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Synthesis of Peptides related to Corticotropin (ACTH). I. Synthesis of a^{1-23NH_2} -ACTH-tricosapeptide Amide[1]

Since the first description of the synthesis of the polypeptide which possess the full *in vivo* corticotropic action of natural ACTH by Hofmann, Yajima, *et al.*[2] in 1961, various polypeptides with corticotropic action have been synthesized.[3]

We now describe an improved synthesis of the tricosapeptide amide[4] corresponding to the first 23 amino acid residues from the amino terminus of ACTH.

The tricosapeptide amide was synthesized by using N^{α}-BOC-, and N^{ε}-Z-protecting groups, and the most of intermediary peptide subunits [Z-Ser-Tyr-Ser-$NHNH_2$[5] (I), BOC-Met-Glu-(OtBu)-His-$NHNH_2$ (II), BOC-Phe-Arg(NO_2)-Try-Gly-OH (III), BOC-Lys(Z)-Pro-Val-Gly-OH (IV), BOC-Lys(Z)-Lys(Z)-Arg(NO_2)-Arg(NO_2)-Pro-OH (V), H-Val-Lys(Z)-Val-Tyr-NH_2 (VI)] were built up by a stepwise manner starting from the C-terminal amino acids.[6]

The three peptide subunits (V, IV and III) which have C-terminal glycine or proline were condensed in a step by step manner, by the use of pentachlorophenyl trichloroacetate (TCAOPCP) method,[7] to the C-terminal subunit VI, and then the other two subunits (II and I) were subsequently coupled by the azide method to avoid racemization. Finally, all protecting groups were deblocked by Sakakibara's hydrogen fluoride method.[8]

By using TCAOPCP method, the partially protected pentapeptide V [mp 94—97° (decomp.), $[\alpha]_D^{21}$ −30.6° (DMF), *Anal.* Calcd. for $C_{50}H_{75}O_{16}N_{15}\cdot H_2O$: C, 51.8; H, 6.7; N, 18.1. Found: C, 51.9; H, 6.9; N, 17.7. Rf^1 0.27] was converted to BOC-Lys(Z)-Lys(Z)-Arg(NO_2)-Arg(NO_2)-Pro-OPCP [Yield 91%, mp 115° (decomp.), Rf^1 0.68], and then condensed with the tetrapeptide amide VI [mp 233—235° (decomp.), $[\alpha]_D^{22}$ −19.5° (DMF). *Anal.* Calcd. for $C_{34}H_{48}O_7N_6$: C, 61.8; H, 7.5; N, 13.1. Found: C, 61.8; H, 7.5; N, 13.0. Rf^2 0.40, Rf^3 0.72] into BOC-Lys(Z)-Lys(Z)-Arg(NO_2)-Arg(NO_2)-Pro-Val-Lys(Z)-Val-Tyr-NH_2 [Yield 76%, mp 189—191° (decomp.), $[\alpha]_D^{22}$ −34.6° (DMF). *Anal.* Calcd. for $C_{83}H_{121}O_{22}N_{21}\cdot H_2O$: C, 55.9; H, 7.0; N, 16.5. Found: C, 55.7; H, 7.0; N, 16.5. Rf^2 0.80, Rf^3 0.95].

After elimination of the BOC-group by acidolysis with cold trifluoroacetic acid, the resulting nonapeptide amide (Rf^2 0.45, Rf^3 0.61) was coupled with BOC-Lys(Z)-Pro-Val-Gly-OPCP [prepared from IV and TCAOPCP. Yield 74% ,mp 188—190° (decomp.), $[\alpha]_D^{23}$ −36.7° (DMF), *Anal.* Calcd. for $C_{37}H_{46}O_9N_5Cl_5$: C, 50.4; H, 5.3; N, 8.0; Cl, 20.1. Found: C, 50.2; H, 5.4; N, 8.0; Cl, 20.3. Rf^1 0.89]. The resulting fully protected tridecapeptide amide BOC-Lys(Z)-Pro-Val-Gly-Lys(Z)-Lys(Z)-Arg(NO_2)-Arg(NO_2)-Pro-Val-Lys(Z)-Val-Tyr-NH_2 [Yield 81%, mp 194—196° (decomp.), $[\alpha]_D^{22}$ −36.0° (DMF). *Anal.* Calcd. for $C_{109}H_{158}O_{28}N_{26}\cdot H_2O$: C, 57.0;

1) Peptides and their derivatives in this communication are of the L-configuration. The following abbreviation are used: Z=benzyloxycarbonyl; BOC=*tert*-butyloxycarbonyl; NO_2=nitro; O*t*Bu=*tert*-butyl ester; OPCP=pentachlorophenyl ester; DCCD=N,N′-dicyclohexylcarbodiimide. The following solvent systems are used for thin layer (Kieselgel G, Merck) chromatography: Rf^1; chloroform–methanol–acetic acid (9:1:0.5 v/v), Rf^2; ethyl acetate–pyridine–acetic acid–water (60:20:6:11 v/v), Rf^3; *n*-butanol–acetic acid–water (4:1:1 v/v), Rf^4; *n*-butanol–pyridine–acetic acid–water (15:10:3:12 v/v).

2) K. Hofmann, H. Yajima, N. Yanaihara, T.Y. Liu and S. Lande, *J. Am. Chem. Soc.*, **83**, 487 (1961).
3) See E. Schröder and K. Lübke, "The Peptide," Vol. II, Academic Press, New York, 1966, p. 194.
4) R. Geiger, K. Sturm and W. Siedel, *Ber.*, **97**, 1207 (1964).
5) K. Hofmann, A. Jöhl, A.E. Furlenmeier and H. Kappeler, *J. Am. Chem. Soc.*, **79**, 1636 (1957); St. Guttmann and R.A. Boissonnas, *Helv. Chim. Acta*, **41**, 1852 (1958).
6) The preparation of these protected peptides will be described in a separate paper.
7) M. Fujino and C. Hatanaka, *Chem. Pharm. Bull.* (Tokyo), **16**, 929 (1968).
8) S. Sakakibara and Y. Shimonishi, *Bull. Chem. Soc. Japan.*, **38**, 1412 (1965); S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada and H. Sugihara, *Bull. Chem. Soc. Japan*, **40**, 2164 (1967); S. Sakakibara, Y. Kishida, R. Nishizawa and Y. Shimonishi, *Bull. Chem. Soc. Japan.*, **41**, 438 (1968).

H, 7.0; N, 15.8. Found: C, 56.7; H, 7.0; N, 15.9. Rf^2 0.75, Rf^3 0.81] was treated with cold trifluoroacetic acid to remove the BOC–group and then condensed with BOC-Phe-Arg(NO_2)-Try-Gly-OPCP [Yield 90.5%, mp 169.5—170.5° (decomp.), $[\alpha]_D^{23}$ −7.8° (DMF). *Anal.* Calcd. for $C_{39}H_{42}O_9N_9Cl_5$: C, 48.9; H, 4.4; N, 13.2; Cl, 18.5. Found: C, 48.7; H, 4.5; N, 13.0; Cl, 18.5. Rf^1 0.70] which was prepared from N-protected tetrapeptide III [mp 155—156° (decomp.), $[\alpha]_D^{26}$ −22.3° (DMF). *Anal.* Calcd. for $C_{33}H_{43}O_7N_9$: C, 55.8; H, 6.1; N, 17.8. Found: C, 55.6; H, 6.1; N, 17.9. Rf^1 0.25] and TCAOPCP to give BOC-Phe-Arg(NO_2)-Try-Gly-Lys(Z)-Pro-Val-Gly-Lys(Z)-Lys(Z)-Arg(NO_2)-Arg(NO_2)-Pro-Val-Lys(Z)-Val-Tyr-NH_2 [Yield 83.3%, mp 186—187° (decomp.), $[\alpha]_D^{21}$ −32.4° (DMF). *Anal.* Calcd. for $C_{137}H_{191}O_{34}N_{35}\cdot 4H_2O$: C, 55.9; H, 6.8; N, 16.7. Found: C, 55.8; H, 6.8; N, 16.7. Amino acid *Anal.*[9] Gly 2.00, Pro 2.00, Val 2.81, Phe 0.94, Lys 3.90, Arg 3.00, Tyr[10] 0.17. Rf^2 0.77, Rf^3 0.91].

After selective elimination of the BOC–group with cold trifluoroacetic acid under nitrogen gas, the resulting heptadecapeptide amide H-Phe-Arg(NO_2)-Try-Gly-Lys(Z)-Pro-Val-Gly-Lys(Z)-Lys(Z)-Arg(NO_2)-Arg(NO_2)-Pro-Val-Lys(Z)-Val-Tyr-NH_2 was condensed with BOC-Met-Glu(OtBu)-His-N_3 [prepared from the corresponding hydrazide II, mp 175—176°, $[\alpha]_D^{23}$ −31.0° (MeOH). *Anal.* Calcd. for $C_{25}H_{43}O_7N_7S$: C, 51.3; H, 7.4; N, 16.7; S, 5.5. Found: C, 51.2; H, 7.3; N, 16.6; S, 5.5, Rf^2 0.50] to give BOC-Met-Glu(OtBu)-His-Phe-Arg(NO_2)-Try-Gly-Lys(Z)-Pro-Val-Gly-Lys(Z)-Lys(Z)-Arg(NO_2)-Arg(NO_2)-Pro-Val-Lys(Z)-Val-Tyr-NH_2 [Yiel d95.8%, mp 202—204° (decomp.), $[\alpha]_D^{22}$ −34.2° (DMF). *Anal.* Calcd. for $C_{157}H_{222}O_{39}N_{40}S$: C, 56.5; H, 6.7; N, 16.8; S, 1.0. Found: C, 55.9; H, 6.8; N, 16.8; S, 1.2. Amino acid *Anal.*[9] Glu 1.18, Gly 2.00, Pro 1.92, Val 2.85, Phe 1.00, Lys 4.00, His 0.98, Arg 2.81, Met[10] 0.62, Tyr[10] 0.18. Rf^2 0.60, Rf^3 0.83].

The fully protected eicosapeptide amide was treated with cold trifluoroacetic acid under nitrogen gas and then coupled with Z-Ser-Tyr-Ser-N_3 (prepared from the corresponding hydrazide I[5] of mp 225—226° (decomp.) and $[\alpha]_D^{23}$ −4.4° (DMF)) into Z-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg(NO_2)-Try-Gly-Lys(Z)-Pro-Val-Gly-Lys(Z)-Lys(Z)-Arg(NO_2)-Arg(NO_2)-Pro-Val-Lys-(Z)-Val-Tyr-NH_2 followed by the purification with column chromatography on silica gel[11] [Yield 86.2%, mp 202—203° (decomp., 176° sinter), $[\alpha]_D^{24}$ −31.8° (DMF). *Anal.* Calcd. for $C_{171}H_{231}O_{45}N_{43}S\cdot 4H_2O$: C, 55.3; H, 6.5; N, 16.2; S, 0.9. Found: C, 55.2; H, 6.5; N, 16.3; S, 0.8. Rf^2 0.38, Rf^3 0.73, Rf^4 0.85]. The resulting N-protected tricosapeptide amide was treated with anhydrous hydrogen fluoride[8,12] to remove the Z-group and NO_2-group, and then treated with Amberlite IRA-410 of an acetate form. The product was purified by column chromatography on CM–cellulose (ammonium acetate buffer).

The purified tricosapeptide amide [Yield 50—65%, $[\alpha]_D^{22}$ −73.0°±3.4° (1% AcOH) (lit.,[4] $[\alpha]_D$ −76.8±1.5°), UV $\lambda_{max}^{0.1N\text{-}NaOH}$ mμ ($E_{1cm}^{1\%}$) 283.5 (24.99), 290 (25.73), Amino acid *Anal.*[9] Ser 2.10, Glu 1.00, Pro 2.00, Gly 2.04, Val 2.80, Met 0.86, Tyr 2.06, Phe 0.99, Lys 4.07, His 1.03, Arg 3.00 (average recovery 98%)] was exhibited as a single spot (ninhydrin, methionine, Ehrlich's, Pauli's, and Sakaguchi's reagents) on thin–layer chromatography (Rf^4 0.55) and behaved as a single component on paper electrophoresis at pH 2.3,[13] 3.6[14] and 6.5[15] and possessed full adrenocorticotropic activity.[16]

9) Acid hydrolysate (6N HCl, 110°, 24 hr). We are grateful to Dr. M. Hori and Mr. I. Yoshida for performing amino acid analyses.
10) Partial alkylation should be occurred within acid hydrolysis. B. Iselin, *Helv. Chim. Acta*, **45**, 1510 (1962).
11) Solvent system: ethyl acetate–pyridine–acetic acid–water (60:20:6:10, v/v).
12) Anisole was used as a scavenger to protect tyrosine and methionine from benzylation, and thioglycolic acid was used to protect methionine and tryptophan from acid oxidation.
13) 2N acetic acid, 600 V, 1 hr. Mobility=Lys×0.98.
14) Pyridine–acetic acid–water (1:10:89, v/v), 400 V, 6 hr. Mobility=Lys×0.84.
15) Pyridine–acetic acid–water (10:0.4:90, v/v), 400 V, 3 hr. Mobility=Lys×0.83.
16) The steroidogenic activity of the synthetic tricosapeptide amide was determined by *in vivo* method (a modified Lipscomb and Nelson method) and compared with the 3rd U.S.P. reference standard; *ca.* 80 units/mg, R. Nakayama and R. Kubota, unpublished.

The synthetic method above described has many advantages; simplicity and good yield of the each steps, all intermediates were good crystallizable materials, and the esterification of the intermediary peptides by the TCAOPCP method was especially favorable compared with DCCD method, because there was no trouble such as acylurea formation or contamination of insoluble dicyclohexylurea.

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Chemical Research Laboratories,
Research & Development Division,
Takeda Chemical Industries, Ltd.,
Juso, Higashiyodogawa-ku, Osaka

MASAHIKO FUJINO
CHITOSHI HATANAKA
OSAMU NISHIMURA

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The Total Amino Acid Sequence of Substance A produced by *Streptomyces carzinostaticus*

Antibacterial polypeptide substance A[1] produced by *Streptomyces carzinostaticus* var. F-41 has a molecular weight of 8440, contains 87 amino acid residues but contains no histidine and methionine. It has an N-terminal amino acid alanine and C-terminal amino acid asparagine and a disulfide bridge[2] which concerned to antibacterial acitivity.

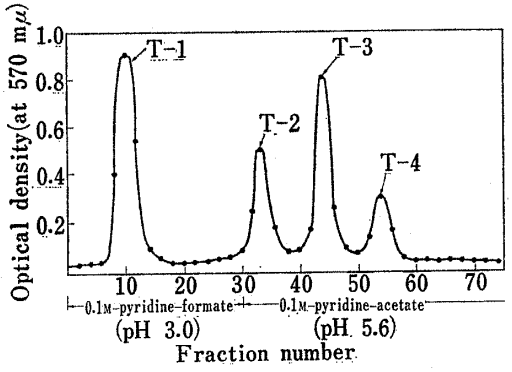

Fig. 1. Chromatogram of the Tryptic Peptides on Amberlite CG-120 Column

380 mg of the lyophilized powder was dissolved in 5 ml of 0.01N-HCl and absorbed on a column of Amberlite CG-120 (1.6×46 cm). The elution was made by the successive change of eluant from 150 ml of 0.1M-pyridine-formate buffer (pH 3.0) to 400 ml of 0.1M-pyridine acetate buffer (pH 5.6). Flow rate was set 0.5 ml/min, and the effluent was collected in 5 ml fraction. The peptide content of effluent was determined by ninhydrin reaction.

The present communication proposes the total amino acid sequence of substance A. In the previous paper,[2] partial acid hydrolysis was performed by the method of Partridge, *et al.*,[3] to obtained seven free amino acids (from A-1 to A-4 in Table I) and eighteen peptide fractions (from A-5 to A-22). These peptide fractions were further separated and purified by paper chromatography and paper electrophoresis, and the amino acid sequence of the each peptide fragment was determined as shown in Table I.

Tryptic digestion of the substance A was carried out by the following procedure. Fourhkndred and thirty mg (5.1×10^{-5} mole) of substance A was dissolved in 20 ml of 0.1M phosphate buffer (pH 7.6) containing 2.0M guanidinium chloride, and incubated with 4.3 mg of trypsin at 37° for 24 hr. After 24 hr

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2) H. Sato, T. Tanimura, T. Nakajima and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **17**, 413 (1969).
3) S.M. Partridge, H.F. Davis and F.S. Adair, *Biochem. J.*, **61**, 11 (1969).