Journal of Medicinal Chemistry

VOLUME 14, NUMBER 10 OCTOBER 1971

Angiotensin II Analogs. 6.[1] Stereochemical Factors in the 5 Position Influencing Pressor Activity. 1

EUGENE C. JORGENSEN,* SAMBASIVA R. RAPAKA, GRAHAM C. WINDRIDGE,

Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94122

AND THOMAS C. LEE

Department of Human Physiology, School of Medicine, University of California, Davis, California 95616

Received November 23, 1970

Analogs of [1-Asn, 5-Ile]angiotensin II were prepared by solid phase synthesis to test the nature of the contribution of isoleucine to pressor activity in the rat. α-Amino acid analogs in the 5 position with unbranched side chains showed relatively low pressor activities, L-Ala (3%), L-α-aminobutyric acid (16%), L-norleucine (21%). High activity was present in 5-substituted analogs with β-branched substituents in aliphatic side chains, L-α-amino-β-ethylvaleric acid (75%), L-alloisoleucine (100%), and in analogs containing β branching as part of a cyclic structure, L-α-cyclopentylglycine (89%), L-α-cyclohexylglycine (114%). These results are consistent with a steric effect, with the β-branching side chains favoring a specific active conformation for the peptide backbone at the 5 position, $\phi \sim -120°$, $\psi \sim +120°$.

Two equiactive naturally occurring variants of angiotensin II are known, [Val^5]angiotensin II and [Ile^5]-angiotensin II. The importance of the amino acid in the 5 position is shown by the greatly descreased biological activity of the synthetic analogs, [Leu^5]angiotensin II (25%)[2] and [Ala^5]angiotensin II (5%).[3] It has been suggested that the side chain of valine or isoleucine in the 5 position may be involved in hydrophobic bonding with the receptor protein.[3,4] In order to test the alternate hypothesis that a steric effect is the most important role of this side chain, a series of analogs of [Asn^1,Ile^5]angiotensin II was prepared, in which the β-branched side chain of Ile in the 5 position was replaced by normal side chains of varying length, or with β-branched aliphatic or alicyclic residues. The amino acid residues incorporated in position 5 were L-alanine (Ala), L-α-aminobutyric acid (Abu), L-norleucine (Nle), L-alloisoleucine (aIle), L-α-amino-β-ethylvaleric acid (Aev), L-α-cyclopentylglycine (Cpg), and L-α-cyclohexylglycine (Chg) (Table I).

Chemistry.—The solid-phase synthesis of the hexapeptide, Val-Tyr(Bzl)-X-His(Bzl)-Pro-Phe-polymer (X = amino acid residue varied) was carried out as previously described.[5,6] Arginine was then incorporated as the Boc-Arg(NO_2) derivative, and Asn as the *p*-nitrophenyl ester of Cbz-Asn. The cleaved and deprotected peptides were purified by NH_4OAc gradient elution from a sulfoethylcellulose (Cellex-SE) column,[5] followed by partition chromatography on Sephadex G-25.[7,8] In the latter system in some peptides the N-terminal Asn was hydrolyzed to form small amounts of the Asp peptides during removal of the solvent on the rotary evaporator. This contaminant was readily separated by rechromatography on sulfoethylcellulose. The problem could be avoided by neutralizing the effluent with AcOH prior to evaporation.

The steric homogeneity of the peptides was evaluated by digestion of acid hydrolysates with L-amino acid oxidase.[6] Digestion of a hydrolysate of Boc-His-(Bzl)-Pro-Phe-polymer showed the presence of about 15% of D-His(Bzl), although the original Boc-His(Bzl) was optically pure. With the exception of XII, the purified octapeptides contained no appreciable D-histidine, indicating that the diasteriomeric peptides were separated during chromatographic procedures. It is interesting to note that this resolution was not

(1) Part 5: E. C. Jorgensen, G. C. Windridge, and T. C. Lee, *J. Med. Chem.*, **14**, 631 (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, Aev stands for L-α-amino-β-ethylvaleric acid, Cpg stands for L-α-cyclopentylglycine, and Chg stands for L-α-cyclohexylglycine.

(2) R. Schwyzer and H. Turrian, *Vitam. Horm. (New York)*, **18**, 237 (1960).

(3) M. C. Khosla, N. C. Chaturvedi, R. R. Smeby, and F. M. Bumpus, *Biochemistry*, **7**, 3417 (1968).

(4) N. C. Chaturvedi, W. K. Park, R. R. Smeby, and F. M. Bumpus, *J. Med. Chem.*, **13**, 177 (1970).

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

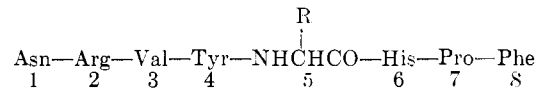
(6) E. C. Jorgensen, G. C. Windridge, and T. C. Lee, *ibid.*, **13**, 352 (1970).

(7) R. R. Smeby, P. A. Khairallah, and F. M. Bumpus, *Nature (London)*, **211**, 1193 (1966).

(8) D. Yamashiro, D. Gillessen, and V. du Vigneaud, *J. Amer. Chem. Soc.*, **88**, 1310 (1966).

TABLE I

PRESSOR ACTIVITIES OF ANGIOTENSIN II ANALOGS IN THE RAT



Amino acid in position 5	R	Activity,[a] %	Duration,[a] %
Ala	CH_3	3	70
Abu	CH_3CH_2	16	70
Nle	$CH_3(CH_2)_3$	21	90
aIle	$CH_3CH_2(CH_3)CH$	100	100
Aev	$(CH_3CH_2)_2CH$	75	100
Cpg	$(CH_2)_4CH$	89	100
Chg	$(CH_2)_5CH$	114	100

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

possible, even on tlc, for XII nor for the less basic analogs reported earlier.[6] The factors which contributed to this unusual amount of racemization during solid-phase synthesis are currently under investigation. No appreciable racemization was detected in other amino acids though the technique does not appear to be sufficiently reproducible to detect less than 2% racemization.

Yields were generally low because of the considerable losses involved in isolating the desired peptides from the D-His-containing peptides and from the hydrogenation side products,[6] even though the desired peptide was, in all cases, the major product. Higher yields could have been achieved by repeatedly rechromatographing the impure fractions, but this was not considered important in the present study.

Bioassay.—The compds were tested for pressor activity in nephrectomized, pentolinium-treated male rats anesthetized with pentobarbital.[9,10] 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 calcd from amino acid analyses, and the biological results are expressed on a molar basis. The pressor activities listed in Table I were calcd from log dose–response curves. Activities calcd by the Bliss statistical method[9,11] agreed with these values within ±5%. All log dose–response curves had slopes which were parallel to the standard angiotensin curve (relative slopes 1.0 ± 0.2) with the exception of the Ala[5] analog (relative slope 1.5) and the Abu[5] analog (relative slope 0.6). The duration of the pressor response was estimated by measuring the widths of the blood pressure peaks at half-height for equipressor doses of standard and of test compound.

Structure–Activity Relationships.—In agreement with previous results,[2,3] a significant decrease in pressor activity resulted from replacement of the β-branched *i*-Pr side chain of valine, or *sec*-Bu side chain of Ile in the 5 position of angiotensin II, with a normal aliphatic group, or with a group which branched at the γ-C. The 3% pressor activity of [Asn[1],Ala[5]]angiotensin II prepared in our studies, agrees well with the 5% value for [Asp[1],Ala[5]]angiotensin II found by Khosla, *et al.*[3] Activity increases with increased bulk of the normal side chain, the Et group of [Asn[1],Abu[5]]angiotensin II imparting 16%, and the *n*-Bu group of [Asn[1],Nle[5]]angiotensin II yielding 21% pressor activity relative to [Asn[1],Val[5]]angiotensin II. The latter result is in good agreement with the 25% pressor activity associated with the *i*-Bu side chain of [Leu[5]]angiotensin II.[2]

When β branching is present in aliphatic or alicyclic amino acids in the 5 position, activity of the octapeptide approximates that of the natural tissue hormone. In the aliphatic series, the stereoisomeric L-alloisoleucine imparts activity equal to that of isoleucine, while the related branching of the 3-pentyl side chain of [Asn[1],-Aev[5]]angiotensin II results in 75% activity. The 89% activity value for the cyclopentyl group of [Asn[1],Cpg[5]]-angiotensin II is further enhanced to 114% by the cyclohexyl side chain of [Asn[1],Chg[5]]angiotensin II.

Discussion

The structure–activity results discussed above may be related to the conformational preference for the section of the polypeptide chain at position 5 in angiotensin II. As shown in Figures 1 and 2, rotation of the planar C_4^α—CONH—C_5^α unit is possible around the N_5–C_5^α bond (defined by ϕ_5), where C_5^α represents the α-C of the residue in the 5 position, while N_5 represents the N of that amino acid residue.[12,13] Similarly, rotation defined by ψ_5 is possible around the C_5^α and C_5' bond, while the C_5^α—CONH—C_6^α segment remains coplanar. The extended conformation is shown in Figure 1, for the Ala residue where $\phi_5 = +180°$ and $\psi_5 = +180°$.[13] This conformation is only possible when branching is not present at C_5^β. Space filling molecular models (Corey–Pauling–Koltun) indicate that glycine would assume the extended conformation readily, while increased steric constraint is introduced by the Me or Et side chains of Ala and α-aminobutyric acid. The presence of the β branching of valine, isoleucine, or of the synthetic analogs of the present study makes the extended conformation impossible due to steric repulsions between the H atoms of the C_5^γ Me or CH_2 groups and the flanking carbonyl O (O_4) and NH (H_6). Space filling models of active analogs varying at position 5 indicate the preferred conformation of Figure 2, when β branching is present in the valyl peptide residue. In this conformation both variable bonds have rotated by about 60° from the extended conformation, to a structure in which minimal steric interactions occur, $\phi = -120 \pm 30°$, $\psi = +120 \pm 30°$. This conformation is a particularly favored one for the highly active [Asn[1],-Chg[5]]angiotensin II if C_5^α is assigned to the expected equatorial position of the cyclohexyl ring. The flanking equatorial $C_5^\gamma H$ atoms are positioned close to the faces of the C_4^α—CONH—C_5^α and C_5^α—CONH—C_6^α planes, and only small variations from the assigned rotational angles are possible. Leach, *et al.*,[14] have made calculations of the steric effects on the peptide backbone by the side chains of α-amino acids. These results support the above conclusions drawn from molecular

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

(10) R. Boucher, R. Veyrat, J. de Champlain, and J. Genest, *Can. Med. Ass. J.*, **90**, 194 (1964).

(11) H. O. Schild, *J. Physiol.*, **101**, 115 (1942).

(12) J. T. Edsall, P. J. Flory, J. C. Kendrew, A. M. Liquori, G. Némethy, G. N. Ramachandran, and H. A. Scheraga, *J. Mol. Biol.*, **15**, 399 (1966).

(13) The abbreviations and symbols used for the description of the conformations of the polypeptide chain are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature, *J. Mol. Biol.*, **52**, 1 (1970).

(14) S. J. Leach, G. Némethy, and H. A. Scheraga, *Biopolymers*, **4**, 369 (1966).

models. For the dipeptide, Gly-Gly, of all the conformations possible if no steric interactions took place, 50% are allowed when steric interactions are considered. In contrast, the unbranched side chain of Gly-Ala permits 16–20% of the possible conformations, while Gly-Nle is reduced to 14%, and Gly-Leu to 11%. The β-branched residues of Gly-Val, Gly-Ile, and Gly-Thr restrict conformations to 5% of those possible without steric interactions. For all of these model compds, the same minimal range of $\phi(-80°$ to $-140°)$ and $\psi(+90°$ to $+150°)$ is present.[13] Although the Tyr[4]-X[5]-His[6] segment of angiotensin II must have secondary steric constraints beyond those defined by the glycyl-peptide models, it is clear that the rotational angles of Leach, *et al.*, agree well with the angles of $\phi = -120°$ and $\psi = +120°$ proposed for β-branched residues in the present series of compounds.

In addition to the steric role for valine and isoleucine in the 5 position the relative importance of hydrophobic character and possible hydrophobic binding to receptor protein is still unclear. If maximal favorable hydrophobic binding is assumed for the structural element NHCH(C(C<)C)CO, all highly active analogs possess this partial structure, while analogs of intermediate activity, such as the Leu[5], Nle[5], and Abu[5] analogs of angiotensin II possess a partial structure, NHCH-(CH_2C)CO. The least active, the Ala[5] analog, shows the least hydrophobic character in the side chain, NHCH(CH_3)CO. In general, however, hydrophobic binding is greater for a normal, than for a branched carbon chain. In the absence of specific steric factors, it would be expected that the order of hydrophobic binding would be Nle > Leu > Ile, while in fact, biological potency is in the reverse order. Further analogs varying more widely in their steric effects and hydrophobic character are currently under study to clarify this point.

For the present, it is concluded that the primary role of Val or Ile in position 5 of angiotensin II, is to govern the stereochemistry of that segment of the polypeptide chain. Biological activity appears to be directly related to the probability that the peptide chain is able to assume an active conformation relative to the probability that it may exist in other conformations which are incapable of interacting with the biological receptor. Other studies[15] have indicated a favored conformation for the carboxyl-terminal tripeptide segment (His-Pro-Phe) of angiotensin II in solution, in which the CO_2^- of phenylalanine is associated with the imidazole ring of histidine. This cyclic structure is further stabilized by a H bond between the Phe NH group and the His carbonyl O. The connecting proline residue, or one which exerts a similar steric constraint, is necessary for their interactions and for biological activity. It appears likely that the Val or Ile residues of position 5 act to further fix the peptide backbone and to facilitate a favorable relative orientation for the alternating aromatic groups shown to be necessary for biological action, the phenolic residue of tyrosine, the imidazole group of His, and the Ph ring of Phe. In addition, a positively charged group, the terminal ammonium group of Asp[1] or the guanido residue of Arg[2], appears to be necessary to augment the inherent low activity of the sterically ordered hexapeptide sequence, Val-Tyr-Ile-His-Pro-Phe.[5]

(15) R. J. Weinkam and E. C. Jorgensen, *J. Amer. Chem. Soc.*, in press.

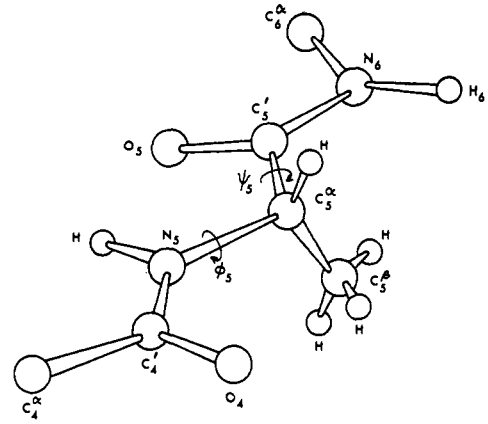


Figure 1.—Perspective drawing of a section of polypeptide chain. The central amino acid residue represents L-alanine in the 5 position of angiotensin II, with connecting peptide units on the amino and carboxyl ends shown in the extended conformation, $\phi_5 = +180°$, $\psi_5 = +180°$. Adopted from a drawing in ref 13.

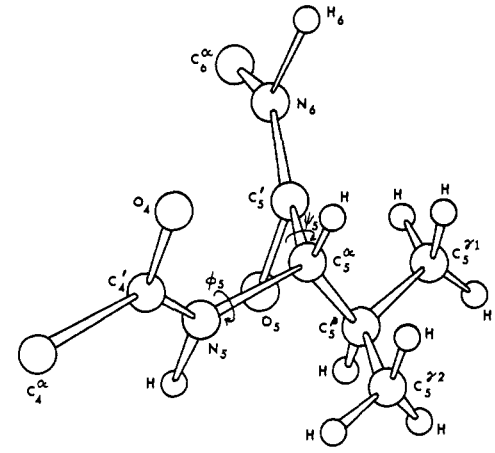


Figure 2.—Perspective drawing of a section of polypeptide chain. The central amino acid residue represents L-valine in the 5 position of angiotensin II, with connecting peptide units on the amino and carboxyl ends shown in a sterically favored conformation, $\phi_5 = -120°$, $\psi_5 = +120°$.

Experimental Section[16]

***N*-*tert*-Butyloxycarbonyl-L-α-amino-β-ethylvaleric Acid** (I).—L-α-Amino-β-ethylvaleric acid[17] (0.725 g, 5 mmoles), MgO (0.2 g, 5 mmoles), NaOH (0.2 g, 5 mmoles), and *tert*-butyloxycarbonyl azide (1.43 g, 10 mmoles) in 10 ml of H_2O and 10 ml of dioxane were stirred at 40–45° for 32 hr. The mixt was evapd to dryness under vacuum at 40–45°. The residue was dissolved

(16) Melting points (Thomas-Hoover Uni-Melt) are corrected. Amino acid anal. (Spinco Model 116 analyzer) were obtd using the standard 4-hr methodology. Peptides were hydrolyzed for 72 hr under N_2 in constant bp HCl contg Gly as an internal standard. Hydrolyses were carried out both in the absence and in the presence of a crystal of PhOH, which protected Tyr from degradation. Peptide content was calcd in terms of free peptide, rather than the hydrated salt. Microanal. 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. Rotations were measured with a Bendix-NPL automatic polarimeter, Type 143A, equipped with a digital readout and printer. 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, MeOH–CH_2Cl_2 (1:1). Unless otherwise noted electrophoresis was carried out on Whatman No. 1 paper at 5000 V using AcOH–HCOOH buffer, pH 1.85 in a Savant apparatus. E_H indicates electrophoretic mobility relative to His = 1.00.

(17) E. Eisler, J. Rudinger, and F. Sorm, *Collect. Czech. Chem. Commun.*, **31**, 4563 (1966).

in 5 ml of H_2O and acidified to pH 3 by the addn of satd citric acid soln. The soln was extd with AcOEt (5 × 50 ml). The AcOEt exts were combined, washed with H_2O (3 × 100 ml), dried (Na_2SO_4), and evapd under vacuum at 40–45° to yield 0.85 g (69%) of white solid. Recrystn from petr ether (30–60°) yielded 0.67 g (55%), mp 91–93°, $[\alpha]^{23}D$ + 11.0° (*c* 3.8, EtOH). Tlc showed one spot (R_f IV, 0.92; R_f V, 0.87) with HCl-ninhydrin.[18] *Anal.* ($C_{12}H_{23}NO_4$) C, H, N.

***N*-*tert*-Butyloxycarbonyl-L-α-cyclohexylglycine (II)** was prepd from 0.78 g (5 mmoles) of L-α-cyclohexylglycine[17] as described for compd I. A white solid was obtained, yield 0.85 g (65%), mp 89–91°. Tlc showed 2 spots [R_f V, 0.95 (major), 0.84 (faint); R_f VI, 0.32 (major), 0.00 (faint); R_f VII, 0.86] with HCl-ninhydrin.

The compd (0.15 g, 0.6 mmole) was dissolved in 3 ml of Et_2O and 0.18 g (1 mmole) of dicyclohexylamine was added. After 5 hr the salt was obtained as white flakes which were recrystd from heptane to yield white crystals, mp 139–141°, $[\alpha]^{23}D$ + 12.8° (*c* 3, EtOH). Tlc showed one spot (R_f VI, 0.32; R_f VII, 0.86) with HCl-ninhydrin. *Anal.* ($C_{13}H_{23}NO_4 \cdot C_{12}H_{23}N$) C, H, N.

***N*-*tert*-Butyloxycarbonyl-L-α-cyclopentylglycine (III)** was prepd from 0.75 g (5 mmoles) of L-α-cyclopentylglycine[17] as described for I. A white solid (0.94 g) was obtained and recrystd from heptane to yield 0.6 g (50%), mp 90–91°, $[\alpha]^{23}D$ −3.0° (*c* 2.5, EtOH). Tlc showed one spot (R_f IV, 0.93; R_f V, 0.74; R_f VII, 0.73) with HCl-ninhydrin. *Anal.* ($C_{12}H_{21}NO_4$) C, H, N.

***N*-*tert*-Butyloxycarbonyl-L-alloisoleucine (IV)** was prepd from 0.92 g (7 mmoles) of L-alloisoleucine (Fox Chemical Co.) as described for I. A white solid (1.2 g, 75%) was obtained, mp 59–62°. Tlc showed one spot (R_f V, 0.93; R_f VI, 0.58; R_f VII, 0.86) with HCl-ninhydrin. For an anal. sample, 0.23 g (1 mmole) of IV and 0.18 g (1 mmole) of dicyclohexylamine were dissolved in Et_2O and held at 0° for 10 hr to yield 0.32 g (78%) of dicyclohexylamine salt, which was recrystd from Et_2O, mp 137–139°, $[\alpha]^{23}D$ + 6.3° (*c* 2, EtOH). Tlc showed one spot (R_f VI, 0.58; R_f VII, 0.86) with HCl-ninhydrin. *Anal.* ($C_{11}H_{21}NO_4 \cdot C_{12}H_{23}N$) C, calcd: 66.95, found: 66.47; H, N.

Boc-His(Bzl)-Pro-Phe-polymer (V).—Boc-Phe-polymer (15.7 g, 3.0 mmoles of Phe) was deprotected, neutralized, and acylated overnight in CH_2Cl_2 with 2.6 g (12 mmoles) of Boc-Pro and 2.5 g (12 mmoles) of DCI as described previously.[5,6] After checking the completeness of the reaction by electrophoresis of a sample of peptide cleaved from the resin with HBr, unreacted amino groups were acetylated,[5] and the peptide was washed, deprotected, and treated with 4.2 g (12 mmoles) of BocHis(Bzl) and 2.5 g (12 mmoles) of DCI in 40 ml of DMF-CH_2Cl_2 (1:1). After 12 hr, about 5% of unreacted dipeptide remained. Acetylation was carried out to block the formation of failure sequences, and to simplify purification. The stock tripeptide-polymer (V) was used for further syntheses.

Asn-Arg-Val-Tyr-Cpg-His-Pro-Phe (VI).—The L-α-cyclopentylglycine[5] analog was prepd from 2.4 g (0.4 mmole) of tripeptide resin (V) and 1.6-mmole portions of Boc-Cpg (III), Boc-Tyr(Bzl), Boc-Val, Boc-Arg(NO_2), and Z-Asn-ONp as described previously,[5,6] with the following exceptions: (a) Boc-Arg(NO_2) was coupled in a mixt of DMF-CH_2Cl_2 (1:1), (b) Z-Asn-ONp was coupled in DMF for 48 hr, (c) acetylation of unreacted amino groups was not carried out on the Arg(NO_2)-containing peptides, and (d) couplings were carried out with DCI, except for the active ester, Z-Asn-ONp.

The protected peptide resin was washed with AcOH (3 × 30 ml) and CF_3COOH (3 × 20 ml) then suspended in 20 ml of CF_3COOH and 1 ml of anisole. HBr (scrubbed with satd resorcinol in C_6H_6 and with $CaCl_2$) was passed through the suspension for 45 min. The resin was filtered and washed with CF_3COOH (2 × 20 ml). The filtrate was evapd at 45° on a rotary evaporator and the residue was lyophilized from 95% AcOH. The resulting powder was washed with dry Et_2O and dried under vacuum over NaOH to give 265 mg. This powder was dissolved in 20 ml of 50% EtOH and 1 ml of AcOH, 270 mg of 10% Pd/C was added and the suspension was stirred under 3 atm of H_2 for 3 days. The mixt was filtered (filter aid) and washed with 50% EtOH. The filtrate was evapd under vacuum at 40° and the residue was lyophilized from 95% AcOH to yield 205 mg of white powder. A 1-mg sample was heated at 110° in 6 *M* HCl (sealed tube) for 24 hr. Electrophoresis (pH 1.85) showed complete absence of His(Bzl).

A 100-mg portion of crude peptide VI was chromatographed on Cellex-SE (NH_4^+),[5] eluting with 1 *M* AcOH for 20 hr, followed by a linear gradient of NH_4OAc in 1 *M* AcOH with a concn change of 10^{-4} *M*/ml. At a flow rate of 28 ml/hr, the main peak as detected by uv absorption at 280 mμ emerged between 940 and 1080 ml. The 45 mg of peptide obtained on evapn and lyophilization contained minor contaminants by tlc. The peptide was dissolved in 5 ml of *sec*-BuOH–3% NH_3 (100:44) and chromatographed on a 3 × 100 cm column of Sephadex G-25[8] which was packed in and eluted with the same solvent system. The flow rate was 12 ml/hr, and alternate 6-ml fractions were tested by tlc for peptide content. Fractions 32–46 were pooled, evapd to dryness after addn of 10 ml of AcOH to the concd soln, and then lyophilized to yield 12 mg of white powder. After further purification *via* the picrate salt[5] there was 8 mg of white powder which showed one Pauly + spot on tlc and electrophoresis: R_f I, 0.28; R_f II, 0.28; E_H 0.73; acid hydrolysate, Asp 0.97, Arg 0.95, Val 1.02, Tyr 0.89, Cpg, 1.08, His 0.95, Pro 1.00, Phe 0.96; acid hydrolysate plus phenol, Asp 0.96 Arg 1.01, Val 1.01, Tyr 1.00, Cpg 1.06, His 0.98, Pro 1.01, Phe 0.97; peptide content 78%. Cpg emerged from the long column of the analyzer 5 ml before Tyr and had a color value which was 91% of that of Leu. A 48-hr acid hydrolysate incubated with *C. adamanteus* L-amino acid oxidase (Worthington) showed the amino acid ratios,[6,19] Asp 0.61, Arg 0.04 Val 0.03, Tyr 0.04, Cpg 0.03, His 0.09 Pro 1.00, Phe 0.04.

Asn-Arg-Val-Tyr-Chg-His-Pro-Phe (VII).—The L-α-cyclohexylglycine[5] analog was prepd from 2.4 g (0.4 mmole) of tripeptide resin V and 1.6-mmole portions of Boc-Chg (II) etc. as described for VI. The yield of crude peptide was 210 mg. A 100-mg portion was chromatographed on Cellex-SE (NH_4^+) as described for VI. The center of the main peak emerging at 870–1050 ml yielded 40 mg of white powder which was further purified on Sephadex G-25 as described for VI. Fractions 51–59 were combined to yield 25 mg which was further purified *via* the picrate salt to yield 18 mg of white powder. This showed one Pauly + spot on tlc and electrophoresis: R_f I, 0.17; R_f II, 0.15; R_f III, 0.50; E_H, 0.72; acid hydrolysate plus phenol, Asp 0.95, Arg 1.00, Val 0.96, Tyr 0.95, Chg 1.02, His 1.01, Pro 1.00, Phe 0.96; peptide content 82%. Chg emerged from the long column of the analyzer 7.4 ml after Phe and had a color value which was 91% of that of Leu. A 48-hr acid hydrolysate incubated with L-amino acid oxidase showed the amino acid ratios,[19] Asp 0.07, Arg 0.04, Val 0.03, Tyr 0.04, Chg 0.02, His 0.07, Pro 1.00, Phe 0.04.

Asn-Arg-Val-Tyr-aIle-His-Pro-Phe (VIII).—The aIle[5] analog was prepd from 1.8 g (0.3 mmole) of tripeptide resin V and 1.2-mmole portions of Boc-aIle (IV) etc. as described for VI. The yield of crude peptide was 135 mg. A 115-mg portion was chromatographed on Cellex-SE (NH_4^+) and the column eluate between 720 and 1000 ml was combined to yield 45 mg of material which was not homogeneous by tlc and electrophoresis. Combined fractions 57–71, as described for VI, from chromatography on Sephadex G-25 yielded 21 mg which was further purified *via* the picrate salt to yield 13 mg of white powder. This showed one Pauly + spot on tlc and electrophoresis: R_f I, 0.07; R_f, II, 0.14; R_f III, 0.55; E_H, 0.74; acid hydrolysate, Asp 1.00, Arg 1.01, Val 1.05, Tyr 0.77, aIle 1.02, His 1.03, Pro 1.03, Phe 1.01; acid hydrolysate plus phenol, Asp 1.03, Arg 1.00, Val 1.01, Tyr 0.97, aIle 0.98, His 0.97, Pro 1.00, Phe 0.98; peptide content 78%. A 48-hr acid hydrolysate incubated with L-amino acid oxidase showed the amino acid ratios,[19] Asp 0.09, Arg 0.04, Val 0.03, Tyr 0.04, aIle 0.03, His 0.08, Pro 1.00, Phe 0.05.

Asn-Arg-Val-Tyr-Nle-His-Pro-Phe (IX).—The L-norleucine[5] analog was prepd from 4.8 g (0.8 mmole) of tripeptide resin V and 3.2-mmole portions of Boc-Nle etc. as described for VI. The yield of crude peptide was 296 mg. Two 100-mg portions were chromatographed on Cellex-SE (NH_4^+), and the portions of the major peak containing minimal contaminants, as seen by tlc, were combined, evapd, and lyophilized. The peptide was then chromatographed on Sephadex G-25, as described for VI. Fractions 31–40 which showed a single spot on tlc were combined,

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

(19) 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. Nle, Chg, Cpg, Aev, Abu, and Ala gave the same results as Ile. Asp is attacked too slowly by L-amino acid oxidase to be checked by this method.

the solns were evapd under vacuum at 40–45° and lyophilized to yield a white powder. This material showed 2 spots of almost equal intensity by tlc and electrophoresis, R_f I, 0.14 (yellow color with ninhydrin); R_f I, 0.13 (blue color with ninhydrin); E_H (pH 6.4), 0.75 (yellow color with ninhydrin) and 0.50 (blue color with ninhydrin). The ninhydrin colors and electrophoretic mobilities indicated conversion of some asparagine peptide to an aspartyl peptide during evaporation at 40–45° since the compd giving a blue ninhydrin reaction was previously absent. To resolve this mixt, 30 mg was chromatographed on a Cellex-SE column packed in H_2O and then was eluted with H_2O for 10 hr at 12 ml/hr. The column was then eluted with a linear gradient of NH_4OAc in H_2O with a concn change of 9×10^{-5} M/ml. The 2 components were completely resolved, the peak with the longer retention time (fractions 53–57, 6 ml each) yielded 18 mg, which on further purification *via* the picrate salt yielded 15 mg of the desired asparagine peptide. This showed one Pauly and ninhydrin + spot on the tlc and electrophoresis: R_f I, 0.14; R_f II, 0.24; R_f III, 0.48; E_H (pH 1.85), 0.81; E_H (pH 6.4), 0.75; acid hydrolysis plus phenol, Asp 0.98, Arg 1.00, Val 1.02, Tyr 1.01, Nle 1.00, His 1.02, Pro 0.98, Phe 0.99; peptide content 78%. A 48-hr acid hydrolysate incubated with L-amino acid oxidase showed the amino acid ratios,[19] Asp 0.12. Arg 0.04 Val 0.03, Tyr 0.04, Nle 0.02, His 0.07, Pro 1.00, Phe 0.04.

The peak with a shorter retention time (Cellex-SE, H_2O-NH_4OAc, fractions 41-44, 6 ml each) yielded 2 mg of a compd, which on picrate treatment gave 1 mg of white powder. This gave a single Pauly + and ninhydrin blue spot on tlc and electrophoresis: R_f I, 0.13; R_f II 0.21; E_H (pH 1.85) 0.81; E_H (pH 6.4), 0.50; acid hydrolysate plus phenol, Asp 1.00, Arg 0.90, Val 1.04, Tyr 1.03, Nle 1.07, His 0.97, Pro 1.04, Phe 0.98; peptide content 69%. The physical properties, color reactions, and amino acid analyses were consistent with those of an aspartyl peptide, Asp- or β-Asp-Arg-Val-Tyr-Nle-His-Pro-Phe.

Asn-Arg-Val-Tyr-Abu-His-Pro-Phe (X).—The L-α-amino-butyric acid[5] analog was prepd from 2.4 g (0.4 mmole) of tripeptide resin V and 1.6-mmole portions of Boc-Abu etc. as described for VI. The yield of crude peptide was 310 mg. A 100-mg portion was purified as described for IX by chromatog on Cellex-SE (NH_4^+). Fractions (10 ml) from 820 to 1150 ml were combined to yield 45 mg. This was chromatographed on Sephadex G-25 (*sec*-BuOH–3% NH_3 as VI) to yield 20 mg, followed by chromatog on Cellex-SE using an NH_4OAc in H_2O gradient with a concn change of 9×10^{-5} M/ml as described for IX. Fractions (6 ml) were collected and monitored at 280 mμ. Two peaks were resolved, the major peak with a longer retention time (fractions 37–39) yielded 7 mg which was further purified *via* the picrate salt to yield 5 mg of white powder. This gave one Pauly and ninhydrin + spot on tlc and electrophoresis: R_f I, 0.12; R_f II, 0.11; R_f III, 0.48; E_H, 0.76; acid hydrolysis plus phenol, Asp 1.00, Arg 1.04, Val 1.02, Tyr 1.03, Abu 1.00, His 0.95, Pro 1.00, Phe 1.02; peptide content 76%. A 48-hr acid hydrolysate incubated with L-amino acid oxidase showed the amino acid ratios,[19] Asp 0.54, Arg 0.04, Val 0.02, Tyr 0.04, Abu 0.02, His 0.09, Pro 1.00, Phe 0.03.

Asn-Arg-Val-Tyr-Aev-His-Pro-Phe (XI).—The L-α-amino-β-ethylvaleric acid[5] analog was prepd from 2.4 g (0.4 mmole) of the tripeptide resin V, and 1.6-mmole portions of Boc-Aev (I) etc. as described for VI. The yield of crude peptide was 204 mg. A 102-mg portion was purified as described for IX by sequential chromatog on Cellex-SE (NH_4OAc in AcOH, yield 60 mg), Sephadex G-25 (*sec*-BuOH-3% NH_3, yield 25 mg), and Cellex-SE (NH_4OAc in H_2O, yield 8 mg). Further purification *via* the picrate salt gave 5 mg of white powder which showed one Pauly and ninhydrin + spot on tlc and electrophoresis: R_f I, 0.14; R_f II, 0.16; R_f III, 0.51; E_H, 0.68; acid hydrolysis plus phenol, Asp 1.01, Arg 0.97, Val 1.00, Tyr 0.95, Aev 1.05, His 0.99, Pro 1.04, Phe 0.97; peptide content 82%. Aev emerged from the long column of the analyzer 15 ml before Tyr and had a color value 86% of that of Leu. A 48-hr acid hydrolysate incubated with L-amino acid oxidase showed the amino acid ratios,[19] Asp 0.73, Arg 0.04, Val 0.02, Tyr 0.03, Aev 0.03, His 0.06, Pro 1.00, Phe 0.03.

Asn-Arg-Val-Tyr-Ala-His-Pro-Phe (XII).—The Ala[5] analog was prepd from 3.0 g (0.5 mmole) of the tripeptide resin V and 2-mmole portions of Boc-Ala etc as described for VI. The related analog, [Asp[1],Ala[5]]angiotensin II has been reported.[3] The yield of crude peptide was 244 mg. A 100-mg portion was purified on a Cellex-SE (NH_4^+) column as described for VI to yield 10 mg which was further purified *via* the picrate salt[5] to yield 8 mg of white powder. This gave one Pauly and ninhydrin + spot by tlc and electrophoresis: R_f I, 0.09; R_f II, 0.12; R_f III, 0.49; E_H, 0.81; acid hydrolysis plus phenol, Asp 0.94, Arg 1.00, Val 1.00 Tyr 1.00, Ala 1.02, His 0.98, Pro 0.98, Phe 1.00; peptide content 76%. A 48-hr acid hydrolysate incubated with L-amino acid oxidase had Asp 0.25, Arg 0.03, Val 0.02, Tyr 0.02, Ala 0.02, His 0.25, Pro 1.00, Phe 0.02.

Acknowledgments.—One of the authors (E. C. J.) is grateful to Professor Robert Schwyzer for his warm hospitality at the Institute for Molecular Biology and Biophysics, Eidgenössische. Technische Hochschule, Zurich, Switzerland, during the preparation of this manuscript, and for the helpful discussions with Professor Schwyzer and Professor Josef Rudinger.