Synthesis of Angiotensin II Analogues by Incorporating β-Homotyrosine or β-Homoisoleucine Residues[1]

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[1-Sarcosine,4-β-homotyrosine]- (I), [5-β-homoisoleucine]- (II), and [1-sarcosine,5-β-homoisoleucine]angiotensin II (III) were synthesized by Merrifield's solid-phase procedure to study the effect on pressor activity and duration of action. The analogues I–III possessed, respectively, 1.98, 2.82, and 29.2% pressor activity of angiotensin II (vagotomized, ganglion-blocked rats by single-injection procedure) and duration of action of 5.5, 6.7, and 4.7 min; the comparative duration of action of an equipressor dose of angiotensin II was 5.2, 6.3, and 5.3 min, respectively. When incubated with leucine aminopeptidase, degradation of II was as fast as that of angiotensin II; this degradation became considerably slower when position 1 was replaced with sarcosine. Incubation of all these analogues with chymotrypsin showed very little or no degradation up to 3 h. The results indicate that an increase in the chain length by one carbon atom in position 4 or 5 of angiotensin II increased resistance to degradation by chymotrypsin without any increase in in vivo duration of action. Further, all analogues showed low pressor activity.

Angiotensin II analogues with competitive antagonistic properties for the parent hormone (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) have proved useful in answering certain questions concerning the mechanism of angiotensin II involvement in the regulation of blood pressure. However, these antagonists are not orally active and can not be used in the long-term management of human hypertension (for detailed reviews, see ref 3–5). Since a short in vivo half-life is presumably due to degradation by peptidases, attempts have been made to make these peptides resistant to enzymatic degradation by replacing the amino acid residues susceptible to enzymatic degradation with *N*-methyl- or α-methyl-substituted amino acids[6-8]. These modifications either reduced the biological activity (e.g., N-methylation) or did not appreciably increase the in vivo half-life. In the present work, we have extended these studies to the incorporation of β-homotyrosine or β-homoisoleucine residues in positions 4 or 5 of angiotensin II, respectively. This approach is based on previous studies which demonstrated that replacement of an α-amino acid residue by the corresponding β-homoamino acid in biologically active peptides (e.g., bradykinin- or angiotensin-converting enzyme inhibitors) yielded analogues that retained significant activity and possessed enhanced resistance to enzymatic degradation.[9,10]

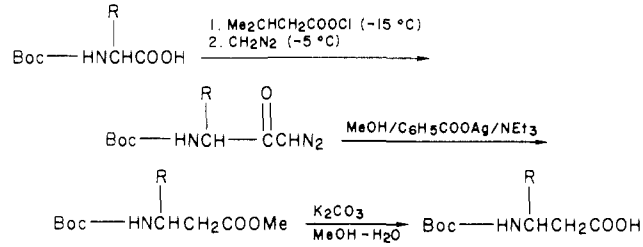
Synthesis of *tert*-butyloxycarbonyl-β-homotyrosine and *tert*-butyloxycarbonyl-β-homoisoleucine was based on the Arndt–Eistert reaction and Wolf rearrangement as described previously for other similar derivatives.[10-12] The experimental conditions adopted are particularly suitable for acid-sensitive blocking groups (cf. Experimental Section and Scheme I). The compounds were crystallized until a steady value of optical rotation was obtained.

The analogues were synthesized by the solid-phase procedure of Merrifield.[13] The protocol used for synthesis and purification was the same as reported previously by Khosla et al.[7] Since the sensitivity of β-homotyrosine and β-homoisoleucine to the ninhydrin test is very low, 3–5 mg of each peptide was hydrolyzed for amino acid analysis. Determination of pressor activity of the analogues was carried out on vagotomized ganglion-blocked rats.[14] Results were expressed as a percentage of the pressor activity of angiotensin II. The duration of action was calculated from the time of injection of the peptide to the time blood pressure levels returned to control values. The duration of action of each analogue was compared to that of an equipressor dose of angiotensin II.

Results and Discussion

Compared to angiotensin II, [Sar[1],βHTyr[4]]-, [βHIle[5]]- and [Sar[1],βHIle[5]]angiotensin II showed 1.98 ± 0.132 (n = 7), 2.82 ± 0.071 (n = 9), and 29.2 ± 3.05% (n = 16) pressor activity. The corresponding duration of action was 5.5, 6.7, and 4.7 min; the comparative duration of action of an equiresponse dose of angiotensin II was 5.2, 6.3, and 5.3 min, respectively.

Scheme I. Synthesis of Boc-Protected β-Homoamino Acids



When incubated with leucine aminopeptidase (cf. Experimental Section), degradation of [βHIle[5]]angiotensin II was as fast as that of angiotensin II. With both these peptides, liberation of aspartic acid from position 1 and arginine from position 2 was detected within 5 min. Contrarily, degradation of sarcosyl analogues and [Sar[1]]angiotensin II[16] was slow, and after 2 h of incubation very little quantities of sarcosine, arginine, or valine were detected. Incubation of all these analogues with chymotrypsin indicated very little or no degradation up to 3 h. Under the same conditions, angiotensin II was completely degraded into two tetrapeptides within 15–20 min of incubation. However, low in vivo duration of action obtained with these analogues seems to indicate that possibly these peptides are rapidly destroyed by peptidases other than those studied here.

Replacement of tyrosine (position 4) with a β-homotyrosine residue or isoleucine (position 5) with a β-homoisoleucine residue in angiotensin II results in an increase in the length of the peptide backbone by one carbon atom. As a result, the side chains of the subsequent amino acids (His, Pro, Phe) and the C-terminal carboxyl group are shifted by one carbon atom. Drastic decrease in pressor activity due to this modification corroborates our previous finding that the positioning of the aromatic side chain and carboxyl group in position 8 is important for full biological activity.[15] In addition, this modification may have disturbed the side-chain conformation of the tyrosine residue or the overall conformation of angiotensin II. Previous studies on the conformation of angiotensin II and its structural analogues suggested that valine (position 3), isoleucine (position 5), and histidine (position 6) serve mainly to correctly align the phenolic ring of tyrosine (position 4) and that any change in this structure

0022-2623/79/1822-1128$01.00/0

may lead to reduced biological activity.[17]

Experimental Section

The peptides were prepared and purified by the procedures described by Khosla et al.[7] TLC was conducted on silica gel or cellulose supported on glass plates. The solvent systems (upper phase) used were: (a) *n*-BuOH–AcOH–H_2O (BAW), 4:1:5 or 4:1:1; (b) *n*-BuOH–AcOH–H_2O–pyridine (BAWP), 30:6:24:20; (c) *n*-BuOH–pyridine–H_2O (BPW), 65:35:35 or 65:35:65; (d) *n*-PrOH–H_2O (PW), 2:1; (e) $CHCl_3$–MeOH–AcOH (CMA), 85:10:5; (f) Me_2CHOH–3% NH_4OH (IPA), 150:66. Ionophoresis was carried out on S & S 2043A filter paper strips in a 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 2.2), and Beckman barbiturate buffer B-2 (pH 8.6). Histidine was run simultaneously as a reference compound and *E*(His) indicates the electrophoretic mobility relative to histidine = 1.00. Location of compounds on chromatograms was carried out with ninhydrin reagent and/or diazotized sulfanilic acid. For locating *tert*-butyloxycarbonyl-protected amino acid derivatives, the plates were first stored in a closed tank saturated with HCl vapors, followed by spraying with ninhydrin. Single symmetrical spots were observed for purified compounds.

Free peptides were hydrolyzed in sealed tubes under N_2 in the presence of phenol in 6 N HCl at 110 °C for 24 h or in a 1:1 mixture of 12 N HCl–propionic acid;[18] peptide polymer esters were invariably hydrolyzed in the latter solvent system. Amino acid analyses were performed on a Jeolco-5AH amino acid analyzer. Optical rotations were determined in a Perkin-Elmer polarimeter, Model 141, equipped with a digital readout. Elemental analyses were performed by Microtech Laboratories, Skokie, Ill. Where analyses are indicated only by symbols of the elements or functions, analytical results obtained for these elements or functions were within ±0.4% of the theoretical values. *tert*-Butyloxycarbonyl-protected amino acids were purchased from Bachem Inc., Torrance, Calif.

(*tert*-Butyloxycarbonyl)-*O*-(2,6-dichlorobenzyl)tyrosyldiazomethane. The procedure for the synthesis of this compound was similar to the one used by Penke et al.[11] and Casaal et al.[12] for the synthesis of Boc-Tyr(Bzl)CHN_2. To a solution of Boc-Tyr(2,6-Cl_2Bzl) (8.8 g, 20 mmol) in Et_2O (70 mL) was added Et_3N (2.8 mL), and the solution was cooled to −15 °C in a dry ice–acetone bath. After 5 min, isobutyl chloroformate (2.8 mL, 20 mmol) was added under magnetic stirring, and the mixture was stirred for another 15 min while the temperature was maintained at −15 °C. A solution of CH_2N_2 in Et_2O (prepared from 10.7 g of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide, "DIAZALD") was now dropped over 25 min at −5 °C, and the mixture was further stirred for 2 h at 0 °C. The solvent was removed in vacuo and the residue extracted with AcOEt. The extract was washed (saturated $NaHCO_3$ solution and H_2O) and dried ($MgSO_4$), and the solvent was removed in vacuo. The residue was crystallized from *n*-hexane–AcOEt to yield 7.3 g (~78%) of Boc-Tyr(2,6-Cl_2Bzl)CHN_2: mp 117 °C; IR spectrum lacked absorption due to the carboxyl OH on the region of 3200–3600 cm^{-1} but showed peaks at 2100 and 1700–1720 cm^{-1}; TLC (silica gel) showed that the product was homogeneous, R_f 0.75 (BAW, 4:1:5), 0.69 (BPW, 65:35:35), 0.70 (PW, 2:1), 0.74 (BAWP).

(*tert*-Butyloxycarbonyl)-*O*-(2,6-dichlorobenzyl)-β-homotyrosine Methyl Ester. To a solution of Boc-Tyr(2,6-Cl_2Bzl)CHN_2 (6.5 g, 14 mmol) in MeOH (50 mL) was added in portions a solution of $C_6H_5CO_2Ag$ (0.4 g) in Et_3N (37 mL) under stirring at room temperature. When the evolution of N_2 had ceased (ca. 3 h), the reaction mixture was treated with activated charcoal, filtered, and washed with hot MeOH, and the combined filtrates were evaporated in vacuo to dryness. The residue was dissolved in AcOEt and then washed (H_2O, saturated NaCl solution) and dried ($MgSO_4$), and the solvent was removed in vacuo. The residue was crystallized from *n*-hexane–MeOH to yield 5.23 g (80%) Boc-βHTyr(2,6-Cl_2Bzl)OMe: mp 126 °C; TLC (silica gel) showed that the product was homogeneous, R_f 0.74 (BAW, 4:1:5), 0.68 (BPW, 65:35:35), 0.68 (PW, 2:1), 0.72 (BAWP).

(*tert*-Butyloxycarbonyl)-*O*-(2,6-dichlorobenzyl)-β-homotyrosine. To a solution of Boc-*O*-(2,6-dichlorobenzyl)-β-homotyrosine methyl ester (6.98 g, 13 mmol) in MeOH (41 mL) and H_2O (2.1 mL) was added K_2CO_3 (2.07 g).[12] The mixture was warmed for 6 h and then MeOH was removed in vacuo. The residue was dissolved in H_2O, acidifed with saturated citric acid solution to pH 4.5, and extracted with AcOEt. The organic phase was washed (H_2O, saturated NaCl solution), dried ($MgSO_4$), and evaporated. The residue was crystallized from AcOEt–hexane to yield (5 g, 84%) Boc-βHTyr(2,6-Cl_2Bzl): mp 156–157 °C; $[\alpha]^{25}_D$ −13.1° (*c* 2, EtOH); TLC (silica gel) R_f 0.72 (BAW, 4:1:5), 0.62 (BPW, 65:35:35), 0.69 (BAWP), 0.67 (PW). Anal. ($C_{22}H_{25}NO_5Cl_2$) C, H, N.

(*tert*-Butyloxycarbonyl)isoleucyldiazomethane. This was obtained by treating an ethereal solution of Boc-Ile (9.3 g, 40 mmol) and NEt_3 with isobutyl chloroformate (5.2 mL, 40 mmol) and CH_2N_2 (obtained from 21.4 g of DIAZALD) as described above for the tyrosyl derivative. Crystallization from hexane yielded 7.68 g (75%) of the desired product: mp 87–88 °C; IR showed peaks at 2100 and 1700–1720 cm^{-1}; TLC (silica gel) R_f 0.72 (BAW, 4:1:5), 0.71 (BPW, 65:35:35), 0.75 (BAWP), 0.61 (PW, 2:1).

(*tert*-Butyloxycarbonyl)-β-homoisoleucine Methyl Ester. This was obtained by treating a solution of Boc-Ile-CHN_2 (7.2 g; 28 mmol) in MeOH (30 mL) with a solution of C_6H_5COOAg (0.8 g) in NEt_3 (7.3 mL) to yield Boc-βHIle-OMe (5.4 g, 80%) as an oil: TLC (silica gel) R_f 0.75 (BAW, 4:1:5), 0.70 (BPW, 65:35:35), 0.91 (CMA, 85:10:5), 0.77 (BAWP), 0.70 (PW).

(*tert*-Butyloxycarbonyl)-β-homoisoleucine. This was obtained by saponifying Boc-βHIle-OMe (5.4 g, 22 mmol) in a mixture of MeOH (67 mL), H_2O (3.4 mL), and K_2CO_3 (3.4 g). Crystallization from hexane yielded 4.04 g (75%) of Boc-βHIle: mp 87–88.5 °C (DCHA salt, mp 130.5–131.5 °C); $[\alpha]^{20}_D$ −9.5° (*c* 2, EtOH); TLC (silica gel) R_f 0.69 (BAW, 4:1:5), 0.64 (BAWP), 0.60 (PW), 0.54 (CMA, 85:10:5). Anal. ($C_{12}H_{13}NO_4$) C, H, N.

[5-β-Homoisoleucine]angiotensin II: TLC (cellulose) R_f 0.32 (BAW, 4:1:5), 0.52 (BPW, 65:35:65), 0.57 (BAWP), 0.66 (IPA, 150:66); TLC (silica gel) R_f 0.05 (BAW, 4:1:5), 0.27 (BPW, 65:35:65), 0.32 (BAWP); *E*(His) 0.69 (pH 2.2), 1.3 (pH 8.6). Amino acid ratio in the acid hydrolysate (12 N HCl–$EtCO_2H$, 1:1, for 48 h at 130 °C): Asp, 1.0; Arg, 1.2; Val, 1.07; Tyr, 0.9; HIle, 1.1; His, 1.0; Pro, 1.0; Phe, 1.0.

[1-Sarcosine,5-β-homoisoleucine]angiotensin II. Initial purification was carried out on AG-1 × 2 (ACO^-), followed by successive column chromatography on Sephadex G-25 (BAW, 4:1:5) and silica gel (EtOH–H_2O, 2:1) columns: TLC (cellulose) R_f 0.36 (BAW, 4:1:5), 0.39 (BPW, 65:35:35), 0.68 (BAWP), 0.5 (PW), 0.83 (IPA, 150:66); TLC (silica gel) R_f 0.08 (CMA, 150:66); *E*(His) 0.96 (pH 2.2), 0.72 (pH 8.6). Amino acid ratio in the acid hydrolysate (6 N HCl, for 24 h at 130 °C): Sar, 1.0; Arg, 1.03; Val, 1.07; Tyr, 0.93; βHIle, 1.07; His, 1.08; Pro, 1.07; Phe, 1.0.

[1-Sarcosine,4-β-homotyrosine]angiotensin II. Purification of this analogue was carried out on ion-exchange (AG-1 × 2, ACO^-) (ammonium acetate buffer, pH 8.6) and Sephadex G-25 (BAW, 4:1:5) columns successively: TLC (cellulose) R_f 0.50 (BAW, 4:1:5), 0.25 (BAW, 4:1:1), 0.69 (BAW, 4:2:5), 0.53 (BPW, 65:35:65), 0.40 (BPW, 65:35:35); *E*(His) 0.68 (pH 2.2), 0.95 (pH 8.6). Amino acid ratio in the acid hydrolysate: Sar, 0.78; Arg, 1.04; Val, 0.90; βHTyr, ~0.95; Ile, 1.0; His, 1.02; Pro, 1.1; Phe, 1.0.

Enzymatic Degradation with Leucine Aminopeptidase (Microsomal, Sigma Chemical Co., 25 units/1.83 mg of Enzyme). Two milligrams of angiotensin II or its analogue was dissolved in 0.4 mL of Tris buffer (0.5 mol, pH 8.5), and the solution was treated with 0.1 mL of $MnCl_2$ solution (0.025 mol). Leucine aminopeptidase (0.2 mg in 0.2 mL distilled water) was added, and the solution was incubated at 37 °C for 1 h. Acetic acid (0.2 mL) was added, the mixture was lyophilized, and the liberated amino acids were detected on TLC and through amino acid analysis.

Enzymatic Degradation with α-Chymotrypsin (Beef Pancreas, Three Times Crystallized, Nutritional Biochemical Corp.). Five milligrams of the peptide was dissolved in 0.5 mL of ammonium acetate buffer (0.1 mol, pH 7.5) and the solution was spotted on TLC (cellulose plates). Chymotrypsin (0.4 mg suspended in 0.4 mL of ammonium acetate buffer) was added, and the mixture was incubated at 37 °C. Acetic acid (0.2 mL) was added and the mixture was lyophilized. The hydrolysate was examined by TLC in suitable solvent systems.

Acknowledgment. This investigation was supported in part by NIH Grant HL-6835 and General Research Support Grant FR-5674. We express our appreciation to Miss Valerie Bilchak for amino acid analysis.

References and Notes

(1) Abbreviated designation of amino acid derivatives and peptides is according to the recommendation of the IUPAC–IUB Commission on Biochemical Nomenclature (IUPAC Information Bulletin No. 26). In addition, the following abbreviations were used: Prd, pyridine; βHTyr, β-homotyrosine; βHIle, β-homoisoleucine.
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Lysosomotropic Agents. 1. Synthesis and Cytotoxic Action of Lysosomotropic Detergents

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Amines whose pK values lie between about 5 and 9 are lysosomotropic because lysosomes are acidic intracellular compartments. If such amines bear long hydrophobic chains, they become detergents upon protonation inside the lysosomes, rupturing the lysosomal membrane and killing the cell. Six types of lysosomotropic amines have been prepared that all behave in the expected manner. They are cytotoxic to all lysosome-bearing cells but not red blood cells, which lack lysosomes. Their mode of action, the effect of alkyl chain length on activity, and the fact that their cytotoxic action appears only above a threshhold intracellular concentration support the belief that they behave as lysosomotropic detergents. Among the potential applications is cancer chemotherapy.

Lysosomotropic drugs are a promising medical development. To date, the most prominent application of this concept, which was originated by De Duve and his coworkers,[1] has been the treatment of leukemia with DNA complexes of adriamycin and daunorubucin.[2] The toxicity of the complexes is much lower than that of the free drugs, which are released within the target cells only after uptake of the complexes by "piggyback endocytosis", followed by intralysosomal digestion of the DNA. Selectivity greater than that of the free drug is achieved because malignant cells are often more pinocytic than normal ones.[2,3]

This is an important discovery. Nevertheless, it seemed desirable to us to avoid altogether the use of exogenous toxic agents, even as a complex, because decomplexation might not always be limited to the intralysosomal milieu. Therefore, the use of the lysosomal enzymes themselves to kill the target cells was investigated.

Lysosomes are membrane-bound organelles containing a variety of hydrolytic enzymes that would be lethal to the host cell if released within it.[1] The goal, then, was to selectively rupture the lysosomal membrane, leaving the cell membrane intact. A good way to break up phospholipid bilayers is with detergents, but ordinary detergents attack the cell membrane first and, therefore, are indiscriminate as to membrane type. Consequently, it was necessary to develop detergents that acted only on lysosomal membranes and none other.

We took advantage of the fact that intralysosomal pH is usually between 3 and 5, i.e., 2–4 pH units lower than that of the crytosol.[1,4] It has long been recognized that an amine whose basicity is such that it is substantially protonated inside but not outside lysosomes, i.e., whose pK is between about 5 and 9, will be lysosomotropic.[1] If in addition it bears a long hydrophobic chain, it will be a lysosomotropic detergent, accumulating inside lysosomes not only because it is thermodynamically more stable there, but also because ionization inhibits its passing out through the lysosomal membrane. At neutral pH in the cytosol or intercellular fluid the largely un-ionized amine will be simply an oily substance without surface-active properties, but upon ionization within the lysosome it will become a detergent, accumulating with (presumably) its hydrophobic tail buried in the hydrocarbon zone of the bilayer and its hydrophilic protonated amine head group

0022-2623/79/1822-1130$01.00/0