# Analogs of Angiotensin II. I. Solid Phase Synthesis[1]

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[5-Ile,8-Tyr]-, [5-Ile,8-(OMe)Tyr]-, [5-Val,8-(OMe)Tyr]-, [4-(OMe)Tyr,5-Ile]-, [4-(OMe)Tyr,5-Val]-, [1-Asp-($NH_2$),4-(OMe)Tyr,5-Val]-, [5-Ile,7-pipecolic acid]-, [5-Ile,8-(3-amino-4-phenyl)butyric acid]-, and [5-Ile,8-(3-amino-3′-phenyl)isobutyric acid]-angiotensins II were synthesized by the solid phase method in yields of 50–63%. All peptides were shown to be homogeneous by C, H, N, analysis, chromatography, and electrophoresis, and amino acid analysis after acid and enzymatic hydrolysis. Introduction of OH or $OCH_3$ on the phenyl ring in position 8 of angiotensin II reduced pressor activity slightly. However, $OCH_3$ in place of the OH of tyrosine in position 4 of angiotensin II caused a drastic reduction of pressor activity. Substitution of an unnatural amino acid in positions 7 or 8 of angiotensin II greatly reduced pressor activity.

The aromatic and the C-terminal carboxyl groups of angiotensin II, H·Asp-Arg-Val-Tyr-Ile (or Val)-His-Pro-Phe·OH, are essential for full biological activity.[2] Since these groups can all lie in close juxtaposition upon folding of the peptide chain, it is of interest to determine the effect of replacement of phenylalanine by various homologs of phenylalanine, thereby changing the relative positions of these groups. Thus, the two homologs of phenylalanine, 3-amino-4-phenylbutyric acid and 3-amino-3′-phenylisobutyric acid, were synthesized and substituted at position 8 of angiotensin II. In the former the $CO_2H$ is separated from the amino group by a $CH_2$ group, where in the latter both Ph and $CO_2H$ groups are removed by $CH_2$. Peptides have also been prepared in which *O*-methyltyrosine has replaced phenylalanine and tyrosine.

Substitution of alanine at position 7 for proline reduces the pressor activity of the peptide 1000-fold. This could be due to the lack of the aliphatic ring of proline and the resulting loss of rigidity. Since the 6-membered ring of pipecolic acid is less planar than the 5-membered ring of proline the peptide bond angles formed from pipecolic possibly may more nearly approach that formed from a primary amino acid. Utilizing this assumption, one should be able to determine the steric importance of the cyclic amino acid in position 7 of angiotensin.

## Results and Discussion

L-3-Amino-4-phenylbutyric acid was synthesized from L-phenylalanine by the procedure of Balenovic, *et al.*[3] Benzyloxycarbonyl-L-phenylalanine chloride was converted into the diazoketone of the higher homolog by treatment with $CH_2N_2$. This was then hydrolyzed in the presence of $Ag_2O$ to L-3-benzyloxycarbonylamino-4-phenylbutyric acid. Subsequent catalytic hydrogenation yielded L-3-amino-4-phenylbutyric acid.

For the synthesis of 3-amino-3′-phenylisobutyric acid, the method of Bohme, *et al.*,[4] was adopted. The Na derivative of diethyl benzylmalonate was prepared and condensed with phthalimidomethylene chloride. The resulting diethyl phthalimidomethylbenzylmalonate was hydrolyzed by heating with concentrated HCl in a sealed tube to give DL-3-amino-3′-phenylisobutyric acid.

The route employed for the synthesis of peptides was essentially the same as described by Marshall and Merrifield[5] for the synthesis of angiotensin II. The butyloxycarbonyl derivative of the C-terminal amino acid of the future peptide was esterified onto chloromethylated polystyrene. This was then introduced into the reaction vessel[6] in which all steps of the synthesis were conducted. The cycle for each amino acid consisted of removal of butyloxycarbonyl group (Boc) by 1 *N* HCl in AcOH, neutralization of the resulting hydrochloride with $Et_3N$ in DMF, and then coupling the free base with the next amino acid using DCI as condensing agent. At the end of all syntheses, the protected octapeptides were removed by bubbling HBr through a suspension of peptide polymer in trifluoroacetic acid and the partially protected peptides were catalytically hydrogenated to free peptides. The compounds at this stage were usually accompanied by minor contaminants, which were removed by partition chromatography on a column of Sephadex G-25 using suitable solvents. All peptides were shown to be homogeneous by paper chromatography and electrophoresis, tlc, C, H, and N analysis, and amino acid analysis after acid and enzymatic hydrolysis. All peptides were completely degraded by leucine aminopeptidase except the histidyl–proline bond and that of DL-3-amino-3′-phenylisobutyric acid. A cruder preparation of hog kidney leucine aminopeptidase will split the histidyl–proline bond more readily. In common with many other angiotensin analogs, these peptides apparently contained varying quantities of acetic acid and water.[6-10]

Biological assay of [5-Ile,7-pipecolic acid]-angiotensin II shows this peptide possessed 1.0% of the pressor activity of natural peptide. The inactivity of [7-pipecolic acid]-angiotensin II proves again the necessity of a specific conformation brought about by proline in this position for biological activity. [5-Ile,8-(3-amino-4-phenyl)butyric acid]angiotensin II, in which the $CO_2H$ is further removed by a $CH_2$, had 10.0% pressor activity of the natural peptide while [5-Ile,-8-DL-(3-amino-3′-phenyl)isobutyric acid]-angiotensin

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(2) For a review of structure–activity relationships of angiotensin analogs see F. M. Bumpus and R. R. Smeby in "Renal Hypertension," I. H. Page and J. W. McCubbin, Ed., Year Book Medical Publishers, Inc., Chicago, Ill., 1968, pp 83–87.

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II, where both $CO_2H$ and Ph groups are removed by a $CH_2$, the pressor activity was lowered further to 0.1%. It is possible that one optical form of this amino acid may have been concentrated in the final peptide. However, because of the low biological activity, no attempts were made to resolve the racemate. These results prove the importance of these essential groups being in proper relative position to be able to bind to a receptor site.

[5-Ile,8-(OMe)Tyr]-angiotensin II and [5-Val,8-(OMe)Tyr]-angiotensin II possess 33% of the pressor activity of the natural peptide. This is less pressor activity than [5-Ile,8-Tyr]-angiotensin II (83%)[11] but greater than that of [5-Ile,8-Ala]-angiotensin II (1%).[12] The pressor activity of the (OMe)Tyr analog has probably been reduced due to the increased steric hindrance of the OMe group compared with either OH or H. However, since the aromatic ring is still present considerable activity has been retained.

[4-(OMe)Tyr,5-Val]-angiotensin II possesses 0.9% of the pressor activity of the natural peptide. This low level of activity is very similar to that of [4-Ala,5-Ile]-angiotensin II (0.3%)[13] and considerably less than [4-Phe,5-Val]-angiotensin II (10%).[14] This drastic reduction in activity upon substitution of *p*-OMe for *p*-OH must be due to steric hindrance rather than the loss of H bonding. The loss of H bonding alone (replacing OH by H) caused a less severe reduction in activity.

The pressor results obtained by group modifications in the positions occupied by aromatic amino acids in angiotensin II can be summarized by arranging the groups in order of decreasing biological activities as follows: position 4: $p\text{-}HOC_6H_4 > C_6H_5 > p\text{-}MeOC_6H_4 = CH_3$; position 8: $C_6H_5 > p\text{-}HOC_6H_4 > p\text{-}MeOC_6H_4 > CH_3$.

Further, the analogs reported here emphasize that as long as the aliphatic side group in position 5 maintains $C_3$ branching, this side group has little effect on biological activity.[15]

The analog [1-Asp($NH_2$),4-(OMe)Tyr,5-Val]-angiotensin II possesses 0.35% of the pressor activity of the natural peptide.

## Experimental Section[16]

The solvent systems used for paper chromatography (PC) and tlc were: (a) BuOH–AcOH–$H_2O$ (4:1:5) (BAW); (b) BuOH–AcOH–pyridine–$H_2O$ (30:6:24:20) (BAPW). Eastman Kodak silica gel sheets, type K 301 R were used for all tlc. The conditions used for paper electrophoresis were: solvent, 95 ml of AcOH and 36 ml of HCOOH diluted to 2 l. with distilled $H_2O$; pH 2.1 at 459 V. Electrophoretic mobilities are reported as the ratio of the distance the peptide moved to the distance L-glutamic acid migrated and abbreviated as $E_G$. All melting points were taken on a Leitz Metzlar hot-stage apparatus and are not corrected. Microanalyses were done by Micro-Tech Laboratories, Skokie, Ill. Amino acid analyses were run on an acid hydrolysate prepared in 6 *N* HCl at 110° for 38 hr. The analyses were performed on a Technicon amino acid autoanalyzer. For enzymatic hydrolysis, 2 mg of the corresponding peptide was dissolved in 3 ml of 0.001 *M* $MgCl_2$ in 0.01 *M* $NaHCO_3$ (pH 8.1) mixed with 60 mg of crude hog kidney leucine peptidase.[17] The mixture was incubated at 37° for 48 hr. Aliquots were taken intermittently and spotted on paper and silica gel thin-layer plates for chromatography on BAW and BAWP ascending solvent systems. While considerable splitting occurred within 1 hr of incubation, complete degradation of two of the peptides to component amino acids required 20 hr. No attempts were made to resolve 3-amino-3'-phenylisobutyric acid. For amino acid analysis following enzymatic hydrolysis, a purified hog kidney leucine aminopeptidase was used (P-L Biochemicals, Inc., Milwaukee, Wis.). The biological activity of all peptides are given in Table I. The pressor assay was performed using a ganglion-blocked, vagotomized rat.[18]

**D,L-Pipecolic Acid.**—D,L-Pipecolic acid synthesized from α-picolinic acid by catalytic hydrogenation was resolved by the method of Schweet, *et al.*,[19] L-pipecolic acid·HCl $[\alpha]^{25}D$ −20.4° (*c* 2.0, $H_2O$); lit.[19] $[\alpha]^{25}D$ −8.13° (*c* 5.0, $H_2O$)).

**Boc-L-pipecolic Acid.**—D,L-Pipecolic acid was converted into its Boc derivative by the method of Schwyzer, *et al.*,[20] in 60% yield, mp 126–127°; $[\alpha]^{25}D$ +20.5° (*c* 1.15, MeOH). Anal. ($C_{11}H_{19}NO_4$) C, H, N.

**Boc-phenylalanine Polymer.**—Boc-phenylalanine (10 mmol) and $Et_3N$ (10 mmol) in EtOH were refluxed with stirring for 24 hr with 10 g of chloromethylated copolystyrene–2.0% divinylbenzene which contained 1.4 mmoles of Cl/g. The polymer ester was removed by filtration, washed with absolute EtOH, $H_2O$, and MeOH, and dried under vacuum. Each gram of polymer was found to contain 0.3 mmol of the amino acid released on hydrolysis of a sample in a mixture of HCl and AcOH.[21]

**Benzyloxycarbonyl-β-benzylaspartylnitroarginylvalyl-*O*-benzyltyrosylisoleucyl-$N^{im}$-benzylhistidylpipecolylphenylalanine Polymer.**—Boc-phenylalanine polymer (5 g containing a total of 1.50 mmol of Boc-phenylalanine) was introduced into a reaction vessel and the following steps were used to introduce each new amino acid residue. (1) Wash with glacial AcOH (3 times with 60 ml). (2) Boc group removed by treatment with 60 ml 1 *N* HCl in AcOH for 30 min. (3) Wash with glacial AcOH (3 times with 60 ml). (4) Wash with absolute EtOH (3 times with 60 ml). (5) Wash with DMF (3 times with 60 ml). (6) Neutralize the HCl salt with 6 ml of $Et_3N$ in 60 ml of DMF for 10 min. (7) Wash with DMF (3 times with 60 ml). (8) Wash with $CH_2Cl_2$ (3 times with 60 ml). (9) With cooling in ice bath add 4.5 mmol of the appropriate Boc-amino acid dissolved in 45 ml of $CH_2Cl_2$ and mix for 10 min. (10) Add 4.5 mmol of DCI dissolved in 15 ml of $CH_2Cl_2$ and shake the mixture 2 hr with cooling in ice, and overnight at room temperature. (11) Wash with $CH_2Cl_2$ (3 times with 60 ml). (12) Wash with EtOH (3 times with 60 ml).

For coupling the amino acids Boc-$N^{im}$-benzylhistidine and Boc-nitroarginine, step 8 was deleted and DMF was used as the solvent in place of $CH_2Cl_2$ in steps 9 through 11.

**Aspartylarginylvalyltyrosylisoleucylhistidylpipecolylphenylalanine ([5-Ile,7-pipecolic Acid]-Angiotensin II).**—The protected peptide polymer was suspended in about 100 ml of trifluoroacetic acid and dry HBr was bubbled through the suspension for 40 min. The polymer was removed by filtration and washed three times with 10-ml portions of trifluoroacetic acid. The combined filtrates were evaporated on a rotary evaporator *in vacuo* at room temperature and the syrupy product obtained was triturated with $Et_2O$. The amorphous powder obtained was collected on a sintered glass funnel, washed well ($Et_2O$), dissolved in 50 ml of MeOH–AcOH–$H_2O$ (10:1:1), and reduced by bubbling $H_2$ through the solution at atmospheric pressure for 36 hr using Pd black (1 g) as the catalyst. After hydrogenation was complete, the catalyst was removed by filtration and the filtrate evaporated to dryness to give 2.45 g of crude compound. This was purified by chromatography on a column of Sephadex G-25 (78 × 4.6 cm)

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(16) All amino acids used were the L isomer other than 3-amino-3'-phenylisobutyric acid. Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements were within ±0.4% of the theoretical values.

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TABLE I

BIOLOGICAL ACTIVITY OF ANGIOTENSIN II ANALOGS

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | Pressor act. % of angiotensin II |
|---|---|---|---|---|---|---|---|---|
| Asp | - Arg - | Val - | Tyr | - Ile - | His - | Pro - | Tyr[a] | 83 |
| [1-Asp, 5-Ile, 8-Tyr]-angiotensin II | | | | | | | | |
| Asp | - Arg - | Val - | Tyr | - Ile - | His - | Pro - | (OMe)Tyr | 33 |
| [1-Asp, 5-Ile, 8-(OMe)Tyr]-angiotensin II | | | | | | | | |
| Asp | - Arg - | Val - | Tyr | - Val - | His - | Pro - | (OMe)Tyr | 33 |
| [1-Asp, 5-Val, 8-(OMe)Tyr]-angiotensin II | | | | | | | | |
| Asp | - Arg - | Val - | (OMe)Tyr - | Ile - | His - | Pro - | Phe | 1.0 |
| [1-Asp, 4-(OMe)Tyr, 5-Ile]-angiotensin II | | | | | | | | |
| Asp | - Arg - | Val - | (OMe)Tyr - | Val - | His - | Pro - | Phe | 0.9 |
| [1-Asp, 4-(OMe)Tyr, 5-Val]-angiotensin II | | | | | | | | |
| Asp($NH_2$) - | Arg - | Val - | (OMe)Tyr - | Val - | His - | Pro - | Phe[b] | 0.4 |
| [1-Asp($NH_2$), 4-(OMe)Tyr, 5-Val]-angiotensin II | | | | | | | | |
| Asp | - Arg - | Val - | Tyr | - Ile - | His - | Pip - | Tyr | 1.0 |
| [7-Pipecolic acid]-angiotensin II | | | | | | | | |
| Asp | - Arg - | Val - | Tyr | - Ile - | His - | Pro - | APB | 10.0 |
| [8-(3-Amino-4-phenylbutyric acid]-angiotensin II | | | | | | | | |
| Asp | - Arg - | Val - | Tyr | - Ile - | His - | Pro - | APIB | 0.1 |
| [8-(3-Amino-3'-phenylisobutyric acid)]-angiotensin II | | | | | | | | |

[a] Schröder and Hempel reported 10–20% of the pressor activity of the [5-Val]-angiotensin II (ref 9). [b] Schröder and Hempel (ref 9) and M. A. Cresswell, R. W. Hanson, and H. D. Law, *J. Chem. Soc.*, 2669 (1967) reported 0.2 and 0.1%, respectively, of the pressor activity of the [5-Val]-angiotensin II.

TABLE II

PHYSICAL CONSTANTS OF ANGIOTENSIN II ANALOGS

| | Chromatography $R_f$ (PC) (BAW) | $R_f$ (tlc) (BAW) | $R_f$ (BAPW) | $E_G$ | $[\alpha]^{23}D$, deg | Mp °C (dec) | Yield % |
|---|---|---|---|---|---|---|---|
| [5-Ile, 8(OMe)Tyr]-angiotensin II | 0.47 | 0.41 | 0.43 | 0.61 | 1.18 | −44.6[a] | 237 | 53 |
| [5-Val, 8(OMe)Tyr]-angiotensin II | 0.42 | 0.37 | 0.32 | 0.54 | 1.23 | −39.3[a] | 207–210 | 55 |
| [4(OMe)Tyr, 5-Ile]-angiotensin II | 0.52 | 0.47 | 0.38 | 0.62 | 1.22 | −52.0[a] | 227 | 51 |
| [4(OMe)Tyr, 5-Val]-angiotensin II | 0.49 | 0.43 | 0.42 | 0.57 | 1.20 | −36.6[a] | 225–227 | 56 |
| [1-Asp($NH_2$), 4(OMe) Tyr, 5-Val]-angiotensin II | 0.49 | 0.44 | 0.41 | 0.63 | 1.20 | −40.6[a] | 205–207 | 50 |
| [5-Ile, 7-Pip]-angiotensin II | 0.52 | 0.55 | 0.35 | 0.46 | 0.96 | −30.2[b] | 224–225 | 62 |
| [5-Ile, 8-APB]-angiotensin II | 0.47 | 0.58 | 0.18 | 0.48 | 1.18 | −43.0[c] | >230 | 48 |
| [5-Ile, 8-APIB]-angiotensin II | 0.43 | 0.54 | 0.17 | 0.48 | 1.13 | 67.6[d] | >225 | 63 |

[a] *c* 1.0, 50% HOAc. [b] *c* 1.0, 0.1 *N* HOAc. [c] *c* 0.86, $H_2O$. [d] *c* 0.71, $H_2O$.

using BuOH–AcOH–$H_2O$ (4:1:5) as the developing solvent.[22] Fractions of 12 ml each were collected. Fractions 90–120 which contained the required octapeptide were pooled, concentrated to 3 ml, filtered through Hyflosupercel, and evaporated to dryness at room temperature *in vacuo*. Addition of dry $Et_2O$ to the residual syrup yielded an amorphous white solid, which was filtered and washed with dry EtOAc and dry $Et_2O$ to give pure title peptide. Physical constants are given in Table II; loss of water at 100°: 2.75%; amino acid ratios found: Asp, 1.04, Arg, 1.12, Val, 1.11, Tyr, 0.99, Ile, 1.00, His, 0.95, pipecolic acid, 1.00, Phe, 0.97. *Anal.* ($C_{51}H_{73}N_{13}O_{12}\cdot CH_3COOH$) C, H, N.

**L-3-Benzyloxycarbonylamino-4-phenylbutyric Acid.**—A solution of 7.9 g (25 mmol) of benzyloxycarbonyl-L-phenylalanine chloride[23] in 50 ml of absolute $Et_2O$ was added at 10° to a solution of $CH_2N_2$ (from 17.5 g of nitrosomethylurea in $Et_2O$). After 12 hr at 10°, excess $CH_2N_2$ was destroyed by addition of AcOH and $Et_2O$ removed under reduced pressure to give 8 g of L-1-diazo-3-benzyloxycarbonylamino-4-phenyl-2-butanone as an oil. It was used as such for the next step.

The diazo ketone (8 g) was dissolved in 50 ml of dioxane and added dropwise with stirring to a mixture of 1 g of freshly prepared $Ag_2O$, 2.5 g of anhydrous $Na_2CO_3$, and 1.5 g of sodium thiosulfate in 100 ml of $H_2O$ at 50°. The mixture was stirred under reflux for an additional hour, cooled, diluted with $H_2O$, and acidified with dilute $HNO_3$. The product, which precipitated from the mixture was filtered and recrystallized from EtOH–$H_2O$; yield: 4.47 g (57%), mp 110–111°. *Anal.* ($C_{18}N_{19}O_4N$) C, H, N.

**L-3-Amino-4-phenylbutyric Acid.**—The benzyloxycarbonyl compound (3.2 g) was dissolved in a mixture of MeOH–AcOH–$H_2O$ and hydrogenated (Pd black). The catalyst was removed by filtration and filtrate evaporated to give the free amino acid; yield: 1.5 g, mp 225–226°; $[\alpha]^{23}D$ +22.1° (*c* 1.5, $H_2O$). *Anal.* ($C_{10}H_{13}NO_2$) C, H, N.

**L-3-Butyloxycarbonylamino-4-phenylbutyric Acid Polymer.**—3-Amino-4-phenylbutyric acid (3.6 g, 20 mmol) and MgO (1.6 g, 40 mmol) were suspended in 50 ml of 50% dioxane, stirred 1 hr at room temperature, and treated with butyloxycarbonyl azide (5.7 g, 40 mmol); after stirring overnight at room tem-

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perature, the mixture was diluted with $H_2O$ (150 ml), the solid removed by filtration, and the filtrate extracted with EtOAc (3 times with 30 ml). The aqueous layer was acidified to pH 4 by addition of cold 10% citric acid. The precipitated oil was extracted into EtOAc which was then washed well with a saturated NaCl solution and dried ($MgSO_4$). It was then evaporated to dryness to give 3.15 g (56%) of 3-butyloxycarbonylamino-4-phenylbutyric acid as a yellowish oil, which was used as such for next step.

A solution of 2.8 g (10 mmol) of the butyloxycarbonyl amino acid and 1.4 ml (10 mmol) of $Et_3N$ in 25 ml of EtOH was added to 10 g of chloromethyl copolystyrene–2% divinylbenzene which contained 1.41 mmol of Cl/g and the mixture stirred at 75–80° for 24 hr. The polymer ester was collected by filtration, washed well with absolute EtOH, $H_2O$, and MeOH, and dried under vacuum. Each gram of polymer was found to contain 0.28 mmol of the amino acid as determined by spectroscopic determination of the amino acid released on hydrolysis of a sample in a mixture of HCl and AcOH.

**Benzyloxycarbonyl-β-benzylaspartylnitroarginylvalyl-*O*-benzyltyrosylisoleucyl-$N^{im}$-benzylhistidylprolyl-3-amino-4-phenylbutyric Acid Polymer.**—The desired sequence was made on the polymer in a manner similar to [7-pipecolic acid]-angiotensin II by stepwise addition of succeeding amino acids to 5 g of 3-butyloxycarbonylamino-4-phenylbutyric acid polymer containing 1.401 mmol of amino acid. A fourfold excess of all amino acids was used for all steps.

**Aspartylarginylvalyltyrosylisoleucylhistidylprolyl-3-amino-4-phenylbutyric Acid ([5-Ile,8-(3-amino-4-phenyl)butyric acid]-angiotensin II).**—The peptide was cleaved from the polymer by HBr in $CF_3CO_2H$ treatment and then hydrogenated in a mixture of MeOH–AcOH–$H_2O$ (50 ml, 10:1:1) at atmospheric pressure for 48 hr using Pd black (1 g). The reaction mixture was then treated in the same manner as for [pipecolic acid]-angiotensin II, to yield 1.10 g of peptide. Chromatography showed a minor spot of low $R_f$ value in addition to one major spot at $R_f$ (BAWP) 0.58. This contaminant was removed by partition chromatography on a column of Sephadex G-25 using BuOH–AcOH–pyridine–$H_2O$ (30:6:24:20) as the developing solvent. Fractions of 10 ml each were collected. Fractions 60–80 which contained the required octapeptide were pooled, concentrated to 3 ml, filtered through Hyflosupercel, and evaporated to dryness at room temperature *in vacuo*. Addition of dry $Et_2O$ to the residual syrup yielded an amorphous white solid, which was dissolved in some DMF and once again precipitated by addition of dry $Et_2O$ to give [5-Ile,8-(3-amino-4-phenyl)butyric acid]-angiotensin II. Physical constants are given in Table II; loss of $H_2O$ on drying at 100° 2.64%; amino acid ratio found: Asp, 0.97; Arg, 1.10; Val, 1.05; Tyr, 0.88; Ile, 1.01; His, 1.00; 3-amino-4-phenylbutyric acid, 0.95. *Anal.* ($C_{51}H_{78}N_{13}O_{12}\cdot CH_3COOH\cdot 2H_2O$) C, H, N.

Leucine aminopeptidase digestion showed a complete hydrolysis to component amino acids within 24 hr.

**Diethyl Phthalimidomethylbenzylmalonate.**—*N*-Chloromethylphthalimide (19.5 g, 100 mmol) in 50 ml of $Et_2O$ was added to a solution of diethyl benzylmalonate (30 g, 120 mmol) and Na (2.3 g, 100 g-atoms) in 150 ml of dry $Et_2O$. The mixture was heated for 2 hr with stirring on a water bath, cooled, and diluted ($H_2O$). The $Et_2O$ layer was dried and concentrated to a residue and this residue triturated with petr ether to give a crystalline solid: yield 30.6 g (76.4%), mp 97–98°; loss of $H_2O$ at 100°, 1.0%. *Anal.* $C_{23}H_{23}NO_6$) C, H, N.

**DL-3-Amino-3′-phenylisobutyric Acid·HCl.**—Phthalimido derivative described above (4.1 g, 10 mmol) and 50 ml of conc HCl were heated for 2 hr in a sealed tube at 170°, cooled, filtered, and evaporated *in vacuo* to yield 1.8 g of crystalline HCl salt. This was recrystallized from MeOH–ether to yield 1.5 g (70%); mp 154–155°. *Anal.* ($C_{11}H_{13}NO_2\cdot HCl$) C, H, N, Cl.

**DL-Butyloxycarbonylamino-3′-phenylisobutyric Acid Polymer.**—DL-3-Amino-3′-phenylisobutyric acid was converted into its Boc derivative by the usual procedure; yield: 64%; mp 106–108°; loss of $H_2O$ at 100° 1.4%. *Anal.* ($C_{15}H_{21}NO_4$) C, H, N.

DL-3-Butyloxycarbonylamino-3′-phenylisobutyric acid (10 mmol) and $Et_3N$ (1.41 ml, 10 mmol) in 20 ml of EtOH were stirred with 10 g of chloromethylated polystyrene–2% divinylbenzene at 75–80° for 20 hr. The polymer ester was filtered *in vacuo*. A sample was hydrolyzed as before in HCl–AcOH and the released amino acid estimated spectrophotometrically. This gave a value of 0.33 mmol of Boc-amino acid/g of polymer.

**Benzyloxycarbonyl-β-benzylaspartylnitroarginylvalyl-*O*-benzyltyrosylisoleucyl-$N^{im}$-benzylhistidylprolyl-3-amino-3′-phenylisobutyric Acid ([5-Ile,8-DL-(3-amino-3′-phenyl)isobutyric acid]-angiotensin II).**—The peptide was cleaved from the polymer by HBr–$CF_3CO_2H$ treatment and then hydrogenated in a mixture of MeOH–AcOH–$H_2O$ (75 ml, 10:2:3) at atmospheric pressure using Pd black. After 48 hr the reaction mixture was processed as for [pipecolic acid]-angiotensin II and the crude compound obtained was chromatographed on a column of Sephadex G-25 using BuOH–AcOH–$H_2O$ (4:1:5). Fractions of 10 ml each were collected. Fractions 87–115 were pooled, concentrated to 5 ml, filtered through Hyflosupercel, and evaporated to near dryness at room temperature *in vacuo*. Addition of dry $Et_2O$ precipitated an amorphous white compound. This was filtered and washed with dry $Et_2O$ to give product; physical constants are given in Table II; loss of $H_2O$ at 100° 2.74%; amino acid ratio found: Asp, 1.10; Arg, 1.02; Val, 1.15; Tyr, 0.96; Ile, 1.09; His, 0.92; Pro, 1.00; 3-amino-3′-phenylisobutyric acid, 1.12. *Anal.* ($C_{51}H_{78}N_{13}O_{12}\cdot CH_3COOH\cdot 2H_2O$) C, H, N.

**Boc-*O*-methyltyrosine Polymer.**—Boc-*O*-methyltyrosine was synthesized according to the procedure of Schwyzer, *et al.*,[26] and the purity was checked by paper and silica gel tlc in two solvent systems: (a) BAW, (b) BAPW. Boc-*O*-methyltyrosine polymer was prepared according to the method of Marshall and Merrifield[5] and analysis[25] of the substituted polymer gave 0.51 mmol of Boc-*O*-methyltyrosine/g of polymer.

**$N^{α}$-Carbonbenzoxy-β-benzylaspartylnitroarginyltyrosylisoleucyl-$N^{im}$-benzylhistidylprolyl-*O*-methyltyrosine Polymer.**—Boc-*O*-methyltyrosine polymer (5.0 g) was placed in the reaction vessel[5] and the desired sequence prepared as before. For imidazole, benzylhistidine, and nitroarginine cycles, 7.7 mmol of these two Boc-amino acids were used. At the end of synthesis, the protected peptide polymer was transferred into a 200-ml flask and dried in desiccator over $P_2O_5$ and paraffin *in vacuo*.

**Aspartylarginylvalyltyrosylisoleucylhistidylprolyl-*O*-methyltyrosine Monoacetate ([5-Ile,8-(OMe)Tyr]-angiotensin II).**—The protected octapeptide polymer (6.8 g) was suspended in 80 ml of anhydrous $F_3CCO_2H$ and HBr gas was bubbled slowly through the suspension with occasional shaking for 30 min at room temperature. The reaction mixture was filtered and the polymer was washed (3 times with 8 ml) with anhydrous $F_3CCO_2H$. The combined filtrate was evaporated to an oil at room temperature *in vacuo*. The peptide was precipitated by the addition of anhydrous $Et_2O$, removed by filtration, and washed (anhydrous $Et_2O$). The partially protected octapeptide (1.5 g) was dissolved in 80 ml of MeOH–AcOH–$H_2O$ (10:1:1) and hydrogenated for 48 hr (Pd black). Catalyst (1 g) was added at the beginning of the reduction and an additional 0.5 g was added after 24 hr. The catalyst was removed by filtration and washed (3 times with 10 ml) with the same solvent mixture. The combined filtrate was evaporated to dryness *in vacuo* at 20° and the residue was dissolved in minimum amount of 50% aqueous AcOH and precipitated with anhydrous $Et_2O$–$Me_2CO$ (1:1) to yield a crude product (1.35 g). This was purified by chromatography on a Sephadex G-25 (coarse) column (4.5 × 80 cm) using BAW as the developing solvent. Fractions of 10 ml were collected. From paper chromatography and uv absorption data, fractions 56–82, 83–120, and 190–217 were pooled and evaporated to dryness at 20° *in vacuo*. The pooled fraction 83–120 (major component) was precipitated from 50% AcOH with $Et_2O$–$Me_2CO$ (1:1) to yield 1.15 g (53%). A sample was reprecipitated from same solvent mixture and dried over $P_2O_5$, NaOH, and paraffin *in vacuo*. Physical constants are given in Table II and amino acid ratios on an acid hydrolysate: Asp, 0.92; Arg, 1.05; Val, 1.00; Tyr, 1.94; Ile, 1.00; His, 0.96; and Pro, 1.08; on an enzymatic hydrolysate: Asp, 0.93; Arg, 1.00; Val, 1.00; Tyr, 1.03; Ile, 0.94; His, 0.81; Pro, 0.78; (OMe)Tyr, 1.05. *Anal.* ($C_{51}H_{78}N_{13}O_{12}\cdot CH_3COOH$) C, H, N.

**Aspartylarginylvalyltyrosylvalylhistidylprolyl-*O*-methyltyrosine Monoacetate ([5-Val,8-(OMe)Tyr]-angiotensin II).**—This free octapeptide was prepared by the above procedure in 55% yield. Physical constants are given in Table II and amino acid ratios on an acid hydrolysate: Asp, 1.00; Arg, 1.05; Val, 2.08; Tyr, 1.98; His, 0.98; Pro, 1.00; on an enzymatic hydrolysate: Asp, 1.05; Arg, 1.00; Val, 1.95; Tyr, 1.00; His, 0.60; Pro, 0.63; (OMe)Tyr, 1.02. *Anal.* ($C_{51}H_{79}N_{13}O_{12}\cdot CH_3COOH\cdot H_2O$) C, H, N.

**Aspartylarginylvalyl-*O*-methyltyrosylisoleucylhistidylprolylphenylalanine Monoacetate ([4-(OMe)Tyr,5-Ile]-angiotensin II.**—The peptide was prepared and purified from carbobenzoxybenzylaspartylnitroarginylvalyl-*O*-methyltyrosylisoleucyl-$N^{im}$-benzylhistidylprolylphenylalanine polymer which was obtained as described above to yield 51% of free octapeptide; see

Table II; amino acid ratios on an acid hydrolysate: Asp, 0.96; Arg, 0.98; Val, 1.00; Tyr, 0.92; Ile, 0.95; His, 1.02; Pro, 1.05; Phe, 1.00; on an enzymatic hydrolysate: Asp, 1.00; Arg, 1.01; Val, 1.08; Ile, 0.98; His, 0.51; Pro, 0.58; (OMe)Tyr, 1.07; Phe, 1.00. *Anal.* ($C_{51}H_{73}N_{13}O_{12}\cdot CH_3COOH$) C, H, N.

**Aspartylarginylvalyl-*O*-methyltyrosylvalylhistidylprolylphenylalanine ([4-(OMe)Tyr,5-Val]-angiotensin II).**—The free octapeptide was prepared as described above to give 56% yield of the desired peptide; see Table II; amino acid ratios on an acid hydrolysate: Asp, 0.92; Arg, 1.06; Val, 2.13; Tyr, 0.98; His, 1.08; Pro, 1.00; Phe, 1.00; on an enzymatic hydrolysate, Asp, 1.00; Arg, 1.01; Val, 2.12; His, 0.58; Pro, 0.61; Phe, 1.00; (OMe)Tyr, 1.00. *Anal.* ($C_{50}H_{71}N_{13}O_{12}\cdot CH_3COOH$) C, H, N.

**Asparaginylarginylvalyl-*O*-methyltyrosylvalylhistidylprolylphenylalanine ([1-Asp($NH_2$),4-(OMe)Tyr,5-Val]-angiotensin II).**—Woodward's Reagent K[24] (0.633 g, 2.5 mmol) was dissolved in 25 ml of DMF with vigorous stirring. At 0°, 0.67 g (2.5 mmol) of carbobenzoxyasparagine and 0.35 ml (2.5 mmol) of $Et_3N$ dissolved in 25 ml of DMF were added. Stirring was continued until the soln cleared (about 3 hr). This was then added to a suspension of 2.5 g of heptapeptide polymer (nitroarginylvalyl-*O*-methyltyrosylvalyl-$N^{im}$-benzylhistidylprolylphenylalanine polymer) suspended in 25 ml of DMF containing 0.35 ml (2.5 mmol) of $Et_3N$. This heptapeptide polymer was prepared as described above. The reaction mixture was shaken in an ice bath for 2 hr, then at room temperature overnight. The resulting protected peptide polymer was collected by filtration, washed [DMF (3 times with 50 ml), absolute EtOH (3 times with 50 ml)], and finally dried over $P_2O_5$ *in vacuo* to yield 2.53 g.

The protected peptide polymer was suspended in 50 ml of anhydrous $F_3CCO_2H$ and a slow stream of HBr was passed through with occasional shaking for 30 min under anhydrous conditions. The suspension was filtered and the polymer was washed (3 times with 8 ml) with anhydrous $F_3CCO_2H$. The combined filtrate was concentrated *in vacuo* at 20° and peptide was precipitated by addition of anhydrous $Et_2O$. The solid was removed by filtration and washed with anhydrous $Et_2O$. This partially protected octapeptide was dissolved in MeOH–AcOH–$H_2O$ (10:1:1) and hydrogenated over Pd black for 48 hr. The peptide was isolated in the usual manner to yield 529 mg of solid. This was purified by chromatography on a Sephadex G-25 (coarse) column by elution with BAW solvent. The peptide emerged mainly as one fraction, was precipitated from 50% AcOH with $Et_2O$–$Me_2CO$ (1:1), and dried over $P_2O_5$, NaOH, and paraffin *in vacuo* to yield 490 mg (50% yield based on 0.84 mmol of N-terminal nitroarginyl heptapeptide with polymer); see Table II; ratios on an enzymatic hydrolysate: Asp($NH_2$), 0.95; Arg, 1.05; Val, 1.92; His, 0.51; Pro, 0.58; Phe, 1.00; (OMe)Tyr, 0.98. *Anal.* $C_{50}H_{72}N_{14}O_{11}\cdot 2CH_3COOH\cdot 2H_2O$ (1200.67): C, 53.97; H, 7.05; N, 16.33. Found: C, 53.42; H, 6.50; N, 16.09.

(24) (a) R. B. Woodward and R. A. Olofson, *J. Amer. Chem. Soc.*, **83**, 1007 (1961). (b) R. B. Woodward, R. A. Olofson and H. Mayer, *ibid.*, 1010 (1961).

# Analogs of Angiotensin II. II. Mechanism of Receptor Interaction[1]

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[5-Ile]-angiotensin II has two sites of action on guinea pig ileum. It directly interacts with receptor sites on smooth muscle, leading to contraction, and also indirectly contracts muscle by interacting with receptor sites on the parasympathetic innervation of the ileum, releasing acetylcholine. An attempt has been made to study these two receptors, using responsiveness of the tissue to analogs of angiotensin II. Substitutions in positions 1–7 showed close correlations between pressor activity, smooth muscle activity, and release of acetylcholine. Substitutions in position 8, however, indicated that [5-Ile,8-(OMe)Tyr]-angiotensin II is about three times more active on guinea pig ileum than on blood pressure, while [5-Ile,8-Tyr]-angiotensin II has roughly the same activity in both. The release of acetylcholine by both peptides was the same; the smooth muscle response to the latter peptide was the same as the parent compound, while response to the former peptide was less than half that of the parent compound. On the other hand [5-Ile,8-Ala]-angiotensin II produced no response on guinea pig ileum similar to its effect on blood pressure, but it inhibited subsequent response to the parent compound. It is assumed that the analog binds to receptor sites producing no excitation but preventing other analogs from interacting. These results have been interpreted by a speculative scheme concerning conformations of muscle and nerve receptors.

The octapeptide, angiotensin II, is known to have a multiplicity of actions.[2] Until recently, angiotensin II analogs were usually assayed either by their pressor responses in ganglion-blocked or nephrectomized rats, or by their musculotropic effects on isolated rat uteri. In general, biological activity in these assay systems was roughly equivalent and this led to an assumption that angiotensin acted only on the smooth muscle cells in these two assay systems, and that the receptor sites on these cells were, at least grossly, similar.

Recently, Peach, Bumpus, and Khairallah[3] reported that angiotensin II inhibited uptake of norepinephrine into sympathetic nerve endings in rabbit heart, at dose levels of less than 50 pg/ml. Angiotensin II analogs substituted in positions 1–7 showed a close correlation between pressor/musculotropic activity and inhibition of uptake. Substitutions in position 8, however, indicated that the benzene ring of phenylalanine was not needed for inhibition of uptake but was required for pressor activity. Substituting phenylalanine by aminophenylbutyric acid or aminophenylisobutyric acid also dissociates inhibition of uptake from the pressor response. The free C-terminal $CO_2H$ was required for both activities. This led to the conclusion that angiotensin receptor sites on sympathetic nerve endings were very similar to those on smooth muscle cells, except that the latter required an aromatic ring structure in position 8, while the former did not.

To study receptor sites on other tissues, guinea pig terminal ileum was used. Response of ileum to angiotensin is biphasic, a rapid component due to release of ACh from the intrinsic parasympathetic nerve ganglia of Meissner and Auerbach or postganglionic nerve end-

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(2) I. H. Page and J. W. McCubbin, Ed., "Renal Hypertension," Year Book Medical Publishers, Inc., Chicago, Ill., Chap. 5 and 9 (1968).

(3) M. J. Peach, F. M. Bumpus, and P. A. Khairallah, *J. Pharmacol. Exp. Ther.*, **167**, 291 (1969).