residue with Et_2O gave **6h** (0.6 g).

***endo*-*N*-(8-Methyl-8-azabicyclo[3.2.1]octan-3-yl)-*N'*-(2-hydroxyphenyl)urea Hydrochloride (6t).** By following the procedure described for the conversion of **10** to **6h**, **6s** (1.9 g, 5 mmol) was converted via its hydrochloride salt to **6t** (0.83 g).

***endo*-*N*-(9-Methyl-9-azabicyclo[3.3.1]nonan-3-yl)-*N'*-phenylurea (11).** By following the procedure outlined for **6b**, phenyl isocyanate (2.4 g, 20 mmol) was converted to title compound 11 (3.6 g).

Pharmacology. The compounds were evaluated for antagonism of the Bezold–Jarisch (BJ) reflex evoked by 5-HT in the anesthetized rat by the method of Fozard.[6,10] Male rats (260–290 g) were anesthetized with urethane (1.25 g/kg ip) and blood pressure and heart rate were recorded. A submaximal dose of 5-HT (6 μg/kg iv) was given repeatedly, and the changes in heart rate were quantified. Compounds were given intravenously prior to administration of 5-HT and the dose required to reduce the 5-HT evoked response to 50% of the control response (ID_{50}) was determined.

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(10) Sanger, G. J. *Br. J. Pharmacol.* **1987**, *91*, 77.

Conformational Restriction of Angiotensin II: Cyclic Analogues Having High Potency

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Cyclic analogues of angiotensin II (AII) were synthesized by connecting the side chains of residues 3 and 5 via a disulfide bridge. Appropriate conformational constraints afforded an analogue, [$Hcy^{3,5}$]AII, having high contractile activity (pD_2 = 8.48 vs 8.81 for AII) and excellent binding affinity (IC_{50} = 2.1 nM vs 2.2 nM for AII). This type of cyclization was also used to prepare a highly potent AII antagonist, [Sar^1,$Hcy^{3,5}$,Ile^8]AII (pA_2 = 9.09 vs 9.17 for [Sar^1,Ile^8]AII; IC_{50} = 0.9 nM vs 1.9 nM for [Sar^1,Ile^8]AII). Model building suggests that this ring structure is consistent with a receptor-bound conformation having any of a variety of three-residue turns, including a γ-turn. In contrast, the receptor-bound conformation of AII does not appear to accommodate a β-turn or an α-helix which includes residues 3–5.

Since its discovery 50 years ago, the linear octapeptide angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe; AII; 1) has been the subject of numerous studies.[2] These investigations have shown that this hormone, being a potent pressor agent, has a vital role in regulation of blood pressure. In order to help establish the relationship between biological activity and conformation, several spectroscopic examinations of 1 and closely related analogues have been reported. These have included nuclear magnetic resonance (NMR) spectroscopy in a variety of solvents[3-8] as well as in the presence of lipid vesicles,[9] circular dichroism,[10-13] and infrared spectroscopy.[14-16] In addition, theoretical approaches[17-19] as well as pH[20-22] and other physicochemical studies[23-27] have also provided insights into the conformation of 1. This work has led to the proposal of several structural models for AII, including conformations which contain an α-helix,[27] a β-turn,[10,14,24] and a γ-turn.[4,24] Other, more complicated structures have also been suggested.[3,18,28] These widely differing conformational analyses serve to emphasize the lack of consensus on the solution conformation of 1. Indeed, Glickson and Marshall et al.[5,29,30] have cogently argued that several of

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(2) Page, I. H. *Hypertension Mechanisms*, Harcourt Brace Jovanovich: New York, 1987; pp 347–470.
(3) Weinkam, R. J.; Jorgensen, E. C. *J. Am. Chem. Soc.* **1971**, *93*, 7038.
(4) Bleich, H. E.; Galardy, R. E.; Printz, M. P. *J. Am. Chem. Soc.* **1973**, *95*, 2041.
(5) Glickson, J. D.; Cunningham, W. D.; Marshall, G. R. *Biochemistry* **1973**, *12*, 3684.
(6) Deslauriers, R.; Paiva, A. C. M.; Schaumburg, K.; Smith, I. C. P. *Biochemistry* **1975**, *14*, 878.
(7) Thiery, C.; Nabedryk-Viala, E.; Fermandjian, S.; Thiery, J. M. *Biochim. Biophys. Acta* **1977**, *494*, 293.
(8) Lenkinski, R. E.; Stephens, R. L.; Krishna, N. R. *Biochemistry* **1981**, *20*, 3122.
(9) Valensin, G.; Delfini, M.; Gaggelli, E. *Biophys. Chem.* **1986**, *24*, 25.
(10) Fermandjian, S.; Morgat, J.-L.; Fromageot, P. *Eur. J. Biochem.* **1971**, *24*, 252.
(11) Greff, D.; Fermandjian, S.; Fromageot, P.; Khosla, M. C.; Smeby, R. R.; Bumpus, F. M. *Eur. J. Biochem.* **1976**, *61*, 297.
(12) Lintner, K.; Fromandjian, S.; Fromageot, P.; Khosla, M. C.; Smeby, R. R.; Bumpus, F. M. *Biochemistry* **1977**, *16*, 806.
(13) Piriou, F.; Lintner, K.; Fermandjian, S.; Fromageot, P.; Khosla, M. C.; Smeby, R. R.; Bumpus, F. M. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 82.
(14) Fermandjian, S.; Fromageot, P.; Tistchenko, A.-M.; Leicknam, J.-P.; Lutz, M. *Eur. J. Biochem.* **1972**, *28*, 174.
(15) Sakarellos, C.; Lintner, K.; Piriou, F.; Fermandjian, S. *Biopolymers* **1983**, *22*, 663.
(16) Surewicz, W. K.; Mantsch, H. H. *J. Am. Chem. Soc.* **1988**, *110*, 4412.
(17) Marshall, G. R.; Bosshard, H. E. *Circ. Res.* **1972**, 30 and 31, Suppl. II, II-143.
(18) De Coen, J.-L.; Ralston, E. *Biopolymers* **1977**, *16*, 1929.
(19) Marchionini, C.; Maigret, B.; Premilat, S. *Biochem. Biophys. Res. Commun.* **1983**, *112*, 339.
(20) Juliano, L.; Paiva, A. C. M. *Biochemistry* **1974**, *13*, 2445.
(21) Fermandjian, S.; Piriou, F.; Sakarellos, C.; Lintner, K.; Khosla, M. C.; Smeby, R. R.; Bumpus, F. M. *Biopolymers* **1981**, *20*, 1971.
(22) Fermandjian, S.; Sakarellos, C.; Piriou, F.; Juy, M.; Toma, F.; Thanh, H. L.; Lintner, K.; Khosla, M. C.; Smeby, R. R.; Bumpus, F. M. *Biopolymers* **1983**, *22*, 227.
(23) Printz, M. P.; Williams, H. P.; Craig, L. C. *Proc. Natl. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 378.
(24) Printz, M. P.; Nemethy, G.; Bleich, H. *Nature (London) New Biol.* **1972**, *237*, 135.
(25) Schiller, P. W. *Can. J. Biochem.* **1977**, *55*, 75.
(26) Schiller, P. W. *Int. J. Pept. Protein Res.* **1980**, *15*, 259.
(27) Smeby, R. R.; Arakawa, K.; Bumpus, F. M.; Marsh, M. M. *Biochim. Biophys. Acta* **1962**, *28*, 550.
(28) Moore, G. J. *Int. J. Pept. Protein Res.* **1985**, *26*, 469.
(29) Marshall, G. R.; Bosshard, H. E.; Vine, W. H.; Glickson, J. D. *Nature (London) New Biology* **1973**, *245*, 125.

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these proposed structures cannot represent the conformation of this hormone in solution.

Because of the high degree of flexibility of 1, it is difficult to unambiguously assign to it a single conformation. In fact, it seems likely that, in solution, this peptide readily interconverts between conformations and so, at equilibrium, exists in several different structural forms.[31] However, when productively associated with its receptor, it is unlikely that 1 can adopt more than a single conformation, having only a relatively narrow range of flexibility. The real issue then becomes which of the solution conformations best approximates the receptor-bound conformation. Assuming a "lock-and-key" mechanism for receptor/ligand interactions, it is expected that (at least) one of the solution conformations will closely approximate the receptor-bound conformation. On the other hand, an induced fit or "zipper" mechanism allows for the possibility that none of the structures found in solution are identical with the conformation that the ligand assumes when interacting with the receptor.

One method of probing the receptor-bound conformation of a ligand is via conformational restriction. Here, structural changes in the molecule afford analogues in which one or more conformational alternatives have been precluded. For example, Marshall and co-workers[32] synthesized analogues of AII in which the proton attached to an α-carbon was replaced by a methyl group. Several of these conformationally restrained, acyclic analogues maintained good biological activity. Attempts to conformationally restrict 1 via cyclization have also been reported. De Coen et al.[33] cyclized [Asn^1,Val^5]AII by forming an amide-linkage between the N- and C-terminii to afford an inactive product. In Moore's[34] laboratory, a weak antagonist was synthesized by replacing residues 1 and 8 with cystine. Most recently, Miranda and Juliano[35] reported the preparation of several analogues having a cystine moiety at various locations. Agonistic activity was observed which varied from 0.01% to 0.17% of that of 1.

Our own interest in the renin–angiotensin system prompted us to undertake a systematic study of the effects of conformational restriction of 1. While the ultimate goal of this research was to discover novel antagonists of AII, we believed that this could be achieved by first developing insights into the conformational requirements necessary for good agonism. As a consequence of this research, we wish to report the synthesis and biological activity of several cyclic analogues of 1.

Chemistry

All peptides described here were prepared by solid-phase peptide synthesis methodology using standard "*t*-Boc" chemistry. Sulfhydryl functional groups were protected during the synthesis with either 4-methylbenzyl (MeB) moieties or with acetamidomethyl (Acm) groups. In the former case, the MeB groups were cleaved during treatment with anhydrous hydrogen fluoride. If the target was a cyclic peptide, those precursors having free sulfhydryl groups were partially purified before oxidation. The cyclized products were further purified and characterized. Peptides containing Acm-protected residues were purified and characterized with the sulfydryl protecting groups intact. The Acm groups were removed in a separate step according to the method of Kamber.[36] Cleavage of the protecting groups under these conditions was accompanied by concomitant oxidation to directly afford cyclic AII analogues. No difficulties were encountered during either synthesis or purification of any of the target peptides.

Because of the difficulty in preparing Boc-homocysteine (Acm), analogues having a homocysteine residue were all synthesized with MeB groups to protect all the thiols. As a consequence of this, the acyclic precursors were not fully purified or characterized and they were not examined in any biological test. However, prior to oxidation of each homocysteine-containing peptide, the high-pressure liquid chromatography (HPLC) retention time of the precursor was clearly different than that of the fully purified and characterized product. Moreover, Ellman's colorimetric test[37] was negative for each of these peptides, indicating that no free sulfhydryl groups were present. Finally, the molecular weight of each peptide, as determined by fast atom bombardment mass spectroscopy (FABMS), was fully consistent with the presence of a disulfide bond. We concluded from this evidence that these analogues are indeed cyclic.

Biology

Contractile activity and AII antagonist activity were determined with rabbit aortic rings. Relative potencies were obtained by comparing the activity recorded to the activity for 1 in a second aortic ring cut from the same preparation. The pD_2 values for AII and AII agonists were calculated from the concentration/response curves while pA_2 was determined according to the method of Arunlakshana and Schild.[38] Receptor binding was determined by measuring competitive displacement of [^{125}I]AII from a rat uterine membrane preparation using nonradioactive analogues.

Results and Discussion

Connecting two residues of an acyclic peptide via a disulfide bond to afford a conformationally restricted, cyclic analogue has been shown to be an effective way of probing the receptor bound conformation of a ligand.[39,40] In some cases, "superagonists" or highly selective agonists were synthesized. However, this approach does require multiple structural changes to be made within a single analogue. As a result, it is often difficult to establish reasons for poor biological activity. This problem becomes particularly acute for peptides in which a large percentage of the residues have been found to be critical for expression of activity. For 1, the side chain functional groups of residues 2, 4, 6, and 8 appear to be important for biological activity.[41-43] Replacement of these residues with cysteine (or

(30) Glickson, J. D.; Dadok, J.; Marshall, G. R. *Biochemistry* **1974**, *13*, 11.
(31) Premilat, S.; Maigret, B. *Biochem. Biophys. Res. Commun.* **1979**, *91*, 534.
(32) Turk, J.; Needleman, P.; Marshall, G. R. *Mol. Pharm.* **1976**, *12*, 217.
(33) De Coen, J. L.; Ralson, E.; Durieux, J. P.; Loffet, A. *Peptides: Chemistry, Structure and Biology*; Walter, R., Meinhofer, J., Eds.; Ann Arbor Science: Ann Arbor, MI, 1975; p 553.
(34) Matsoukas, J. M.; Scanlon, M. N.; Moore, G. J. *J. Med. Chem.* **1984**, *27*, 404.
(35) Miranda, A.; Juliano, L. *Braz. J. Med. Chem.* **1988**, *21*, 903.
(36) Kamber, B. *Helv. Chim. Acta* **1971**, *54*, 927.
(37) Deakin, H.; Ord, M. G.; Stocken, L. A. *Biochem. J.* **1963**, *89*, 296.
(38) Arunlakshana, O.; Schild, H. O. *Br. J. Pharm.* **1959**, *14*, 48.
(39) Hruby, V. J. *Conformationally Directed Drug Design*; Vida, J. A., Gordon, M., Eds.; American Chemical Society: Washington, DC, 1984; p 9.
(40) Schiller, P. W.; Eggeman, B.; DiMaio, J.; Lemieux, C.; Nguyen, T. M. D. *Biochem. Biophys. Res. Commun.* **1984**, *118*, 131.
(41) Reference 1, p 429.
(42) Bumpus, F. M.; Khosla, M. C. *Hypertension—Physiopathology and Treatment*; Genest, J., Koiw, E., Kuchel, O., Eds.; McGraw-Hill: New York, 1977; p 183.

Table I. Contractile Activities and Binding Affinities of AII Analogues with AII as the Standard

	angiotensin II (AII) analogue	contractile activity: pD_2[b] ±SE	contractile activity: relative potency[c] ±SE	n	binding potency[a] IC_{50} (nM)
1	AII	8.81 ± 0.08	1.0	14	2.2
2	[Cys[3]]AII	7.68 ± 0.06	0.092 ± 0.013	2	15
3	[Cys[5]]AII	7.21 ± 0.06	0.031 ± 0.004	2	20
4a	[Cys(Acm)[3,5]]AII	<5.0	<0.0001	4	10000
4b	[Cys[3,5]]AII	<5.0	<0.0001	4	43
5a	[D-Cys(Acm)[3], Cys(Acm)[5]]AII	NA[d]	NA[d]	2	>10000
5b	[D-Cys[3],Cys[5]]AII	NA[d]	NA[d]	2	NB[e]
6a	[Cys(Acm)[3], D-Cys(Acm)[5]]AII	NA[d]	NA[d]	2	10000
6b	[Cys[3],D-Cys[5]]AII	NA[d]	NA[d]	2	10000
7	[Pen[3],Cys[5]]AII	~5	~0.0001	6	58
8	[Cys[3],Pen[5]]AII	5.80 ± 0.06[f]	0.001 ± 0.0007	3	1.3
9	[Pen[3,5]]AII	~5	~0.0001	6	2.6
10	[Hcy[3],Cys[5]]AII	5.91 ± 0.14	0.0010 ± 0.00081	4	10
11	[Cys[3],Hcy[5]]AII	6.98 ± 0.07	0.012 ± 0.0041	4	13
12	[Hcy[3,5]]AII	8.48 ± 0.14	0.49 ± 0.16	4	2.1
13	[Sar[1],Ile[3],Hcy[3,5]]AII	NA[d]	NA[d]	2	0.9[g]
		(pA_2 = 9.09 ± 0.19)[h]			

[a] Concentration of analogue needed to produce 50% inhibition in specific binding of [^{125}I]AII to a rat uterine membrane preparation. Data represent the mean of triplicate determinations (SE ± 15%). A second, low-affinity, subclass of binding sites was also observed in some instances. These data were not included for clarity. [b] Calculated from the concentration/response curves for each peptide tested. [c] Calculated relative to the concentration/response curves of AII measured on a paired rabbit aortic ring at the same time that the concentration/response curves of the analogue were obtained. [d] NA = no activity observed up to 10 μM concentration of the analogue. [e] NB = no displacement of radioligand observed at an analogue concentration of 10 μM. [f] The pD_2 value for one of the four replicates of 8 could not be determined due to an incomplete concentration/response curve. To permit statistical analysis, this replicate was not included here. [g] The IC_{50} for [Sar[1],Ile[3]]AII was found to be 1.9 nM. [h] Calculated according to the method of Arunlakshana and Schild;[38] n = 4.

cystine) may result in substantial loss of activity, even if the backbone conformation closely approximates that of the natural product. Residue 5 (and perhaps to a lesser extent residue 3) also serves an important role.[22,42] However, here the steric bulk of these side chains may function to properly orient the backbone for effective receptor/ligand interactions. If true, then conformational restriction by replacement of one or both of these residues with cystine could still afford active analogues.

Regoli et al.[43] previously determined that replacement of valine at residue 3 with alanine afforded an analogue having reduced, but still good, potency (relative potency = 0.08). Similar results were obtained upon substitution of isoleucine at residue 5 with alanine (relative potency = 0.06). In our laboratories, analogues **2** and **3**, having a cysteine moiety at residues 3 or 5, respectively, show equivalent decreases in potency (see Table I). These results confirm that the presence of cysteine at either of these positions does not result in unexpected biological activity. Incorporation of (Acm-protected) cysteine moieties at both positions 3 and 5 afford an acyclic analogue (**4a**) having very weak, but reproducible, biological activity. At a concentration of 10 μM, full contractile activity is not obtained and so values for the pD_2 cannot be calculated from the concentration/response curves (see Figure 1). Biological testing of these analogues at higher concentrations proved to be impractical due to tachyphylaxis. The presence of the two Acm protecting groups makes it difficult to interpret these data. The loss of activity may be a function of replacement of residues 3 and 5 with cysteines. Alternatively, the steric bulk and/or polarity of the Acm groups may be the cause of the contractile activity.

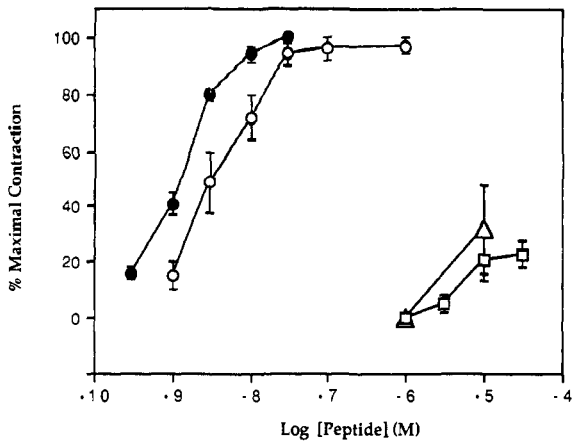

Figure 1. Concentration/contraction curves in isolated rabbit aortic rings for peptide **1** (●; n = 12), peptide **4a** (△; n = 2), peptide **4b** (□; n = 4), and peptide **12** (○; n = 3). Values represent means ± SE.

Upon deprotection and cyclization of **4a**, the contractile activity of the resulting cyclic peptide (**4b**) did not appreciably change. It is interesting to note that, although binding affinity is 1 order of magnitude lower than that of **1**, it is still substantially higher than that of **4a**. This may indicate a partial antagonism associated with **4b**. This observed binding affinity suggests that the conformational restrictions imposed on this molecule still allow for effective (but not optimal) receptor/ligand interactions. We were encouraged by this observation and decided to investigate further by preparing analogues which might restore contractile activity.

Conformational constraints were varied somewhat by replacing L-cysteine at positions 3 or 5 with the corresponding D-isomer. Neither of these cyclic analogues (**5b**, **6b**) or their acyclic precursors (**5a**, **6a**) showed any contractile potency at concentrations of up to 10 μM. Only extremely weak binding to the AII receptor was observed for any of these analogues. Within this series, an L,L-cystine residue at the 3/5 position (**4b**) appears to provide the most effective conformational restrictions.

Incorporation of penicillamine at these sites did result in a modest improvement in biological activity. While the maximal response plateau was not always obtained for analogues **7**, **8**, or **9** at a concentration of 10 μM, a complete concentration/response curve was obtained in one or more of the replicates of each analogue. Despite weak contractile activities, the binding affinities, especially of **8** and **9**, were unexpectedly high. These results may indicate the presence of some partial antagonist character of these analogues, although this was not explicitly examined. Disulfide bridges containing 1 or 2 penicillamine residues are known[44,45] to be more sterically crowded than the corresponding analogues having a cystine group. It was previously shown[17,21,22] that the constraints imposed by a β-substituted side chain at position 5 are important for expression of full pressor activity. Potency falls in the absence of such a substituent and this may explain, at least partially, the reduced activity found with **3** as well as with **4a** and **4b**. Replacement of both β-protons of the half-cystine at position 5 of **4b** with methyl groups does improve biological activity, but this analogue (**8**) is still 3 orders of magnitude less potent than **1**.

(43) Regoli, D.; Park, W. K.; Rioux, F. *Pharmacol. Rev.* **1974**, *26*, 69.

(44) Meraldi, J.-P.; Yamamoto, D.; Hruby, V. J.; Brewster, A. I. R. *Peptides: Chemistry, Structure and Biology*; Walter, R., Meinhofer, J., Eds.; Ann Arbor Science: Ann Arbor, MI, 1975; p 803.

(45) Hruby, V. J. *Life Sci.* **1982**, *31*, 189.

The conformational requirements of AII at its receptor were also probed by varying ring size. Analogue **4b** contains a medium-sized ring (11 atoms) and cyclic structures of this size still exhibit considerable transannular and large-angle strain. Strain energy attributed to these factors may be somewhat relieved by the presence of two peptide bonds within the ring, but endocyclic bonds having double bond character also have associated strain energies. Ring flexibility can be increased and strain can be reduced by enlarging the ring. Here, this was accomplished by replacing the half-cystine groups of **4b** with 1 or 2 half-homocystine moieties. Inserting one additional methylene unit into the ring afforded analogues (**10**, **11**) which are more potent than **4b** by at least 1 or 2 orders of magnitude. The location of the methylene group did appear to be important for contractile potency (but apparently not for binding affinity). Analogue **11**, having a half-homocystine at residue 5, is 1 full order of magnitude more potent than the isomer (**10**) in which the half-homocystine residue is located at position 3. Increasing the ring size to 13 atoms results in an additional increase in biological potency. In fact, **12** has nearly the same contractile activity as the natural product, **1** (see Table I, Figure 1). This peptide also binds to the receptor with over 2-fold greater affinity than **1**.

We feel that it is appropriate to interpret the dramatically increased potency of **12** as being the result of increased effectiveness of the interactions of this AII analogue with its receptor. Although the conformational constraints imposed on **12** are certainly less than those of **4b**, this peptide is, relative to the acyclic natural product, still quite constrained at those residues which are endocyclic (i.e., residues 3–5). This steric crowding provides insights into the structure of the biologically active state of this portion of the molecule. γ-Turns, have been found[46] to be stabilized by the presence of cystine at residues i and (i + 2). Spectral evidence indicated the presence of two hydrogen bonds in the cyclic tripeptide (Boc)Cys-Ala-CysNHMe; a 3 → 1 C_7 hydrogen bond and a 1 → 3 C_{11} hydrogen bond. From CPK model building, it appears that a classic γ-turn[47] can be constructed at residues 3–5 of **12** having both of these hydrogen bonds. Such a model has been previously proposed for AII by Printz et al.[4,24] Their reported structure readily accommodates a homocystine moiety spanning residues 3 and 5 with only minor changes in the dihedral angles of the backbone.

In a similar fashion, β-turns have also been stabilized by a disulfide linkage connecting residues i and (i + 3).[48] The homocystine group in analogue **12** does permit a 4-residue turn between residues 2, 3, 4, and 5 or between residues 3, 4, 5, and 6. However, molecular models show that both of these structures are strained and quite perturbed from any of the recognized β-turns. For example, the 4 → 1 C_{10} hydrogen bond typically found in a β-turn is highly distorted in both of these counter turns by twisting of the entire turn structure. Also, the backbone dihedral angles, especially at residues (i + 1) and (i + 2), differ substantially from the ideal.

It appears unlikely from model building that **12** can accommodate an α-helical structure involving residues 3–5. The side chains of residues 3 and 5 would be on opposite faces of a helix and connecting them via a disulfide bond would appear to involve unacceptable distortions of the dihedral angles and disruption of the hydrogen bonds.

It is not necessary for the receptor bound conformation of AII to contain only readily recognized structural features. Several somewhat more complex models of AII have been reported.[13,17,19,28] For example, after examining the biological activity of several conformationally restricted analogues, Marshall and Bosshard[17] predicted "probable backbone angles of the biologically active conformation of angiotensin II". While these predicted dihedral angles do not indicate a γ-turn between residues 3 and 5, they are readily accommodated by **12**. Since both this work and the present study probe the structure of AII at its receptor, we feel that the structural information obtained is complementary.

These structural insights do not permit a prediction of the complete receptor-bound conformation of AII. However, there is certainly sufficient information to permit the design of a conformationally restricted AII antagonist. Several reported[49,50] peptide antagonists of AII result from replacement of residue 1 with sarcosine and position 8 with an amino acid, such as alanine or isoleucine, which has an aliphatic side chain. This suggested to us that combining these features with the conformational constraints found in **12** might afford an AII antagonist. In fact, upon synthesis, **13** was found to be a potent inhibitor of AII with a pA_2 of 9.09. No agonistic activity was observed up to 10 μM. This compares favorably to the reported[50] potency of the acyclic analogue [Sar^1,Ile^8]AII (pA_2 = 9.17; 1.0% residual pressor activity). The binding affinity is actually better than that of [Sar^1,Ile^8]AII. Clearly, the structural rigidity imposed by a homocystine residue at position 3/5 allows for the synthesis of very effective AII antagonists as well as highly potent agonists.

Conclusions

Cyclization of AII across residues 3 and 5 has resulted in an agonist (**12**) having similar contractile activity and binding affinity to the natural product. This ring structure was also used to design an effective AII inhibitor (**13**). While these peptides are conformationally restricted, they are still sufficiently flexible that several different conformational alternatives are possible. This includes variations of a γ-turn as well as other structures which are less rigidly defined. Some flexibility may actually be an important feature in receptor recognition since the AII conformation may change during the process of binding to its receptor. This is one explanation for the dramatic increase in contractile activity as ring size increases from 11 to 13 atoms. Alternatively, it is possible that the "active" conformation is precluded by the smaller ring and this certainly remains an area for further study. The data presented here does not permit the unambiguous determination of the receptor-bound structure. However, several structural elements, including an α-helix and the variety of β-turns,[51] appear to be unlikely, at least to the extent that they include residues 3–5.

This work also supports the contention that steric hin-

(46) Kishore, R.; Balaram, P. *Biopolymers* **1985**, *24*, 2041.
(47) Nemethy, G.; Printz, M. P. *Macromolecules* **1972**, *5*, 755.
(48) Ravi, A.; Balaram, P. *Tetrahedron* **1984**, *40*, 2577.
(49) Bumpus, F. M. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1977**, *36*, 2128.
(50) Aumelas, A.; Sakarellos, C.; Lintner, K.; Fermandjian, S.; Khosla, M. C.; Smeby, R. R.; Bumpus, F. M. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 1881.
(51) After completion of this manuscript, the synthesis and biological testing of [Sar^1,Hcy^{3,5},Ile^8]AII was reported elsewhere (Sugg, E. E.; Dolan, C. A.; Patchett, A. A.; Chang, R. S. L.; Faust, K. A.; Lotti, V. J. *Peptides: Chemistry, Structure and Biology*; Rivier, J. E., Marshall, G. R., Eds.; ESCOM Science Publishers B. V.: Leiden, The Netherlands, 1990; p 305). The antagonistic activity was nearly identical with that reported here. They concluded that [Sar^1,Cys^{3,5},Ile^8]AII adopts a β-turn conformation.

drance exerted by a β-substituted side chain on residue 5 (and perhaps also residue 3) is important for receptor recognition. If the constraints imposed by these side chains are mimicked by cyclization across these residues, then the same biological activity can be realized in the absence of β-disubstitution at both of these sites.

Knowledge of the receptor bound conformation of AII will provide useful insights which may allow for the rational design of novel antagonists. This research represents a step in this direction, but additional studies will be required in order to take full advantage of this information. Additional structure/activity relationship (SAR) studies to further probe the effects of cyclization in this region of the molecule, both in terms of ring size and in the nature of the bridging group, will permit a more detailed analysis of the structural requirements for optimal receptor/ligand interactions. In addition, we anticipate that energy calculations may allow for a better understanding of the ring conformation as well as more effective interfacing of this information with those SAR studies already available.

Experimental Section

Compositional analysis data were collected from 6 M HCl hydrosylates (vapor phase, 110 °C, 24 h) with ninhydrin-based analysis performed on a Beckman 6300 high-performance analyzer. Positive ion fast atom bombardment mass spectroscopy (FABMS) was carried out with a VG-ZAB SE double-focusing mass spectrometer. The FAB beam consisted of xenon neutrals at an acceleration of 9 kV (1 mA). The mass spectrometer scanned at a resolution of 1000. The FAB matrix used was a mixture of glycerol/thioglycerol/HCl/dimethyl sulfoxide. HPLC was carried out on a Waters system consisting of two Model 510 pumps connected to a Model 680 automated gradient controller. Additional solvent mixing was accomplished by an Axxiom Model 400 mixer. Column effluent was monitored at 220 nm by a Waters Model 481 spectrophotometer and peak area was measured with a Waters Model 740 integrator. All eluents for both high-pressure and medium-pressure liquid chromatography were HPLC grade, and 0.05% trifluoroacetic acid (spectrograde) was added.

Peptide Synthesis. Solid-phase peptide syntheses were carried out on 1% cross-linked divinyl benzene resins (0.3–0.5 mequiv/g) with an ABI Model 430A peptide synthesizer. Unless otherwise specified, all protected amino acids, reagents, and solvents were purchased from ABI and used directly with no further purification. The resins (Boc-Phe-O-resin or Boc-Ile-O-resin) were purchased from Peninsula Laboratories, Inc. (Boc)sarcosine was also purchased from Peninsula Laboratories and used directly with no further purification. Boc-*N*-(benzyloxy)-L-histidine, Boc-*S*-(acetamidomethyl)-L-cysteine, Boc-*S*-(acetamidomethyl)-D-cysteine, and Boc-*S*-(methoxybenzyl)-L-penicillamine were purchased from Bachem, Inc. and used directly with no further purification. Boc-*S*-(p-methylbenzyl)homocysteine was purchased from Chemalog and used directly with no further purification. The peptides were removed from resin and deprotected with anhydrous hydrogen fluoride (HF; 10 mL/g resin-bound peptide) containing anisole (1 mL/g) and 2-mercaptopyridine (0.1 g/g) at 0 °C for 1 h on a Peptide Institute Type I HF-Reaction Apparatus. After evaporation of the HF, the residue was triturated with ether (6 × 50 mL). The residue was then washed with aqueous acetic acid (50 mL of 30% AcOH followed by 2 × 50 mL of 5% AcOH). The aqueous fractions were combined and lyophilized. The crude peptides were dissolved in 0.1 M tris(hydroxymethyl)aminomethane (Tris) buffer (pH 8; 250 mL/g). An equal weight of dithiothreitol (DTT) was added and the resulting solution was stirred at room temperature for 0.5 h before being filtered, acidified, and lyophilized. The crude reduced peptides were then purified via HPLC on a C_{18} silica gel column (Vydac; 22 mm i.d. × 250 mm, 15–20 μm, 300 A) eluting with a linear gradient of 15–35% acetonitrile over 25 min at a flow rate of 9 mL/min.

Cyclization. The acyclic analogues were taken up in 80/20 acetic acid/water (0.5 mL/mg peptide) and an equal volume of 0.04 M iodine solution (80/20 acetic acid/water) was added. The resulting dark brown solution was stirred at room temperature until the reactions were complete as determined by HPLC (5 min to 2 h). The reaction mixtures were diluted with an equal volume of water and extracted with ether until no color remained in the aqueous phase. The organic layer was washed once with water and the aqueous layers were combined and concentrated in vacuo at room temperature to about half of the original volume. Lyophilization left a (usually) light yellow solid which was purified via HPLC as described above to afford the desired product as a white solid. Yields are unoptimized. Greater emphasis was placed on peptide purity, which resulted in decreased yields. Moreover, only a sufficient quantity of peptide was purified to complete the necessary analyses/assays. All peptides were purified to greater than 97% purity as measured by analytical HPLC. Amino acid analyses and FABMS were in agreement with the expected results.

Biological Assays. Functional Assays. The agonist and antagonist activities of these peptides were measured with rabbit aortic rings. Male New Zealand white rabbits (2–2.5 kg) were sacrificed with an overdose of pentobarbitol and exsanguinated via the carotid arteries. The thoracic aorta was removed, cleaned of adherent fat and connective tissue, and then cut into 3-mm ring segments. The endothelium was removed from the rings by gently sliding a rolled-up piece of filter paper into the vessel lumen. The rings were then mounted in a water-jacketed tissue bath maintained at 37 °C between a moveable and a fixed stainless steel wire, with the moveable end attached to an FT03 Grass transducer coupled to a Model 7D Grass Polygraph for recording isometric force responses. The bath was filled with 20 mL of oxygenated (95% O_2/5% CO_2) Krebs solution of the following composition (mM): NaCl (130), $NaHCO_3$ (15), KCl (5), NaH_2PO_4 (1.2), $MgSO_4$ (1.2), $CaCl_2$ (2.5), and glucose (11.4). For the agonist assay, paired rings from the same rabbit aorta were exposed to increasing concentrations of either AII or the test peptides (at 30-min intervals), during which time the tissue was washed with fresh Krebs solution (3 × 20 mL). For the measurements of antagonistic activity, paired rings from the same rabbit aorta were again used; one ring was exposed to increasing concentrations of AII (at 30 min intervals) and a second ring was exposed to increasing concentrations of AII in the presence of the compound acting as an antagonist. The compound tested for antagonist activity was added 5 min prior to the addition of AII. The concentration/response curves for AII in the presence of the antagonist were evaluated in terms of the percent of the maximal contraction of the control ring exposed only to AII.

Receptor Binding Assay. Rat uterine membranes were prepared from fresh tissue. All procedures were performed at 4 °C. Uteri were stripped of fat and homogenized using a Polytron for 25 s in 20 volumes of ice-cold Dulbecco's phosphate-buffered saline containing 5 mM EDTA. The homogenate was centrifuged at 1500*g* for 20 min, and the supernatent was recentrifuged at 100000*g* for 60 min. The pellet was resuspended in buffer consisting of 2 mM EGTA and 50 mM Tris-HCl (pH 7.5) to a final protein concentration of 4 mg/mL. Protein concentration was measured by using the method of Bradford.[52]

Assay tubes contained 0.25 mL of a solution containing 5 mM $MgCl_2$, 2 mM EGTA, 0.5% bovine serum albumin, 50 mM Tris-HCl (pH 7.5), and [^{125}I]AII (approximately 10^5 cpm) in the absence or presence of unlabeled ligand. The reaction was initiated by the addition of 50 μg of membrane protein and the mixture was incubated at 25 °C for 30 min. The incubation was terminated with ice-cold 50 mM Tris-HCl (pH 7.5) and the mixture was filtered to separate membrane-bound labeled peptide from free ligand. The incubation tube and filter were washed with ice-cold buffer. Filters were assayed for radioactivity in a Micromedic γ-counter. Nonspecific binding was defined as binding in the presence of 10 μM unlabeled AII. Specific binding was calculated as total binding minus nonspecific binding. Binding data were analyzed by a nonlinear least-squares curve fitting program. This method also showed the presence of a second, low-affinity binding site. No attempt was made to determine the function, if any, of this subclass of binding sites. For clarity, these data were not presented.

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(52) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.

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Registry No. 1, 4474-91-3; 2, 126898-68-8; 3, 126898-69-9; 4a, 126898-70-2; 4b, 126898-71-3; 5a, 126898-72-4; 5b, 126898-73-5; 6a, 126999-84-6; 6b, 126999-85-7; 7, 126925-39-1; 7 (cyclic), 126925-36-8; 8, 126925-40-4; 8 (cyclic), 126925-37-9; 9, 126898-76-8; 9 (cyclic), 126925-38-0; 10, 126898-77-9; 10 (cyclic), 126898-74-6; 11, 126898-78-0; 11 (cyclic), 126898-75-7; 12, 126898-79-1; 12 (cyclic), 126898-66-6; 13, 126898-80-4; 13 (cyclic), 126898-67-7.

Supplementary Material Available: Table listing the amino acid analysis and FABMS for compounds 2-13 (1 page). Ordering information is given on any current masthead page.

Crystallographic Studies of Angiotensin Converting Enzyme Inhibitors and Analysis of Preferred Zinc Coordination Geometry

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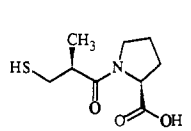
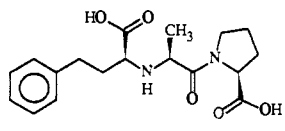
The molecular structures of two potent inhibitors of angiotensin converting enzyme (ACE, EC 3.4.15.1, dipeptidyl carboxypeptidase), ketoace, (5*S*)-5-benzamido-4-oxo-6-phenylhexanoyl-L-proline, and (1*S*,2*R*)-1-[[2-(benzoylthio)-cyclopentyl]carbonyl]-L-proline were determined by X-ray diffraction methods. The distances between the binding functions in both crystal structures are in agreement with the experimental results for the hypertension drug captopril and the enzyme substrate hippuryl-L-histidyl-L-leucine. The modified peptide skeletons of both inhibitors adopt extended conformations with the proline amide bond trans. Crystallographic data have been used to determine the coordination geometry for zinc–sulfhydryl and zinc–carbonyl interactions. Coordination distances and bond angles are found to be different from values assumed in models of the angiotensin converting enzyme active site. No preferred torsion angles for a zinc–sulfhydryl inhibitor interaction can be identified. Superposition of the crystallographic structures of four ACE ligands shows that the observed extended conformations place the pharmacophores, zinc atom ligand, carbonyl oxygen atom, and carboxyl group, in juxtaposition and provide an alternative model for the interaction of ligands with the ACE active site.

Angiotensin converting enzyme (ACE, EC 3.4.15.1, dipeptidyl carboxypeptidase) is the regulatory zinc protease in the renin-angiotensin system. ACE converts the decapeptide Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu to the vasoconstrictive octapeptide angiotensin II by cleaving the C-terminal dipeptide.[1] In addition, the converting enzyme hydrolyzes the nonapeptide bradykinin, which has hypotensive activity.[2] Thus, both enzymatic reactions result in elevation of blood pressure. Clinical studies have shown that inhibitors of the converting enzyme are effective in the treatment of essential hypertension and congestive heart failure. Captopril (1),[3,4] a mercaptoalkanoyl amino acid, was the first orally active ACE inhibitor that possessed clinical usefulness. Substitution of the zinc binding sulfhydryl moiety in captopril by carboxyl,[5] carbonyl,[6] or phosphonic acid ligands[7] enhanced both activity and binding specificity for the inhibitors, such as for the hypertension drug enalaprilat (2).[5]

We report the first crystal structure for a representative of the carbonyl ligand class, ketoace (3),[6] and the structure

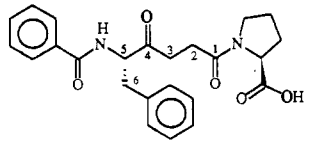


1 captopril

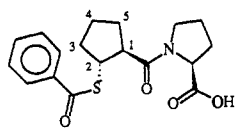


2 enalaprilat

for a semirigid analogue of captopril, 4.[8] Both molecular structures assist in the prediction of the binding conformation for ACE inhibitors and in the modeling of the active-site architecture of the converting enzyme. Ketoace, (5*S*)-5-benzamido-4-oxo-6-phenylhexanoyl-L-proline (3), is a derivative of the tripeptide ACE inhibitor Bz-Phe-Gly-Pro, in which the labile peptide amide bond of the model peptide is replaced by a more stable ketomethylene group. The nonpeptide analogue is 130 times more potent



3 ketoace



4

in vitro than the peptide[6] and 3-fold more potent than captopril,[9] although it has slow binding kinetics in vitro.[10] In vivo activity has been observed by continuous iv infusion;[9,11] however, ketoace is less active when given either

(1) Skeggs, L. T.; Marsh, W. H.; Kahn, J. R., Shumway, N. P. *J. Exp. Med.* **1954**, *99*, 275–282.
(2) Yang, H. Y. T.; Erdoes, E. G.; Levin, Y. *Biochem. Biophys. Acta* **1970**, *214*, 374–376.
(3) Ondetti, M. A.; Rubin, B.; Cushman, D. W. *Science* **1977**, *196*, 441–444.
(4) Cushman, D. W.; Cheung, H. S.; Sabo, E. F.; Ondetti, M. A. *Biochemistry* **1977**, *16*, 5484–5491.
(5) Patchett, A. A.; Harris, E.; Tristram, E. W.; Wyvratt, M. J.; Wu, M. T.; Taub, D.; Peterson, E. R.; Ikeler, T. J.; ten Broeke, J.; Payne, N. G.; Ondeyka, D. L.; Thorsett, E. D.; Greenlee, W. J.; Lohr, N. S.; Hoffsommer, R. D.; Joshua, H.; Ruyle, W. V.; Rothrack, J. W.; Aster, S. D.; Maycock, A. L.; Robinson, F. M.; Hirschmann, R. F.; Sweet, C. S.; Ulm, E. H.; Gross, D. M.; Vassil, T. C.; Stone, C. A. *Nature (London)* **1980**, *288*, 280–283.
(6) Almquist, R. G.; Chao, W. R.; Ellis, M. E.; Johnson, H. R. *J. Med. Chem.* **1980**, *23*, 1392–1398.
(7) Petrillo, E. W., Jr.; Powell, J. R.; Cushman, D. W.; Ondetti, M. A. *Clin. Exp. Hypertens., Part A* **1987**, *A9*, 235–241.
(8) Ciabatti, R.; Padova, G.; Bellasio, E.; Tarzia, G.; Depaoli, A.; Battaglia, F.; Cellentani, M.; Barone, D.; Baldoli, E. *J. Med. Chem.* **1986**, *29*, 411–417.
(9) Almquist, R. G.; Crase, J.; Jennings-White, C.; Meyer, R. F.; Hoefle, M. L.; Smith, R. D.; Essenburg, A. D.; Kaplan, H. R. *J. Med. Chem.* **1982**, *25*, 1292–1299.
(10) Grobelny, D.; Galardy, E. *Biochemistry* **1986**, *25*, 1072–1078.
(11) Meyer, R. F.; Nicolaides, E. D.; Tinney, F. J.; Lunney, E. A.; Holmes, A.; Hoefle, M. L.; Smith, R. D.; Essenburg, A. D.; Kaplan, H. R.; Almquist, R. G. *J. Med. Chem.* **1981**, *24*, 964–969.

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