Synthesis of Analogs of [8-Isoleucine]angiotensin II with Variations in Position 1†

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[Des-Asp^1,Ile^8]-, [Pro^1,Ile^8]-, [MePhe^1,Ile^8]-, [Me$_2$Gly^1,Ile^8]-, [MeAla^1,Ile^8]-, [MeIle^1,Ile^8]-, and [Gac^1,Ile^8]angiotensin II, synthesized by Merrifield's solid-phase procedure, possess 0.4, 0.7, 0.9, 0.6, 1.0, 0.7, and 0.7% pressor activity of angiotensin II (vagotomized, ganglion-blocked rats) and pA_2 values (rabbit aortic strips) of 8.04, 8.52, 8.34, 8.87, 9.03, 8.73, and 9.21, respectively. The comparative pA_2 value obtained for [Sar^1,Ile^8]angiotensin II was 9.17. These results suggest that antagonism to contractile response of angiotensin II was reduced when position 1 in [Sar^1,Ile^8]angiotensin II was replaced by a hydrogen atom or a cyclic imino acid (proline). Antagonistic activity was also reduced when the α-carbon atom of sarcosine was substituted with an aromatic side chain (*N*-methylphenylalanine). In contrast, the presence of a branched aliphatic residue at the α-carbon atom (*N*-methylisoleucine), an additional methyl group at the nitrogen atom of sarcosine (dimethylglycine), or replacement of sarcosine by a highly basic moiety (guanidineacetic acid) had little effect on the *in vitro* antagonistic potency of the parent compound.

The 8-substituted analogs of angiotensin II possess variable agonist properties, some extremely minor while others approach that of the parent hormone.[2,3] We have previously demonstrated that the more closely the side group in position 8 resembles the aromatic group found in natural angiotensin II (phenylalanine) the greater the potency for myotropic action.[2,3]

Of the many modifications of the position 8, substitution with isoleucine or leucine produced the most potent antagonists.[2-9] Replacement of other side groups, in addition to that of the 8 position, may change the potency of the antagonist by either modifying its resistance to degradation by plasma enzymes or by changing its binding to a receptor.[10] Sarcosine in the 1 position of [Ala^8]angiotensin II enhanced its antagonistic properties.[11] It was first suggested that the introduction of sarcosine in the 1 position of 8-substituted angiotensin antagonists increases the *in vivo* potency by increasing the biological half-life of the peptide.[12] Studies on [Sar^1]angiotensin II, however, showed that the introduction of the N-methylated amino acid into this position not only made the peptide more resistant to degradation by plasma angiotensinase A but may also have increased its binding to the receptor site on aortic muscle.[13,14]

To obtain further information concerning the role of both the side group and N-alkylation in the 1 position, we synthesized analogs of [Ile^8]angiotensin II (Table I) in which sarcosine was replaced by (a) *N*-methylphenylalanine (IV), *N*-methylalanine (VI), or *N*-methylisoleucine (VII) to study the effect of substitution of an aromatic or aliphatic side chain on the α-carbon atom of sarcosine; (b) proline (III) to investigate the nature of the secondary amino group required for potent antagonist; (c) dimethylglycine (V) to study the influence of a tertiary amino group in this position; (d) guanidineacetic acid (VIII) to determine the influence of a highly basic group in position 1. [Des-Asp^1,Ile^8]angiotensin II (II) was synthesized to study the importance of chain length in these peptides.

The analogs were synthesized by the solid-phase procedure of Merrifield[15] (*cf.* Experimental Section). Comparative pressor activity of these analogs and their antagonism to contractile activity of angiotensin II are given in Table I.

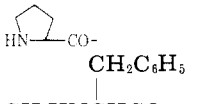
Biological Results. Table I describes the biological properties of the various antagonists of angiotensin II. Inhibition of contractile activity of angiotensin II was studied on isolated, spirally cut rabbit aortic strips[16] (*cf.* Experimental Section). pA_2 values were calculated according to the method described by Arunlakshana and Schild.[17] In the range of concentrations used (10^{-10}, 10^{-9}, and 10^{-8} *M*), log dose–response curves obtained with each compound were parallel and shifted to the right and the compounds were easily washed after 15–30 min, suggesting that these analogs are competitive antagonists of angiotensin II. However, when log $(x - 1)$ was plotted against $-\log$ [B], the slope of the line, *n*, of most of these antagonists deviates greatly from one, suggesting that these compounds are not competitive antagonists. Regoli, *et al.*,[18] obtained slope values (n), as estimated from their published graph, of 0.3 and 0.5 for [Gly^8]- and [Leu^8]angiotensin II, respectively. While testing [Sar^1,Leu^8]angiotensin II on rat stomach strips Regoli, *et al.*,[19] found evidence that it is a noncompetitive antagonist. With the present knowledge, it is difficult to explain the significance of the deviation of the slope value from unity in these peptides. One can speculate that the analogs are interacting with tissue sites other than the receptor sites, making less of the analogs available to the receptors.

Structure–Activity Relationships. Earlier studies from our laboratories indicated that the presence of an α-nitrogen atom at the N terminus and its basicity are both important for maximum antagonistic activity in the 8-substituted analogs of angiotensin II.[10] Present results indicate that antagonism to contractile response to angiotensin II was reduced when position 1 in [Sar^1,Ile^8]angiotensin II was replaced by a hydrogen atom or a cyclic imino acid (proline). Antagonistic activity was also reduced when the α-carbon atom of sarcosine was substituted with an aromatic side chain (*N*-methylphenylalanine). In contrast, the presence of a branched aliphatic residue at α-carbon atom (*N*-methylisoleucine), an additional methyl group at the nitrogen atom of sarcosine (dimethylglycine), or replacement of sarcosine by a highly basic moiety (guanidineacetic acid) had little effect on the *in vitro* antagonistic potency of the parent compound. However, the comparative duration of the antagonistic effect of [Me$_2$Gly^1,Ile^8]angiotensin II, both *in vitro* and *in vivo* studies, was found to be short (*ca.* 20–30 min) (Turker, *et al.*, unpublished results).

A preliminary investigation in dogs with [Des-Asp^1,-Ile^8]angiotensin II reveals that although this heptapeptide is less potent than [Sar^1,Ile^8]angiotensin II as antagonist of the pressor response of angiotensin II, it is tenfold more potent as an inhibitor for the release of aldosterone (Bravo, *et al.*, unpublished results). It is of interest to note that the corresponding C-terminal heptapeptide of angiotensin II ([Des-Asp^1]angiotensin II) has 15% pressor

† Presented in part at the 165th National Meeting of the American Chemical Society, Dallas, Texas, April 8–13, 1973.[1] Abbreviated designation of amino acid derivatives and peptides is according to the recommendation of the IUPAC-IUB Commission (IUPAC Information Bulletin No. 26). In addition, the following abbreviations were used: Sar = sarcosine, MeAla = *N*-methylalanine, MeIle = *N*-methylisoleucine, MePhe = *N*-methylphenylalanine, Me$_2$Gly = dimethylglycine, Gac = guanidineacetic acid.

Table I. Biological Properties of Analogs of [Ile^8]angiotensin II with Variations in Position 1

Compd no.	Residue in position 1	n	pA_2	Pressor activity[a]
I	CH_3HNCH_2CO–	2.13 ± 0.10	9.17 ± 0.04 (6)	1.0
II	H–	0.71 ± 0.06	8.04 ± 0.09 (5)	0.4
III		0.66 ± 0.06	8.52 ± 0.13 (5)	0.7
IV	$CH_3HNCHCO$– $CH_2C_6H_5$	0.56 ± 0.04	8.34 ± 0.35 (6)	0.9
V	$(CH_3)_2NCH_2CO$–	1.26 ± 0.08	8.87 ± 0.04 (5)	0.6
VI	$CH_3HNCHCO$– $CH_3CHCH_2CH_3$	2.03 ± 0.29	9.03 ± 0.07 (5)	1.0
VII	$CH_3HNCHCO$–	2.15 ± 0.06	8.73 ± 0.09 (5)	0.7
VIII	$H_2NC(=NH)NHCH_2CO$–	0.86 ± 0.08	9.21 ± 0.15 (6)	0.7

[a] Relative to [Asp^1,Ile^5]angiotensin II = 100.

activity of angiotensin II[20,21] but it is as active as angiotensin II in stimulating aldosterone secretion from adrenal cortex.[22] These results suggest functional differences in the receptors in the adrenal cortex and vascular smooth muscle[23] and corroborate the findings that the C-terminal heptapeptide, a metabolite of angiotensin II, is more important for the release of aldosterone than the corresponding octapeptide.[22]

Experimental Section

Solvents used for ascending tlc were (a) *n*-BuOH–AcOH–H_2O (BAW) (4:1:5); (b) *n*-BuOH–AcOH–H_2O–Pyr (BAWP) (30:6:24:20); (c) *n*-BuOH–AcOEt–AcOH–H_2O (BEAW) (1:1:1:1); (d) *n*-BuOH–Pyr–H_2O (BPW) (10:2:5); (e) *n*-PrOH–H_2O (1:1). Ascending tlc was conducted on cellulose supported on glass plates (Brinkmann celplate-12). Ionophoresis was carried out on filter paper strips on S & S 2043A filter paper strips in Beckman electrophoresis cell (Durrum type) Model R, series D at 400 V, using HCO_2H–AcOH buffer prepared by diluting 60 ml of HCO_2H and 240 ml of AcOH to 2 l. with distilled H_2O (pH 1.9) and Beckman barbiturate buffer B-2 (pH 8.6). Histidine was used as a reference compound and *E*(His) indicates the electrophoretic mobility relative to histidine = 1.00. Detection of the compound on chromatograms was carried out with ninhydrin and/or with diazotized sulfanilic acid. The free peptides were hydrolyzed in sealed tubes under N_2 in 6 *N* HCl at 110° for 24 hr in the presence of phenol. Amino acid analyses were performed on Jeolco-5AH amino acid analyzer. Elemental analyses were performed by Micro-Tech Laboratories, Skokie, Ill.; where analyses are indicated only by symbols of the elements or functions, analytical results obtained for those elements or functions were within ±0.4% of the theoretical values. L-Amino acid oxidase from the venom of *Agkistrodon p. piscivorus* was purchased from General Biochemicals. Angiotensin analogs reported in this paper contain L-isoleucine in position 5.

Synthesis and Purification of Analogs. The protected heptapeptide polymer [Boc-Arg(NO_2)-Val-Tyr(Bzl)-Ile-His(Bzl)-Pro-Ile-polymer ester] was synthesized by the solid-phase procedure of Merrifield.[15] After having ascertained the purity[10] of the free heptapeptide ([Des-Asp^1,Ile^8]angiotensin II), the above protected heptapeptide polymer was used as the starting material for the synthesis of various octapeptides reported in this paper. The protocol used for the synthesis and purification was the same as previously described by us[10] except that precautions were taken to avoid racemization by (a) carrying out coupling at each stage at 0–5° (the apparatus used for this purpose was of the manual type[24]); (b) using ethyl 2-hydroximino-2-cyanoacetate[25] as an additive to minimize racemization[26] of histidine during the coupling of Boc-His(Bzl).

Coupling of dimethylglycine appeared difficult due to insolubility of this amino acid in most organic solvents. Even after three couplings of a suspension of this amino acid in DMF, the polymer gave positive ninhydrin reaction due to free amino groups. However, this difficulty was overcome by coupling through activation with *N*-ethyl-5-phenylisoxazolium 3′-sulfonate.[27] The active ester was soluble in DMF and after two couplings the peptide polymer gave a ninhydrin-negative test.[28] This latter test was routinely used to ensure complete coupling of all *tert*-butyloxycarbonyl-*N*-alkylamino acids or guanidineacetic acid at the N terminus. Guanidineacetic acid was protected as its nitro derivative.

Fractions in the column chromatography were cut without regard for yield to obtain the desired compound in the pure form and no attempt was made to rechromatograph the minor fractions for identification purposes. The homogeneity of the compounds was determined by (a) electrophoresis at pH 8.6 and 1.95; (b) thin-layer chromatography in five solvent systems of different pH, and (c) amino acid analysis.

tert-Butyloxycarbonyl-*N*-methylamino acids were synthesized by a modified procedure of Olsen,[29] as previously reported by us for *tert*-butyloxycarbonyl-*N*-methylphenylalanine,[30] except that CH_3I was added in three lots over a period of 50 hr.

tert-Butyloxycarbonyl-*N*-methylisoleucine was obtained as clear oil: tlc (silica gel) R_f 0.71 (90:10 $CHCl_3$–AcOH), R_f 0.48 (95:5 $CHCl_3$–AcOH), R_f 0.86 (85:10:5 $CHCl_3$–MeOH–AcOH). *Anal.* ($C_{12}H_{23}NO_4$) C, H, N.

[Des-Asp^1,Ile^8]angiotensin II: tlc (cellulose) R_f 0.50 (BAW), R_f 0.72 (BEAW), R_f 0.17 (BPW), R_f 0.76 (BAWP), R_f 0.81 (PW); *E*(His) 0.83 (pH 1.95), *E*(His) 1.05 (pH 8.6). Amino acid ratio in the acid hydrolysate: Arg 0.95, Val 1.00, Tyr 1.00, Ile 1.91, His 0.92, Pro 0.99, and after incubation with L-amino acid oxidase His, 0.05, Pro 1.00.

[Pro^1,Ile^8]angiotensin II: tlc (cellulose) R_f 0.50 (BAW), R_f 0.79 (BEAW), R_f 0.14 (BPW), R_f 0.75 (BAWP), R_f 0.74 (PW); *E*(His) 0.81 (pH 1.95), *E*(His) 1.0 (pH 8.6). Amino acid ratio in the acid hydrolysate: Pro 2.03, Arg 0.91, Val 1.00, Tyr 1.10, Ile 2.02, His 0.90.

[MePhe^1,Ile^8]angiotensin II: tlc (cellulose) R_f 0.66 (BAW), R_f 0.82 (BEAW), R_f 0.44 (BPW), R_f 0.82 (BAWP), R_f 0.75 (PW); *E*(His) 0.85 (pH 1.95), *E*(His) 0.96 (pH 8.6). Amino acid ratio in the acid hydrolysate: Arg 0.97, Val 1.06, Tyr 0.90, Ile 2.12, His 1.00, Pro 1.00. The chromatographic peak for *N*-methylphenylalanine merged into that of isoleucine and since color intensity of this amino acid is low only a qualitative estimation could be carried out to establish its presence.

[Me$_2$Gly^1,Ile^8]angiotensin II. The protected heptapeptide polymer [Boc-Arg(NO_2)-Val-Tyr(OBzl)-Ile-His(Bzl)-Pro-Ile-P] (4 g) was deprotected in the usual way. The trifluoroacetate salt was neutralized (NEt_3–DMF) and the peptide polymer coupled with dimethylglycine activated with Woodward's Reagent K as follows. Me_2Gly·HCl salt (700 mg) was suspended in DMF and cooled to 0° under magnetic stirring. NEt_3 (1.4 ml) was added and after 5 min of stirring Woodward's Reagent K[27] (1.265 g) was added in small portions over a period of 10 min. The mixture was stirred for 1 hr at 0° when all of the reagent went into solution. The solution was filtered and the filtrate added to the resin. After stirring overnight, it was filtered and washed (DMF) and the coupling step repeated. At the end (when the resin did not give color with ninhydrin) the resin was washed with DMF and 10% NEt_3 in DMF to wash off brownish color from the resin. The analog was worked up and purified by the general procedure: tlc (cellulose)

R_f 0.37 (BAW), R_f 0.68 (BEAW), R_f 0.00 (BPW), R_f 0.62 (BAWP), R_f 0.69 (PW); E(His) 0.86 (pH 1.95), E(His) 0.95 (pH 8.6). Amino acid ratio in the acid hydrolysate: Arg 1.02, Val 1.03, Tyr 1.03, Ile 1.92, His 0.88, Pro 1.00.

[MeAla^1,Ile^8]angiotensin II: tlc (cellulose) R_f 0.52 (BAW), R_f 0.74 (BEAW), R_f 0.12 (BPW), R_f 0.71 (BAWP), R_f 0.89 (PW); E(His) 0.82 (pH 1.95), E(His) 1.0 (pH 8.6). Amino acid ratio in the acid hydrolysate: Arg 0.90, Val 1.00, Tyr 1.07, Ile 2.05, His 1.04, Pro 0.91.

[MeIle^1,Ile^8]angiotensin II: tlc (cellulose) R_f 0.75 (BAW), R_f 0.86 (BEAW), R_f 0.48 (BPW), R_f 0.89 (BAWP), R_f 0.93 (PW); E(His) 0.71 (pH 1.95), E(His) 1.12 (pH 8.6). Amino acid ratio in the acid hydrolysate: Arg 0.91, Val 1.04, Tyr 1.00, Ile 1.80, His 1.00, Pro 1.04. The peak for N-methylisoleucine merged into that of valine (retention time 86 min); since the color intensity of this amino acid with ninhydrin was 150-fold less as compared to valine, only a qualitative estimation could be carried out.

Nitroguanidineacetic Acid. Guanidineacetic acid was nitrated[31] by the following procedure. Guanidineacetic acid (10 g) was added in portions to a cooled (−10°) mixture of fuming H_2SO_4 (40 ml) and fuming HNO_3 (40 ml) over 1 hr with vigorous stirring. The mixture was further stirred at −10° for 3 hr when a clear colorless solution was obtained. The mixture was poured onto crushed ice in a beaker, and the pH of the aqueous solution adjusted to 8 by dropwise addition of concentrated NH_4OH at 0–5°. (The solid lumps were broken while maintaining the pH at 8.) The pH was again adjusted to 3 with dilute HCl, the mixture was allowed to stand for 10 min at 5°, and the colorless solid filtered on a sintered funnel and washed with H_2O and 1 N HCl. The residual amorphous powder was crystallized from H_2O (or MeOH) to give 8 g of nitroguanidineacetic acid: mp 186–187°; tlc (silica gel) R_f 0.18 (BAW), R_f 0.26 (BAWP), R_f 0.1 (BPW). Spots were detected with iodine vapor; nitroguanidineacetic acid gave a negative test with Sakaguchi reagent. *Anal.* ($C_3H_6N_4O_4$) C, H, N.

[Gac^1,Ile^8]angiotensin II: tlc (cellulose) R_f 0.57 (BAW), R_f 0.75 (BEAW), R_f 0.05 (BPW), R_f 0.78 (BAWP), R_f 0.70 (PW); E(His) 0.84 (pH 1.95), E(His) 0.84 (pH 8.6). Amino acid ratio in the acid hydrolysate: Arg 0.98, Val 1.00, Tyr 1.02, Ile 1.98, His 0.89, Pro 1.06. The free peptide gave a ninhydrin-negative test.

Pharmacological Evaluation. The pressor activity was determined by pressor assay in vagotomized, ganglion-blocked rats by the procedure reported by Pickens, *et al.*[32] The results given in Table I represent mean values obtained for three or four pressor responses at different dose levels and for angiotensin II controls and are relative to [Asp^1,Ile^5]angiotensin II = 100. Sufficient time was given for complete recovery between each injection in order to minimize any tachyphylactic effect which may be present.

Inhibition of contractile activity of angiotensin II was studied on isolated, spirally cut rabbit aortic strips.[16] pA_2 values were calculated according to the method described by Arunlakshana and Schild[17] by the equation: $\log (x - 1) = n \log B + \log K_2$. First a log dose–response curve for angiotensin II was determined on rabbit aortic strips, before and in the presence of the analogs. Next, the dose-ratio of equal response to angiotensin II was calculated in the presence of various analogs at several molar concentrations of the antagonist in the range of 10^{-10}, 10^{-9}, and 10^{-8} M. This was followed by plotting the log dose–ratio minus one [$\log (x - 1)$] against the negative logarithm of the molar concentration of the antagonist [−log B]. The point on the abscissa that is intercepted by the plotted line is designated as the pA_2 value. The pA_2 value given in Table I represents mean values obtained for five to six separate experiments with each antagonist using [Asp^1,Ile^5]angiotensin II as the agonist.

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