Novel Cyclic Analogs of Angiotensin II with Cyclization between Positions 5 and 7: Conformational and Biological Implications

Wei-Jun Zhang,[†] Gregory V. Nikiforovich,*[,‡] Jacqueline Pérodin,[§] Darren E. Richard,[§] Emanuel Escher,[§] and Garland R. Marshall[†,‡]

Department of Molecular Biology and Pharmacology and Center for Molecular Design, Washington University, St. Louis, Missouri 63130, and Faculté de médecine[c], Université de Sherbrooke, Canada J1H 5N4

Received October 16, 1995[⊗]

To study the conformational features of molecular recognition of angiotensin II (Asp[1]-Arg[2]-Val[3]-Tyr[4]-Val/Ile[5]-His[6]-Pro[7]-Phe[8], AII), the synthesis and biological testing of several cyclic analogs of AII cyclized between positions 5 and 7 have been performed. The synthesized analogs were Sar[1]-Arg[2]-Val[3]-Tyr[4]-cyclo(Cys[5]-His[6]-Pen[7])-Phe[8] **(3)**, Sar[1]-Arg[2]-Val[3]-Tyr[4]-cyclo(Asp[5]-His[6]-Apt[7])-Phe[8] **(4)**, Sar[1]-Arg[2]-Val[3]-Tyr[4]-cyclo(Glu[5]-His[6]-Apt[7])-Phe[8] **(5)**, Sar[1]-Arg[2]-Val[3]-Tyr[4]-cyclo(Cys[5]-His[6]-Mpt[7])-Phe[8] **(6)**, Sar[1]-Arg[2]-Val[3]-Tyr[4]-cyclo(Cys[5]-His[6]-Mpc[7])-Phe[8] **(7)**, Sar[1]-Arg[2]-Val[3]-Tyr[4]-cyclo(Hcy[5]-His[6]-Mpt[7])-Phe[8] **(8)**, and Sar[1]-Arg[2]-Val[3]-Tyr[4]-cyclo(Hcy[5]-His[6]-Mpc[7])-Phe[8] **(9)**, where Apt stands for 4-amino-*trans*-proline, and Mpt and Mpc for 4-mercapto-*trans*- and -*cis*-prolines, respectively. Compound **(9)** showed good affinity at AT-1 receptors, namely a K_D = 20 nM. In functional assays, it showed the characteristics of a weak partial agonist with a relative affinity of 0.26% of that for AII and an intrinsic efficacy, αE, of 0.42. Molecular modeling suggested a possible explanation for this finding: the relatively strong binding and the weak partial agonistic activity of compound **9** are due to interaction with AT-1 receptor of only two functionally important groups, namely, the side chains of the His[6] and Phe[8] residues.

Introduction

During the past two decades, extensive structure−activity studies of angiotensin II (Asp[1]-Arg[2]-Val[3]-Tyr[4]-Val/Ile[5]-His[6]-Pro[7]-Phe[8], AII, AT, angiotensin, **1**), the known pressor and myotropic agent, have been performed to establish a realistic model for the biologically active conformation of AII, i.e., for the conformation it adopts when binding specific receptors (the "receptor-bound" conformation). Conformational studies of AII have been performed by a variety of approaches (e.g., see Duncia et al.[1] and references therein) including physicochemical measurements, energy calculations, and the synthesis and biological testing of conformationally constrained analogs (see Nikiforovich[2]). Several cyclic analogs of AII, mostly stabilized by either lactam[3−5] or disulfide bonds[6] between different backbone positions, were synthesized from 1975 until 1994. Unfortunately, all of these compounds proved to be virtually inactive.

A breakthrough in the study of cyclic AII analogs came with the synthesis of compounds containing a Hcy[3]-Hcy[5] cycle[7,8] which showed practically the same binding potency at AT-1 receptors as AII itself. The cycle in question allows the peptide backbone to adopt a β-turn in the region of the Tyr[4] and Val[5] residues, which had been suggested earlier by independent molecular modeling studies.[9,10] Further development of this model resulted in new potent cyclic analogs, namely cyclo[Sar[1], Cys/Hcy[3], Mpt[5]]AII.[11] However, both energy calculations and NMR data favored an "open turn" model for the cycles of the two Mpt-containing analogs, with $\phi \approx -130°$, $\psi \approx 60°$ for the Tyr[4] residue.[12] This discrepancy suggested that the biologically active conformation(s) of AII and its analogs should not be considered solely in terms of backbone structures, i.e., different kinds of turns, peptide chain reversals, etc., and an attempt was, therefore, made to find a common spatial arrangement of important functional groups for receptor interaction in low-energy conformers of AII and its active analogs. Using the assumption that the aromatic moieties of Tyr[4], His[6], and Phe[8] residues and C-terminal carboxyl were important for biological activity (see Regoli et al.[13]), a model was developed for the receptor-bound conformation of AII[14] which was the basis for the design of a new analog, [Sar[1], D-Tyr[4], Pro[5]]-AII **(2)**. The compound is an agonist with a relatively good affinity toward AT-1 receptors (K_D = 58 nM) and with an EC_{50} of 94 nM (see Tables 1 and 2).

Despite this success of this new model for the biologically active conformation of AII,[14] its verification by experimental methods is still far from complete. Whereas conformations of the AII 3−5 fragment, which bears the crucial Tyr aromatic ring, were studied quite thoroughly by synthesis and biological testing of cyclic analogs (see above) as well as by NMR of these conformationally restricted analogs,[12] fragment AII 5−7 which includes the His imidazole moiety has not been thoroughly investigated with conformational constraints. Therefore, the present study describes the synthesis, biological testing, and molecular modeling studies of several cyclic analogs of AII cyclized between positions 5 and 7, namely, Sar[1]-Arg[2]-Val[3]-Tyr[4]-cyclo(Cys[5]-His[6]-Pen[7])-Phe[8] **(3)**, Sar[1]-Arg[2]-Val[3]-Tyr[4]-cyclo(Asp[5]-His[6]-Apt[7])-Phe[8] **(4)**, Sar[1]-Arg[2]-Val[3]-Tyr[4]-cyclo(Glu[5]-His[6]-Apt[7])-Phe[8] **(5)**, Sar[1]-Arg[2]-Val[3]-Tyr[4]-cyclo(Cys[5]-His[6]-Mpt[7])-Phe[8] **(6)**, Sar[1]-Arg[2]-Val[3]-Tyr[4]-cyclo(Cys[5]-His[6]-Mpc[7])-Phe[8] **(7)**, Sar[1]-Arg[2]-Val[3]-Tyr[4]-cyclo(Hcy[5]-His[6]-Mpt[7])-Phe[8] **(8)**, and Sar[1]-Arg[2]-Val[3]-Tyr[4]-cyclo(Hcy[5]-His[6]-Mpc[7])-Phe[8] **(9)**, where Apt stands for 4-amino-*trans*-proline and Mpt and Mpc for 4-mercapto-*trans*- and -*cis*-prolines, respectively.

[†] Department of Molecular Biology and Pharmacology, Washington University.
[‡] Université de Sherbrooke.
[§] Center for Molecular Design, Washington University.
[⊗] Abstract published in *Advance ACS Abstracts,* June 1, 1996.

S0022-2623(95)00774-6 CCC: $12.00 © 1996 American Chemical Society

Table 1. Binding Data for Angiotensin Analogs

compound	K_D (nM)[a]	relative affinity	Hill coefficient
angiotensin (**1**)	2.0	100	0.97
[Sar^1]AII (control)	0.54	370	1.0
[Sar^1, D-Tyr^4, Pro^5]AII (**2**)	58 ± 15	3.4	1.33
cyclo[Sar^1, Cys^5, Pen^7]AII (**3**)	>10000		
cyclo[Sar^1, Asp^5, Apt^7]AII (**4**)	>10000		
cyclo[Sar^1, Glu^5, Apt^7]AII (**5**)	>10000		
cyclo[Sar^1, Cys^5, Mpt^7]AII (**6**)	>10000		
cyclo[Sar^1, Cys^5, Mpc^7]AII (**7**)	2300 ± 150	0.09	0.77
cyclo[Sar^1, Hcy^5, Mpt^7]AII (**8**)	750 ± 150	0.27	1.12
cyclo[Sar^1, Hcy^5, Mpc^7]AII (**9**)	20 ± 8	10	1.24

[a] K_D values were recalculated from IC_{50} values according to Cheng and Prussof.[26]

Results

Synthesis. Peptides were synthesized by the standard solid phase peptide synthesis techniques using Merrifield resin and Boc strategy. Couplings used 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TbTu), or (benzotriazol-1-yloxy)tris(dimethylamine)phosphonium hexafluorophosphate (Bop) in the presence of HOBt and DIEA, or diisopropylcarbodiimide (DIC) with HOBt. After synthesis, the peptides were cleaved from the resin using HF containing 2.5% anisole and several drops of diethyl mercaptan. After cleavage, the oxidation of the mercaptoproline-containing analogs, **6**−**9**, to form the disulfide bridges was done with two different methods: (A) the iodine method, using I_2, dilute concentration, and low temperature for a short time or (B) the DMSO method,[15] which is more gentle and produces less side products, at the cost of a slower reaction. For the aminoproline-containing analogs, **4** and **5**, Boc-Glu(OFm) and Boc-Asp(OFm) derivatives were used in position 5, and 4-*trans*-aminoproline was used in position 7. After the base-labile side chains of Asp and Glu were removed by 20% piperidine/DMF, the peptides were cyclized on the polymer using Bop as coupling reagent together with HOBt and DIEA in DMF. Completion of the reaction was checked by the Kaiser test. For analog **3**, Boc-β,β-dimethyl-Cys(Mob)-OH, Boc-Pen(Mob)OH, was used in position 7 in the synthesis and was cyclized with Cys in position 5 after HF cleavage (Mob is methyloxybenzyl). In this case, the oxidation was carried out with the iodine method only. In all cases, the crude peptides were purified by reversed phase chromatography. Their homogeneity was established by analytical HPLC, and their molecular weights were confirmed by Fab mass spectrometry. The Apt, Mpt, and Mpc residues used were the pure L-enantiomers.

Biological Data. A. Binding Studies. The results for radioligand binding assays at AT-1 receptors are listed in Table 1. It is evident that just two compounds, the linear peptide **2**, and one of the two cyclic peptides with the largest ring size, namely **9**, showed levels of binding more or less comparable to that of AII itself (compound **1**). Accordingly, compounds **2** and **9**, which have the highest affinities, were submitted to further functional assays. Molecular modeling studies were also performed for compounds **7** and **9** (see below). Compound **2** has been previously studied by molecular modeling.[14]

B. Functional Assays. Table 2 reports the activities of compounds **2** and **9** in contraction assays performed using isolated rabbit aorta. Whereas the linear compound **2** behaves as a full agonist of lesser potency, the biological response of the cyclic compound **9** is much more complicated. Figure 1 shows the dose−response curves of compound **9** in comparison to AII itself. The curve for compound **9** is a typical one for a weak partial agonist. It is noteworthy also that the cyclic compound **9** invokes a very slow contractile response. It takes about 8 times longer for this compound to produce its maximal response in comparison with AII itself; similar effects were observed with some AII-related compounds previously.[29]

Table 2. Functional Data for Angiotensin Analogs

compound	EC_{50} (nM)	relative affinity	αE
angiotensin (**1**)	4.6 ± 1.8	100	1.0
[Sar^1]AII (control)	0.35 ± 0.05	1306	1.0
[Sar^1, D-Tyr^4, Pro^5]AII (**2**)	94 ± 32	4.9	1.0
cyclo[Sar^1, Hcy^5, Mpc^7]AII (**9**)	1767 ± 551	0.26	0.42 ± 0.08

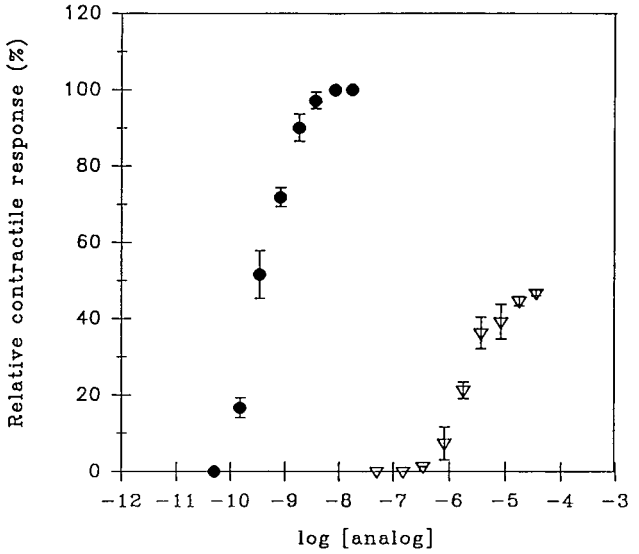

Figure 1. Concentration−response curve of cyclo[Sar^1, Hcy^5, Mpc^7]-AII on rabbit aorta strips (▽, $n = 3$; ●, [Sar^1, Val^5]AII, $n = 3$).

Table 3. Dihedral Angles of Low-Energy Ring Conformations of Compounds **9** and **7**

compound	conformer type	Hcy/Cys^5 ϕ	Hcy/Cys^5 ψ	His^6 ω	His^6 ϕ	His^6 ψ	Mpc^7 ω	Mpc^7 ϕ	Mpc^7 ψ
9	I	−153	141	−179	−68	−34	145	−75	82
	II	−81	82	171	−107	45	98	−75	90
	III	−75	130	175	−130	41	92	−75	97
	IV	−53	−57	175	58	38	69	−75	121
7	I	−162	104	158	−96	35	102	−75	96

Molecular Modeling. Energy calculations on compounds **9** and **7** (data related to compound **7** are given in brackets) yielded 128 (90) different conformations of the peptide backbone which satisfy the energy criterion: $\Delta E = E - E_{min} \leq 10$ kcal/mol. However, the variety of conformations for cyclic moieties within this set of low-energy conformers is much more limited. Namely, there are only 4 (1) conformer(s) for compounds **9** (**7**) based on the values of the ψ_5, ω_{56}, ϕ_6, ψ_6, and ω_{67} dihedral angles, i.e., by those backbone angles located within the cyclic moiety. Dihedral angle values for each type of the low-energy ring structures for both compounds are listed in Table 3.

The low-energy conformers obtained were subjected to a geometric comparison procedure which, for a pair

of conformers belonging to different molecules, included an assessment of the best fit[25] of the spatial arrangement of the chosen atomic centers. Generally, two conformers were regarded as geometrically similar when the corresponding rms value was less than 1.0 Å. Different atomic centers were used for different comparisons (see below).

Discussion

The biological data obtained suggest that conformational limitations imposed by the Pro residue in position 7 are of importance for binding (the drastic loss of affinity for compound **3**), which confirms the same conclusion made on the basis of extensive structure−function data for linear analogs of AII with replacements in position 7.[16] The data of Table 1 suggest also that the disulfide linkage, which is more flexible than the lactam, is more favorable for binding (cf. results for compounds **5** and **8** with the same sizes of cyclic moieties). However, the most interesting observation is the finding of compound **9** that possesses significant potency in binding assays and behaves as a weak partial agonist in functional assays.

All compounds listed in Table 1 retain the functional groups that are known to be most important for the manifestation of AII activity, namely, the side chains of the Tyr, His, and Phe residues, and the C-terminal carboxyl. Therefore, the differences in binding potencies observed in Table 1 for compounds **2**−**9** can be attributed mostly to conformational changes caused by cyclization. In other words, our suggestion is that, for instance, possible low-energy conformations of the potent compound **9** should be compatible with the receptor-bound conformation(s) of AII, whereas low-energy conformations for the nonpotent compound **7** are not despite the fact that the compounds differ only by one valence bond in their cyclic moieties (Hcy for compound **9** *vs* Cys for compound **7**). To validate this suggestion, we have performed geometrical comparison of low-energy conformers for compounds **7** and **9** with low-energy conformers of AII itself (the set of 56 low-energy conformers of AII has been found previously[14]).

The 4−8 fragment of AII is the most important for binding as it contains all those side chains found important for recognition, and it seems natural to perform the comparison at the level of this fragment. Accordingly, the spatial arrangements of all C^{α} and C^{β} atoms in this fragment, as well as of the carbon atom in the C-terminal carboxyl, in all low-energy conformers of compounds **7** and **9** were compared to the same arrangements in all 56 low-energy conformer of AII. Such an approach has been applied earlier to the linear compound **2**, which showed similarity of some low-energy conformers of compound **2** to the proposed receptor-bound conformer of AII.[14] In our case, however, none of the low-energy conformers of either compound **7** or **9** showed strong similarity to any of the low-energy conformers of AII at the level of the 4−8 fragment (i.e., in all cases the rms values were larger than 3 Å for the atom comparisons made). The same negative results were obtained for the smaller tetrapeptide fragments 4−7 and 5−8 (using the same type of atomic centers for comparison). Only tripeptide fragments 4−6 and 6−8 of both compounds **7** and **9** show geometrical similarity to the corresponding tripeptides of AII. It is noteworthy that among the many low-energy 3D structures of AII showing similarity to the 4−6 fragment for compounds **7** and **9**, there are 33 conformers similar to both compounds. On the contrary, the only structure of AII showing strong similarity to fragment 6−8 of the nonpotent compound **7** is not the same as the 12 other AII structures showing similarity to fragment 6−8 of the potent compound **9**. Among those 12 structures, there is also a conformer corresponding to the proposed receptor-bound conformation of AII.[14]

These results indicate that the high affinity of compound **9** toward AT-1 receptors cannot be explained by assuming that the binding mode of compound **9** at AT-1 receptors involves interaction of *all* the functionally important groups with the receptor. Perhaps, only *some* of these groups bind to the receptor in the same mode as AII. If it so, the corresponding groups should be located in fragment 6−8, and not in fragment 4−6, which showed similarity to AII conformers for both the nonpotent compound **7** and the potent compound **9**. The same observation could rationalize the partial agonist response of compound **9**, keeping in mind the data on dose−response curves of rabbit aorta and rat stomach strips, which were obtained in the early 1970s using AII analogs with single substitutions of Tyr, His, or Phe residues by alanine.[13] These data clearly indicated that the efficacy of the AII analogs is virtually abolished in the case of [Ala^8]AII, is significantly reduced for [Ala^4]-AII, and is much less reduced (intrinsic efficacy, αE = 0.8−1.0) for [Ala^6]AII. In other words, out of three functionally important side chains, the His residue is more important for receptor binding than for receptor stimulation. The other two residues, Phe and Tyr, both are involved in receptor stimulation as well, the Phe residue being most important in this respect. (This conclusion is in agreement with studies on angiotensin peptide antagonists.[17,18]) Therefore, the relatively strong binding and the weak partial agonistic activity of compound **9** may be due to interaction with the AT-1 receptor of only two functionally important groups, namely, the side chains of the His and Phe residues. Other possibilities might account, for instance, for the difference between binding to AT receptors in bovine adrenal cortexes (binding assays) and rabbit aortas (functional assays).

An alternative explanation based on different binding sites for peptide agonists and antagonists is also possible. In this case two binding sites for compound **9** are postulated: one which stabilizes the agonist state of the receptor and the other which stabilizes the antagonist state. The binding measurements determine an average of the two states of the receptor, while the functional assay only measures binding to the agonist state of the receptor. In this model, partial agonism is based on the relative affinity of a compound for the two states of the receptor. The effect of mutations of the AT-1 receptor which impact binding affinities of angiotensin and peptide antagonists differentially[30] supports this type of model. Until compound **9** has been screened against such mutations (work in progress), however, the assumption of a common binding mode for agonist and partial agonists remains operative.

Our suggestion of the mode of interaction of compound **9** does not contradict the proposed model of the receptor-

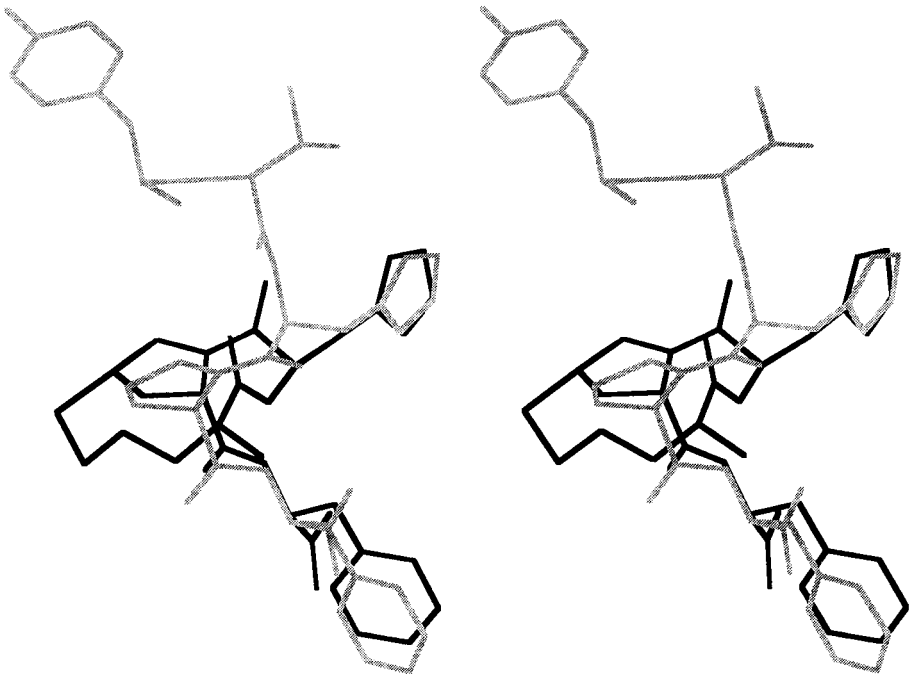

Figure 2. Stereoview of best overlap of low-energy conformers of compound **9** (in bold, fragment 6−8) with the proposed model of AII receptor-bound conformation[14] (in gray, only fragment 4−8 is shown). Only fragments 6−8 were overlapped. All hydrogens are omitted.

bound conformation of AII,[14] since this model is among the low-energy conformers of AII compatible to low-energy conformers of compound **9** at the level of fragment 6−8 only, i.e., AII and compound **9** can overlap in the spatial arrangement of C^{α} and C^{β} atoms of residues 6−9, as well as of the C-terminal carboxyl carbon atom. Those conformers of compound **9** belong to the family of low-energy conformers of the cyclic moiety described as the last entry in Table 3. The corresponding overlap is depicted in Figure 2. The rotamers for the His and Phe side chains of compound **9** shown in Figure 2 were among those obtained at the last step of calculations (see the Experimental Section). This particular spatial arrangement of side chains differs from the lowest-energy one by 8.5 kcal/mol for compound **9** and by 3.8 kcal/mol for AII, both values still being in the allowed "low-energy" range. (Note that this range reflects all uncertainties inherent to any energy calculation, i.e., neglecting the heterogeneous environment when binding to the receptor, ignoring the entropic effects of desolvation and freezing of rotatable bonds, etc.) The conformer of compound **9** shown in Figure 2 would allow interaction with the receptor with "correct" placement of the His and Phe moieties to account for the binding affinity and with an "incorrect" position of the Tyr side chain to account for the decreased efficacy. Interestingly, the same interpretation is also valid for the model of the receptor-bound conformer of AII proposed by other authors.[27,28]

Summarizing, three main conclusions can be proposed based on the results of this study. First, an angiotensin analog possessing a good binding affinity (20 nM, 10% of angiotensin II) to AT-1 receptors with a new type of cyclization in the C-terminal 5−7 fragment has been prepared with constraints placed on this segment for the receptor-bound conformation. Second, the biological studies of this analog combined with molecular modeling can be rationalized with the models of the receptor-bound conformation of AII proposed earlier. Third, these findings suggest using the conformer of the cyclo-(Hcy[5]-His[6]-Mpc[7])-Phe[8] fragment depicted in Figure 2 as a template for the design of a "true" peptidomimetic (those binding to the same receptor site as a parent peptide). Indeed, one can expect that in this case replacement of the crucial Phe aromatic side chain in compound **9** by an aliphatic moiety (like in the case of saralasin) would lead to a compound with antagonistic properties. This would provide a test of the same recognition site for peptide agonists and antagonists.

Experimental Section

A. Synthesis. General. Peptides were synthesized on the Merrifield resin, either manually, or using the ACT200 peptide synthesizer (Advanced Chemtech, Louisville, KY). Homoserine was purchased from Schweizerhall (South Plainfield, NJ) as were *N*-α-Boc-*S*-(4-methoxybenzyl)cysteine and *N*-α-Boc-(4-MeBzl)homocysteine. *N*-α-Boc-Pro(*cis*-4-*S*-MeBzl) and *N*-α-Boc-Pro(*trans*-4-*S*-MeBzl) were prepared based on the literature[19] or by the more efficient stereoselective procedure of Kolodziej et al.[31] *N*-α-Boc-Pro(*trans*-4-NH-Fmoc) was prepared according to the literature.[32] Other Boc-amino acids and HOBt were purchased from Advanced ChemTech (Louisville, KY). TbTu, HbTu, and Bop were purchased from Richelieu Biotechnologies (St-Hyacinthe, Canada). Boc-L-Asp-*β*-fluorenylmethyl ester, Boc-L-Glu-*γ*-fluorenylmethyl ester, and Boc-*S*-(*p*-methoxybenzyl)-L-penicillamine were purchased from Bachem (Torrance, CA). Peptides were cleaved from the solid support using HF containing 5−10% anisole and several drops of ethanedithiol at 0 °C for 1 h. The amino acids used and their protecting groups were Arg(Tos), Tyr(2-Cl-Bzl), Cys(MeBzl), His(Bom), Boc-Asp-*β*-Fm ester, Boc-Glu-*γ*-Fm ester and Boc-Apt(Fmoc), i.e., Boc-Pro(*trans*-4-NH-Fmoc). Peptides containing Arg were first purified with ion exchange resin (Bio-Rex70 Bio-Lab) and then by HPLC chromatography using a Rainin Instruments (Woburn, MA) Model HPXL equipped with a Vydac C_{18} column (5 μM, 10 × 250 mm). The mobile phase consisted of two solvents: A (0.1% TFA in water) and B (acetonitrile), and retention time are given for each peptide. The purity of the peptides was determined using analytical HPLC (SP8800 Spectra-physics, Houston, TX) with a C_{18} column (5 μM, 4.3 × 250 mm). The mobile phase was as follows: A (0.05% TFA in water) and B (0.038% TFA in 10%

water/90% acetonitrile). For amino acid analysis, the peptides were hydrolyzed in the gas phase using 6 N HCl (containing several drops of phenol for Tyr) at 110 °C for 24 h. The hydrolyzed peptides were subjected to a standard dabsylating procedure and were analyzed using a Beckman amino acid analyzer (Model 126, Fullerton, CA). Peptides containing mercapto amino acids were oxidized prior to amino acid analysis with performic acid prepared in situ.

Compound 7: Sar-Arg-Val-Tyr-cyclo(Cys-His-Mpc)-Phe. The crude compound from HF cleavage was oxidized by two main techniques.

(A) The Iodine Method. A 100 mg sample of the crude peptide was dissolved in a mixture of 350 mL of MeOH, 10 mL of AcOH, and 50 mL of H_2O at 5 °C. A solution of I_2/AcOH (0.1 M, 4−40 equiv) was added until a deep brown solution resulted. The solution was adjusted to pH 4 with AcOH. After 20 min, cold ascorbic acid was added to quench the iodine color, and the pH was adjusted to 5.5 with NH_4OH. Biogel resin (H^+) was added to the solution, and it was stirred at 0−5 °C for 1.5 h. The mixture was filtered, and the resin was washed with methanol, water/0.5% AcOH and water. The peptide was removed from the resin using 50% AcOH and water, and the solution was lyophilized and purified by HPLC to yield 21 mg of compound with a purity of 99.8% by analytical HPLC. Analytical data: t_R = 13.61 min (Vydac C_{18} column, gradient 5−45% B, t = 25 min, V = 1.5 mL/min); MS (FAB) m/z 1022.2; amino acid analysis (24 h hydrolysis) Sar (1) 1, Arg (1) 1, Val (1) 0.95, Tyr (1) 1, Cys (1) 0.67, His (1) 1, Mpc (1) 0.6, Phe (1) 0.95. Since the percentages of Cys and Mpc/Mpt were found to be low when using the regular hydrolysis, the amino acid analysis was repeated with the peptide being oxidized with performic acid prior to analysis. This gave the following data: Sar (1) 0.9, Arg (1) 0.9, Val (1) 1, Tyr (1) 0.5, Cys (1) 1, His (1) 0.9, Mpc (1) 1, Phe (1) 1. In this case, Tyr was low.

(B) The DMSO Method. Crude compound (10 mg) was washed three times using ether−mercaptoethanol, 98:2, to remove any organic scavengers. The remaining solid was extracted with 1.4 mL of 25% AcOH and again by 3 mL of 5% AcOH. The combined acetic acid extracts were diluted to 10 mL to a give a final AcOH concentration of 5%. The pH was adjusted to 6.5 using ammonium carbonate, and 20% by volume of DMSO was added to this solution which contained the deprotected peptide at a concentration of approximately 1 mg/mL. The progress of the oxidation reaction was monitored by analytical C_{18} RP-HPLC. The reaction was complete after 10 h. This reaction generated fewer byproducts than the iodine method. The solution was lyophilized and purified using preparative C_{18} RP-HPLC. Two milligrams of cyclic monomer with a purity of 99.2% was obtained. This product was found to be identical to that prepared using the iodine method by RP-HPLC.

Compound 6: Sar-Arg-Val-Tyr-cyclo(Cys-His-Mpt)-Phe. The crude peptide after cleavage (80 mg) was dissolved in 80% acetic acid (60 mL), and several more milliliters of AcOH was added. The solution was poured into methanol (350 mL) chilled to 0 °C, and I_2/AcOH solution (0.1 M, 32.5 mL) was added until the solution became a deep brown color. It was stirred for 20 min at 0−5 °C, and the same procedure was done as for compound **7**. After HF, the crude sample showed a large peak in HPLC and several very small peaks. The purification yielded 15 mg of compound (**6**) with a purity of 99.5%. Analytical data: t_R = 15.17 min (Vidac C_{18}, gradient 5−45% B, t = 25 min, V = 1.5 mL/min); MS (FAB) m/z 1022 (M + 1); amino acid analysis: Sar (1) 0.8, Arg (1) 0.7, Val (1) 1, Tyr (1) 0.9, Cys (1) 1.1, His (1) 1.1, Mpt (1) 0.92.

Compound 9: Sar-Arg-Val-Tyr-cyclo(Hcy-His-Mpc)-Phe. This compound was synthesized twice. The first time, 140 mg of the sample was dissolved, after the HF cleavage, in 70 mL of 80% AcOH, 7 mL of AcOH, and 400 mL of methanol at 5 °C. A large excess of 0.1 M I_2/AcOH was added to the solution until it became deep brown. Then the mixture was stirred for 20 min. The other steps followed the same purification procedure as described above. After purification by HPLC, 11.3 mg of the pure sample was obtained (purity of 99%). Analytical data: t_R = 14.36 min (Vydac C_{18} column; gradient 5−45% B; t = 25 min; V = 1.5 mL/min); MS (FAB) 1036.6; amino acid analysis Sar (1) 1.2, Arg (1) 1.2, Val (1) 1, Tyr (1) 1, Hcy (1) 1, His (1) 1, Mpc (1) 1.1, Phe (1) 1. The second time, the sample was dissolved in 20% AcOH (80 mL) and was poured into methanol (350 mL). The mixture was stirred at 0−5 °C, and 0.1 M I_2/AcOH solution was added until the mixture became dilute brown. The mixture was stirred at this temperature for 30 min and for an additional 10 min at room temperature. Then, the purification procedure described above for the large excess of I_2 was used. Only 7 mg of the pure sample was obtained after lyophilization and purification by RP-HPLC. The weaker oxidation condition gave less yield and more byproducts than the stronger condition. Generally, oxidation of mercaptoproline-containing peptides appeared more efficient at the stronger iodine concentration and for the shorter time.

Compound 8: Sar-Arg-Val-Tyr-cyclo(Hcy-His-Mpt)-Phe. After HF cleavage, 100 mg of the sample was dissolved in 80% AcOH (100 mL), and the solution was poured into methanol (350 mL). The mixture was stirred at 0−5 °C, and I_2/AcOH solution (0.1 M) was added until the mixture was deep brown. The stirring was continued for 20 min, and the product was isolated as described for the iodine method above. The final sample (10.5 mg) was obtained with a purity of 99.1% Analytical data: t_R = 14.0 min (Vydac C_{18} column; gradient 5−45% B; t = 25 min; V = 1.5 mL/min), MS (FAB) 1036.5; amino acid analysis Sar (1) 1.1, Arg (1) 1.1, Val (1) 1, Tyr (1) 1.1, Hcy (1) 1.05, His (1) 1.1, Mpt (1) 0.9, Phe (1) 1.1.

Compounds 4 and 5: Sar-Arg-Val-Tyr-cyclo(Asp-His-Apt)-Phe and Sar-Arg-Val-Tyr-cyclo(Glu-His-Apt)-Phe. The cyclization procedure for compound **4** was as follows: 0.3 mM Boc-Asp-His(Bom)-Apt-Phe resin obtained by removal of the Fmoc and OFm protecting groups with piperidine was swollen in DMF. Bop (3 equiv) and DIEA (6 equiv) were added. The mixture was shaken for 10 h and then washed with DMF (6 × 1 min), C_2H_5OH (2 × 1 min), and CH_2Cl_2 (6 × 1 min). The Kaiser test showed a deep blue color. This procedure was repeated twice, but cyclization was still incomplete. The coupling reagents were then changed to DCCI (6 equiv) and HOBT (6 equiv) in CH_2Cl_2, and the mixture was shaken for 10 h. After washing, a quantitative ninydrin test showed 1.3% of the α-amino group which had not been coupled. The resin was treated with acetic anhydride (0.12 mL), DIEA (0.23 mL), and a catalytic amount of (dimethylamino)pyridine in DMF. The mixture was shaken for 2 h. The quantitative ninhydrin test showed 0.2% uncoupled amine. In the case of compound **5**, 0.6 mM Boc-Glu-His(Bom)-Apt-Phe resin was treated by the same procedure as for **4**. The reaction time needed for cyclization was shorter, perhaps due to the larger ring size in compound **5**. The Kaiser test showed a deep blue color after 10 h of reaction with Bop reagent. The procedure was repeated twice, but the Kaiser test still showed the color. Then the reagent was changed to DCCI and HOBt, and the cyclization reaction was performed for 10 h. The ninhydrin test showed 0.6% of free amine. After an additional 2 h, the ninhydrin test showed 0.075% of free amine. Since the reagent used in the Kaiser test contains pyridine, samples were heated at 110 °C for 3 min. In these conditions, the Fmoc protective group of some amino acids was removed, and a weak gray blue color appeared in the Kaiser test. Peptide resins of compounds **4** and **5** were subsequently coupled after cyclization with Boc-Tyr(2ClBzl), Boc-Val, Boc-Arg(Tos), and Boc-Sar. The 100 mg of peptide resin cleaved by HF yielded approximately 40 mg of the crude peptide samples. One hundred milligrams of each sample was purified by HPLC to get 14 mg of the pure sample for the Asp-containing compound and 16.5 mg for the Glu-containing compound. Analytical data (Vydac C_{18} column, gradient 5−45% B over 25 min, V = 1 mL/min): cyclo[Sar^1,-Asp^5,Apt^7]AII; t_R = 14.6 min; MS (FAB) 1002; amino acid analysis Sar (1) 1.1, Arg (1) 0.93, Val (1) 1, Tyr (1) 0.9, Asp (1) 1.0, His (1) 1.05, Phe (1) 1.0, Apt (1) 1.1. Cyclo[Sar^1, Glu^5, Apt^7]AII; t_R = 16.4 min; MS (FAB) 1016; amino acid analysis Sar (1) 1.1, Arg (1) 1.0, Val (1) 0.9, Tyr (1) 0.9, Glu (1) 1.0, His (1) 1.0, Phe (1) 1.0, Apt (1) 1.2.

Compound 3: Sar-Arg-Val-Tyr-cyclo(Cys-His-Pen)-Phe (0.25 mM) was synthesized using the Boc strategy and the TbTu coupling method. Then 130 mg of crude sample was obtained

after HF treatment which yielded 28 mg of pure producet after HPLC purification. The purity by analytical RP-HPLC was 99%. Analytical data (Vydac C_{18} column, gradient 5−45% B over 25 min, $V = 1$ mL/min): $t_R = 14.30$ min; MS (FAB) m/z 1024 (M + 1); amino acid analysis Sar (1) 1, Arg (1) 1.05, Val (1) 1, Tyr (1) 1, Cys (1) 0.8, His (1) 1.1, Pen (ND), Phe (1) 1.

B. Biological Testing. Binding of AII Analogs. Materials. Bovine adrenal glands were obtained from a nearby slaughterhouse. AII was purchased from Sigma (St. Louis, MO). PD123319 (1-[[4-(dimethylamino)-3-methylphenyl]methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]-pyridine-6-carboxylic acid), a specific AT-2 recetor blocker, was a generous gift of Parke-Davis Warner-Lambert (Ann Arbor, MI). [^{125}I]AII (2000 Ci/mmol) was prepared with iodogen (Pierce, Rockford, IL) as described by Fraker and Speck.[20] The product was purified to apparent homogeneity by HPLC (reverse-phase C-18), and the specific radioactivity was determined by self-displacement in the binding system.

Preparation of Membranes. Bovine adrenal cortexes (dissected free of medullary tissue) were homogenized with eight stokes of a Dounce homogenizer (loose pestle) in a medium containing 110 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 25 mM Tris-HCl pH 7.2, 5 mM KH_2PO_4, 1 mM dithiothreitol, and 2 mM EGTA. After centrifugation at 500*g* for 15 min, the supernatant was centrifuged at 35000*g* for 20 min. The pellet was resuspended in the same medium without EGTA supplemented with glycerol (14% v/v) and sorbitol (1.4% w/v), at a concentration of 20−30 mg of protein/mL). The membranes were stored at −70 °C until used for AII binding studies.

AII Competition Studies. AII binding studies were performed in the presence of 25 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM $MgCl_2$, and 0.1% bovine serum albumin (BSA) in a total assay volume of 500 μL. PD123319 (5 μM) was also added to the medium in order to inhibit AT-2 binding. Membranes (25 μg of protein) were incubated for 45 min at room temperature. Binding of [^{125}I]AII (0.05 nM) was challenged with increasing concentrations of AII or AII analogs. Bound radioactivity was separated from free ligand by filtration through GF/C filters presoaked in the binding buffer. The nonspecific binding was mesured in the presence of 1 μM AII. The receptor-bound radioactivity was analyzed by γ counting.

Biological Assays on Rabbit Aorta Strips. New Zealand rabbits of either sex (1.5−2.5 kg) were killed by stunning and carotid exsanguination. The thoracic aorta was rapidly removed after a full-length thoracic laparotomy and immersed in oxygenated Krebs solution. The excised aorta was mounted onto a glass rod, and adipose tissue was removed and then cut into 5 mm rings. The tissues were suspended in 5 mL organ baths containing continuously oxygenated Krebs solution, changed every 15 min and maintained at 37 °C. An initial tension of 2 g was applied and repeatedly readjusted until stabilized (averaging a 90 min incubation period). Tissue contractions were measured isometrically using force transducers (Grass FT03) and recorded with a Grass polygraph (Grass, Quincy, Mass).

C. Molecular Modeling. Energy calculations for the cyclic compounds **9** and **7** (data related to compound **7** are given in parentheses) were performed using the ECEPP potential field.[21,22] Dihedral angles were the only variables in the process of energy minimization, since rigid valence geometry with a planar *trans*-peptide bonds was assumed (both *trans* and *cis* peptide bonds were examined for the Mpc residue; in this case, as well as for ω angles (dihedral angles of peptide bonds) within the cyclic moiety, the ω_{56} and ω_{67} dihedral angles were allowed to rotate). The valence geometry and atomic charges at the mercaptoprolines were calculated by the use of the SYBYL program (the Tripos force field). Aliphatic and aromatic hydrogens were generally included in united atomic centers of CH_n type; only H^α atoms and H^δ atoms of prolines were described explicitly. The calculation protocol consists of five build-up calculation steps from cyclic 5−7 fragments to 4−8, 3−8 (this step was omitted for compound **7**), 2−8 fragments, and, finally, to the entire molecule. At the first step of the calculations, all possible combinations of local minima of *E*, *F*, *C*, *A*, and *A** types (according to the Zimmerman's notation[23]) for the peptide backbone for each amino acid residue were considered. Generally, the same minima were considered also for each residue, which was addded at any sequential step of the buildup procedure. For Pro and Mpc residues, minima of *F*, *C*, and *A* types were considered. The minima of *E*, *A*, *E**, and *A** types were considered for the N-terminal Sar[1] residues, and the minima of *E*, *C*, and *A** types were considered for the C-terminal Phe[8] residues. Two filters were used to eliminate conformers from further consideration. First, only the backbone structures selected at the previous step by $E - E_{min} < \Delta E = 10$ kcal/mol were considered at subsequent steps. Second, from the set of low-energy structures obtained at the previous step only those differing by more than 40° in at least one value of any backbone dihedral angle were selected for the next step. In this way, at each of the five steps, the following numbers of low-energy backbone conformers were selected for compounds **9** and **7**: 16 (13), 108 (78), 195, 68 (130), and 128 (90). The dihedral angle values of side chain groups (χ_i's) and of the terminal groups of the backbone (ϕ_1 and ψ_8) were optimized before energy minimization to achieve their most favorable spatial arrangements according to an algorithm described elsewhere.[24] After that, energy minimization involved all dihedral angles. Some additional calculations were performed for compound **9**. Namely, three possible rotamers of the χ_1 dihedral angle (g^+, t, and g^-) for the Tyr[4], His[6], and Phe[8] residues were separately considered for those backbone structures compatible to the backbone structures of the 6−8 fragment of AII. These calculations yield 16 conformers within the energy range of 10 kcal/mol.

Acknowledgment. This work was supported by NIH Grants GM24483 and HL54085 (Washington University) and by the Medical Research Council of Canada. The authors are grateful to Dr. David Chalmers for his valuable help in the preparation of the manuscript.

References

(1) Duncia, J. V.; Chiu, A. T.; Carini, D. J.; Gregory, G. B.; Johnson, A. L.; Price, W. A.; Wells, G. J.; Wong, P. C.; Calabrese, J. C.; Timmermans, P. B. M. W. M. The Discovery of Potent Nonpeptide Angiotensin II Receptor Antagonists: A New Class of Potent Antihypertensives. *J. Med. Chem.* **1990**, *33*, 1312−1329.

(2) Nikiforovich, G. V. Computational molecular modeling in peptide design. *Int. J. Pept. Protein Res.* **1994**, *44*, 513−531.

(3) Chipens, G.; Nikiforovich, G.; Mutulis, F.; Veretennikova, N.; Vosekalna, I.; Sosnov, A.; Polevaya, L.; Ancans, J.; Mishlyakova, N.; Liepinsh, E.; Sekacis, I.; Breslav, M. Cyclic analogs of linear peptides. In *Peptides. Structure and Biological Function. Proceedings of the Sixth American Peptide Symposium*; Gross, E., Meienhofer, J., Eds.; Pierce Chemical Co.: Rockford, IL, 1979; pp 567−570.

(4) De Coen, J.-L.; Ralston, E.; Durieus, J. P.; Loffet, A. Cyclic angiotensin. In *Peptides: Chemistry, Structure and Biology. Proceedings of the 4th American Peptide Symposium*; Walter, R., Meienhofer, J., Eds.; Pierce Chemical Co.: Ann Arbor, MI, 1975; pp 553−558.

(5) Matsoukas, J. M.; Hondrelis, J.; Agelis, G.; Barlos, K.; Gatos, D.; Ganter, R.; Moore, D.; Moore, G. J. Novel Synthesis of Cyclic Amide-Linked Analogues of Angiotensins II and III. *J. Med. Chem.* **1994**, *37*, 2958−2969.

(6) Miranda, A.; Juliano, L. Conformationally restricted analogs af angiotensin II: Titration and biological activity. *Braz. J. Med. Biol. Res.* **1988**, *21*, 903−914.

(7) Spear, K. L.; Brown, M. S.; Reinhard, E. J.; McMahon, E. G.; Olins, G. M.; Palomo, M. A.; Patton, D. R. Conformational Restriction of Angiotensin II: Cyclic Analogues Having High Potency. *J. Med. Chem.* **1990**, *33*, 1935−1940.

(8) Sugg, E. E.; Dolan, C. A.; Patchett, A. A.; Chang, R. S. L.; Faust, K. A.; Lotti, V. J. Cyclic Disulfide Analogs of [Sar[1], Ile[8]]-Angiotensin II. In *Peptides: Chemistry, Structure and Biology. Proceedings of the Eleventh American Peptide Symposium*; Rivier, J., Marshall, G. R., Eds.; ESCOM Publishers: Leiden, 1989; pp 305−306.

(9) Turk, J.; Needleman, P.; Marshall, G. R. Analogues of Angiotensin II with Restricted Conformational Freedom, Including a New Antagonist. *Mol. Pharmacol.* **1976**, *12*, 217−224.

(10) Chipens, G. I.; Ancan, Y. E.; Nikiforovich, G. V.; Balodis, Y. Y.; Makarova, N. A. Synthesis and Conformational Analysis of New Angiotensin Analogues Containing Aza-α-Homoamino Acids. In

Peptides 1978; Siemion, I. Z., Kupryszewski, G., Eds.; Wroclaw University Press: Wroclaw, 1979; pp 415−419.
(11) Plucinska, K.; Kataoka, T.; Yodo, M.; Cody, W. L.; He, J. X.; Humblet, C.; Lu, G. H.; Lunney, E.; Major, T. C.; Panek, R. L.; Schelkun, P.; Skeean, R.; Marshall, G. R. Multiple Binding Modes for The Receptor-Bound Conformations of Cyclic AII Agonists. *J. Med. Chem.* **1993**, *36*, 1902−1913.
(12) Nikiforovich, G. V.; Kao, J. L.-F.; Plucinska, K.; Zhang, W. J.; Marshall, G. R. Conformational Analysis of Two Cyclic Analogs of Angiotensin: Implications for the Biologically Active Conformation. *Biochemistry* **1994**, *33*, 3591−3598.
(13) Regoli, D.; Park, W. K.; Rioux, F. Pharmacology of Angiotensin. *Pharm. Rev.* **1974**, *26*, 69−123.
(14) Nikiforovich, G. V.; Marshall, G. R. Three-dimensional recognition requirements for angiotensin agonists: A novel solution for an old problem. *Biochem. Biophys. Res. Commun.* **1993**, *195*, 222−228.
(15) Tam, J. P.; Wu, C.-R.; Liu, W.; Zhong, J. W. Disulfide Bond Formation in Peptides by Dimethyl Sulfoxide. Scope and Application. *J. Am. Chem. Soc.* **1991**, *113*, 6657.
(16) Samanen, J.; Cash, T.; Narindray, D.; Brandeis, E.; Adams, W., Jr; Weideman, H.; Yellin, T. An Investigation of Angiotensin II Agonist and Antagonist Analogues with 5,5-Dimethylthiazolidine-4-carboxylic Acid and Other Constrained Amino Acids. *J. Med. Chem.* **1991**, *34*, 3036−3043.
(17) Marshall, G. R.; Vine, W.; Needleman, P. A specific competitive inhibitor of angiotensin II. *Proc. Natl. Ac. Sci. U.S.A.* **1970**, *67*, 1624−1630.
(18) Samanen, J.; Cash, T.; Narindray, D.; Brandeis, E.; Yellin, T.; Regoli, D. The Role of Position 4 in Angiotensin II Antagonism: A Structure-Activity Study. *J. Med. Chem.* **1989**, *32*, 1366−1370.
(19) Eswarakrishnan, V.; Lamar, F. Sulfenic acids and related compounds. 13. Unsymmetrical disulfides based on methyl-4-mercapto-butanesulfinate and 4(S)- or 4(R)-mercaptoprolines. *J. Org. Chem.* **1981**, *46*, 4182.
(20) Fraker, P. J.; Speck, J. C. Protein and cell iodination with a sparingly soluble chloroamine, 1,3,4,6-tetrachloro-3α,6α-diphenylglycoloryl. *Biochem. Biophys. Res. Commun.* **1978**, *80*, 849−857.
(21) Dunfield, L. G.; Burgess, A. W.; Scheraga, H. A. Energy Parameters in Polypeptides. 8. Empirical Potential Energy Algorithm for the Conformational Analysis of Large Molecules. *J. Phys. Chem.* **1978**, *82*, 2609−2616.
(22) Nemethy, G.; Pottle, M. S.; Scheraga, H. A. Energy Parameters in Polypeptides. 9. Updating of Geometrical Parameters, Non-bonded Interactions, and Hydrogen Bond Interactions for the Naturally Occuring Amino Acids. *J. Phys. Chem.* **1983**, *87*, 1883−1887.
(23) Zimmerman, S. S.; Scheraga, H. A. Influence of Local Interactions on Protein Structure. I. Conformational Energy Studies of N-Acetyl-N′-Methylamides of Pro-X and X-Pro Dipeptides. *Biopolymers* **1977**, *16*, 811−843.
(24) Nikiforovich, G. V.; Hruby, V. J.; Prakash, O.; Gehrig, C. A. Topographical Requirements for Delta-Selective Opioid Peptides. *Biopolymers* **1991**, *31*, 941−955.
(25) Nyburg, S. C. Some uses of a best molecular fit routine. *Acta Crystallogr.* **1974**, *B30* (part I), 251−253.
(26) Cheng, Y.; Prussof, W. M. Relationship between the inhibition constant (Ki) and the concentration of inhibitor which causes 50% inhibition (IC50) of an enzymatic reaction. *Biochem. Pharmacol.* **1978**, *22*, 3099−3108.
(27) Samanen, J. M.; Peishoff, C. E.; Keenan, R. M.; Weinstock, J. Refinement of a molecular model of angiotensin II employed in the discovery of potent nonpeptide antagonists. *Bioorg. Med. Chem. Lett.* **1992**, *3*, 909−914.
(28) Joseph, M. P.; Maigret, B.; Scheraga, H. A. Proposals for the angiotensin II receptor-bound conformation by comparative computer modeling of AII and cyclic analogs. *Int. J. Pept. Protein Res.* **1995**, *46*, 514−526.
(29) Bosse, R.; Gerold, M.; Fischli, W.; Holck, M.; Escher, E. An Angiotensin with Prolonged Action and Blood Pressure-Lowering Properties *J. Cardiovasc. Pharmacol.* **1990**, *16* (suppl. 4), S50−S55.
(30) Hjorth, S. A.; Schambye, H. T.; Greenlee, W. J.; Schwartz, T. W. Identification of peptide binding residues in the extracellular domains of the AT_1 receptor. *J. Biol. Chem.* **1994**, *269*, 30953−30959.
(31) Kolodziej, S. A.; Nikiforovich, G. V.; Skeean, R.; Lignon, M.-F.; Martinez, J.; Marshall, G. R. Ac-[3- and 4-Alkylthioproline[31]]-CCK_4 Analogs: Synthesis and Implications for the CCK-B Receptor-Bound Conformation. *J. Med. Chem.* **1995**, *38*, 137−149.
(32) Webb, T. R.; Eigenbrot, C. Conformationally Restricted Arginine Analogues. *J. Org. Chem.* **1991**, *56*, 3009−3016.

JM9507744