Angiotensin II Analogs. 7.[1] Stereochemical Factors in the 5 Position Influencing Pressor Activity. 2

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Analogs of [1-asparagine-5-isoleucine]angiotensin II were prepared by solid-phase synthesis to further test the proposal that a steric effect on the peptide backbone is the primary role of the amino acid residue in the 5 position which contributes to the pressor effect of the hormone. The varying steric effects introduced by the α-amino acids used in place of Ile correlated well with a conformational preference at the 5 position of $\phi \sim -120°$, $\psi \sim +120°$ for high pressor activity. α-Amino acids in the 5 position with little steric constraint showed low activity, sarcosine (1%), α-aminoisobutyric acid (0.9%). [Asn[1], D-Pro[5]]angiotensin II was inactive (0.05%), while the related rigid ring analogs containing the proper stereochemical elements showed significant activity; L-Pro (10%) and cycloleucine (24%).

Angiotensin II is the most potent naturally occurring pressor substance known. The carboxyl terminal hexapeptide 3–8 sequence [Val-Tyr-Ile (or Val)-His-Pro-Phe] carries the features essential for pressor and for myotropic activities. The low pressor activity of this sequence (0.3%)[2,3] is enhanced to full hormonal activity by the N-terminal dipeptide, Asp-Arg, whose functional residues appear to be the spatially equivalent guanido group of arginine and the terminal ammonium group of aspartic acid.[4]

The functionally important hexapeptide 3–8 is composed of amino acid residues with alternating aliphatic and aromatic side chains. The aromatic residues (Tyr,[4] His,[6] Phe[8]) appear to be the essential elements for pressor activity, while the primary role of the aliphatic residues (Val[3], Ile[5] or Val[5], Pro[7]) appears to be steric, in limiting the peptide backbone to conformations which facilitate a favorable relative orientation of the aromatic residues. A highly fixed portion of this sequence may be the carboxyl terminal tripeptide segment, His-Pro-Phe, in which the CO_2^- of Phe appears to be associated with the imidazole ring of His, and the Phe NH may form a hydrogen bond with the CO group of His.[5] This intramolecular interaction requires the connecting Pro residue, or one which exerts a similar steric constraint.

A steric role for the Val or Ile residues of position 5 was indicated by studies of analogs of [Asn[1],Ile[5]]-angiotensin II.[1] α-Amino acids in the 5 position imparted relatively low pressor activities if they possessed unbranched side chains, whereas the presence of β branching in aliphatic or alicyclic side chains resulted in activities approximating that of the natural hormone.

These results were consistent with a steric effect at the 5 position which favored a specific active peptide backbone conformation, $\phi_5 = -120 \pm 30°$, $\psi_5 = +120 \pm 30°$.[6] The rotational angle $\phi_5 = -120°$ places the amide NH and α-CH protons of the Ile residue trans to each other, in agreement with the value for this angle estimated by nmr[5] for the related peptide, Gly-Val-Tyr-Ile-His-Pro-Phe. One consequence of such a conformation would be to facilitate an orientation of the phenolic residue of Tyr[4] to a position in space relative to the fixed His-Pro-Phe sequence,[5] in which a productive interaction with the biological receptor could occur. However, the biological significance of the proposed solution conformation of the His-Pro-Phe sequence has not yet been proven.

In order to provide further information concerning the steric role of the amino acid residue in the 5 position, the following amino acids were incorporated in place of Ile in [Asn,[1]Ile[5]]angiotensin II: α-aminoisobutyric acid (Aib), sarcosine (Sar, N-methylglycine), L-proline (Pro), D-proline (D-Pro), and cycloleucine (Cle, α-aminocyclopentanecarboxylic acid).

Chemistry.—The solid-phase peptide synthesis of the protected octapeptides, Z-Asn-Arg(NO_2)-Val-Tyr-(Bzl)-X-His(Bzl)-Pro-Phe-polymer (X = amino acid residue varied), was carried out as previously described.[1,3,4] The peptides were cleaved from the polymer, hydrogenated, and purified as described earlier.[4]

A side product lacking tyrosine was isolated during the purification of [Asn,[1]Pro[5]]angiotensin II. This may represent a significant failure sequence[7] during solid-phase synthesis or a reduction of the aromatic ring during removal of protecting groups by catalytic hydrogenolysis. Amino acid analysis of an acid hydrolysate of the peptide showed no cyclohexylalanine, a potential reduction product. Hexahydrotyrosine, if formed, is not stable under the conditions of acid hydrolysis, and would not be detected.[8]

(1) Part 6: E. C. Jorgensen, S. R. Rapaka, G. C. Windridge, and T. C. Lee, *J. Med. Chem.*, **14**, 899 (1971). This investigation was supported in part by Public Health Service Research Grant AM 08066 from the National Institute of Arthritis and Metabolic Diseases. The abbreviations used to denote amino acid derivatives and peptides are those recommended in *J. Biol. Chem.*, **241**, 2491 (1966); **242**, 555 (1967). In addition, Aib stands for α-aminoisobutyric acid, and Cle stands for cycloleucine (1-aminocyclopentanecarboxylic acid).

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(3) E. C. Jorgensen, G. C. Windridge, and T. C. Lee, *J. Med. Chem.*, **13**, 352 (1970).

(4) E. C. Jorgensen, G. C. Windridge, W. Patton, and T. C. Lee, *ibid.*, **12**, 733 (1969).

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(6) The abbreviations, symbols, and conventions used to describe conformations of the polypeptide chain are those recommended by the IUPAC–IUB Commission on Biochemical Nomenclature, *J. Mol. Biol.*, **52**, 1 (1970).

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Oxidation of acid hydrolysates of the peptides with *Crotalus adamanteus* L-amino acid oxidase was used to evaluate the optical purity of the constituent amino acids.[1,3] The results indicated that the peptides were essentially optically homogeneous as far as can be evaluated by this method except for IV and V in which removal of the D-His-containing contaminant[1,3] could not be achieved. Proline, which is not attacked by this enzyme, has previously been shown to be optically pure in a related peptide prepared in the same manner.[3]

Bioassay.—The compds were tested for pressor activity in nephrectomized, pentolinium-treated male rats as described previously.[1,9] All log dose–response curves had slopes which were parallel to the standard angiotensin curve (relative slopes 1.0 ± 0.1). Activities and durations of action relative to [Asn,[1]Val[5]]angiotensin II are shown in Table I.

TABLE I

PRESSOR ACTIVITIES OF ANGIOTENSIN II ANALOGS IN THE RAT

Asn—Arg—Val—Tyr—X—His—Pro—Phe
1 2 3 4 5 6 7 8

Amino acid in position 5	X	Activity,[a] %	Duration,[a] %
Aib	$NHC(CH_3)_2CO$	0.9	120
Sar	$N(CH_3)CH_2CO$	1.0	120
Pro	$N(CH_2)_3CHCO$	10.0	90
D-Pro	$N(CH_2)_3HCCO$	0.05	100
Cle	$NHC(CH_2)_4CO$	24.0	100

[a] Relative to [Asn,[1]Val[5]]angiotensin II (Hypertensin-CIBA) = 100 on a molar basis.

Structure–Activity Relationships and Discussion.—Replacement of Ile by Aib or Sar in the 5 position results in pressor activities of about 1%. Space filling molecular models (Corey–Pauling–Koltun) show that the fully extended conformation possible for Gly or Ala,[1] is prevented by the steric interaction between the *N*-Me group and the carbonyl O in the Sar[5] analog. However, little steric constraint exists beyond this, for when the N–C^α bond angle is $\phi = +$ or $-120°$, there is virtually free rotation about the C^α–CO bond, ψ. Similarly, when $\psi = +$ or $-120°$, rotation about the ϕ bond is freely possible, limited only by carbonyl O interactions on adjacent residues. This lack of steric constraint about C_5^α accounts for the low biological activity of the Sar analog. The α,α-Me_2 groups of Aib impart considerably more steric constraint than do the Sar or Gly residues, but the rotational angles favored are quite different from the desirable ones of $\phi = -120°$, $\psi = +120°$, which are prevented by the steric effects of the geminal methyl groups. The fully extended form, $\phi = +180°$, $\psi = +180°$, is possible. The possibility for many unfavorable conformations, and the energy barrier to favorable ones, as well as the lack of an asymmetric α substituent to provide a unique direction to the peptide backbone, are factors which account for the low activity of the Aib analog.

L-Pro in the 5 position imparts moderate activity (10%). The coplanar nature of the pyrrolidine ring and the acyl group attached to the ring N ($\phi = -60°$, C^αR cis to NR) provides a sterically fixed point of reference. Although this appears to be a somewhat unfavorable angle for ϕ, the freedom of rotation available to the C^α–CO bond may partially compensate. Models show that this bond may rotate freely about $\psi = +120°$ (C^α–H trans to C–O). In this position the peptide backbone is reasonably close to that for the configuration, $\phi = -120°$, $\psi = +120°$. The slight deviations in bond angles, and slightly altered position of the alicyclic side chain and carbonyl oxygen atom relative to Ile, may account for the reduced, but still significant, activity of the Pro analog. The D-Pro analog is essentially inactive (0.05%). Here, the peptide backbone is forced to turn away from the favored structure, so that the Tyr[4] residue would be on the opposite side of the peptide chain from His.[6]

The relatively high activity of the Cle (α-aminocyclopentanecarboxylic acid) analog (24%) provides additional support for a sterically favorable conformation. The fixed positions of the β-CH_2 of the cyclopentane ring appear to prevent the extended conformation possible for the related acyclic Aib analog. Sterically favored angles appear to be $\phi = -60°$ (C^αR cis to NH) or $\phi = +60°$ (C^αR′ cis to NH); $\psi = +60°$ (C^αR cis to CO), $\psi = -60°$ (C^αR′ cis to CO) or $\psi = +180°$ (C^αN cis to CO). Although 6 combinations of favored angles are possible, one of these, $\phi = -60°$, $\psi = +60°$, is readily achieved and presents a peptide bond angle and hydrocarbon side chain virtually identical with that of Ile or Val ($\phi = -120°$, $\psi = +120°$).

Summary.—The amino acids used in the present study, consisting of α,α-dialkyl, *N*-Me, cyclic, and L and D enantiomers, provide a wide range of steric effects on the peptide backbone at the 5 position of angiotensin II. The pattern of biological activity associated with these varying types and degrees of steric constraint is consistent with a favored peptide backbone conformation of $\phi_5 = -120°$, $\psi_5 = +120°$.

Experimental Section[10]

N-*tert*-**Butyloxycarbonyl-α-aminoisobutyric Acid** (I).—α-Aminoisobutyric acid (2.06 g, 20 mmoles), MgO (0.8 g, 20 mmoles), NaOH (0.8 g, 20 mmoles), and *tert*-butyloxycarbonyl azide (5.7 g, 40 mmol) in 40 ml of H_2O and 40 ml of dioxane were stirred at 40–45° for 3 days. The mixt was evapd to dryness under vacuum at 40–45°. The residue was dissolved in 20 ml of H_2O and acidified to pH 3 with a satd soln of citric acid. The soln was extd with AcOEt, the exts were washed with H_2O, dried (Na_2SO_4), and evapd under vacuum. Crystn from Et_2O yielded 0.75 g (12%), mp 117–118°. Tlc showed one spot with HCl-ninhydrin,[11] R_f V, 0.30; R_f VI, 0.34. *Anal.* ($C_9H_{17}NO_4$) C, H, N.

(9) T. A. Assaykeen, K. Otsuka, and W. F. Ganong, *Proc. Soc. Exp. Biol. Med.*, **127**, 306 (1968).

(10) Melting points (Thomas-Hoover Uni-Melt) are corrected. Amino acid analyses (Spinco Model 116 Analyzer) were obtd using the standard 4-hr methodology. Peptides were hydrolyzed for 72 hr under N_2 in constant boiling HCl containing Gly as an internal standard. Hydrolyses were carried out both with and without the addition of a crystal of PhOH, which protected Tyr from degradation. Peptide content was calcd in terms of free peptide, rather than the hydrated salt. Microanalyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley, Calif. Where analyses are indicated only by symbols of the elements, anal. results obtained were within ± 0.4% of the theor values. Precoated silica gel G plates (E. Merck) were used for tlc with the solvent systems: I, *sec*-BuOH–3% NH_3 (100:44); II, *n*-BuOH–AcOH–H_2O (4:1:5); III, *n*-BuOH–pyridine–AcOH–H_2O (15:10:3:12); IV, *i*-Pr_2O–$CHCl_3$–AcOH (6:3:1); V, *n*-BuOH–AcOH–H_2O (4:1:1); VI, xylene–pyridine–AcOH (100:15:5); VII, EtOH–CH_2Cl_2 (1:1). Electrophoresis was carried out on Whatman No. 1 paper at 5 kV using AcOH–HCOOH buffer, pH 1.85 in a Savant apparatus. E_H indicates electrophoretic mobility relative to histidine = 1.00.

(11) E. C. Jorgensen, G. C. Windridge, and T. C. Lee, *J. Med. Chem.*, **13**, 744 (1970).

N-tert-**Butyloxycarbonylcycloleucine** (II) was prepd from 1.29 g (10 mmoles) of cycloleucine (α-aminocyclopentanecarboxylic acid) as described for I. A white solid was obtd, 0.4 g (17%), recrystn from AcOEt–heptane, mp 131–133°. Tlc showed one spot with HCl-ninhydrin, R_f IV, 0.70; R_f VII, 0.71. *Anal.* ($C_{11}H_{19}NO_4$) C, H, N.

Asn-Arg-Val-Tyr-Aib-His-Pro-Phe (III) was prepd from 2.4 g (0.4 mmole) of Boc-His(Bzl)-Pro-Phe-polymer, 0.33 g (1.6 mmoles) of Boc-Aib (I), and 0.33 g (1.6 mmoles) of DCI as described previously.[3,4] Fourfold excesses of Boc-Tyr(Bzl), Boc-Val, Boc-Arg(NO_2), and Z-Asn-ONp were then coupled.[1] The peptide was cleaved from the resin with HBr in CF_3COOH and filtered, and the filtrate was evapd to dryness. The residue was lyophilized from 95% AcOH to yield a white powder which was stirred in 50% aq EtOH contg 5% AcOH over 10% Pd/C under 3 atm of H_2 for 3 days. The filtrate was evapd under vacuum at 40°, and the residue was lyophilized from 95% AcOH to yield 130 mg. A 90-mg portion of crude peptide was dissolved in 2 ml of 1 *M* AcOH and applied to a sulfoethylcellulose column [1.5 × 75 cm, Cellex-SE (NH_4^+) Bio-Rad Laboratories, Richmond, Calif.] which had been packed in 1 *M* AcOH. The column was operated at room temp, and was eluted at 12 ml/hr with 1 *M* AcOH for 20 hr. A linear gradient of NH_4OAc in 1 *M* AcOH was then started using a concn change of 10^{-4} *M*/ml. The effluent was monitored at 280 mμ, and 10-ml fractions were collected. Initial fractions of the main peak were found to be homogeneous by tlc. These fractions (850–880 ml) were combined, evapd, and lyophilized to yield 10 mg which was further purified *via* the picrate salt[4] to yield 6 mg of white powder. The peptide showed one Pauly + spot on tlc and electrophoresis: R_f I, 0.07; R_f II, 0.12; R_f III, 0.50; E_H, 0.77; acid hydrolysis, Asp 0.94, Arg 0.91, Val 1.00, Tyr 0.69, Aib 1.03, His 0.90, Pro 1.01, Phe 0.92; acid hydrolysis plus phenol, Asp 1.00, Arg 1.00, Val 1.00, Tyr 0.99, Aib 1.00, His 1.00, Pro 1.03, Phe 0.98; peptide content 48%. Aib emerged 30 ml before Val from the long column of the analyzer and had a color value which was 7.5% of that of Leu. A 48-hr acid hydrolysate incubated with *C. adamanteus* L-amino acid oxidase (Worthington) showed the amino acid ratios,[3,12] Asp 0.54, Arg 0.06, Val 0.03, Tyr 0.05, Aib 0.70, His 0.08, Pro 1.00, Phe 0.05.

Asn-Arg-Val-Tyr-Sar-His-Pro-Phe (IV).—The Sar[5] (*N*-methylglycine) analog was prepd from 1.8 g (0.3 mmole) of Boc-His-(Bzl)-Pro-Phe-polymer and 1.2-mmole portions of Boc-Sar[13,14] and DCI followed by chain extension, cleavage from the resin, and deprotection by hydrogenation, as described for III. The yield of crude peptide was 73 mg. This was chromatographed on Cellex-SE (NH_4^+) as described for III. Fractions from the main peak eluting at 910–960 ml were homogeneous by tlc. These were evapd and lyophilized to yield 10 mg which was further purified *via* the picrate salt[11] to yield 6.5 mg of white powder. This showed one Pauly + spot on tlc and electrophoresis: R_f I, 0.08; R_f II, 0.08; E_H, 0.76; acid hydrolysis: Asp 1.00, Arg 1.01, Val 1.08, Tyr 0.81, Sar 1.10, His 1.00, Pro 1.04, Phe 0.98; acid hydrolysis plus phenol, Asp 1.02, Arg 0.98, Val 1.02, Tyr 0.98, Sar 0.98, His 1.00, Pro 1.02, Phe 1.00; peptide content 80%. Sar emerged 14 ml before Pro and had a color value which was 9.4% of that of Leu. A 48-hr acid hydrolysate incubated with L-amino acid oxidase showed the amino acid ratios,[12] Asp 0.60, Arg 0.03, Val 0.02, Tyr 0.05, Sar 1.00, His 0.17, Pro 1.00, Phe 0.02.

Asn-Arg-Val-Tyr-Pro-His-Pro-Phe (V).—The L-Pro[5] analog was prepd from 1.8 g (0.3 mmole) of Boc-His(Bzl)-Pro-Phe-polymer and 1.2-mmole portions of Boc-Pro and DCI, followed by chain extension, cleavage from the resin, and hydrogenation as described for III. A 100-mg portion of the crude peptide (total 264 mg) was chromatographed on Cellex-SE (NH_4^+) as described for III. Early fractions of the main peak (540–560 ml) were homogeneous by tlc. These were evapd and lyophilized from 95% AcOH to yield 12 mg, which was further purified *via* the picrate salt[11] to yield 9 mg of white powder. Fractions of the main peak which were not homogeneous by tlc were combined, evapd, and lyophilized from 95% AcOH to yield a white powder. This was dissolved in a few ml of *sec*-BuOH–3% NH_3 (100:44) and chromatographed on a 3 × 100 cm column of Sephadex G-25 which was packed in and eluted by the same solvent system. The flow rate was 12 ml/hr, and alternate 6-ml fractions were tested by tlc for peptide content. Initial fractions of the peptide-containing eluate were homogeneous by tlc (R_f I, 0.15) and were found to contain a peptide lacking in Tyr (see below). This compd was sepd from later fractions which were also homogeneous by tlc (R_f I, 0.11). The latter fractions were combined, evapd, and lyophilized, followed by further purification *via* the picrate salt[11] to yield 15 mg of white powder. This showed one Pauly + spot on tlc and electrophoresis: R_f I, 0.12; R_f II, 0.19; E_H,0.68; acid hydrolysis, Asp 1.02, Arg 0.94, Val 1.04, Tyr 0.81, Pro 2.00, His 0.96, Phe 1.00; acid hydrolysis plus phenol, Asp 1.02, Arg 0.96, Val 1.02, Tyr 1.00, Pro 2.05, His 0.96, Phe 1.00. A 48-hr acid hydrolysate incubated with L-amino acid oxidase showed the amino acid ratios,[12] Asp 0.60, Arg 0.04, Val 0.02, Tyr 0.04, Pro 2.00, His 0.16, Phe 0.04.

Isolation of a Tyrosine-Free Peptide from V.—The early fractions from partition chromatog of V on Sephadex G-25 were combined, evapd, and lyophilized to yield 7 mg, which on further purification *via* the picrate salt gave 4 mg of white powder. Tlc showed a single Pauly + spot, R_f II, 0.53, and two spots, R_f I, 0.15 (major) and R_f I, 0.11 (trace). Electrophoresis showed a single Pauly + spot: E_H, 0.70; acid hydrolysis plus phenol, Asp 0.96, Arg 0.96, Val 1.00, Tyr 0.03, His 1.03, Pro 2.14, Phe 0.99.

Asn-Arg-Val-Tyr-D-Pro-His-Pro-Phe (VI).—The D-Pro[5] analog was prepd from 2.4 g (0.4 mmole) of Boc-His(Bzl)-Pro-Phe-polymer and 1.6-mmole portions of Boc-D-Pro and DCI, followed by chain extension, cleavage from the resin, and hydrogenation as described for III. The yield of crude peptide was 264 mg. A 100-mg portion was chromatogd on a 3 × 100 cm column of Sephadex G-25 packed in *sec*-BuOH 3% NH_3 (100:44) and eluted with the same solvent system as described for V. Fractions (6 ml) were collected. The flow rate was 12 ml/hr. A broad peptide-contg peak was not homogeneous by tlc, so fractions 37–96 were combined to yield 35 mg which was chromatogd on Cellex-SE (NH_4^+) as described for III. Fractions from the main peak which were eluted at 960–1000 ml were combined, evapd, and lyophilized to yield 12 mg. This was further purified *via* the picrate salt[11] to give 7.5 mg of white powder which showed one spot on tlc and electrophoresis: R_f I, 0.14; R_f II, 0.14; R_f III, 0.47; E_H, 0.69, acid hydrolysis plus phenol, Asp 1.09, Arg 0.97, Val 1.00, Tyr 0.97, Pro 1.92, His 0.97, Phe 0.97; peptide content 75%. A 48-hr acid hydrolysate incubated with L-amino acid oxidase showed the amino acid ratios,[12] Asp 0.58, Arg 0.04, Val 0.03, Tyr 0.05, Pro 2.00, His 0.10, Phe 0.04.

Asn-Arg-Val-Tyr-Cle-His-Pro-Phe (VII).—The cycloleucine[5] analog was prepd from 3.6 g (0.6 mmole) of Boc-His(Bzl)-Pro-Phe-polymer and 2.4-mmole portions of Boc-Cle (II) and DCI, followed by chain extension, cleavage from the resin, and hydrogenation as described for III. The yield of crude peptide was 350 mg. A 100-mg portion was chromatogd on Cellex-SE (NH_4^+) as described for III. Fractions from the main peak eluting at 760–810 ml were homogeneous by tlc. These were evapd and lyophilized to yield 22 mg which was further purified *via* the picrate salt[11] to yield 15 mg of white powder. This showed one Pauly + spot on tlc and electrophoresis: R_f I, 0.16; R_f II, 0.21; R_f III, 0.44; E_H, 0.78; acid hydrolysis, Asp 0.99, Arg 0.97, Val 1.10, Tyr 0.62, Cle 1.14, His 1.00, Pro 1.12, Phe 1.03; acid hydrolysis plus phenol, Asp 1.02, Arg 0.98, Val 1.03, Tyr 0.93, Cle 1.00, His 0.98, Pro 1.03, Phe 1.00; peptide content 71%. Cle emerged from the long column of the analyzer 14 ml before Tyr and had a color value which was 17% of that of Leu. A 48-hr acid hydrolysate incubated with L-amino acid oxidase showed the amino acid ratios,[12] Asp 0.07, Arg 0.04, Val 0.02, Tyr 0.05, Cle 1.03, His 0.10, Pro 1.00, Phe 0.04.

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(12) A mixt of amino acids subjected to the same hydrolytic and enzymatic procedures had Arg 0.04, Val 0.02, Tyr 0.04, Ile 0.02, His 0.08, Pro 1.00, Phe 0.04. L-Aspartic acid is attacked too slowly by L-amino acid oxidase to be checked by this method.

(13) E. Schnabel, *Justus Liebigs Ann. Chem.*, **702**, 188 (1967).

(14) E. Schnabel, H. Herzog, P. Hoffmann, E. Klauke, and I. Ugi, *ibid.*, **716**, 175 (1968).