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Synthesis of Peptides related to Corticotropin (ACTH). VIII.[1] Synthesis of α^{1-24}-ACTH[2]

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A tetracosapeptide (α^{1-24}-ACTH) corresponding to the first 24-amino acid sequence of corticotropin (ACTH) was synthesized by a classical method. The ester exchange method using pentachlorophenyl trichloroacetate (TCAOPCP) was employed successfully for condensation of the main fragments having C-terminal glycine and proline. All protecting groups of the tetracosapeptide were removed using the hydrogen fluoride method. The synthetic peptide thus obtained exhibited an activity of *ca.* 90 units/mg in *in vivo* steroidogenic assay.

Since the first publication of the synthesis of the tricosapeptide, which possesses full corticotropic activity, by Hofmann *et al.*,[4] in 1961, various polypeptides have been synthesized in order to clarify the structure-function relationship of adrenocorticotropin, and the relative importance of the amino acid residues in the ACTH molecule have been elucidated.[5,6] Previously, we reported briefly about the syntheses and biological activities of α^{1-24}-ACTH, its analogues with an amino peptidase-resistant amino acid at the N-terminal position of α^{1-24}-ACTH (β-alanine^{1}-, γ-aminobutyric acid^{1}-, sacrosine^{1}- and proline^{-1}-α^{1-24}-ACTH), and lysine^{1}-α^{1-24}-ACTH.[7] The strategy for the synthesis of these analogues was different from those reported by other investigators.[5]

In this paper, we describe in detail the synthesis of α^{1-24}-ACTH by two routes (A and B). In route A, as shown in Chart 1, the main strategy for the synthesis of the tetracosapeptide is as follows: 1) benzyloxycarbonyl and nitro groups were used to protect the ε-amino function of the lysine residues and the guanido function of the arginine residues, respectively; 2) the peptide fragments having glycine or proline at the C-termini were pentachlorophenylated by the ester exchange method[8] using pentachlorophenyl trichloroacetate (TCAOPCP); 3) in

1) Part VII: M. Fujino, C. Hatanaka, and O. Nishimura, *Chem. Pharm. Bull.* (Tokyo), **19**, 1075 (1971).
2) The amino acids, peptides and their derivatives (except glycine) in this paper were of the L-configuration. Their abbreviated designations are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 2485 (1966); *ibid.*, **6**, 362 (1967). Other abbreviations used are: NO_2, nitro; IBOC, *l*-iso-bornyloxycarbonyl; OPCP, pentachlorophenyl ester; ODNP, 2,4-dinitrophenyl ester; OTCP, 2,4,5-trichlorophenyl ester; OSu, N-hydroxysuccinimide ester; DCC, N,N'-dicyclohexylcarbodiimide; HONB, N-hydroxy-5-norbornene-endo-2,3-dicarboximide; TCAOPCP, pentachlorophenyl trichloroacetate; TFA, trifluoroacetic acid; *p*-TsOH, *p*-toluenesulfonic acid.
3) Location: *Jusohonmachi, Yodogawa-ku, Osaka, 532, Japan.*
4) K. Hofmann, H. Yajima, N. Yanaihara, T.Y. Liu, and S. Lande, *J. Am. Chem. Soc.*, **83**, 487 (1961).
5) For a review, see E. Schröder and Lübke, "The Peptides," Vol. II, Academic Press, New York, 1966, p. 194.
6) *a*) R. Geiger, K. Sturm, G. Vogel and W. Siedel, *Z. Naturforsch.*, **19B**, 858 (1964); *b*) H. Otsuka, K. Inouye, M. Kanayama, and F. Shinozaki, *Bull. Chem. Soc. Japan*, **38**, 679, 1563 (1965); *c*) R. A. Boissonnas, S. Guttmann, and J. Pless, *Experientia*, **22**, 526 (1966); *d*) S. Guttmann, J. Pless, and R.A. Boissonnas, "Peptides," ed. H.C. Beyerman, A. Van De Linde, and W. Maassen Van Den Brink, North-Holland Publ. Co., Amsterdam, 1967, p. 221; e) H. Kappeler, B. Riniker, W. Rittel, P.A. Desaulles, R. Maier, B. Schär, and M. Staehelin, *ibid.*, p. 214; *f*) W. Rittel, "Pharmacology of Hormonal Polypeptides and Proteins," Plenum Press, New York, 1968, p. 35.
7) M. Fujino, C. Hatanaka, and O. Nishimura, *Chem. Pharm. Bull.* (Tokyo), **18**, 1288 (1970).
8) M. Fujino and C. Hatanaka, *Chem. Pharm. Bull.* (Tokyo), **16**, 929 (1968).

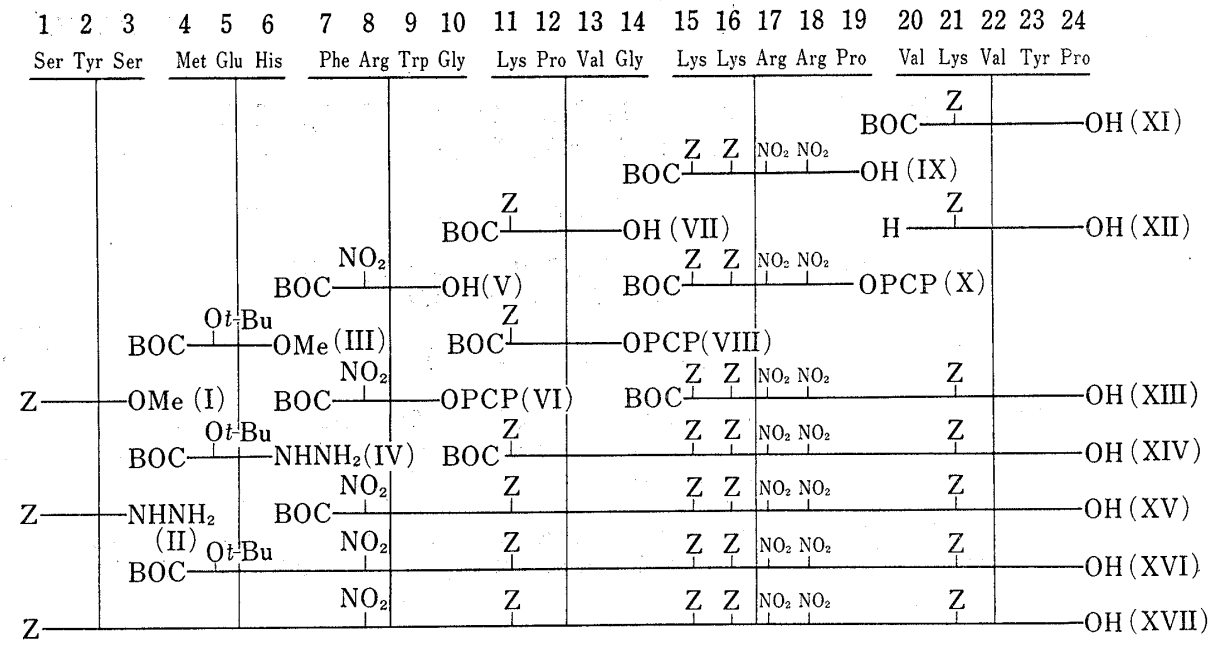

Chart 1. Synthetic Route A to N-Protected Tetracosapeptide

the fragment condensation, the carboxyl group of proline at position 24 at ACTH was protected by the salt formation with triethylamine; and 4) all the protecting groups of the tetracosapeptide (XVII) were removed by the hydrogen fluoride method.[9]

The N-protected C-terminal pentapeptide subunit (XI) was synthesized as shown in Chart 2. The syntheses of the other protected peptide subunits (I, II, III, IV, V, VII and IX[10a]), and the active esters (VI, VIII and X[10b]) have been described in detail in previous papers.

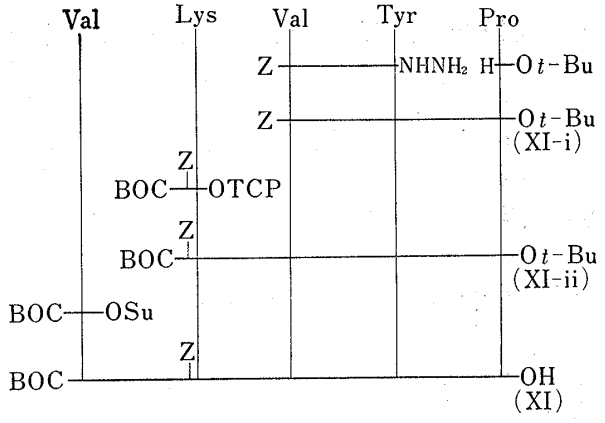

Chart 2. Synthetic Route to N-Protected C-Terminal Pentapeptide

The N-protected decapeptide (XIII) was prepared by coupling BOC-Lys(Z)-Lys(Z)-Arg(NO_2)-Arg(NO_2)-Pro-OPCP (X) with the C-terminal pentapeptide (XII) obtained by trifluoroacetic acid (TFA) treatment of BOC-Val-Lys(Z)-Val-Tyr-PrO-OH (XI). The N-protected decapeptide (XIII) obtained was treated with TFA at below 15° for 15 min to remove the BOC protecting group and the resulting partially protected decapeptide was reacted with BOC-Lys(Z)-Pro-Val-Gly-OPCP (VIII) to get the N-protected tetradecapeptide (XIV). Compound XIV was obtained as a precipitate from acetic acid–ethanol in good yield and exhibited satisfactory purity. It was found that ε-benzyloxycarbonyl groups of lysines contained in the peptide derivatives underwent partial deblocking, when the derivatives were treated with TFA over 20°.

The N-protected tetradecapeptide (XIV) was treated by TFA to give the partially protected tetradecapeptide, which was further acylated with BOC-Phe-Arg(NO_2)-Trp-Gly-OPCP

9) 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, *ibid.*, **41**, 438 (1968); S. Sakakibara, N. Nakamizo, Y. Kishida, and S. Yoshimura, *ibid.*, **41**, 1477 (1968).

10) *a*) M. Fujino, O. Nishimura, and C. Hatanaka, *Chem. Pharm. Bull.* (Tokyo), **17**, 2135 (1969); *b*) M. Fujino, C. Hatanaka, and O. Nishimura, *ibid.*, **18**, 771 (1970).

(VI). The octadecapeptide (XV) thus obtained was precipitated from acetic acid–water and then from acetic acid–ethanol to give an analytically pure sample in good yield. The octadecapeptide (XV) was then treated carefully with cold TFA containing 1.0% thioglycolic acid under nitrogen and the resulting partially protected octadecapeptide was allowed to react with BOC-Met-Glu(O*t*-Bu)-His-$NHNH_2$ (IV) *via* the corresponding azide. The resulting N-protected heneicosapeptide (XVI) was purified by column chromatography on silica gel with ethyl acetate–pyridine–acetic acid–water (30: 10: 3: 5 v/v) as solvent. The N-protected tetracosapeptide (XVII) was prepared by coupling Z-Ser-Tyr-Ser-$NHNH_2$ (II) *via* the corresponding azide with the partially protected heneicosapeptide which had been obtained by treating XVI with TFA. XVII was purified in a similar fashion to that used for the N-protected heneicosapeptide (XVI).

Finally, the N-protected tetracosapeptide (XVII) was treated at 0° for one hour with anhydrous hydrogen fluoride which contained an excess of anisole, thioglycolic acid, methionine and tryptophan to avoid undesirable side reactions. The resulting peptide in the form of the hydrogen fluoride was converted into the corresponding acetate by being passed through a column of Amberlite IRA-400 (acetate form). The final product was purified by column chromatography on carboxymethylcellulose using a gradient elution from 0.1M ammonium acetate buffer to 0.5M ammonium acetate buffer, and then on Amberlite XAD-2 resin using a exponential gradient elution of 50% ethanol–0.2M ammonium acetate containing 0.5% acetic acid.

In route B,[11] the N-protected tetracosapeptide (XXIX) was synthesized by condensation of the two main fragments (XXVII and XXVIII). The partially protected N-terminal decapeptide (XXVII) was prepared as shown in Chart 3, and the partially protected C-terminal tetradecapeptide (XXVIII) was prepared by treating the N-protected tetradecapeptide (XVI in Chart 1) with 4N HCl-acetic acid.

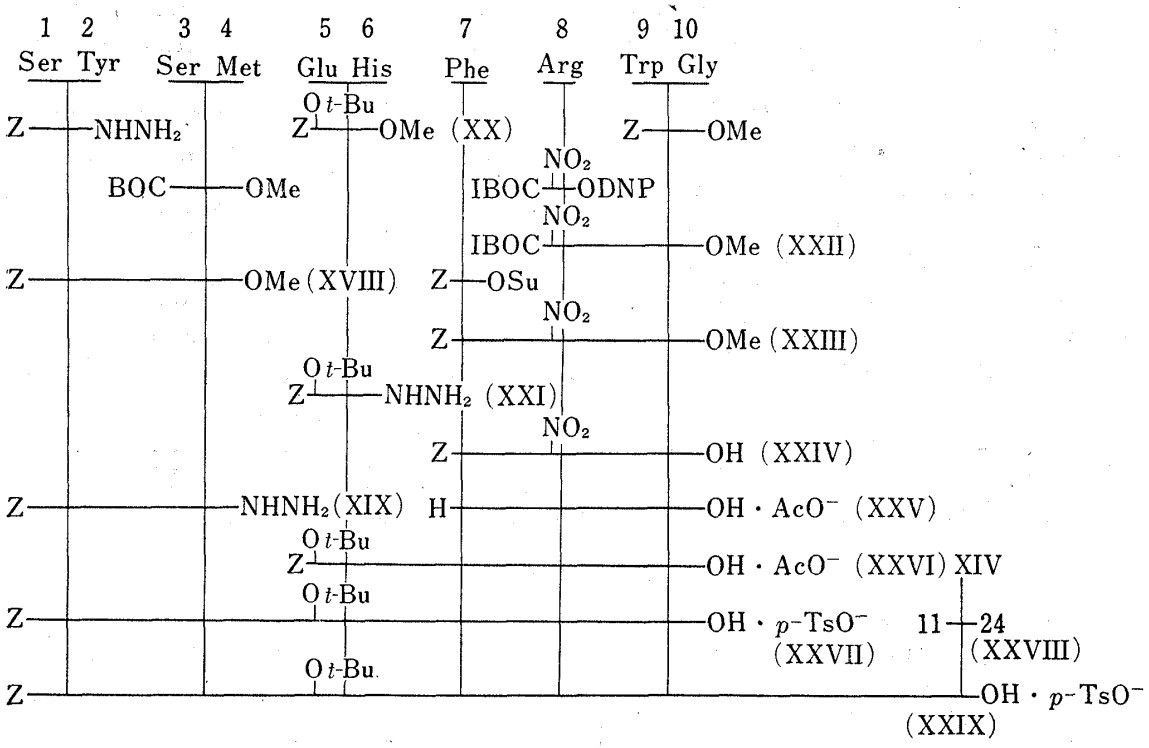

Chart 3. Synthetic Route B to N-Protected Tetracosapeptide

11) O. Nishimura and M. Fujino, "Proceedings of the 10th Symposium on Peptide Chemistry," J. Noguchi ed., Protein Research Foundation, Osaka, Japan, 1972, p. 154.

The C-terminal subunit (Z-Phe-Arg(NO_2)-Trp-Gly-OMe, XXIII) of the main fragment (XXVII) was prepared in a manner essentially similar to that described by Hofmann *et al.*,[12] except for the N-protecting group. IBOC-Arg(NO_2)-Trp-Gly-OMe (XXII) was prepared in excellent yield by the reaction of IBOC-Arg(NO_2)-ODNP[13] with the dipeptide ester obtained by catalytic hydrogenolysis of Z-Trp-Gly-OMe. The resulting N-protected tripeptide ester (XXII) was treated with TFA containing thioglycolic acid to give the partially protected tripeptide ester, which further reacted with Z-Phe-OSu to give Z-Phe-Arg(NO_2)-Trp-Gly-OMe (XXIII) in crystalline form. This compound XXIII was saponified to give the N-protected tetrapeptide (XXIV), which was catalytically hydrogenolyzed in 80% acetic acid at 50° to give H-Phe-Arg$^+$-Trp-Gly-OH·AcO$^-$ (XXV). Compound XXV was allowed to react with Z-Glu(O*t*-Bu)-His-$NHNH_2$ (XXI) *via* the corresponding azide to give Z-Glu(O*t*-Bu)-His-Phe-Arg$^+$-Trp-Gly-OH·AcO$^-$ (XXVII) in good yield. Compound XXVI was then subjected to catalytic hydrogenolysis to yield the partially protected hexapeptide. Coupling of the resulting hexapeptide with the tetrapeptide hydrazide (XIX) was performed after conversion of the hydrazide to the corresponding azide by $NaNO_2$ treatment, yielding the partially protected decapeptide (XXVII). Compound XIX was obtained by treating the corresponding tetrapeptide ester (XVIII) with hydrazine.

In the synthesis of the partially protected tetracosapeptide (XXIX) by condensation of the two main fragments (XXVII and XXVIII), the active ester methods using 2,4,5-trichlorophenyl ester, 2,4-dinitrophenyl ester, pentachlorophenyl ester, N-hydroxysuccinimide ester and N-hydroxy-5-norbornene-2,3-dicarboximide ester[14] were examined. Among these active esters, the 2,4,5-trichlorophenyl ester was most effective and the partially protected tetracosapeptide (XXIX) yield was 82.7%. HONB ester was also found to be effective, and a coupling yield of 45.4% was found in this case. The 2,4-dinitrophenyl ester, however, could not be isolated. This can be attributed to remarkable degradation of the active ester. The pentachlorophenyl ester also seemed to be labile and the coupling yield was poor (22%). Furthermore, the N-hydroxysuccinimide ester could not be isolated, because formation of succinimidoxycarbonyl β-alanine N-hydroxysuccinimide ester[15] predominates over formation of the N-hydroxysuccinimide ester of XXVII.

Finally, the partially protected tetracosapeptide (XXIX) was treated at 0° for 30 min with anhydrous hydrogen fluoride which contained an excess of anisole, skatole and methyl ethyl sulfide to avoid undesirable side reactions. The resulting crude peptide was purified by column chromatography on carboxymethylcellulose and Amberlite XAD-2 in the same manner as described previously in route A.

The tetracosapeptide (α^{1-24}-ACTH) obtained in both routes were found to be homogeneous by thin-layer chromatography (TLC), paper chromatography and paper electrophoresis. The amino acid composition of an enzymatic digest and an acid hydrolysate were determined with a Hitachi model KLA-3B autoanalyzer and the result agreed well with the theoretical values. Steroidogenic potencies of both synthetic α^{1-24}-ACTH were determined in a dexamethasone-blocked rat after 15 min of administration by a modified Lipscomb and Nelson method; both compounds possessed an activity of 90 units/mg compared with 3rd U.S.P.

Experimental

All melting points were taken by the capillary method and are uncorrected. TLC was carried out on Merck's silica gel G with a solvent system of Rf^1, $CHCl_3$–MeOH–AcOH (9:1:0.5); Rf^2, AcOEt–pyridine–

12) K. Hofmann and S. Lande, *J. Am. Chem. Soc.*, **82**, 2289 (1961).
13) M. Fujino, S. Shinagawa, O. Nishimura, and T. Fukuda, *Chem. Pharm. Bull.* (Tokyo), **20**, 1017 (1972).
14) M. Fujino, S. Kobayashi, T. Fukuda, M. Obayashi, S. Shinagawa, and O. Nishimura, *Chem. Pharm. Bull.* (Tokyo), **22**, 1857 (1974).
15) H. Gross and L. Bilk, "Peptides," North-Holland Publishing Co., Amsterdam, p. 156; H. Gross and L. Bilk, *Tetrahedron*, **24**, 6935 (1968); F. Weygand, W. Steglich, and N. Chytil, *Z. Naturforsh.*, **23B**, 1391 (1968).

AcOH–H_2O (30:10:3:5); Rf^3, *n*-BuOH–AcOH–H_2O (4:1:1); Rf^4, *n*-BuOH–pyridine–AcOH–H_2O (15:10:3:12); Rf^5, *n*-BuOH–AcOEt–AcOH–H_2O (1:1:1:1); Rf^6, *n*-BuOH–AcOH–H_2O (4:2:5). In the azide method, the entire operation was carried out in a cold room at 3°. Evaporations were performed in rotary evaporators under reduced pressure at a temperature of 40—45°. Evaporation of TFA, which was used to remove the BOC protecting group, was carried out below 15°.

Z-Val-Tyr-Pro-O*t*-Bu (XI-i)[16]——An ice-cold aqueous 5 N $NaNO_2$ solution (7 ml) was added slowly to an ice-cold stirred solution of Z–Val–Tyr–$NHNH_2$ (12.9 g, 30 mmoles) in dimethylformamide (DMF) (120 ml) and 2 N HCl (60 ml) at −10°. After being stirred for 5 min at −5°, the solution was diluted with cold water (120 ml) and the separated oily azide was extracted with ice-cold AcOEt. The extract was washed with ice-cold 4% $NaHCO_3$ and then H_2O, and dried over anhyd. Na_2SO_4. The azide solution thus obtained was added to an ice-cold solution of H–Pro–O*t*-Bu, which was prepared from HCl·Pro–O*t*-Bu (7.74 g, 30 mmoles) and Et_3N (4.2 ml, 30 mmoles) in AcOEt (90 ml), and the mixture was stirred for 20 hr at 3°. The solvent was evaporated and the resulting crystalline solid was triturated with hot AcOEt: yield 9.73 g (57%); mp 174—175°; $[\alpha]_D^{23}$ −70.3° (c=1.5 in MeOH). *Anal.* Calcd. for $C_{31}H_{41}O_7N_3$: C, 65.59; H, 7.28; N, 7.40. Found: C, 65.31; H, 7.32; N, 7.56.

BOC-Lys(Z)-Val-Tyr-Pro-O*t*-Bu (XI-ii)——Compound XI-i (9.7 g, 17 mmoles) in MeOH (250 ml) was hydrogenated over Pd-black (0.9 g). The catalyst was filtered off, and the filtrate was concentrated to give an oily residue, which was allowed to react with BOC–Lys(Z)–OTCP (9.52 g, 17 mmoles) in DMF (100 ml) for 17 hr at room temperature. The mixture was diluted with ice-cold 10% $AcONH_4$ and the resulting oil was extracted with AcOEt. The extract was washed with 4% $NaHCO_3$ and H_2O, dried over anhyd. Na_2SO_4, and evaporated. Addition of petroleum benzine to the residue gave a fine precipitate, which was reprecipitated from ether–petroleum benzine: yield 12.2 g (89%); mp 107—110°; $[\alpha]_D^{24}$ −63.5° (c=1.0 in MeOH); Rf^1 0.62. *Anal.* Calcd. for $C_{42}H_{61}O_{10}N_5$: C, 63.38; H, 7.72; N, 8.80. Found: C, 62.58; H, 7.67; N, 8.78.

BOC-Val-Lys(Z)-Val-Tyr-Pro-OH (XI)——Compound XI-ii (2.4 g, 3 mmoles) was treated with TFA (10 ml) at below 15° for 25 min and TFA was evaporated. Addition of dry ether to the residue yielded a precipitate, which was collected by filtration, washed with dry ether and dried over NaOH under reduced pressure: Rf^2 0.59; Rf^3 0.71. The resulting powder and Et_3N (0.84 ml) were dissolved in a mixture of DMF (14 ml) and H_2O (5 ml), and allowed to react with BOC–Val–OSu (1.13 g, 3 mmoles) (prepared by an ester exchange reaction[17] from BOC–Val–OH and N-hydroxysuccinimide dichloroacetate in pyridine at 50° for 40 min) with vigorous stirring for 6 hr. Addition of AcOH (1 ml) and H_2O (50 ml) to the reaction mixture yielded a precipitate, which was collected by filtration, washed with H_2O and reprecipitated from MeOH–ether: yield 2.4 g (84%); mp 143—146° (decomp.); $[\alpha]_D^{23}$ −65.4° (c=1.0 in DMF); Rf^1 0.65; Rf^2 0.80. *Anal.* Calcd. for $C_{42}H_{60}O_{11}N_6$: C, 61.14; H, 7.33; N, 10.19. Found: C, 61.20; H, 7.59; N, 9.91.

BOC-Lys(Z)-Lys(Z)-Arg(NO_2)-Arg(NO_2)-Pro-Val-Lys(Z)-Val-Tyr-Pro-OH (XIII)——Compound XI 3.3 g, 4 mmoles) was dissolved in TFA (15 ml), and the solution was kept at 10—15° for 20 min. TFA was concentrated to half volume, and dry ether was added to the residual solution to obtain solid material. The precipitate was filtered, washed with dry ether and dried over NaOH under reduced pressure: (XII). Rf^2 0.46, Rf^3 0.60, Rf^4 0.73. The dried powder was dissolved in DMF (50 ml) together with BOC–Lys(Z)–Lys(Z)–Arg(NO_2)–Arg(NO_2)–Pro–OPCP[10b] (X) (6.12 g, 4.4 mmoles) and Et_3N (1.2 ml). After being stirred for 12 hr, ether (150 ml) containing AcOH (1 ml) was added to precipitate the crude product, which was purified by reprecipitation from EtOH–ether: yield 6.2 g (82%); mp 168° (decomp., sintering at 127°); $[\alpha]_D^{23}$ −36.7° (c=1.0 in DMF); Rf^1 0.61; Rf^2 0.81; Rf^3 0.89. *Anal.* Calcd. for $C_{88}H_{121}O_{24}N_{21}\cdot H_2O$: C, 56.18; H, 6.91; N, 15.64. Found: C, 55.99; H, 6.91; N, 15.58.

Boc-Lys(Z)-Pro-Val-Gly-Lys(Z)-Lys(Z)-Arg(NO_2)-Arg(NO_2)-Pro-Val-Lys(Z)-Val-Tyr-Pro-OH (XIV)——A solution of XIII (2.32 g, 1.5 mmoles) in TFA was kept standing at 10—15° for 20 min and treated as described in the synthesis of XIII to give the tetradecapeptide trifluoroacetate. This compound was dissolved in DMF (20 ml) together with BOC–Lys(Z)–Pro–Val–Gly–OPCP[10b] (VIII) (1.59 g, 1.8 mmoles) and Et_3N (1.2 ml). The mixture was stirred for 12 hr at room temperature, then AcOH (1 ml) and cold ether (60 ml) were added to give a precipitate, which was filtered, washed with ether, and reprecipitated from AcOH–EtOH: yield 3.10 g (86%); mp 170° (decomp., sintering at 142—147°); $[\alpha]_D^{23}$ −36.7° (c=1.0 in DMF). Rf^2 0.70, Rf^3 0.78. *Anal.* Calcd. for $C_{114}H_{164}O_{30}N_{26}\cdot 2H_2O$: C, 56.68; H, 7.01; N, 15.08. Found: C, 56.50; H, 7.02; N, 15.06.

BOC-Phe-Arg(NO_2)-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 (XV)——A solution of XIV (2.64 g, 1.1 mmoles) in TFA (15 ml) was treated as described in the synthesis of XIV to give the octadecapeptide trifluoroacetate: Rf^2 0.41; Rf^3 0.61; Rf^4 0.72. The dried powder was dissolved in DMF (13 ml) together with BOC–Phe–Arg(NO_2)–Trp–Gly–OPCP[10b] (VI) (1.3 g, 1.3 mmoles) and Et_3N (0.26 ml). The mixture was stirred for 12 hr, then cold ether (50 ml) containing AcOH (1 ml) was added to precipitate the crude product, which was purified by reprecipitation from AcOH–

16) H. Kappeler and R. Schwyzer, *Helv. Chim. Acta*, 44, 1136 (1961).
17) M. Fujino and C. Hatanaka, "Proc. of the 5th Symposium on Peptide Chemistry," S. Sakakibara ed., Protein Research Foundation, Osaka, Japan, 1967, p. 13; *Ann. Report Takeda Res. Lab.*, 28, 12 (1969).

H_2O and then from AcOH–EtOH: yield 3.19 g (96%); mp 166—167° (decomp.,sintering at 149—158°); $[\alpha]_D^{23}$ −36.0° (c=1.0 in DMF). Rf^2 0.58; Rf^3 0.64; Rf^4 0.78. *Anal.* Calcd. for $C_{142}H_{197}O_{36}N_{35}\cdot H_2O$: C, 56.05; H, 6.79; N, 16.14. Found: C, 56.17; H, 6.77; N, 16.04. Amino acid *anal.*[18]: Lys 3.8 (4); Pro 2.9 (3); Gly 2.0 (2); Val 3.0 (3); Phe 1.1 (1). Average recovery 91%.

BOC-Met-Glu(O*t*-Bu)-His-Phe-Arg(NO_2)-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 (XVI)——An ice-cold aqueous 1 N $NaNO_2$ solution (1.2 ml) was added slowly to a solution of BOC–Met–Glu(O*t*-Bu)–His $NHNH_2$[10a] (IV) (600 mg, 1 mmoles) in a mixture of 1 N HCl (10 ml) and H_2O saturated with NaCl (12 ml) at −10°. The mixture was stirred for 15 min at −5°, and then $NaHCO_3$ (600 mg) was added. The azide was extracted with ice-cold AcOEt and the extract was washed with ice-cold 4% $NaHCO_3$ and H_2O, and dried over anhyd. Na_2SO_4. The azide solution was added to the solution of the octadecapeptide trifluoroacetate [prepared by treatment of compound XV (2.13 g, 0.7 mmole) with TFA containing 1% thioglycolic acid at 10—15° for 20 min under nitrogen] and Et_3N (0.25 ml) in DMF (40 ml) at −10°. The reaction mixture was stirred for 4 hr at −5° and then for an additional 12 hr at 3°. The additional azide solution prepared from 120 mg (0.2 mmole) of IV was added and the reaction mixture was stirred for additional 24 hr at 3°. Addition of AcOH (1 ml) and ether (200 ml) containing 0.2% thioglycolic acid to the reaction mixture precipitated the crude product, which was dissolved in a mixture of AcOEt–pyridine–AcOH–H_2O (30: 10: 3: 5) and then applied to a column (4×35 cm) of silica gel and eluted with the same solvent. The main fractions were pooled and concentrated. To the residue, ice water was added to give a precipitate, which was filtered and triturated with 2% AcOH, then washed with H_2O: yield 2.09 g (87%); mp 172—177° (decomp., sintering at 160°); $[\alpha]_D^{22}$ −36.0° (c=1.0 in DMF). Rf^2 0.54; Rf^3 0.64; Rf^4 0.90. *Anal.* Calcd. for $C_{157}H_{222}O_{39}N_{40}S$: C, 56.82; H, 6.71; N, 16.36; S, 0.94. Found: C, 56.74; H, 6.79; N, 16.30; S, 1.18. Amino acid *anal.*[18]: Lys 3.84 (4); His 0.94 (1); Glu 1.12 (1); Pro 2.70 (3); Gly 1.98 (2); Val 3.00 (3); Phe 1.00 (1). Average recovery 90%.

Z-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg(NO_2)-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 (XVII)——An ice-cooled aqueous 4 N $NaNO_2$ solution (0.15 ml) was added to a solution of Z–Ser–Tyr–Ser–$NHNH_2$[10a] (II) (252 mg, 0.5 mmole) in 6 N HCl (0.4 ml) and DMF (7 ml) at −10°. The mixture was stirred for 30 min at −10° and ice-cold Et_3N (0.3 ml) was added. The azide solution, which was dried over anhyd. Na_2SO_4, was then added to a solution of 10% Et_3N in DMF (1.7 ml) and the heneicosapeptide trifluoroacetate [obtained by TFA treatment of compound XVI (1.38 g, 0.4 mmole) in a similar fashion to that described in the synthesis of compound XVI]. The reaction mixture was stirred at −5° for 6 hr and then at 3° for additional 12 hr. Additional azide solution, which was prepared from 126 mg (0.25 mmole) of II, was added to the reaction mixture and the solution was stirred at 3° for 24 hr. Next, AcOH (1 ml) and ether (100 ml) containing 0.1% thioglycolic acid were added and the mixture was left to stand in a refrigerator. The resulting oil was separated by decantation of the supernatant and addition of AcOEt to the residual oil gave a precipitate, which was collected by filtration and washed with AcOEt. The resulting powder was dissolved in AcOEt–pyridine–AcOH–H_2O (30: 10: 3: 5) and applied to a column (3.5×27 cm) of silica gel, which was eluted with the same solvent. The main fractions were pooled and concentrated. Addition of cold water to the residue gave a precipitate, which was collected by filtration and washed with 1% aqueous AcOH containing 0.1% thioglycolic acid and then with H_2O. Yield 1.11 g (74%); mp 197—203° (sintering at 164—168°); $[\alpha]_D^{22}$ −32.8° (c=1.0 in DMF). Rf^2 0.37; Rf^3 0.46; Rf^4 0.81. *Anal.* Calcd. for $C_{176}H_{237}O_{47}S\cdot 2H_2O$: C, 55.99; H, 6.43; N, 15.95; S, 0.85. Found: C, 56.03; H, 6.43; N, 16.02; S, 1.08.

α^{1-24}-ACTH——The N-protected tetracosapeptide (XVII) (200 mg) was treated with 20 ml of anhyd. hydrogen fluoride in the presence of anisole (0.3 ml), thioglycolic acid (0.05 ml), Met (200 mg), Trp (200 mg) and TFA (1 ml) at 0° for 60 min. The hydrogen fluoride and TFA were removed under reduced pressure leaving a residue, which was dried in a desiccator over NaOH. The dried material was dissolved in H_2O (20 ml) and insoluble material was removed by filtration with diatomaceous earth. The filtrate was passed through a column of Amberlite IRA-400 (AcO^-, 30 ml) and the column was washed with H_2O. Effluent and washings were combined and evaporated to dryness. The residue was dissolved in 2% aqueous thioglycolic acid (10 ml) and the solution was kept at 50° for 20 hr under nitrogen. The resulting solution was diluted with H_2O (200 ml) and applied to a column (1.5×40 cm) of carboxymethylcellulose, which was washed successively with ammonium acetate buffer, 0.01 M (200 ml) and 0.1 M (200 ml), and then eluted with an exponential gradient from 0.1 M ammonium acetate buffer (500 ml) to 0.5 M ammonium acetate buffer (500 ml). The effluent was monitored at 280 mμ, and the fractions corresponding to the principal peak were collected. The pooled solution was applied to a column of Amberlite XAD-2 (50—200 mesh), which was first washed with 0.1 M ammonium acetate buffer (300 ml) and then eluted with an exponential gradient from 0.1 M ammonium acetate buffer (600 ml) to 50% EtOH (600 ml) containing AcOH (30 ml). The fractions corresponding to the principal peak were pooled and lyophilized to constant weight after EtOH had been evaporated; yield 68 mg; $[\alpha]_D^{23}$ −85.4° (c=1.0 in 1% AcOH). TLC: Rf^6, 0.22 (Woelm precoated silica gel

18) Partial alkylation of Tyr and Met, and degradation of Arg(NO_2) would occur during acid hydrolysis (110°, 24 hr, in 5.7 N HCl). See B. Iselin, *Helv. Chim. Acta*, **45**, 1510 (1962).

F 254/366); paper chromatography: Rf^4, 0.23 (Whatman No. 1); paper electrophoresis: pH 1.9 (acetic acid–80% formic acid–water, 15:5:80), 450 V, 1 hr, −8.5 cm; pH 3.6 (pyridine–acetic acid–water, 1:10:89), 400 V, 4 hr, −7.5 cm. UV $\lambda_{max}^{0.1NaOH}$ mμ ($E_{1cm}^{1\%}$) 283.0 (24.75), 289.5 (25.47). Tyr/Trp=1.93 (UV). Amino acid ratios in an acid hydrolysate: Lys 4.00 (4); His 0.94 (1); Arg 2.95 (3); Ser 2.10 (2); Glu 1.05 (1); Pro 2.87 (3); Gly 2.00 (2); Val 3.00 (3); Met 0.75 (1); Tyr 2.00 (2); Phe 2.00 (2); average recovery 96%. Amino acid ratios in an aminopeptidase-M digest (E/s=1.000 mμ/1 μmole, 37°, 20 hr): Lys (3.25 (4); His 0.97 (1); Arg 2.25 (3); Trp 1.02 (1); Ser 2.00 (2); Glu 1.01 (1); Gly 2.02 (2); Val 2.98 (3); Met 1.01 (1); Tyr 2.20 (2); Phe 1.00 (1). Average recovery 92%.

IBOC-Arg(NO_2)-Trp-Gly-OMe (XXII)——To an ice-cold solution of IBOC–Arg(NO_2)–OH[13] (12.0 g, 30 mmoles) and 2,4-dinitrophenol (6.1 g, 33 mmoles) in CH_3CN (70 ml) and tetrahydrofuran (THF) (30 ml), dicyclohexylcarbodiimide (DCC) (6.8 g, 30 mmoles) was added and the reaction mixture was stirred for 6 hr. The mixture was filtered and the filtrate was added to a solution of H–Trp–Gly–OMe in MeOH [prepared by catalytic hydrogenation of Z–Trp–Gly–OMe[10] (13.4 g, 33 mmoles)]. After being stirred for 30 hr at room temperature, the solutions was concentrated to give an oily material, which was dissolved in AcOEt, washed with 0.5 N HCl and H_2O, dried over anhyd. Na_2SO_4, and then evaporated. Addition of ether to the residue gave a precipitate, which was recrystallized from benzene: yield 18.9 g (96%); mp 105—115° (decomp.); $[\alpha]_D^{22}$ −23.9° (c=1.0 in DMF). *Anal.* Calcd. for $C_{31}H_{44}O_8N_8 \cdot 2/3C_6H_6$: C, 59.25; H, 6.82; N, 15.80. Found: C, 59.20; H, 6.99; N, 15.79.

Z-Phe-Arg(NO_2)-Trp-Gly-OMe[12] (XXIII)——IBOC–Arg(NO_2)–Trp–Gly–OMe (XXII) (16.4 g, 25 mmoles) was treated with TFA containing 1% thioglycolic acid (50 ml) for 15 min at room temperature and TFA was evaporated to half volume. Upon addition of dry ether, the tetrapeptide trifluoroacetate precipitated, and was collected and dried. The dried powder of the resulting trifluoroacetate was dissolved in DMF (30 ml) together with Z–Phe–OSu (9.9 g, 25 mmoles) and Et_3N (3.5 ml). After being stirred for 3 hr at room temperature, the reaction mixture was poured into H_2O (1.3 liters), and then extracted with AcOEt. The extracts were washed with 1 N HCl, 5% $NaHCO_3$ and H_2O, then dried over anhyd. Na_2SO_4, and evaporated. Addition of ether to the residue gave a precipitate, which was crystallized from MeOH: yield 14.5 g (76%); mp 130° (decomp.); $[\alpha]_D^{22}$ −19.1° (c=1.02 in DMF). *Anal.* Calcd. for $C_{37}H_{43}O_9N_9 \cdot H_2O$: C, 57.28; H, 5.85; N, 16.27. Found: C, 57.62; H, 6.02; N, 15.93.

Z-Phe-Arg(NO_2)-Trp-Gly-OH[12] (XXIV)——To compound XXIII (13.95 g, 18 mmoles) in a mixture of MeOH (90 ml) and DMF (10 ml), 1 N NaOH (27 ml) was added at 0° and the mixture was stirred for 1 hr at room temperature. Addition of 1 N HCl (27.5 ml) and H_2O (500 ml) to the reaction mixture gave a precipitate, which was recrystallized from MeOH: yield 11.5 g (87%); mp 195—197°; $[\alpha]_D^{22}$ −18.4° (c=0.99 in DMF). *Anal.* Calcd. for $C_{36}H_{91}O_9N_9 \cdot 1/2H_2O$: C, 57.43; H, 5.62; N, 16.74. Found: C, 57.20, H, 5.72; N, 16.81.

H-Phe-Arg$^+$-Trp-Gly-OH•AcO$^-$ (XXV)[12]——Compound XXIV (29.7 g, 40 mmoles) in 80% AcOH over a Pd-black at 50° for 8 hr. The catalyst was filtered off and the filtrate was evaporated, giving a solid material, which was recrystallized from H_2O: yield 90%; mp 236—238°; *Anal.* Calcd. for $C_{28}H_{36}O_5N_5 \cdot CH_3COOH \cdot 1/2H_2O$: C, 56.86; H, 6.52; N, 17.68. Found: C, 56.85; H, 6.53; N, 17.01.

Z-Glu(O*t*-Bu)-His-Phe-Arg$^+$-Trp-Gly-OH•AcO$^-$ (XXVI)——An ice-cold aqueous 5 N $NaNO_2$ solution (6.4 ml, 31.6 mmoles) was added slowly to a solution of Z-Glu(Ot-Bu)–His–$NHNH_2$[19] (12.7 g, 26 mmoles) in NaCl-satd. H_2O (100 ml) and 1 N HCl (140 ml) at −5°. The mixture was stirred for 3 min at −5°, and then ice-cold 2 N $NaHCO_3$ (140 ml) and AcOEt (100 ml) were added. The AcOEt layer was washed twice with cold H_2O and dried over anhyd. Na_2SO_4. The azide solution was added to a solution of H–Phe–Arg$^+$–Trp–Gly–OH–AcO$^-$ (12.5 g, 20 mmoles) and Et_3N (5.6 ml) in DMF (120 ml), then stirred for 24 hr at 0—3°. Addition of AcOEt (1 liter) containing AcOH (10 ml) to the reaction mixture gave a precipitate which was recrystallized from MeOH–H_2O (3:2): yield 18.8 g (87%); mp 203—205° (decomp.); $[\alpha]_D^{35}$ −20.9° (c=0.98 in DMF). *Anal.* Calcd. for $C_{51}H_{64}O_{11}N_{12} \cdot CH_3COOH \cdot H_2O$: C, 57.91; H, 6.41; N, 15.29. Found: C, 57.96; H, 6.01; H, 15.66.

Z-Ser-Tyr-Ser-Met-Glu(O*t*-Bu)-His-Phe-Arg$^+$-Trp-Gly-OH•*p*-TsO$^-$ (XXVII)——Compound XXVI (13.5 g, 12.5 mmoles) in 80% AcOH (200 ml) was hydrogenated over a Pd catalyst. The catalyst was filtered off and the filtrate was evaporated, leaving an oily residue. Addition of acetone to the residue gave a precipitate, which was collected and dried in a desiccator over NaOH. Yield 13.0 g. The dried powder was dissolved in DMF (100 ml) together with Et_3N (3.5 ml, 25 mmoles).

On the other hand, an ice-cold aqueous 2 N $NaNO_2$ (8.25 ml) was added slowly to a solution of Z–Ser–Tyr–Ser–Met–$NHNH_2$[20] (XIX) (9.55 g, 15 mmoles) in the mixture of DMF (100 ml) and 2 N HCl (22.5 ml) at −5°. After being stirred for 15 min at −5°, the reaction mixture was cooled to −30° and Et_3N (6.3 ml) was added. The azide solution was added to a solution of the above hexapeptide salt in DMF, and the mixture was stirred for 24 hr at 0—3°. AcOEt (1.8 liters) containing AcOH (10 ml) was added to precipitate the

19) R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, **44**, 1991 (1961).
20) C.H. Li, J. Meienhofer, E. Schnabel, D. Chung, T.B. Lo, and J. Ramachandran, *J. Am. Chem. Soc.*, **83**, 4449 (1961); C.H. Li, J. Ramachandran, D. Chung, and B. Gorup, *ibid.*, **86**, 2703 (1964).

crude product, which was collected, then washed with hot 5% aqueous AcOH and recrystallized from MeOH–H_2O (8:2): yield 15.0 g (77%); mp 203—204° (decomp.); $[\alpha]_D^{22}$ −19.1° (c=0.48 in DMF). *Anal.* Calcd. for $C_{71}H_{92}O_{18}N_{16}S \cdot CH_3COOH \cdot 2H_2O$: C, 55.26; H, 6.36; N, 14.13; S, 2.07. Found: C, 55.42; H, 6.33; N, 13.95; S, 2.08.

To a solution of the partially protected decapeptide acetate (7.75 g, 5 mmoles) thus obtained in DMF (70 ml), *p*-toluenesulfonic acid monohydrate (950 mg, 5 mmoles) was added. AcOEt–ether (1:1, 400 ml) was added to obtain XXVII as a precipitate: yield 8.22 g (99%); mp 170—175° (decomp.); $[\alpha]_D^{23}$ −15.2° (c=1.01 in DMF). *Anal.* Calcd. for $C_{78}H_{100}O_{21}N_{16}S_2 \cdot 5H_2O$: C, 53.47; H, 6.55; N, 12.85; S, 3.65. Found: C, 53.47; H, 5.95; N, 12.63; S, 3.96.

H-Lys(Z)-Pro-Val-Gly-Lys(Z)-Lys(Z)-Arg(NO_2)-Arg(NO_2)-Pro-Val-Lys(Z)-Val-Tyr-Pro-OH (XXVIII)——To a solution of compound XIV (50 g, 21 mmoles) in AcOH (200 ml), 4N HCl–AcOH (200 ml) was added and the solution was stirred for 20 min at room temperature. Ether was added to obtain a precipitate, which was filtered, washed with ether, and dried in desiccator over NaOH. Yield 50 g. The resulting powder was dissolved in a mixture of AcOEt–pyridine–AcOH–H_2O (30:5:3:5) (150 ml), and the solution was applied to a column (6.5×70 cm) of silica gel, which was eluted with the same solvent. The main fractions were pooled and the solvent was evaporated. Addition of ether to the residue gave a precipitate: yield 25.0 g. The other fractions containing XXVIII were further purified in the same manner to give XXVIII: yield 13.2 g. Total yield 38.2 g (78%). mp 110—115° (decomp.); $[\alpha]_D^{23}$ −36.3° (c=0.96 in DMF). *Anal.* Calcd. for $C_{109}H_{156}O_{28}N_{26} \cdot 2H_2O$: C, 56.57; H, 6.96; N, 15.72. Found: C, 56.32; H, 6.96; N, 15.72. Amino acid *anal.*:[18] Lys 4.24 (4); Arg 1.24 (2); Pro 3.05 (3); Gly 1.00 (1); Val 3.30 (3); Tyr 0.95 (1); average recovery 95%.

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*-TsO$^-$ (XXIX)——A solution of compound XXVII (13.0 g, 7.82 mmoles), TCP (6.17 g, 31.28 mmoles) and DCC (6.47 g, 31.28 mmoles) in DMF (80 ml) was stirred for 8 hr at room temperature. Additional TCP (6.18 g) and DCC (6.47 g) were added and the mixture was stirred for an additional 20 hr. The DC urea formed was removed by filtration and the filtrate was poured into a mixture of AcOEt and ether (1:1). The resulting precipitate was collected by filtration: yield 13.8 g (96%). The TCP ester of compound XXVII thus obtained was added to a solution of compound XXVIII (13.0 g, 5.61 mmoles) and N-ethylmorpholine (0.715 ml, 5.61 mmoles) in DMF (100 ml) and the reaction mixture was stirred for 20 hr at room temperature and then for 3 hr at 40—50°. The DC urea formed was removed by filtration. Ether (600 ml) containing AcOH (6.0 ml) was added to the filtrate to give a precipitate, which was reprecipitated from MeOH–H_2O (8:2): yield 12.8 g (83%); mp 187—190°; $[\alpha]_D^{22}$ −32.9° (c=0.64 ih DMF). *Anal.* Calcd. for $C_{180}H_{246}O_{45}N_{42}S \cdot C_7H_8O_3S \cdot 6H_2O$: C, 55.72; H, 6.50; N, 14.60; S, 1.59. Found: C, 55.74; H, 6.53; N, 14.13; S, 1.55. Amino acid *anal.*[18]: Lys 4.38 (4); His 1.00 (1); Arg 2.56 (3); Ser 2.00 (2); Glu 1.00 (1); Pro 3.19 (3); Gly 2.06 (2); Val 3.25 (3); Met 0.44 (1); Try 1.82 (2); Phe 1.00 (1). Average recovery: 96%.

α^{1-24}-ACTH——The N-partially protected tetracosapeptide *p*-toluene sulfonate (XXIX) (8.5 g) was treated with 100 ml of anhyd. hydrogen fluoride in the presence of anisole (19.0 ml), skatole (2.9 g) and methyl ethyl sulfide (5.0 ml) at 0° for 30 min. The hydrogen fluoride was removed under reduced pressure, giving a residue which was dried in a desiccator over NaOH. The dried material was dissolved in H_2O and the solution was extracted with *n*-BuOH. The aqueous layer was passed through a column of Amberlite IRA-400 (AcO^-, 420 ml) and the column was washed with H_2O. Effluent and washings were combined and lyophilized to yield 7.4 g of crude α^{1-24}-ACTH. The crude material was dissolved in 3% aqueous mercaptoethanol (50 ml) and the solution was kept at 50° for 20 hr under nitrogen. The resulting solution was diluted with H_2O (500 ml) and applied to a column (4.0×35 cm) of carboxymethylcellulose which was washed successively with H_2O and 0.1 M ammonium acetate buffer (5 liters) and eluted with 0.3 M ammonium acetate buffer. The effluent was applied to a column (4.5×10 cm) of Amberlite XAD-2 (50—200 mesh). For elution, an exponential gradient of 50% EtOH (900 ml) containing AcOH (4.5 ml) to 0.1 M ammonium acetate buffer (1500 ml) was used and the main fractions were pooled and lyophilized to constant weight after EtOH had been evaporated: yield 1.7 g, $[\alpha]_D^{26}$ −87.0° (c=0.25 in 1% AcOH). TLC: Rf^6 0.22 (Woelm precoated silica gel F 254/366); paper chromatography: Rf^4 0.23 (Whatman No. 1); paper electrophoresis: pH 1.9 (acetic acid–80% formic acid–water, 15:5:80), 450 V, 1 hr, −8.5 cm; pH 3.6 (pyridine–acetic acid–water, 1:10:89), 400 V, 4 hr, −7.5 cm. UV $\lambda_{max}^{0.1\,NaOH}$ mμ ($E_{1cm}^{1\%}$) 283.0 (25.8), 289.5 (27.3). Tyr/Trp=1.92 (UV). Amino acid ratios in an acid hydrolysate: Lys 4.09 (4); His 1.08 (1); Arg 2.93 (3); Ser 2.08 (2); Glu 1.00 (1); Pro 3.00 (3); Gly 1.95 (2); Val 3.04 (3); Met 0.77 (1); Tyr 2.06 (2); Phe 1.00 (1). Average recovery: 90%. Amino acid ratios in an aminopeptides-M digest: Lys 3.20 (4); His 1.00 (1); Arg 2.20 (3); Trp 1.03 (1); Ser 2.02 (2); Glu 1.00 (1); Gly 2.03 (2); Val 3.03 (3); Met 1.00 (1); Tyr 2.30 (2); Phe 1.00 (1). Average recovery: 90%.

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