Journal of Medicinal Chemistry

VOLUME 15, NUMBER 7 JULY 1972

Solid-Phase Synthesis of Seryltyrosylserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycine Hydrazide and Its N^{α}-*tert*-Butyloxycarbonyl Derivative and Their Melanotropic Activity

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Two melanotropically active decapeptides, Ser-Tyr-Ser-Met-Gln-His-Phe-Arg-Trp-Gly-$NHNH_2$ (III) and its Boc derivative (II), have been synthesized by the solid-phase method. Cleavage of the fully protected peptide from the resin was achieved with hydrazine in dimethylformamide. All the protecting groups except the NH_2-terminal Boc group were removed with sodium in liquid ammonia to give peptide II. The Boc group in peptide II was removed with trifluoroacetic acid to obtain peptide III.

The decapeptides seryltyrosylserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycine hydrazide (III) and its N^{α}-Boc† derivative (II) are analogs of a decapeptide[1,2] occurring in α-MSH and ACTH (see Figure 1). The synthesis of the decapeptide, Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly, α^{1-10}-ACTH, has been achieved earlier by classical techniques.[3,4] We report herein the synthesis of peptides II and III by the solid-phase method.[5]

Figure 2 presents an outline of the synthetic scheme which followed essentially the Merrifield procedure[5] as described previously.[6,7] After the first cycle, removal of the Boc group was effected by HCl–dioxane solution containing 1% β-mercaptoethanol to protect tryptophan from acid oxidation as suggested by Marshall.[8] The postcoupling reaction with acetic anhydride[9] was used to ensure complete acylation of free α-amino groups. Cleavage of the protected decapeptide from resin was accomplished with hydrazine in dimethylformamide.[10] Protected decapeptide was submitted to countercurrent distribution in the solvent system, acetic acid–chloroform–0.01 *M* ammonium acetate (8:7:4, by volume), which gave one minor and one major peak with partition coefficients of 0.02 and 0.19, respectively, as shown in Figure 3. The major peak, which represents the desired product (I, Figure 2) gave a single spot in thin-layer chromatography on silica gel which was positive to the chlorine test[11] but negative to the ninhydrin as expected. The amino acid composition of the material, as analyzed according to the procedure of Spackman, *et al.*,[12] agreed with the theoretical values.[3] The yield of I based on starting Boc-Gly-OCH_2-R was 33%. Peptide I was treated with sodium in liquid ammonia[13] and the product was purified by chromatography on carboxymethylcellulose[14] (Figure 4) to give peptide II with 46% yield. Peptide II was shown to be homogenous by paper electrophoresis and amino acid analysis.

Peptide II was treated with trifluoroacetic acid and the product was submitted to chromatography on carboxymethylcellulose. As shown in Figure 5, it gave essentially a single peak; the recovered material (III) gave a single spot in paper electrophoresis and thin-layer chromatography on silica gel. In addition, digestion of III with chymotrypsin gave the four expected peptide fragments as revealed by paper electrophoresis: Ser-Tyr, Ser-Met-Glu-His-Phe, Arg-Trp, Gly-$NHNH_2$. Reaction of the chymotryptic digest with leucineaminopeptidase liberated all amino acids as expected.

Bioassay data for melanocyte stimulating activity *in vivo*[15] in hypophysectomized *Rana pipiens* and *in vitro*[16] using frog skins are summarized in Table I. It may be noted in the *in vivo* assay that 1 μg of III produced a change of melanophore index from 1+ to 3+ within 1 hr whereas II required only 0.1 μg. Apparently acylation of NH_2-terminal serine with the Boc group increases activity tenfold, to nearly the potency of ACTH. This difference was also observed by the *in vitro* assay. In comparison with α^{1-10}-ACTH, it is evident that replacement of the carboxyl group with a hydrazide group does not change the *in vitro* MSH potency but enhances the *in vivo* activity. On the other hand, acylation of the NH_2-terminal serine with the Boc group increases both the *in vivo* and *in vitro* activity. The observation is in agreement with an earlier report[17] that deacylation of α-MSH decreases melanotropic activity.

Experimental Section

Boc-*O*-benzylseryl-*O*-benzyltyrosyl-*O*-benzylserylmethionylglutaminyl-*im*-benzylhistidylphenylalanyl-N^{G}-tosylarginyltryptophylglycyl Resin. Boc-glycyl resin[6] (1.88 g; 0.84 mmole of glycine) was treated by the following steps: (1) three washings with 15-ml portions of dioxane; (2) cleavage of the Boc group by addition of 5.8 *N* HCl–dioxane to make a final concentration of 3.7 *N* with the dioxane which was held up by the resin, and shaking for 20 min in

†Abbreviations: Boc, *tert*-butyloxycarbonyl; MSH, melanocyte-stimulating hormone, melanotropin; ACTH, adrenocorticotropic hormone. All amino acids occurring in the peptides mentioned in this paper are of the L configuration with the exception of glycine.

ACTH: H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys . . . Phe-COOH
1 2 3 4 5 6 7 8 9 10 11 39

α-MSH: Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2
1 2 3 4 5 6 7 8 9 10 11 12 13

II: BOC-Ser-Tyr-Ser-Met-Gln-His-Phe-Arg-Trp-Gly-$NHNH_2$
1 2 3 4 5 6 7 8 9 10

III: H-Ser-Tyr-Ser-Met-Gln-His-Phe-Arg-Trp-Gly-$NHNH_2$
1 2 3 4 5 6 7 8 9 10

Figure 1. Structural comparison of ACTH, α-MSH, peptides II and III.

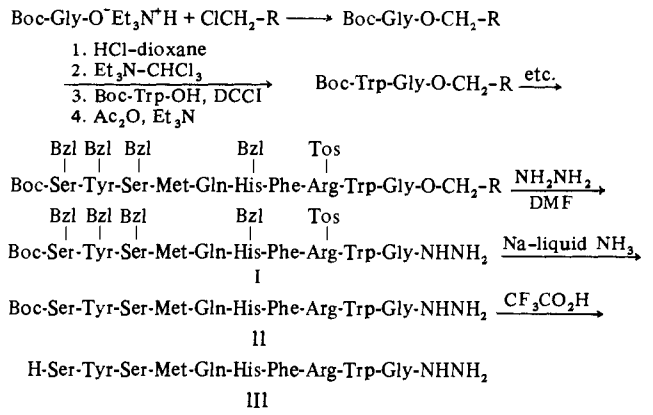

Figure 2. Outline of the synthesis of decapeptide hydrazide (III) and N^{α}-BOC-decapeptide hydrazide (II) by solid phase procedure; R, polystyrene-2% divinylbenzene resin; Bzl, benzyl; Tos, *p*-toluenesulfonyl; DCCI, *N,N'*-dicyclohexylcarbodimide; DMF, dimethylformamide.

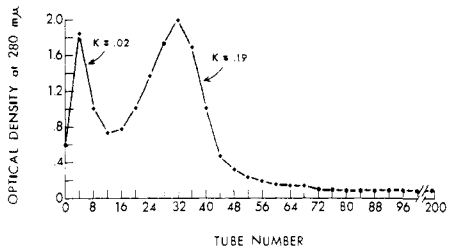

Figure 3. Countercurrent distribution of the partially protected decapeptide hydrazide. Solvent system, acetic acid-chloroform-0.01 *M* ammonium acetate (8:7:4 by volume); 5 ml in each phase; 24°.

the first cycle and 30 min for the succeeding cycles; (3) three washings with 15-ml portions of dioxane; (4) three washings with 15-ml portions of absolute ethanol; (5) three washings with 15-ml portions of chloroform; (6) neutralization of the hydrochloride salt with 15 ml of chloroform and 1.5 ml of triethylamine for 10 min; (7) three washings with 15-ml portions of chloroform; (8) three washings with 15-ml portions of dichloromethane; (9) addition of 3.5 mmoles of Boc amino acid in 9 ml of dichloromethane and shaking for 10 min; (10) addition of 3.5 mmoles of *N,N'*-dicyclohexylcarbodiimide[18] in 6 ml of dichloromethane and shaking for 5 hr; (11) three washings with 15-ml portions of dimethylformamide; (12) acetylation by addition of 0.6 ml of acetic anhydride and 0.4 ml of triethylamine in 15 ml of dimethylformamide and shaking for 20 min; (13) three washings with 15-ml portions of dimethylformamide; (14) one washing with a 15-ml portion of absolute ethanol; (15) three washings with 15-ml portions of acetic acid; (16) three washings with 15-ml portions of ethanol.

After the first cycle, 0.15 ml of β-mercaptoethanol was added to the HCl–dioxane solution. The Boc group of glutamine was removed by the action of 50% trifluoroacetic acid in dichloromethane for 15 min. Glutamine *p*-nitrophenyl ester was employed, and the coupling time for this amino acid was 24 hr. For Boc-Trp-OH, Boc-N^{G}-Tos-Arg-OH,[19,20] Boc-*im*-Bzl-His[20]-OH, and Boc-Gln-ONP, 5% dimethylformamide–dichloromethane, 10% dimethylformamide–dichloromethane, and for the last two amino acids 100% dimethylformamide, respectively, were used for the solution of Boc amino acid. For Boc-*im*-Bzl-His-OH and Boc-Gln-ONP, 7 mmoles was used for coupling. After nine cycles, the resin was dried under vacuum.

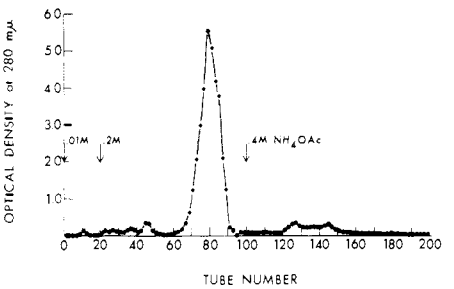

Figure 4. Chromatography of peptide II on carboxymethylcellulose column (1 × 55 cm). The initial buffer was 0.01 *M* ammonium acetate, pH 4.6. After 20 tubes (5 ml/tube), a gradient with respect to pH and concentration of salt was started by introducing 0.2 *M* ammonium acetate buffer of pH 6.7 through a 500-ml mixing flask containing the starting buffer. Later, the gradient was increased by substituting 0.4 *M* ammonium acetate of pH 6.7 as the solution flowing into the mixing flask.

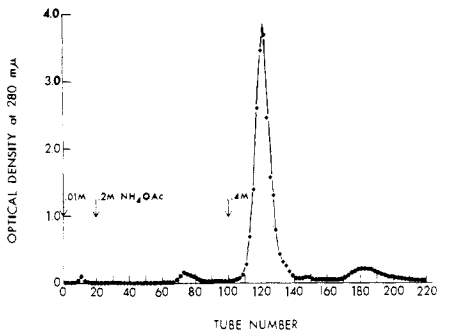

Figure 5. Chromatography of peptide III on carboxymethylcellulose column (1 × 55 cm). Conditions were the same as in Figure 4.

Table I. Melanotropic Activity of Ovine ACTH, α-MSH, and Synthetic Peptides

Peptide	Melanocyte stimulating activity	
	in vitro, units/g	*in vivo*, μg[a]
Ovine ACTH	2×10^{7}	0.1
α-MSH	1×10^{10}	0.01
α^{-1-10}-ACTH	1×10^{6}	5
II	1×10^{7}	0.1
III	1×10^{6}	1

[a] The dose produces a change in melanophore index in hypophysectomized *Rana pipiens* from 1+ to + within 1 hr.

N^{α}-Boc-*O*-benzylseryl-*O*-benzyltyrosyl-*O*-benzylserylmethionylglutaminyl-*im*-benzylhistidylphenylalanyl-N^{G}-tosylarginyltryptophylglycine Hydrazide (I). The above protected decapeptide resin was suspended in a solution of 15 ml of dimethylformamide plus 1 ml of hydrazine. After stirring vigorously for 24 hr at room temperature, the mixture was filtered and the resin residue was washed with three 10-ml portions of dimethylformamide. The combined filtrate was evaporated to a small volume; the protected peptide was precipitated by addition of water. The precipitate was collected and dried under vacuum. The crude product was subjected to countercurrent distribution for 200 transfers (Figure 3) in a system consisting of acetic acid-chloroform-0.01 *M* ammonium acetate (8:7:4 by volume). The two peaks [major peak (K = 0.19, tubes 18–52) and minor peak (K = 0.02, tubes 1–10)] were isolated and evaporated to obtain respectively 672 and 157 mg of peptides.

Amino acid analysis[12] of the major peak (peptide I) showed: $Ser_{1.82}Tyr_{0.90}Met_{0.83}Glu_{1.0}Phe_{1.03}Arg_{1.12}Gly_{0.93}$. Elementary analysis of the material was carried out. *Anal.* ($C_{99}H_{119}N_{19}O_{18}S_2$ (1927): C, 61.7; H, 6.2; N, 13.8. Found: C, 60.9; H, 6.4; N, 13.5

N^{α}-Boc-seryltyrosylserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycine Hydrazide (II). Protected decapeptide hydrazide (I) (200 mg) was dissolved in 250 ml of ammonia, freshly distilled from sodium. Small pieces of sodium were added until a permament blue color remained for 20 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 Boc-decapeptide hydrazide was purified by chromatography on carboxymethylcellulose using gradient elution with ammonium acetate to yield, after four lyophilizations, 82 mg of Boc-decapeptide hydrazide (II).

Paper electrophoresis of II in pyridine–acetate buffer (pH 3.7, 400 V, 5 hr) showed one ninhydrin-negative, Pauly-positive spot at a mobility relative to lysine, R_f 0.41. In collidine acetate buffer (pH 6.9, 400 V, 5 hr), there was also only one ninhydrin-negative, Pauly-positive spot with a mobility relative to lysine, R_f 0.39. Thin-layer chromatography of II on silica gel (*n*-butyl alcohol–acetic acid–water, 4:1:1 by volume, and *n*-butyl alcohol–acetic acid–water–pyridine, 15:3:12:10 by volume) showed one ninhydrin-negative, Pauly-positive spot at R_f 0.1 and 0.58, respectively.

Amino acid analysis of II gave: $Ser_{1.64}Tyr_{0.97}Met_{1.01}Glu_{1.00}His_{0.93}Phe_{0.96}Arg_{1.00}Gly_{0.92}$. Chymotrypsin digestion (enzyme–substrate ratio, 1/50, w/w; pH 8.5 Tris buffer, 37°, 24 hr) of the peptide gave four fragments as revealed by paper electrophoresis in pyridine acetate buffer (pH 3.7, 400 V, 5 hr). One of the fragments was a negatively charged, ninhydrin-negative, Ehrlich-negative, Pauly-positive spot at a mobility relative to lysine, R_f 0.22, which is probably *tert*-butyloxycarbonylseryltyrosine. The other three were positively charged spots: a ninhydrin-positive, Pauly-negative, Ehrlich-negative spot; a ninhydrin-positive, Pauly-positive, Ehrlich-negative spot; and a ninhydrin-positive, Pauly-negative, Ehrlich-positive spot. The relative mobility of these three spots to lysine were respectively R_f 0.11, 0.46, and 0.63, which correspond respectively to glycine hydrazide, serylmethionylglutaminylhistidylphenylalanine, and arginyltryptophan.

Seryltyrosylserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycine Hydrazide (III). The above Boc-decapeptide hydrazide (II, 81 mg) was dissolved in 10 ml of trifluoroacetic acid and stored under nitrogen for 30 min. Trifluoroacetic acid was removed under vacuum, the residue was dissolved in small amounts of acetic acid, and the product was precipitated by ethyl ether; the peptide was collected and washed with ether and dried. The peptide purified by carboxymethylcellulose chromatography using ammonium acetate gradient elution (Figure 5) yielded, after 4 lyophilizations, 47 mg of III.

Paper electrophoresis of III in pyridine acetate buffer (pH 3.7, 400 V, 5 hr) showed one ninhydrin-positive, Pauly-positive spot at a mobility relative to lysine, R_f 0.68. Paper electrophoresis of III in collidine acetate buffer (pH 6.9, 400 V, 5 hr) showed one ninhydrin-positive, Pauly-positive spot at a mobility relative to lysine, R_f 0.57. Thin-layer chromatography on silica gel (*n*-butyl alcohol–acetic acid–water, 4:1:1 by volume, and *n*-butyl alcohol–acetic acid–water–pyridine, 15:3:12:10 by volume) showed one ninhydrin-positive, Pauly-positive spot at R_f values of 0.04 and 0.51, respectively. Amino acid analysis of an acid hydrolysate of III gave: $Ser_{1.60}Tyr_{0.99}Met_{1.00}Glu_{1.00}His_{1.04}Phe_{0.99}Arg_{1.10}Gly_{0.95}$.

Chymotryptic digestion (24 hr, 37°) of III gave four fragments which were identified by paper electrophoresis in pyridine acetate buffer (pH 3.7, 400 V, 5 hr): (1) one ninhydrin-positive, Pauly-negative, and Ehrlich-negative spot; (2) one ninhydrin-positive, Pauly-positive, and Ehrlich-negative spot; (3) one ninhydrin-positive, Pauly-positive, and Ehrlich-negative spot; and (4) one ninhydrin-positive, Pauly-negative, and Ehrlich-positive spot. The respective mobilities (R_f) of these fragments relative to lysine were 0.11, 0.19, 0.46, and 0.63 which correspond respectively to glycine hydrazide, seryltyrosine, serylmethionylglutaminylhistidylphenylalanine, and arginyltryptophan. After inactivation of chymotrypsin at 100° for 15 min, the mixture was digested with leucine aminopeptidase (enzyme–substrate ratio, 1/20, w/w; pH 8.5 Tris buffer containing 0.01 *M* $MgCl_2$, 37°, 48 hr); the total digest was submitted to amino acid analysis and gave the following values: $(Ser + Gln)_{2.55}Tyr_{1.00}Met_{1.00}His_{0.81}Phe_{0.73}Arg_{1.00}Trp_{1.00}Gly_{0.90}$.

Biological Assays. The melanocyte-stimulating activities were determined by the procedure described by Shizume, *et al.*,[16] using isolated skins of *Rana pipiens*. *In vivo* activities were measured with hypophysectomized *Rana pipiens* (not more than 4 days after operation) as described by Hogben and Slome.[15] The data in Table I give also the potency for α_S-ACTH,[21] synthetic α^{1-10}-ACTH, and α-MSH.[22] Both α_S-ACTH and α^{1-10}-ACTH were assayed alongside with peptide II and III. The value for α-MSH was taken from ref 22.

Acknowledgment. We thank Drs. J. Blake and D. Yamashiro for helpful suggestions. This work was supported in part by grants from the National Institutes of U. S. Public Health Research Service (GM-2907).

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