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# Angiotensin II. Synthesis and Biological Activity of 6-(1-Methylhistidine) and 6-(3-Methylhistidine) Analogs

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The synthesis and purification of [1-MeHis$^6$]angiotensin II and [3-MeHis$^6$]angiotensin II are described. The [3-MeHis$^6$] analog was about 5% as active as angiotensin II in contracting isolated smooth muscle preparations, as a vasopressor agent, and in releasing prostaglandin from isolated perfused rabbit kidneys. [1-MeHis$^6$]angiotensin II had little or no biological activity. Neither of the analogs had any antagonistic activity. The effect of pH on the uterine sensitivity to histidine analogs and to *N*-acetyl angiotensin II indicates that neither the state of protonation of the α-amino or imidazole groups nor a conformation charge associated with titration of these groups accounts for the increased sensitivity of the uterus to angiotensin at alkaline pH.

The histidine moiety of angiotensin II (AII) has been implicated in the pH-dependent sensitivity of uterine strips to angiotensin analogs[1-3] and to the development of *in vitro* tachyphylaxis.[2] Substitution of the imidazole ring of histidine by a methyl group has been reported for the hypothalamic thyrotropin releasing factor[4] (pGlu-His-Pro-$NH_2$, TRF) and the hypothalamic luteinizing hormone releasing factor[5] (pGlu-His-Try-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-$NH_2$, LRF). [3-MeHis$^2$]-TRF was found to have 800% of the biological activity of TRF whereas its isomer [1-MeHis$^2$]-TRF is essentially inactive. [1-MeHis$^2$]-LRF and [3-MeHis$^2$]-LRF were found to have 3 and 6%, respectively, of LRF biological activity. Thus, the role of nitrogen 1 and nitrogen 3 of the imidazole ring of histidine in TRF and LRF seems to be different. This is substantiated by the fact that substitution by Gly or elimination of His$^2$-residue in LRF yielded two analogs exhibiting definitive inhibitory biological properties.[6] In order to examine further the role of histidine in biologically active peptides, we have synthesized [1-MeHis$^6$]- and [3-MeHis$^6$]angiotensin II and report here their biological properties.

## Results†

The two octapeptides [1-MeHis$^6$]-AII and [3-MeHis$^6$]-AII were synthesized by the solid-phase methodology; these two analogs were evaluated for their biological activity.

**Oxytocic Effects of Angiotensin Analogs.** In isolated rat uterine strips, at pH 6.8, [3-MeHis$^6$]-AII was about 10% as active as AII, whereas [1-MeHis$^6$]-AII had only low levels of biological activity (0.1%) (Table I). Incubation of uterine strips with 2 μg/ml of [1-MeHis$^6$]-AII did not alter the AII oxytocic response. Under more alkaline conditions (pH 8.1) the AII and [3-MeHis$^6$]-AII dose-response curves were shifted to the left indicating a twofold increase in sensitivity. There was no change in [1-MeHis$^6$]-AII response at pH 8.1.

**Pressor Effects.** [3-MeHis$^6$]-AII was about 5% as active as AII in elevating rat blood pressure and the [1-MeHis$^6$]-AII was only 0.05% as active (Table II). Thus, the relative activity in the intact animal and uterine strips was about the same. Neither MeHis analog inhibited AII pressor responses nor lowered renal hypertension in rats (not shown).

**Prostaglandin Release.** For release of a half-maximal amount of prostaglandin-like substance (PLS) and for a half-maximal contraction of a rat stomach strip, a dose of 5-10 μg of [3-MeHis$^6$]-AII was required (1.5-3% of AII), whereas the [1-MeHis] analog was inactive in both systems (Table III). The [3-MeHis] analog was somewhat more effective in releasing PLS from the kidney than in directly contracting rat stomach strips. Infusion of [1-MeHis$^6$]-AII did not inhibit the AII induced release of PLS.

Table I. Comparison of the Spasmogenic Action of Angiotensin Analogs on Isolated Rat Uterine Strips at pH 6.8 and 8.1[a]

| | Amt of analog needed to produce a half-maximal uterine contraction, ng/ml | |
|---|---|---|
| Peptide analogs | pH 6.8 | pH 8.1 |
| AII | 25 | 7 |
| *N*-Acetyl-AII | 25 | 8 |
| [3-MeHis$^6$]-AII | 100 | 48 |
| [1-MeHis$^6$]-AII | 10,000 | 10,000 |

[a] The maximal response achieved with the angiotensin analogs was 2.5 g of uterine tension. The values represent the means determined on strips isolated from five animals. The standard errors were 15% or less. Each strip served as its own control, so that AII dose-response curves were tested before and after the MeHis$^6$ analogs. The oxytocic effects of the analogs were completely reversible. None of the analogs possessed AII inhibitory activity at either pH.

Table II. Pressor Response of Rats to AII Analogs[a]

| Peptide analogs | $\Delta_{25}$, μg/kg |
|---|---|
| AII | 0.1 (8) |
| [3-MeHis$^6$]-AII | 2.0 (4) |
| [1-MeHis$^6$]-AII | 200 (4) |

[a] $\Delta_{25}$ indicates the dosage of analog required to produce a 25% increase in mean blood pressure. The $\Delta_{25}$ was obtained from the graphic plot of the dose-response curves for each analog. These curves were parallel to the pressor curve for angiotensin II. The standard errors were 10% or less. The number in parentheses indicates the number of animals tested.

† The following abbreviations are utilized in this manuscript: PLS, prostaglandin-like substance; $P_4T_8$, [Phe$^4$,Tyr$^8$]angiotensin II; and AII, angiotensin II.

Table III. Comparison of Angiotensin Analogs as Direct Smooth Muscle Spasmogenics or as Inducers of Prostaglandin-Like Substance (PLS) Released from the Perfused Rabbit[a]

| Peptide analogs | Amt of analog needed to produce a half-maximal contraction | |
|---|---|---|
| | Direct assay on RSS, μg | Through kidney assayed on CR, μg |
| [Asn$^1$,Val$^5$]-AII | 0.15 | 0.15 |
| [*N*-Acetyl-Asn$^1$,Val$^5$]-AII | 0.15 | 0.15 |
| [Asp$^1$,Val$^5$]-AI | 15 | 1.5 |
| [3-MeHis$^6$]-AII | 10 | 5 |
| [1-MeHis$^6$]-AII | No response at 100 | No response at 100 |
| [Des-Phe$^8$]-AII | 150 | 150 |

[a]The ability of angiotensin analogs to directly contract smooth muscle was measured on rat stomach strips (RSS), whereas chick rectum strips (CR), which do not respond to the direct application of angiotensin, do contract with PLS released from the perfused rabbit kidney treated with angiotensin. Contractions were measured with an F-50 myograph (physiograph) with the gain of the amplifier set so a half-maximal response was a 2-cm contraction. At this gain 10 ng of $PGE_1$ or $PGE_2$ standards caused a 3-cm contraction. Each angiotensin analog was tested in at least four separate perfused kidney preparations. The same maximal response was achieved with all the AII analogs except the [1-MeHis$^6$]-AII which was inactive at the highest dose tested.

## Discussion

The effects of angiotensin analogs on uterus strip are partially dependent on the condition of the experiment. In alkaline de Jalon's solution, uterine strips possess some spontaneous contractility and are more sensitive to AII.[1] The angiotensin antagonists [Phe$^4$,Tyr$^8$]-AII, [*p*-fluoro-Phe$^4$]-AII, and [Ile$^8$]-AI were antagonists at pH 6.8 and agonists at pH 8.6. The antagonists [Ile$^8$]-AII and [Cys$^8$]-AII were about ten times less potent under alkaline condition.[3]

It was previously assumed that the imidazole of His$^6$ was the only amino acid undergoing significant changes in ionization at this pH. There was also no evidence reported for a conformational change in angiotensin II in this pH range. Recently, Glickson, *et al.*,[7] have shown that not only the imidazole group (pH 6.26 ± 0.04) but also the α-amino group (pH 6.98 ± 0.04) undergo significant changes in ionization in this range. In addition, there are definite indications of a conformational transition seen by proton nmr with a pH of 6.6 ± 0.2 which would imply involvement of either or both the α-amino and imidazole groups. More recent studies with $^{19}F$ nmr by Vine, *et al.*,[8] have shown a similar conformation change in [*p*-fluoro-Phe$^8$]-AII.

The involvement of the α-amino group was examined by the preparation of acetyl AII which has been previously described.[9] The activities of this derivative paralleled that of AII in its biological activity and showed the increase in activity with the increase in pH (not shown). This implies that the AII derivative in which the α-amino group is unprotonated is not the more active species.

The AII histidine at pH 6.8 is partially protonated but it would be almost entirely unprotonated at pH 8.6 and the latter form might possibly be the biologically more active form. Substitution of (3-pyrazolyl)alanine (p*K* = 2.3) for the histidine (imidazole p*K* = 6.7) in AII, however, produces an analog which changes its degree of protonation very little when added to strips at pH 8.6 (instead of at pH 6.8). There was, however, still an increase in sensitivity of the uterus to this analog upon changing to the more alkaline conditions.[1] The [3-MeHis$^6$]-AII had a similar increase in sensitivity under alkaline conditions but, interestingly, the [1-MeHis$^6$]-AII was unaffected by the change in pH. This does not reflect the more basic character of the 1-MeHis (p*K* = 6.56) compared with His (p*K* = 6.0) as the 3-MeHis has a similar p*K* (6.48).[4] The extremely low level of activity of [1-MeHis$^6$]-AII makes it difficult to place much weight on observations with this analog. These results indicate that the state of the ionization of the imidazole group does not account for changes seen in the sensitivity of the uterus to angiotensin as a function of pH.

Stewart and Freer[2] have observed that tachyphylaxis to AII developed rapidly in uterine strips at pH 6.8 but there was no tachyphylaxis at pH 7.4 or 8.9. Tachyphylaxis was also obtained with [Ile$^5$,Orn$^6$]-AII and [Ile$^5$,Arg$^6$]-AII but not with [Val$^5$,pyr(3)-Ala$^6$]-AII. Their results indicate that a positive charge on the side chain at position 6 is required for tachyphylaxis. It was noted that substitution of (3-pyrazolyl)alanine for histidine in $P_4T_8$ abolished all of the inhibitory activity even at pH 6.8 (not shown). It appears that there is similarity between conditions (*i.e.*, low pH) necessary to produce tachyphylaxis with several AII analogs and inhibition with [Phe$^4$,Tyr$^8$]-AII. The relationship between tachyphylaxis and inhibition is not so clear, however, since the [Orn$^6$] and [Arg$^6$] analogs apparently do not inhibit AII even at pH 6.8 (R. Freer, personal communication). There may also be a strict steric requirement for inhibition as there is for the oxytocic effect.

The results obtained with [3-MeHis$^6$]-AII confirm the results seen with [(3-pyrazolyl)alanine$^6$]-AII.[1] Together they imply that the state of ionization of the imidazole group of [His$^6$] does not effect the interaction of angiotensin with its receptor. Similarly, the increase in sensitivity seen with acetyl-AII (in uterine strips in alkaline media, analogous to the enhanced AII potency) also implies a lack of participation of the α-amino group in the pH dependence. The absence of participation of either groups (histidine$^6$ or the α-amino group) makes it highly unlikely that the solution conformational change associated with the titration of either/or both groups is involved. This leaves a change in the receptor itself or a change in some subsequent process in the stimulus-response coupling as the mechanism for increased sensitivity to AII in the uterus.

## Experimental Section

Homogeneity of the peptides was demonstrated by thin-layer chromatography on Eastman chromatogram sheets (6061 silica gel with fluorescent indicator) in seven solvent systems: (1) 1-butanol-pyridine-0.1 *N* acetic acid, 5:3:11 (upper phase); (2) ethyl acetate-pyridine-acetic acid-water, 5:5:1:3; (3) isoamyl alcohol-pyridine-water, 7:7:6; (4) 2-propanol-1 *N* ammonia, 2:1; (5) 2-propanol-1 *N* acetic acid, 2:1; (6) butanol-acetic acid-water, 4:1:5 (upper phase); (7) butanol-2-propanol-ethyl acetate-1 *N* ammonia, 1:1:2.5:1. Uv, $I_2$, ninhydrin spray, and Pauly reagent were successively used.

Amino acid analyses were performed on peptide hydrolysates (6 *N* HCl + 0.5% thioglycolic acid at 100° in evacuated sealed tubes for 48 hr) using a Beckman/Spinco Model 119 amino acid analyzer. Peak areas were determined by an Infotronics Model CRS-100A electronic integrator. The starting materials‡ were bought from BACHEM and were found to be homogeneous and optically pure. Nmr spectra were obtained with a Jeolco HNM-PS-100 spectrometer. $D_2O$ was used as solvent. Optical rotations were measured on a Perkin-Elmer Model 141 polarimeter.

**Syntheses.** Polystyrene cross-linked with 1% divinylbenzene resin (Bio-Rad SX-1) was chloromethylated according to standard procedures;[10] 0.8 mequiv of Cl/g of resin was obtained. Esterification was performed as described by Stewart and Young;[10] 0.15 mequiv of Boc-Phe/g of resin was obtained. Deblocking was achieved in 20 min by TFA–$CH_2Cl_2$ (50:50) followed by neutralization by 15% $Et_3N$ in DMF. Successive coupling of each amino acid (3 molar excess) was mediated by dicyclohexylcarbodiimide (3 molar excess) for 30 min.

‡Starting protected amino acids: α-Boc-L-Asp-β-benzyl ester; α-Boc-L-nitro-Arg; α-Boc-L-Val; α-Boc-*O*-benzyl-L-Tyr; α-Boc-L-Ile; α-Boc-L-1-MeHis; α-Boc-L-3-MeHis; α-Boc-L-Pro; α-Boc-L-Phe.

The reaction was controlled by the ninhydrin test of Kaiser, *et al.*[11] Ile was the only residue that had to be recoupled under these conditions. Washes included DMF, dioxane, $CH_2Cl_2$, and MeOH. A 1-hr HF treatment at 0° in the presence of anisole deblocked and cleaved the peptide resin. HF was thoroughly eliminated under high vacuum. The crude peptides mixed with the resin were extracted with 0.2 *N* AcOH that was lyophylized. Purification was achieved in one step by partition chromatography (system 6) (100 mg of crude mixture) on a Sephadex G25 column (1.2 × 100 cm). Cuts emphasizing purity more than yield were made on the basis of a ninhydrin test.

**Asp-Arg-Val-Tyr-Ile-1-MeHis-Pro-Phe-OH.** Amino acid analysis gave Asp, 1.00; Arg, 1.05; Val, 1.00; Tyr, 1.00; Ile, 0.90; 1-MeHis, 1.00; Pro, 0.95; Phe, 1.10. $R_f$ systems: 0.34, 1; 0.56, 2; 0.32, 3; 0.71, 4; 0.40, 5; 0.39, 6; 0.26, 7. $[\alpha]^{25}D$ −55° (*c* 0.40, 1% AcOH).

**Asp-Arg-Val-Tyr-Ile-3-MeHis-Pro-Phe-OH.** Amino acid analysis gave Asp, 1.00; Arg, 1.05; Val, 0.95; Tyr, 1.00; Ile, 1.00; 3-MeHis, 1.10; Pro, 0.95; Phe, 1.20. $R_f$ systems: 0.35, 1; 0.57, 2; 0.33, 3; 0.67, 4; 0.38, 5; 0.40, 6; 0.27, 7. $[\alpha]^{25}D$ −54° (*c* 0.66, 1% AcOH).

Nmr showed for both compounds sharp absorptions for im-2-H and im-4-H; sharp AB system for the four aromatic protons of tyrosine and one singlet for the five protons of phenylalanine; $\alpha$-$CH_2$-Gly and the $CH_3$ groups of Ileu were also easily recognizable. All protons integrated properly. Acetyl-Asn-Arg-Val-Tyr-Val-His-Pro-Phe-OH was prepared from [Asn[1],Val[5]]-AII (Hypertensin, Ciba, the generous gift of Dr. B. Riniker) by acetylation according to Riordan and Vallee.[12] The material was purified by preparative thin-layer chromatography (Analtech) in system 6.

**Methods.** Isolated uterine strips were prepared as previously described.[3] The tissue was then suspended in a 5-ml tissue bath at room temperature in de Jalon's solution[13] (calcium 0.5 m*M*) and aerated with either 95% $O_2$–5% $CO_2$ (pH 6.8) or with 100% $O_2$ (pH 8.1).

**Blood Pressure Determinations.** Blood pressure was measured in albino rats (150–200 g) anesthetized with sodium pentobartibal (30 mg/kg iv) and treated with phenoxybenzamine (30 mg/kg) and propranolol (15 mg/kg) by recording from the carotid artery (P-1000 A, linear-core physiograph transducer). The agent (50 μl) was injected into the cannulated jugular vein. An AII dose–response curve was determined before and after the administration of each analog.

**Prostaglandin Release.** The release of prostaglandin-like substance (PLS) was measured from isolated rabbit kidney perfused with Krebs solution at 37° and at a constant flow of 8 ml/min. The venous effluent was superfused over a series of isolated assay tissues chosen for their sensitivity to prostaglandins (chick rectum and rat stomach strip) and to AII (rat stomach strip) as previously described.[14]

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# Diamidino-α,ω-diphenoxyalkanes. Structure–Activity Relationships for the Inhibition of Thrombin, Pancreatic Kallikrein,† and Trypsin

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A series of diamidino-α,ω-diphenoxyalkanes was synthesized and examined for inhibitory activity against thrombin, pancreatic kallikrein, and trypsin. Modification of the compounds included lengthening of the alkane chain, variation in the position of the amidino groups, and the substitution of halogen on the benzene rings. The compounds function as active-site-directed reversible inhibitors, and each molecule appears to bind to only a single enzyme molecule. In an amidase assay at pH 8.1 and 37°, bovine thrombin was most effectively inhibited by 2′,2″-diiodo-4′,4″-diamidino-1,8-diphenoxyoctane ($K_i$ = 1.1 × $10^{-6}$ *M*). Under the same conditions, 2′,2″-diiodo-4′,4″-diamidino-1,5-diphenoxypentane proved to be the most active inhibitor of pancreatic kallikrein and trypsin with $K_i$ values of 4.5 × $10^{-7}$ and 3.4 × $10^{-7}$ *M*, respectively. The latter compound also was most effective in blocking the clotting activity of human thrombin.

In body tissues and body fluids proteases play a causative or adjunctive role in a number of important disease processes, such as inflammation, thrombosis, and complement-dependent immune reactions. Control of the hyperproteolytic activity offers itself as an obvious approach to alleviation of the abnormal conditions, and efforts in this direction have been made especially in the prevention and treatment of thromboembolic disorders which are among the leading causes of morbidity and mortality in man. To reduce the clotting tendency two methods have been

† Kallikrein is a registered trademark assigned to Farbenfabriken Bayer AG, Leverkusen, Federal Republic of Germany.

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