December, 1976] BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN, VOL. 49 (12), 3615—3619 (1976) 3615

Synthesis of Corticotropin Peptides. X. The Synthesis of ACTH(1—18)-OH, ACTH(1—18)-NH$_2$ and ACTH(1—19)-NH$_2$[*]

Ken Inouye, Fusako Shinozaki, Makoto Kanayama, and Hideo Otsuka
Shionogi Research Laboratory, Shionogi & Co., Ltd., Fukushima-ku, Osaka 553

(Received June 29, 1976)

The final coupling, deprotection and purification processes for the synthesis of octadecapeptides, H–Ser–Tyr–Ser–Met–Glu–His–Phe–Arg–Trp–Gly–Lys–Pro–Val–Gly–Lys–Lys–Arg–Arg–OH(I) and H–Ser–Tyr–Ser–Met–Glu–His–Phe–Arg–Trp–Gly–Lys–Pro–Val–Gly–Lys–Lys–Arg–Arg–NH$_2$ (II), corresponding to the first eighteen amino acid residues of corticotropin (ACTH) are described, in which the *N*-hydroxysuccinimide ester of the N-terminal decapeptide derivative is coupled with the C-terminal octapeptide and octapeptide amide derivatives to give the two protected end products. The same decapeptide active ester is also utilized to the synthesis of a nonadecapeptide, H–Ser–Tyr–Ser–Met–Glu–His–Phe–Arg–Trp–Gly–Lys–Pro–Val–Gly–Lys–Lys–Arg–Arg–Pro–NH$_2$ (III), identical with the first nineteen amino acid residues of ACTH. The *in vivo* steroidogenic potencies of the synthetic peptides are compared with that of the synthetic human ACTH (α_h-ACTH) as reference to show that the relative potencies of I, II, and III *vs.* α_h-ACTH are 0.14, 0.48, and 0.47, respectively, as estimated on a molar basis.

In early 1960's considerable effort was devoted to the synthesis of peptides related to corticotropin (ACTH) to obtain a picture of the structural features essential for eliciting the adrenal-stimulating activity. Thus, ACTH(1—19)-OH,[1] ACTH(1—20)-NH$_2$,[2] ACTH(1—23)-OH,[3] and ACTH(1—24)-OH[4] were synthesized in three different laboratories, and their activities were found to be comparable enough to that of the native hormone. However, a heptadecapeptide ACTH(1—17)-OH[5] was only 15% as active as the nonadecapeptide ACTH(1—19)-OH. We also demonstrated that a hexadecapeptide [Gly^1, Arg^{15,16}]-ACTH(1—16)-OH[6] and a heptadecapeptide [Gly^1, Arg^{16}]-ACTH(1—17)-NH$_2$[7] were only slightly active in adrenal steroidogenesis. These observations suggested that the integrity of the unique Lys–Lys–Arg–Arg sequence in positions 15—18 of the ACTH molecule was crucial for eliciting a high degree of activity. In order to ascertain this point and to find the smallest peptide possessing a moderate adrenal-stimulating potency, we decided to synthesize an octadecapeptide, H–Ser–Tyr–Ser–Met–Glu–His–Phe–Arg–Trp–Gly–Lys–Pro–Val–Gly–Lys–Lys–Arg–Arg–OH, ACTH(1—18)-OH (I), and an octadecapeptide amide, H–Ser–Tyr–Ser–Met–Glu–His–Phe–Arg–Trp–Gly–Lys–Pro–Val–Gly–Lys–Lys–Arg–Arg–NH$_2$, ACTH(1—18)-NH$_2$ (II). A preliminary account of the synthetic and biological studies of peptides I and II has appeared.[8] The syntheses of the N-terminal decapeptide derivative, Boc–Ser–Tyr–Ser–Met–Glu(OBu^t)–His–Phe–Arg(H$^+$)–Trp–Gly–O$^-$ (IV),[9] and the C-terminal octapeptide derivatives, H–Lys(Boc)–Pro–Val–Gly–Lys(Boc)–Lys(Boc)–Arg–Arg–OH (V) and H–Lys(Boc)–Pro–Val–Gly–Lys(Boc)–Lys(Boc)–Arg–Arg–NH$_2$ (VI),[10] have already been reported. In this paper the final coupling, deprotection and purification processes to lead to the production of peptides I and II are described in detail. In addition, the synthesis of a nonadecapeptide amide, H–Ser–Tyr–Ser–Met–Glu–His–Phe–Arg–Trp–Gly–Lys–Pro–Val–Gly–Lys–Lys–Arg–Arg–Pro–NH$_2$, ACTH(1—19)-NH$_2$ (III), is also a subject of this paper.

Synthesis of ACTH(1—18)-OH (I) and ACTH(1—18)-NH$_2$ (II). The final coupling reaction to produce the protected octadecapeptides was carried out by the *N*-hydroxysuccinimide (HOSu) ester method[11] in essentially the same manner as described in the synthesis of [Gly^1]-ACTH(1—18)-NH$_2$.[12] The protected decapeptide (IV) was converted into the hydrochloride Boc–Ser–Tyr–Ser–Met–Glu(OBu^t)–His–Phe–Arg(HCl)–Trp–Gly–OH and this was esterified with HOSu by means of dicyclohexylcarbodiimide (DCC). For the synthesis of peptide I, the resulting decapeptide active ester hydrochloride was allowed to react with the acetate salt of the N^α-free octapeptide derivative (V) in the presence of triethylamine to give Boc–Ser–Tyr–Ser–Met–Glu(OBu^t)–His–Phe–Arg–Trp–Gly–Lys(Boc)–Pro–Val–Gly–Lys(Boc)–Lys(Boc)–Arg–Arg–OH. The protected octadecapeptide thus obtained was treated with trifluoroacetic acid in the presence of anisole and 2-mercaptoethanol as

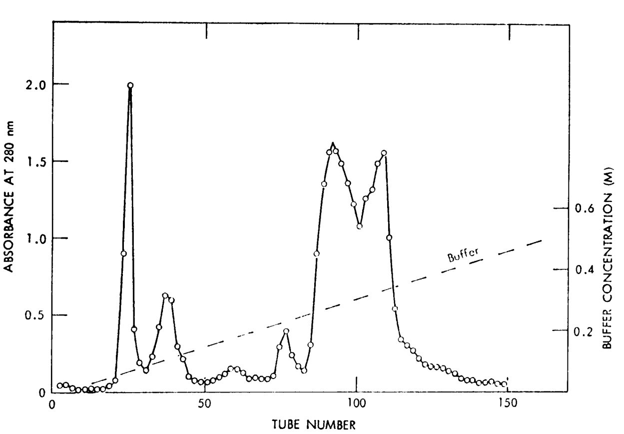

Fig. 1. Carboxymethyl cellulose column chromatography of crude synthetic ACTH(1—18)-OH: sample, 0.28 g; column, Serva, 0.7 meq/g, 1.7×21 cm; buffer, 0—0.6 M ammonium acetate (pH 6.0), 2000 ml; fractionation, 10 ml/tube.

---

[*] All the amino acid residues mentioned in this communication are of the L-configuration. Abbreviations used are those recommended by the IUPAC-IUB Commission of Biochemical Nomenclature [*Biochemistry*, **5**, 2485 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1972)].

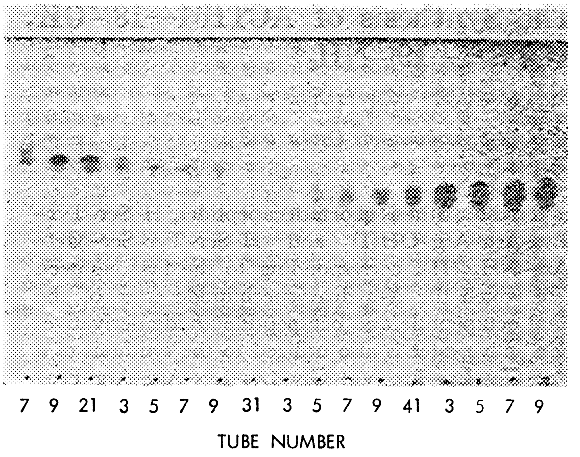

Fig. 2. Examination of fractions by TLC in the partition chromatography of partially purified ACTH(1—18)-OH. Partition chromatography: sample, 0.16 g, derived from tubes 86—115 in Fig. 1; column, Sephadex G-25 (medium), 2.2×50 cm; solvent, 1-butanol–acetic acid–pyridine–water (12:3:4:6); fractionation, 4.4 ml/tube. TLC: on a cellulose plate (precoated, Merck) with 1-butanol–acetic acid–pyridine–water (15 : 3 : 10 : 15) as solvent; detection, Ehrlich reagent.

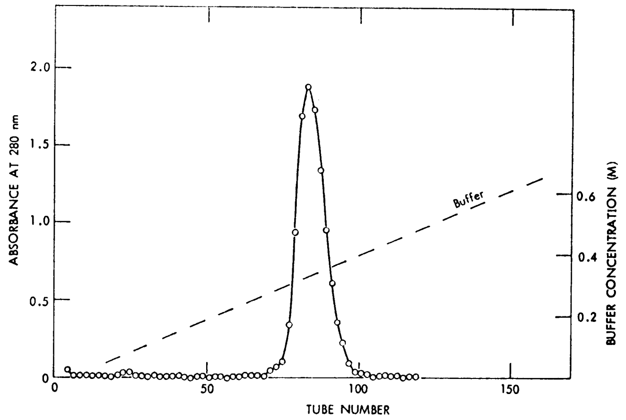

Fig. 3. Rechromatography of partially purified synthetic ACTH(1—18)-OH on a carboxymethyl cellulose column: sample, 0.11 g, fractionated by partition chromatography (Fig. 2, see Experimental for details); column, CM-52 (Whatman), 1.7×18 cm; buffer, 0—0.6 M ammonium acetate (pH 6.0), 1500 ml; fractionation, 10 ml/tube.

scavengers to liberate the desired peptide (I) in the form of trifluoroacetate. This was converted into the corresponding acetate by the treatment with Amberlite CG-400 (acetate form). The resulting crude preparation of peptide I was then purified by carboxymethyl cellulose column chromatography and by partition chromatography on a Sephadex G-25 column (Fig. 1—3). The partition chromatography was performed with a homogeneous mixture, 1-butanol–acetic acid–pyridine–water (12 : 3 : 4 : 6), as solvent[13] and the collected fractions were monitored by the Folin-Ciocalteu method[14] or by TLC more effectively (Fig. 2). The synthesis of peptide II was accomplished in the same manner as described above except that the acetate salt of VI was used as the amine component in the coupling reaction. The overall yields for the final coupling, deprotection and purification processes of peptide I and peptide II were 37 and 26%, respectively.

The synthetic octadecapeptides thus obtained were found to be homogeneous to ninhydrin and the Ehrlich reagent in TLC. Their acid hydrolysates contained the constituent amino acids in the ratios expected by theory with the exception of tryptophan. The tryptophan-tyrosine ratio in intact I and II was determined spectrophotometrically to be unity.[15]

Synthesis of ACTH(1—19)-NH$_2$ (III). The C-terminal tetrapeptide sequence Lys–Arg–Arg–Pro–NH$_2$ was synthesized by the step-by-step procedure starting with H–Pro–NH$_2$ derived from Z–Pro–NH$_2$[16] by catalytic hydrogenolysis. The two arginines were incorporated in the form of Z–Arg(NO$_2$) by the DCC method and by the mixed anhydride procedure, respectively, in combination with the removal of benzyloxycarbonyl (Z) group with hydrogen bromide in acetic acid. The resulting N^α-free tripeptide was then allowed to react with Z–Lys(Boc)–OSu[10] to afford Z–Lys(Boc)–Arg(NO$_2$)–Arg(NO$_2$)–Pro–NH$_2$ (IX). Compound IX was hydrogenolyzed for removal of the Z and two nitro groups and the product was coupled with Z–Lys(Boc)–Pro–Val–Gly–Lys(Boc)–N$_3$[10] to give a nonapeptide, Z–Lys(Boc)–Pro–Val–Gly–Lys(Boc)–Lys(Boc)–Arg–Arg–Pro–NH$_2$ (X), corresponding to positions 11—19 of ACTH.

Compound X was hydrogenolyzed and the resulting N^α-free nonapeptide was made react with the decapeptide *N*-hydroxysuccinimide ester, derived from IV, to yield Boc–Ser–Tyr–Ser–Met–Glu(OBu^t)–His–Phe–Arg–Trp–Gly–Lys(Boc)–Pro–Val–Gly–Lys(Boc)–Lys(Boc)–Arg–Arg–Pro–NH$_2$. This protected nonadecapeptide was treated with trifluoroacetic acid in the presence of anisole and 2-mercaptoethanol as scavengers to liberate peptide III, which was, after conversion into the acetate form, purified by partition chromatography and by ion-exchange chromatography in essentially the same manner as described for peptides I and II. The overall yield for the final coupling, deprotection and purification processes of peptide III was 38%. The purified material behaved as a single component in TLC and its acid hydrolysate contained the constituent amino acids in the ratios expected by theory with the exception of tryptophan. The tryptophan-tyrosine ratio in intact III was determined spectrophotometrically to be unity.[15]

The octadecapeptide amide (II) and the nonadecapeptide amide (III) have been synthesized by two other groups independently.[17,18] The optical rotations of our preparations of peptides II and III were found to differ significantly from those reported by Ramachandran *et al.*[17] Bajusz and Medzihradszky[18] also pointed out this lack of identity in optical rotation between their peptides and those of Ramachandran *et al.*

Biological Activity of ACTH(1—18)-OH (I), ACTH(1—18)-NH$_2$ (II) and ACTH(1—19)-NH$_2$ (III).

TABLE 1. STEROIDOGENIC ACTIVITY OF SYNTHETIC PEPTIDES[19)]

Compound	Molecular weight[a)]	*In vivo* steroidogenic activity	
		units/mg[b)]	units/μmol
ACTH(1—18)-OH (I)	2250	26.8	60.3
ACTH(1—18)-NH_2 (II)	2249	96.7	217
ACTH(1—19)-NH_2 (III)	2346	90.4	212
Synthetic α_h-ACTH[20)]	4541	98.6	448

a) As anhydrous free base. b) Third USP Corticotropin Reference Standard was used as reference.

The synthetic peptides obtained above were assayed for the *in vivo* steroidogenic activity by a method based on the elevated levels of 11-hydroxycorticosteroids in the adrenal venous plasma of the hypophysectomized rat in response to intravenous administration of the test sample. The results are shown in Table 1,[19)] in which the potencies of peptides I, II, and III are compared with that of the synthetic human hormone (α_h-ACTH).[20)]

Table 1 first shows that the octadecapeptide amide (II) is three to four times more active than the corresponding peptide acid (I) in the *in vivo* adrenal steroidogenesis. The similar potentiation effect associated with the C-terminal amidation has been reported with ACTH(1—17)-NH_2 and ACTH(1—19)-NH_2.[17)] Imura *et al.* has found that peptide II is inactivated more slowly than peptide I in fresh human plasma.[21)] This finding would offer evidence to support the view that the observed higher potency of the peptide amide is a consequence of the increased stability in the biological systems. When the steroidogenic activity was assayed in isolated rat adrenal cells, peptide I and peptide II were found to exhibit virtually the same degree of potency.[22)] This is probably due to the absence of carboxypeptidase action in the assay system employed.

Table 1 next shows that ACTH(1—18)-NH_2 (II) and ACTH(1—19)-NH_2 (III) exhibit almost the same potency and they are as active as the synthetic α_h-ACTH when compared by weight. In the table, the potencies are also expressed in terms of units per μmole to compare the synthetic fragments with α_h-ACTH taking account of their molecular weights. Thus, the relative potencies *vs.* α_h-ACTH may be calculated as 0.48 and 0.47 for II and III, respectively, indicating that these fragment peptides are nearly half as active as the intact hormone, as compared on a molar basis. Ramachandran *et al.* also demonstrated that ACTH(1—18)-NH_2 and ACTH(1—19)-NH_2 possessed almost identical potency in the *in vivo* steroidogenesis.[17)] From their data the relative potencies *vs.* sheep ACTH (481 USP units/μmol)[17)] of the octadecapeptide amide and the nonadecapeptide amide are estimated to be 0.62 and 0.72, respectively.

The biological data obtained above clearly indicate that all the structural features required for the manifestation of steroidogenic activity reside within the first eighteen amino acid sequence of the ACTH molecule. Since it has been reported that ACTH(1—17)-NH_2 is only 23% as active as ACTH(1—18)-NH_2,[17)] the octadecapeptide should be the smallest ACTH fragment that is comparable to the intact hormone as a steroidogenic agent. As mentioned above, the C-terminal amide group in ACTH(1—18)-NH_2 appears to be effective only for the stabilization of the peptide *in vivo*. It has been shown that a natural ACTH and a synthetic 39 amino acid peptide are inactivated more slowly than the fragment peptides such as ACTH(1—18)-NH_2, ACTH(1—19)-OH and ACTH(1—26)-OH.[21)] Therefore, the C-terminal portion of the ACTH molecule may not be involved in the process of adrenal stimulation, but may play a role in stabilizing the N-terminal half of the hormone in the physiological environment.

Experimental

Thin layer chromatography was (TLC) performed on precoated "silica gel" plates (Kieselgel 60 F_{254}, Merck) or precoated "cellulose" plates (Cellulose F_{254}, Merck).

H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-OH Acetate, ACTH(1—18)-OH (I).[8)] To a solution of IV (0.16 g, 0.11 mmol)[9)] in *N*,*N*-dimethylformamide (DMF, 4 ml) previously chilled in an ice-bath was added M hydrochloric acid (0.3 ml) and the solution was introduced into ice-cold ethyl acetate (50 ml). The resulting precipitates of the decapeptide hydrochloride (0.17 g) was dissolved in DMF (4 ml) together with *N*-hydroxysuccinimide (HOSu, 0.050 g, 0.44 mmol) and to this was added dicyclohexylcarbodiimide (DCC, 0.091 g, 0.44 mmol) at 0 °C. The mixture was kept at 4 °C overnight, after which it was introduced into ethly acetate-ether (1:1, 100 ml) to give the decapeptide active ester hydrochloride as amorphous precipitates (0.18 g). The active ester thus obtained was added to a solution of the octapeptide acetate [V, prepared from the N^{α}-benzyloxycarbonyl (*Z*) derivative (0.16 g, 0.1 mmol) by catalytic hydrogenolysis][10)] and triethylamine (0.056 ml, 0.4 mmol) in DMF (6 ml) and the mixture was kept at 4 °C for 2 days. The product was isolated as precipitates which formed when the reaction mixture was introduced into ethyl acetate (50 ml). The crude protected octadecapeptide thus obtained (0.32 g) was mixed with anisole (0.2 ml) and 2-mercaptoethanol (0.2 ml) and chilled in an ice-bath. To this was added trifluoroacetic acid (5 ml) and the mixture was kept at room temperature for 60 min. The precipitates which formed upon addition of ether were filtered off, washed with ether and dried *in vacuo*. This was then dissolved in water and the solution was passed through a column (1.7×6 cm) of Amberlite CG-400 (acetate form) with additional portions of water. The aqueous solutions were combined and lyophilized to give the deprotected octadecapeptide (0.28 g).

For purification the crude peptide obtained above was chromatographed on a carboxymethyl cellulose column using an ammonium acetate buffer (Fig. 1). The fractions corresponding to a main peak (tubes 86—115) were pooled, evaporated *in vacuo* at a bath temperature of 50 °C and lyophilized (0.16 g). This material was then submitted to partition chromatography on a column of Sephadex G-25 (medium) with 1-butanol-acetic acid-pyridine-water (12:3:4:6) as solvent. Tubes 41—65 containing the desired peptide as a single component, as examined by means of TLC (cellulose) with 1-butanol-acetic acid-pyridine-water (15:3:10:15) as solvent and the Ehrlich reagent as reagent for locating peptide spots (Fig. 2), were combined and evaporated *in vacuo* at a bath temperatuer of 45 °C and the residue was lyophilized (0.10 g). Tubes 31—40 containing a less polar impurity in addition to the desired peptide were combined and evaporated *in vacuo*. The residue was rechromatographed on a Sephadex G-25 column (1.7×30 cm) in the same manner as above to give an additional amount of the homogeneous peptide (0.01 g). These preparations were combined (0.11 g) and rechromatographed on a carboxymethyl cellulose column using an ammonium acetate buffer (Fig. 3). The fractions corresponding to a single peak (tubes 77—100) were combined, evaporated and lyophilized to afford the pure octadecapeptide (I) as colorless fluffy powder; yield 0.10 g (37%), $[\alpha]_{\mathrm{D}}^{25.5}$ $-61.2\pm1.9°$ (c 0.5, 0.1 M acetic acid). $\lambda_{\mathrm{max}}^{0.1\mathrm{M}\ \mathrm{HCl}}$ 280 nm ($E_{1\mathrm{cm}}^{1\%}$ 25.1), $\lambda_{\mathrm{shoulder}}^{0.1\mathrm{M}\ \mathrm{HCl}}$ 288 nm ($E_{1\mathrm{cm}}^{1\%}$ 19.0); $\lambda_{\mathrm{max}}^{0.1\mathrm{M}\ \mathrm{NaOH}}$ 282 nm ($E_{1\mathrm{cm}}^{1\%}$ 25.8), 289 nm ($E_{1\mathrm{cm}}^{1\%}$ 25.1). TLC (cellulose): a single component (ninhydrin and Ehrlich reagents) in 1-butanol–acetic acid–pyridine–water (15:3:10:15). Amino acid ratios in acid hydrolysate (figures in parentheses represent theoretical values): Lys 2.94 (3), His 1.00 (1), Arg 3.00 (3), Ser 1.72 (2), Glu 1.03 (1), Pro 0.95 (1), Gly 2.00 (2), Val 1.00 (1), Met 0.98 (1), Tyr 0.98 (1), Phe 0.98 (1). The Trp/Tyr ratio in intact I was 0.98 as determined spectrophotometrically.15)

H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-NH$_2$ *Acetate, ACTH(1—18)-NH*$_2$ *(II).*8) To a solution of the octapeptide amide acetate [VI, prepared from the N^{α}-Z derivative (0.18 g, 0.12 mmol) by catalytic hydrogenolysis]10) and triethylamine (0.033 ml, 0.24 mmol) in DMF (5 ml) was added the decapeptide *N*-hydroxysuccinimide ester [prepared from IV (0.19 g, 0.13 mmol)9) in exactly the same manner as described above] and the mixture was kept at 4 °C for 2 days. The crude protected octadecapeptide obtained (0.34 g) was, in the presence of anisole (0.2 ml) and 2-mercaptoethanol (0.2 ml), treated with trifluoroacetic acid (4 ml) at room temperature for 65 min to give the deblocked peptide (0.32 g).

The crude peptide (acetate form) obtained above was chromatographed on a column (1.7×20 cm) of carboxymethyl cellulose (Serva, 0.7 meq/g) using an ammonium acetate buffer (pH 6.0, 2000 ml) with a linear concentration gradient of 0—0.6 M. Ten-ml fractions were collected and their absorption at 280 nm was measured. The fractions corresponding to a main peak (tubes 96—127) were pooled, evaporated *in vacuo* at a bath temperature of 50 °C and lyophilized (0.11 g). This material was then submitted to partition chromatography on a column (2.7×24 cm) of Sephadex G-25 (medium) with 1-butanol–acetic acid–pyridine–water (12:3:4:6) as solvent and 5 ml-fractions were collected. The Folin-Ciocalteu method14) was effected for monitoring the peptide content of the fractions. Tubes 14—45 were combined and evaporated *in vacuo* at a bath temperature of 45 °C to give a residue which was lyophilized (0.1 g). The partially purified peptide was rechromatographed on a column (1.7×20 cm) of carboxymethyl cellulose (CM-52, Whatman) using an ammonium acetate buffer (pH 6.0, 1500 ml) with a linear concentration gradient of 0—0.6 M. Ten-ml fractions were collected and those corresponding to a single peak (tubes 138—165) were combined, evaporated *in vacuo* and lyophilized to give the pure octadecapeptide amide (II) as colorless fluffy powder; yield 0.086 g (26%), $[\alpha]_{\mathrm{D}}^{25}$ $-61.5\pm2.4°$ (c 0.4, 0.1 M acetic acid). Lit, $[\alpha]_{\mathrm{D}}^{25}$ $-92.7°$ (c 0.1, 0.1 M acetic acid);17) $[\alpha]_{\mathrm{D}}^{20}$ $-54.0°$ (c 0.1, 0.1 M acetic acid).18) $\lambda_{\mathrm{max}}^{0.1\mathrm{M}\ \mathrm{HCl}}$ 280 nm ($E_{1\mathrm{cm}}^{1\%}$ 25.8), $\lambda_{\mathrm{shoulder}}^{0.1\mathrm{M}\ \mathrm{HCl}}$ 288 nm ($E_{1\mathrm{cm}}^{1\%}$ 19.6); $\lambda_{\mathrm{max}}^{0.1\mathrm{M}\ \mathrm{NaOH}}$ 282 nm ($E_{1\mathrm{cm}}^{1\%}$ 26.9), 289 nm ($E_{1\mathrm{cm}}^{1\%}$ 26.0). TLC (cellulose): a single component (ninhydrin and Ehrlich reagents) in 1-butanol–acetic acid–pyridine–water (15 : 3 : 10 : 15). Amino acid ratios in acid hydrolysate: Lys 3.02 (3), His 1.04 (1), Arg 3.07 (3), Ser 1.80 (2), Glu 1.04 (1), Pro 1.04 (1) Gly 2.02 (2), Val 1.00 (1), Met 1.00 (1), Tyr 1.00 (1), Phe 0.98 (1). The Trp/Tyr ratio in intact II was 0.94 as determined spectrophotometrically.15)

H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-NH$_2$ *Acetate, ACTH(1—19)-NH*$_2$ *(III).* Compound X (0.17 g, 0.1 mmol) was hydrogenolyzed over palladium in methanol containing acetic acid (0.5 ml) for 3 h. Removal of the solvent by evaporation *in vacuo* followed by lyophilization from acetic acid gave the N^{α}-free nonapeptide derivative (0.17 g). This was dissolved in DMF (3 ml) and triethylamine (0.042 ml, 0.3 mmol) was added. To this solution was added the decapeptide *N*-hydroxysuccinimide ester [prepared from IV (0.16 g, 0.11 mmol)9) as described above] and the mixture was kept at 4 °C for 2 days. The crude protected nonadecapeptide obtained (0.32 g) was, in the presence of anisole (0.2 ml) and 2-mercaptoethanol (0.2 ml), treated with trifluoroacetic acid (5 ml) at room temperature for 60 min to give the deblocked peptide (0.30 g).

The crude peptide obtained above was, after treatment with Amberlite CG-400 (acetate), submitted to a column (1.7×30 cm) of carboxymethyl cellulose (Serva, 0.7 meq/g) using an ammonium acetate buffer (pH 6.0, 2000 ml) with a linear concentration gradient of 0—0.6 M. The fractions (10 ml/tube) corresponding to a main peak (tubes 106—160) were pooled and lyophilized (0.19 g). This material was chromatographed on a Sephadex G-25 column (medium, 1.7×45 cm) with 1-butanol–acetic acid–pyridine–water (12: 3 : 4 : 6) as solvent and 4.5 ml-fractions were collected. The fractions containing the desired peptide as a single component (tubes 35—47), as examined by TLC, were combined (0.13 g). For final purification this was rechromatographed on a carboxymethyl cellulose column (CM-52, Whatman, 1.7×30 cm) using an ammonium acetate buffer (pH 6.0, 2000 ml) with a linear concentration gradient of 0—0.6 M. The franctions (10 ml/tube) corresponding to a single peak (tubes 158—185) were combined, evaporated and lyophilized to afford the pure nonadecapeptide (III) as colorless fluffy powder; yield 0.11 g (38%), $[\alpha]_{\mathrm{D}}^{26}$ $-69.4\pm2.1°$ (c 0.5, 0.1 M acetic acid). Lit, $[\alpha]_{\mathrm{D}}^{25}$ $-102.6°$ (c 0.5, 0.1 M acetic acid);17) $[\alpha]_{\mathrm{D}}^{20}$ $-50.0°$ (c 0.5, 0.1M acetic acid).18) $\lambda_{\mathrm{max}}^{0.1\mathrm{M}\ \mathrm{HCl}}$ 280 nm ($E_{1\mathrm{cm}}^{1\%}$ 23.6), $\lambda_{\mathrm{shoulder}}^{0.1\mathrm{M}\ \mathrm{HCl}}$ 288 nm ($E_{1\mathrm{cm}}^{1\%}$ 17.8); $\lambda_{\mathrm{max}}^{0.1\mathrm{M}\ \mathrm{NaOH}}$ 282 nm ($E_{1\mathrm{cm}}^{1\%}$ 24.1), 289 nm ($E_{1\mathrm{cm}}^{1\%}$ 23.3). Tlc (cellulose): a single component (ninhydrin and Ehrlich reagents) in 1-butanol–acetic acid–pyridine–water (15:3:10:15). Amino acid ratios in acid hydrolysate: Lys 2.98 (3), His 1.00 (1), Arg 3.02 (3), Ser 1.71 (2), Glu 1.06 (1), Pro 1.91 (2), Gly 1.98 (2), Val 1.00 (1), Met 0.98 (1), Tyr 1.00 (1), Phe 0.96 (1). The Trp/Tyr ratio in intact III was 0.95 as determined spectrophotometrically.15)

Z-Arg(NO$_2$*)-Pro-NH*$_2$ *(VII).* Z-Pro-NH$_2$ (3.43 g, 13.8 mmol)16) was hydrogenolyzed over palladium in methanol for 4 h. The resulting H-Pro-NH$_2$ (crystalline free base) and Z-Arg(NO$_2$)-OH (4.42 g, 12.5 mmol) were coupled with DCC (2.58 g, 12.5 mmol) in DMF-acetonitrile (1 : 5, 48 ml) at room temperature overnight. The product was precipitated from methanol-ethyl acetate and then crystallized from methanol; yield 3.14 g (56%), mp 173—174 °C, $[\alpha]_{\mathrm{D}}^{25.5}$ $-19.6\pm0.6°$ (c 1.0, DMF).

Found: C, 49.89; H, 6.03; N, 21.69%. Calcd for $C_{19}H_{27}N_7O_6$: C, 50.77; H, 6.05; N, 21.82%.

Z-Arg(NO$_2$*)-Arg(NO*$_2$*)-Pro-NH*$_2$ *(VIII).* Compound VII (2.65 g, 5.9 mmol) was treated with 25% hydrogen bromide in acetic acid (10 ml) at room temperature for 60 min to give H-Arg(NO$_2$)-Pro-NH$_2$ hydrobromide which was precipitated by the addition of ether (2.85 g).

A solution of Z-Arg(NO_2)-OH (1.77 g, 5 mmol) and tributylamine (1.31 ml, 5.5 mmol) in anhydrous tetrahydrofuran (20 ml) was chilled in an ice-salt bath and ethyl chloroformate (0.53 ml, 5.5 mmol) was added dropwise. The mixture was stirred at the same temperature for 5 min, after which an ice-cold solution of the dipeptide amide hydrobromide obtained above and tributylamine (3.58 ml, 15 mmol) in tetrahydrofuran-water (3 : 1, 20 ml) was introduced. The reaction mixture was stirred at −10—0 °C for 3 h followed by evaporation *in vacuo* at a bath temperature of 40 °C. The sirupy residue, dissolved in 1-butanol–ethyl acetate (1 : 3), was washed with M hydrochloric acid and M sodium hydrogencarbonate, and evaporated *in vacuo*. The crude product thus obtained was chromatographed on a column of silica gel (30 g, Kieselgel 60, Merck) with ethyl acetate–acetic acid–water (4 : 1 : 1) as solvent. The fractions containing the desired product as a major component were collected and evaporated *in vacuo* at a bath temperature of 40 °C, and the residue was precipitated from methanol-ether. The chromatography was repeated until the precipitates became completely homogeneous as examined by TLC on a silica gel plate with ethyl acetate–acetic acid–water (4:1:1) as solvent; yield 0.98 g, $[\alpha]_D^{25.5}$ −36.4±0.8° (*c*, 1.0, methanol).

Found: C, 46.18; H, 6.39; N, 23.82%. Calcd for $C_{25}H_{38}N_{12}O_9\cdot 2CH_3OH$: C, 45.37; H, 6.49; N, 23.52%.

Z-Lys(Boc)-Arg(NO_2)-Arg(NO_2)-Pro-NH_2 (IX). Compound VIII (0.78 g, 1.2 mmol) was treated with 25% hydrogen bromide in acetic acid (10 ml) at room temperature for 90 min to give H–Arg(NO_2)–Arg(NO_2)–Pro–NH_2 hydrobromide (0.93 g). This was allowed to react with Z-Lys(Boc)-OSu (0.57 g, 1.2 mmol)[10] in DMF in the presence of triethylamine (0.56 ml, 4.0 mmol) at 4 °C overnight. The solvent was evaporated *in vacuo* and the residue dissolved in 1-butanol-ethyl acetate (1:3) was washed with ice-cold M hydrochloric acid and M sodium hydrogencarbonate and evaporated *in vacuo*. The residue was solidified by treatment with ether; yield 0.91 g (87%), $[\alpha]_D^{25}$ −38.9±0.8° (*c* 1.0, methanol). TLC (silica gel): homogeneous (sulfuric acid) in ethyl acetate-acetic acid-water (4 : 1 : 1).

Found: C, 48.76; H, 7.05, N, 21.36%. Calcd for $C_{36}H_{58}N_{14}O_{12}\cdot CH_3OH$: C, 48.78; H, 6.86; N, 21.53%.

Z-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-NH_2 Acetate (X). Compound IX (0.66 g, 0.75 mmol) was hydrogenolyzed over palladium in 20% acetic acid in methanol (10 ml) for 7 h to give the N^α-free tetrapeptide H–Lys(Boc)–Arg–Arg–Pro–NH_2. A 0.60 g-portion (0.65 mmol) was dissolved in tetrahydrofuran–water (5 : 2, 7 ml) and triethylamine (0.20 ml) was added. The resulting solution was combined with an ice-cold solution of Z-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-N_3 [derived from the corresponding hydrazide (0.89 g, 1mmol)][10] in 90% tetrahydrofuran and the mixture was stirred at 4 °C overnight followed by evaporation *in vacuo*. To this were added ethyl acetate (15 ml) and M acetic acid (15 ml) and the mixture was shaken vigorously. The aqueous phase which separated was extracted repeatedly with ethyl acetate. The organic solutions were combined and evaporated *in vacuo*. The residue was again distributed between ethyl acetate (15 ml) and M acetic acid (15 ml), and the organic phase was extracted with M acetic acid repeatedly. The aqueous solutions were combined and evaporated *in vacuo* at a bath temperature of 45 °C. The residue was redissolved in M acetic acid (15 ml) and the solution was extracted with water-saturated 1-butanol repeatedly. The butanol extracts were combined and evaporated *in vacuo* at a bath temperature of 45 °C. The resulting sirupy residue was precipitated from methanol-ether and was then lyophilized to give X; yield 0.78 g (73%), $[\alpha]_D^{25}$ −53.5±0.9° (*c* 1.0, methanol). Tlc (silica gel): homogeneous (ninhydrin, after pretreatment with hydrochloric acid) in ethyl acetate-acetic acid-water (3 : 1 : 1).

Found: C, 51.78; H, 8.00; N, 16.08%. Calcd for $C_{70}H_{119}N_{19}O_{17}\cdot 2CH_3COOH\cdot 4H_2O$: C, 52.56; H, 8.05; N, 15.74%.

References

1) C. H. Li, J. Meienhofer, E. Schnabel, D. Chung, T-B. Lo, and J. Ramachandran, *J. Am. Chem. Soc.*, **82**, 5760 (1960); **83**, 4449 (1961).

2) K. Hofmann, H. Yajima, T-Y. Liu, N. Yanaihara, C. Yanaihara, and J. L. Humes, *J. Am. Chem. Soc.*, **84**, 1054 (1962); **84**, 4481 (1962).

3) K. Hofmann, H. Yajima, T-Y. Liu, and N. Yanaihara, *J. Am. Chem. Soc.*, **83**, 487 (1961); **84**, 4475 (1962).

4) H. Kappeler and R. Schwyzer, *Helv. Chim. Acta*, **44**, 1136 (1961); R. Schwyzer and H. Kappeler, *ibid.*, **46**, 1550 (1963).

5) C. H. Li, D. Chung, J. Ramachandran, and B. Gorup, *J. Am. Chem. Soc.*, **86**, 2703 (1964); **84**, 2460 (1962).

6) H. Otsuka, K. Inouye, M. Kanayama, and F. Shinozaki, *Bull. Chem. Soc. Jpn.*, **38**, 679 (1965).

7) H. Otsuka, K. Inouye, M. Kanayama, and F. Shinozaki, *Bull. Chem. Soc. Jpn.*, **38**, 1563 (1965).

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

9) H. Otsuka, K. Inouye, F. Shinozaki, and M. Kanayama, *Bull. Chem. Soc. Jpn.*, **39**, 1171 (1966).

10) H. Otsuka, K. Inouye, M. Kanayama, and F. Shinozaki, *Bull. Chem. Soc. Jpn.*, **39**, 882 (1966).

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

12) H. Otsuka, M. Shin, Y. Kinomura, and K. Inouye, *Bull. Chem. Soc. Jpn.*, **43**, 196 (1970).

13) An effective use of a homogeneous solvent system in partition chromatography was demonstrated previously: K. Inouye, *Bull. Chem. Soc. Jpn.*, **38**, 1148 (1965).

14) J. L. Bailey, "Techniques in Protein Chemistry," Elsevier, Amsterdam-London-New York (1967), p. 340.

15) 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).

16) K. Inouye, K. Namba, and H. Otsuka, *Bull. Chem. Soc. Jpn.*, **44**, 1689 (1971).

17) J. Ramachandran, D. Chung, and C. H. Li, *J. Am. Chem. Soc.*, **87**, 2696 (1965).

18) S. Bajusz and K. Medzihradszky, "Peptides," Proceedings of the 8th European Peptide Symposium, Noordwijk, The Netherlands, 1966, H. C. Beyerman, A. van de Linde, and W. Maassen van den Brink (eds.), North-Holland, Amsterdam (1967), p. 209.

19) The assays were performed by Dr. Akira Tnaka and Mr. Kunihiro Odaguchi of this laboratory. We thank Dr. Tanaka for permission to include these data in the present communication.

20) K. Inouye, K. Watanabe, and H. Otsuka, *Bull. Chem. Soc. Jpn.*, in press.

21) H. Imura, H. Matsuyama, S. Matsukura, T. Miyake, and M. Fukase, *Endocrinol.*, **80**, 599 (1967).

22) M. Nakamura, *J. Biochem.* (Tokyo), **71**, 1029 (1972).