An Investigation of Angiotensin II Agonist and Antagonist Analogues with 5,5-Dimethylthiazolidine-4-carboxylic Acid and Other Constrained Amino Acids†

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To probe the receptor-bound conformational requirements of angiotensin II (ANG II) octapeptide agonists and antagonists, the synthesis and biological activities of [Sar^1]ANG II agonist and [Sar^1,X^8]ANG II antagonist analogues (X^8 = Ile, D-Phe, or Aib) bearing conformational constraints in positions 3, 5, and 7 were investigated and compared with previous literature efforts. The conformational constraints that were examined include Pro, Dtc (5,5-dimethylthiazolidine-4-carboxylic acid), Aib, Cle, (NMe)Ala, (NMe)Ile, and the lactam modification, L,L-lactam-Phe, previously described by Freidinger et al. (*J. Org. Chem.* **1982**, *47*, 104-109). Both [Sar^1,(NMe)Ala^3 and Pro^3]ANG II retained agonist activity, while only [Sar^1,(NMe)Ala^3,Ile^8]ANG II retained antagonist activity. [Sar^1,Dtc^5]ANG II displayed superior agonist activity, while both [Sar^1,Dtc^5 and Cle^5,Ile^8]ANG II displayed superior antagonist activity. In contrast to position 5, Dtc^7 substitution for Pro^7 of either [Sar^1]ANG II or [Sar^1,Ile^8]ANG II gave analogues with reduced activities. These results are consistent with the hypothesis that conformations of [Sar^1]ANG II and [Sar^1,Ile^8]ANG II containing a C7 conformation in position 7 are preferred for both ANG II agonist and antagonist activity. Incorporation of the L,L-lactam-Phe modification into [Sar^1]ANG II gives a pure ANG II antagonist (pA_2 8.3), comparable to saralasin (pA_2 8.6). In positions 3, 5, and 7 the conformational requirements for the ANG II agonist [Sar^1]ANG II and the ANG II antagonist [Sar^1,Ile^8]ANG II may be different. Individual substitution of (NMe)Ala^3, Dtc^5, D-Phe^8 and Aib^8 [[Sar^1,Aib^8]ANG II: Khosla et al. *J. Med. Chem.* **1977**, *20*, 1051-1055] into [Sar^1,Ile^8]ANG II gives analogues that retain antagonist activity. Multiple substitutions of these types of residues into [Sar^1,Ile^8]ANG II gives analogue **45** [Sar^1,(NMe)Ala^3,Dtc^5,Aib^8]ANG II, **46** [Sar^1(NMe)Ala^3,D-Phe^8]AII, and **47** [Sar^1,Dtc^5,D-Phe^8]AII, which display considerably reduced antagonist activity. In ANG II antagonists the construction of highly constrained analogues may not be possible by the additive substitution of "preferred" constrained amino acids into a single analogue.

Introduction

Linear peptides such as the pressor peptide hormone angiotensin II (ANG II) are capable of adopting a large number of conformations.[1-5] As a result, the ^{1}H NMR spectrum of ANG II is best interpreted as a representation of a large set of these conformations.[2] The interaction of ANG II with its template of membrane-bound protein receptor binding groups, however, selects out a subset of these conformations, the size of which is presumed to be quite small. Determination of the receptor-bound conformation(s) of ANG II could significantly advance the rational design of potent ANG II antagonists.

For a number of biologically active peptides the incorporation of conformationally constrained amino acids has given rise to analogues with improved biological activities.[6] Presumably, a constrained analogue that retains biological activity is represented in solution by a set of conformations that is smaller than that of the native unconstrained peptide, and that set of constrained analogue conformations in solution contains a larger fraction of the receptor bound conformation(s). With regard to the octapeptide ANG II, conformational constraints may well need to be incorporated into several positions along the backbone to give a highly constrained analogue before the ^{1}H NMR spectrum can provide information that will be of use in model building. Indeed, in certain cases[6] multiple substitutions of constrained amino acids have given potent analogues with sufficient rigidity to display discrete solution conformations that are suggestive of bioactive conformation. Peptides bearing conformational constraints have often displayed superior stability to enzymatic degradation as well.[6] It was the goal of this study, therefore, to search for highly constrained ANG II analogues bearing multiple substitutions of constrained amino acids that retain high degrees of biological activity. The discovery of such highly constrained ANG II analogues could then be evaluated for enzymatic stability and spectroscopic

† The abbreviations for natural amino acids and nomenclature for peptide structures follow the recommendations for peptide structures follow the recommendations of the IUPAC-IUB commission on Biochemical Nomenclature [*J. Biol. Chem.* **1971**, *247*, 977). Abbreviations for nonnative amino acids include Aib = α-aminoisobutyric acid, or (αMe)Ala; (αMe)Tyr = α-methyltyrosine; Cle = cycloleucine, or 1-aminocyclopentanecarboxylic acid; (dehydro)Phe = 2-amino-3-phenylacrylic acid; Dtc = 5,5-dimethylthiazolidine-4-carboxylic acid; L,L-lactam-Phe = 2-[(*S*)-3-amino-2-oxo-1-pyrrolidinyl]-(*S*)-3-phenylpropionic acid; (NMe)Ala = *N*-methylalanine, (NMe)Ile = *N*-methylisoleucine; (SMe)Pen = *S*-methylpenicillamine; Tpr = thioproline, or thiazolidine-4-carboxylic acid; (O)Tpr = 1-oxothiazolidine-4-carboxylic acid. Other abbreviations in this paper include TEA = triethylamine, TFA = trifluoroacetic acid, DCC = *N*,*N*′-dicyclohexylcarbodiimide, and σ-Br-Z = 2-bromobenzyloxycarbonyl.

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Table I. Activities of [Asp[1]]ANG II Analogues in the Literature

no.	Asp–Arg–X–Tyr–Y–His–Z–Phe (1 2 3 4 5 6 7 8) X[3]	Y[5]	Z[7]	agonist: in vitro[a] rabbit aorta	agonist: in vivo[b] rat BP
1	Val	Ile	Pro	100	100
2	Ala			30 (RU)[c]	80.5,[d] 68,[d] 35[e]
3	Pro			80 (RU)[f]	53,[g], 40[e]
4	Aib			–	1.0[e]
5	Cle			–	1–2[h]
6		Ala		5.0 (RU)[c]	4.9[i]
7		Nle		–	21[j]
8		Pro		–	10[k]
9		Aib		–	1.0,[e] 0.9[k]
10		Cle		–	24[k]
11			Ala	0.1 (RU)[c]	1.5,[i] 7.5[e]
12		Val	(NMe)Ala	4.3 (GPI)[l]	22[l]
13			Aib	–	1.0[e]
14			Cle	–	1–1.5[h]

[a] Agonist activity in vitro, expressed as percent activity relative to ANG II, was measured in the rabbit aorta strip assay unless shown otherwise (RU = rat uterus, GPI = guinea pig ileum) according to methods described in the references. [b] Agonist ANG II like activity in vivo, expressed as percent activity relative to ANG II, was measured in the rat blood pressure assay described in references listed in the table. [c] Reference 7. [d] Reference 9. [e] Reference 12. [f] Reference 10. [g] Reference 11. [h] Reference 13. [i] Reference 8. [j] Reference 14. [k] Reference 15. [l] Reference 16.

Table II. Activities of [Sar[1]]ANG II Agonist Analogues Substituted in Position 3

no.	Sar–Arg–X–Tyr–Ile–His–Pro–Phe (1 2 3 4 5 6 7 8) X[3]	agonist: in vitro[a] rabbit aorta	agonist: in vivo[b] rat BP
14	Val	180 ± 14	125 ± 15
15	Ala	38 ± 4.2	–
16	(NMe)Ala	120 ± 15	–

[a] Agonist ANG II like activity in vitro, expressed as percent activity relative to ANG II, pD_2 8.5, was measured in the rabbit aorta strip assay (n = 5) according to the method of Rioux et al.[31] [b] Agonist ANG II like activity in vivo, expressed as percent activity, was measured in the rat blood pressure assay (n = 5) according to the method of Regoli et al.[32]

evidence of bioactive conformation(s).

Previous studies of ANG II analogues bearing conformationally constrained amino acids suggested that the presence of proline in either position 3 or 7 was not detrimental to agonist activity, whereas incorporation of proline into position 5 gave an analogue with reduced agonist activity, Table I.[7-16] Previous studies have also shown that the conformational requirements for ANG II agonists may differ from ANG II antagonists, e.g. [Sar[1],Phe[8]]ANG II is a potent agonist whereas [Sar[1],D-Phe[8]]ANG II is a potent antagonist.[17] Certain conformationally constrained amino acids have been incorporated into ANG II antagonists, e.g., [Sar[1],Ile[8]]ANG II,[11,18-20] but a thorough examination has not been reported.

Conformational Constraints Employed in This Study. This paper extends the previous studies to include a greater variety of constrained amino acids into positions 3, 5, and 7 of both [Sar[1]]ANG II and the potent ANG II antagonist [Sar[1],Ile[8]]ANG II to further examine the receptor-bound conformational requirements of both ANG II agonists and antagonists. These analogues bear three types of conformationally constrained amino acids that constrain conformation in different ways: N^{α}-alkyl amino acids,[21,22] i.e. (NMe)Ala, C^{α}-alkyl amino acids,[23] i.e. Aib,

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Table III. Activities of [Sar[1],Ile[8]]ANG II Antagonist Analogues Substituted in Position 3

no.	Sar–Arg–X–Tyr–Ile–His–Pro–Ile (1 2 3 4 5 6 7 8) X[3]	agonist: in vivo[a] ANG II like[c]	agonist: in vivo[b] ANG II like[d]	antagonist: in vitro pA_2	antagonist: in vivo ID_{50}[e]
17	Val	0	10.2 ± 0.8	9.1	10.0 ± 2.0
18	Ala	0	7.5 ± 0.8	8.3	25.0 ± 3.2
19	Pro	1.5[f]	–	7.17[f]	–
20	Aib	0	3.0 ± 0.4	7.0	50 ± 4.8
21	(NMe)Ala	0	18.0 ± 2.3	8.9	25.0 ± 3.1

[a] Agonist "ANG II like" activity and antagonist activity, pA_2, were measured in the in vitro rabbit aorta strip assay ($n = 5$) according to the method of Rioux et al.[31] [b] Residual "ANG II like" activity and antagonist activity, ID_{50}, were measured in vivo, in the rat blood pressure assay ($n = 5$) described by Regoli et al.[32] [c] ANG II like activity in vitro is expressed as percent activity relative to ANG II, pD_2 8.5 ($n = 5$). [d] ANG II like activity in vivo is expressed by the mmHg of blood pressure increase produced by a one microgram bolus intravenous injection of compound ($n = 5$). [e] ID_{50} in ng/rat per min (using 250-g rats). [f] Reference 9.

Table IV. Activities of ANG II Agonist Analogues Substituted in Position 5

no.	Sar–Arg–Val–Tyr–Y–His–Pro–Phe (1 2 3 4 5 6 7 8) Y[5]	agonist: in vitro[a] rabbit aorta	agonist: in vivo[b] rat BP
14	Ile	180 ± 14	125 ± 15
22	Cle	70 ± 11	–
23	Dtc	250 ± 18	100 ± 13
24	(SMe)Pen	80 ± 10	–
25	(NMe)Ile	20.0 ± 1.9	–

[a] Agonist ANG II like activity in vitro, expressed as percent activity relative to ANG II, pD_2 8.5, was measured in the rabbit aorta strip assay ($n = 5$) according to the method of Rioux et al.[31] [b] Agonist ANG II like activity in vivo, expressed as percent activity, was measured in the rat blood pressure assay ($n = 5$) according to the method of Regoli et al.[32]

Cle, and (αMe)Tyr, and N^{α} to C^{α} cyclic amino acids, i.e. Pro, Dtc[24,25] and the lactam modification,[26] L,L-lactam-Phe, previously described by Freidinger.

The Importance of the Side Chain. Since the loss or retention of biological activity is the measure of importance of a conformational constraint, the substitution should retain the native side chain or at least mimick some of the physical properties of the side chain, e.g. hydrophilicity, ionic charge, steric volume. In the present set of analogues, the native amino acids in positions 3, (Val), 5 (Ile), and 7 (Pro) contain lipophilic side chains. The following contrained amino acids display comparable lipophilicities: Pro[7] may be compared with (NMe)Ala and Aib (π_R 0.653, 0.618, and 0.618, respectively); while Val[3] and Ile[5] should be compared with Dtc, (NMe)Ile and Cle are comparable (π_R 1.238, 1.766, 1.632, 1.372, and 2.168, respectively).[27] Peptide couplings involving *N*-alkyl and α-alkyl amino acids can be difficult especially when the coupling amino acids contain bulky side chains, e.g. Val, Ile. In that event the shorter constrained amino acid, e.g. (NMe)Ala or Aib, may be incorporated more readily, but these analogues must be compared to Ala to account for the lack of side chain. Where possible we attempted to employ the full side chain.

Positions 4 and 8 have been examined previously[17,28-30] with analogues bearing NMe and C^{α}Me and were not reexamined here. In both of these positions, C^{α}Me analogues were superior.

Most of the ANG II analogues that bear conformational constraints in the literature were designed as Phe[8] agonists (Table I). The present study examines modifications to both Phe[8] agonists and Ile[8] antagonists to search for modifications that may differentially affect agonist and antagonist activity.

Results and Discussion

A number of analogues of [Sar[1]]ANG II, henceforth refered to as *agonist analogues*, were prepared that contain modifications of the position 3 Val, Table II, the position 5 Ile, Table IV, and the position 7 Pro, Table VI. These analogues were evaluated for ANG II-like agonist activity in the in vitro rabbit aorta strip assay[31] and in vivo for pressor activity.[32] Similarly, a number of analogues of

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Table V. Activities of [Sar[1],Ile[8]]ANG II Antagonist Analogues Substituted in Position 5

no.	Sar–Arg–Val–Tyr–Y–His–Pro–Ile (1 2 3 4 5 6 7 8) Y[5]	agonist: in vitro[a] ANG II like[c]	agonist: in vivo[b] ANG II like[d]	antagonist: in vitro pA_2	antagonist: in vivo ID_{50}[e]
17	Ile	0	10.2 ± 0.8	9.1	10.0 ± 2.0
26	Ala	0	5.0 ± 0.7	7.5	50.0 ± 5.4
27	Aib	0	12.5 ± 1.3	7.7	25.0 ± 3.7
28	Pro	0	7.0 ± 1.0	8.7	20.0 ± 3.5
29	(NMe)Ile	0[f]	–	7.64[f]	–
30	Cle	0	10.0 ± 1.3	8.3	10.0 ± 1.2
31	Dtc	0	11.4 ± 0.95	9.0	25.0 ± 3.2
32	(SMe)Pen	0	12.5 ± 1.1	9.0	20.0 ± 0.71
33	Tyr	0	2.5 ± 0.42	9.6	50.0 ± 5.5
34	(αMe)Tyr	0	0[g]	8.25	100 ± 14.5

[a] Agonist "ANG II like" activity and antagonist activity, pA_2, were measured in the in vitro rabbit aorta strip assay (n = 5) according to the method of Rioux et al.[31] [b] Residual "ANG II like" activity and antagonist activity, ID_{50}, were measured in vivo, in the rat blood pressure assay (n = 5) described by Regoli et al.[32] [c] ANG II like activity in vitro is expressed as percent activity relative to ANG II, pD_2 8.5 (n = 5). [d] ANG II like activity in vivo is expressed by the mmHg of blood pressure increase produced by a one microgram bolus intravenous injection of compound (n = 5). [e] ID_{50} in ng/rat per min (using 250-g rats). [f] Reference 20. [g] Analogue 34 displays residual ANG II like activity at higher doses, e.g. 2.5 mm at 10 μg.

Table VI. Activities of [Sar[1]]ANG II Agonist Analogues Substituted in Position 7

no.	Sar–Arg–Val–Tyr–Ile–His–Z–Phe (1 2 3 4 5 6 7 8) Z[7]	agonist: in vitro[a] rabbit aorta	agonist: in vivo[b] rat BP
14	Pro	180 ± 14 160 (RU)[c]	125 ± 15
35	Tpr	238 (RU)[c]	
36	Dtc	4.0 ± 0.5	
37	Aib	18.0 ± 1.7	

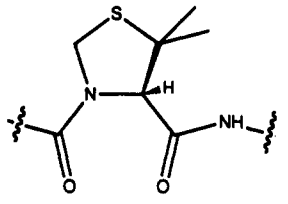
[a] Agonist ANG II like activity in vitro, expressed as percent activity relative to ANG II, pD_2 8.5, was measured in the rabbit aorta strip assay (n = 5) according to the method of Rioux et al.[31] [b] Agonist ANG II like activity in vivo, expressed as percent activity, was measured in the rat blood pressure assay (n = 5) according to the method of Regoli et al.[32] [c] Reference 36.

[Sar[1],X[8]]ANG II, henceforth refered to as *antagonist analogues*, were prepared that contain modifications of the position 3 Val, Table III, the position 5 Ile, Table V, and the position 7 Pro, Table VII. In most of these antagonist analogues X[8] = Ile, but in some analogues X[8] = D-Phe, Ala, or Aib, which give antagonists of comparable activity to Ile[8], Table VIII. These analogues were evaluated for their ability to antagonize the effects of ANG II in the in vitro rabbit aorta strip assay and the in vivo rat pressor assay.

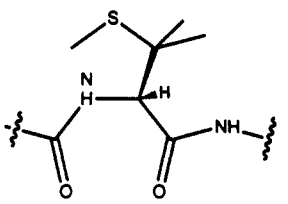
Position 3 of ANG II Agonists. Previous studies with ANG II agonists evaluated the effects of Pro[3], Aib[3], and Cle[3] substitution, Table I. The only modification which retained most of the agonist activity of ANG II was Pro[3] in analogue **3**. The present study extended position 3 modifications to include (NMe)Ala[3] in analogue **16**, which was superior to the Ala[3] analogue **15**, but less than the Val[3] analogue **14**, Table II. It appears, therefore, that either type of modification, Pro or (NMe)Ala, may be deployed in position 3 of ANG II agonists.

Position 3 of ANG II Antagonists. Pro[3], Aib[3], and (NMe)Ala[3] were examined in the ANG II antagonist [Sar[1],Ile[8]]ANG II, analogue **17**, Table III. The Pro[3] analogue **19** (previously reported in ref 9) and Aib[3] analogue **20** displayed greater than 1 order of magnitude lower potency than either the Val[3] analogue **17** or Ala[3] analogue **18**. The (NMe)Ala[3] analogue **21**, on the other hand, was more potent than the Ala[3] analogue **18** and almost as potent as the Val[3] analogue **17**. These results suggest that the structural requirements for ANG II antagonists may be more stringent in position 3 than for ANG II agonists.

Position 5 of ANG II Agonists. In previous studies, Table I, the Pro[5] agonist analogue **8** retained a level of potency comparable to the Ala[5] agonist **6**. The Aib[5] analogue **9** was somewhat less active than the Ala[5] agonist **6**. The more lipophilic Cle[5] analogue **10** was identical with the Nle[5] analogue **7** but less active than the native Ile[5] analogue **1**. In general, ANG II agonists that lack a β-branched amino acid in position 5 (e.g. Nle[5]) are less active than those that bear substitutions that are β-branched (e.g., Ile[5]).[14,15,18,33] In the present study, Table IV, the comparable Cle[5] analogue **22** was also less active than the native Ile[5] agonist **14**. An appropriately β-branched version of Cle was not available, but Dtc, a β-branched version of Pro was available. The Dtc[5] analogue **23** was a superagonist in vitro but displayed in vivo activity that merely paralleled the native Ile[5] agonist **14**. (SMe)Pen was also examined since it is a β-branched amino acid that contains the same number of carbon atoms as Dtc but lacks the five-membered ring structure. The (SMe)Pen[5] analogue **24**, however, was less active than the native Ile[5] agonist, suggesting that structural constraint imposed by the five-membered ring in Dtc is important in position 5. In this position we were able to incorporate an NMe amino acid with the native side chain, i.e. (NMe)Ile, in position 5 of the agonist analogue **25**. This modification, however, resulted in a loss of activity.



Dtc



(SMe)Pen

Position 5 of ANG II Antagonists. Khosla et al.[20] had been able to prepare the (NMe)Ile[5] antagonist **29**, Table V, but like the (NMe)Ile[5] agonist **25** in Table IV, it was less active than the native Ile[5] compound **17**. As with the

(32) Regoli, D.; Park, W. K. The Pressor and Myotropic Effects and the Antagonistic Properties of Several Analogs of Angiotensin II. *Can. J. Physiol. Pharmacol.* **1972**, *50*, 99–112.

(33) Nadasi, L.; Medzihradszky, K. The Use of a Steric Parameter (Y_γ) in QSAR Calculations for Peptide Hormones. *Peptides* **1983**, *4*, 137–144.

Table VII. Activities of [Sar[1],Ile[8]]ANG II Antagonist Analogues Substituted in Position 7

no.	Sar–Arg–Val–Tyr–Ile–His–Y–Ile (1 2 3 4 5 6 7 8) Y[7]	agonist: in vitro[a] ANG II like[c]	agonist: in vivo[b] ANG II like[d]	antagonist: in vitro pA_2	antagonist: in vivo ID_{50}[e]
17	Pro	0	10.2 ± 0.94	9.1 8.6 (RU)[f]	10.0 ± 0.71
38	(NMe)Ala	0	10.0 ± 1.2	8.4	20.0 ± 3.1
39	Aib	0	20.0 ± 1.9	8.0	60 ± 8.5
40	Tpr	0	15.0 ± 1.7	9.7 7.2 (RU)[f]	12.5 ± 1.9
41	(O)Tpr	0	-	6.7	-
42	Dtc	4.0	40 ● 6.3	7.5	250 ± 32
43	L,L-lactam-Phe	0	0[g]	8.3	100 ± 18.5

[a] Agonist "ANG II like" activity and antagonist activity, pA_2, were measured in the in vitro rabbit aorta strip assay (n = 5) according to the method of Rioux et al.[31] [b] Residual "ANG II like" activity and antagonist activity, ID_{50}, were measured in vivo, in the rat blood pressure assay (n = 5) described by Regoli et al.[32] [c] ANG II like activity in vitro is expressed as percent activity relative to ANG II, pD_2 8.5 (n = 5). [d] ANG II like activity in vivo is expressed by the mmHg of blood pressure increase produced by a one microgram bolus intravenous injection of compound (n = 5). [e] ID_{50} in ng/rat per min (using 250-g rats). [f] RU = rat uterus, ref 36. [g] Agonist activity was not observed at 1 μg/rat, but at higher doses, e.g. 5.0 mmHg at 5 μg.

Table VIII. Comparison of ANG II Analogues Bearing Single and Multiple Conformational Constraints

no.	Sar–Arg–W–Tyr–X–His–Y–Z– (1 2 3 4 5 6 7 8) W[3]	X[5]	Y[7] Z[8]	agonist: in vitro[a] ANG II like[c]	agonist: in vivo[b] ANG II like[d]	antagonist: in vitro pA_2	antagonist: in vivo ID_{50}[e]
21	(NMe)Ala	Ile	Pro-Ile	0	18.0 ● 2.3	8.9	25.0 ± 3.1
31	Val	Dtc	Pro-Ile	0	11.4 ● 0.95	9.0	25.0 ± 3.2
44	Val	Ile	Pro-Aib	0[f]	11.0[f]	8.7[f]	19.76 (DR)[f]
45	Val	Ile	Pro-D-Phe	0[g]	7.5 ● 0.6[g]	9.0[g]	12.5 ± 2.3[g]
46	(NMe)Ala	Dtc	Pro-Aib	0	-	≪6.0	-
47	(NMe)Ala	Ile	Pro-D-Phe	0	10	<6.0	100
48	Val	Dtc	Pro-D-Phe	0	5	7.5	1000
compared to:							
17	Val	Ile	Pro-Ile	0	10.2 ± 0.8	9.1	10.0 ± 2.0
49	Val	Ile	Pro-Ala	0	10.0 ± 0.7	8.6	15.0 ± 3.0
				0.50[f]	15.11[f]	8.61[f]	7.92 (DR)[f]

[a] Agonist "ANG II like" activity and antagonist activity, pA_2, were measured in the in vitro rabbit aorta strip assay (n = 5) according to the method of Rioux et al.[31] [b] Residual "ANG II like" activity and antagonist activity, ID_{50}, were measured in vivo, in the rat blood pressure assay (n = 5) described by Regoli et al.[32] [c] ANG II like activity in vitro is expressed as percent activity relative to ANG II, pD_2 8.5 (n = 5). [d] ANG II like activity in vivo is expressed by the mmHg of blood pressure increase produced by a one microgram bolus intravenous injection of compound (n = 5). [e] ID_{50} in ng/rat per min (using 250-g rats). [f] Described by Khosla et al.[50] DR = dose ratio, in vivo ANG II like activity with 250 ng/kg per min for 3 min. [g] Described by Samanen et al.[17]

ANG II agonists, the less lipophilic Aib[5] and Pro[5] antagonists **27** and **28** were less active than the corresponding Cle[5] and Dtc[5] antagonists **30** and **31**. It is interesting to note that, in contrast to the position 5 agonist analogues, the (SMe)Pen[5] antagonist is equipotent with the Ile[5] antagonist **17**. This suggests that the five-membered ring structure of Dtc may be less important in position 5 of ANG II antagonists than in position 5 of ANG II agonists. We had previously shown[34] that ANG II antagonists do not require a β-branched amino acid in position 5 and that aromatic amino acids such as Tyr give antagonists with enhanced activity in vitro, e.g. the Tyr[5] antagonist **33**. In this case the α-alkyl amino acid (αMe)Tyr[5] gives an analogue **34**, which is less active than the α-H substituted analogue **33**. It is important to note that while the Cle[5] antagonist **30** was less potent that the native β-branched Ile[5] antagonist **17** in vitro, it is equipotent in vivo.

Position 7 of ANG II Agonists.[35] Previous studies had examined (NMe)Ala, Aib, and Cle substitutions into position 7 of ANG II. While the (NMe)Ala[7] analogue had retained more agonist activity than either the Aib[7] or Cle[7] agonist **13** and **14**, the native proline was nonetheless superior, analogue **1**. In the present series of analogues, Aib[7] substitution still gives an agonist **37** that is less active than the native Pro[7] agonist **14**. The Dtc[7] agonist **36** is also considerably less active than the Pro[7] agonist **14**. Previously published spectroscopic and theoretical data shows that Dtc-containing peptide are unable of adopting the C7 conformation but can adopt α helical and more extended conformations. Thus, while ANG II may be capable of adopting a C7 conformation about Pro[7], it may not be able to adopt an α helical conformation about position 7 that either Dtc or Aib peptides can adopt. As mentioned earlier, this conclusion must be tempered by the alternate possibility that the β-methyl groups in Dtc or the α-methyl group in Aib could be interacting negatively with the receptor. A third explanation for the low activity displayed by the Dtc[7] agonist **36** could implicate the γ-sulfur atom in Dtc, but the analogous Tpr[7] agonist **35** was shown previously[36] to be more active than the Pro[7] agonist **14**, suggesting a positive receptor interaction with the γ-sulfur atom.

Position 7 of ANG II Antagonists.[35] In ANG II antagonists (NMe)Ala[7] and Aib[7] substitutions give analogues **38** and **39** with reduced antagonist activity over the parent

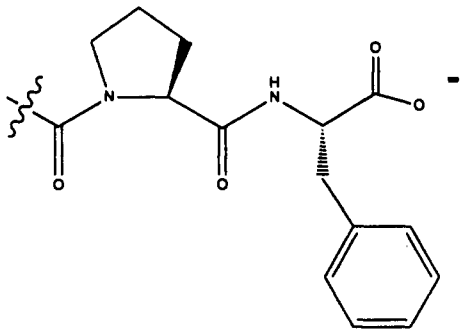
(34) Samanen, J.; Narindray, D.; Cash, T.; Brandeis, E.; Adams, W., Jr.; Yellin, T.; Regoli, D. Potent Angiotensin II Antagonists with Non-β-Branched Amino Acids in Position 5. *J. Med. Chem.* **1989**, *32*, 466–472.

(35) This work was initially communicated in Samanen, J.; Cash, T.; Narindray, D.; Yellin, T.; Regoli, D. The Substitution of Conformationally Constrained Amino Acids into Position 7 of Angiotensin II (Ang II) Agonist and Antagonist Analogs. *Peptides, Chemistry, Structure and Biology; Proceedings of the Eleventh American Peptide Symposium*; Rivier, J., Ed.; ESCOM Inc.: Leiden, 1990; pp 307–309.

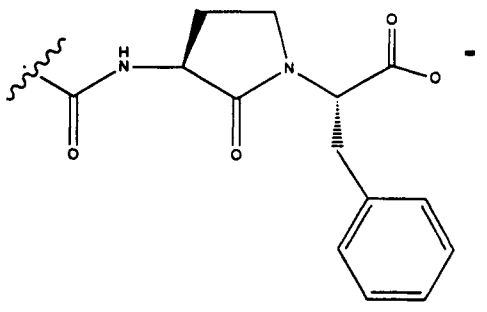
(36) Moore, G. J. Angiotensins II and III: The Functional Role of the Penultimate Proline Residue. *Peptides, Synthesis, Structure, Function; Proceedings of the Seventh American Peptide Symposium*; Rich, D. H., Gross, E., Eds.; Pierce Chemical Co.: Rockford, IL, 1981; pp 245–248.

Pro[7] antagonist 17. As with ANG II agonists, Dtc[7] substitution gave an antagonist 42 with reduced antagonist activity. In the rat uterus, the previously reported Tpr[7] antagonist 40 was somewhat less active than the native Pro[7] antagonist 17.[36] In the rabbit aorta, however, this analogue appears to be more potent than 17. Again, while ANG II may be capable of adopting a C7 conformation about Pro[7], it may not be able to adopt an α helical conformation that either Dtc or Aib peptides can adopt, since the corresponding Dtc[7] and Aib[7] peptides were less active.

We also examined[37] the lactam modification previously described by Freidinger[26] in position 7. Even though the modification was incorporated into a Phe[8] analogue, 43, the compound was devoid of agonist activity in vitro and in vivo but was a pure antagonist, with a level of activity (pA_2 8.3) comparable in vitro to saralasin (pA_2 8.6). The partial agonist activity typically seen in ANG II antagonists in vivo was also reduced (no increase in blood pressure upon bolus injection of 1 μg of peptide). The in vivo potency was lower than that of saralasin in vivo (IC_{50} 100 vs 25 ng/rat per min),[37] perhaps due to the lack of a secondary amino acid in position 7 that typically blocks degradation by converting enzyme.[38] The known different



-Pro-Phe



-L,L-lactam-Phe

conformational effects of Pro versus the lactam[26] suggests that at least two different types of conformations about position 7 are acceptable for high affinity receptor interaction by ANG II antagonists. This contrasts the agonist data for which one set of constraints was preferred at position 7, which is consistent with the hypothesis that ANG II agonists adopt a single conformation about the C-terminus.[39]

Multiple Substitutions of "Preferred" Conformational Constraints in ANG II Antagonists. Individually, (NMe)Ala[3], Dtc[5], D-Phe[8], and Aib[8] substitutions into [Sar[1],Ile[8]]ANG II give analogues with retained antagonist activity, Table VIII. Cosubstitution of these types of residues into [Sar[1]]ANG II gives 46 [Sar[1],(NMe)-Ala[3],Dtc[5],Aib[8]]ANG II, which is devoid of agonist or antagonist activity, 47 [Sar[1],(NMe)Ala[3],D-Phe[8]]ANG II, which displayed a minor amount of antagonist activity in vivo, and 48 [Sar[1],Dtc[5],D-Phe[8]]ANG II, which displayed a minor amount of antagonist activity in vitro, Table VIII. Since we were searching for potent constrained ANG II antagonists, this excercise was not performed in a [Phe[8]]ANG II agonist. While the data from Tables I–VII could be employed in an analysis of bioactive conformation of ANG II agonist and antagonists, the fact that three analogues bearing different combinations of "preferred" conformational constrants, 46–48, were all inactive as agonists or antagonists, challenges simple interpretations of the data for positions 3, 5, and 8. Even though the multiple incorporation of "preferred" conformational constraints has led to highly constrained potent analogues of native peptides such as somatostatin[40] and LHRH,[41] the simple addition of constrained amino acids to ANG II may be unproductive. Synthesis of all possible combinations in ANG II agonists and ANG II antagonists have not been attempted. Nonetheless, the present results bode ill for the identification of a highly constrained ANG II agonist or antagonist octapeptide. One possible interpretation of the inactivity of these analogues may be that the constraints collectively lock the peptide into the final ligand conformation that lacks sufficient flexibility for a dynamic receptor binding process or "zipper" mechanism[42] that has been previously proposed for flexible peptide ligand and receptor interaction. If the zipper mechanism applies to ANG II receptor interaction, then a certain degree of flexibility will need to be retained in ANG II octapeptide antagonists to maintain high receptor affinity.

Since highly constrained biologically active analogues were not discovered, the analogues were not rigorously evaluated for stability to enzymatic degradation. Also, a thorough spectroscopic evaluation of the ANG II analogues was not attempted. While some of the data presented may relate ultimately to ANG II bioactive conformation, it is clear that this information may not yet be incorporated into detailed models of bioactive conformation. Certain critical pieces of the puzzle remain to be identified before coherent ANG II agonist and antagonist bioactive conformations are discovered.

Experimental Section

tert-Butyloxycarbonyl amino acids and peptide reagents were obtained from Bachem Fine Chemicals, Inc., Protein Research

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(42) Burgen, A. S. V.; Roberts, G. C. K.; Feeney, J. Binding of Flexible Ligands to Macromolecules. *Nature* 1975, *253*, 753–755.

Table IX. ANG II Analogue Data[a]

	amino acid analysis[b]								TLC R_f			HPLC		
no.	1	2	3	4	5	6	7	8	A	B	C	solvent % CH_3CN	K'	% purity
14	Sar	Arg	Val	Tyr	Ile	His	Pro	Phe	0.22	0.58	0.28	20	2.60	>98
	(+)	0.98	1.00	1.01	0.99	1.02	1.01	0.99						
15	Sar	Arg	Ala	Tyr	Ile	His	Pro	Phe	0.18	0.32	0.44	38	8.0	95
	(+)	1.01	0.97	1.03	0.98	1.01	0.97	1.03						
16	Sar	Arg	(NMe)Ala	Tyr	Ile	His	Pro	Phe	0.31	0.66	0.67	32	3.85	>98
	(+)	1.01	(+)	1.02	0.97	0.99	1.01	1.02						
17	Sar	Arg	Val	Tyr	Ile*	His	Pro	Ile*	0.19	0.67	0.75	20	2.80	>98
	(+)	0.99	0.97	0.98	1.01	1.07	0.99	1.01						
18	Sar	Arg	Ala	Tyr	Ile*	His	Pro	Ile*	0.18	0.27	0.63	38	3.31	>98
	(+)	1.01	1.00	1.02	0.98	1.01	0.98	0.98						
20	Sar	Arg	Aib	Tyr	Ile*	His	Pro	Ile*	0.12	0.36	0.57	30	2.75	>98
	(+)	0.96	(+)	1.03	0.96	1.03	1.05	0.96						
21	Sar	Arg	(NMe)Ala	Tyr	Ile*	His	Pro	Ile*	0.16	0.36	0.52	40	0.72	>98
	(+)	1.01	(+)	1.02	0.97	1.02	0.99	0.97						
22	Sar	Arg	Val	Tyr	Cle	His	Pro	Ile	0.18	0.42	0.59	25	3.55	>98
	(+)	1.00	1.02	1.01	(+)	1.01	0.98	1.00						
23	Sar	Arg	Val	Tyr	Dtc	His	Pro	Ile	0.18	0.43	0.50	20	5.1	>98
	(+)	1.00	1.03	1.00	(+)	0.95	1.02	1.01						
24	Sar	Arg	Val	Tyr	(SMe)Pen	His	Pro	Ile	0.12	0.31	0.41	23	3.9	>98
	(+)	1.04	1.02	1.03	(+)	0.97	0.90	1.03						
25	Sar	Arg	Val	Tyr	(NMe)Ile	His	Pro	Ile	0.17	0.48	0.44	20	7.7	>98
	(+)	1.05	1.00	1.02	(+)	1.01	0.92	1.00						
26	Sar	Arg	Val	Tyr	Ala	His	Pro	Ile	0.10	0.31	0.40	30	2.05	98
	(+)	1.02	1.01	1.02	0.98	1.01	0.98	0.99						
27	Sar	Arg	Val	Tyr	Aib	His	Pro	Ile	0.14	0.35	0.42	30	2.71	>98
	(+)	0.98	0.98	0.99	(+)	1.01	1.02	1.02						
28	Sar	Arg	Val	Tyr	Pro*	His	Pro*	Ile	0.04	0.19	0.52	20	2.45	>98
	(+)	1.04	1.02	1.01	0.92	1.01	0.92	0.95						
30	Sar	Arg	Val	Tyr	Cle	His	Pro	Ile	0.14	0.36	0.58	17	19.1	>98
	(+)	1.02	0.99	0.98	(+)	1.01	0.98	1.02						
31	Sar	Arg	Val	Tyr	Dtc	His	Pro	Ile	0.16	0.44	0.58	20	3.6	>98
	(+)	1.01	1.03	0.99	(+)	0.97	1.01	0.99						
32	Sar	Arg	Val	Tyr	(SMe)Pen	His	Pro	Ile	0.20	0.44	0.53	20	3.7	>98
	(+)	1.02	1.01	1.01	(+)	0.96	0.99	1.01						
33	Sar	Arg	Val	Tyr*	Tyr*	His	Pro	Ile	0.20	0.48	0.73	20	2.14	90
	(+)	0.98	0.99	1.00	1.00	1.03	1.03	0.97						
34	Sar	Arg	Val	Tyr	(αMe)Tyr	His	Pro	Ile	0.04	0.32	0.54	20	2.93	98
	(+)	1.03	0.96	0.97	(+)	1.06	0.78	1.19						
36	Sar	Arg	Val	Tyr	Ile	His	Dtc	Phe	0.21	0.60	0.53	30	2.40	95
	(+)	1.02	1.02	1.00	0.96	1.00	(+)	1.00						
37	Sar	Arg	Val	Tyr	Ile	His	Aib	Phe	0.17	0.47	0.58	25	2.5	>98
	(+)	1.00	1.01	1.00	0.99	1.00	(+)	1.00						
38	Sar	Arg	Val	Tyr	Ile*	His	(NMe)Ala	Ile*	0.10	0.25	0.32	38	10.2	>98
	(+)	1.01	1.01	1.02	0.98	0.99	(+)	0.98						
39	Sar	Arg	Val	Tyr	Ile*	His	Aib	Ile*	0.20	0.34	0.58	43	1.8	>98
	(+)	1.01	1.01	1.01	0.97	1.02	(+)	0.97						
40	Sar	Arg	Val	Tyr	Ile*	His	Tpr	Ile*	0.19	0.49	0.54	20	2.3	>98
	(+)	1.04	1.01	0.99	0.96	1.04	(+)	0.96						
41[c]	Sar	Arg	Val	Tyr	Ile*	His	(O)Tpr	Ile*	0.05	0.27	0.50	25	1.6	>98
	(+)	1.04	1.01	0.99	0.96	1.04	(+)	0.96						
42	Sar	Arg	Val	Tyr	Ile*	His	Dtc	Ile*	0.28	0.74	0.84	30	1.9	>98
	(+)	1.05	1.06	1.01	0.91	1.04	(+)	0.91						
43	Sar	Arg	Val	Tyr	Ile	His	lactam-Phe		0.23	0.61	0.45	20	10.3	88
	(+)	1.00	0.79	1.01	0.96	1.02	(+)							
45	Sar	Arg	Val	Tyr	Ile	His	Pro	D-Phe	0.19	0.55	0.76	23	8.6	96
	(+)	1.01	1.01	1.02	0.96	1.02	0.99	1.02						
46[d]	Sar	Arg	(NMe)Ala	Tyr	Dtc	His	Pro	Aib	0.01	0.21	0.22	20	3.50	98
	(+)	0.94	(+)	1.00	(+)	1.01	1.00	(+)						
47[e]	Sar	Arg	(NMe)Ala	Tyr	Ile	His	Pro	D-Phe	0.43	0.73	0.89	12	1.8	>98
	(+)		(–)[e]	1.04	0.96	1.04	1.03	1.04						
48[f]	Sar	Arg	Val	Tyr	Dtc	His	Pro	D-Phe	0.21	0.61	0.57	22	3.8	>98
	(+)	1.12	1.12	0.57	(+)	0.99	1.11	1.11						
49	Sar	Arg	Val	Tyr	Ile	His	Pro	Ala	0.06	0.29		10	5.86	>98
	(+)	1.04	1.00	1.04	0.99	1.01	0.94	0.97						

[a] See text for details of analytical procedures. [b] Amino acid analysis expressed in molar ratios of the D,L amino acids in the peptides. [c] FAB $(M + H)^+$ 1002. [d] FAB $(M + H)^+$ 957. [e] Arg-(NMe)Ala absent, presumably due to lack of hydrolysis. Structure is correct, however, via FABMS $(M + H)^+$ 988. [f] FAB $(M + H)^+$ 1032. (+) = Amino acid present in roughly 1 molar equiv (in cases where quantitation is difficult). * = Amino acid present in two positions; value expressed is half the experimental value.

Foundation, or Chemical Dynamics Corporation and were used without further purification. Boc-Dtc,[34] Boc-(SMe)Pen,[34] Boc-(NMe)Ile,[20] and Boc(2,6-Cl_2Bzl,αMe)Tyr[20] and were prepared by standard procedures from commercially available amino acids. Boc-L,L-lactam-Phe was prepared via the literature procedure.[26] The enantiomeric Boc-D,L-lactam-Phe, however, could not be obtained by this procedure.

Peptide Synthesis and Purification. All peptides were prepared by the solid-phase method on Beckman 990-B Peptide synthesizers.[43,44] The C-terminal residue was esterified to a

chloromethylated copolymer of polystyrene and 2% divinylbenzene (Bio-Rad) via a cesium salt procedure.[45] The degree of substitution was determined by amino acid analysis of a hydrolysate obtained by treating the amino acid resin with HCl–PrOH (1:1) at 120 °C for 3 h.[46] Routine deprotection of Boc-amino protecting groups was accomplished with 30% TFA in CH_2Cl_2 and neutralization with 10% TEA in CH_2Cl_2. Coupling of each amino acid was performed with a 2.5 M excess of *tert*-butyloxycarbonyl amino acid and DCC in CH_2Cl_2 with completeness of reaction monitored by the ninhydrin test.[47] Side chain protecting groups were as follows: Arg, tosyl; Tyr, σ-Br-Z or 2,6-Cl_2Bzl; His, tosyl.

In most cases coupling was complete after 2 h. If the Ninhydrin test remained positive, a recoupling cycle was performed. After the last coupling and deprotection the peptide was cleaved from resin by treatment with anhydrous HF containing 50% (v/v) anisole at 0 °C for 60 min. After vacuum evaporation of HF the resin was rinsed with Et_2O to remove anisole and then rinsed with glacial HOAc and filtered. The filtrate was diluted with water and lyophilized to a powder of crude peptide material.

The crude peptide were purified to homogeneity either by (a) partitioning through 200 transfers of countercurrent distribution in *n*-BuOH–HOAc–H_2O (4:1:5), (b) by partition chromatography[48] on Sephadex G-15 in *n*-BuOH–HOAc–H_2O (4:1:5), or (c) reversed-phase semipreparative HPLC[49] on a Whatman C^{18} column using the appropriate solvent mixture of CH_3CN/0.1 N NH_4OAc, pH 4. The volumes of chromatographic fractions containing pure peptide by TLC were reduced by partial rotary evaporation and dried to powders by lyophilization to constant weight.

Homogeneity of each peptide was determined by the following methods: (a) Amino acid analysis of 72-h acid hydrolysis (6 N HCl, 110 °C) performed on a Beckman Model 120C analyzer. (b) Analytical TLC on silica gel plates with solvent systems A = *n*-BuOH–AcOH–H_2O (4:1:5), B = *n*-BuOH–AcOH–H_2O–EtOAc (1:1:1:1), and C = *n*-BuOH–AcOH–H_2O–pyridine (15:3:12:10), visualizing spots with Pauly reagent.[47] (c) Analytical reversed-phase HPLC on a C_{18} silica gel column using the appropriate CH_3CN–0.1 N NH_4OAc (pH 4) mixture, following elution by UV (250-nm detection). (d) FAB mass spectrometry performed on a VG ZAB-1F-HF mass spectrometer with a standard FAB source employing a glycerol matrix, in cases where more than two unnatural amino acids are present in the structure. Analytical data for all peptides are listed in Table IX.

[Sar^1,(O)Tpr^7,Ile^8]ANG II, 41. Solid $NaIO_4$ (28.2 mg, 0.132 mmol) was added to an aqueous solution (100 μL) of [Sar^1,Tpr^7,Ile^8]ANG II, **40** (98.5 mg, 0.1 mmol). After the mixture was stirred for 2 h, TLC indicated the disappearance of **40**, and the solution was lyophilized. Rotary evaporation and lyophilization of the fractions analyzed by TLC to contain pure **41** from Sephadex G-25 partition chromatography, using an *n*-BuOH–AcOH–H_2O (4:1:5) solvent mixture, gave **41** as a fluffy white powder (18.3 mg); FAB mass spectrum $(M^+H)^+$ 1002; other analytical data displayed in Table IX.

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Registry No. **1**, 4474-91-3; **2**, 13761-29-0; **3**, 19729-16-9; **4**, 135145-55-0; **5**, 37578-26-0; **6**, 135145-56-1; **7**, 135145-57-2; **8**, 135145-58-3; **9**, 135145-59-4; **10**, 135145-60-7; **11**, 135145-61-8; **12**, 135145-62-9; **13**, 135145-63-0; **15**, 135145-65-2; **16**, 135145-66-3; **17**, 37827-06-8; **18**, 135145-67-4; **19**, 43021-22-3; **20**, 135145-68-5; **21**, 135145-69-6; **22**, 135145-70-9; **23**, 117940-36-0; **24**, 117940-32-6; **25**, 135145-71-0; **26**, 117918-12-4; **27**, 135145-72-1; **28**, 117918-21-5; **29**, 57817-46-6; **30**, 135145-73-2; **31**, 117940-37-1; **32**, 117940-33-7; **33**, 117918-14-6; **34**, 135145-74-3; **35**, 82018-92-6; **36**, 135145-75-4; **37**, 135145-76-5; **38**, 71381-73-2; **39**, 135145-77-6; **40**, 82018-99-3; **41**, 135145-78-7; **42**, 135145-79-8; **43**, 135145-80-1; **44**, 63146-96-3; **45**, 111821-39-7; **46**, 135189-65-0; **47**, 135267-98-0; **48**, 135355-13-4; **49**, 38027-95-1; Asp-Arg-Cle-Tyr-Val-His-Cle-Phe, 135145-64-1; Sar-Arg-Val-Tyr-Ile-His-Pro-Phe, 51833-69-3; angiotensin II, 11128-99-7.

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