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# Angiotensin II Analogs. 10.[1a,†] Stereochemical Factors in the 5 Position Influencing Pressor Activity

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[1-Asparagine,5-threonine]angiotensin II and [1-asparagine,5-*O*-methylthreonine]angiotensin II were prepared by solid-phase synthesis as analogs with β-branched side chains significantly different in lipophilic character from the naturally occurring peptides, which have valine or isoleucine in the 5 position. In the rat pressor assay, compared on a molar basis with [1-asparagine,5-valine]angiotensin II, the 5-threonine analog was 10% as potent and the 5-*O*-methylthreonine analog was 118% as potent. The high degree of lipophilic character which valine or isoleucine imparts to the 5 position of angiotensin II is unnecessary for pressor activity.

The importance of the steric effects of the side chain in the 5 position of angiotensin II has been established through the replacement of the naturally occurring valine or isoleucine residues by a wide variety of aliphatic and alicyclic amino acids.[2-5] Analogs retaining the β-branching feature all exhibited high pressor activity, whereas those devoid of the steric restraint of β branching showed much lower activity even though their side chains approximated the hydrophobicities of the valine or isoleucine side chains. All of the β-branched analogs which have been reported possess a high degree of hydrophobicity and thus do not permit the assessment of a possible hydrophobic role for the side chains in addition to their steric role. Among the less active, non-β-branched analogs, there is a regular increase in pressor activity with increasing chain length which might be due to increased lipophilic character. Recently, conformations have been proposed for angiotensin II in which there is a hydrophobic role suggested for the 5-position side chain.[6] The present work represents an attempt to evaluate this possible hydrophobic role of the 5-position side chain by synthesizing analogs which retain the steric effect of β branching with side chains which are less hydrophobic. Threonine has been used as a hydrophilic analog of valine in which one of the terminal methyl groups has been replaced isosterically by a hydroxyl group. *O*-Methylthreonine has been used as a more hydrophilic analog of isoleucine in which the terminal ethyl group has been replaced by a methoxyl group.

**Chemistry.** [Asn[1],Thr[5]]angiotensin II was synthesized by three solid-phase routes differing in the choice of protecting groups for histidine and arginine. The first of these utilized Boc-His(Bzl) and Boc-Arg($NO_2$) as described previously.[4] The product of this synthesis required laborious purification to remove the D-His containing diastereomer[7] and other closely related impurities presumably arising during hydrogenolytic debenzylation. Without rechromatographing the inhomogeneous fractions, the yield was very poor. Since experiments had indicated that there was far less risk of racemization with protecting groups which suppress the basicity of the imidazole ring of histidine,[8] the synthesis was repeated using Boc-His(Dnp) in combination with Boc-Arg($NO_2$). The chief problems with this approach were the difficulty of removing all traces of thiol prior to hydrogenolysis of the nitro group from Arg($NO_2$) and side reactions during hydrogenolysis. The use of liquid HF for removal of the nitro group was not attempted since with the decision to use liquid HF, a more practical approach becomes possible in which all protecting groups are removed during cleavage of the peptide from the resin by HF. This third approach utilized Boc-His(Tos) in combination with Aoc-Arg(Tos). The use of the tosyl group rather than the nitro group for arginine protection was an arbitrary one; however, a recent report[9] has indicated the superiority of Arg(Tos) over Arg($NO_2$) when liquid HF is utilized. This third approach was most successful for the synthesis of [Asn[1],Thr[5]]angiotensin II and was used also for [Asn[1],Thr(Me)[5]]angiotensin II. However, a significant amount of a tyrosine-deficient peptide was observed which may have resulted from Friedel-Crafts type benzylation or tosylation of the tyrosine ring despite the presence of a large excess of anisole as a scavenger.

Purification of both analogs was achieved by chromatography on microcrystalline carboxymethylcellulose with an $NH_4Ac$ gradient. Fractions were cut without regard for yield in order to obtain the purest possible material, and no attempt was made to rechromatograph impure fractions although this could be done to increase the yield of pure product.

**Bioassay.** The compounds were tested for pressor activity in nephrectomized, pentolinium-treated male rats anesthetized with pentobarbital.[10,11] 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 calculated from amino acid analyses. The pressor activities listed in Table I are expressed on a molar basis. In a single responsive rat, three or four pressor responses exceeding 10 mm were obtained at each of two or more dose levels for test compounds and for angiotensin II controls. The Thr(Me)[5] analog was tested independently in three rats, yielding results of 117, 118, and 129% the activity of angiotensin II. Two of the preparations of [Asn[1],Thr[5]]angiotensin II (**2a,b**) were

Table I. Pressor Activities of Angiotensin II Analogs in the Rat

| Analog | Pressor activity[a] |
|---|---|
| [Asn[1],Thr[5]]angiotensin II (2a) | 8 |
| [Asn[1],Thr[5]]angiotensin II (2b) | 9 |
| [Asn[1],Thr[5]]angiotensin II (2c) | 11 |
| [Asn[1],Thr(Me)[5]]angiotensin II (3) | 118 |

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

† The abbreviations used to denote amino acids and peptides are those recommended.[1b] In addition, Aev stands for L-α-amino-β-ethylvaleric acid, Cpg stands for L-α-cyclopentylglycine, Chg stands for L-α-cyclohexylglycine, and Aoc stands for *tert*-amyloxycarbonyl.

Table II. Comparison of Side-Chain Lipophilicities with Pressor Activities of 5-Position Analogs of [Asn¹]angiotensin II

| 5-Position amino acid | Side-chain structure | Hansch $\pi$ value[a] | Pressor activity of peptide, % |
|---|---|---|---|
| Thr | $-CH(OH)CH_3$ | −0.29 | 10 |
| Thr(Me) | $-CH(OCH_3)CH_3$ | +0.40 | 118 |
| Val | $-CH(CH_3)_2$ | +1.37 | 100 |
| Ile | $-CH(CH_3)CH_2CH_3$ | +1.87 | 100 |
| *a*-Ile | $-CH(CH_2CH_3)CH_3$ | +1.87 | 100 |
| Cpg | $-CH(CH_2)_4$ | +2.14 | 89 |
| Aev | $-CH(CH_2CH_3)_2$ | +2.30 | 75 |
| Chg | $-CH(CH_2)_5$ | +2.51 | 114 |

[a] See ref 12 and 13.

compared in the same rat, while the third preparation (2c) was tested in a different rat.

**Structure-Activity Relationships.** Table II shows a comparison of the pressor activities of the analogs reported in this paper and those reported earlier[4] with the lipophilic character of the 5-position side chains as measured by Hansch $\pi$ values.[12,13] These data show that there is no simple correlation between pressor activity and the degree of lipophilicity of the 5-position side chain. A broad range of lipophilicity is tolerated; in fact, the most potent analogs are at opposite ends of the lipophilicity spectrum. If the 5-position side chain is involved in a productive hydrophobic interaction with other hydrophobic groups in the molecule and/or the receptor, the requirement for lipophilic character must be rather small, with greater lipophilicity having no further effect. Clearly, the high degree of hydrophobic character which valine or isoleucine imparts to the 5 position of angiotensin II is not essential for pressor activity. It is not clear if a certain amount of hydrophobic character is necessary for the 5 position. The reduced pressor activity of [Asn¹,Thr⁵]-angiotensin II suggests that excessive hydrophilic character in the 5 position might be unfavorable. However, this reduction in activity could be due to hydrogen bonding of the hydroxyl group with groups within the peptide resulting in an altered conformation or with groups in the receptor resulting in misalignment. Other hydrophilic analogs will have to be prepared and tested in order to distinguish between the two possibilities of a general hydrophilic effect *vs.* a specific detrimental effect of the hydroxyl group.

## Experimental Section

Acetone was reagent grade, dried over $Na_2SO_4$. Melting points (Thomas-Hoover Uni-Melt) are corrected. Amino acid analyses (Spinco Model 116 analyzer) were obtained using the standard 4-hr methodology. Peptides were hydrolyzed for 48 hr under $N_2$ in constant boiling HCl containing 1% PhOH as a scavenger and Ala as an internal standard. Peptide content was calculated for the diacetates. Microanalyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley, Calif. Where analyses are indicated only by the symbols of the elements, analytical results obtained were within ±0.4% of the theoretical values. Precoated silica gel G plates (E. Merck) were used for tlc with the following solvent systems: I, xylene–pyridine–AcOH (100:15:5); II, *n*-BuOH–AcOH–$H_2O$ (4:1:5); III, *sec*-BuOH–3% $NH_4OH$ (100:44). Electrophoresis was carried out on Whatman No. 1 paper at 5 kV using AcOH–HCOOH buffer, pH 1.85, in a Savant apparatus. Peptides were detected on paper and tlc with Pauly's reagent. Nmr was carried out on a Jeolco-JMN-4H-100 instrument.

Dicyclohexylammonium *N-tert*-Butyloxycarbonyl-*O*-methyl-L-threoninate (1). The procedure is based on that described for the benzyloxycarbonyl compound.[14] To a solution of 4.4 g (20 mmol) of *N-tert*-butyloxycarbonyl-L-threonine in 30 ml of $Me_2CO$ were added 30 ml of MeI and 30 g of $Ag_2O$. The suspension was stirred at 23° for 24 hr. Tlc showed no remaining *N-tert*-butyloxycarbonyl-L-threonine ($R_f^I$ 0.19) but a mixture of equal parts of methyl *N-tert*-butyloxycarbonyl-L-threoninate ($R_f^I$ 0.50) and methyl *N-tert*-butyloxycarbonyl-*O*-methyl-L-threoninate ($R_f^I$ 0.61). Methylation was repeated three more times until ether formation was 90%.‡ The reaction mixture was filtered, the $Ag_2O$ washed ($Me_2CO$), and the filtrate evaporated. The residual oil was dissolved in 80 ml of $Me_2CO$ and the solution stirred for 3 hr at 23° with 12 ml of 2 *N* NaOH. After removing the $Me_2CO$ (rotary evaporator, 30°), the solution was diluted with 70 ml of $H_2O$ and extracted with AcOEt (2 × 20 ml). The aqueous phase was acidified to pH 2 with saturated citric acid solution and extracted with AcOEt (3 × 50 ml). The AcOEt extracts were combined, washed with $H_2O$ (2 × 10 ml), dried ($MgSO_4$), and decolorized (Norite). After filtration and evaporation *in vacuo* there was 3.7 g. This was dissolved in 50 ml of $Et_2O$ and filtered, and 3.4 ml (20 mmol) of dicyclohexylamine was added. The salt (5.5 g) was obtained in two crops and recrystallized from *tert*-BuOH (15 ml). The crystals were collected by centrifugal filtration, washed with *tert*-BuOH (5 ml) and heptane (10 ml), and dried giving 4.0 g (48%), mp 160–161°. *Anal.* ($C_{22}H_{42}N_2O_5$) C, H, N.

**Asn-Arg-Val-Tyr-Thr-His-Pro-Phe (Method 1) (2a).** This synthesis from 0.3 mmol of Boc-His(Bzl)-Pro-Phe-polymer was carried out as described previously,[4] giving 196 mg. A 100-mg portion was purified by chromatography on Cellex-SE, Sephadex G-25, and again on Cellex-SE as described previously,[4] giving 7.5 mg (4%) of chromatographically homogeneous material, $R_f^{II}$ 0.09 and $R_f^{III}$ 0.11. An acid hydrolysate had Asp 1.00, Arg 1.09, Val 0.98, Tyr 0.99, Thr 0.90,§ His 0.90, Pro 1.12, and Phe 1.02. A 48-hr hydrolysate incubated with L-amino acid oxidase[7] had Asp 0.34, Arg 0.04, Val 0.02, Tyr 0.04, Thr 0.32, His 0.07, Pro 1.00, and Phe 0.04.

**Asn-Arg-Val-Tyr-Thr-His-Pro-Phe (Method 2) (2b).** Boc-Phe-polymer (15 g, 3 mmol) was deprotected and neutralized by shaking with the following sequence of solvents and reagents: (1) $CH_2Cl_2$, 3 × 75 ml, 2 min each; (2) 50% $CF_3COOH$–$CH_2Cl_2$, 75 ml, 10 min; (3) 50% $CF_3COOH$–$CH_2Cl_2$, 75 ml, 30 min; (4) $CH_2Cl_2$, 3 × 75 ml, 2 min each; (5) EtOH, 3 × 75 ml, 2 min each; (6) DMF, 3 × 75 ml, 2 min each; (7) 10% $Et_3N$–DMF, 75 ml, 10 min; (8) DMF, 3 × 75 ml, 2 min each; (9) $CH_2Cl_2$, 3 × 75 ml, 2 min each. The resulting Phe-polymer was shaken for 1 hr with a solution of 2.58 g (12 mmol) of Boc-Pro in 50 ml of $CH_2Cl_2$; then 2.47 g (12 mmol) of DDC was added and shaking continued for 24 hr. After washing with $CH_2Cl_2$ (3 × 75 ml) the polymer was shaken for 1 hr with 5 ml of $Ac_2O$ and 5 ml of $Et_3N$ in 75 ml of $CH_2Cl_2$ and then washed as follows: $CH_2Cl_2$, 2 × 75 ml, 2 min each; 50% EtOH–$CH_2Cl_2$, 75 ml, 10 min; EtOH, 2 × 75 ml, 2 min each.

The above reaction scheme was repeated using 3.8 g (9 mmol) of Boc-His(Dnp) which was allowed to equilibrate with the polymer for 10 min before adding 1.85 g (9 mmol) of DCC. Once His(Dnp) was incorporated, the polymer became dark brown during subsequent acetylation or neutralization steps. The polymer was washed with $CH_2Cl_2$ (6 × 75 ml, 2 min each) and dried *in vacuo* at 23°.

An aliquot of the Boc-tripeptide-polymer equivalent to 0.3 mmol of tripeptide was converted to Arg($NO_2$)-Val-Tyr(Bzl)-Thr(Bzl)-His(Dnp)-Pro-Phe-polymer trifluoroacetate by chain extension using 0.9-mmol portions of the appropriate Boc-amino acids and DCC. The procedure described above was followed using volumes one-tenth as large. Boc-Arg($NO_2$) was coupled in a mixture of 0.5 ml of DMF and 6.5 ml of $CH_2Cl_2$. This coupling was repeated since electrophoresis of the peptide obtained by cleaving a small aliquot of the heptapeptide-polymer showed incomplete reaction at this stage. Acetylation was omitted after Arg($NO_2$) had been incorporated. After neutralization, the heptapeptide-polymer was shaken for 4 days with 700 mg (1.8 mmol) of Z-Asn-ONp in 7 ml of purified DMF.[16] The polymer was washed with DMF (3 × 10 ml), $CH_2Cl_2$ (6 × 10 ml), 50% $CF_3COOH$–$CH_2Cl_2$ (2 × 10 ml), and $CF_3COOH$ (10 ml). The polymer was suspended in 10 ml of $CF_3COOH$ + 1 ml of anisole and HBr was bubbled through the suspension for 2 hr. The reaction mixture was filtered and the polymer was washed with $CF_3COOH$ (4 × 10 ml). The filtrates were combined and evaporated to an oil *in vacuo* at 23° and this oil was lyophilized from AcOH giving 400 mg of yellow powder.

A 220-mg portion of this crude, partially protected peptide was dissolved in 3 ml of DMF. The solution was made alkaline with 0.1 ml of concentrated $NH_4OH$; then 1 ml of $HSCH_2CH_2OH$ was added. After 24 hr, the peptide was precipitated with $Et_2O$ and then reprecipitated twice from DMF solution with $C_6H_6$. After drying, there was 170 mg of beige powder. Attempts to remove the $NO_2$ group

‡ In an earlier small-scale experiment using a different lot of $Ag_2O$ from the same manufacturer (MCB), etherification was accomplished much more readily. $Ag_2O$ freshly prepared by the method of Helferich and Klein[15] did not improve the yields.

§ Uncorrected for hydrolytic losses.

from this product by catalytic hydrogenation failed, presumably because some sulfur-containing contaminants remained. The crude material was subjected to CCD in 1-BuOH–AcOH–$H_2O$ (4:1:5) through 20 transfers. Tubes 1–10 gave 120 mg of pale yellow powder on lyophilization. This product in 95% AcOH was hydrogenated over 100 mg of 10% Pd/C at 3 atm of $H_2$ during 20 hr, giving 110 mg.

A solution of 110 mg of the crude peptide in 5 ml of 0.01 *M* $NH_4Ac$ was applied to a 2.5 × 94 cm column of carboxymethylcellulose (Whatman CM-52, $NH_4^+$) packed in the same buffer. The column was eluted at 45 ml/hr with an $NH_4Ac$ gradient ($dC/dV = 10^{-4}$ *M*/ml from 0.01 *M*). The effluent was monitored at 280 mμ and chromatographically homogeneous fractions from the main peak were pooled and lyophilized giving 29 mg (15%) of white powder, $R_f^{II}$ 0.10 and $R_f^{III}$ 0.14. An acid hydrolysate had Asp 0.99, Arg 0.96, Val 1.01, Tyr 0.99, Thr 0.90,§ His 0.96, Pro 1.01, Phe 1.01, and peptide content 86%. An L-amino acid oxidase digest of the acid hydrolysate[7] had Asp 0.48, Arg 0.05, Val 0.01, Tyr 0.06, Thr 0.53, His 0.08, Pro 1.00, and Phe 0.05.#

In addition to the desired peptide, there was present in a later peak 11 mg of a peptide containing an unknown amino acid in place of tyrosine. The elution volume of this amino acid on the amino acid analyzer did not correspond to any of the common amino acids; it was close to, but not identical with, that of hexahydrophenylalanine.

**Asn-Arg-Val-Tyr-Thr-His-Pro-Phe (Method 3) (2c).** Using the procedure outlined in **2b**, 0.3 mmol of Boc-Phe-polymer was acylated successively with 1.2-mmol portions of Boc-Pro, Boc-His(Tos), Boc-Thr(Bzl), Boc-Tyr(Bzl), Boc-Val, and Aoc-Arg(Tos) using DCC as the condensing reagent. The heptapeptide-polymer was acylated with Z-Asn-ONp as described for **2b** and, after washing with $CH_2Cl_2$ (6 × 10 ml), the polymer was dried *in vacuo*.

Half of the protected octapeptide-polymer was treated with 10 ml of liquid HF at 0° for 1 hr in the presence of 1 ml of anisole.[17] After removing the HF under high vacuum, the polymer was extracted with 95% AcOH and the estracts were lyophilized giving 260 mg of white powder.

A solution of 130 mg of the crude peptide in 10 ml of 0.03 *M* $NH_4Ac$ was chromatographed on a 2.5 × 93 cm column of CM-52 (Whatman) packed in the same buffer. The column was eluted at 48 ml/hr with an $NH_4Ac$ gradient ($dC/dV = 10^{-4}$ *M*/ml from 0.03 *M*). The effluent was monitored at 280 mμ and the chromatographically homogeneous fractions from the main peak were pooled and lyophilized giving 41 mg (47%) of white powder, $R_f^{II}$ 0.08 and $R_f^{III}$ 0.12. An acid hydrolysate had Asp 1.01, Arg 1.00, Val 1.00, Tyr 1.00, Thr 0.91,§ His 0.98, Pro 1.01, Phe 1.01, and peptide content 91%. An L-amino acid oxidase digest[7] of the acid hydrolysate had Asp 0.88, Arg 0.04, Val 0.02, Tyr 0.05, Thr 0.74, His 0.05, Pro 1.00, and Phe 0.04.# An aminopeptidase-M digest[18] had Asn 1.0,** Arg 1.00, Val 0.98, Try 1.00, Thr 1.0,** and peptide content 91%. Digestion was incomplete beyond Thr because of the resistance of the His-Pro bond to aminopeptidase-M.[19,20]

In addition to the desired peptide, there was present in a later peak 15 mg of a tyrosine-deficient peptide. This peptide had the same electrophoretic mobility at pH 1.85 as the desired octapeptide indicating a similar molecular weight but migrated more rapidly on tlc, $R_f^{II}$ 0.16 and $R_f^{III}$ 0.17.

**Asn-Arg-Val-Tyr-Thr(Me)-His-Pro-Phe (3).** This peptide was synthesized and purified as described for 2c, starting with 0.2 mmol of Boc-Phe-polymer, using Boc-Thr(Me) in place of Boc-Thr. After drying, the octapeptide was cleaved from the polymer with HF as before giving 250 mg.

A solution of 100 mg of the crude peptide in 5 ml of 0.03 *M* $NH_4Ac$ was chromatographed as described for 2c. Chromatographically homogeneous fractions were pooled and lyophilized giving 24 mg (24%) of white powder, $R_f^{II}$ 0.07 and $R_f^{III}$ 0.14. An acid hydrolysate had Asp 1.03, Arg 1.08, Val 1.00, Tyr 1.00, Thr + Thr(Me) 0.90,§ His 0.99, Pro 1.01, Phe 1.00, and peptide content 94%. An L-amino acid oxidase digest of the acid hydrolysate had Asp 0.85, Arg 0.08, Val 0.02, Tyr 0.06, Thr + Thr(Me) 0.73, His 0.10, Pro 1.00, and Phe 0.06.# An aminopeptidase-M digest[18] had Asn 1.0,** Arg 1.00, Val 0.97, Tyr 1.00, Thr(Me) 1.0,** and peptide content 92%.

Thr(Me) coeluted with Thr on the amino acid analyzer and gave approximately the same color yield with ninhydrin. Under the conditions employed for acid hydrolysis of the peptides, ~80% conversion of Thr(Me) to Thr occurs as judged from tlc; thus the amino acid remaining after L-amino acid oxidase digestion is presumably Thr, a poor substrate for the enzyme, rather than Thr(Me) which is a good substrate. The 100-MHz nmr spectrum of this peptide in $D_2O$ showed a strong singlet at 3.25 ppm (OMe) which was absent from the spectrum of **2**.

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#A mixture of amino acids subjected to the same hydrolytic and enzymatic procedures had Arg 0.04, Val 0.02, Tyr 0.04, His 0.08, Pro 1.00, and Phe 0.04. Asp and Thr are attacked too slowly by L-amino oxidase to be checked by this method.

**Overlap of Asn and Thr or Thr(Me) prevented a more accurate determination of these values.