# THE INFLUENCE OF CERTAIN SUBSTITUTIONS ON 1 AND 2 POSITIONS OF ANGIOTENSIN II ON BIOLOGICAL RESPONSES*

Paul CORDOPATIS[a], Urania BELTÉ[a], Dimitrios THEODOROPOULOS[a], Roger BOSSÉ[b] and Emanuel ESCHER[b]

[a] *Department of Chemistry, University of Patras, Patras 26200, Greece and*
[b] *Department of Pharmacology, University of Sherbrooke, Sherbrooke, Quebec J1H, 5N4, Canada*

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*Dedicated to the memory of Dr Karel Bláha.*

Position 1 modified analogues of angiotensin II, as well as two analogues with additional elimination of Arg in position 2, were prepared by means of solid phase peptide synthesis. These compounds, namely [Ser$^1$]AT, [DOPA$^1$]AT, [Cys$^1$]AT, [Cys(Acm)$^1$]AT, [N-MeAib$^1$]AT, [Aib$^1$,Aib$^2$]AT and [GABA$^1$, GABA$^2$]AT, were tested in the classical smooth-muscle assay (rabbit aorta strip) and, for binding potency, on bovine adrenal membranes and possessed a $pD_2$ value of 7.23, 6.79, 6.43, 5.80, 6.43 and $IC_{50}$ ($10^{-10}$ mol $l^{-1}$) value of 31.6, 23.2, nd, nd, 170.0, respectively. The last two analogues were found to be totally inactive.

Angiotensin II [AT, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe]** is one of the best known peptide hormones[2,3]. Its functions are mainly blood pressure control and are expressed at several sites, to mention only the most important: contraction of blood vessels, stimulation of aldosterone secretion from adrenals, actions on central nervous system stimulating thirst perception and central mediated blood pressure increase. Many studies have shown the importance of the individual amino acid residues using structure–activity relationships.

Position 1 of angiotensin II seems only to govern the affinity and has been designed as not essential in the past[4]. This position is, however, an ideal anchoring function in this very important hormone and the most active analogues are bearing N-methylglycine (sarcosin) in this position[5,6]. This has been explained by increased intrinsic activity or affinity for the receptor site,

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** Abbreviations are according to the IUPAC–IUB Commission for biochemical nomenclature[1]; additional abbreviations are explained in the text. All optically active amino acids are of the L-configuration.

modified conformation with subsequent biological activity alteration, as well as by decreased rate of degradation due to plasma aminopeptidases. Side chain modifications of the 1-aspartyl residue with bulky alkyl groups gave analogues with full intrinsic activity but lower potency[7,8] which is attributed to a possible steric interference with essential amino acid residues, like 3-Val and 4-Tyr. Interesting enough, replacement of 1-Asp with α-aminoisobutyric acid (Aib) resulted in the analogue with higher potency than Ciba-Hypertensin but with about 60% relative activity in comparison to [Asp[1]]AT (ref.[9]). It must be noted that in the case of angiotensin II antagonists, N-methylation in position 1, as in [N-MeAsn[1], Ile[8]]AT, led to an analogue which was equipotent to [Sar[1], Ile[8]]AT (ref.[10]).

This study was undertaken in order to gain further knowledge about the functions of position 1 and, more generally, of the N-terminus part of AT. For these reasons, a series of angiotensin II analogues bearing small alterations on this N-terminal amino acid was synthesized by conventional solid phase methodology[11]. The series includes [N-MeAib[1]]AT, [DOPA[1]]AT, [Ser[1]]AT, [Cys[1]]AT and [Cys(Acm)[1]]AT. Two analogues like [Aib[1], Aib[2]]AT and [GABA[1], GABA[2]]AT (GABA: γ-aminobutyric acid) with additional elimination of Arg (the position 2 amino acid) were also prepared.

## EXPERIMENTAL

$N^{\alpha}$-Butyloxycarbonyl-protected amino acids and peptide reagents were obtained from Bachem AG, Switzerland, and Protein Research Foundation, Japan or prepared in our laboratory. All solvents and reagents used for solid-phase synthesis were of analytical quality and were redistilled before use. The protocol used for the solid-phase synthesis[11] was the same as previously described[9,12]. N-MeAib, DCC and HOBt (4 molar excess, 1:1:1) in $CH_2Cl_2$–DMF (4:1) were shaken with the resin overnight until a negative ninhydrin test was obtained. For the incorporation of L-DOPA, coupling was performed with 3 equivalents of Boc-L-DOPA, DCC and HOBt for 5 h. The following side-chain protected amino acid derivatives were used: Boc-His(Tos), Boc-Tyr(2-BrZ), Boc-Arg($NO_2$), Boc-Cys(Acm), Boc-Ser(Bzl). Boc-L-DOPA was prepared according to Banerjee and Ressler[15] from L-DOPA-OMe.HCl which was converted to Boc-L-DOPA-OMe by the method of Nagasawa et al[16]. Cleavage of the peptides from the resin and removal of the protecting groups was accomplished with HF containing 10 vol.% of anisole for 1 h at 0 °C.

After removal of HF and drying under vacuum the resin was washed several times with ether and then extracted with 2 M acetic acid. Lyophilization of the latter extract yielded the crude peptide. The analogues were purified by gel chromatography on Sephadex G-15 using 0.2 M acetic acid as the eluent, followed by partition chromatography in a glass column (2.5 × 90 cm), on Sephadex G-25 (fine) saturated with the lower phase of the system 1-butanol–acetic acid–water (4:1:5), and eluted with the upper (organic) phase. Final purification was achieved by preparative HPLC on reversed-phase support C-18 with 5–35% gradient of $CH_3CN$ in 0.05% TFA–water. All products gave single spots on TLC (Merck precoated silica gel plates, type G60-F254) in the solvent systems: (A) 1-butanol–acetic acid–water (4:1:5 upper phase) and (B) 1-butanol–acetic acid–water–pyridine (15:3:10:6). Elemental analyses were performed on a Hewlett–Packard Mod. 185 analyzer and the

data (C, H, N) fall within ±0.4% of the theoretical values. The amino acid analyses were carried out on a Beckman 119 amino acid analyzer after acid hydrolysis in 6 M HCl (in the presence of cresol and mercaptoethanol) at 100 °C for 20 h in vacuo. They exhibited the following molar ratios: [Ser$^1$]AT: Ser 1.0; Arg 1.02; Val 1.03; Tyr 0.95; Ile 0.92; His 0.97; Pro 1.07; Phe 1.04. [Cys$^1$]AT: Cys 0.92; Arg 1.03; Val 1.02; Tyr 1.00; Ile 0.98; His 0.97; Pro 1.04; Phe 1.04. [Cys(Acm)$^1$]AT: Cys 0.93; Arg 1.03; Val 1.03; Tyr 1.00; Ile 0.98; His 0.96; Pro 1.03; Phe 1.04. [Aib$^1$, Aib$^2$]AT: Aib 1.92; Val 1.02; Tyr 0.98; Ile 1.00; His 0.97; Pro 1.06; Phe 1.05. Aib emerged 30 ml before Val from the long column of the analyzer and had a colour value which was 8% of that of Ile. In order to obtain a reasonable peak size a large amount of the peptide was hydrolyzed. [DOPA$^1$]AT: L-DOPA, present; Arg 1.01; Val 1.06; Tyr 0.97; Ile 1.00; His 0.96; Pro 0.97; Phe 1.03. A standard of L-DOPA was treated under the same hydrolysis conditions and was shown, by comparison with an untreated standard, to be partially destroyed. [N-MeAib$^1$]AT: N-MeAib, present; Arg 1.02; Val 1.02; Tyr 0.95; Ile 1.00; His 0.97; Pro 1.01; Phe 1.03.[GABA$^1$, GABA$^2$]AT: GABA, present; Val 1.05; Tyr 0.97; Ile 1.00; His 0.94; Pro 1.08; Phe 1.03.

N-MeAib and GABA ratios were not calculated because of the difficulties in detection of these compounds. The presence of N-MeAib or GABA in the structure of the synthesized peptides was determined by TLC of the hydrolysates (silica gel; two dimension solvent systems: (A) and (C) phenol–water, (3:1, v/v); ninhydrin detection).

The compounds were tested on rabbit aorta strips (in vitro) and for binding in bovine adrenocortical membranes[17]. The results of the two biological assays are present in Table I.

TABLE I
Comparison of the biological activities of angiotensin II and its 1 and 1,2-positions analogues on two pharmacological preparations[a]

| Analogue | In vitro, rabbit aorta | | Binding, bovine adrenocortical membranes | | Ref. |
|---|---|---|---|---|---|
| | $pD_2$ | RA, % | $IC_{50}$ ($10^{-10}$ mol/l) | RA, % | |
| [Asp$^1$]AT | 8.19 | 100.0 | 8.34 | 100.0 | 2,3 |
| [Asn$^1$]AT | 7.92 | 54.0 | 9.70 | 86.0 | 2,3 |
| [Sar$^1$]AT | 8.70 | 324.0 | 3.00 | 278.0 | 5 |
| [Aib$^1$]AT | 7.95 | 58.0 | 6.80 | 122.0 | 9 |
| [N-MeAib$^1$]AT | 6.43 | 1.7 | 170.00 | 4.9 | *b* |
| [DOPA$^1$]AT | 6.79 | 4.0 | 23.20 | 36.0 | *b* |
| [Ser$^1$]AT | 7.23 | 11.0 | 31.60 | 26.0 | *b* |
| [Cys$^1$]AT | 6.43 | 1.7 | nd | nd | *b* |
| [Cys(Acm)$^1$]AT | 5.80 | 0.4 | nd | nd | *b* |
| [Aib$^1$, Aib$^2$]AT | inactive | | inactive | | *b* |
| [GABA$^1$, GABA$^2$]AT | inactive | | inactive | | *b* |

[a] Key: $pD_2$ is the negative log of the dose that produces half-maximum contraction; RA is the relative affinity; the $IC_{50}$ of each analogue is the concentration of the peptide that displaces 50% of the radioactive label [$^{125}$I Tyr$^4$]AT from the receptor. The relative affinity indicated is the relationship between the $IC_{50}$ of AT and the $IC_{50}$ of the tested compound. [b] This paper.

## RESULTS AND DISCUSSION

We have shown that whilst the replacement of 1-aspartyl residue of AT with Aib gives an analogue ([Aib$^1$]AT) with high potency and relative affinity, the increased basicity of its N-terminal amino group by methylation, e.g. [N-MeAib$^1$]AT, contrary to the expectations, reduces both potency and the binding properties (Table I).

In order to explore further the role of the β-carboxyl group in position 1 of AT, we have replaced it with the sulfhydryl group, free ([Cys$^1$]AT) or substituted ([Cys(Acm)$^1$]AT), and the hydroxyl group ([Ser$^1$]AT). The last analogue exhibits a $pD_2$ of 7.23 whilst in [DOPA$^1$]AT, with a bulky and flat aromatic ring, the biological responses are further reduced ($pD_2$ 6.79). On the other hand, the [β-Malyl$^1$]AT analogue[12] in which the α-amino group has been replaced by a hydroxyl group, shows a $pD_2$ of 7.06 and a relative affinity 7.04% in comparison to [Asp$^1$]AT, tested under the same experimental conditions as all other analogues. Replacement of the β-carboxyl group with SH or S-Acm group almost abolishes the in vitro activity whilst the binding potency to adrenocortical membranes is not detectable.

Double substitution in positions 1 and 2 by Aib and GABA, gave the octapeptide analogues [Aib$^1$, Aib$^2$]AT and [GABA$^1$, GABA$^2$]AT which were found to be totally inactive in the two pharmacological tests. This is in correlation with the assumption that the guanidino group of Arg represents the feature in the N-terminal dipeptide which is essentially responsible for the high pressor activity of angiotensin II relative to the hexapeptide, Val-Tyr-Ile-His--Pro-Phe (ref.[13]).

Thus, it appears, at present, that:

(i) An increase in basicity at the N-terminal nitrogen atom of angiotensin II is not the only reason for increased potency[14].

(ii) Bulky groups in position 1 may affect the spatial orientation of essential residues like 3-Val and 4-Tyr. This in turn could produce changes in the preferred backbone conformation of the hormone and explain differences in biological activities observed.

However, these findings illustrate the complexity of the attempts to generalize the biological role of a single side chain group like the α-amino and the β-carboxyl groups of angiotensin II.

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