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Synthesis of Peptides related to Corticotropin (ACTH). IX.[1] Application of N-Hydroxy-5-norbornene-2,3-dicarboximide Active Ester Procedure to the Synthesis of Human Adrenocorticotropic Hormone (α_h-ACTH)[2,3]

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Human adrenocorticotropic hormone (α_h-ACTH), H–Ser–Tyr–Ser–Met–Glu–His–Phe–Arg–Trp–Gly–Lys–Pro–Val–Gly–Lys–Lys–Arg–Arg–Pro–Val–Lys–Val–Tyr–Pro–Asn–Gly–Ala–Glu–Asp–Glu–Ser–Ala–Glu–Ala–Phe–Pro–Leu–Glu–Phe–OH, was synthesized by using N-hydroxy-5-norbornene-2,3-dicarboximide (HONB) with N,N′-dicyclohexylcarbodiimide (DCC) for the stepwise elongation and fragment condensation.

The synthetic α_h-ACTH, purified on a column of carboxymethylcellulose, Amberlite XAD-2 resin and Bio-Gel P-6, was found to be homogeneous by various criteria and exhibits full biological activity (*ca.* 145 units/mg) in *in vitro* steroidogenic assay.

Recently, the revised structure of human adrenocorticotropic hormone (α_h-ACTH, Fig. 1) was reported by L. Gráf, *et al.*[5] and B. Riniker, *et al.*[6] Since then, several groups published the total syntheses of this hormone by conventional methods[7,8] and by solid phase technique.[9]

H–Ser–Tyr–Ser–Met–Glu–His–Phe–Arg–Trp–Gly–Lys–Pro–Val–Gly–Lys–Lys–Arg–Arg–Pro–Val–
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Lys–Val–Tyr–Pro–Asn–Gly–Ala–Glu–Asp–Glu–Ser–Ala–Glu–Ala–Phe–Pro–Leu–Glu–Phe–OH
21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39

Fig. 1. Structure of α_h-ACTH

In our current studies on the synthesis of corticotropin-related peptides, we also accomplished the total synthesis of α_h-ACTH by the method different from the above authors.

As shown in Fig. 2, two fragments bearing the protecting groups all removable by hydrogen fluoride,[10] served to build up the entire sequence of α_h-ACTH. Of these two fragments, the N-terminal-protected tetracosapeptide (I) was previously synthesized.[1]

1) Part VIII: C. Hatanaka, O. Nishimura, and M. Fujino, *Chem. Pharm. Bull.* (Tokyo), **23**, 1017 (1975).
2) Part of this work was presented by O. Nishimura and M. Fujino, Proc. 10th Symposium on Peptide Chemistry in Japan, ed. by J. Noguchi, 1972, p. 154.
3) Amino acids, peptides and their derivatives mentioned in this paper are of the L-configuration. The abbreviations used are those recommended by the IUPAC-IUB Commission of Biochemistry on Nomenclature in July 1965 and July 1966: *Biochemistry*, **5**, 2485 (1966); *ibid.*, **6**, 362 (1967).
Other abbreviations used are: NO_2=nitro; OPCP=pentachlorophenyl ester; ODNP=2,4-dinitrophenyl ester; ONB=N-hydroxy-5-norbornene-2,3-dicarboximide ester; DCC=N,N′-dicyclohexylcarbodimide; HONB=N-hydroxy-5-norbornene-2,3-dicarboximide; *p*-TsOH=*p*-toluenesulfonic acid.
4) Location: *Jusohonmachi, Yodogawa-ku, Osaka, 532, Japan.*
5) L. Gráf, S. Bajusz, A. Patty, E. Barat, and G. Csek, *Acta Biochim. et Biophys. Scad. Sci. Hung.*, **6**, 415 (1971).
6) B. Riniker, P. Sieber, W. Rittel, and H. Zuber, *Nature, New Biol.*, **235**, 114 (1972).
7) P. Sieber, W. Rittel, and B. Riniker, *Helv. Chim. Acta*, **55**, 1243 (1972).
8) L. Kisfaludy, M. Löw, T. Szirtes, I. Schon, M. Sarkozi, S. Bajusz, A. Turan, R. Beke, A. Juhasz, L. Graf, and K. Medzihradszky, Proc. 3rd American Peptide Symposium, ed. by J. Meienhofer, 1972, p. 299.
9) D. Yamashiro and C.H. Li, *J. Am. Chem. Soc.*, **95**, 1310 (1973).
10) S. Sakakibara and Y. Shomonishi, *Bull. Chem. Soc. Japan*, **38**, 1412 (1965).

(I) Z-Ser-Tyr-Ser-Met-Glu(O^{t}Bu)-His-Phe-Arg-Trp-Gly-Lys(Z)-Pro-Val-Gly-Lys(Z)-Lys(Z)-
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Arg(NO$_2$)-Arg(NO$_2$)-Pro-Val-Lys(Z)-Val-Tyr-Pro-OH·p-TsOH
17 18 19 20 21 22 23 24

(II) Z-Asn-Gly-Ala-Glu(O^{t}Bu)-Asp(O^{t}Bu)-Glu(O^{t}Bu)-Ser-Ala-Glu(O^{t}Bu)-Ala-Phe-Pro-Leu-
25 26 27 28 29 30 31 32 33 34 35 36 37
Glu(O^{t}Bu)-Phe-O^{t}Bu
38 39

Fig. 2. Synthetic Fragments

The synthetic route to the protected C-terminal pentadecapeptide ester (II) was newly established according to the scheme in Chart 1. The N-hydroxy-5-norbornene-2,3-dicarboximide (HONB)[11] active ester was used extensively for this purpose since, as mentioned previously, this active ester procedure has many advantageous features, such as suppression of racemization and β-alanine derivative formation.[12,13] In addition, formation of N-acylurea and the nitrile compound in asparagine is prevented by this procedure.

The tripeptide ester Z-Leu-Glu(O^{t}Bu)-Phe-O^{t}Bu (IV)[14] and the tripeptide active ester Z-Ala-Phe-Pro-OPCP (V) were first prepared. For the synthesis of IV, Z-Glu(O^{t}Bu)-ONB was coupled with H-Phe-O^{t}Bu to give Z-Glu(O^{t}Bu)-Phe-O^{t}Bu (III)[14] in crystalline state. Compound III was subjected to hydrogenation using palladium black as catalyst and the resulting dipeptide ester, H-Glu(O^{t}Bu)-Phe-O^{t}Bu, was acylated with Z-Leu-ONB to give IV in crystalline form. For the synthesis of V, Z-Phe-ONB was first coupled with H-Pro-O^{t}Bu to give Z-Phe-Pro-O^{t}Bu as an oil. Hydrogenation of this dipeptide ester using palladium black as catalyst gave H-Phe-Pro-O^{t}Bu, which was then coupled with Z-Ala-ONB. Treatment of the resulting oily tripeptide ester, Z-Ala-Phe-Pro-O^{t}Bu, with trifluoroacetic acid afforded the corresponding acid, which was converted into V by pentachlorophenyltrichloroacetate.[15]

To assemble the two peptide units thus obtained, IV was subjected to catalytic hydrogenation and the resulting H-Leu-Glu(O^{t}Bu)-Phe-O^{t}Bu was allowed to react with V at room temperature for 48 hr. The desired protected hexapeptide ester, Z-Ala-Phe-Pro-Leu-Glu-(O^{t}Bu)-Phe-O^{t}Bu (VI), was isolated in satisfactory yield.

Next, a combination of catalytic hydrogenation and the HONB method was applied to lengthen the peptide chain of VI in stepwise manner to the tridecapeptide stage, except for the Ser residue which was incorporated as 2,4-dinitrophenylester.[16] All intermediates, Z-Glu(O^{t}Bu)-Ala-Phe-Pro-Leu-Glu(O^{t}Bu)-Phe-O^{t}Bu (VII),[14] Z-Ala-Glu(O^{t}Bu)-Ala-Phe-Pro-Leu-Glu(O^{t}Bu)-Phe-O^{t}Bu (VIII),[14] Z-Ser-Ala-Glu-(O^{t}Bu)-Ala-Phe-Pro-Leu-Glu(O^{t}Bu)-Phe-O^{t}Bu (IX),[7] Z-Glu(O^{t}Bu)-Ser-Ala-Glu(O^{t}Bu)-Ala-Phe-Pro-Leu-Glu(O^{t}Bu)-Phe-O^{t}Bu (X),[7] Z-Asp(O^{t}Bu)-Glu(O^{t}Bu)-Ser-Ala-Glu(O^{t}Bu)-Ala-Phe-Pro-Leu-Glu(O^{t}Bu)-Phe-O^{t}Bu (XI),[7] Z-Glu(O^{t}Bu)-Asp(O^{t}Bu)-Glu(O^{t}Bu)-Ser-Ala-Glu(O^{t}Bu)-Ala-Phe-Pro-Leu-Glu(O^{t}Bu)-Phe-O^{t}Bu (XII)[7] and the protected tridecapeptide ester, Z-Ala-Glu(O^{t}Bu)-Asp(O^{t}Bu)-Glu(O^{t}Bu)-Ser-Ala-Glu(O^{t}Bu)-Ala-Phe-Pro-Leu-Glu(O^{t}Bu)-Phe-O^{t}Bu (XIII),[7] were isolated in analytically pure state.

The next step involved incorporation of the Asn-Gly moiety into XIII. As mentioned by L. Gráf, *et al.*,[5] this Asn-Gly bond is sensitive to alkaline conditions. Therefore, we prepared

11) M. Fujino, S. Kobayashi, M. Obayashi, T. Fukuda, S. Shinagawa, and O. Nishimura, *Chem. Pharm. Bull.* (Tokyo), **22**, 1857 (1974).
12) M. Löw and L. Kisfaludy, *Acta Chim. Acad. Sci. Hung.*, **44**, 61 (1965).
13) H. Gross and L. Bilk, *Tetrahedron*, **24**, 1391 (1968).
14) R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, **49**, 134 (1966).
15) M. Fujino and C. Hatanaka, *Chem. Pharm. Bull.* (Tokyo), **16**, 929 (1968).
16) F. Morchiori, R. Rocchi, and E. Scoffone, *Gazz. Chim. Ital.*, **93**, 834 (1963).

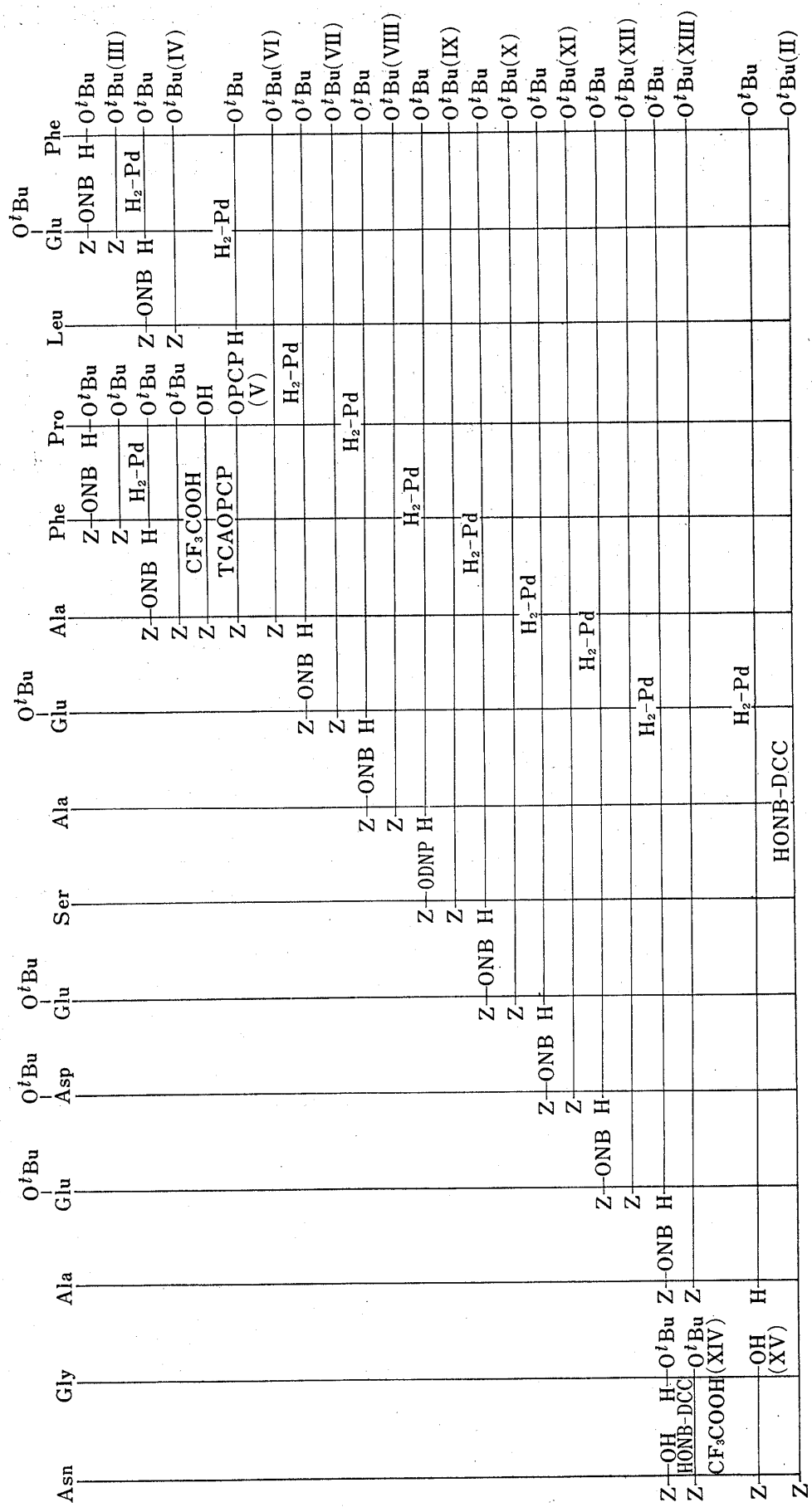

Chart 1

Z=carbobenzoxy, O^{t}Bu=t-butylester, TCAOPCP=pentachlorophenyltrichloroacetate, other abbreviations used are presented in ref. 3

Z-Asn-Gly-OH (XV) by a method different from that of S.J. Leach and H. Lindly[17] without exposure to any alkaline conditions.

This dipeptide (XV) was obtained by trifluoroacetic acid treatment of Z-Asn-Gly-O^{t}Bu (XIV), which had been synthesized by coupling Z-Asn-OH with H-Gly-O^{t}Bu *via* its HONB active ester.

Z-Asn-Gly-OH, thus synthesized, was converted into the corresponding HONB ester, which was condensed with the N$^\alpha$-deprotected XIII to yield the protected pentadecapeptide ester (II) in crystalline state. Its homogeneity was established by thin-layer chromatography, elemental analysis and amino acid analysis.

The C-terminal-protected pentadecapeptide II for the synthesis of α_h-ACTH was thus obtained by adopting the HONB active ester method in satisfactory yields at each coupling step.

In order to construct the entire amino acid sequence of α_h-ACTH, I, which has been described previously, and H-Asn-Gly-Ala-Glu(O^{t}Bu)-Asp(O^{t}Bu)-Glu(O^{t}Bu)-Ser-Ala-Glu(O^{t}Bu)-Ala-Phe-Pro-Leu-Glu(O^{t}Bu)-Phe-O^{t}Bu obtained by catalytic hydrogenation of II, were condensed, as shown in Chart 2, by DCC in the presence of HONB at room temperature for two days.

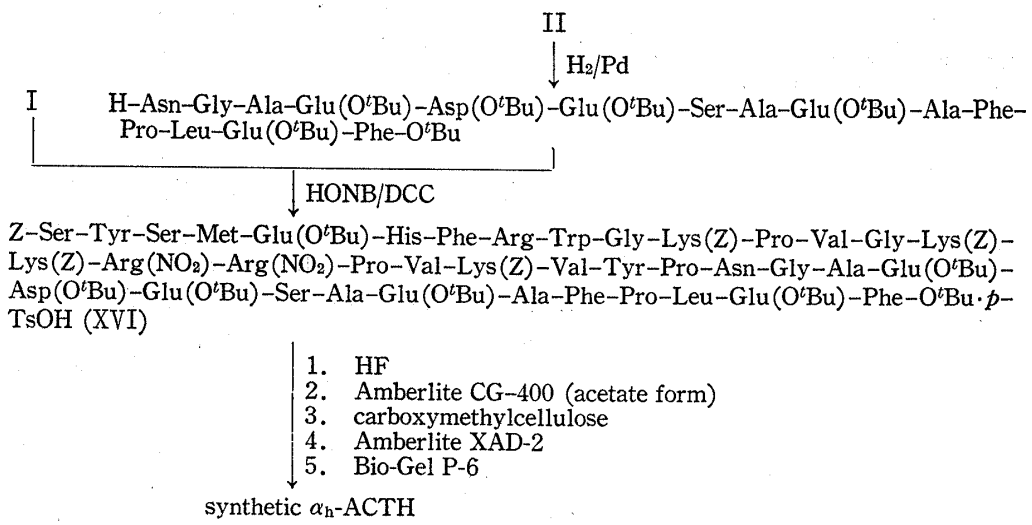

Chart 2. Synthesis of α_h-ACTH

The resulting crude protected nonatriacontapeptide ester, Z-Ser-Tyr-Ser-Met-Glu(O^{t}Bu)-His-Phe-Arg-Trp-Gly-Lys(Z)-Pro-Val-Gly-Lys(Z)-Lys(Z)-Arg(NO$_2$)-Arg(NO$_2$)-Pro-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(O^{t}Bu)-Asp(O^{t}Bu)-Glu(O^{t}Bu)-Ser-Ala-Glu(O^{t}Bu)-Ala-Phe-Pro-Leu-Glu(O^{t}Bu)-Phe-O^{t}Bu·*p*-Tos-OH (XVI), was purified by reprecipitation from hot 80% aqueous methanol as analytically pure material. The purified protected nonatriacontapeptide ester (XVI) was treated with hydrogen fluoride in the presence of anisole, skatole, methylethylsulfide and thioglycolic acid at 0° for one hour to remove all protecting groups. Addition of these scavengers is well known to be effective in preventing the possible alkylation of Met, Tyr and Trp residues during the deblocking procedure.

The resulting crude synthetic α_h-ACTH was converted into the corresponding acetate by being passed through a column of Amberlite CG-400 (acetate form). This acetate was chromatographed on carboxymethyl-cellulose using a gradient elution with an ammonium acetate buffer. A typical chromatogram is illustrated in Fig. 3.

17) S.J. Leach and H. Lindly, *Australian J. Chem.*, 7, 173 (1954).

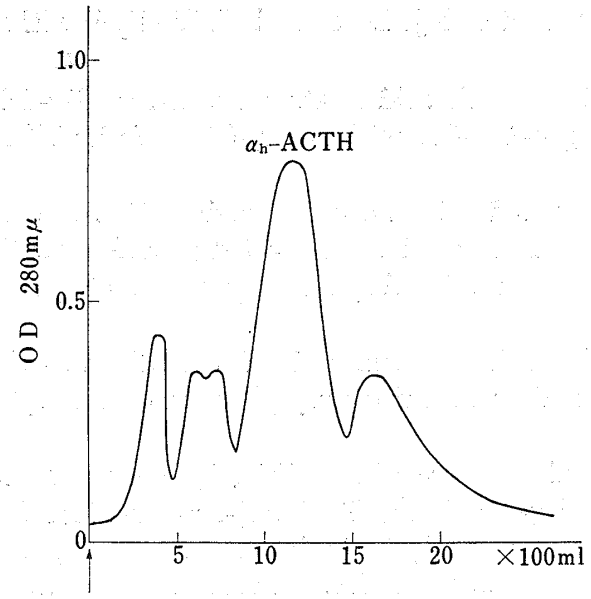

Fig. 3. Carboxymethylcellulose Chromatography of Crude Synthetic α_h-ACTH

Arrow indicates where the gradient elution was started by introducing 0.2M (pH 6.9) ammonium acetate buffer through a 2000 ml mixing chamber containing 0.01M (pH 6.9) ammonium acetate buffer.
column: 3.0×24.0 cm

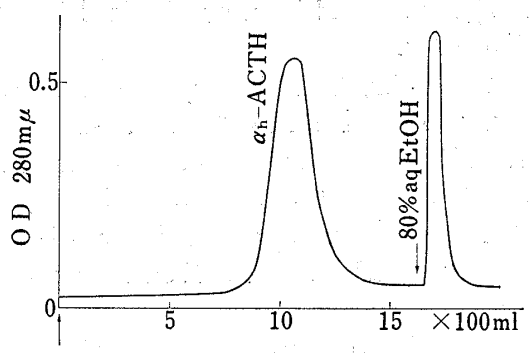

Fig. 4. Amberlite XAD-2 Chromatography of Partially Purified Synthetic α_h-ACTH

Arrow indicates where the gradient elution was started by introducing 80% aqueous ethanol (885 ml, containing 1% acetic acid) through a 1650 ml mixing chamber containing 0.1M ammonium acetate buffer.
column: 2.5×7.5 cm

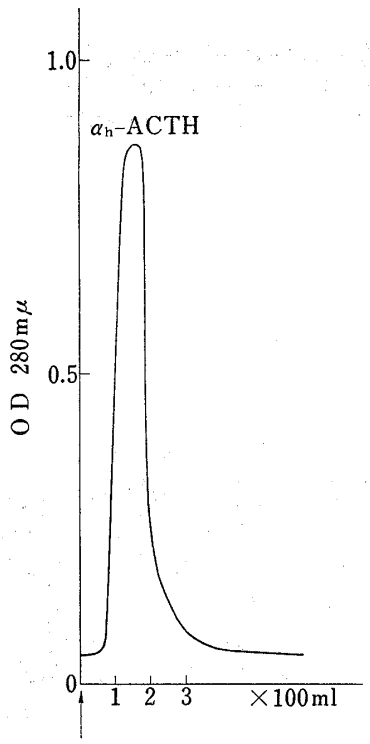

Fig. 5. Gel Filtration on Bio-Gel P-6 of Purified Synthetic α_h-ACTH

Arrow indicates where the elution was started by introducing 1N acetic acid.
column: 3.0×48.0 cm

The fractions corresponding to the main peak were collected and then applied to a column of Amberlite XAD-2 for desalting and further purification. The desired compound was eluted with aqueous ethanol (see Fig. 4).

The material eluted by 80% aqueous ethanol exhibited a yellow color when tested with Ehrlich's reagent. This seems to be a degradation product of the Trp residue formed during the HF treatment. Therefore, this column chromatography of Amberlite XAD-2 was quite effective for separating such a Trp-degradated product.

The desired fractions obtained above were subjected to chromatography on Bio-Gel P-6 (Fig. 5) using dilute acetic acid as an eluant for further desalting.

Highly purified synthetic α_h-ACTH thus obtained was found to be homogeneous by various criteria, such as thin-layer chromatography, paper chromatography and electrophoresis. The amino acid analysis on an acid hydrolysate of the synthetic material also agreed well with theoretical values.

This synthetic α_h-ACTH was not digested completely by using aminopeptidese M alone, presumably because of the presence of Pro residue[18]

18) E.C. Jorgenson, G.C. Windrigde, W. Patton, and T.C. Lee, *J. Med. Chem.*, **12**, 733 (1969).

and its higher molecularity. Therefore, enzymic hydrolysis was carried out with the combination of pronase P and aminopeptidase M by modifying the method of C. Bennet, *et al.*[19]

It is worthwhile to note that a reasonable recovery of Trp was obtained in this enzymic hydrolysis, although Trp is destroyed in the usual acid hydrolysis. Also, the Asn peak overlapped the Ser peak. However, Asp was determined as one mole in this hydrolysis and two moles in acid hydrolysis. These results indicate that the Asn residue in our synthesis of α_h-ACTH remained intact throughout the synthesis.

A study on the *in vitro* steroidogenic activity carried out by the procedure of Sayers, *et al.*,[20,21] revealed that the synthetic α_h-ACTH possessed an activity of *ca.* 145 units/mg compared with the 3rd U.S.P. reference standard.

The experimental results presented here demonstrated that our synthetic α_h-ACTH is highly pure and, in addition, the HONB active ester is a useful tool for the preparation of complicated peptides.

Experimental

All melting points were taken by the capillary method and are uncorrected. Product purity was tested by thin-layer chromatography. Solvent system used were: $CHCl_3$–MeOH–AcOH (9: 1: 0.5, Rf^1), AcOEt–pyridine–AcOH–H_2O (60: 20: 6: 10, Rf^2), *n*-BuOH–AcOH–H_2O (4: 1: 1, Rf^3), *n*-BuOH–pyridine–AcOH–H_2O (30: 20: 6: 24, Rf^4).

Z-Glu(O^{t}Bu)-Phe-O^{t}Bu (III)——Z-Phe-O^{t}Bu (21.3 g, 0.06 mole) was dissolved in 200 ml of MeOH and hydrogenated over Pd-black catalyst (2.0 g) for 8 hr. The Pd catalyst was filtered off and the filtrate was evaporated *in vacuo*. The resulting residue and Z-Glu(O^{t}Bu)-ONB [prepared from Z-Glu(O^{t}Bu)-OH (20.2 g, 0.06 mole), HONB (10.8 g, 0.06 mole) and DCC (12.4 g, 0.06 mole) in dioxane (100 ml)] were dissolved in 70 ml of dioxane. After being stirred for 12 hr at room temperature, the reaction mixture was evaporated *in vacuo* and the resulting oily residue was dissolved in AcOEt. The AcOEt layer was washed successively with water, 0.2 N HCl and 5% $NaHCO_3$ and dried over anhydr. Na_2SO_4. The AcOEt solution was evaporated *in vacuo* and the resulting oily residue was crystallized by addition of hexane. The product was recrystallized from AcOEt–hexane: yield 28.0 g (86.4%); mp 131.0—131.5° (lit.[14] mp 131.5—132.5°); $[\alpha]_D^{22}$ −11.2° (c=0.99 in DMF). Rf^1 0.96. *Anal.* Calcd. for $C_{30}H_{40}O_7N_2$: C, 66.64; H, 7.38; N, 5.15. Found: C. 66.91; H, 7.86; N, 5.16.

Z-Leu-Glu(O^{t}Bu)-Phe-O^{t}Bu (IV)——Compound III (16.2 g, 0.03 mole) was dissolved in 200 ml of MeOH and hydrogenated over Pd-black (1.5 g) as catalyst for 5 hr. The Pd catalyst was filtered off and the filtrate was evaporated *in vacuo*. The resulting residue and Z-Leu-ONB [prepared from Z-Leu-OH (8.00 g, 0.03 mole), HONB (5.40 g, 0.03 mole) and DCC (6.18 g, 0.03 mole) in dioxane (50 ml)] were dissolved in 50 ml of DMF and the reaction mixture was stirred for 12 hr at room temperature. The reaction mixture was evaporated *in vacuo* and the resulting residue was dissolved in AcOEt. The AcOEt layer was washed successively with 0.2 N HCl and 5% $NaHCO_3$ in the usual manner and dried over anhydr. Na_2SO_4. The AcOEt solution was evaporated *in vacuo* and the resulting oily residue was crystallized by addition of hexane. The product was recrystallized from AcOEt–hexane: yield 16.4 g (83.7%); mp 113—115° (lit.[14] mp 116.5—117.5°); $[\alpha]_D^{22}$ −14.3° (c=1.04 in DMF); Rf^1 0.93. *Anal.* Calcd. for $C_{36}H_{51}O_8N_3$: C, 66.13; H, 7.86; N, 6.42. Found: C, 66.26; H, 8.17; N, 6.53.

Z-Ala-Phe-Pro-OPCP (V)——Z-Pro-O^{t}Bu (15.5 g, 0.05 mole) was dissolved in 200 ml of MeOH and hydrogenated over Pd-black (1.5 g) for 8 hr. The Pd catalyst was filtered off and the filtrate was evaporated *in vacuo*. The resulting residue and Z-Phe-ONB [prepared from Z-Phe-OH (15.0 g, 0.05 mole), HONB (9.0 g, 0.05 mole) and DCC (10.3 g, 0.05 mole) in dioxane (100 ml)] were dissolved in 100 ml of dioxane and the reaction mixture was stirred for 12 hr at room temperature. The reaction mixture was evaporated *in vacuo* and the resulting residue was dissolved in AcOEt. The AcOEt layer was washed with 0.2 N HCl and 5% $NaHCO_3$ in the usual manner and dried over anhydr. Na_2SO_4. The AcOEt solution was evaporated *in vacuo* to obtain Z-Phe-Pro-O^{t}Bu as oil; yield, 21.0 g (90.0%); Rf^1 0.93.

This oily dipeptide ester was dissolved in 200 ml of MeOH and hydrogenated over Pd-black (1.5 g) for 6 hr. The Pd catalyst was filtered off and the filtrate was evaporated *in vacuo*. The resulting residue and Z-Ala-ONB [prepared from Z-Ala-OH (10.0 g, 0.045 mole), HONB (8.06 g, 0.045 mole) and DCC (9.27 g, 0.045 mole) in dioxane (100 ml)] were dissolved in 100 ml of dioxane. The reaction mixture was stirred for 12 hr at room temperature. The solvent was evaporated *in vacuo* and the resulting residue was dissolved

19) C. Bennet, W.H. Königsberg, and G.M. Edelmann, *Biochemistry*, **9**, 3181 (1970).
20) G. Sayers, R.L. Swallow, and N.D. Giordano, *Endocrinology*, **88**, 1063 (1971).
21) We thank Dr. R. Nakayama, and Mr. M. Shikata of these laboratories for the biological assay.

in AcOEt. The AcOEt layer was washed with 0.2 N HCl and 5% $NaHCO_3$ in the usual manner and dried over anhydr. Na_2SO_4. The AcOEt solution was evaporated *in vacuo* to dryness. Z–Ala–Phe–Pro–O^{t}Bu was obtained as oil; yield, 23.1 g (100%); Rf^1 0.89. This oily tripeptide ester was treated with trifluoroacetic acid (150 ml) for 1 hr at 10° and trifluoroacetic acid was evaporated *in vacuo*. The resulting residue was dissolved in AcOEt and the AcOEt layer was washed three times with water and dried over anhydr. Na_2SO_4. The AcOEt solution was then evaporated *in vacuo* to give Z–Ala–Phe–Pro–OH as an oil; yield, 21.2 g (100%); Rf^1 0.75. This oily tripeptide was dissolved in 40 ml of DMF together with triethylamine (6.3 ml, 0.045 mole) and pentachlorophenyltrichloroacetate[15] (24.7 g, 0.06 mole) under cooling. The reaction mixture was allowed to stand for 3 hr at room temperature and then poured into 300 ml of cold water. The separated oily product was washed with water and crystallized from EtOH. Recrystallization from EtOH yielded 20.6 g (64.0% based on Z–Pro–O^{t}Bu) of V; mp 175—177°; $[\alpha]_D^{21}$ −36.7° (c=0.99 in DMF); Rf^1 0.91. *Anal.* Calcd. for $C_{31}H_{28}O_6N_3Cl_5$: C, 52.00; H, 3.94; N, 5.85; Cl, 23.79. Found: C, 52.03; H, 3.91; N, 5.97; Cl, 23.79.

Z-Ala-Phe-Pro-Leu-Glu(O^{t}Bu)-Phe-O^{t}Bu (VI)——Compound IV (13.1 g, 0.02 mole) was dissolved in 200 ml of MeOH and hydrogenated over Pd-black (1.5 g) for 5 hr. The Pd catalyst was filtered off and the filtrate was evaporated *in vacuo* to dryness. The resulting residue and compound V (14.3 g, 0.02 mole) were dissolved in 100 ml of dioxane and stirred for 2 days at room temperature. The solvent was evaporated *in vacuo* and the resulting residue was dissolved in AcOEt. The AcOEt layer was washed with 0.2 N HCl and dried over anhydr. Na_2SO_4. The AcOEt solution was evaporated *in vacuo* and the resulting oily residue was precipitated by addition of ether and hexane. The product was purified by reprecipitation from AcOEt–hexane; yield, 19.4 g (100%); mp 145.0—147.0°; $[\alpha]_D^{22}$ −38.6° (c=1.01 in DMF); Rf^1 0.74. *Anal.* Calcd. for $C_{53}H_{72}O_{11}N_6 \cdot H_2O$: C, 64.48; H, 7.55; N, 8.51. Found: C, 64.35; H, 7.38; N, 8.45.

Z-Glu(O^{t}Bu)-Ala-Phe-Pro-Leu-Glu(O^{t}Bu)-Phe-O^{t}Bu (VII)——Compound VI (18.4 g, 0.019 mole) was dissolved in 200 ml of MeOH and hydrogenated over Pd-black (1.5 g) for 5 hr. The Pd catalyst was filtered off and the filtrate was evaporated *in vacuo* to dryness. The resulting residue and Z–Glu(O^{t}Bu)–ONB [prepared from Z–Glu(O^{t}Bu)–OH (7.09 g, 0.021 mole), HONB (3.78 g, 0.021 mole) and DCC (4.33 g, 0.021 mole) in dioxane (50 ml)] were dissolved in 100 ml of dioxane and stirred for 12 hr at room temperature. The solvent was evaporated *in vacuo* and the resulting residue was dissolved in AcOEt. The AcOEt layer was washed successively with 0.2 N HCl and 5% $NaHCO_3$ and dried over anhydr. Na_2SO_4. The AcOEt solution was evaporated *in vacuo* and the resulting crystalline residue was triturated with ether. The precipitate was collected by filtration and recrystallized from AcOEt; yield 19.45 g (89.1%); mp 168—170° (decomp.) (lit.[14] mp 172—173°); $[\alpha]_D^{21}$ −34.4° (c=1.03 in DMF); Rf^1 0.72. *Anal.* Calcd. for $C_{62}H_{87}O_{14}N_7$: C, 64.51; H, 7.60; N, 8.49. Found: C, 64.04; H, 7.73; N, 8.39.

Z-Ala-Glu(O^{t}Bu)-Ala-Phe-Pro-Leu-Glu(O^{t}Bu)-Phe-O^{t}Bu (VIII)——Compound VII (18.5 g, 0.016 mole) was dissolved in 200 ml of MeOH and hydrogenated over Pd-black (1.5 g) for 8 hr. The Pd catalyst was filtered off and the filtrate was evaporated *in vacuo* to dryness. The resulting residue and Z–Ala–ONB [prepared from Z–Ala–OH (4.46 g, 0.02 mole), HONB (3.48 g, 0.02 mole) and DCC (4.12 g, 0.02 mole) in dioxane (30 ml)] were dissolved in 100 ml of dioxane and stirred for 12 hr at room temperature. The solvent was evaporated *in vacuo* and the resulting residue was triturated with water. The precipitate was crystallized from AcOEt; yield 18.8 g (96.0%); mp 185—186° (decomp.); $[\alpha]_D^{23}$ −34.9° (c=1.05 in DMF); Rf^1 0.70. *Anal.* Calcd. for $C_{65}H_{92}O_{15}N_8$: C, 63.70; H, 7.56; N, 9.14. Found: C, 63.42; H, 7.54; N, 9.18.

Z-Ser-Ala-Glu(O^{t}Bu)-Ala-Phe-Pro-Leu-Glu(O^{t}Bu)-Phe-O^{t}Bu (IX)——Compound VIII (12.3 g, 0.01 mole) was dissolved in 200 ml of 80% aqueous acetic acid and hydrogenated over Pd-black (1.0 g) for 8 hr. The Pd catalyst was filtered off and the filtrate was evaporated *in vacuo* to dryness. The resulting residue and Z–Ser–ODNP (5.30 g, 0.013 mole) were dissolved in 50 ml of DMF and stirred for 12 hr at room temperature. The reaction mixture was poured into 850 ml of ether and the resulting precipitate was collected by filtration. The precipitate was purified by reprecipitation from AcOEt–ether: yield 11.5 g (87.7%); mp 181—183° (decomp.); $[\alpha]_D^{23}$ −32.9° (c=1.04 in DMF); Rf^1 0.67. *Anal.* Calcd. for $C_{68}H_{97}O_{17}N_9$: C, 62.22; H, 7.44; N, 9.60. Found: C, 62.11; H, 7.88; N, 9.77.

Z-Glu(O^{t}Bu)-Ser-Ala-Glu(O^{t}Bu)-Ala-Phe-Pro-Leu-Glu(O^{t}Bu)-Phe-O^{t}Bu (X)——Compound IX (9.45 g, 7.2 mmoles) was dissolved in a mixture (200 ml) of *n*-butanol–MeOH–water (1 : 1 : 1) and hydrogenated over Pd-black (1.0 g) for 8 hr. The Pd catalyst was filtered off and the filtrate was evaporated *in vacuo* to dryness. The resulting residue and Z–Glu(O^{t}Bu)–ONB [prepared from Z–Glu(O^{t}Bu)–OH (3.64 g, 10.8 mmoles), HONB (1.94 g, 10.8 mmoles) and DCC (2.23 g, 10.8 mmoles) in dioxane (30 ml)] were dissolved in 50 ml of DMF and stirred for 12 hr at room temperature. The reaction mixture was poured into 700 ml of water. The resulting precipitate was collected by filtration. The precipitate was purified by reprecipitation from AcOEt–ether; yield 10.3 g (95.4%); mp 189—190° (decomp.); $[\alpha]_D^{23}$ −29.7° (c=0.98 in DMF); Rf^1 0.65. *Anal.* Calcd. for $C_{77}H_{112}O_{20}N_{10}$: C, 61.74; H, 7.53; N, 9.35. Found: C, 61.09; H, 7.61; N, 9.23.

Z-Asp(O^{t}Bu)-Glu(O^{t}Bu)-Ser-Ala-Glu(O^{t}Bu)-Ala-Phe-Pro-Leu-Glu(O^{t}Bu)-Phe-O^{t}Bu (XI)——Compound X (9.0 g, 6.0 mmoles) was dissolved in a mixture (150 ml) of *n*-butanol–MeOH–water (1 : 1 : 1) and hydrogenated over Pd-black (1.0 g) for 8 hr. The Pd catalyst was filtered off and the filtrate was evaporated *in vacuo* to dryness. The resulting residue and Z–Asp(O^{t}Bu)–ONB [prepared from Z–Asp(O^{t}Bu)–

OH (2.91 g, 9 mmoles), HONB (1.62 g, 9 mmoles) and DCC (1.85 g, 9 mmoles) in dioxane (30 ml)] were dissolved in 50 ml of DMF and stirred for 12 hr at room temperature. The reaction mixture was poured into 750 ml of water and the resulting precipitate was collected by filtration. The precipitate was purified by reprecipitation from EtOH–ether and then AcOEt–ether: yield 9.2 g, (92.0%) mp 194—195° (decomp.); $[\alpha]_D^{23}$ −29.8° (c=1.05 in DMF); Rf^1 0.67. *Anal.* Calcd. for $C_{85}H_{125}O_{23}N_{11}$: C, 61.17; H, 7.54; N, 9.23. Found: C, 61.01; H, 7.67; N, 9.26.

Z-Glu(O^{t}Bu)-Asp(O^{t}Bu)-Glu(O^{t}Bu)-Ser-Ala-Glu(O^{t}Bu)-Ala-Phe-Pro-Leu-Glu(O^{t}Bu)-Phe-O^{t}Bu (XII)——Compound XI (8.35 g, 5 mmoles) was dissolved in a mixture (100 ml) of *n*-butanol–MeOH–water (1: 1: 1) and hydrogenated over Pd-black (1.0 g) for 8 hr. The Pd catalyst was filtered off and the filtrate was evaporated *in vacuo* to dryness. The resulting residue and Z–Glu(O^{t}Bu)–ONB [prepared from Z–Glu(O^{t}Bu)–OH (2.36 g, 7 mmoles), HONB (1.26 g, 7 mmoles) and DCC (1.44 g, 7 mmoles) in dioxane (30 ml)] were dissolved in 50 ml of DMF and stirred for 12 hr at room temperature. The reaction mixture was poured into 750 ml of water and the resulting precipitate was collected by filtration. The precipitate was purified by reprecipitation from EtOH–hexane and then recrystallization from 70% aqueous MeOH: yield 8.0 g (86.3%); mp 215—216° (decomp.); $[\alpha]_D^{23}$ −27.2° (c=1.07 in DMF), Rf^1 0.71. *Anal.* Calcd. for $C_{94}H_{140}O_{26}N_{12}$: C, 60.88; H, 7.61; N, 9.06. Found: C, 60.37; H, 7.48; N, 9.09.

Z-Ala-Glu(O^{t}Bu)-Asp(O^{t}Bu)-Glu(O^{t}Bu)-Ser-Ala-Glu(O^{t}Bu)-Ala-Phe-Pro-Leu-Glu(O^{t}Bu)-Phe-O^{t}Bu (XIII)——Compound XII (6.5 g, 3.5 mmoles) was dissolved in a mixture (130 ml) of *n*-butanol–MeOH–water (1: 1: 1) and hydrogenated over Pd-black (0.7 g) for 8 hr. The Pd catalyst was filtered off and the filtrate was evaporated *in vacuo* to dryness. The resulting residue and Z–Ala–ONB [prepared from Z–Ala–OH (1.12 g, 5 mmoles), HONB (895 mg, 5 mmoles) and DCC (1.03 g, 5 mmoles) in dioxane (30 ml)] were dissolved in 50 ml of DMF and stirred for 12 hr at room temperature. The reaction mixture was poured into 750 ml of water and the resulting precipitate was collected by filtration. The precipitate was purified by reprecipitation from EtOH–hexane and then recrystallization from 70% aqueous EtOH: yield 5.8 g (86.1%); mp 225—226° (decomp.); $[\alpha]_D^{24}$ −25.4° (c=1.04); Rf^1 0.67. *Anal.* Calcd. for $C_{97}H_{145}O_{27}N_{13}$: C, 60.51; H, 7.59; N, 9.45. Found: C, 60.13; H, 7.59; N, 9.22.

Z-Asn-Gly-O^{t}Bu (XIV)——Z–Asn–OH (5.33 g, 0.02 mole), H–Gly–O^{t}Bu (2.62 g, 0.02 mole) and HONB (3.58 g, 0.02 mole) were dissolved in 30 ml of DMF and to this DCC (4.12 g, 0.02 mole) was added under cooling. The reaction mixture was stirred for 1 hr at 0° and for 12 hr at room temperature and then filtered. The filtrate was diluted with water (500 ml). The oily substance separated was extracted with AcOEt. The AcOEt layer was washed successively with 0.2 N HCl, then 5% $NaHCO_3$ in the usual manner. The solvent was evaporated *in vacuo* and the resulting residue was triturated with ether. The crystalline precipitate was recrystallized from 50% aqueous MeOH: yield 4.6 g (60.7%); mp 149—150° (decomp.); $[\alpha]_D^{23}$ −6.0° (c=0.48 in DMF); Rf^1 0.68. *Anal.* Calcd. for $C_{18}H_{25}O_6N_3$: C, 56.98; H, 6.64; N, 11.08. Found: C, 56.93; H, 6.57; N, 11.05.

Z-Asn-Gly-OH (XV)——Compound XIV (3.8 g, 0.01 mmole) was dissolved in 50 ml of trifluoroacetic acid and the solution was stirred for 45 min at room temperature. Trifluoroacetic acid was evaporated *in vacuo* and the resulting residue was triturated with ether. The crystalline precipitate was collected by filtration and recrystallized from chloroform and then 50% aqueous MeOH: yield 3.0 g (93.0%); mp 165—168° (lit.[17] mp 166—169°); $[\alpha]_D^{23}$ −3.4° (c=1.05 in DMF), Rf^1 0.06, Rf^2 0.29. *Anal.* Calcd. for $C_{14}H_{17}O_6N_3$: C, 52.01; H, 5.30; N, 13.00. Found: C, 51.68; H, 5.31; N, 12.95.

Z-Asn-Gly-Ala-Glu(O^{t}Bu)-Asp(O^{t}Bu)-Glu(O^{t}Bu)-Ser-Ala-Glu(O^{t}Bu)-Ala-Phe-Pro-Leu-Glu(O^{t}Bu)-Phe-O^{t}Bu (II)——Compound XIII (2.5 g, 1.3 mmoles) was dissolved in a mixture (70 ml) of *n*-butanol–MeOH–water (1: 1: 1) and hydrogenated over Pd-black (0.3 g) for 8 hr. The Pd catalyst was filtered off and the filtrate was evaporated *in vacuo* to dryness. The resulting residue, compound XV (504 mg, 1.56 mmoles) and HONB (281 mg, 1.56 mmoles) were dissolved in 20 ml of DMF and to this DCC (321 mg, 1.5 mmoles) was added under cooling. After being stirred for 12 hr at room temperature, the reaction mixture was filtered and diluted with cold water (500 ml). The resulting precipitate was collected by filtration and recrystallized from 50% aqueous EtOH and then 70% aqueous EtOH: yield 2.2 g (80.9%); mp 237—238° (decomp.); $[\alpha]_D^{23}$ −16.9° (c=0.95 in DMF); Rf^1 0.66. *Anal.* Calcd. for $C_{103}H_{154}O_{30}N_{16}H_{20}\cdot H_2O$: C, 58.50; H, 7.43; N, 10.59. Found: C, 58.48; H, 7.43; N, 10.53. Amino acid *Anal.* (6 N HCl, 110°, 24 hr): Asp, 1.98 (2); Ser, 0.95 (1); Glu, 3.95 (4); Pro, 1.03 (1); Gly, 1.08 (1); Ala, 3.10 (3); Leu, 0.97 (1); Phe, 2.02 (2). Average recovery 100%.

H-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe-OH [α_h-ACTH (25—39)]——Compound II (315 mg) and anisole (0.5 ml) were made to settle in a container made of difuron and treated with anhydrous hydrogen fluoride (approximately 10 ml) in an ice bath for 1 hr. Hydrogen fluoride was removed *in vacuo*. The pasty residue was dried over NaOH pellets *in vacuo* for 20 hr, and then dissolved in 30 ml of water and washed with *n*-butanol. The solution was passed through a column (1.0 cm × 3.0 cm) of Amberlite IRA-400 (acetate form). The column was washed with water (100 ml) and then the product was eluted with 30% aqueous acetic acid and the eluate (100 ml) was pooled and then lyophilized (200 mg). This crude peptide was dissolved in 2 ml of 1 N acetic acid and applied to a column (1.5 cm × 100 cm) of Sephadex LH-20, which was eluted with 1 N acetic acid. The desired fractions were pooled and lyophilized:

yield 178 mg; $[\alpha]_D^{27}$ −86.8° (c=0.25 in 1% acetic acid); Rf^3 0.15, Rf^4 0.30. Paper chromatography, Rf^4 0.32. Amino acid *Anal.* (6 N HCl, 110°, 24 hr): Asp, 2.02 (2); Ser, 0.92 (1); Glu, 3.86 (4); Pro, 1.02 (1); Gly, 1.00 (1); Ala, 3.05 (3); Leu, 0.98 (1); Phe, 2.02 (2). Average recovery 81.0% (calculated as anhydrous form).

Z-Ser-Tyr-Ser-Met-Glu(O^{t}Bu)-His-Phe-Arg-Trp-Gly-Lys(Z)-Pro-Val-Gly-Lys(Z)-Lys(Z)-Arg(NO$_2$)-Arg(NO$_2$)-Pro-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(O^{t}Bu)-Asp(O^{t}Bu)-Glu(O^{t}Bu)-Ser-Ala-Glu(O^{t}Bu)-Ala-Phe-Pro-Leu-Glu(O^{t}Bu)-Phe-O^{t}Bu·*p*-Ts-OH (XVI)——Compound II (1.05 g, 0.5 mmoles) was dissolved in a mixture (70 ml) of *n*-butanol–MeOH–water (1: 1: 1) and hydrogenated over Pd-black (0.1 g) for 8 hr at 40°. The Pd catalyst was filtered off and the filtrate was evaporated *in vacuo* to dryness. The resulting residue, which had been dried over P_2O_5 for 12 hr, Z–Ser–Tyr–Ser–Met–Glu(O^{t}Bu)–His–Phe–Arg–Trp–Gly–Lys(Z)–Pro–Val–Gly–Lys(Z)–Lys(Z)–Arg(NO$_2$)–Arg(NO$_2$)–Pro–Val–Lys(Z)–Val–Tyr–Pro–OH·*p*–Ts–OH (I)[1] (1.96 g, 0.5 mmole) and HONB (108 mg, 0.6 mmole) were dissolved in 20 ml of DMF and to this DCC (413 mg, 2.0 mmoles) was added at room temperature. The reaction mixture was stirred for 48 hr at room temperature. The formed urea was filtered off and then the filtrate was poured into 400 ml of ether. The resulting precipitate was collected by filtration and then purified by reprecipitation from 80% aqueous MeOH: yield 2.6 g (88.4%); mp 208—210° (decomp.); $[\alpha]_D^{26}$ −24.1° (c=0.61 in DMF); Rf^2 0.40. *Anal.* Calcd. for $C_{282}H_{400}O_{75}N_{58}S_2 \cdot 13H_2O$: C, 55.52; H, 7.03; N, 13.32; S, 1.05. Found: C, 55.30; H, 6.96; N, 13.39; S, 1.15. Amino acid *Anal.*. (6 N HCl containing one drop of phenol, 110°, 24 hr): Lys, 3.95 (4); His, 1.15 (1); Arg, 2.10 (3); Asp, 1.95 (2); Ser, 2.95 (3); Glu, 5.05 (5); Pro, 4.00 (4); Gly, 3.05 (3); Ala, 3.00 (3); Val, 3.00 (3); Met, 1.05 (1); Leu, 0.95 (1); Tyr, 2.00 (2); Phe, 3.10 (3). Average recovery 89.0%.

H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe-OH (α_h-ACTH)——Compound XVI (1.3 g), skatole (250 mg), anisole (2 ml), methylethylsulfide (2 ml) and thioglycolic acid (0.02 ml) were made to settle in a container made of difron and treated with anhydrous hydrogen fluoride (approximately 25 ml) in an ice bath for 1 hr. Hydrogen fluoride was removed *in vacuo*. The brownish pasty residue was dissolved in 30 ml of water and the solution was washed with a mixture of *n*-butanol–AcOEt (1: 1). The solution was passed through a column (3.5 cm × 15.0 cm) of Amberlite CG-400 (acetate form) and 400 ml of the eluate was pooled and lyophilized (1.1 g). The lyophilized material was dissolved in 20 ml of 3% mercaptoethanol solution and incubated for 23 hr at 50° under nitrogen. The solution was diluted with 400 ml of H_2O and applied to a column (3.0 cm × 24.0 cm) of carboxymethylcellulose, which was first washed with water (500 ml) and then with 0.01 N ammonium acetate buffer (500 ml, pH 6.9). The product was eluted with an exponential gradient from 0.01 N ammonium acetate buffer (2000 ml, pH 6.9) to 0.2 N ammonium acetate buffer (pH 6.9). The desired fractions (900—1400 ml) were pooled and applied to a column (2.5 cm × 7.5 cm) of Amberlite XAD-2 (50—200 mesh). The column was washed with 0.1 N ammonium acetate (400 ml) and the product was eluted with exponential gradient from 0.1 N ammonium acetate (1650 ml) to 80% aqueous EtOH (885 ml) containing 1% acetic acid. The desired fractions (890—1190 ml) were pooled, evaporated and lyophilized (141 mg).

The lyophilized material was dissolved in 10 ml of 1 N acetic acid and the solution was applied to a column of Bio-Gel P-6 (3.0 cm × 48.0 cm), which was eluted with the same solvent. The desired fractions (90—230 ml) were collected and lyophilized to constant weight: yield 133 mg; $[\alpha]_D^{24}$ −87.6° (c=0.26 in 1% acetic acid); Rf^4 0.51. Paper chromatography, Rf^4 0.19. Amino acid *Anal.* (6 N HCl, 110°, 24 hr): Lys, 3.88 (4); His, 1.03 (1); Arg, 2.91 (3); Asp, 2.12 (2); Ser, 2.85 (3); Glu, 5.03 (5); Pro, 4.06 (4); Gly, 3.09 (3); Ala, 3.15 (3); Val, 2.97 (3); Met, 1.03 (1); Leu, 1.03 (1); Tyr, 2.00 (2); Phe, 2.97 (3); average recovery 92.8% (calculated as nonatriacontapeptide heptaacetate hexadecahydrate). UV $\lambda_{max}^{0.1N\ NaOH}$ mμ ($E_{1cm}^{1\%}$) 282.5 (16.51), 290.0 (16.97), Tyr/Trp=2.03. Paper electrophoresis: pH 2.3 (2 N acetic acid, 500 V, 1 hr) −5.9 cm, pH 3.6 (pyridine–acetic acid–water=1: 10: 89, 500 V, 2 hr) −1.5 cm, pH 6.5 (pyridine–acetic acid–water=10: 0.4: 90, 500 V, 4 hr) −2.3 cm.

Enzymatic Digestion of Synthetic α_h-ACTH——Synthetic α_h-ACTH (2.5 mg) was dissolved in 1.0 ml of 0.1 M Tris–HCl buffer (pH 7.4), and to this 1.0 ml of 10% aqueous ethanol solution of pronase P (0.2 mg, Kaken Kagaku Co. Ltd.) was added . The digestion mixture was incubated for 23 hr at 39° and heated in a water bath at 100° for 5 min.

A portion(0.1 ml) of a solution of aminopeptidase M (0.1 mg, Röhm and Hass) was added to this digestion mixture, which was incubated for 23 hr at 39° and diluted with sodium citrate buffer pH 2.2 (2.0 ml) and a portion (1 ml) of this reaction mixture was subjected to amino acid analysis.

The results were as follows: Lys, 3.27 (4); His, 0.93 (1); Arg, 2.13 (3); Trp, 1.00 (1); Asp, 0.93 (1); Ser+Asn, 4.07 (4); Glu, 4.80 (5); Gly, 3.23 (3); Ala, 3.27 (3); Val, 3.27 (3); Met, 0.93 (1); Leu, 1.13 (1); Tyr, 1.27 (2); Phe, 2.13 (3). Average recovery 93.7% (calculated as heptaacetate hexadecahydrate).

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