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Adrenocorticotropin. 40. The Synthesis of a Protected Nonapeptide and of a Biologically Active Nonadecapeptide Related to Adrenocorticotropic Hormone. [5-Glutamine]adrenocorticotropin-(1-19)†

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The solid phase synthesis of a protected nonapeptide I, H-Lys(Tos)-Pro-Val-Gly-Lys(Tos)-Lys(Tos)-Arg(Tos)-Arg(Tos)-Pro-OH, is described. Peptide I was coupled with a previously described protected decapeptide hydrazide. After the removal of the protecting groups, and purification by repeated ion-exchange chromatography, a biologically active nonadecapeptide, H-Ser-Tyr-Ser-Met-Gln-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-OH, corresponding to the glutamine analog of the first 19 amino acid residues in the ACTH molecule is obtained.

The nonadecapeptide, Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro, corresponding to the first 19 amino acid residues in the adrenocorticotropin (ACTH) molecule,[1] has previously been synthesized[2] by conventional procedures; the synthesis of its glutamine analog has also been described in preliminary reports.[2,3] This paper describes the synthesis of the glutamine analog (designated as [5-glutamine]-$\alpha^{1\text{-}19}$-ACTH) by coupling the protected nonapeptide I, H-Lys(Tos)-Pro-Val-Gly-Lys(Tos)-Lys(Tos)-Arg(Tos)-Arg(Tos)-Pro-OH with the protected decapeptide hydrazide II, Boc-Ser(Bzl)-Tyr(Bzl)-Ser(Bzl)-Met-Gln-His(Bzl)-Phe-Arg(Tos)-Trp-Gly-$NHNH_2$, by the azide procedure. Both I and II were synthesized by the solid phase method.[4,‡] The synthesis of II has been previously described.[6] The synthesis of I is described herein.

The synthesis of the fully protected nonapeptide I followed essentially the Merrifield procedure[4] with some modifications[7] as indicated in Figure 1. Peptide I was cleaved from the resin with HBr in F_3CCO_2H. After evaporation, the partially protected nonapeptide was submitted to countercurrent distribution in the system AcOH-$CHCl_3$-0.01 *M* NH_4OAc (7:7:4, v/v) to give one major peak with partition coefficient (K) of 0.70. The recovered material from the major peak showed one ninhydrin-positive spot by tlc on silica gel; amino acid analysis[8] gave the values $Lys_{2.5}$ $Pro_{2.1}$ $Val_{1.0}Gly_{1.0}Arg_{2.0}$ which are in agreement with the structure of I. The yield of I based on starting Boc-Pro-OCH_2-Ⓡ was 36%.

For the synthesis of the protected nonadecapeptide III (see Figure 2) the reaction of I with an excess of protected decapeptide hydrazide[6] II was carried out in DMF by the azide procedure. The reaction product (crude nonadecapeptide III) was washed with MeOH to remove unreacted nonapeptide I; to remove all protecting groups the remaining mixture was first treated with F_3CCO_2H and then with Na in liq NH_3.[9] The nonadecapeptide IV was isolated by desalting on Amberlite IRC-50 resin[10] followed by chromatography on CM-cellulose[11] using continuous gradient elution with NH_4OAc. Rechromatography on CM-cellulose gave IV in 8% yield (based on I).

Peptide IV was found to be homogeneous by paper electrophoresis and tlc on silica gel. The amino acid composition

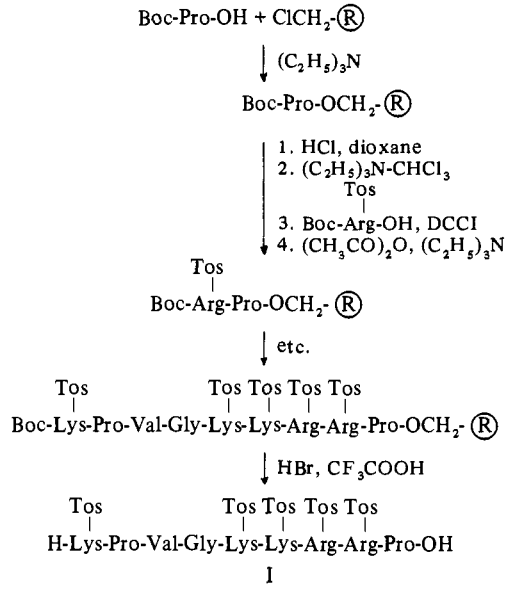

Figure 1. An outline for the solid-phase synthesis of protected nonapeptide I.

†For paper XXXIX, see reference 8; all amino acids occurring in the peptide mentioned in this paper are of the L configuration with the exception of glycine.

‡The coupling of 2 blocked fragments, both of which were synthesized by the solid-phase method, has recently been reported by Visser, *et al.*[5]

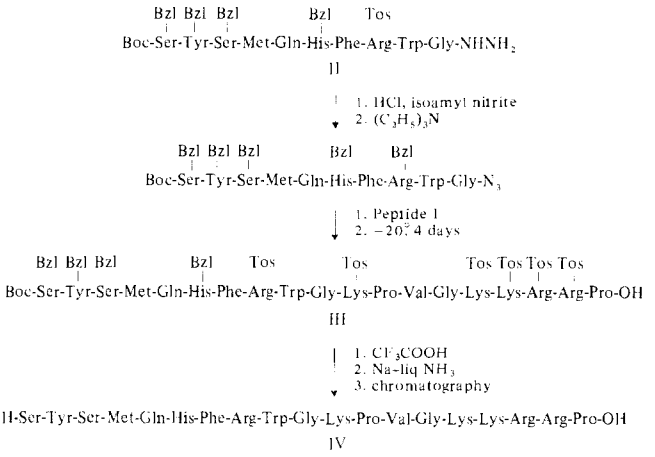

Figure 2. An outline for the synthesis of nonadecapeptide IV.

of an acid hydrolysate of IV was determined by the chromatographic method[8] and gave values (Table I) as theoretically expected. Peptide IV was submitted to enzymatic digestions with a combination of chymotrypsin, trypsin, and leucine aminopeptidase; amino acid analysis[8] of the digest gave values in good agreement with the theory (Table I) except for Arg, Lys, and Pro which were low as expected from the specificity of the enzymes. It is important to note that the analysis showed the presence of one Trp residue in IV.

Adrenal-stimulating potency of IV was assayed by a modified *in vivo* procedure[12] and found to exhibit 31 units/mg when compared with the natural hormone. By the frog skin[13] assay *in vitro*, peptide IV was shown to possess an MSH activity of 1×10^7 units/g. Furthermore, in hypophysectomized frog assay[14] *in vivo*, a dose of 0.1 μg of peptide IV produced a change in melanophore index from 1+ to 3+ within 1 hr. The biological activities of IV are summarized and compared with natural[15] α_S-ACTH and synthetic[16] α^{1-19}-ACTH in Table II. It may be seen that [5-glutamine]-α^{1-19}-ACTH (IV) is about one-third as active as α^{1-19}-ACTH in adrenal steroidogenic effect but possesses the same MSH potency as α^{1-19}-ACTH. Apparently, a substitution of glutamine in position 5 for Glu decreases adrenocorticotropic activity and does not alter the intrinsic melanotropic activity.

Table I. Amino Acid Composition of the Synthetic α^{1-19}-ACTH

Amino acid	Acid hydrolysate	Enzymic digest	Theoretical
Ser	1.9	3.0	2
Glu-NH_2	0		1
Tyr	1.0	1.0	1
Meth	1.1	0.9	1
Glu	1.1	0	0
His	1.0	0.8	1
Phe	1.0	0.8	1
Arg	2.8	2.3	3
Trp	0	1.0	1
Gly	2.1	1.8	2
Lys	2.8	2.3	3
Pro	2.0	0.6	2
Val	1.0	1.0	1

Table II. Biological Activities of α^{1-19}-ACTH and [5-Glutamine]-α^{1-19}-ACTH

Biological effect	α_S-ACTH	α^{1-19}-ACTH	[5-Glutamine]-α^{1-19}-ACTH
In vivo adrenal steroidogenesis, units/mg	100	106.8[b]	31.2[c]
In vivo MSH activity, units/g	1×10^7	1×10^7	1×10^7
In vivo MSH activity,[a] μg	0.1	0.1	0.1

[a] The dose produces a change in melanophore index in hypophysectomized *Rana pipiens* of from 1+ to 3+ within 1 hr. [b] Four point assay: λ = 0.12 with 95% confidence limit of 71.2–145.2. [c] Four point assay: λ = 0.16 with 95% confidence limit of 19.4–47.7.

Experimental Section

Boc-N^{ϵ}-TsLys-Pro-Val-Gly-N^{ϵ}-TsLys-N^{ϵ}-TsLys-N^{G}-TsArg-N^{G}-TsArg-Pro-resin. Boc-Pro resin (2.1 g) contg 0.4 mmoles/g of Pro (prepd from Bio-beads 5X-2, 200–400 mesh by standard procedures[4]) was treated for the incorporation of each amino acid by the following steps: (1) 3 washings with 15-ml portions of dioxane; (2) cleavage of the Boc group by the addn of 5.8 *M* HCl–dioxane to make a final concn of 3.7 *M*; the mixt was shaken for 20 min in the first cycle and 30 min in the subsequent cycles; (3) 3 washings with 15-ml portions of dioxane; (4) 3 washings with 15-ml portions of abs EtOH; (5) 3 washings with 15-ml portions of $CHCl_3$; (6) neutralization of the HCl salt with 15 ml of $CHCl_3$ and 1.5 ml of Et_3N for 10 min; (7) 3 washings with 15-ml portions of $CHCl_3$; (8) 3 washings with 15-ml portions of CH_2Cl_2; (9) addn of 3.2 mmoles of Boc-amino acids in 9 ml of CH_2Cl_2 and shaking for 10 min; (10) addn of 3.2 mmoles of dicyclohexylcarbodiimide[17] in 6 ml of CH_2Cl_2 and shaking for 5 hr; (11) 3 washings with 15-ml portions of DMF; (12) acetylation by addn of 0.6 ml of Ac_2O and 0.4 ml of Et_3N in 15 ml of DMF and shaking for 20 min; (13) 3 washings with 15 ml of DMF; (14) 1 washing with a 15-ml portion of abs EtOH; (15) 3 washings with 15-ml portions of AcOH; (16) 3 washings with 15-ml portions of abs EtOH. DMF–CH_2Cl_2 (1:9) was used to dissolve Boc-Arg (Tos)-OH.[18] After 8 cycles, the resin was dried under vacuum (yield, 3.15 g).

N^{ϵ}-TsLys-Pro-Val-Gly-N^{ϵ}-TsLys-N^{ϵ}-TsLys-N^{G}-TsArg-N^{G}-TsArg-Pro (I). The above protected nonapeptide resin was suspended in 16 ml of (1:1) F_3CCO_2H–CH_2Cl_2 and 1 ml of anisole. Anhyd HBr was bubbled into this suspension, while shaking for 1 hr. The mixt was filtered, the resin was washed with two 10-ml portions of F_3CCO_2H, and the combined filtrate was evapd to a residue which was submitted to countercurrent distribution for 100 transfers in AcOH–$CHCl_3$–0.01 *M* NH_4OAc (7:7:4, v/v). The material from the major peak (tubes 31–55) with $K = 0.70$ was isolated by evapn to yield 615 mg (36%) of peptide I. Tlc on silica gel in 3 solvent systems [*n*-BuOH–AcOH–H_2O (4:1:1, v/v), pyridine–AcOH–H_2O (50:30:15, v/v) and *n*-BuOH–pyridine–AcOH–H_2O (15:10:3:12, v/v)] gave single ninhydrin-positive spots with R_f values of 0.40, 0.90, and 0.62, respectively. Amino acid analysis of an acid hydrolysate showed $Lys_{2.5}Pro_{2.1}Val_{1.0}Gly_{1.0}Arg_{2.0}$.

H-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-OH. Peptide I (100 mg) was dissolved in 150 ml of liq NH_3, freshly dist twice from Na. Small pieces of Na were added until a permanent blue color remained for 10 min. The soln was evapd to dryness, and the peptide was dissolved in 0.1 *M* of AcOH. For desalting the soln was placed on an Amberlite IRC-50 column (3 × 10 cm) and eluted with pyridine–AcOH–H_2O (30:4:66, v/v). The lyophilized crude nonapeptide was purified by CM-cellulose chromatog, using NH_4OAc gradient elution,[16] to yield, after 3 lyophilizations, 22.4 mg of the free nonapeptide. Paper electrophoresis of the free peptide in pyridine acetate buffer of pH 3.7 for 5 hr at 400 V showed one ninhydrin-positive spot with a mobility relative to lysine of R_f 1.19. Amino acid analysis of an acid hydrolysate gave $Lys_{3.2}Pro_{2.0}Val_{1.0}Gly_{1.0}Arg_{2.1}$.

H-Ser-Tyr-Ser-Met-Gln-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-OH, IV. II[6] (410 mg, 212 μmoles) was dissolved in 5 ml of DMF and cooled to −20°. To the cold soln of II, 0.25 ml of HCl (4.7 *N*) in THF and 0.037 ml *i*-AmONO were added; it was stored under N_2 for 30 min and then cooled to −40° and neutralized with Et_3N. A solution of I (321 mg, 175 μmoles) in 3 ml of DMF was added. The temp of the mixt was slowly raised to −20°. The mixt was stored at this temp for 4 days. After evapn of the solvents the product was isolated by trituration with H_2O and dried *in vacuo*. Tlc of the product on silica gel in the solvent systems BuOH–AcOH–H_2O (4:1:1, v/v) and BuOH–pyridine–AcOH–H_2O (15:10:3:12, v/v) gave 1 ninhydrin-positive and 2 ninhydrin-negative spots which correspond to peptides I, II, and III, respectively. The nonapeptide derivative I was removed by extn with MeOH. Tlc of the MeOH–

insol material on silica gel in the BuOH-AcOH-H_2O (4:1:1, v/v) system gave 2 ninhydrin-negative and Cl-positive spots with R_f values of 0.60 and 0.36 which corresponds to peptides II and III.

The MeOH-insol material (270 mg) was dissolved in 20 ml of F_3CCO_2H and stored under N_2 for 60 min. The solvent was removed *in vacuo* and the residue was dissolved in 350 ml of freshly distilled liq NH_3 and treated with small pieces of Na until the blue color persisted for 20 min. NH_3 was evapd and the residue was dissolved in 25 ml of 0.1 *N* AcOH and desalted on an IRC-50 column as described earlier.[18] The crude nonadecapeptide was eluted with pyridine-AcOH-H_2O (30:4:66, v/v) and isolated by lyophilization to yield 146 mg of material. This was further purified by column chromatog on CM-cellulose as previously described.[16] The purified product was rechromatographed on CM-cellulose to yield 32 mg of the nonadecapeptide IV (peptide content based on uv spectra, 80%; 8% yield based on protected nonapeptide).

Peptide IV was found to be homogeneous by electrophoresis (400 v, 5 hr) on paper: at pH 3.7 (pyridine acetate buffer), mobility relative to lysine 0.85; at pH 6.9 (collidine acetate buffer), mobility relative to lysine 0.68. The amino acid composition of an acid hydrolysate of IV as determined by chromatog was in good agreement with theoretically expected values (Table I). A sample of IV was submitted to enzymic digest, first with a mixt of trypsin and chymotrypsin (enzyme/substrate, 1:50; pH 8.5, 37°, 48 hr), and then with leucine aminopeptidase (enzyme/substrate, 1:25; pH 8.5, 37°, 48 hr). The amino acid anal. of the digest gave values (Table I) as expected from the specificity of these enzymes.

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On the Role of the Histidine Moiety in the Structure of the Thyrotropin-Releasing Hormone†

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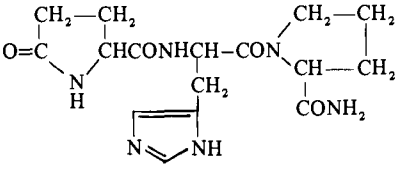
pGlu-Phe-Pro-NH_2 (**2**), an analog of the thyrotropin-releasing hormone (TRH, 1), where Phe replaces His, has been synthesized and found to have nanogram activity *in vivo* which is up to 10% that of TRH. Characteristic of TRH, pGlu-Phe-Pro-NH_2 is inactivated by serum and inhibited by triiodothyronine; therefore, it closely simulates TRH. pGlu-Phe-Pro-NH_2 is apparently the most potent analog of TRH where one of its natural amino acid moieties is replaced by another common and natural amino acid. It is considered that: (a) both the π electrons and the basicity of His may be functional for ultimate release of thyrotropin; (b) release may consist of both complexing and an ionic mechanism involving a negatively charged group of the receptor site. pGlu-Trp-Pro-NH_2 (**3**) and pGlu-Tyr-Pro-NH_2 (**4**) having both aromaticity and functionality in the second amino acid were also synthesized, but these analogs did not release TSH even at extremely high dose levels. To study possible inhibition of TRH by an analog, pGlu-Phe-3Hyp-NH_2 (**5**) was synthesized. **5** did not inhibit the activity of TRH and neither did the tripeptides **3** and **4**.

Chang, *et al.*,[1] and Bowers, *et al.*,[2] have reported the synthesis and hormonal activities of analogs of the thyrotropin-releasing hormone (TRH), which is pyroglutamylhistidylprolinamide (pGlu-His-Pro-NH_2)[3] (**1**). These analogs consisted of modifications in the proline moiety of TRH, and several showed hormonal activity.

Active analogs were found when the moiety of Pro-NH_2 was modified to Pro-$NHCH_3$ and Pro-OCH_3. Hormonal activity was retained when this Pro-NH_2 moiety was replaced by Ala-NH_2, Abu-NH_2, Val-NH_2, and Leu-NH_2. These latter analogs were considered as "open proline" moieties having 3-6 C atoms, in comparison with the 5 C atoms of proline.

The initial finding[2] that pGlu-^{im}Bzl-His-Pro-NH_2 showed about 0.1% of the activity of TRH became very interesting.



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†Hypothalamic Hormones. 32.