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Analogues of Angiotensin II with Restricted Conformational Freedom, Including a New Antagonist

JOHN TURK,[1] PHILIP NEEDLEMAN,[2] AND GARLAND R. MARSHALL[3]

Departments of Biological Chemistry, Pharmacology, and Physiology and Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110

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SUMMARY

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Two analogues of angiotensin II (AII) and one analogue of the AII antagonist [1-sarcosine, 8-valine]-angiotensin II ([Sar[1], Val[8]]-AII) have been synthesized which contain a methyl group in the place of a proton on α-carbon 4 or 8. Theoretical studies indicate that these analogues should have restricted conformational freedom. The relatively high activity of the position 4 analogue in the rat uterus and blood pressure assays, when interpreted in the light of previous structure-activity studies, allows the tentative assignment of the torsional angles ϕ and ψ at position 4 in the receptor-bound conformation of AII. These values differ from those determined for AII in solution. The position 8 analogue, [Sar[1],Val(αMe)[8]]-AII is itself an antagonist and is 7 times more potent *in vivo* than [Sar[1],Val[8]]-AII.

INTRODUCTION

Information about the spatial disposition of critical chemical functions of angiotensin II as it interacts with its receptor may prove useful in the rational design of analogues with desirable pharmacological properties, such as increased agonistic or antagonistic potency, duration of action, or organ selectivity. Direct study of peptide-receptor complexes is not feasible at the present time, however, because receptor isolation and characterization are in an early stage. An indirect approach to the elucidation of the receptor-bound conformation of AII[4] is to generate analogues with restricted, partially defined conformations and to use their biological activity as an index of how closely the introduced conformational constraints approximate the binding constraints imposed by the receptor.

In this regard, Marshall and co-workers (1-3) and subsequently others (4, 5) have shown from theoretical studies on the allowed torsional angles of model peptides

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[1] Graduate Fellow, Medical Scientist Training Program (GM-02016), National Institutes of Health.

[2] Recipient of Research Career Development Award HL-195-86 from the National Institutes of Health.

[3] Established Investigator of the American Heart Association (AHA-70-111).

[4] The abbreviations used are: AII, angiotensin II; Sar-, sarcosine(yl-); Boc-, *tert*-butoxycarbonyl-; Phe(αMe), α-methylphenylalanine(yl-); Val(αMe), α-methylvaline(yl-).

that the replacement of the α-proton of an amino acid residue with a methyl group results in a dramatic reduction of the conformational space available to the backbone of the peptide chain at the position where that residue occurs. Peptide hormone analogues containing α-methylamino acid residues should therefore correspond closely to "conformational analogues" of the hormone, i.e., analogues which have a primary structure essentially identical with that of the native hormone but which are capable of adopting only a more restricted set of conformations. Two such analogues of AII were synthesized and assayed biologically in this study.

Peptides containing α-methylamino acids are also of interest because of their potential resistance to the action of proteolytic enzymes. Model compounds containing α-methylamino acids are known to be resistant to the action of carboxypeptidase A, chymotrypsin, and hog renal acylase (6–9), and their apparently reduced susceptibility to chemical hydrolysis (10, 11) suggests that they may resist attack by other enzymes as well. For this reason an α-methylamino acid-containing analogue of a potent antagonist of AII was synthesized and assayed. One of the principal limitations to the utility of presently existing antagonists of AII is their transient lifetime (12). Conferring resistance to proteolysis at key positions in the sequence could prolong the duration of action of these molecules.

MATERIALS AND METHODS

Materials. [Asn^1,Val^5]-AII was a gift of Ciba-Geigy, Inc. [Sar^1,Ile^8]-AII was purchased from Beckman Instruments. All *tert*-butoxycarbonylamino acids were obtained from Bachem, except for Boc-Phe(αMe) and Boc-Val(αMe), which were prepared as described elsewhere (13). The Boc-L-phenylalanyl-substituted resin was synthesized by the method of Marshall and Merrifield (14), as was the Boc-Phe(αMe)-resin, except that in the latter case a reflux period of 3 days was used to achieve quantitative attachment of the amino acid. The Boc-Val(αMe)-resin could not be prepared in this way and was instead prepared by the method of Gisin (15), but the incubation period was increased to 3 weeks to achieve quantitative attachment.

Analytical methods. Thin-layer chromatography was performed on 0.25-mm silica gel G plates (Analtech) in the following four systems: 1-butanol–pyridine–acetic acid–H_2O, 15:10:3:12; 1-propanol–H_2O, 2:1; *sec*-butyl alcohol–3% NH_4OH, 100:44; and 1-butanol–acetic acid–H_2O, 4:1:1. Mobilities in these systems are designated R_{F1}, R_{F2}, R_{F3}, and R_{F4}, respectively. Thin-layer plates were developed by ninhydrin and Clorox-starch sprays (16). Peptides were hydrolyzed by the method of Westall *et al.* (17) for 4 and 12 hr. Amino acid analyses were performed on a Spinco 120 C analyzer. The peak for Val(αMe) appears after valine, and its color constant is 7.6% of that for valine. The peak for Phe(αMe) cochromatographs with that for phenylalanine, and since its color constant is only 12.3% of that for phenylalanine, it is also obscured by tyrosine. To quantitate the amount of Phe(αMe) present in peptides containing phenylalanine or tyrosine, it is necessary to remove the latter two amino acids from the peptide hydrolysate enzymatically. This is accomplished by successive 12-hr digestions at 37° with L-amino acid oxidase (0.2 mg/mg of peptide) at pH 6.5 in 0.2 M Tris buffer with 0.1 M KCl, and then with D-amino acid oxidase (0.05 mg/mg of peptide) plus catalase (0.05 mg/mg of peptide) in the same buffer at pH 8.3. The amount of Phe(αMe) can then be calculated with respect to either aspartic acid or proline, both of which are inert to the above treatment.

Boc-β-benzyl-L-aspartyl-nitro-L-arginyl-L-valyl-O-benzyl-L-tyrosyl-L-valyl-imidazolylbenzyl-L-histidyl-L-prolyl-L-α-methyl-phenylalanyl-resin (I). A total of 3.0 g of 0.23 mEq/g of BocPhe(αMe)-resin was placed in the reaction vessel and subjected to two 30-min deprotection steps with 25% trifluoroacetic acid in dichloromethane. Each step was preceded by three swelling steps in dichloromethane, three shrinking steps in 95% *tert*-butyl alcohol–5% dichloromethane, and three swelling steps in dichloromethane. The

deprotected Phe(αMe)-resin was then subjected to two 4-hr couplings with 6-fold excesses of Boc-proline with the appropriate shrinkage-swelling cycle preceding and following each step. From this point on, the synthesis proceeded by the customary cycle of operations (14).

L-Aspartyl-nitro-L-arginyl-L-valyl-L-tyrosyl-L-valyl-imidazolylbenzyl-L-histidyl-L-prolyl-L-α-methylphenylalanine (II). Compound I was suspended in 40 ml of anhydrous trifluoroacetic acid, and a slow stream of HBr was bubbled through the suspension for 45 min at room temperature. The suspension was filtered, and the resulting solution was then concentrated under reduced pressure, diluted with H_2O, and lyophilized. The yield was 670 mg (97%).

L-Aspartyl-L-arginyl-L-valyl-L-tyrosyl-L-valyl-L-histidyl-L-prolyl-L-α-methylphenylalanine ([Phe(αMe)⁸]-AII). Compound II and 500 mg of 5% palladium on barium sulfate were hydrogenated in 30 ml of methanol–acetic acid–H_2O (10:1:1) at 50 psi for 48 hr. The solution was filtered, concentrated under reduced pressure, diluted with H_2O, and lyophilized. The yield was 605 mg (88%). The crude octapeptide was purified by 180 transfers in a 1-butanol–acetic acid–H_2O (4:1:5) countercurrent distribution system. The location of the peptide was determined by the method of Lowry *et al.* (18), and it was found to be distributed symmetrically in tubes 52–88 (k = 0.65). Thin-layer chromatography was performed on the contents of each of these tubes, and the contents of tubes 56–80 were combined, concentrated under reduced pressure, diluted with H_2O, and lyophilized. The yield was 250 mg [36% based on the Phe(αMe) attached to the resin]. Amino acid analysis showed Asp 0.96, Pro 1.00, Val 2.04, Tyr 1.00, Phe(αMe) 1.07, Arg 0.96, His 0.91. One spot was seen in four thin-layer chromatographic systems: R_{F1} = 0.65, R_{F2} = 0.54, R_{F3} = 0.26, and R_{F4} = 0.11.

Boc-β-benzyl-L-aspartyl-nitro-L-arginyl-L-valyl-L-α-methylphenylalanyl-L-valyl-imidazolyltosyl-L-histidyl-L-prolyl-L-phenylalanyl-resin (III). This compound was prepared from 3.0 g of 0.38 mEq/g of Boc-phenylalanyl-resin in the same manner as for compound I, except that two 4-hr couplings with 6-fold excesses of Boc-amino acid were used to attach Boc-Phe(αMe) to the deprotected peptidyl-resin. Two deprotections were then performed before the next Boc-amino acid was added, and the latter was attached by two 4-hr couplings with 6-fold excesses of Boc-amino acid.

L-Aspartyl-L-arginyl-L-valyl-L-α-methylphenylalanyl-L-valyl-L-histidyl-L-prolyl-L-phenylalanine ([Phe(αMe)⁴]AII). Compound III was dried and placed in a polypropylene reaction vessel of an HF apparatus (19). Anisole (3 ml) and anhydrous HF (20 ml) were added, and the suspension was stirred for 50 min at 0°. The HF was removed by a stream of nitrogen and then under vacuum. The dry peptide and resin were washed with ethyl acetate and then extracted with 1% acetic acid. The extract was lyophilized to give 1075 mg (93%) of crude peptide, which was then purified by 180 transfers in a butanol–acetic acid–H_2O (4:1:5) countercurrent distribution system. The location of uncontaminated peptide was determined as above, and 506 mg of peptide (44% of the theoretical yield) were harvested from tubes 40–64 (k = 0.41). Amino acid analysis showed Asp 1.00, Pro 1.04, Val 2.16, Phe 0.93, Phe(αMe) 1.18, His 0.92, Arg 0.96. One spot was seen in four thin-layer chromatographic systems: R_{F1} = 0.65, R_{F2} = 0.54, R_{F3} = 0.31, and R_{F4} = 0.09.

Boc-L-sarcosyl-L-arginyl-L-valyl-O-benzyl-L-tyrosyl-L-valyl-imidazolyltosyl-L-histidyl-L-prolyl-L-α-methylvalyl-resin (IV). This compound was prepared in the same manner as compound I, except that the Boc-Val(αMe)-resin was triply deprotected and subjected to three 6-hr couplings with 9-fold excesses of Boc-proline.

L-Sarcosyl-L-arginyl-L-valyl-L-tyrosyl-L-valyl-L-histidyl-L-prolyl-L-α-methylvaline ([Sar¹,Val(αMe)⁸]-AII). Compound IV was subjected to the same cleavage-deprotection procedure as compound III. The yield of crude peptide was 370 mg (95%). This material was subjected to 560 transfers in the same countercurrent distribution system used above. A total of

180 mg of peptide was harvested from tubes 64–80 (k = 0.15) and then subjected to 180 transfers in a 1-butanol–pyridine–acetic acid–H_2O (8:2:1:9) countercurrent distribution system. Peptide harvested from tubes 28–44 (k = 0.25) weighed 130 mg (33%). Amino acid analysis showed Sar 1.02, Pro 1.00, Val(αMe) 0.97, Val 2.02, Tyr 1.02, His 0.97, Arg 0.98. One spot was observed in four thin-layer chromatographic systems: R_{F1} = 0.63, R_{F2} = 0.06, R_{F3} = 0.22, R_{F4} = 0.07.

Enzymatic digestion in vitro. Solutions of 0.01 mg/ml of AII and [Phe(αMe)^8]-AII in 0.05 M phosphate buffer (pH 7.4) were digested with 4 μg/ml (4 units) of bovine carboxypeptidase A (diisopropyl fluorophosphate) (Sigma Chemical Company) at 37°. Aliquots of the solutions were assayed after intervals of 0–60 min for residual activity in rat uterus.

Bioassays. The oxytocic activity of the analogues was measured as described elsewhere (20). The incubation period with the antagonist was 15 min. Blood pressures were measured in urethane (1.25 g/kg)-anesthetized Zivic-Miller male rats (400–600 g) by recording from the left femoral artery with a Physiograph linear core transducer. The agent was injected into the cannulated right jugular vein by means of a Hamilton syringe automatic dispensing apparatus. An AII dose-response curve was determined before and after the administration of each analogue, so that each animal served as its own control. Various concentrations (0.02–0.2 mg/ml) of the antagonists were infused into the cannulated left jugular vein for 15 min before and during the challenging doses of AII at a rate of 20 μl/min.

RESULTS

The three newly synthesized analogues are [Phe(αMe)^4]-AII, [Phe(αMe)^8]-AII, and [Sar^1,Val(αMe)^8]-AII. The syntheses of [Val(αMe)^3]-AII and [Val(αMe)^5]-AII were attempted also, but it was found that Boc-Val(αMe) is an extremely inactive acylating agent in solid-phase synthesis when activated by the usual coupling agents. The syntheses of these two analogues may require solution-phase synthetic methods or more drastic coupling procedures, such as the acyl chloride method. The biological activities of the new analogues are summarized in Table 1. Plots which illustrate the data from which the values in Table 1 were derived are presented in Figs. 1 and 2.

As can be seen from the data, [Phe(αMe)^8]-AII is essentially as potent an agonist as AII both *in vivo* (80%) and *in vitro* (100%). The presence of a methyl group on the position 8 α-carbon does not, therefore, result in a significant decrease in agonistic potency. This also seems to be the case at position 4, since [Phe(αMe)^4]-AII is 19% as active as AII *in vitro* and 9%

TABLE 1

Agonistic activity *in vitro* is expressed as (ratio of the dose of AII required to produce a half-maximal response to that of the analogue required to produce the same response) times 100. The expression of agonistic activity *in vivo* is essentially the same, except that it is based on the dose of agent required to produce a 25-mm increase in mean blood pressure. $pA_{x=2}$ is the negative logarithm of the molar concentration of inhibitor that requires twice the control dose of AII to be used in order to obtain a half-maximal response. The maximal response decreases with increasing inhibitor concentration. $pA_{h=2}$ is the negative logarithm of the molar concentration of inhibitor that decreases the maximal response by a factor of 2. ID_{50} is the infusion rate *in vivo* of inhibitor that requires twice the control dose of AII to be used in order to produce a 25-mm increase in blood pressure.

A. Angiotensin II agonists

Analogue	Activity on rat uterus	Rat pressor activity
	%	%
AII	100	100
[Phe(αMe)^8]-AII	100	80
[Phe(αMe)^4]-AII	19	9
[Sar^1,Val(αMe)^8]-AII	<0.01	<0.1

B. Angiotensin II antagonists

Analogue	Rat uterus		Rat pressor ID_{50}
	$pA_{x=2}$	$pA_{h=2}$	
			pmole/kg/min
[Sar^1,Ile^8]-AII	8.92	8.42	0.03[a]
[Sar^1,Val(αMe)^8]-AII	8.75	8.34	0.03
[Sar^1,Val^8]-AII			0.21[a]

[a] Values of Regoli *et al.* (12).

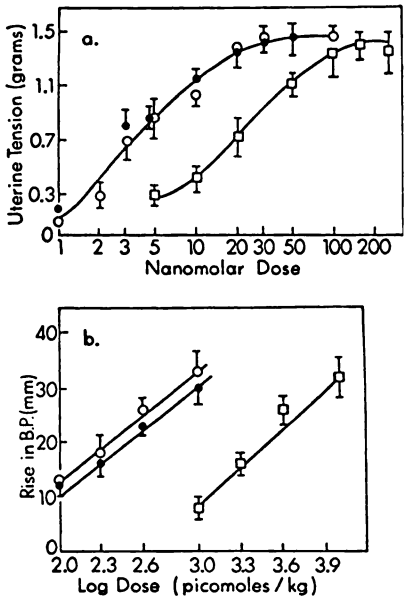

FIG. 1. *Dose-response curves for AII* (○), *[Phe(αMe)⁸]-AII* (●), *and [Phe(αMe)⁴]-AII* (□) *in (a) rat uterus and (b) rat blood pressure assays*

Four determinations were made in each of four animals, for a total of 16 separate determinations. The mean value from each animal was averaged with the others. Standard errors of the mean are indicated by bars (n = 4).

as active *in vivo*. The moderate reduction in activity is probably not due to the presence of the 4-α-methyl group, but to the absence of a phenolic hydroxyl at that position, since the activity of [Phe⁴]-AII has been reported as 10–20% *in vivo* and 3% *in vitro* (12, 21). Technical difficulties with the protection of α-methyltyrosine have so far prevented the synthesis of [4-α-methyltyrosine]-AII. [Phe(αMe)⁸]-AII proved to be quite resistant to the action of carboxypeptidase A, retaining 100% activity after an incubation time of 1 hr under conditions described in MATERIALS AND METHODS. Under the same conditions native AII retained less than 1% activity after 30 min (data not shown).

[Sar¹,Val(αMe)⁸]-AII is an excellent inhibitor of AII both *in vivo* and *in vitro*, with a potency essentially equivalent to that of [Sar¹,Ile⁸]-AII. [Sar¹,Val(αMe)⁸]-AII does not produce any displacement of the bradykinin dose-response curve in rat uterus (data not shown) and has no agonistic activity in that preparation at concentrations up to 10 μg/ml. At high infusion levels the analogue does evoke a transient pressor response *in vivo*, but the residual activity is no more than 0.1%. Like other inhibitors bearing the 1-sarcosyl substitution (12), [Sar¹,Val(αMe)⁸]-AII does not act as a true competitive inhibitor *in vitro*, since it depresses the maximum response attainable and continues to exert an inhibitory effect for some time after washout. This may be due to slow rates of association with and dissociation from the receptor. For this reason a relatively long incubation period was used in the assays, and pA_h as well as pA_x values (22) were determined. In this situation the pA_2 values are simply empirical characterizations of antagonistic potency and do not have the thermodynamic significance of a dissociation constant. They are reproducible, however, provided that the same incubation time is used in each determination. As shown in Fig. 3, the recovery of responsiveness to AII *in vivo* after an infusion of [Sar¹,Val(αMe)⁸]-AII is slightly slower than after an infusion of [Sar¹,Ile⁸]-AII.

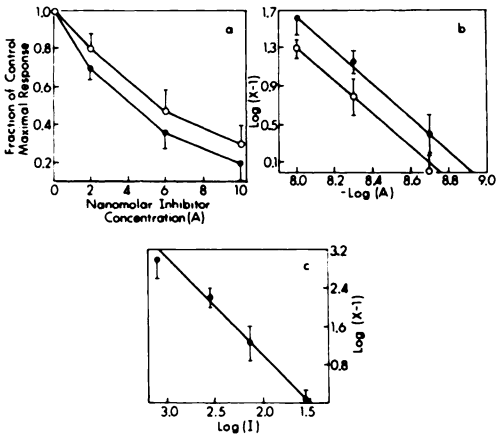

FIG. 2. *Characterization of inhibition by [Sar¹,-Val(αMe)⁸]-AII* (●) *and [Sar¹,Ile⁸]-AII* (○) *as (a) depression of maximal response in rat uterus, (b) displacement of midpoint of dose-response curve to the right on the dosage axis, and (c) displacement to the right on the dosage axis of dose of AII required to produce 25-mm rise in mean blood pressure*

Bars represent standard errors (n = 4). *A*, nanomolar inhibitor concentration; *I*, infusion rate of inhibitor, in picomoles per kilogram per minute.

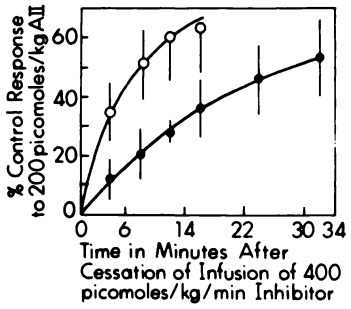

FIG. 3. *Time course of recovery of responsiveness in vivo to a dose of exogenous AII after 1-hr infusion of [Sar¹,Val(αMe)⁸]-AII (●) or [Sar¹,Ile⁸]-AII (○)*

Complete recovery was achieved after 70–120 min in the case of the former, and after 30–50 min in the case of the latter. Bars represent standard errors (n = 4).

DISCUSSION

The finding that [Phe(αMe)⁴]-AII is essentially as active as an isofunctional analogue which does not bear an α-methyl group in position 4, namely, [Phe⁴]-AII, suggests that the conformational restrictions imposed by the α-methyl group are compatible with the conformational requirements that must be met by AII at position 4 in order to bind to its receptor and elicit a physiological response. The conformational constraints imposed by the α-methyl group have been fully described elsewhere (1–5), but they can be summarized by saying that the allowable values for the torsional angles ϕ and ψ which describe the backbone conformation of a peptide (23) fall into two small areas centered around (ϕ = −57°, ψ = −47°) and (ϕ = +57°, ψ = +47°). Other sets of angles are sterically forbidden because they would require simultaneous occupancy of the same space by more than 1 atom.

The assignment of torsional angles at position 4 can be made more specific. The conformational constraints imposed by a cyclic N-methylamino acid in position 5 are well tolerated when the alterations of the native isopropyl side chain are taken into account, since [Asn¹,Pro⁵]-AII retains 10% of the potency of AII compared to the 5% potency of [Ala⁵]-AII (24, 25). A prolyl residue places the following constraints on the torsional angles of the preceding residue in the peptide chain: (ϕ = −57°, ψ = −47°) or (ϕ < 0°, ψ > 0°). Taken together with the conditions established in the last paragraph, this implies that in the receptor-bound conformation of AII the torsional angles at position 4 lie close to (ϕ = −57°, ψ = −47°).

The calculated possible values for ϕ_4 based on the position 4 $NHC_\alpha H$ vicinal coupling constant determined from NMR studies of AII in aqueous solution are (−157°, −83°, 36°, and 84°) (1). None of these values lies close to ϕ_4 = −57° (or to +57°). This implies that the time-averaged solution conformation of the AII backbone at position 4 is different from the receptor-bound backbone conformation at position 4. A similar situation is thought to occur at both positions 3 and 5, based on studies with other series of analogues (1). This suggests that AII may undergo a conformational change on binding to its receptor, or that the receptor may selectively bind one of several species of conformers that coexist in solution. Notice that this approach bears only on backbone conformation at selected positions, and not on side chain conformation.

The constraints imposed by the α-methyl group in position 8 are considerably relaxed, since that is the COOH-terminal position in the peptide chain, and steric interactions with the atoms of a succeeding residue are nonexistent.[5] The failure of the 8-α-methyl group to interfere with binding is established by the high potencies of [Phe(αMe)⁸]-AII and [Sar¹, Val(αMe)⁸], however.

[Sar¹,Val(αMe)⁸]-AII was prepared in an attempt to produce an antagonist of AII with a prolonged duration of action. Studies on model substrates of carboxypeptidase A have shown that substrates whose ultimate residue is Phe(αMe) are cleaved 50 times less rapidly than are substrates whose ultimate residue is phenylalanine (9). In addition, [Phe(αMe)⁸]-AII is not degraded by carboxypeptidase A under conditions in which AII is completely de-

[5] G. R. Marshall and H. E. Bosshard, unpublished observations.

stroyed, as described above. It was thus hoped that the half-life of [Sar^1, Val(αMe)^8]-AII would be prolonged from minutes to hours. This would require that the 7—8 cleavage be rate-limiting in the metabolism of 1-sarcosine-containing analogues of AII. [Sar^1,Val(αMe)^8]-AII is 7 times more potent *in vivo* than the isofunctional analogue [Sar^1,Val^8]-AII (12), which may reflect a higher steady-state accumulation of the former compound at a given rate of infusion, as a result of slower destruction. As seen in Fig. 3, however, the duration of action of the analogue is increased relative to that of [Sar^1,Ile^8]-AII only by a factor of 3.

Some prolongation of action is thus observed, but it is small. There are two possible explanations. First, the 7—8 scission is normally the rate-limiting step in the destruction of 1-sarcosine-containing analogues of AII, but either (a) the carboxypeptidase that catalyzes the cleavage is hindered less by the α-methyl group than is carboxypeptidase A or (b) the rate of the next most rapid cleavage is only 3 times less than that for the unprotected 7—8 bond. Alternatively, cleavage at a site other than the 7—8 bond may limit the lifetime of the 1—8 peptide. If the fragment produced by this cleavage retained some activity, and if its longevity were increased by the 8-α-methyl group, a small prolongation of action would still be observed. Since fragments 2—8 and 3—8 of AII are known to retain some agonistic activity (26), similar fragments of antagonists may retain some antagonistic activity. Extending protection to sites in addition to the 7—8 bond by α- or N-methylation may permit the construction of long-acting AII antagonists. Bonds 1—2, 2—3, and 4—5 are known to be cleaved in peripheral vascular beds (27, 28).

In summary, three analogues of angiotensin II have been synthesized which contain optically active α-methylamino acids. Such analogues have reduced conformational freedom of the backbone, owing to the steric bulk of the methyl group. The relatively high biological activity of [Phe(αMe)^4]-AII, when interpreted in the light of previous structure-activity studies, allows tentative assignment of the torsional angles ϕ and ψ at position 4 in the receptor-bound conformation of AII. These values do not correspond to those deduced for AII in solution by NMR techniques. This suggests that a conformational change may occur on binding, or that the receptor selectively complexes with only some of multiple coexisting solution conformers. In addition, [Sar^1,Val(αMe)^8]-AII is an inhibitor whose potency *in vivo* is 7 times that of the isofunctional analogue that does not bear an 8-α-methyl group, namely, [Sar^1,Val^8]-AII. The 8-α-methyl substitution is of potential utility in the construction of antagonists with increased potency and, if alternative sites of cleavage can be protected, duration of action.

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