Solid-Phase Peptide Synthesis of [L-Alanine^3-L-isoleucine^5]-angiotensin II*

M. C. Khosla,† Robert R. Smeby, and F. Merlin Bumpus

ABSTRACT: [L-Alanine^3-L-isoleucine^5]-angiotensin II was synthesized by stepwise elongation of the peptide chain on copolystyrene–divinylbenzene using dicyclohexylcarbodiimide as the condensing agent. The purified end product was obtained in 55% yield and possessed 68% of the pressor activity of [isoleucine^5]-angiotensin II.

The synthesis of [L-alanine^3-L-isoleucine^5]-angiotensin II was undertaken for the following reasons. First, change of side-chain branching from the β-carbon atom (valine) to the γ-carbon atom (leucine) in position 3 of angiotensin II did not affect the biological activity (Schwyzer *et al.*, 1957; Schwyzer, 1961) but a similar change in position 5 reduced the activity to 25% (Schwyzer *et al.*, 1957; Schwyzer, 1961). It seemed of interest to remove all side-chain branching in position 3 by replacing valine with alanine. Second, the study of aliphatic amino acids offered a new approach in structure–activity relationship of peptide hormones in view of the fact that the aliphatic side chains in position 3 of oxytocin have a positive function in binding the hormone molecule to a receptor (Rudinger and Krejci, 1962; Nesvadba *et al.*, 1963). Third, it would provide additional evidence for the usefulness and general applicability of solid-phase peptide synthesis.

Results and Discussion

The synthetic route employed was essentially the same as described by Marshall and Merrifield (1965) for the synthesis of angiotensin, except that condensations with dicyclohexylcarbodiimide were conducted at low temperatures and the final product was purified by column chromatography.

t-Butyloxycarbonylphenylalanine was esterified onto chloromethylated polymer, as usual, and the cycle for each amino acid consisted of removal of the protecting *t*-butyloxycarbonyl group (*t*-BOC)[1] with 1 N hydrochloric acid in acetic acid, neutralization of the resulting hydrochloride salt with triethylamine in dimethylformamide, and coupling the free base with *t*-BOC-amino acids. *N*,*N*′-Dicyclohexylcarbodiimide was used as the condensing agent and methylene chloride was used as the solvent for condensation in all the steps, except those utilizing *t*-butyloxycarbonyl-nitroarginine and *t*-butyloxycarbonyl-*N*-imidazole–benzyl-L-histidine. These were condensed in dimethylformamide due to greater solubility of these intermediates in this solvent. Excess reagents and by-products were removed by washing successively with methylene chloride, ethanol, and acetic acid. The protected octapeptide was cleaved from the polymer by bubbling hydrogen bromide through a suspension of the peptide–polymer in trifluoroacetic acid. This treatment also removed the benzyl groups from both the aspartic acid and tyrosine residues. The protecting N^{im}-benzyl group of histidine and nitro group of arginine were then removed by catalytic hydrogenation over palladium black.

The crude mixture, so obtained, contained one major and three minor components demonstrated as spots on paper and thin layer chromatography and was separated into its components by chromatography on a column of Sephadex G-25 in *n*-butyl alcohol–acetic acid–water–pyridine system. The homogeneity of [L-alanine^3-isoleucine^5]-angiotensin II was determined by paper electrophoresis and paper and thin layer chromatography. Amino acid composition and elemental analysis were determined and were found to be within the expected range. The peptide was completely degraded to component amino acids by partially purified leucine aminopeptidase and, perhaps owing to the presence of prolinase and prolidase in the crude enzyme (Schwarz and Bumpus, 1959), the degradation did not stop when histidine became N-terminal amino acid (Riniker, 1964). Since considerable splitting of N-terminal aspartic acid took place within 1 hr of incubation, a rearrangement of N-terminal α- to β-aspartyl residue, either during synthesis or during the removal of the peptide from the polymer, was ruled out. The biological assay showed that [alanine^3]-angiotensin II possessed 68% of the pressor activity of the natural angiotensin II.

The results obtained indicate that aliphatic residues, branched or straight chained, in position 3 are not biologically specific as is the case on position 5. Further, solid-phase synthesis appears to be an elegant

* From the Research Division, Cleveland Clinic Foundation, Cleveland, Ohio 44106. *Received December 8, 1966.* Supported in part by Grant HE-6835 from the National Heart Institute.

† Present address: Central Drug Research Institute, Lucknow, India.

[1] Abbreviations used: *t*-BOC, *t*-butyloxycarbonyl group; DMF, dimethylformamide; MPW, methyl ethyl ketone–pyridine–water; BAWP, *n*-butyl alcohol–acetic acid–water–pyridine; BAW, *n*-butyl alcohol–acetic acid–water.

and quick procedure to obtain biologically active peptides, and it appears that the low yields and impure products are not due to a faulty coupling but to the final step in which the peptide is removed from the polymer. As already discussed by Marshall and Merrifield (1965), exposure of peptide polymer to HBr for less than 25–30 min results in lower yields but purer products, while exposure for 1 hr or longer to obtain quantitative cleavage yields a complex mixture of products not hydrolyzable by leucine aminopeptidase despite the fact that no β-aspartic acid derivative was detectable. This step warrants further investigation to improve solid-phase peptide synthesis.

Experimental Section

t-Butyloxycarbonylamino acids were synthesized according to the procedure of Schwyzer *et al.* (1959). Purity was checked by paper and thin layer chromatography (tlc) and through optical rotation.

Solvents used for ascending paper chromatography (PC) on No. 1 Whatman filter paper and tlc were: (a) *n*-butyl alcohol–acetic acid–water (BAW) (4:1:5), (b) methyl ethyl ketone–pyridine–water (MPW) (40:10:16), and (c) *n*-butyl alcohol–acetic acid–water–pyridine (BAWP) (30:6:24:20). Tlc was carried out on Eastman Kodak silica gel chromatogram sheets type $K_{301}R_2$.

Ionophoresis was carried out on filter paper strips supplied with Beckman electrophoresis cell (Durrum type) Model R, series D, at 450 v, using formic acid–acetic acid buffer (pH 1.9) for 3 hr at room temperature. E = 1.22 Glu indicates that the substance migrated 1.22 times the distance migrated by glutamic acid. Protected or semiprotected compounds were detected by spraying the chromatograms with ninhydrin and/or diazotized sulfanilic acid. For amino acid analysis, the samples were hydrolyzed in 6 N HCl in a sealed tube (under nitrogen) at 110° for 36 hr and the analyses were performed on a Technicon amino acid Auto-Analyzer.

t-BOC-phenylalanine Polymer. A solution of 2.65 g (10 mmoles) of *t*-BOC-phenylalanine and 1.4 ml (10 mmoles) of triethylamine in 25 ml of ethanol was added to 10.00 g of the chloromethylated copolystyrene–2% divinylbenzene (Merrifield, 1963) which contained 5.02% chlorine and the mixture was stirred at 80° for 24 hr. The esterified polymer was removed by filtration, washed with ethanol and water, and dried under vacuum. The total amount of *t*-BOC-phenylalanine esterified onto the polymer was determined by weight increase (1.34 g) of the polymer and by spectrophotometric estimation of the unreacted phenylalanine in the filtrate. The esterified polymer was found to contain 0.47 mmole of *t*-BOC-phenylalanine/g of polymer (or a total of 5 mmoles of *t*-BOC-phenylalanine residue). The latter method was found to be particularly suitable for all the aromatic amino acids, and both these methods gave quantitative and comparable results and were quicker than the amino acid analysis method employed by Merrifield (1963) and Marshall and Merrifield (1965).

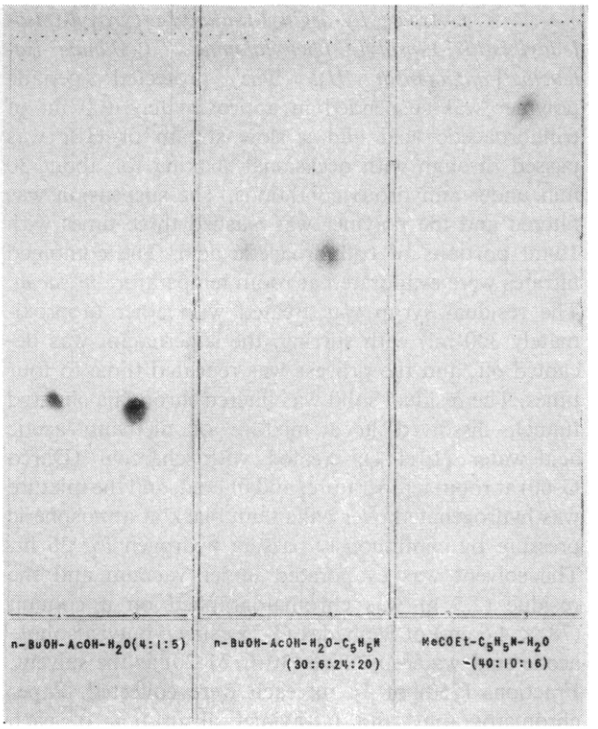

FIGURE 1: Thin layer chromatograms of [L-alanine[3]-L-isoleucine[5]]-angiotensin II in different solvent systems.

t-Butyloxycarbonyl-β-benzyl-L-aspartyl-nitro-L-arginyl-L-alanyl-O-benzyl-L-tyrosyl-L-isoleucyl-N^{im}-benzyl-L-histidyl-L-prolyl-L-phenylalanyl Polymer. *t*-BOC-phenylalanyl polymer obtained above was transferred quantitatively to a reaction vessel and the following cycle of reactions was used to introduce each new residue: (1) washed (3 × 75 ml) with glacial acetic acid, (2) *t*-BOC group was cleaved by 1 N HCl in glacial acetic acid (75 ml) for 30 min, (3) washed (3 × 75 ml) with glacial acetic acid, (4) washed (3 × 75 ml) with absolute ethanol, (5) washed (3 × 75 ml) with dimethylformamide, (6) neutralized the hydrochloride salt with 7.5 ml of triethylamine in 75 ml of dimethylformamide for 10–15 min, (7) washed (3 × 75 ml) with DMF, (8) washed (3 × 75 ml) with methylene chloride, (9) introduced 12 mmoles of the appropriate *t*-BOC-amino acid in 75 ml of methylene chloride with ice cooling and allowed to mix for 10 min, (10) introduced 12 mmoles of *N,N'*-dicyclohexylcarbodiimide in 20 ml of methylene chloride and the mixture was shaken 2 hr with cooling by ice–water mixture and then overnight at room temperature, (11) washed (3 × 75 ml) with methylene chloride, and (12) washed (3 × 75 ml) with ethanol. For N^{im}-benzyl-L-histidine and nitro-L-arginine cycles, step 8 was deleted and dimethylformamide was substituted for methylene chloride in steps 9–11. At the end of synthesis, the polymer was now transferred into a 300-ml round-bottom flask and dried overnight in a vacuum desiccator over phosphorus pentoxide.

L-*Aspartyl*-L-*arginyl*-L-*alanyl*-L-*tyrosyl*-L-*isoleucylhistidyl*-L-*prolyl*-L-*phenylalanine* ([*Alanine*^{3}-*isoleucine*^{5}]-*angiotensin II*). The protected peptide polymer was suspended in approximately 100 ml of trifluoroacetic acid and a slow stream of HBr was passed through with occasional shaking for about 30 min under anhydrous conditions. The suspension was filtered and the polymer was washed three times with 10-ml portions of trifluoroacetic acid. The combined filtrates were evaporated at room temperature *in vacuo*. The residual syrup was treated with ether (approximately 300 ml) with stirring, the supernatant was decanted off, and the process was repeated three to four times. The residual solid was filtered through a sintered funnel, dissolved in a mixture of methanol–acetic acid–water (10:1:1), treated with charcoal (Darco G-60) at room temperature, and filtered, and the mixture was hydrogenated over palladium black at atmospheric pressure by continuously passing hydrogen for 36 hr. The solvent was evaporated under vacuum and the residue (2.5 g) was chromatographed on a column (78 × 4.6 cm) of Sephadex G-25 using *n*-butyl alcohol–acetic acid–water–pyridine (30:6:24:20) as the solvent. Fractions (250) of 14 ml each were collected. Paper chromatography and ultraviolet absorption of each fraction showed four distinct compounds and fractions 35–52, 53–69, 70–100, and 200–215 were pooled and evaporated to dryness under vacuum.

The residue from fractions 70–100 (major component) was washed several times with ethanol and ethyl acetate, dissolved in 2 ml of deionized water, and filtered through Hyflo-Super-Cel, and the filtrate was reduced in volume in a rotary evaporator. An amorphous solid was precipitated from this residual syrup with ethanol to give (55% yield based on 5 mmoles of *t*-BOC-phenylalanine which was esterified on the polymer) [alanine^3-isoleucine^5]-angiotensin II, mp 203–206° dec, very hygroscopic, loss of H_2O at 100°, 4.88%, $[\alpha]_D^{23}$ −69.4° (*c* 0.5, 1 N AcOH), PC R_F BAW 0.35 and R_F BAWP 0.52, tlc R_F BAW 0.37, R_F BAWP 0.62, and R_F MPW 0.87; E = 1.22 Glu. As shown in the tlc chromatograms with minimal and three-times minimal of the sample employed, respectively, single homogeneous spots were obtained identified both with ninhydrin and diazotized sulfanilic acid reagents.

Quantitative amino acid determination gave the following molar ratio: Asp, 1.1; Arg, 1.01; Ala, 1.13; Tyr, 0.72; Ile, 1.06; His, 1.00; Pro, 1.17; Phe, 1.02 (in a separate experiment, the molar ratio of tyrosine was found to be fairly low when valyltyrosine was hydrolyzed under similar conditions). *Anal.* Calcd for $C_{48}H_{67}N_{13}O_{12}$ (1018.12): N, 17.88. Found: N, 17.70.

For enzymatic hydrolysis, 2 mg of the peptide was dissolved in 3 ml of 0.001 M $MgCl_2$ in 0.01 M $NaHCO_3$ (pH 8.1), and the solution was treated with 60 mg of partially purified leucine aminopeptidase (Schwarz and Bumpus, 1959). The mixture was triturated well with a glass rod and incubated at 37° for 48 hr. Aliquots were taken intermittently and spotted on paper and silica gel thin layers for chromatography and the chromatograms were developed in BAW and BAWP ascending systems. The results obtained indicated that considerable amount of N-terminal aspartic acid was cleaved within 1 hr of incubation and the degradation to component amino acids took place within 20 hr. The biological activity was determined by pressor assay in vagotomized, ganglion-blocked rat (Pickens *et al.*, 1965), and [alanine^3-isoleucine^5]-angiotensin II showed 68% of the pressor activity of [asparagine^1-valine^5]-angiotensin II (Hypertensin, CIBA).

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