and [Sar^1,Dtc^5]-AII (10% and 250%, respectively[19]), in which the amide hydrogen involved in the proposed γ-turn conformation has been replaced by a methylene group of the ring, makes recognition dependent of a γ-turn conformation centered on residue 4 highly unlikely.

β-Turns. The results of Kataoka *et al.*[7] suggest that the four residues of the 2–5 sequence of the cyclic analogs of Spear *et al.*[5] could accommodate a type III′ β-turn, while the 3–6 sequence could accomodate type I and III β-turns with other β-turns types being sterically unlikely. This analysis suggests the possibility that the receptor-bound conformation of AII is a β-turn which is centered at residues 3 and 4 or 4 and 5, consistent with previous suggestions. Spear *et al.*[5] had ruled out this possibility on the basis of CPK molecular model building as follows: "However, molecular models show that both of these structures (referring to a four-residue turn between residues 2–5 or 3–6) are strained and quite perturbed from any recognized β-turns." One cannot be confident that all classes of β-turns were examined, especially considering the results of Kataoka *et al.*[7]

The incorporation of mercaptoproline analogs in positions 3 and 5 for construction of the disulfide constraint provide more restricted bicyclic AII analogs, such as c[Sar^1,MPc^3,Hcy^5]-AII, to probe the likelihood of a bound conformation with a turn centered on residues 3–5. This compound has relatively high affinity for AII receptors (see Table I). The MPc^3-Tyr^4-Hcy^5 disulfide-bridged substructure of this analog can accommodate types I and III β-turns centered at residues 4 and 5.[7] The inclusion of the cyclic disulfide and proline constraint of c[Sar^1,-MPc^3,Hcy^5]-AII eliminates, however, a type III′ β-turn at residues 3 and 4.

In addition, the excellent activities of analogs **1**, c[Sar^1,-Hcy^3,MPt^5[-AII, and **3**, c[Sar^1,Cys^3,MPt^5]-AII (Figure 2 and Table I), imply that their conformational constraint is compatible with recognition and activation by the AII receptor. Analog **3**, which contains the peptide sequence in positions 3–5 modeled by the tripeptide, Cys-Ala-MPt, is extremely restricted to essentially one conformation, eliminating both γ- and β-turns at the 20° torsional scan used by Kataoka *et al.*[7] The Tyr-4 residue may assume a conformation compatible with left-handed α-helical values (at least, the central alanine of Cys-Ala-MPt probably does according to Kataoka *et al.*[7]) when bound to the receptor. All other active, constrained AII monomer analogs in the literature which we have examined can assume conformations which are consistent with this hypothesis. On the other hand, the use of a grid search with torsional increments of 20° may have missed allowed conformations associated with alternate conformations such as the γ-turn. We have examined the possible conformations of Hcy-Ala-MPt and Cys-Ala-MPt at a finer torsional increment (10°) and find the γ-turn to be excluded for the central Ala residue as would be expected for residues preceding a proline (see Figure 3b,c). The right-handed α-helical area and β-turns are also precluded by the cyclization, and not by the sequence alone as they are allowed for the noncyclic peptide (Figure 3a) with the set of parameters used. Only 15 conformers were sterically allowed (Figure 3c) for c[Cys-Ala-MPt] with two sets of values for the central residue clustered around the left-handed helical values and an alternate conformation (which we will refer to as the "open turn") with torsional values of Φ = −130°, Ψ = 65°, intermediate between torsional angles for an inverse γ-turn and the β-sheet. All β-turn conformations for c[Cys-Ala-MPt] with Ala at the $i+1$ position of the turn were excluded on the basis of the allowed set of torsional angles. A low-energy conformer, the "open turn", of this peptide segment incorporated into angiotensin II containing similar torsional angles (Φ_4 = −130°, Ψ_4 = 65°) has been found in independent studies of the low-energy conformations of AII analogs (Nikiforovich and Marshall, unpublished) which allows a common three-dimensional arrangement of the Tyr-4, His-6, and Phe-8 side chains as well as the C-terminal carboxylate. Preliminary NMR and CD studies of **1**, c[Sar^1,Hcy^3,MPt^5]-AII, and **3**, c[Sar^1,Cys^3,MPt^5]-AII, support this "open turn" conformation (Φ_4 = −130°, Ψ_4 = 65°) in DMSO as predominate (Plucinska, Kao, Nikiforovich, and Marshall, unpublished). This would suggest these two conformers, the left-handed helix, and the "open turn" of c[Sar^1,Cys^3,MPt^5]-AII as probable candidates for the receptor-bound conformation of the tripeptide segment of this analog and, perhaps, for this segment of other active monomer analogs.

How committed should we be to the assumption of common bindings modes with identical backbone conformations for all active analogs? In view of much of what we know about ligand–protein interactions,[24] this is clearly an untenable assumption in general. It is clear, however, that the backbone conformations of different analogs do not have to be identical for successful presentation of the essential side chains in a correct three-dimensional pattern for recognition. This view is reinforced when one considers the activity of the antiparallel dimer of 3–5′,5–3′,bis[Sar^1,-Cys^{3,5}]-AII. Conformational studies of this compound and the monomer have clearly shown that the dimer has an extended antiparallel sheet conformation[25] for residues 3–5 (Φ = −139°, Ψ = 135°). The diagnostic chemical shifts seen in the NMR studies[20,25,26] of the antiparallel dimer of 1–3′,3–1′,bis[Boc-Cys-Ala-Cys-NHCH$_3$], of which the crystal structure (Ala, Φ = −155°, Ψ = 162°) has been



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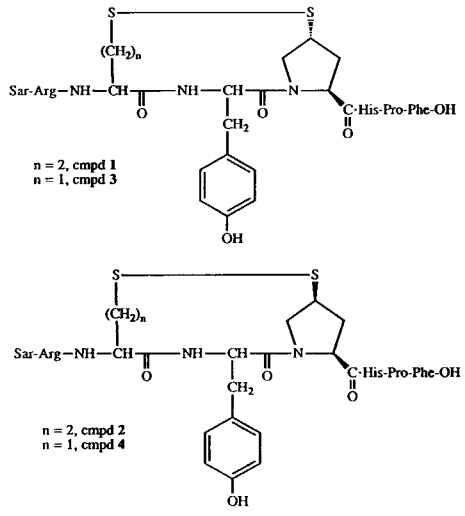
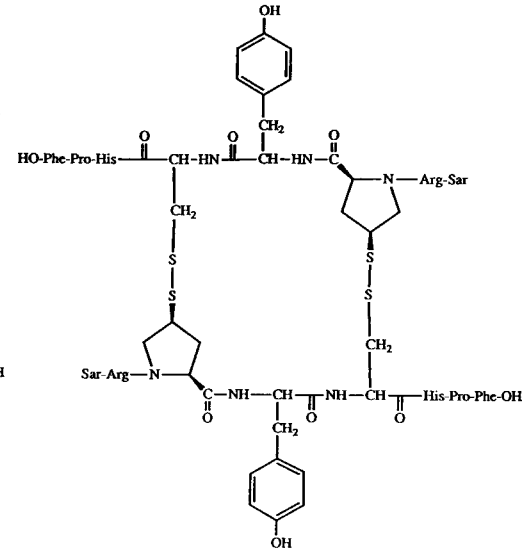
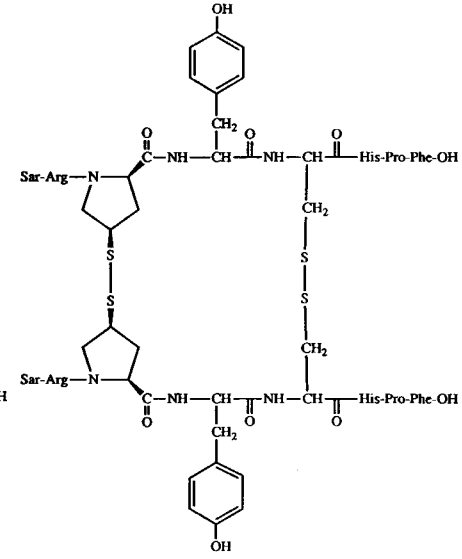
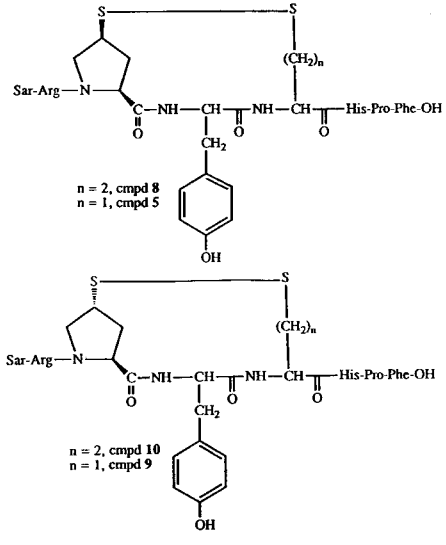


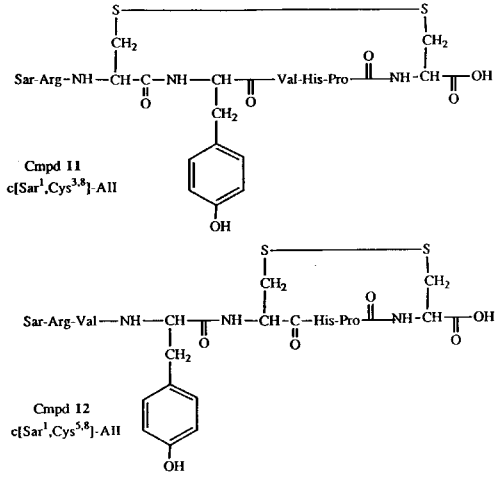


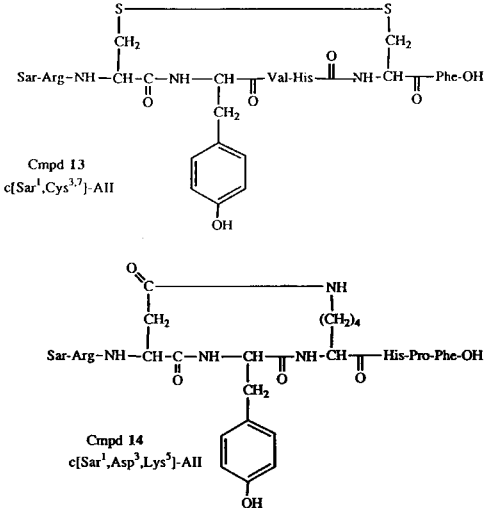
Cmpd **6**, 3-5',5-3',bis-c[Sar^1,Mpc^3,Cys^5]-AII, antiparallel dimer



Cmpd **7**, 3-3',5-5',bis-c[Sar^1,Mpc^3,Cys^5]-AII, parallel dimer







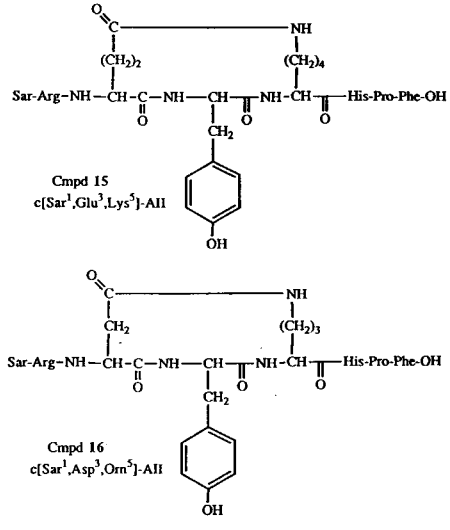


Figure 2. Structures of cyclic AII analogs.

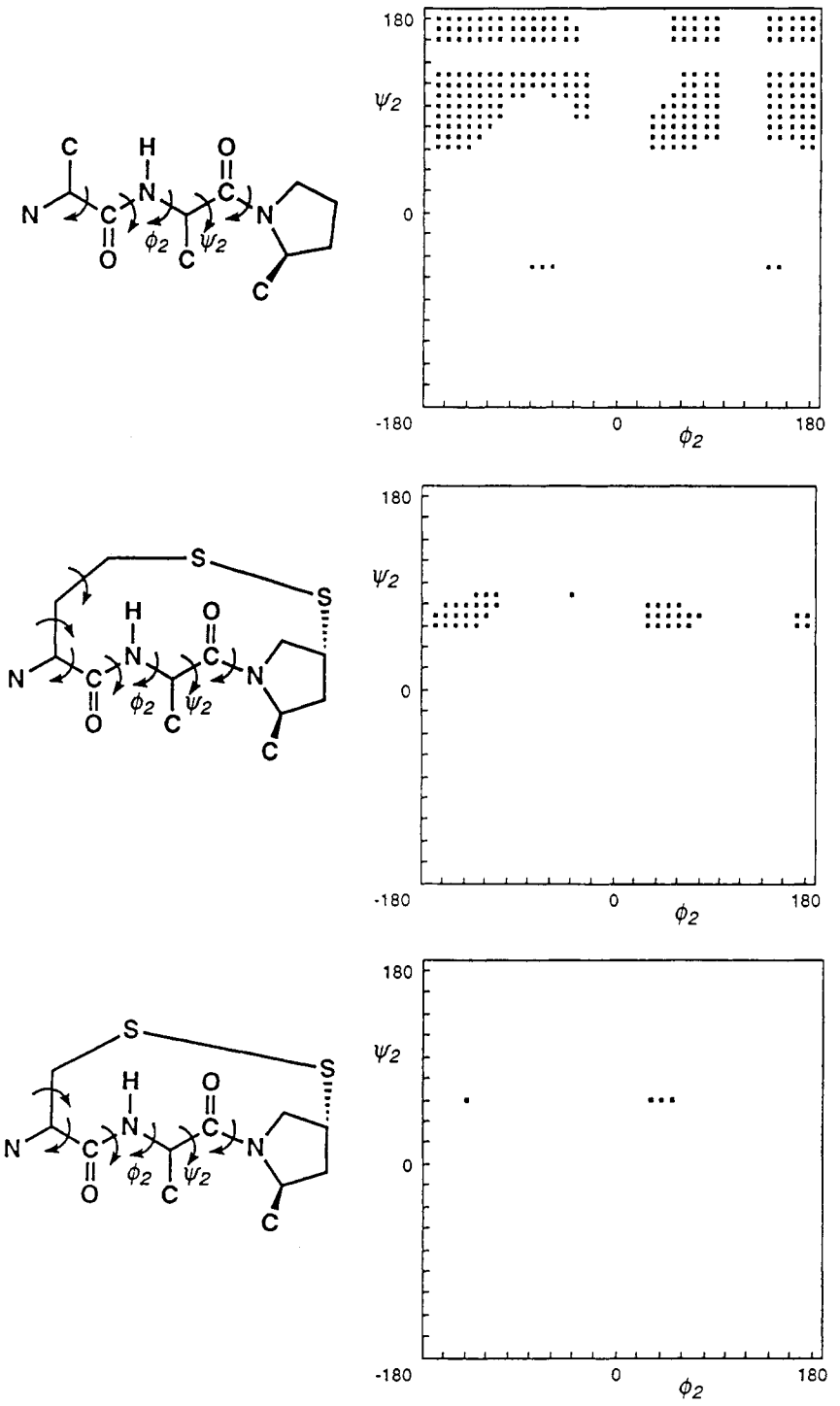


Figure 3. Ramanchandran plot of Ala-2 residue from conformational analysis of the tripeptides, Ac-Ala-Ala-Pro-$NHCH_3$ (a), Ac-c[Hcy-Ala-MPt]-$NHCH_3$ (b), and Ac-c[Cys-Ala-MPt]-$NHCH_3$ (c).

determined,[27] are also seen in the NMR spectra of 3–5′,5–3′,bis[Sar[1],Cys[3,5]]-AII (Plucinska, Kao, Nikiforovich, and Marshall, unpublished). This would imply that the dimer with an extended conformation in the region of the Tyr-4 residue is recognized at the receptor as are the cyclic monomers where the disulfide constraint has been shown to force the peptide to fold back upon itself. If one abandons the requirement for similar backbone conformations, the next level of analysis imposes the pharmacophore hypothesis; i.e., active analogs position crucial functional groups, the side chains, in a similar three-dimensional pattern. In the case of AII, the crucial residues required for optimal biological activity are the phenol ring of Tyr-4, the imidazole ring of His-6, the aromatic ring, and carboxylate of Phe-8. Preliminary conformational analyses (Alvey and Marshall, unpublished) of the antiparallel 3–5′,5–3′,bis[Sar[1],Cys[3,5]]-AII compared with c[Sar[1],Hcy[3],MPt[5]]-AII and [MeF[4]]-AII shows that this is a feasible hypothesis with a common three-dimensional pattern for the crucial side chain residues being accessible despite differences in local backbone conformations of these analogs. A different backbone conformation of the dimer is capable of presenting the three critical groups by some combination of the two sets of side chain residues available to the dimer when compared with the cyclic monomers. Initial studies suggest that the Tyr residue of one monomer combines with the His and Phe residues from the other monomer of the dimer to present an

analogous three-dimensional arrangement of the side chain residues to that of a cyclic monomer. Additional support for this interpretation comes from the relative activities of the three analogs containing the common sequence Sar-Arg-Mpc-Tyr-Cys-His-Pro-Phe. The cyclized monomer (5) binds poorly (IC_{50} = 4700 nM) to the AT1 receptor, while both the parallel (7, IC_{50} = 190 nM) and antiparallel (6, IC_{50} = 110 nM) dimers bind much better. One assumes that some combination of the two sets of pharmacophoric elements in the two dimers complement the receptor. Adding one additional methylene group to the ring of the monomer, c[Sar[1],Mpc[3],Hcy[5]]-AII (8, IC_{50} = 110 nM), enhances the affinity by almost 10-fold, presumably by allowing a more complementary arrangement of the critical pharmacophoric elements.

Nonadditivity of Modifications. The recognition that the backbone conformation is not a crucial recognition element provides a basis for understanding nonadditivity in constrained analogs.[19] An example from AII is the set, [Pro[3]]-AII, [Pro[5]]-AII, and [Pro[3,5]]-AII, where each of the singly substituted compounds retains significant activity and the analog containing the two modifications is essentially inactive (Marshall, unpublished). Each of the single constraints is probably suboptimal for interaction with the receptor, with the peptide adopting a different backbone conformation to correctly position the crucial recognition elements. Neither of these two different receptor-bound conformations of the singly substituted analogs can accomodate the other additional substitution, and the side chains cannot assume the correct three-dimensional orientation in [Pro[3,5]]-AII.

Angiotensin II–Antibody Complex. The only potentially relevant three-dimensional information on a bound conformation of AII comes from the crystal structure of angiotensin II–antibody (mAb-131) complex which has recently been solved[28] at 3-Å resolution. To develop the anti-anti-idotypic antibody used in the complex, an antibody (mAb-31) against angiotensin served as an antigen for a second antibody which has been shown to bind to the AII receptor. This is thought to imply that the second antibody mimics the receptor-bound conformation of AII on its surface. A third antibody (mAb-131) was raised against the second antibody supposedly mimicking the AII receptor. mAb-131 binds AII with a measured affinity of 1.3×10^{-10} M, and the complex between AII and mAb-131 was felt to be representative of the AII–receptor complex. The conformation of AII in the complex has two turns, a chain reversal involving Ile-5, His-6, and Pro-7, which is most highly buried in the complex, and another turn involving Asp-1 and Arg-2. The binding cavity of the antibody is both deep and narrow and AII folds into a compact structure that fills it completely, completing the convex surface of the protein. While this conformation is consistent with some of the models of AII previously proposed based on structure–activity studies and solution studies alone, it is difficult to reconcile with the biological activity of cyclized AII analogs prepared in this study.

A previous study[29] of a complex between an antibody (Fab D1.3) against lysozyme and an anti-idiotope Fab (E225) defined a 13-residue idiotope. The complex of lysozyme with Fab D1.3 was also determined allowing direct comparison between its binding site, or paratope, and the idiotope which binds to E225. Of the 13 residues comprising the idiotope, only seven are in common with the paratope. In other words, only some of the residues of D1.3 which are recognized by lysozyme are also recognized by E225. This implies that mimicry at the atomic level is not essential for anti-idiotopes, and the concepts underlying rigid transfer of antigen-templates are inconsistent with the inherent adaptability of antibody binding sites. It is not surprising, therefore, that molecular details of this antibody complex do not seem to correlate with the conclusions derived from structure–activity studies.

Conclusions

Novel angiotensin II analogs have been prepared in which mercaptoproline analogs have been substituted at positions 3 and 5 and cyclized by a disulfide to the other position containing either a Cys or Hcy residue. Assuming a common binding mode for the set of analogs, the high activity seen excludes all the conformations previously proposed as that bound to the receptor. Nevertheless, a conformation in which Tyr-4 assumes torsional values associated with the left-handed helix is a viable candidate for the receptor-bound conformation of AII cyclic monomer peptides based on the analysis of side chain constraints in tripeptides by Kataoka *et al.*[7] Additional calculations (Nikiforovich, Alvey, and Marshall, unpublished) suggest an alternate receptor-bound conformation, "open turn" ($\Phi_4 = -130°, \Psi_4 = 65°$), for the Tyr-4 position. The activity associated with the parallel and antiparallel dimers as well as the cyclic monomers implies that the dimer with an extended conformation in the region of the Tyr-4 residue is also recognized at the receptor as well as are cyclic monomers where the disulfide constraint has been shown to force the peptide to fold back upon itself. This observation challenges the previous assumptions that common backbone conformations for AII are involved in receptor interaction and focuses attention of the three-dimensional arrangement of critical side chain functionality as the key to molecular recognition of these AII analogs by the receptor.

Experimental Section

Materials. [Tyrosyl-3,5-^{3}H(N)]angiotensin II (specific activity 59.9 Ci/mmol) and [^{125}I]-Tyr[4]-angiotensin II (2200 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Angiotensin peptides (Ang I, II, and III and saralasin) were obtained from U.S. Biochemicals (Cleveland, OH). DUP 753 was prepared according to methods described in the patent literature.[30] Homocysteine was purchased from Schweitzerhall as *N*-α-Boc-*S*-(4-methylbenzyl)homocysteine.

Boc-Pro(*cis*-4-S-Bzl). The *cis*-4-mercaptoproline derivative was prepared from hydroxyproline through the tosyl intermediate as follows: 100 mL of sodium ethoxide, 21 wt % in EtOH (18 g, 270 mmol NaOEt) was added to a round-bottom flask under N_2 and chilled to 0 °C in an ice–salt bath. Benzyl mercaptan (33 mL, 35 g, 280 mmol) was added by syringe (temperature <5 °C). This solution was slowly added by a cannula to a previously chilled solution of *N*-formyl-*trans*-4-(tosyloxy)proline methyl ester (48.2 g, 140 mmol) in 100 mL of EtOH under N_2 taking care to maintain the temperature below 5 °C. After completion of the addition, the mixture was allowed to warm to room temperature while stirring for 24 h. The reaction mixture was vacuum filtered and the filtrate concentrated on a rotary evaporator to an oil which was then diluted in H_2O and extracted three times with CH_2Cl_2. The combined organic phase was dried over $MgSO_4$, filtered, and concentrated by rotary evaporation. The crude product was flash chromatographed on 1 kg of silica using hexane/EtOAc, 1:1, as solvent. The eluant was gradually changed to 100% EtOAc and then EtOAc/MeOH, 95:5. 16.8 g (41%) of crude product

(*N*-formyl-*cis*-4-(benzylthio)proline methyl ester) was obtained. NMR indicated a mixture of methyl and ethyl esters.

The crude intermediate (16.8 g) was hydrolyzed by refluxing under N_2 in 3 N HCl (202 mL) for 3.5 h. Two-thirds of the solution was removed by rotary evaporation, and the solids which formed were collected by vacuum filtration and dried under high vacuum at 35 °C; 10.6 g (67% yield) of crude *cis*-4-(benzylthio)-proline hydrochloride was isolated. ^{1}H NMR (DMSO): δ 1.19–3.55 (5H, several m), 3.85 (2H, s), 4.29–4.39 (1H, t), 7.35 (5H, s).

Crude *cis*-4-(benzylthio)proline hydrochloride (10.6 g, 45 mmol) was combined with 10.7 g of di-*tert*-butyldicarbonate and 160 mL of CH_2Cl_2 and stirred under N_2 and chilled to 0 °C in an ice–salt bath. Triethylamine (14.9 g, 20.5 mL, 147 mmol) was added dropwise by syringe. After the addition was completed, the ice bath was removed and the reaction mixture stirred for 24 h. The reaction was diluted with ether and washed with 1 N HCl, and the organic layer was dried over $MgSO_4$, filtered, and concentrated by rotary evaporation to a yellow oil. Crystals formed within 5 min and were collected by vacuum filtration, washed with ether/hexane, 1:1, and vacuum-dried to obtain 11.1 g (74% yield). Mp: 134.8–136.5 °C. $[\alpha]^{22}_D$ = −57.99° (1% in MeOH). ^{1}H NMR ($CDCl_3$): δ 1.40–1.55 (9H, 2 s), 1.9–3.98 (5 H, several m), 3.75 (2H, s), 4.18–4.38 (1H, m), 7.32 (5H, s). Anal. Calcd for $C_{17}H_{23}NO_4S$: C, 60.51; H, 6.87; N, 4.15. Found: C, 60.19; H, 6.93; N, 4.05.

Boc-Pro(*trans*-4-S-Bzl). This compound was prepared in an analogous fashion to the *cis*-derivative from the epimer of 4-hydroxyproline (Eswarakrishnan and Lamar[31]). Mp: 99.5–100 °C. $[\alpha]^{22}_D$ = −12.84° (1% in MeOH). ^{1}H NMR ($CDCl_3$): δ 1.40–1.50 (9H, 2 s), 1.92–3.89 (5H, several m), 3.75 (2H, s), 4.30–4.40 (1H, m), 7.35 (5H, s). Anal. Calcd for $C_{17}H_{23}NO_4S$: C, 60.51; H, 6.87; N, 4.15. Found: C, 60.15; H, 6.88; N, 3.95.

Boc-Pro(*cis*-4-S-*p*-MeBzl). This compound was prepared in an analogous fashion, substituting MeOH for EtOH and NaOMe for NaOEt in the procedure, using *p*-methylbenzyl mercaptan. Mp: 99–100 °C. $[\alpha]^{22}_D$ = −57.0° (1% in MeOH). ^{1}H NMR ($CDCl_3$): δ 1.39–1.44 (9H, 2 s), 1.9–3.98 (5H, several m), 2.33 (3H, s), 3.71 (2H, s), 4.19–4.32 (1H, m), 7.1–7.25 (4H, q). Anal. Calcd for $C_{18}H_{25}NO_4S$: C, 61.51; H, 7.17; N, 3.99. Found: C, 61.33; H, 7.23; N, 3.83.

Boc-Pro(*trans*-4-S-*p*-MeBzl). This compound was prepared in an analogous fashion, substituting MeOH for EtOH and NaOMe for NaOEt in the procedure, using *p*-methylbenzyl mercaptan. Mp: 116.5–117 °C. $[\alpha]^{22}_D$ = −120° (1% in MeOH). ^{1}H NMR ($CDCl_3$): δ 1.4–1.48 (9H, 2 s), 1.99–3.9 (5H, several m), 2.32 (3H, s), 3.74 (2H, s), 4.3–4.48 (1H, m), 7.08–7.23 (4H, q). Anal. Calcd for $C_{18}H_{25}NO_4S$: C, 61.51; H, 7.17; N, 3.99. Found: C, 61.53; H, 7.25; N, 3.73.

Boc-Pro(*cis*-4-S-*p*-MeOBzl)·DCHA. This compound was prepared in an analogous fashion using *p*-methoxybenzyl mercaptan and isolated as the dicyclohexylamine (DCHA) salt. Mp: 179–180 °C. $[\alpha]^{22}_D$ = −43.0° (1% in MeOH). ^{1}H NMR ($CDCl_3$): δ 1.4 (9H, s), 1.1–4.4 (29H, several m), 3.71 (2H, s), 3.8 (3H, s), 6.81–6.9 (2H, d), 7.19–7.27 (2H, d). Anal. Calcd for $C_{30}H_{46}N_2O_5S$: C, 65.66; H, 8.82; N, 5.0. Found: C, 65.55; H, 8.76; N, 5.10.

Boc-Pro(*trans*-4-S-*p*-MeOBzl)·DCHA. This compound was prepared in an analogous fashion using *p*-methoxybenzyl mercaptan and isolated as the dicyclohexylamine salt. Mp: 179–180 °C. $[\alpha]^{22}_D$ = −11.0° (1% in MeOH). ^{1}H NMR ($CDCl_3$): δ 1.4 (9H, s), 1.1–4.3 (29H, several m), 3.7 (2H, s), 3.8 (3H, s), 6.8–6.88 (2H, d), 7.18–7.25 (2H, d). Anal. Calcd for $C_{30}H_{46}N_2O_5S$: C, 65.66; H, 8.82; N, 5.0. Found: C, 65.90; H, 19.0; N, 5.28.

Peptide Synthesis of Cyclic Position 3–5 Disulfide Angiotensin Analogs. The peptides were synthesized in either manual or automatic mode (Model ACT200 peptide synthesizer) using Boc amino acids and coupling reagents (DIC, TBTU, HBTU, or BOP) were used in DMF solution in the presence of HOBT (2 equiv). DIPEA was added to keep the reaction mixture at pH 6–7. Acylating amino acids were used in 2-fold excess. Double coupling was applied routinely and the reaction time was 2–3 hr. 40% TFA/CH_2Cl_2 in the presence of 2.5% anisole was used as the deprotecting reagent. A shrink–swell procedure was applied between couplings by washing the resin with ethanol. The HF cleavage procedure included ethanedithiol (0.5%) and anisole (10%) and was performed at 0 °C. Boc amino acids and HOBT were purchased from Advanced Chem Tech. TBTU, HBTU, and BOP were purchased from Richelieu Biotechnologies. The amino acids were used with permanent protecting groups as Arg(Tos), Tyr(2BrZ), Cys(Acm), Cys(Mbzl), and His(Tos). Boc-Pro(*cis*-4-*S*-MBzl) and Boc-Pro(*trans*-4-*S*-MBzl) were prepared based on literature procedures.[28] Phenylalanine was attached to a 0.5 mequiv/g substituted Merrifield resin in the presence of KF using the Horiki procedure.[32] The peptides were purified with ion exchange resin (Bio-Rex 70, Bio-Rad Laboratories) and by HPLC chromatography using a Beckman Instrument Model 332 HPLC equipped with a Vydac C18 column (5 μm, 10 × 250 mm). The mobile phase consists of two solvents: A (0.05% TFA in water) and B (0.038% TFA in 10% water and 90% acetonitrile). The purity of the peptides was established by analytical HPLC (a Spectra Physics instrument equipped with a Vydac C18 column, 5 μm, 4.6 × 250). The hydrolysis of the peptides was performed in the gas phase using 6 N HCl in the presence of phenol at 110 °C for 24 h. The hydrolyzed amino acids were subjected to a standard dabsylation procedure and the amino acid analyses were done on Beckman Model 126 amino acid analyzer. The peptides having two different mercapto amino acids were oxidized prior to amino acid analysis with performic acid prepared *in situ*. The oxidation of homocysteine and mercaptoproline peptides required very mild conditions; the postoxidation product had to be lyophilized without preliminary concentration under vacuum as is routinely done with cysteine (with such a procedure, recovery of homocysteic or mercaptoprolinesulfonic acids were negligable). The oxidized mercapto amino acids are labeled in the amino acid analyses with an asterisk. Syntheses for compounds previously reported in the literature are not given. FAB-MS spectra were recorded on a Finnigan 3300 spectrometer equipped with a capillaritron gas gun from Phrasor Scientific (Duarte, CA) in acidified glycerol as a matrix.

3–5′,5–3′,Bis[Sar^1,Cys^{3,5}]-AII (0). [Sar^1,Cys^{3,5}]-AII was prepared by standard solid-phase synthetic procedures and cleaved by HF. To a stirred solution of 12 mg (0.012 mol) of crude peptide in 20 mL of H_2O, ammonia (0.03 M) was added slowly to adjust the pH to 8.5. An excess of a solution (0.002 N) of $K_3[Fe(CN)_6]$ was gradually added, and the reaction was monitored with Ellmann's reagent. After completion of the reaction, diluted AcOH was slowly added to lower the pH to 5.5. AG4-X4, an anion exchange resin in the acetate form, was added and the mixture stirred for 1 h after which the resin was removed by filtration and washed, and the filtrates were combined. The cation exchange resin, BioRex 70, H^+ form, was added and the mixture stirred for 1.5 h. The resin was then filtered and washed with H_2O, MeOH, 0.5% AcOH, and H_2O. The arginine-containing peptide was removed by 50% AcOH and the solution lyophilized after dilution with H_2O. The crude product was purified by HPLC (Vydac C18; gradient, 18–35% B; v = 4 mL/min; t = 40 min). After a repeat purification, 2.3 mg (19%) of antiparallel dimer was obtained with a purity greater than 98%. HPLC$_{anal.}$: t_R = 14.5 min; gradient, 5–40% B; t = 25 min, flow = 1.5 mL/min. Amino acid analysis: Sar (0.80), Arg (0.98), Cys (2.0), Tyr (0.80), His (0.99), Pro (0.89), Phe (1.00). FAB-MS ($C_{88}H_{118}N_{26}O_{20}S_4$): M^+/e = 1990 (partially reduced dimer), M^+/e = 996 (reduced monomer).

c[Sar^1,Hcy^3,Mpt^5]-AII (1). Procedure I: Iodine Oxidation. Crude [Sar^1,Hcy^3,Mpt^5]-AII·3HF (100 mg, 91 μmol) was dissolved in a mixture of AcOH (5 mL) and H_2O (25 mL). MeOH (350 mL) was added and temperature of the solution was lowered to 0 °C. Iodine solution in methanol (10^2 M) was slowly added to the stirred reaction mixture until a light brown color was obtained. Cooling was stopped and the oxidation continued over the next 35 min. Ascorbic acid was added to quench any excess of iodine and the pH value of the solution was adjusted carefully to 5.5 with dilute NH_4OH. The procedure to eliminate non-arginine peptides from the reaction mixture was identical to that described for c[Sar^1,Cys^{3,5}]-AII (0). The crude peptide was purified using HPLC (Vydac C_{18}; gradient 18–25% B; v = 4 mL/min; t = 40 min) to yield 13 mg (13.8%) of c[Sar^1,Hcy^3,Mpt^5]-AII with an HPLC purity of 98.5%. HPLC$_{anal.}$: t_R = 12.5 min; gradient, 5–45% B, t = 25 min, flow = 5 mL/min. Amino acid analysis: Sar (0.90), Arg (1.00), Hcy (0.92), Tyr (1.00), Mpt (1.03),

His (0.89), Pro (0.89), Phe (0.92). FAB-MS ($C_{47}H_{63}N_{13}O_{10}S_2$): $M^+/e + 1 = 1034$.

Procedure II: Potassium Ferricyanide Oxidation. Crude [Sar^1,Hcy^3,Mpt^5]-AII·3HF (15 mg, 13 μmol) was dissolved in H_2O (30 mL). Dilute NH_4OH (0.03 M) was added slowly to adjust the pH to 8. An excess of $K_3[Fe(CN)_6]$ solution (0.002 M) was gradually added and the reaction was followed by Ellman's reagent. All steps of the purification procedure were the same as described for the cyclic antiparallel dimer (3–5′,5–3′,bis[Sar^1,-Cys^{3,5}]-AII) below. The HPLC conditions were identical to that for the iodine oxidation above. c[Sar^1,Hcy^3,Mpt^5]-AII (0.3 mg, 2.3% yield) was obtained (this product was identical with the cyclic compound formed by iodine oxidation). Note: no indication of a significant amount of cyclic dimer formations was observed in the course of the reaction (HPLC traces did not show the peaks characterized by the retention time between 14 and 16 min in the gradient described above), nor was a tendency for cyclodimerization noted during the purification.

c[Sar^1,Hcy^3,Mpc^5]-AII (2). Procedure I: Iodine Oxidation. Crude [Sar^1,Hcy^3,Mpc^5]-AII·3HF (100 mg, 91 μmol) was oxidized and purified as described for [Sar^1,Hcy^3,Mpt^5]-AII (1). c[Sar^1,Hcy^3,Mpc^5]-AII (13.5 mg, 14% yield) was obtained with a purity of 98.8%. $HPLC_{anal.}$: t_R = 12.7 min; Vydac C_{18}; gradient, 5–45% B; t = 25 min, v = 1.5 mL/min. Amino acid analysis: Sar (0.92), Arg (0.98), Hcy (1.01)*, Tyr (0.96), Mpc (0.98)*, His (0.97), Pro (1.00), Phe (1.00). FAB-MS ($C_{47}H_{63}N_{13}O_{10}S_2$): $M^+/e + 1 = 1034$.

Procedure II: Potassium Ferricyanide Oxidation. The oxidation procedure of [Sar^1,Hcy^3,Mpc^5]-AII followed exactly the conditions described for the *trans* isomer (1). However, from examination of HPLC chromatograms, oligomeric products were the predominate product. Oxidation of these peptides, i.e., [Sar^1,-Hcy^3,Mpt^5]-AII (1) and [Sar^1,Hcy^3,Mpc^5]-AII (2), under the conditions of fast disulfide–sulfide exchange (thermodynamic control) exemplifies the relative stability of the two cyclic disulfides as estimated by theoretical calculations.[7] Analogs having Hcy (or Cys) in the third position exhibit a higher propensity for cyclization when the fifth position is occupied by Mpt than by Mpc.

c[Sar^1,Cys^3,Mpt^5]-AII (3). Procedure I: Iodine Oxidation with Two Free Sulfhydryl Groups. Crude [Sar^1,Cys^3,Mpc^5]-AII·3HF (100 mg, 92 μmol) was subjected to cyclization using iodine as the oxidant under the conditions described for c[Sar^1,-Hcy^3,Mpt^5]-AII (1). After HPLC purification (Vydac C_{18}; gradient, 15–30% B; v = 4 mL/min; t = 40 min), 7 mg (7.3% yield) of c[Sar^1,Cys^3,Mpt^5]-AII was obtained with HPLC purity of 98.5%. $HPLC_{anal.}$: t_R = 10.45 min; Vydac C_{18}; gradient, 5–45% B; t = 25 min; v = 1.5 mL/min. Amino acid analysis: Sar (1.09), Arg (0.95), Cys (1.00)*, Tyr (1.00), Mpt (1.04)*, His (0.93), Pro (1.14), Phe (1.04). FAB-MS ($C_{46}H_{61}N_{13}O_{10}S_2$): $M^+/e + 1 = 1020$.

Procedure II: One Cysteine Sulfhydryl Function Was Protected with Acm. The Acm protection of one sulfhydryl group of cysteine was introduced in order to facilitate the cyclization of the rather constrained, 11-membered ring of the heterocycle derived from the "Cys-Tyr-Mpc" unit, as was predicted theoretically and by inspection of Drieding models. According Kamber et al.,[10] the pair of SH + S(Acm) serves as a better substrate for iodine oxidation based on the high reactivity of the pair SI + S(Acm). Crude [Sar^1,Cys(Acm)^3,Mpt^5]-AII·3HF (100 mg, 86 μmol) was subjected to the cyclization reaction as described above for two free sulfhydryl groups. No visible progress of reaction was observed by HPLC. Increasing the iodine concentration resulted, after HPLC purification, in only a 4.5 mg (5.1%) yield of c[Sar^1,Cys^3,Mpt^5]-AII as characterized above.

c[Sar^1,Cys^3,Mpc^5]-AII (4). Procedure I: Substrate with Two Free Sulfhydryl Groups. Crude [Sar^1,Cys^3,Mpc^5]-AII·3HF (100 mg, 92 μmol) was oxidized with iodine under the conditions described for c[Sar^1,Hcy^3,Mpt^5]-AII (1). The reaction went to completion in under 5 min. After routine ion exchange on Bio-Rex 70 resin and HPLC purification procedures (Vydac C_{18}; gradient, 16–30% B; v = 4 mL/min; t = 40 min), 19.5 mg (20.8% yield) of c[Sar^1,Cys^3,Mpc^5]-AII was obtained with a purity near 100%. $HPLC_{anal.}$: t_R = 11 min; Vydac C_{18}; gradient, 5–45% B; t = 25 min; v = 1.5 mL/min. Amino acid analysis: Sar (0.83), Arg (1.10), Cys (1.11)*, Tyr (1.07), Mpc (1.09)*, His (1.03), Pro (0.98), Phe (1.00). FAB-MS ($C_{46}H_{61}N_{13}O_{10}S_2$): $M^+/e + 1 = 1020$.

Procedure II: One Cysteine Sulfhydryl Function Was Protected with Acm. Crude [Sar^1,Cys(Acm)^3,Mpc^5]-AII·3HF (100 mg, 86 μmol) was subjected to the above described oxidation conditions; however, no reaction progress was noticed after 1 h. Thus, the conditions were changed, and iodine was added to maintain a deep brown color of the solution. After 30 min, the iodine was quenched with ascorbic acid and products isolated as above. The main product was the linear dimer 5–5′,bis[Sar^1,-Cys(Acm)^3,Mpc^5]-AII, after HPLC purification (Vydac C_{18}; gradient, 15.5–30% B; t = 40 min; v = 4 mL/min which yielded 12.5 mg. HPLC: t_R = 13 min; Vydac C_{18}; gradient, 5–45% B; t = 25 min; v = 1.5 mL/min. FAB-MS ($C_{98}H_{134}N_{28}O_{22}S_4$): $M^+/e + 2 = 2184$. The cyclic monomer was not detected in the reaction mixture. Attempts to oxidize this linear dimer under more severe conditions (80% AcOH, very high iodine concentration) resulted in extensive polymerization. The cyclic dimer was not detected.

Procedure III: One Cysteine Sulfhydryl Function Was Protected with Acm. Crude [Sar^1,Cys(Acm)^3,Mpc^5]-AII·3HF (10 mg, 8.6 μmol) was dissolved in 80% AcOH (150 mL) and the solution cooled to 5 °C. Iodine in methanol (10^2 M) was added to maintain a deep, brown color of the reaction mixture. Cooling was removed, and after 30 min, the iodine was reduced with ascorbic acid and the excess acetic acid was removed under vacuum. After purification under the conditions described for 1, 1.5 mg (14% yield) of cyclic monomer was obtained with approximate purity of 99%.

Note: the constrained ring was formed much more easily when oxidized with a high concentration of iodine in 80% acetic acid, if Acm was introduced as a protecting group on one of the cysteines.

c[Sar^1,Mpc^3,Cys^5]-AII (5). Procedure I: One Cysteine Sulfhydryl Function Was Protected with Acm. Crude [Sar^1,-Mpc^3,Cys(Acm)^5]-AII·3HF (10 mg, 86 μmol) was cyclized by iodine oxidation as described above. The crude, postreaction mixture was purified by ion exchange and HPLC chromatography (Vydac C_{18}; gradient, 15–45% B; v = 4 mL/min; t = 40 min). The following three products were isolated.

c[Sar^1,Mpc^3,Cys^5]-AII. Yield: 2 mg (2.2%). $HPLC_{anal.}$: t_R = 10.8 min; Vydac C_{18}; gradient, 5–45% B; v = 1.5 mL/min. FAB-MS ($C_{46}H_{61}N_{13}O_{10}S_2$): $M^+/e + 1 = 1020$. Amino acid analysis: Sar (1.00), Arg (1.06), Mpc (0.85)*, Tyr (0.89), Cys (1.00)*, His (0.90), Pro (1.04), Phe (1.00).

Note: during lyophylization and purification, very fast cyclodimerization of the cyclic monomer was observed by HPLC chromatography (oligomer and polymerization reaction could not be excluded as well), and as a result, the antiparallel dimer, 3–5′,5–3′,bis[Sar^1,Mpc^3,Cys^5]-AII was formed. Thus, the amount of cyclic monomer obtained in this reaction did not reflect the true initial yield of the cyclization reaction.

3–5′,5–3′,Bis[Sar^1,Mpc^3,Cys^5]-AII (6). Yield: 2 mg (2.2%). $HPLC_{anal.}$: t_R = 11.7 min (the conditions as above). Amino acid analysis: Sar (0.99), Arg (0.97), Mpc (1.03)*, Tyr (089), Cys (0.99)*, His (0.95), Pro (1.02), Phe (1.00); FAB-MS ($C_{92}H_{122}N_{26}O_{20}S_4$): $M^+/e + 1 = 2039$ (dimer), $M^+/e + 1 = 1020$ (this peak arises from fast fragmentation of the cyclic dimer by FAB; the intensity of this peak was much higher than the peak of molecular ion. α-Chymotrypsin hydrolysis confirmed the antiparallel dimer assignment (see below).

3–3′,Bis[Sar^1,Mpc^3,Cys(Acm)^5]-AII. Yield: 10 mg (10.4%). $HPLC_{anal.}$: t_R = 12.9 min (the conditions were the same as above described). FAB-MS ($C_{98}H_{134}N_{28}O_{22}S_4$): $M^+/e + 1 = 2183$ (linear dimer with two Acm protecting function), $M^+/e + 2 = 1093$ (this ion is formed from disulfide bridge cleavage during mass registration), $M^+/e + 2 = 1020$ (very low intensity peak of monomer formed from the contamination of the sample with the traces of parallel dimer; the intensity of parallel dimer peak of these type of compounds is very low and was not detectable in such small concentrations. Increases in iodine concentration during oxidation did not change the composition of the reaction mixture).

Procedure II: One Cysteine Sulfhydryl Function Was Protected with Acm. Crude [Sar^1,Mpc^3,Cys(Acm)^5]-AII·3HF (35 mg, 30 μmol) was oxidized using a high concentration of iodine in 80% AcOH. After 45 min, the reaction was terminated by adding ascorbic acid and purification was routinely performed as above. Three products were isolated: a cyclic monomer (2

mg, 6.3% yield, three times higher than in procedure I), a linear dimer with two cysteines still protected with Acm groups, and a parallel dimer, 3-3',bis[Sar^1,Mpc^3,Cys(Acm)^5]-AII, as identified below (see next procedure). No antiparallel, cyclic dimer was detected during this oxidation procedure.

3-3',5-5',Bis[Sar^1,Mpc^3,Cys^5]-AII (7). Purified 3-3',bis-[Sar^1,Mpc^3,Cys(Acm)^5]-AII (8.5 mg, 3.8 μmol) was dissolved in 100 mL of 80% AcOH and the solution was cooled to 5 °C. Iodine in methanol (10^2 M) was added to maintain a deep, brown color of the reaction mixture (about 35 mL). The reaction was terminated with ascorbic acid after 30 min and the excess acetic acid removed under vacuum. The HPLC purification (Vydac C_{18}; gradient, 16–23% B; v = 4 mL/min; t = 40 min) of oxidized product gave 1.5 mg (19% yield) of cyclic, parallel dimer, 3-3',5-5',bis[Sar^1,Mpc^3,Cys^5]-AII. HPLC$_{anal.}$: t_R = 13.2; Vydac C_{18}; gradient, 18–35% B; t = 25 min; v = 1.5 mL/min. Amino acid analysis: Sar (0.89), Arg (1.01), Mpc (0.97)*, Tyr (0.98), Cys (1.00)*, His (0.92), Pro (1.03), Phe (1.00). FAB-MS ($C_{97}H_{122}N_{26}O_{20}S_4$): M^+/e = 2038 (cyclic dimer), $M^+/e + 1$ = 1020 (this ion belong to a monomer formed during mass registration), $m^+/e + 2$ = 1093 (very low intensity peak formed from fragmentation of traces of the substrate present in the purified material). α-Chymotrypsin hydrolysis (see below) confirmed that the cyclic dimer has the parallel arrangement.

c[Sar^1,Mpc^3,Hcy^5]-AII (8). Crude [Sar^1,Mpc^3,Hcy^5]-AII·3HF (100 mg, 91 μmol) was oxidized under the conditions described for [Sar^1,Hcy^3,Mpt^5]-AII (1). After 40 min, the reaction was terminated with ascorbic acid and purification was routinely performed. The HPLC purification (Vydac C_{18}; gradient, 16–25% B; v = 4 mL/min; t = 40 min) gave 13 mg (13.8% yield) of cyclic monomer with a purity of approximately 99%. HPLC$_{anal.}$: t_R = 10.8 min; Vydac C_{18}; gradient, 5–45% B; t = 25 min; v = 1.5 mL/min. Amino acid analysis: Sar (0.85), Arg (0.94), Mpc (0.97)*, Tyr (1.02), Hcy (1.00)*, His (0.97), Pro (0.93), Phe (1.00). FAB-MS ($C_{47}H_{63}N_{13}O_{10}S_2$): $M^+/e + 1$ = 1034.

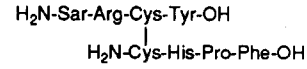
c[Sar^1,Mpt^3,Cys^5]-AII (9). Crude [Sar^1,Mpt^3,Cys^5]-AII·3HF (100 mg, 91 μmol) was dissolved in a mixture of AcOH (10 mL) and H_2O (25 mL). Methanol (350 mL) was added and the temperature lowered to 5 °C. Iodine in methanol (10^2 M) was added to keep the color of the reaction mixture deep brown. After 30 min, the crude arginine peptides were isolated on Bio-Rex 70 resin and lyophilized. The concentration of cyclic monomer formed during the oxidation, as observed on HPLC, was dramatically decreased after lyophilization. Further loss of product was noticed during purification by HPLC (Vydac C_{18}; gradient, 17–35% B; t = 40 min; v = 4 mL/min). The overall yield of c[Sar^1,Mpt^3,Cys^5]-AII was 4 mg (4.2%). HPLC$_{anal.}$: t_R = 11.4 min; gradient, 5–45% B; v = 1.5 mL/min; t = 25 min. Amino acid analysis: Sar (1.10), Arg (1.02), Mpt (1.00)*, Tyr (1.02), Cys (1.12)*, His (0.97), Pro (0.88), Phe (1.00). FAB-MS ($C_{40}H_{61}N_{13}O_{10}S_2$): $M^+/e + 1$ = 1020.

c[Sar^1,Mpt^3,Hcy^5]-AII (10). Crude [Sar^1,Mpt^3,Hcy^5]-AII·3HF (140 mg, 130 μmol) was oxidized as described above. Oligomerization of the cyclic monomer occurred very rapidly during HPLC purification, as happened with the Cys^5 analog. The yield of cyclic monomer after HPLC purification (Vydac C_{18}; gradient, 17–30% B; t = 40 min; v = 4 mL/min) was 7 mg (5.1%) of 98.8% purity. HPLC$_{anal.}$: t_R = 11.9 min; Vydac C_{18}; gradient, 5–45% B; v = 1.5 mL/min.

Note: The two cyclic peptides, c[Sar^1,Mpt^3,Hcy^5]-AII (10) and c[Sar^1,Mpt^3,Cys^5]-AII (9), were very unstable as predicted theoretically as well as by inspection of Dreiding models. No tendency to form cyclic dimers was observed as both cyclic monomers were transformed during purification procedures; HPLC traces indicate formation of oligomeric products.

α-Chymotrypsin Hydrolysis[33] To Determine Dimer Orientation. 0.1 mg of substrate was dissolved in 2 mL of phosphate buffer (pH = 8 or 6), at 25 °C and 0.1 mg of α-chymotrypsin was added. To follow the progress of the reaction, 50 μL of the hydrolysate was dissolved in 600 μL of 0.005% TFA and an HPLC analysis was performed.

(1) 3-5',5-3',Bis[Sar^1,Cys^{3,5}]-AII (Antiparallel, Cyclic Dimer). The hydrolysis was performed at pH = 6 for 15 min. Only one peak was observed, t_R = 12.46 min, on HPLC (Vydac C_{18}; gradient, 5–30% B; t = 25 min; v = 1.5 mL/min; substrate t_R = 17.74 min) consistent with an antiparallel dimer with the most susceptible bond for hydrolysis occurring between tyrosine-4 and cysteine-5 (See Figure 2). As a result, two identical products are formed, both containing an unsymmetric, disulfide bond:



Note: when the hydrolysis was monitored after 2 min, three peaks were observed on HPLC: t_R = 12.46 min (both Tyr-Cys peptide bonds hydrolyzed), t_R = 17.74 min (substrate), and t_R = 18.38 min (product of partial hydrolysis; only one Tyr-Cys peptide bond broken).

(2) 3-5',5-3',Bis[Sar^1,Mpc^3,Cys^5]-AII (Antiparallel, Cyclic Dimer). The hydrolysis was performed at pH = 8 for 2 h; the reaction was much slower than the previous case. HPLC: Vydac C_{18}; gradient, 5–45% B; t = 25 min; v = 1.5 mL/min. As would be predicted for the antiparallel dimer, only one hydrolysis peak was observed, t_R = 9.41 min, as a result of Tyr-Cys peptide bond cleavage. The retention time of the parent dimer was 11.7 min. FAB-MS: $M^+/e + 1$ = 1038, $M^+/e + 1$ = 537 (Sar-Arg-Mpc-Tyr), $M^+/e + 1$ = 502 (Cys-His-Pro-Phe).

(3) 3-3',5-5',Bis[Sar^1,Mpc^3,Cys^5]-AII (Parallel, Cyclic Dimer). The hydrolysis was performed at pH = 8 for 5 min. The HPLC traces were identical over 2 h of examination. HPLC: Vydac C_{18}; gradient, 5–45% B; t = 25 min; v = 1.5 mL/min. Two main and one minor peaks were observed: t_R = 7.85, 8.90 (minor), and 12.71 min. After examination of a hydrolysate of the linear precursor (see next experiment), the peaks were identified as follows. t_R = 7.85 min, bis[Sar-Arg-Mpc-Tyr]; FAB-MS: $M^+/e + 1$ = 1073, $M^+/e + 1$ = 537. t_R = 12.71 min, bis[Cys-His-Pro-Phe]; FAB-MS: $M^+/e + 1$ = 1003, $M^+/e + 1$ = 502. t_R = 8.90 min (minor peak), Cys(Acm)-His-Pro-Phe (this fragment arose from traces of the linear precursor of the cyclic dimer which was present in the purified material).

(4) 3-3',Bis[Sar^1,Mpc^3,Cys(Acm)^5]-AII (Linear Precursor of Cyclic, Parallel Dimer). The hydrolysis was performed at pH = 8 for 1.5 h. HPLC: Vydac C_{18}; gradient, 5–45% B; t = 25 min; v = 1.5 mL/min. Two major and a third, minor peak were observed: t_R = 7.74, 8.75, and 12.48 min (minor peak). They were identified as bis[Sar-Arg-Mpc-Tyr], t_R = 7.74 min; Cys-(Acm)-His-Pro-Phe, t_R = 8.75 min, and bis[Cys-His-Pro-Phe], t_R = 12.48 min (minor peak). This peptide fragment arose from contamination of the substrate with the parallel dimer.

Note: The HPLC trace of the mixture of the CT hydrolyzates of 3-3',5-5',bis[Sar^1,Mpc^3,Cys^5]-AII and 3-3',bis[Sar^1,Mpc^3,Cys-(Acm)^5]-AII showed only three peaks corresponding exactly to the fragments described above.

Peptide Synthesis of Cyclic 3-8, 5-8, and 3-7 Disulfide Angiotensin Analogs (11–13). The peptides were prepared by solid-phase peptide synthetic techniques on appropriately substituted PAM resins, using an Applied Biosystems 430A instrument with N^{α}-Boc protected amino acids, orthogonally protected as follows: tosyl (Tos) for Arg, *para*-methylbenzyl (MeBzl) for cysteine, [(2-bromobenzyl)oxy]carbonyl (2Br-Z) for tyrosine, and (benzyloxy)methyl (Bom) or 2,4-dinitrophenyl (Dnp) for histidine. All amino acids were coupled as the symmetrical anhydride (DCC-mediated, 2-fold excess except for Boc-Arg(Tos) and Boc-His(Bom), which were coupled as the HOBt-activated esters (4-fold excess). The N^{α}-Boc protecting groups were removed with two treatments of 50% trifluoroacetic acid (TFA) in DCM (2 and 20 min each). For peptides utilizing His(DNP), the DNP group was removed prior to final removal of the N-terminal Boc group by treatment with 10% thiophenol in DMF (1 × 5 min, 1 × 60 min) and then washed with DMF (2×), MeOH (3×), H_2O (2×), DMF (3×), and DCM (3×). After removal of the N-terminal Boc group as above, the protected peptide resin was cleaved and completely deprotected by treatment with anhydrous hydrogen fluoride (HF, 90%) with dimethyl sulfide (DMS, 5%) and anisole (5%) at 0 °C for 60 min. The resin was washed with ethyl ether (3 × 50 mL), and extracted with 30% aqueous acetic acid (3 × 50 mL). The peptide extract was concentrated under reduced pressure, resuspended in water (100 mL), and lyophilized. The crude peptide mixture was suspended in water (0.2 mg/mL), and the pH adjusted to 8.5 and maintained with dilute ammonium hydroxide. The solution was tritated with 0.01 N aqueous

potassium ferricyanide until a yellow color was achieved (1 h). The pH was then adjusted to 5.5 with 10% aqueous HOAc. After addition of anion-exchange resin (AG4-X4 acetate form, 10 equiv), the solution was stirred for 1 h, filtered, concentrated under reduced pressure, and lyophilized. The crude peptide mixture was purified by preparative HPLC on a Vydac 218TP1022 (2.2 × 25.0 cm) with a mobile phase of 0.1% aqueous trifluoroacetic acid and increasing concentrations of 0.1% trifluoroacetic acid in acetonitrile. Fractions were combined based upon analytical HPLC evaluation and concentrated under reduced pressure to remove the acetonitrile and lyophilized. The purified peptides were analyzed for homogeneity and structural integrity by analytical HPLC, capillary zone electrophoresis, proton NMR, and fast atom bombardment mass spectrometry (FAB-MS).

Peptide Synthesis of Cyclic Lys-5 Lactam Angiotensin Analogs (14–15). The peptides were prepared by solid-phase peptide synthetic techniques on N^{α}-Boc-Phe-PAM resins using an Applied Biosystems 430A instrument with N^{α}-Boc protected amino acids, orthogonally protected as follows: Tos for Arg, 2Br-Z for tyrosine, Bom for histidine, fluorenylmethyl (Fm) for aspartic or glutamic acid, and fluorenylmethyloxycarbonyl (Fmoc) for lysine. All amino acids were coupled as the symmetrical anhydride (DCC-mediated, 2-fold excess except for Boc-Arg-(Tos) and Boc-His(Bom), which were coupled as the HOBt-activated esters (4-fold excess). The N^{α}-Boc protecting groups were removed with two treatments of 50% trifluoroacetic acid (TFA) in DCM (2 and 20 min each) and the N^{α}-Fmoc protecting groups were removed with two treatments of 20% piperidine in DMF (10 min each). Prior to removal of the N^{α}-Fmoc protecting group of asapartic or glutamic acid in position 3, the peptide resin was transferred to a manual shaker, treated with 20% piperidine in DMF (2 × 10 min), and washed with DMF (3×), 10% MeOH (3×), and DMF (3×). The side chain lactam was formed by treatment with N,N'-diisopropylcarbodiimide (2 mmol) and N-hydroxytriazole (2 mmol) in DMF. After shaking overnight, ninhydrin indicated that the cyclization was approximately 90% complete. Fresh reagents were added, mixed for an additional 4 h, washed with DMF (3×), and dried under vacuum, and the syntheses were completed on the 430A synthesizer. The protected peptide resin was cleaved and completely deprotected by treatment with anhydrous hydrogen fluoride (HF, 90%) with dimethyl sulfide (DMS, 5%) and anisole (5%) at 0 °C for 60 min. The resin was washed with ethyl ether (3 × 50 mL) and extracted with 30% aqueous acetic acid (3 × 50 mL). The peptide extract was concentrated under reduced pressure, resuspended in water (100 mL) and lyophilized. The crude peptide mixture was purified by preparative HPLC on a Vydac 218TP1022 (2.2 × 25.0 cm) with a mobile phase of 0.1% aqueous trifluoroacetic acid and increasing concentrations of 0.1% trifluoroacetic acid in acetonitrile. Fractions were combined on the basis of analytical HPLC evaluation, concentrated under reduced pressure to remove the acetonitrile, and lyophilized. The purified peptides were analyzed for homogeneity and structural integrity by analytical HPLC, capillary zone electrophoresis, proton NMR, and fast atom bombardment mass spectrometry (FAB-MS).

Peptide Synthesis of Cyclic Orn-5 Lactam Angiotensin Analogs (16). The peptides were prepared by solid-phase peptide synthetic techniques on N^{α}-Boc-Phe-PAM resins using an Applied Biosystems 430A instrument with N^{α}-Boc- and Fmoc-protected amino acids, orthogonally protected as follows: Boc-Arg(Tos), Boc-His(Bom), Fmoc-Asp(tBu), Fmoc-Tyr(Bzl), and Fmoc-Orn(Boc). All amino acids were coupled as the symmetrical anhydride (DCC-mediated, 2-fold excess), except for Boc-Arg-(Tos) and Boc-His(Bom), which were coupled as the HOBt-activated esters (4-fold excess). The N^{α}-Boc protecting groups were removed with two treatments of 50% trifluoroacetic acid (TFA) in DCM (2 and 20 min each), and the N^{α}-Fmoc protecting groups were removed with two treatments of 20% piperidine in DMF (10 min each). Prior to removal of the N^{α}-Fmoc protecting group of aspartic acid, the peptide resin was transferred to a manual shaker, treated with 50% TFA in DMF (2 × 10 min), and washed with DMF (3×), 10% DIEA in DCM (2 × 2 min), and DMF (3×). The side chain lactam was formed by treatment with N,N'-diisopropylcarbodiimide (2 mmol) and N-hydroxytriazole (2 mmol) in DMF. After shaking overnight, a ninhydrin test indicated that the cyclization was approximately 90% complete. Fresh reagents were added, mixed for an additional 4 h, and washed with DMF (3×), and the N-terminal Fmoc group was removed as above. The resin was washed with DMF (3×) and DCM (3×) and dried under reduced pressure and the synthesis was completed on the 430A synthesizer. The protected peptide resin was cleaved and completely deprotected by treatment with anhydrous hydrogen fluoride (HF, 90%) with dimethyl sulfide (DMS, 5%) and anisole (5%) at 0 °C for 60 min. The resin was washed with ethyl ether (3 × 50 mL) and extracted with 30% aqueous acetic acid (3 × 50 mL). The peptide extract was concentrated under reduced pressure, resuspended in water (100 mL), and lyophilized. The crude peptide mixture was purified by preparative HPLC on a Vydac 218TP1022 (2.2 × 25.0 cm) with a mobile phase of 0.1% aqueous trifluoroacetic acid and increasing concentrations of 0.1% trifluoroacetic acid in acetonitrile. Fractions were combined on the basis of analytical HPLC evaluation, concentrated under reduced pressure to remove the acetonitrile, and lyophilized. The purified peptide was analyzed for homogeneity and structural integrity by analytical HPLC, capillary zone electrophoresis, proton NMR, and fast atom bombardment mass spectrometry (FAB-MS).

Angiotensin II-Type 1 (AT1) Receptor Binding Assay. Rat liver membranes were prepared freshly according to a modification of the method of Dudley et al.[11] Binding of [^{3}H]angiotensin II to membranes was conducted in a final volume of 0.25 mL of 50 mM NaH_2PO_4 (pH 7.1) supplemented with 100 μM bacitracin, 0.2% BSA, 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM EGTA, containing 5 mg of original tissue weight of homogenate, 0.5 nM [^{3}H]angiotensin II, and test compound. 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 48R cell harvester. Filters were washed three times with 4 mL of Tris buffer (pH 7.4), then transferred to scintillation vials for counting. Nonspecific binding was defined as radioactivity retained on the filters in the presence of 10 μM saralasin, and specific binding was defined as total binding minus nonspecific binding. IC_{50} values were calculated by weighted nonlinear regression curve-fitting to the mass action equation.[34]

Angiotensin II-Type 2 (AT2) Receptor Binding Assay. Rabbit uterine membranes were prepared freshly according to the method of Dudley *et al.*[11] with the exception that the tissue was disrupted in 20 volumes of ice-cold 20 mM NaH_2PO_4 (pH 7.1). Binding of [^{125}I]angiotensin II to membranes was carried out in a final volume of 0.25 mL of 50 mM NaH_2PO_4 (pH 7.1) supplemented with 100 μM bacitracin, 0.2% BSA, 100 mM NaCl, 10 mM $MgCl_2$, 1 μM EGTA, and 1 μM DUP 753 (to eliminate AT1 binding sites) containing 0.05 nM [^{125}I]angiotensin II, 5 mg of original tissue weight of homogenate, and test compound. Samples were incubated at 25 °C for 3 h, binding was terminated by filtration, and samples were processed as above except that washed filters were transferred to glass tubes and counted in a γ counter.

Vascular Contractility Studies. Vascular rings were prepared from rabbit thoracic aortas as previously described[11] and mounted in 20-mL organ baths containing Krebs-bicarbonate solution maintained at 37 °C and gassed continuously with 5% CO_2 in oxygen. The resting tension was adjusted to 4.0 g and tissues were allowed a 90-min equilibration period before experiments were initiated. The peptides were added cumulatively to the ring preparations until a maximal response was achieved. EC_{50} values were determined graphically from the curves and defined as the concentration of peptide which produces 50% of the maximal contractile response.

Molecular Modeling. The effects of these and other side-chain cyclizations between residues i and $i + 2$ on restriction of the allowed backbone conformations have been modeled using a systematic search approach. The SYBYL molecular modeling program[35] was used to sample all sterically allowed conformations of the cyclic ring systems at a 20° or smaller increment. The use of the relatively rigid proline ring to fix the side chain used in cyclization has a dramatic effect on reducing the conformational flexibility on the resultant bicyclic ring systems and further defines the receptor-bound conformation of AII. Analysis (Kataoka *et al.*[7]) of the accessible conformations available to the

ring systems composed of Cys-Ala-Cys, or Hcy-Ala-Hcy, shows a dramatic restriction of conformations to helical values for the central Ala residue in the case of Cys-Ala-Cys. In the case of Hcy-Ala-Hcy, a Ramachandran plot for the central Ala residue is essentially no different than that for Ac-Ala-NH-CH_3. This implies that the cyclization through the longer Hcy residues imposes little conformational constraint on the backbone conformation of the single, central residue. The degrees of freedom lost upon cyclization have been compromised by the additional bonds in the cycle. To further understand these effects, compatibility of cyclic constraints with β-turn conformations was examined (see Kataoka *et al.*[7]). Identical methodology with appropriately scaled van der Waals radii[36] was used to examine the details of the conformational effects of disulfide cyclization of Hcy-Ala-Mpt and Cys-Ala-Mpt at a 10° torsional increment, where the conformational restriction is quite severe. The proline amide bond was examined for both *cis*- and *trans*-isomeric states.

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