Angiotensin II Analogues. 12. Role of the Aromatic Ring of Position 8 Phenylalanine in Pressor Activity[1]

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To evaluate the relative contributions to pressor activity of lipophilic and aromatic character for the phenyl ring of position 8 phenylalanine in [Asn[1],Ile[5]]angiotensin II, the benzyl side chain was replaced by a variety of normal or branched aliphatic and substituted aromatic residues. A conformationally constrained analogue in which the 2-aminoindane-2-carboxylic acid (Ind) replaced the phenylalanine residue was prepared in order to examine the steric requirement for the aromatic ring for pressor activity. The analogues were synthesized by the solid-phase method and had the following pressor activities in the rat: Ahp[8], 11.5%, Nle[8], 7.3%; Leu[8], 1.2%; Ind[8], 0.1%; Phe(4-NH_2)[8], 52.5%; Phe(4-NO_2)[8], 15%; and the disubstituted analogues [Sar[1],Leu[8]]AII, 2.5%; [des-Asp[1],D-Ala[2],Leu[8]]AII, 1.4%; [Tyr(3-Bzl)[4],Phe(4-NO_2)[8]]AII, 0.2%. In the absence of aromatic character, higher lipophilicity of the analogues resulted in higher pressor activity. However, aromaticity was more important than lipophilic character and was necessary for full activity. Steric interference, caused by a bulky substituent on the ring or by branching of the aliphatic residue, resulted in reduced potency. When the sizes of the substituents were comparable, the aromatic, π-electron-enriched analogues were more active than the π-electron-deficient analogues. The spatial orientation of the ring relative to the peptide backbone was critical for the pressor effect. [Ind[8]]AII was essentially lacking in pressor activity and was more potent than [Leu[8]]AII as an AII antagonist, in spite of its aromatic nature. Substitution of sarcosine in position 1 greatly enhanced the potency and duration of antagonist activity. [Sar[1],Leu[8]]AII was more potent and longer acting than [Ind[8]]AII as an angiotensin II inhibitor. These results suggest that incorporation of a conformationally constrained aromatic ring in position 8 of angiotensin analogues can be an effective approach to the development of potent inhibitors with low pressor activity.

As a potent, naturally occurring pressor substance, angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe, AII) is implicated in the etiology of high renin subclasses of renal, renovascular, and malignant hypertension[2-4] and in primary aldosteronism.[5] To correct such pathologic states and to assess the mechanism of other essential hypertensions,[5-7] angiotensin II inhibitors[8,9] like saralasin, [Sar[1],Val[5],Ala[8]]AII, have been used experimentally. However, for either clinical diagnosis or treatment of hypertension, these inhibitors are limited in their usefulness because of their short duration of action and their retention of some pressor activity which could precipitate cardiac failure.[6,7,10] In order to produce a selective angiotensin II antagonist with minimal pressor activity, it is necessary to establish the structural features responsible for agonist and antagonist effects.

Extensive structure–activity studies of angiotensin II analogues have shown that the residues in positions 1–7 define the specificity, intensity, and duration of the biological effect, while the nature of the residue in position 8 establishes the hormone analogue as an agonist or antagonist.[11-15] Smooth-muscle contracting and pressor activities are decreased and antagonistic activities predominate when the side chain of phenylalanine in position 8 is displaced from its regular position by insertion of a methylene group in the peptide backbone.[16] Antagonists are also produced when the aromatic residues of positions 4 and 8 are transposed[17] or when the spatial orientation of the aromatic ring is changed by replacement of L-phenylalanine with the D isomer.[18] However, inhibitory activity is produced most effectively when the aromatic ring is replaced by an aliphatic or alicyclic side chain.[11] [Ala[8]]AII, prepared earlier by Bumpus[19] and by Park,[20] was characterized by Khairallah,[21] Türker,[22] and Gagnon[18] as the first of the potent angiotensin II antagonists in studies in vitro. The [Ala[8]]AII analogue was found by Park and Regoli[23] and by Pals[24] to antagonize the rat pressor effect of angiotensin II in vivo. These observations have stimulated the synthesis and biological evaluation of a wide variety of angiotensin II analogues in which the L-phenylalanine residue of position 8 has been replaced.[25-31] Because the potential interactions of the phenyl ring with its receptor involve its hydrophobic nature, its aromatic character, and its spatial alignment relative to other residues, it is desirable to evaluate the respective contribution of each feature to the pressor activity of angiotensin II.

We report the synthesis and biological evaluation of angiotensin II analogues with the para position of phenylalanine substituted by either the electron-withdrawing nitro group or the electron-donating amino group. Furthermore, the phenyl group was replaced by the linear, aliphatic side chains of norleucine and heptyline,[32] which have lipophilic character similar to that of phenylalanine. The branch-chained leucine, which is similar to norleucine in lipophilicity, was incorporated into the peptide to test the effect of branching on pressor activity. [Sar[1],Leu[8]]AII and [Des-Asp[1],D-Ala[2],Leu[8]]AII were prepared to introduce a *N*-methyl or a D-amino acid at the N terminus in order to reduce degradative metabolism and to prolong the effect of the peptides. A conformationally constrained analogue, 8-indane(amino acid)angiotensin II, in which the phenylalanine ring is preserved but its rotation is restricted, was prepared to test the importance of the spatial orientation of the phenyl ring for pressor activity.

Biological Results. The compounds were tested for pressor activity in nephrectomized, pentolinium-treated male rats that had been anesthetized with pentobarbital.[33,34] The peptides were dissolved in normal saline containing 0.1% polyvinylpyrrolidone in siliconized glass containers. The molarities of peptide solutions, including the angiotensin standard, were based on peptide content found from amino acid analyses of the peptide hydrolysates. Pressor activities calculated on a molar basis for the compounds from the present study are listed in Table I.

Discussion

When the pressor activities of linear aliphatic analogues of angiotensin II are tabulated (Table II), higher activities are found for position-8 substituents with higher values

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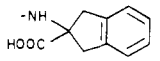
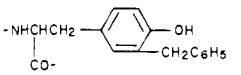
Table I. Relative Pressor Activities of [Asn¹,Ile⁵]Angiotensin II Analogues in the Rat

angiotensin analogues	struct of position 8 substit	additional substitution	pressor act.
[Asn¹,Val⁵]AII (angiotensin II)	-NHCHCH$_2$-C$_6$H$_5$ (COOH)		100
[Asn¹,Ile⁵,Ahp⁸]AII	-NHCH(CH$_2$)$_4$CH$_3$ (COOH)		11.5
[Asn¹,Ile⁵,Nle⁸]AII	-NHCH(CH$_2$)$_3$CH$_3$ (COOH)		7.3[a]
[Asn¹,Ile⁵,Leu⁸]AII	-NHCHCH$_2$CH(CH$_3$)$_2$ (COOH)		1.2
[Sar¹,Ile⁵,Leu⁸]AII	-NHCHCH$_2$CH(CH$_3$)$_2$ (COOH)	NHCH$_2$CO- (CH$_3$)[c]	2.5[b]
[Des-Asp¹,D-Ala²,Ile⁵,Leu⁸]AII	-NHCHCH$_2$CH(CH$_3$)$_2$ (COOH)	D-NH$_2$CH(CH$_3$)CO-[d]	1.4
[Asn¹,Ile⁵,Ind⁸]AII			0.1
[Asn¹,Ile⁵,Phe(4-NH$_2$)⁸]AII	-NHCHCH$_2$-C$_6$H$_4$-NH$_2$ (COOH)		52.5
[Asn¹,Ile⁵,Phe(4-NO$_2$)⁸]AII	-NHCHCH$_2$-C$_6$H$_4$-NO$_2$ (COOH)		15
[Asn¹,Tyr(3-Bzl)⁴,Ile⁵,Phe(4-NO$_2$)⁸]AII	-NHCHCH$_2$-C$_6$H$_4$-NO$_2$ (COOH)		0.16

[a] [Asp¹,Ile⁵,Nle⁸]AII was reported by W. K. Park, C. Choi, F. Rioux, and D. Regoli, *Can. J. Biochem.*, 52, 113 (1974), to possess 1% of the pressor activity of [Asp¹,Ile⁵]AII. [b] After completion of this work, the long-lasting inhibitory properties of [Sar¹,Ile⁵,Leu⁸]AII in vivo and in vitro were reported by D. Regoli, F. Rioux, and W. K. Park, *Rev. Can. Biol.*, 31, 73 (1972). [c] Position 1. [d] Position 1 deletion and position 2.

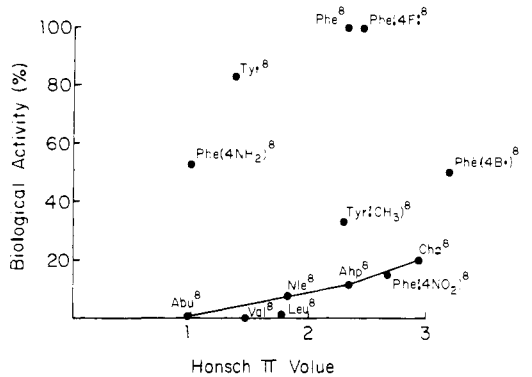


Figure 1. Biological activities of angiotensin II analogues and Hansch π values of the substituents in position 8.

of the partition coefficient π, which measures the relative lipophilicities of substituents. Among angiotensin II analogues substituted with aminobutyric acid,[25] norleucine, aminoheptanoic acid, and cyclohexylalanine,[16] a linear relationship is observed between pressor activities and π values (Figure 1). Furthermore, concurrent administration of a single dose of [Nle⁸]AII or of [Ahp⁸]AII with angiotensin II gave an additive pressor effect. These results suggest that activation of the receptor is proportional to the degree of hydrophobic bonding between the position-8 side chain and the receptor.

When the effect of branching on pressor activity is examined, even though the branched leucine has a π value similar to that of the linear norleucine, [Leu⁸]AII is only one-sixth as active as [Nle⁸]AII. Furthermore, the branched-chain analogue [Leu⁸]AII inhibits the pressor response to angiotensin II. The observation that branching

Table II. Pressor Activities of Position 8 Analogues and Physical-Chemical Characters of their Substituents

substit	pressor act.	π value[a]	chain character	ref	para substituents: electronic character	para substituents: covalent radii, Å[c]	
aliphatic analogues							
Abu⁸	0.5[b]	0.95	linear	25			
Val⁸	0.5	1.46	branched	25			
Leu⁸	1.2	1.74	branched				
Nle⁸	7.3	1.83	linear				
Ahp⁸	11.5	2.34	linear				
Cha⁸	20	2.95	cyclic	16			
aromatic analogues							
Phe(4-F)⁸	100	2.47			e⁻ withdrawing	1.33	37
Tyr⁸	83	1.38			e⁻ donating	2.33	38
Phe(4-NH$_2$)⁸	52.5	1.00			e⁻ donating	2.48	
Phe(4-Br)⁸	50	3.19			e⁻ withdrawing	1.85	39
Tyr(CH$_3$)⁸	33	2.31			e⁻ donating	3.88	40
Phe(4-NO$_2$)⁸	15	2.67			e⁻ withdrawing	2.69	
angiotension II							
Phe⁸	100	2.33	aromatic			1.08	

[a] The π value for phenylalanine was based on Φ-CH$_2$ +2.69 and glycine −0.36 to give +2.33. π values for para substituted phenylalanines were adjusted according to the difference between benzene and para-substituted benzene. The above values were taken from J. Iwasa, T. Fujita, and C. Hansch, *J. Med. Chem.*, 8, 150 (1965). Values for aliphatic substituents were adjusted according to T. Fujita, J. Iwasa, and C. Hansch, *J. Am. Chem. Soc.*, 86, 5157 (1964). [b] Oxytocic activity. [c] Covalent radii were calculated from the sums of bond lengths according to A. J. Gordon and R. A. Ford, "The Chemists' Companion", Wiley, New York, 1972, p 109.

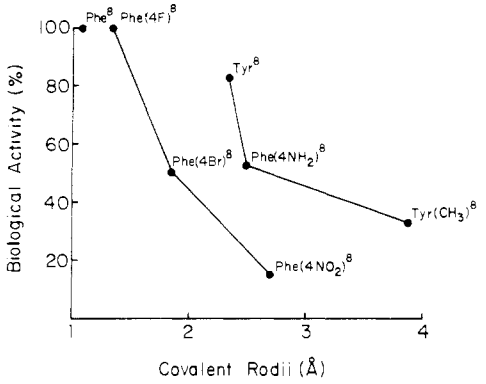


Figure 2. The effect of the size of the ring substituent on biological activity of angiotensin II analogues substituted in the 8 position.

of the side chain can decrease the agonist activity and enhance the inhibitory effect agrees with results on 8-leucine substitutions by other investigators[16,25,31,35] and is consistent with the finding of antagonistic activities for other branched analogues, [Val^8]AII and [Ile^8]AII.[16,25,27,31,36] These findings imply that the part of the receptor which is normally activated by the compact and planar aromatic ring of phenylalanine is probably also planar. Thus, the flexible linear analogues, such as [Nle^8]AII and [Ahp^8]AII, may align properly with the planar region of the receptor in a hydrophobic interaction which activates the receptor. In contrast, γ branching may cause distorted alignment of the nonplanar side chain of [Leu^8]AII with the planar region of the receptor. This could lead to inadequate hydrophobic bonding and interference with optimal activation of the receptor by angiotensin II.

Additional support for an aromatic and planar receptor region is implied by the observation that the similarly shaped but more hydrophobic cyclohexylalanine (Cha) residue in [Cha^8]AII produces an analogue only one-fifth as active as angiotensin II[16] (Table II). Moreover, aromatic analogues are usually more active than aliphatic analogues with the same lipophilicities or π values (Figure 1). These results imply that the aromatic analogue may elicit the pressor response through an aromatic–aromatic interaction with the receptor, which is much more effective than an aliphatic–aromatic interaction.

Although no simple correlation could be drawn between biological activities and the electronegativities of the aromatic substituents,[37-40] higher activities were found for analogues with less bulky substituents on the para position of the aromatic ring (Table II). When the biological activities of these analogues and the covalent radii of the para substituents were plotted, two lines emerged as shown in Figure 2. Since the nitro and the amino groups are similar in size (Table II), they probably cause a comparable degree of steric repulsion. However, the electron-enriched analogues such as [Phe(4-NH_2)^8]AII are more active than the electron-deficient analogues such as [Phe(4-NO_2)^8]AII (Figure 2), in spite of the higher lipophilicity of the Phe(4-NO_2) relative to the Phe(4-NH_2) residue. These results further suggest that the π electron density of the aromatic phenyl ring is more important than its hydrophobic character in activating the angiotensin receptor and that steric interference is detrimental to the activation interactions.

The effect of steric interference is further illustrated with the indane(amino acid) analogue, in which the phenylalanine ring is covalently linked to the α carbon of the peptide backbone through an o-methylene group, giving a rigid aromatic ring lacking the rotational freedom of the phenylalanine ring. [Ind^8]AII is a potent angiotensin antagonist with extremely low pressor activity (0.1%). In order to evaluate the inhibitory property of [Ind^8]AII, the potency and duration of action of this antagonist were compared with those of [Leu^8]AII, which is a potent monosubstituted inhibitor of angiotensin II. With a single dose of 4000 pmol, [Ind^8]AII antagonized the rat pressor effect of 20 pmol of AII, which returned to one-half of its pretreatment level after 8 min. When normotensive, sham-operated rats that had been anesthetized with urethan were given simultaneous infusions of [Ind^8]AII with AII at a dose ratio of 20:1 2000 pmol of [Ind^8]AII/100 pmol of AII per minute, the pressor response to AII was completely eliminated. When [Ind^8]AII infusion was discontinued, the mean pressure rose but did not return to pretreatment level. When infusions of [Ind^8]AII at 4000 pmol/min were administered to two kidney, renal hypertensive rats that had been anesthetized with urethan, the mean blood pressure was reduced from 165 to 105 mmHg, and the pressor response to a single injection of 200 pmol of AII, which produced a 70 mmHg rise before treatment, was abolished. Fifteen minutes after [Ind^8]AII infusion was discontinued, a single injection of 200 pmol of AII raised the blood pressure in the rats by 35 mmHg. After 45 min, the mean blood pressure returned to the control value of 165 mmHg. In contrast, [Leu^8]AII and [des-Asp^1,D-Ala^2,Leu^8]AII had, respectively, 1.4 and 1.2% pressor activities. Infusion of [Leu^8]AII at 75 pmol/min reduced the pressor responses toward single doses of 2, 4, and 8 pmol of AII to 55–80% of pretreatment level. Neither of the leucine-8 analogues showed a prolonged effect.

As an angiotensin II inhibitor, the disubstituted [Sar^1,Leu^8]AII[41-46] caused more of the undesirable pressor rise (2.5%) than [Ind^8]AII (0.1%). At a single injection of 400 pmol of [Sar^1,Leu^8]AII, the pressor responses to single doses of 3, 6, and 12 pmol of AII were significantly inhibited and returned to one-half of the pretreatment level after 30 min. This prolonged inhibitory effect was also observed when [Sar^1,Leu^8]AII was coinfused with AII at a dose ratio of 20:1 (1400 pmol of [Sar^1,Leu^8]AII/70 pmol of AII per minute). During 30 min of coinfusion, the pressor effect of AII was reduced by 67%, which rose to one-half of pretreatment level 45 min after [Sar^1,Leu^8]AII infusion was discontinued.

These results indicate that, although [Ind^8]AII is more active and longer acting than [Leu^8]AII in inhibiting the pressor effect of AII, the introduction of sarcosine rendered [Sar^1,Leu^8]AII more potent and longer acting than [Ind^8]AII. Their respective durations of action ($T_{1/2}$) were 45 and 15 min. Sarcosine substitution[47] has been found to increase the affinity of angiotensin II analogues to the receptor,[44,48] as well as reducing their degradation by angiotensinase A[48] and aminopeptidase.[44] Since the stereospecific and exclusively L-oriented carboxypeptidase is another major inactivation enzyme of angiotension II, the prolonged inhibitory effect of [Ind^8]AII may result from similar proteolytic resistance of the optically inactive indane(amino acid) to carboxypeptidase inactivation. Combination of the unique properties of sarcosine and indane(amino acid) could be useful for the development of potent inhibitors resistant to metabolic degradation.

Conclusions. Our results suggest that phenylalanine in position 8 activates the angiotensin II receptor primarily through its aromatic ring π electrons and that hydrophobic bonding may mimick this interaction to some extent. Previously, the aliphatic partial agonists [Ala^8]AII and [Abu^8]AII were postulated[31] to activate a specific part of

the receptor. Our results demonstrate a proportional increase of pressor activity with increasing lipophilic character of the linear, aliphatic analogues. Steric requirements for the pressor response are shown by the decreased pressor activities of aromatic rings with distorted orientations, bulky substituents, or by branching of the aliphatic chain.

Since either lipophilic or aromatic character can initiate the undersirable pressor component of an antagonist action, one approach to potent inhibitors with minimal pressor activity is the incorporation of lipophobic and nonaromatic residues into position 8 of angiotensin II. The reported potent antagonism[28,49,50] of the hydrophilic–aliphatic analogues [Sar^1,Ser^8]AII, [Sar^1,Thr^8]AII, and [Sar^1,Thr(Me)^8]AII support this conclusion. Alternatively, results with the [Ind^8]AII analogue demonstrate that distortion of the proper alignment of the aromatic ring with the receptor by a conformationally rigid analogue can be an effective approach in developing potent inhibitors with extremely low pressor activity. Because the more hydrophilic [Sar^1,Thr^8]AII analogue induced less initial pressor response than did [Sar^1,Thr(Me)^8]AII,[49] introduction of hydrophilic character into conformationally constrained inhibitors may provide an attractive direction for more selective and potent inhibitors of angiotensin II.

Chemistry. The indane(amino acid) (Ind) was prepared using the Strecker method.[51,52] L-Aminoheptanoic acid (Ahp) was synthesized and resolved according to Birnbaum et al.[32] Boc-Ind and Boc-Ahp were prepared by the Schnabel procedure.[53]

The peptides were synthesized using the solid-phase method,[54] with the Boc-protected amino acids incorporated in a stepwise manner by dicyclohexylcarbodiimide. The side chains were protected as Boc-His(Bzl), Boc-Tyr(Bzl), and Boc-Arg(NO_2), and Z-Asn was introduced as the active *p*-nitrophenyl ester. The C-terminal Boc-protected amino acids were attached to the resin through the triethylammonium salts, and the completed peptide-resins were cleaved with HBr/TFA, followed by catalytic hydrogenation to remove the remaining side-chain protecting groups.[55] The protective groups Boc-His(Tos), Boc-Tyr(Bzl), and Aoc-Arg(Tos) were utilized for the side chains of the [Phe(4-NO_2)^8]AII analogue to avoid hydrogenation, and they were removed by liquid HF.[56] A rearranged side product,[57,58] [Tyr(3-Bzl)^4,Phe(4-NO_2)^8]AII, was identified and isolated in 30% yield. The aminophenylalanine analogue [Phe(4-NH_2)^8]AII was prepared by hydrogenation of [Phe(4-NO_2)^8]AII.

The peptides were purified by ion-exchange chromatography, partition chromatography, countercurrent distribution, or a combination of the above until they were homogeneous to TLC and electrophoresis and gave the expected amino acid ratios. The optical purity of the peptides was examined by L-amino acid oxidase digestion of the peptide hydrolysates; undigestible D amino acids were evaluated from amino acid analysis.[59]

Experimental Section

Melting points (Thomas-Hoover Uni-melt) are uncorrected. Microanalyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley. Where analyses are indicated only by symbols of the elements, analytical results obtained for these elements were within ±0.4% of the theoretical values. Rotations were measured with a Bendix-NPL automatic polarimeter, Type 143A, equipped with a digital readout and printer. Precoated silica gel 60 F254 on glass plates (E. Merck) were used for TLC with the following solvent systems: (I) *sec*-butyl alcohol–3% NH_4OH (100:44), (II) butanol–acetic acid–H_2O (4:1:5, upper phase), (III) xylene–pyridine–acetic acid (100:15:5). Electrophoresis was carried out on Whatman no. 1 paper (0.16-mm thick) at 5000 V using AcOH–HCOOH buffer, pH 1.85, in a Savant apparatus. E_H indicates electrophoretic mobility relative to histidine = 1.00. Peptides were hydrolyzed for 48 h under N_2 in constant-boiling HCl containing D-alanine as an internal standard, except in [des-Asp^1,D-Ala^2,Ile^5,Leu^8]AII where D-Arg·HCl was used as the internal standard. Hydrolyses were carried out in the presence of phenol to protect tyrosine from degradation. Amino acid analyses (Spinco Model 116 analyzer) were obtained using the standard 4-h methodology. Peptide content was calculated in terms of free peptide.

***N*-(*tert*-Butyloxycarbonyl)-2-aminoindane-2-carboxylic Acid (Boc-Ind; 1).** The 2-aminoindane-2-carboxylic acid[51,52] (1.77 g, 10 mmol) was dissolved in 50 mL of 1:1 H_2O–dioxane and 0.8 g (20 mmol) of NaOH. *tert*-Butyloxycarbonyl azide (3.88 g, 27 mmol) was added to the solution. The mixture was stirred at 40 °C and was maintained at pH 10 by the addition of 10% NaOH for 24 h. The reaction mixture was washed with Et_2O and acidified to pH 3.5 with saturated citric acid solution. The aqueous solution was extracted with EtOAc, and the EtOAc extracts were combined, washed with H_2O, dried ($MgSO_4$), and evaporated under vacuum. Crystallization from EtOAc–petroleum ether gave 0.5 g (21%): TLC R_f (II) 0.72, R_f (III) 0.41; mp 168–169 °C. Anal. ($C_{15}H_{19}NO_4$) C, H, N.

***N*-(*tert*-Butyloxycarbonyl)-L-α-aminoheptanoic Acid Dicyclohexylammonium Salt (Boc-Ahp·DCHA; 2).** L-α-Aminoheptanoic acid[32] (4.35 g, 30 mmol) was dissolved in 2 N NaOH to which *tert*-butyloxycarbonyl azide (8.6 g, 60 mmol) in 10 mL of dioxane was added, and the mixture was stirred at pH 11 and room temperature for 2 days. The reaction mixture was washed with Et_2O, acidified to pH 1 with 6 N HCl, and extracted with Et_2O. The Et_2O extracts were dried (Na_2SO_4) and evaporated under vacuum at 50 °C to a yellow oil. The oil was dissolved in petroleum ether (bp 30–60 °C), and dicyclohexylamine was added until the solution was basic. The precipitate was recrystallized from heptane–petroleum ether, giving 93% yield: TLC R_f (II) 0.64, R_f (III) 0.52; mp 135–135.5 °C; $[\alpha]^{23}_D$ +9.61° (*c* 2.06, MeOH). Anal. ($C_{24}H_{46}N_2O_4$) C, H, N.

Asn-Arg-Val-Tyr-Ile-His-Pro-Ind (3). A solution of 1.30 g (4.7 mmol) of *N*-(*tert*-butyloxycarbonyl)-2-aminoindane-2-carboxylic acid (1) and 0.47 g (4.7 mmol) of Et_3N in 10 mL of absolute EtOH was added to 6.0 g of chloromethylated copolystyrene–2% divinylbenzene which contained 1.4 mmol of Cl/g of resin. The suspension was stirred at reflux temperature for 48 h. The esterified resin was filtered; washed with EtOH, EtOH–H_2O, MeOH–H_2O, and MeOH; and dried in vacuo. Amino acid analysis following hydrolysis in 6 N HCl showed that the resin contained 0.40 mmol of Ind/g.

The esterified resin (2.5 g, 1 mmol) was deprotected, neutralized, and acylated overnight in CH_2Cl_2 with Boc-Pro (0.52 g, 2.4 mmol) and DCC (0.50 g, 2.4 mmol). A sample of the peptide was cleaved from the resin with HBr/TFA and analyzed by electrophoresis for completion of the reaction. Unreacted amino groups were acetylated, and the resin was washed. The cycle of deprotection, neutralization, and acylation was repeated to give the product peptide-resin as described previously.[60,61] Cleavage from the resin by HBr/TFA gave 900 mg of partially protected peptide. Hydrogenolysis of a 430-mg portion with 430 mg of 10% Pd/C under 2.9 atm of H_2 for 50 h gave 370 mg of **3**. A 193-mg portion was chromatographed on a 2.5 × 90 cm column of carboxymethylcellulose (Whatman CM-52, NH_4^+) and eluted with an NH_4OAc gradient. The eluate was monitored at 280 and 254 nm and was analyzed by TLC and electrophoresis. Fractions containing the peptide were combined to give 67 mg, which was not homogeneous to TLC. The peptide (61 mg) was further purified by countercurrent distribution for 320 transfers between the upper and lower phases of the mixture of butyl alcohol–*tert*-butyl alcohol–0.7 M NH_4OAc (5:2:5). The fraction of K = 0.64 were combined. The solvent was removed, and the peptide was lyophilized and purified through the picrate salt[60] to give 34 mg of white powder: TLC R_f (I) 0.14, R_f (II) 0.17; E_H 0.59. An acid hydrolysate had the following amino acid ratios: Asp, 0.98; Arg, 1.01; Val, 1.03; Tyr, 1.02; Ile, 0.96; His, 1.01; Pro, 0.99; Ind, 1.00; peptide content 92%. When eluted with pH 4.30 (0.20 N) sodium citrate buffer for 30 min and subsequently with pH 5.26 (0.35 N) sodium citrate buffer for 60 min, the indane(amino acid) emerged from the short column

at 11 mL (19.5 min) with a color value of 15.2% that of arginine, and histidine came out at 34 mL (58.5 min). A 48-h acid hydrolysate incubated with *C. adamanteus* L-amino acid oxidase[59] for 48 h showed the following amino acid ratios; Asp, 0.84; Arg, 0.05; Val, 0.02; Tyr, 0.04; Ile, 0.00; His, 0.11; Pro, 1.00; Ind, 0.97. A mixture of amino acids subjected to the same hydrolytic and enzymatic procedures had the following amino acid ratios: Arg, 0.04; Val, 0.02; Tyr, 0.04; Ile, 0.02; His, 0.08; Ind, 1.00; Ahp, 0.02; Nle, 0.02; Leu, 0.02; Sar, 1.00; Ala, 0.02; Phe(4-NO_2), 0.04; Phe(4-NH_2), 0.00. Asparagine is attacked too slowly by L-amino acid oxidase to be checked by this method.

Asn-Arg-Val-Tyr-His-Pro-Ahp (4). Boc-Ahp was esterified to the resin as described for **3**, giving 0.30 mmol of Ahp/g of resin. Synthesis was carried out on 0.20 mmol of Boc-Ahp-polymer, giving 177 mg of peptide. A 149-mg portion was chromatographed on a 2.5 × 100 cm column of CM-52 (NH_4^+) and eluted with an NH_4OAc gradient. The appropriate fractions were combined, and the peptide was further purified through the picrate salt[60] to give 46 mg of white powder: TLC R_f (I) 0.16, R_f (II) 0.15, E_H 0.70. An acid hydrolysate had the following amino acid ratios: Asp, 1.03; Arg, 1.03; Val, 1.04; Tyr, 0.98; Ile, 1.00; His, 0.97; Pro, 1.00; Ahp 0.98; peptide content 81%. Ahp emerged from the long column of the analyzer, at 14 mL (12 min), after Tyr and had a color value which was 106% that of Ile. A 48-h acid hydrolysate incubated with L-amino acid oxidase showed the following amino acid ratios: Asp, 0.81; Arg, 0.04; Val, 0.01; Tyr, 0.05; Ile, 0.02; His, 0.08; Pro, 1.00; Ahp, 0.03.

Asn-Arg-Val-Tyr-Ile-His-Pro-Nle (5). Boc-Nle was esterified to the resin as described for **3**, giving 0.19 mmol of Nle/g of resin. Synthesis was carried out on 0.2 mmol of Boc-Nle-polymer, giving 135 mg of peptide. This was chromatographed on a 2.5 × 100 cm column of CM-52 (NH_4^+) and eluted with an NH_4OAc gradient. The appropriate fractions were combined, and the peptide was further purified through the picrate salt,[60] giving 48 mg of white powder: TLC R_f (I) 0.16, R_f (II) 0.13, E_H 0.74. An acid hydrolysate had the following amino acid ratios: Asp, 0.97; Arg, 1.01; Val, 1.03; Tyr, 1.00; Ile, 1.00; His, 1.02; Pro, 0.97; Nle, 1.01; peptide content 82%. A 48-h acid hydrolysate incubated with L-amino acid oxidase for 48 h showed the following amino acid ratios: Asp, 0.80; Arg, 0.02; Val, 0.02; Tyr, 0.05; Ile, 0.03; His, 0.09; Pro, 1.00; Nle, 0.04.

Boc-Val-Tyr(Bzl)-Ile-His(Bzl)-Pro-Leu-polymer (6). Boc-Leu-polymer was prepared from 2.40 g (10 mmol) of Boc-Leu·0.5H_2O, and compound **6** was prepared from 5.56 g (1.5 mmol of Leu) of Boc-Leu-polymer as described for **3**.

Boc-Arg(NO_2)-Val-Tyr(Bzl)-Ile-His(Bzl)-Pro-Leu-polymer (7). Compound **6** (0.75 mmol) was deprotected, neutralized, and acylated overnight with Boc-Arg(NO_2) (0.96 g, 3.0 mmol) and DCC (0.62 g, 3.0 mmol).

Asn-Arg-Val-Tyr-Ile-His-Pro-Leu (8). The deprotected and neutralized **7** (0.375 mmol) was acylated in purified DMF with 0.87 g (2.25 mmol) of Z-Asn-ONp for 72 h. Cleavage of the peptide-resin by HBr/TFA gave 425 mg of peptide. A portion (301 mg) of this partially protected peptide was hydrogenated to give 225 mg. The crude peptide (152 mg) was chromatographed on a 2.5 × 10 column of CM-52 (NH_4^+) and eluted with an NH_4OAc gradient. The appropriate fractions were combined, and the peptide was further purified through the picrate salt[60] to give 72 mg of white powder: TLC R_f (I) 0.15, R_f (II) 0.13, E_H 0.74. An acid hydrolysate had the following amino acid ratios: Asp, 1.03; Arg, 1.02; Val, 0.98; Tyr, 0.98; Ile, 0.97; His, 0.98; Pro, 1.03; Leu, 1.03; peptide content 88%. A 48-h acid hydrolysate incubated with L-amino acid oxidase showed the following amino acid ratios: Asp, 0.53; Arg, 0.03; Val, 0.01; Tyr, 0.03; Ile, 0.02; His, 0.07; Pro, 1.00; Leu, 0.03.

Sar-Arg-Val-Tyr-Ile-His-Pro-Leu (9). The deprotected and neutralized compound **7** (0.375 mmol) was acylated overnight in CH_2Cl_2 with 0.28 g (1.5 mmol) of Boc-Sar and 0.31 g (1.5 mmol) of DCC, giving 460 mg of partially protected peptide, and a portion (300 mg) was hydrogenated to give 260 mg. The crude peptide (152 mg) was chromatographed on a 2.5 × 100 cm column of CM-52 (NH_4^+) and eluted with an NH_4OAc gradient. The appropriate fractions were combined, and the peptide was further purified through the picrate salt[60] to give 56 mg of white powder: TLC R_f (I) 0.17, R_f (II) 0.13, E_H 0.72. An acid hydrolysate had the following amino acid ratios: Sar, 1.02; Arg, 1.00; Val, 1.02; Tyr, 0.97; Ile, 0.97; His, 1.03; Pro, 0.98; Leu, 1.03; peptide content 84%. A 48-h acid hydrolysate incubated with L-amino acid oxidase showed the following amino acid ratios: Sar, 1.03; Arg, 0.04; Val, 0.02; Tyr, 0.06; Ile, 0.03; His, 0.11; Pro, 1.00; Leu, 0.03.

D-Ala-Val-Tyr-Ile-His-Pro-Leu (10). Compound **6** (0.375 mmol) was deprotected, neutralized, and acylated overnight in CH_2Cl_2 with 0.28 g (1.5 mmol) of Boc-D-Ala and 0.31 g (1.5 mmol) of DCC, giving 283 mg of partially protected peptide, which was hydrogenated to give 243 mg. The peptide (101 mg) was purified by countercurrent distribution for 200 transfers between the upper and lower phases of the mixture of butyl alcohol–*tert*-butyl alcohol–H_2O (5:2:5). Fractions of K = 0.26 were combined to give 35 mg, which was not homogenous. A portion (26 mg) was chromatographed on a 2.5 × 100 cm column of CM-52 (NH_4^+) and eluted with an NH_4OAc gradient. The appropriate fractions were combined to give 19 mg of peptide. Further purification through the picrate salt[60] gave 8 mg of white powder: TLC R_f (I) 0.26, R_f (II) 0.27, E_H 0.58. An acid hydrolysate had the following amino acid ratios: Ala, 1.01; Val, 0.98; Tyr, 0.98; Ile, 0.97; His, 1.04; Pro, 0.99; Leu, 1.02; peptide content 70%. A 48-h acid hydrolysate incubated with L-amino acid oxidase showed the following amino acid ratios: Ala, 0.96; Val, 0.02; Tyr, 0.04; Ile, 0.02; His, 0.09; Pro, 1.00; Leu, 0.03.

Asn-Arg-Val-Tyr-Ile-His-Pro-Phe(4-NO_2) (11). Boc-Phe(4-NO_2) was esterified to the resin, giving 0.22 mmol of Phe(4-NO_2)/g of resin. Synthesis was carried out on 0.88 mmol of Boc-Phe(4-NO_2)-polymer as described for **3**, except that Boc-His(Tos) instead of Boc-His(Bzl) was incorporated. Cleavage of the peptide-polymer with liquid HF at 0 °C for 1 h in the presence of anisole removed all protecting groups and gave 948 mg of peptide. The crude peptide (352 mg) was purified by countercurrent distribution for 600 transfers between the upper and lower phases of the mixture of butanol–acetic acid–water (4:1:5). Fractions of K = 0.12 were combined, and the peptide was further purified through the picrate salt[60] to give 80 mg: TLC R_f (I) 0.16, R_f (II) 0.11, E_H 0.64. An acid hydrolysate had the following amino acid ratios: Asp, 0.99; Arg, 1.02; Val, 1.00; Tyr, 1.03; Ile, 0.98; His, 1.00; Pro, 0.99; Phe(4-NO_2), 1.01; peptide content 93%. A 48-h acid hydrolysate incubated with L-amino acid oxidase for 48 h showed the following amino acid ratios: Asp, 0.80; Arg, 0.04; Val, 0.01; Tyr, 0.03; Ile, 0.02; His, 0.08; Pro, 1.00; Phe(4-NO_2), 0.05. A sample of Phe(4-NO_2)·H_2O obtained from treatment of Boc-Phe(4-NO_2) with HCl/HOAc and crystallization from H_2O was shown to be over 99.5% of the L isomer when incubated with L-amino acid oxidase and gave 4% of D-amino acid when it was treated under the hydrolytic conditions for peptides. When the short column was eluted with pH 5.26 (0.35 N) sodium citrate buffer, Phe(4-NO_2) emerged at 11 mL (18.5 min) with a color value 82% that of arginine, which came out at 31 mL (54 min).

Asn-Arg-Val-Tyr(3-Bzl)-Ile-His-Pro-Phe(4-NO_2) (12). Fractions of K = 0.38 from CCD purification of the previous crude mixture (**11**; 352 mg) were combined, and the peptide was further purified through the picrate salt[60] to give 35 mg: TLC R_f (I) 0.19, R_f (II) 0.16, E_H 0.60. An acid hydrolysate had the following amino acid ratios: Asp, 1.02; Arg, 0.99; Val, 1.01; Tyr, 0.04; Tyr(3-Bzl), 1.00; Ile, 0.99; His, 1.01; Pro, 0.99; Phe(4-NO_2), 1.00; peptide content 94%. A 48-h acid hydrolysate incubated with L-amino acid oxidase for 60 h showed the following amino acid ratios: Asp, 0.70; Arg, 0.05; Val, 0.01; Tyr, 0; Tyr(3-Bzl), 0.12; Ile, 0.02; His, 0.07; Pro, 1.00; Phe(4-NO_2), 0.05. When eluted with pH 6.60 (0.2 N) sodium citrate buffer, Tyr(3-Bzl) emerged from the short column at 31 min, with a color value 92% that of Arg. Under these conditions, both Phe(4-NO_2) and His came out at 18 min. Elution of the short column with pH 5.26 (0.35 N) sodium citrate buffer was necessary to differentiate the two amino acids and to determine their ratios. Intramolecular rearrangement of *o*-benzyltyrosine to 3-benzyltyrosine during HF treatment has been reported previously.[57,58] The yield of product indicated that approximately 30% rearrangement had occurred under the cleavage conditions.

Asn-Arg-Val-Tyr-Ile-His-Pro-Phe(4-NH_2) (13). Compound **11** (30 mg) was dissolved in 4 mL of 2:25:25 AcOH–CH_3OH–H_2O and hydrogenated with 15 mg of 10% Pd/C under 1 atm of H_2 for 14 h to give 30 mg. Chromatography of the peptide on a 2.5 × 90 cm column of CM-52 (NH_4^+) and elution with an NH_4OAc

gradient did not give a homogeneous product. The peptide was purified by countercurrent distribution for 700 transfers between the upper and lower phases of butanol–acetic acid–0.2 M NH_4OAc (4:1:5). Fractions of K = 0.19 were combined, and the peptide was further purified through the picrate salt[60] to give 15 mg: TLC R_f (I) 0.15, R_f (II) 0.04, E_H 0.80. An acid hydrolysate had the following amino acid ratios: Asp, 0.98; Arg, 1.00; Val, 1.00; Tyr, 1.02; Ile, 0.98; His, 1.02; Pro, 1.02; Phe(4-NH_2), 1.00; peptide content 89%. A 48-h acid hydrolysate incubated with L-amino acid oxidase gave the following amino acid ratios: Asp, 0.79; Arg, 0.03; Val, 0.01; Tyr, 0.03; Ile, 0.02; His, 0.11; Pro, 1.00; Phe(4-NH_2), 0.06. Phe(4-NH_2) emerged from the short column at 15.5 min, with a color value 96.5% that of Arg.

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References and Notes

(1) (a) The abbreviations used to denote amino acids and peptides are those recommended by the IUPAC–IUB Commission on Biochemical Nomenclature [*J. Biol. Chem.*, **247**, 911 (1972), and *Biochemistry*, **6**, 362 (1967)]. Other abbreviations include: Ahp, L-2-aminoheptanoic acid; Ind, 2-aminoindane-2-carboxylic acid; Phe(4-NO_2), 4-nitrophenylalanine; Phe(4-NH_2), 4-aminophenylalanine; Tyr(3-Bzl), 3-benzyltyrosine; DCC, *N,N*-dicyclohexylcarbodiimide; DCHA, dicyclohexylammonium salt; AII, angiotensin II, Asp-Arg-Val-Tyr-Ile(or Val)-His-Pro-Phe. (b) For paper 11 in this series, see E. C. Jorgensen, I. C. Kiraly-Olah, T. C. Lee, and G. C. Windridge, *J. Med. Chem.*, **17**, 323 (1974). (c) This report was presented in part at the 3rd American Peptide Symposium, 1972.
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Angiotensin II Analogues. 13. Role of the Hydroxyl Group of Position 4 Tyrosine in Pressor Activity[1]

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In order to determine the features of the phenolic ring in position 4 of [Asn[1],Ile[5]]angiotensin II that contribute to pressor activity, analogues with selected aromatic substituents were synthesized by the solid-phase method. They showed pressor activities in the rat: [Asn[1],Phe(4-NH_2)[4]]AII, 24%; [Asn[1],Phe(4-NO_2)[4]]AII, 0.1%; [Asn[1],Tyr(3,5-Me_2)[4]]AII, 2.2%; [Asn[1],D-Tyr(3,5-Me_2)[4]]AII, 1.4%. These results indicate that the activity contributed by the aromatic character of the phenyl ring in the side chain of position 4 is enhanced by a group in the para position that may function as a proton donor in hydrogen-bond formation. Bulky substituents ortho to this hydrogen-bonding group decrease activity by steric interference with hydrogen-bond formation. Bulky groups than cannot act as hydrogen donors in the para position of the aromatic ring drastically decrease the activating effect of the aromatic ring on pressor activity.

The tyrosine side chain of a peptide hormone is capable of participating in many types of interactions with structural components of its receptor. The phenolic hydroxyl group can form hydrogen bonds by proton donation or acceptance, or the aromatic ring can interact with other aromatic, hydrophobic, or polarizable systems. In angiotension II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) physical measurements by direct titration,[2] UV,[3] ^{1}H NMR,[4] and ^{13}C NMR[5] all indicate a normal pK_a of about 10 for the tyrosine phenolic group. These measurements further indicate the absence of any intramolecular interaction of the tyrosine hydroxyl group in the conformation of angiotensin II in solution. However, replacement of the hydroxyl group of tyrosine by a fluorine atom results in the loss of pressor activity and the acquisition of antagonistic activity.[6] This finding suggests a specific role for the tyrosine hydroxyl group at the hormone receptor, acting either directly or through its electronic effects on the aromatic ring.

In this study, we have prepared analogues of [Asn[1],-Ile[5]]angiotensin II with unnatural aromatic amino acids replacing the tyrosine in position 4. The *p*-aminophenylalanine and *p*-nitrophenylalanine analogues were prepared because of their hydrogen-bond donor and acceptor properties and to include analogues with electron-donating and electron-withdrawing substituent effects on the aromatic ring. The 3,5-dimethyltyrosine analogue was prepared because it retains the structural features of tyrosine, modified by bulky electron-donating groups flanking the phenolic hydroxyl function.

The peptides were synthesized using the solid-phase method,[7] with the Boc-protected amino acids incorporated in a stepwise manner by dicyclohexylcarbodiimide. Boc-DL-3,5-dimethyltyrosine was incorporated into the peptide without protection of the phenolic group. The resulting diastereomeric peptides [Asn[1],Tyr(3,5-Me_2)[4]]AII and [Asn[1],D-Tyr(3,5-Me_2)[4]]AII were separated by a combination of column chromatography and countercurrent distribution. The individual diastereomers were characterized by L-amino acid oxidase digestion of the peptide hydrolysates. The aminophenylalanine analogue [Asn[1],Phe(4-NH_2)[4]]AII was prepared by hydrogenation of [Asn[1],Phe(4-NO_2)[4]]AII.

Biological Results. The compounds were tested for pressor activity in nephrectomized, pentolinium-treated male rats that were anesthetized with pentobarbital.[8,9] The peptides were dissolved in normal saline containing 0.1% polyvinylpyrrolidone in siliconized glass containers. The molarities of the peptide solutions, including the angiotensin standard, were based on peptide content found from amino acid analyses of the peptide hydrolysates. Pressor activities calculated on a molar basis for the compounds from the present study are listed in Table I.

Discussion

The replacement of the tyrosine group in position 4 of angiotensin II by aliphatic residues results in loss of pressor activitiy as shown by the alanine[10,11] (0.3%), 1-aminocyclopentanecarboxylic acid[12] (0.1%), and glutamic acid[13](0.1%) analogues. The phenyl ring in the absence of the phenolic hydroxyl group still contributes significantly to pressor activity, as shown by the phenylalanine analogue,[14] with 10% the activity of angiotensin II.

The amino analogue [Asn[1],Phe(4-NH_2)[4]]AII prepared in this study is 24% as active as angiotensin II in the rat pressor assay (Table I). A recent report by Escher[15] of [Sar[1],Phe(4-NH_2)[4],Val[5]]AII showed this analogue to possess 15% of the activity of [Sar[1],Val[5]]AII in vitro

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