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Synthesis of Corticotropin Peptides. IX. Synthesis of [1-Glycine]-ACTH(1—18)-Octadecapeptide Amide

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The synthesis is decribed of an octadecapeptide, glycyl-tyrosyl-seryl-methionyl-glutamyl-histidyl-phenyl-alanyl-arginyl-tryptophyl-glycyl-lysyl-prolyl-valyl-glycyl-lysyl-lysyl-arginyl-arginine amide, corresponding to the first eighteen amino acid residues of corticotropin except for the amino end which has been replaced by a glycine for the serine in the native hormone. The protected end product was obtained by two different coupling procedures, the active ester and the catalyzed carbodiimide, from the amino-terminal decapeptide and the octapeptide of carboxyl end. The over-all yields of the peptide, for the final coupling, deblocking and purification steps, in the active ester and the carbodiimide procedures were approximately forty and thirty per cent, respectively. The synthetic octadecapeptide amide has been shown to possess a high adrenal corticotropic activity, which is comparable to that of the corresponding l-serine octadecapeptide amide, and a lipolytic activity which is significantly lower than that of the l-serine analog.

In a previous communication we reported the synthesis of a corticotropin peptide, ACTH(1—18)-amide (I), which was shown to possess a high steroidogenic potency.[1] In 1963 Lebovitz and Engel reported that the amino-terminal serine of porcine corticotropin could be converted into a glycine without loss of corticotropic activity.[2] This implies that the hydroxymethyl group of amino-terminal serine is not essential for the activity in case of the native hormone. In connection with this finding, the synthesis of a peptide, corresponding to the first eighteen amino acid residues of adrenocorticotropin (ACTH) except for the amino end which was substituted by a glycine for the serine in the parent peptide I, was undertaken as an attempt to prepare analogs of corticotropin peptides. Here will be described the synthesis of the octadecapeptide amide, glycyl-tyrosyl-seryl-methionyl-glutamyl-histidyl-phenylalanyl-arginyl-tryptophyl-glycyl-lysyl-prolyl-valyl-glycyl-lysyl-lysyl-arginyl-arginine amide (II), which may be designated as [Gly[1]]-ACTH(1—18)-NH_2 for abbreviation.[3]

In the final reaction leading to the protected end product, the fragment corresponding to the first ten amino acid residues was coupled with the one corresponding to the rest of the molecule by two different procedures, the *N*-hydroxysuccinimide-catalyzed carbodiimide method[4] and the active ester method.[5] The syntheses of both fragments as intermediates have been already reported.[6]

A protected decapeptide Boc-Gly-Tyr-Ser-Met-Glu(OBu^t)-His-Phe-Arg-Trp-Gly-OH (III)[6a] and a partially protected octapeptide H-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-NH_2 (IV),[6b] which had been converted into the hydrochloride, were coupled with *N*,*N*′-dicyclohexylcarbodiimide (DCC) in the presence of *N*-hydroxysuccinimide as a catalyst to lead to the formation of a protected octadecapeptide Boc-Gly-Tyr-Ser-Met-Glu(OBu^t)-His-Phe-Arg-Trp-Gly-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-NH_2 (V). The crude product was submitted for purification to chromatography on a column of carboxymethyl cellulose (CMC) in the 50% *t*-butanol - 2M ammonium acetate (pH 5.70) system (Fig. 1). The purified V was obtained in a yield of 50% and was found to be homogeneous in thin-layer chromatography. The protecting groups of V were then removed by treatment with 90% trifluoroacetic acid to liberate the octadecapeptide II. The crude product was, after having been converted into the acetate by treatment with an anion-exchange resin (acetate form), purified on

1) H. Otsuka, K. Inouye, F. Shinozaki and M. Kanayama, *J. Biochem.* (Tokyo), **58**, 512 (1965).

2) H. E. Lebovitz and F. L. Engel, *Endocrinol.*, **73**, 573 (1963).

3) All amino acid residues are of the L-configuration. The abbreviated designation of amino acids, peptides and their derivatives accords with the proposal of the IUPAC-IUB Commission on Biochemical Nomenclature.

4) E. Wünsch and F. Drees, *Chem. Ber.*, **99**, 110 (1966).

5) G. W. Anderson, J. E. Zimmerman and F. M. Callahan, *J. Amer. Chem. Soc.*, **86**, 1839 (1964); *ibid.*, **85**, 3039 (1963).

6) a) H. Otsuka, K. Inouye, F. Shinozaki and M. Kanayama, This Bulletin, **39**, 1171 (1966); b) H. Otsuka, K. Inouye, M. Kanayama and F. Shinozaki, *ibid.*, **39**, 882 (1966).

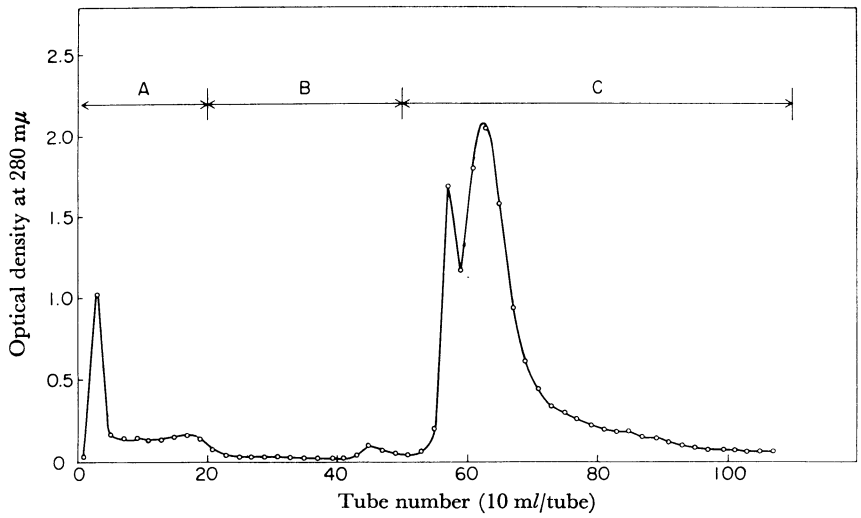

Fig. 1. Chromatography of protected octadecapeptide amide (V) on a carboxymethyl cellulose column. Material: crude product, 0.139 g. Column: carboxymethyl cellulose (Serva, 0.44 meq/g), 1.6×9 cm. Solvent: A, 50% *t*-butanol; B, 50% *t*-butanol - 2M ammonium acetate, pH 5.70 (99 : 1, 150 ml) through a mixing chamber containing 50% *t*-butanol (150 ml); C, 50% *t*-butanol - 2M ammonium acetate, pH 5.70 (98: 2, 300 ml) through a mixing chamber containing 50% *t*-butanol - 2M ammonium acetate, pH 5.70 (99 : 1, 300 ml).

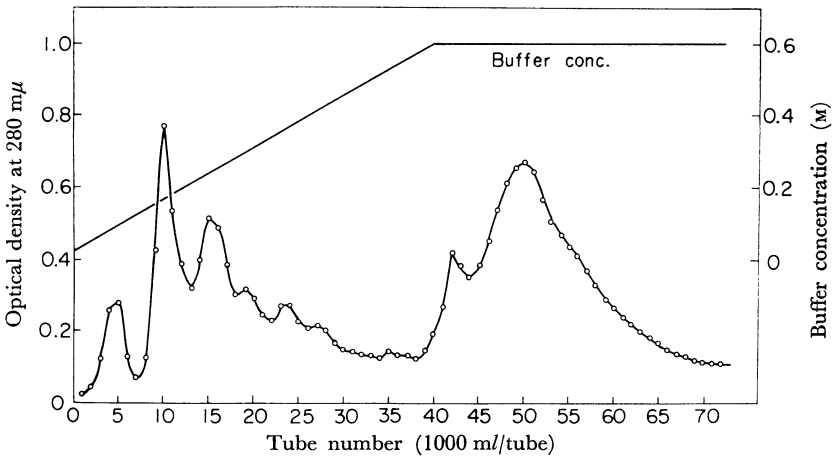

Fig. 2. Chromatography of octadecapeptide amide (II) on a carboxymethyl cellulose column.[7] Material: product obtained from crude protected octadecapeptide amide (V, 11.8 g). Column: carboxymethyl cellulose (Brown, 0.78 meq/g), 6.9×78 cm. Solvent: ammonium acetate buffer (pH 6.5).

a CMC column in the ammonium acetate buffers with successive concentration and pH gradients. The purified material was recovered from the main peak in a yield of 59%.

In an alternative procedure to obtain peptide II, decapeptide III was first esterified with *N*-hydroxysuccinimide by the DCC method[5] and the resulting active ester isolated was coupled with the acetate of octapeptide IV in the presence of triethylamine. The product was, without further purification, treated with 90% trifluoroacetic acid to remove the protecting groups. The crude octadecapeptide trifluoroacetate was converted by treatment with Amberlite IRA-400 (acetate form) into the acetate,

7) The data were taken from part of an experiment performed by Dr. Ikuo Kikkawa and staff of this Laboratory under the supervision of one of us (H.O.). We thank Dr. Kikkawa for permission to include this synthesis in the present communication.

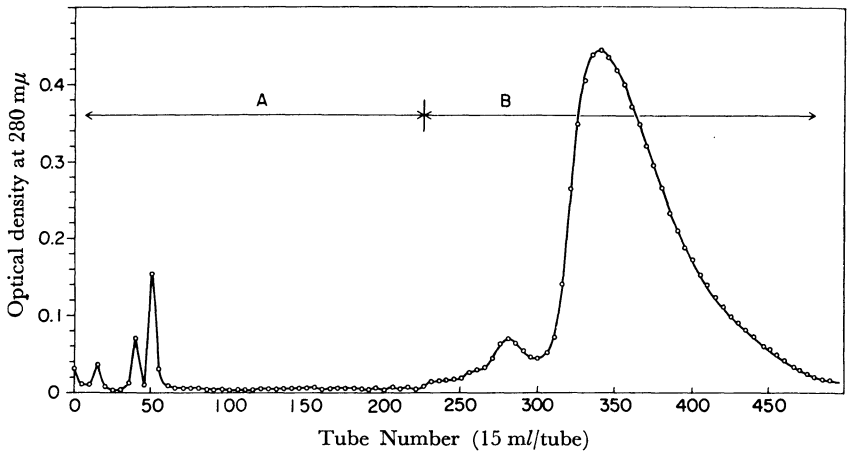

Fig. 3. Rechromatography of octadecapeptide amide (II) on a carboxymethyl cellulose column.[7] Material: partially purified II, 0.300 g. Column: carboxymethyl cellolose (Brown, 0.78 meq/g), 3.8×28 cm. Solvent: A, ammonium acetate (pH 6.5) with a linear concentration gradient of 0.025—0.6M, 3300 ml; B, 0.6M ammonium acetate (pH 6.8).

which was, after having been incubated with mercaptoethanol, submitted to chromatography on a CMC column using the ammonium acetate buffer (pH6.5) with a linear concentration gradient (Fig. 2). The octadecapeptide II was thus obtained in an over-all yield of 50% for the final coupling step. Further purification was carried out on a CMC column in the same manner as above in a recovery of 80% (Fig. 3).

In both cases peptide II obtained was found to be homogeneous to ninhydrin, Pauly, Ehrlich, Sakaguchi and methionine (PtI_6'') reagents in paper chromatography. Quantitative amino acid analysis[8] showed that an acid hydrolysate of the product contained the individual amino acids in the ratios expected by theory with the exception of tryptophan. The tryptophan-tyrosine ratio was determined spectrophotometrically to be unity.[9]

The biological properties of the octadecapeptide II are compared in Table 1 with those of the parent peptide I;[10] the latter has been reported.[1] The lipolytic activity of the Gly^1-analog (II) was found to be significantly lower than that of the Ser^1-peptide (I) and is on the same order of magnitude as that of a native corticotropin when assayed in the rat.[13] The adrenal stimulating activity of peptide II was assayed by three different procedures for the comparison with peptide I. A slightly higher activity was obtained with peptide II as compared with peptide I, when assayed by both *in vivo*[11] and *in vitro*[12] steroidogenesis procedures, whereas peptide II was found to be slightly less active than peptide I when assayed by the adrenal ascorbic acid depletion method. The synthesis of ACTH(1—23)-NH_2

Table 1. Biological activities of the synthetic octadecapeptides[10]

Peptide	Adrenal corticotropic[a] (USP units/mg)			Lipolytic[13] (Minimal effective dose, μg)	
	In vivo[b]	*In vivo*[11]	*In vitro*[12]	Rabbit	Rat
ACTH(1—18)-NH_2 (I)	45.4	62—173	10.6	0.000004	0.003
[Gly^1]-ACTH(1—18)-NH_2 (II)	21.8	161	14.9—20.2	0.00035	0.012

a) Potencies are represented in terms of the third USP Corticotropin Unit.
b) According to USP, XVII, 147 (1965).

8) D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).
9) D. H. Beaven and E. R. Holiday, *Adv. Protein Chem.*, **7**, 319 (1952); T. W. Goodwin and R. A. Morton, *Biochem. J.*, **40**, 628 (1946).
10) The authors are much indebted to Dr. Akira Tanaka of this Laboratory for these biological assays: A. Tanaka, *Saishin Igaku* (Tokyo), **23**, 911 (1968).
11) A modification of Lipscomb and Nelson (H. S. Lipscomb and D. H. Nelson, *Endocrinol.*, **71**, 13 (1962)): A. Tanaka and C. H. Li, *Endocrinol. Japonica*, **13**, 180 (1966).
12) M. A. Saffran and A. V. Schally, *Endocrinol.*, **56**, 523 (1955).
13) A. Tanaka, B. T. Pickering and C. H. Li, *Arch. Biochem. Biophys.*, **99**, 294 (1962).

and the corresponding Gly[1]-analog was recently reported by Geiger *et al.*[14] They gave the latter a little higher value of activity as compared with that of the former, using the adrenal ascorbic acid depletion method.

Experimental

Glycyl-tyrosyl-seryl-methionyl-glutamyl-histidyl-phenylalanyl-arginyl-tryptophyl-glycyl-lysyl-prolyl-valyl-glycyl-lysyl-lysyl-arginyl-arginine Amide (II). *a) By the N-Hydroxysuccinimide-catalyzed Carbodiimide Method.* The partially protected octapeptide, H-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys (Boc)-Arg-Arg-$NH_2 \cdot 3CH_3COOH$,[6b] (IV, 0.074 g, 0.05 mmol) was dissolved in 3 m*l* of ice-cold 0.05N hydrochloric acid and the solution was immediately lyophilyzed to give the corresponding trihydrochloride. The protected decapeptide, Boc-Gly-Tyr-Ser-Met-Glu (OBu^t)-His-Phe-Arg-Trp-Gly-OH,[6a] (III, 0.080 g, 0.055 mmol) was dissolved in 1.5 m*l* of dimethylformamide together with the octapeptide hydrochloride obtained above, and to this solution were added *N*-hydroxysuccinimide (0.013 g, 0.11 mmol) and *N,N'*-dicyclohexylcarbodiimide (DCC, 0.014 g, 0.07 mmol) at 0°C. The reaction mixture was stirred at 0°C for one hour and at room temperature for 48 hr. *N,N'*-Dicyclohexylurea (DCU) which formed was filterred off and the filtrate was introduced into ice-cold ethyl acetate (100 m*l*). The precipitates which separated were filtered off, washed with cold ethyl acetate, and dried over phosphorus pentoxide *in vacuo* to afford 0.139 g of the crude product. This crude material (0.139 g) was dissolved in 5 m*l* of 50% *t*-butanol and to this was added Amberlite CG-4B (acetate form, 1.1 cc), and the mixture was shaken for 30 min. After removal of the resin, M mercaptoethanol (0.5 m*l*) was added to the filtrate. The mixture was, after having been incubated at 37°C overnight, subjected to chromatography on a column (1.6×9 cm) of carboxymethyl cellulose (Serva, 0.44 meq/g), using successively the following solvent systems: 50% *t*-butanol (200 m*l*); the one with a linear gradient obtained from 50% *t*-butanol (150 m*l*) and 50% *t*-butanol-2M ammonium acetate, pH 5.70 (99 : 1, 150 m*l*); the one with a linear gradient obtained from 50% *t*-butanol-2M ammonium acetate, pH 5.70 (99 : 1, 300 m*l*) and 50% *t*-butanol-2M ammonium acetate, pH 5.70 (98 : 2, 300 m*l*). The results of chromatography are shown in Fig. 1. Fractions corresponding to the main peak (tubes 55—70) were pooled and the bulk of the solvent was removed *in vacuo*. The residue was lyophilized to give the protected octadecapeptide (V); yield 0.105 g (50% on the weight basis). The material was found to be unreactive to ninhydrin and it behaved as a single component to Pauly and Sakaguchi reagents in thin-layer chromatography (Silica gel G, *n*-butanol-acetic acid-water=4 : 1 : 2 by volume as solvent).

To remove the protecting groups the purified peptide V (0.140 g) was treated with 90% trifluoroacetic acid (2 m*l*) at room temperature for 60 min. After the solvent had been removed *in vacuo* over potassium hydroxide pellets, the residue was dissolved in water, (5 m*l*). The aqueous solution was shaken with Amberlite CG-4B (acetate form, 2.5 cc) for one hour, and then the resin was removed by filtration. To the filtrate M mercaptoethanol (1 m*l*) was added and the mixture was, after having been kept at 37°C overnight, subjected to chromatography on a column (1.6×11 cm) of carboxymethyl cellulose (Serva, 0.44 meq/g), using successively the following ammonium acetate buffers: the one with a linear gradient obtained from 0.075M, pH 5.80 (400 m*l*) and 0.25M, pH 7.00 (400 m*l*); the one with a linear gradient obtained from 0.25M, pH 7.00 (450 m*l*) and 0.40M, pH 8.60 (450 m*l*); 0.40M, pH 8.60 (450 m*l*). Fractions (10 m*l*/tube) corresponding to the main peak (tubes 70—120) were combined and the bulk of the solvent was removed *in vacuo*. The residue was lyophilized repeatedly to constant weight and dried over phosphorus pentoxide *in vacuo* at 60°C to give the pure octadecapeptide (II); yield 0.070 g (59% for the deblocking step, as determined spectrophotometrically),[15] homogeneous (R_f=0.09) to ninhydrin, Pauly, Ehrlich, Sakaguchi and methionine (PtI_6'') reagents in paper chromatography (*n*-butanol-acetic acid-water=4 : 1 : 2 by volume as solvent). $[\alpha]_D^{23.5}$ −51.4±1.9° (*c* 0.472, 0.1N acetic acid). $\lambda_{max}^{0.1N\ NaOH}$=281 mμ (ε 6750), 288 mμ (ε 6490). Amino acid ratios in acid hydrolysate:[8] Ser 0.93, Glu 0.96, Pro 0.98, Gly 3.25, Val 1.00, Met 1.00, Tyr 1.05, Phe 1.00, Lys 3.23, His 1.07, NH_3 1.39, Arg 2.95, Trp 0.46 (decomposed partially with acid). The Trp/Tyr ratio in intact II was determined spectrophotometrically to be 0.91 : 1.[9]

b) By the Active Ester Method.[7] To a solution of Boc-Gly-Tyr-Ser-Met-Glu(OBu^t)-His-Phe-Arg-Trp-Gly-OH[6a] (III, 8.04 g, 5.2 mmol) in 240 m*l* of dimethylformamide were successively added N hydrochloric acid (5.2 m*l*), *N*-hydroxysuccinimide (2.4 g, 20.8 mmol) and DCC (4.3 g, 20.8 mmol) at an ice-salt bath temperature. The mixture was stirred at 4°C for 20 hr. Additional quantities of the succinimide (2.4 g) and DCC (4.3 g) were added and the stirring was continued for 5 hr. After removal of DCU by filtration the filtrate was introduced into 2000 m*l* of ethyl acetate-ether (1 : 1 by volume). The resulting precipitates were filtered off, washed with ethyl acetate-ether and dried *in vacuo* to yield the protected decapeptide active ester hydrochloride; 9.38 g.

H-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-$NH_2 \cdot 3CH_3COOH$[6b] (IV, 4.73 g, 3.1 mmol) and triethylamine (4.8 m*l*) were dissolved in dimethylformamide (150 m*l*) and to this solution was added the decapeptide active ester obtained above (9.38 g) at 0°C. The mixture was stirred at 4°C for 4 days, and was then poured into ice-cold ethyl acetate to separate the crude protected octadecapeptide (V), which was filtered off, washed with cold ethyl acetate and dried *in vacuo*; yield 13.11 g.

To this crude product (11.8 g) was added 90% trifluoroacetic acid (120 m*l*) and the mixture was stirred at 0°C for 15 min and at room temperature for 75 min. After the solvent had been removed *in vacuo*, the residue was dried over potassium hydroxide pellets *in vacuo* and was then dissolved in water (*ca.* 50 m*l*). The solution was applied to a column (4.4×30 cm) of Amberlite IRA-400 (acetate form) and the column was washed with water. The effluents were combined and concentrated

14) R. Geiger, K. Sturm, G. Vogel and W. Siedel, *Z. Naturforsch.*, **19b**, 858 (1964).

15) The over-all yield of peptide, calculated on the basis of the amine component used for the final coupling step, is therefore 29.5%.

in vacuo to about 400 m*l*, and to this was added 25 m*l* of M mercaptoethanol. The mixture was, after incubation at 37°C overnight, submitted to a column (6.9×78 cm) of carboxymethyl cellulose (Brown, 0.78 meq/g). The column was eluted with an ammonium acetate buffer (pH 6.5) having a linear concentration gradient of 0.025—0.6M to collect 40 *l* of fractions and then with 0.6M ammonium acetate buffer (pH 6.5) until the desired peptide totally emerged (Fig. 2). Fractions corresponding to the main peak (tubes 44—72) were combined and the bulk of the solvent was removed *in vacuo*. Repeated lyophilization of the residue gave the octadecapeptide (II) as fluffy powder. The material was dried over phosphorus pentoxide at 55°C *in vacuo* to yield 3.17 g (50.2% on the basis of the amine component used for the coupling step, as determined spectrophotometrically); homogeneous to ninhydrin, Pauly and Sakaguchi reagents in paper chromatography (*n*-butanol-acetic acid-pyridine-water=30 : 6 : 20 : 24 by volume (BAPW) as solvent). $[\alpha]_D^{23}$ $-53.6\pm1.9°$ (*c* 0.494, 0.1N acetic acid). Amino acid ratios in acid hydrolysate:[8] Ser 0.96, Glu 0.98, Pro 1.04, Gly 3.00, Val 0.99, Met 0.99, Tyr 0.99, Phe 1.02, Lys 3.00, His 0.99, Arg 3.06.

A 0.3 g-portion of the peptide obtained above was submitted, for further purification, to a column (3.8×28 cm) of carboxymethyl cellulose (Brown, 0.78 meq/g), using the ammonium acetate buffer (pH 6.5) with a linear concentration gradient of 0.025—0.6M (3300 m*l*) followed by 0.6M ammonium acetate (pH 6.8). The results of this rechromatography are shown in Fig. 3. Fractions corresponding to the main peak (tubes 310—480) were pooled, concentrated and lyophilyzed to obtain the pure peptide II; 0.24 g, $[\alpha]_D^{23}$ $-55.3\pm1.9°$ (*c* 0.510, 0.1N acetic acid). Homogeneous to ninhydrin, Pauly, Ehrlich, Sakaguchi and methionine (PtI_6'') reagents in paper chromatography (BAPW) and in paper electrophoresis (600 V/36 cm, in 2N acetic acid). Amino acid ratios in acid hydrolysate:[8] Ser 0.94, Glu 1.01, Pro 0.99, Gly 3.00, Val 1.00, Met 0.98, Tyr 0.98, Phe 0.97, Lys 2.90, His 0.97, Arg 2.97.

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