Adrenocorticotropins. XXXIX. The Solid Phase Synthesis of Methionylglutamylhistidylphenylalanylarginyltryptophylglycine[1]

James Blake and Choh Hao Li

Contribution from the Hormone Research Laboratory, School of Medicine, University of California, San Francisco, California 94122.
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Abstract: The heptapeptide, methionylglutamylhistidylphenylalanylarginyltryptophylglycine (I), has been synthesized by the solid phase method. Cleavage of the fully protected peptide from the resin was achieved with hydrogen bromide in trifluoroacetic acid containing 1% mercaptoethanol; the product was completely deblocked with sodium in liquid ammonia to give the biologically active peptide I.

The recent successes in peptide synthesis using the solid phase method[2-4] have led us to investigate this procedure for the synthesis of a peptide related to pituitary hormones. The heptapeptide, methionylglutamylhistidylphenylalanylarginyltryptophylglycine (I), occurs in the structures of melanotropins (MSH),[5] adrenocorticotropins (ACTH),[5] and lipotropins (LPH)[6,7] as shown in Figure 1. It has been synthesized by the classical procedure[8] and shown to possess melanocyte-stimulating as well as ACTH-releasing activities. Since peptide I contains methionine, glutamic acid, histidine, arginine, and tryptophan, which are known to present problems in peptide synthesis, it was deemed of interest to synthesize I by the solid phase method.

The synthesis of peptide I followed essentially the Merrifield procedure[3] with the modifications as indicated in Figure 2. After the first cycle, deblocking was accomplished with the addition of β-mercaptoethanol to the hydrogen chloride–dioxane solution to protect tryptophan from acid oxidation.[9] The post-coupling treatment with acetic anhydride, as originally suggested by Merrifield,[2] was used to ensure complete acylation of free amine. The acetylation procedure was shown to consume 97% of free amine in 20 min; therefore, any contaminants that might arise from incomplete coupling (of the BOC amino acids) would be primarily N-acetyl peptides rather than free amine peptides differing from I only by the absence of one amino acid residue. Since N-acetyl peptides should be more readily separable from I (because of charge difference) than free amine peptides, the acetylation procedure should yield a purer product.

Cleavage of the protected heptapeptide from the resin was accomplished with hydrogen bromide in trifluoroacetic acid;[3] methyl ethyl sulfide was used as a scavenger to protect methionine from benzylation[10,11] and β-mercaptoethanol was used to protect tryptophan from acid oxidation.[9] The trifluoroacetic acid solution of partially protected heptapeptide was evaporated and submitted to countercurrent distribution in the system *n*-butyl alcohol–acetic acid–water to give two peaks in a ratio of 3:1, with partition coefficient (K) equaling 2.2 and 5.8, respectively (Figure 3). The major peak (K = 2.2) showed one ninhydrin-positive spot by thin layer chromatography on silica gel; the amino acid analysis[12] of the material recovered from the major peak gave the values $Met_{0.90}Glu_{0.89}Phe_{1.08}Arg_{0.95}Gly_{1.00}$ which are in agreement with the structure of methionylglutamyl-*im*-benzylhistidylphenylalanyl-N^G-tosylarginyltryptophylglycine (II). The yield of II based on starting BOC-Gly-OCH_2Ⓟ was 30%. Thin layer chromatography of the material in the minor peak (Figure 3; K = 5.8) showed one ninhydrin-positive spot corresponding to peptide II and a stream of ninhydrin-negative, chlorine-positive material with mobility of higher R_f value; amino acid analysis showed $Met_{0.40}Glu_{0.54}Phe_{0.88}Arg_{1.02}Gly_{1.00}$. The amino acid analysis, thin layer chromatography, and behavior in countercurrent distribution (higher K than peptide II) are all in agreement with the conclusion that the material in the minor peak is composed of N-acetyl peptides (plus peptide II).

Peptide II was treated with sodium in liquid ammonia[13] and the product was purified by carboxymethylcellulose chromatography[14] (Figure 4) to give peptide I in a 59% yield (18% over-all yield). Peptide I was shown to be pure by paper electrophoresis and thin layer chromatography on silica gel. Amino acid analysis of an acid hydrolysate and a leucine aminopeptidase digest of peptide I gave $Met_{0.90}Glu_{0.99}His_{1.01}Phe_{1.00}Arg_{0.98}Gly_{1.00}$ and $Met_{1.09}Glu_{1.06}His_{0.96}Phe_{1.01}Arg_{0.96}Try_{1.08}Gly_{1.00}$, respectively.

MSH assay by the *in vivo* procedure[15] showed that a dose of 1 μg of peptide I produced a change in

(1) (a) For paper XXXVIII, see J. Blake and C. H. Li, *Biochim. Biophys. Acta*, 147, 386 (1967). (b) All amino acids occurring in the peptide mentioned in this paper are of the L configuration with the exception of glycine.
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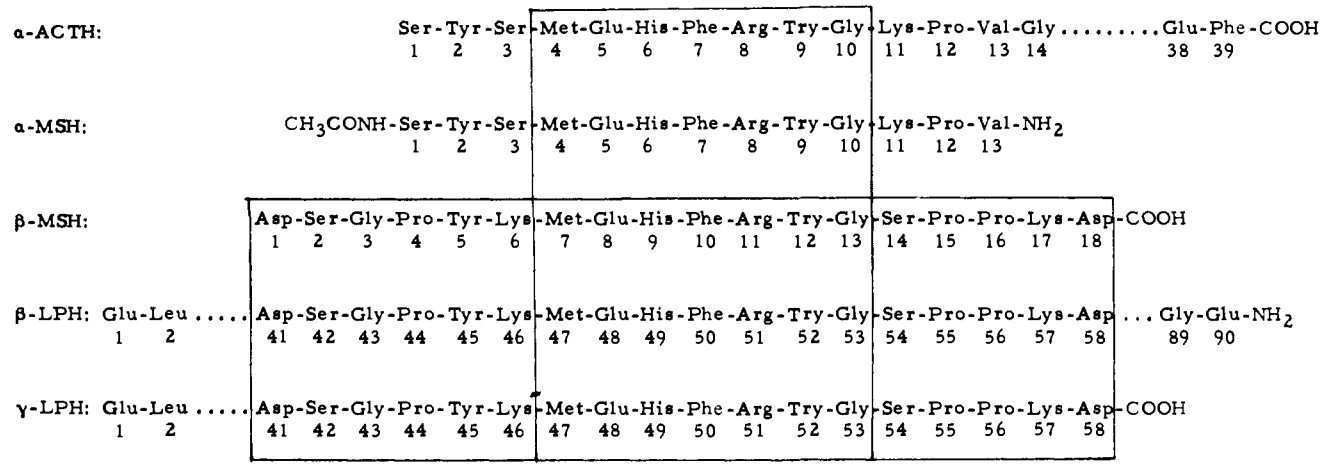

Figure 1. Structural comparison of sheep pituitary α-MSH, β-MSH, α-ACTH, β-LPH, and γ-LPH.

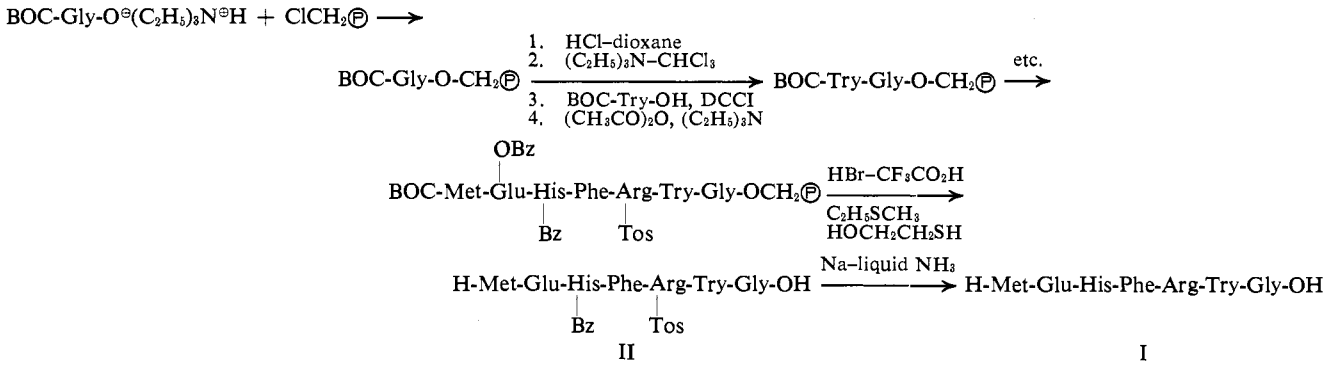

Figure 2. Outline of the synthesis of methionylglutamylhistidylphenylalanylarginyltryptophylglycine by the solid phase procedure; BOC-, *t*-butyloxycarbonyl; P, polystyrene–2% divinylbenzene copolymer; Bz, benzyl; Tos, *p*-toluenesulfonyl; DCCI, N,N′-dicyclohexylcarbodiimide.

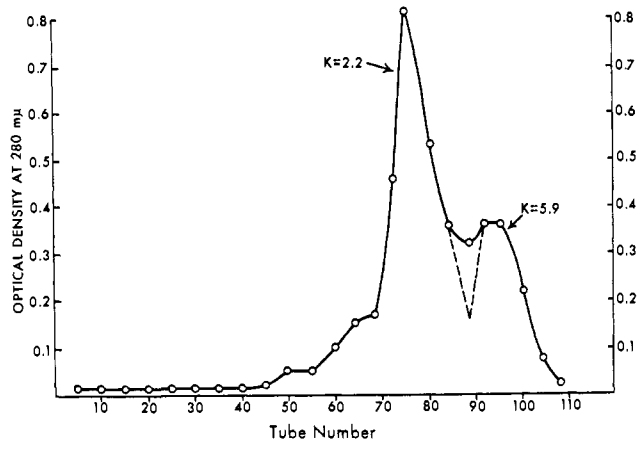

Figure 3. Countercurrent distribution of the partially protected heptapeptide II in the system *n*-butyl alcohol–acetic acid–water (4:1:5 by volume; 5-ml volume of each phase). Distribution was carried out at 24°.

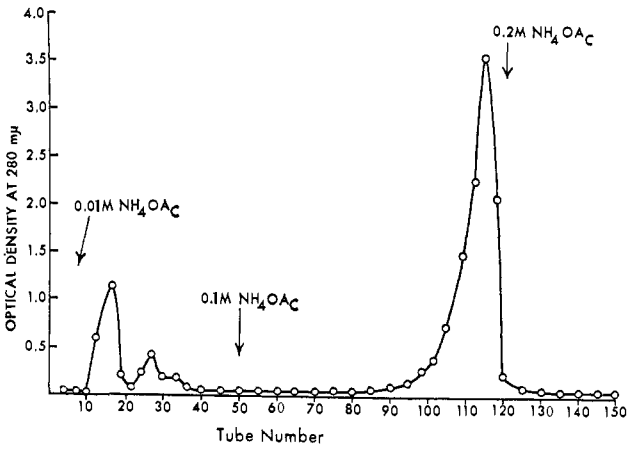

Figure 4. Carboxymethylcellulose chromatography (column, 1.4 × 55 cm) of heptapeptide I. The initial buffer was 0.01 *M* ammonium acetate, pH 4.5. After 50 tubes (4 ml/tube), a gradient with respect to pH and concentration of salt was started by introducing 0.1 *M* ammonium acetate buffer of pH 6.7 through a 500-ml mixing flask containing the starting buffer. Later, the gradient was increased by substituting 0.2 *M* ammonium acetate of pH 6.7 as the solution flowing into the mixing flask.

melanophore index in hypophysectomized *Rana pipiens* from 1+ to 3+ within 1 hr.

Experimental Section

BOC-glycyl Resin. To a solution of 4.0 g (22.9 mmol) of BOC-glycine and 3.0 ml (21.6 mmol) of triethylamine in 66 ml of anhydrous ethanol was added 15 g of Bio-beads S·X-2, 200–400 mesh, chloromethylated[16] at 0.72 mequiv/g. The mixture was stirred under reflux for 22 hr and filtered, and the resin was exhaustively washed

(16) Commercially available from Bio-Rad Laboratories, Richmond, Calif.

with ethanol, water, and methanol. A sample of the dried BOC-glycyl resin was hydrolyzed in 1:1 dioxane–constant-boiling HCl, and amino acid analysis showed the glycine content to be 0.31 mmol/g.

BOC-methionyl-γ-benzylglutamyl-*im*-benzylhistidylphenylalanyl-N^G-tosylarginyltryptophylglycyl Resin. A portion of BOC-glycyl resin (1.72 g, 0.53 mmol of glycine) was treated by the following steps: (1) three washings with 10-ml portions of dioxane; (2) cleavage of the BOC group by addition of 8.5 ml of 5.8 *N* HCl–dioxane and shaking for 40 min;[17] (3) three washings with 10-ml portions of dioxane; (4) three washings with 8-ml portions of ethanol; (5) three washings with 8-ml portions of chloroform; (6) neutralization of the hydrochloride with 8 ml of chloroform and 1 ml of triethylamine for 10 min; (7) three washings with 10-ml portions of chloroform; (8) three washings with 10-ml portions of dichloromethane; (9) addition of 2.0 mmol of BOC-amino acid in 6 ml of dichloromethane and shaking for 10 min; (10) addition of 2.1 mmol of N,N′-dicyclohexylcarbodiimide[18] in 2 ml of dichloromethane and shaking for 2 hr; (11) three washings with 8-ml portions of dimethylformamide; (12) acetylation by addition of 0.5 ml of acetic anhydride, 0.3 ml of triethylamine, and 8 ml of dimethylformamide, and shaking for 20 min; (13) three washings with 8-ml portions of dimethylformamide; (14) one washing with 8 ml of ethanol; (15) three washings with 8-ml portions of acetic acid; (16) three washings with 8-ml portions of ethanol.

After the first cycle, 0.1 ml of β-mercaptoethanol was added to the HCl–dioxane solution. For BOC-Try-OH, BOC-N^G-Tos-Arg-OH,[19,20] and BOC-Im-Bz-His-OH,[20] 5% dimethylformamide–dichloromethane, 10% dimethylformamide–dichloromethane, and 100% dimethylformamide, respectively, were used for solution of the BOC amino acid. For BOC-*im*-Bz-His-OH, 2.5 mmol was used for coupling. After six cycles, the resin was dried under vacuum.

Methionylglutamylhistidylphenylalanylarginyltryptophylglycine (I). The above protected heptapeptide resin (1.07 g, 0.24 mmol of peptide) was suspended in 8 ml of trifluoroacetic acid, 1 ml of methyl ethyl sulfide, and 0.1 ml of β-mercaptoethanol. Anhydrous hydrogen bromide was bubbled into this suspension, while shaking, for 30 min. The mixture was filtered, the resin was washed with three 7-ml portions of trifluoroacetic acid, and the combined filtrate was evaporated to a residue which was submitted to countercurrent distribution for 100 transfers in the system consisting of *n*-butyl alcohol–acetic acid–water (4:1:5 by volume). The major peak, $K = 2.2$, tubes 68–84 (Figure 3), was isolated and evaporated to 88 mg (0.073 mmol, 30%) of a glassy residue, methionylglutamyl-*im*-benzylhistidylphenylalanyl-N^G-tosylarginyltryptophylglycine (II). Thin layer chromatography on silica gel (*n*-butyl alcohol–acetic acid–water, 4:1:1) showed one ninhydrin-positive spot at R_f 0.4. Amino acid analysis of an acid hydrolysate showed $Met_{0.90}Glu_{0.89}Phe_{1.08}Arg_{0.95}Gly_{1.00}$.

Peptide II was dissolved in 100 ml of ammonia, freshly distilled from sodium. Small pieces of sodium were added until a permanent blue color remained for 30 min. The solution was evaporated to dryness, and the residue was desalted on IRC-50 resin and eluted with pyridine–acetic acid–water (30:4:66). The lyophilized crude heptapeptide was purified by carboxymethylcellulose chromatography, using ammonium acetate gradient elution, to yield, after three lyophilizations, 42 mg of heptapeptide I (peptide content 98%, 0.043 mmol, 59% yield from peptide II), $[\alpha]^{25}D$ −20° (*c* 0.8, 0.1 *N* acetic acid).

Paper electrophoresis in pyridine acetate buffer (pH 3.7, 400 V, 4 hr) showed one ninhydrin-positive, Pauly-positive spot at a mobility relative to lysine, R_f 0.58. Thin layer chromatography on silica gel (*n*-butyl alcohol–acetic acid–water, 4:1:1 by volume) showed one ninhydrin-positive spot at R_f 0.1. Amino acid analysis of an acid hydrolysate and leucine aminopeptidase digest (pH 8, 24 hr, 37°) gave $Met_{0.90}Glu_{0.99}His_{1.01}Phe_{1.00}Arg_{0.98}Gly_{1.00}$ and $Met_{1.09}Glu_{1.06}His_{0.96}Phe_{1.01}Arg_{0.96}Try_{1.08}Gly_{1.00}$, respectively.

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(17) After filtration of the dioxane washes, the resin holds *ca.* 5 ml of dioxane which dilutes the added HCl to an effective concentration of 3.65 *N* in the dioxane–resin mixture.

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