

**Studies on Peptides. LX.<sup>1,2)</sup> Synthesis of the Nonatriacontapeptide  
corresponding to the Entire Amino Acid Sequence of  
Bovine Adrenocorticotrophic Hormone**

KANAME KOYAMA, HIROKI KAWATANI, HARUAKI YAJIMA,<sup>3a)</sup>  
MASAHIKO FUJINO, and OSAMU NISHIMURA<sup>3b)</sup>

*Faculty of Pharmaceutical Sciences, Kyoto University<sup>3a)</sup> and Medical  
Research Laboratory, Takeda Chemical Ind. Ltd.<sup>3b)</sup>*

(Received December 2, 1975)

The nonatriacontapeptide corresponding to the newly revised amino acid sequence of bovine adrenocorticotrophic hormone was synthesized by successive condensation of 3 peptide fragments; Z(OMe)-(15—19)-OH, Z(OMe)-(11—14)-OH and Z-(1—10)-OH, with H-(20—39)-OBzl, a synthetic intermediate of bovine type corticotropin-like intermediate lobe peptide. The synthetic peptide exhibited the *in vivo* steroidogenic activity of 93.8 IU/mg.

Recently the amino acid sequence of bovine adrenocorticotrophic hormone (ACTH) proposed earlier in 1965<sup>4)</sup> was reexamined by Li,<sup>5)</sup> and its revised sequence for the residue 25 to 30 has been published. More recently, Johl, *et al.*<sup>6)</sup> concluded that this revised sequence of bovine ACTH is identical with that of the respective peptide of ovine origin.

We wish to report the synthesis of the nonatriacontapeptide corresponding to the revised sequence of bovine ACTH (I) which represents the adrenocorticotrophic principle of two mammalian species. For this synthesis, the partially protected eicosapeptide ester (II), a synthetic intermediate of bovine ACTH-like intermediate lobe peptide (CLIP),<sup>7)</sup> served as an amino component, with which two available peptide fragments, III (sequence 15—19) and IV (sequence 11—14) used for our previous synthesis of porcine ACTH<sup>8)</sup> were successively condensed. However, in this synthesis, the decapeptide unit V (sequence 1—10) derived from condensation of V-a (sequence 1—4) and V-b (sequence 5—10) was taken as one fragment as shown in Fig. 1. To prepare the fragment V-b, Z-Glu(OBu<sup>t</sup>)-OH, instead of Z(OMe)-Glu(OBzl)-OH, was employed. By this route, the fragment V could be prepared without exposing the Trp-containing peptide to trifluoroacetic acid (TFA).<sup>9)</sup>

Among these fragments, the eicosapeptide ester, H-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Ser-Ala-Gln-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl (II), was synthesized with a minor modification. Z(OMe)-Glu(OBzl)-Ser-Ala-Gln-Ala-Phe-Pro-OH was first prepared as illustrated in Fig. 2. Z-Ser-Ala-NHNH<sub>2</sub> was condensed with

- 1) Part LIX: H. Yajima, K. Koyama, Y. Kiso, A. Tanaka, and H. Nakamura, *Chem. Pharm. Bull.* (Tokyo), **24**, 492 (1976).
- 2) Amino acids, peptides and their derivatives mentioned in this paper are of the L-configuration. Abbreviations used are those recommended by IUPAC-IUB Commission of Biochemical Nomenclature: *Biochem.*, **5**, 2485 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1972). Z = benzyloxycarbonyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, Tos = *p*-toluenesulfonyl, OBzl = benzyl ester, ONP = *p*-nitrophenyl ester.
- 3) Location: a) *Sakyo-ku, Kyoto, 606, Japan*; b) *Yodogawa-ku, Osaka, 532, Japan*.
- 4) C.H. Li, J.S. Dixon, and D. Chung, *J. Am. Chem. Soc.*, **80**, 2587 (1958); *idem*, *Biochim. Biophys. Acta*, **46**, 324 (1961).
- 5) C.H. Li, *Biochem. Biophys. Res. Commun.*, **49**, 835 (1972).
- 6) A. Johl, B. Riniker, and L.S. Hulliger, *FEBS Letters*, **45**, 172 (1974).
- 7) H. Kawatani and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **22**, 1872 (1974).
- 8) H. Yajima, K. Koyama, Y. Kiso, A. Tanaka, and M. Nakamura, *Chem. Pharm. Bull.* (Tokyo), **24**, 492 (1976).
- 9) F. Weygand and K. Hunger, *Chem. Ber.*, **98**, 1 (1962).

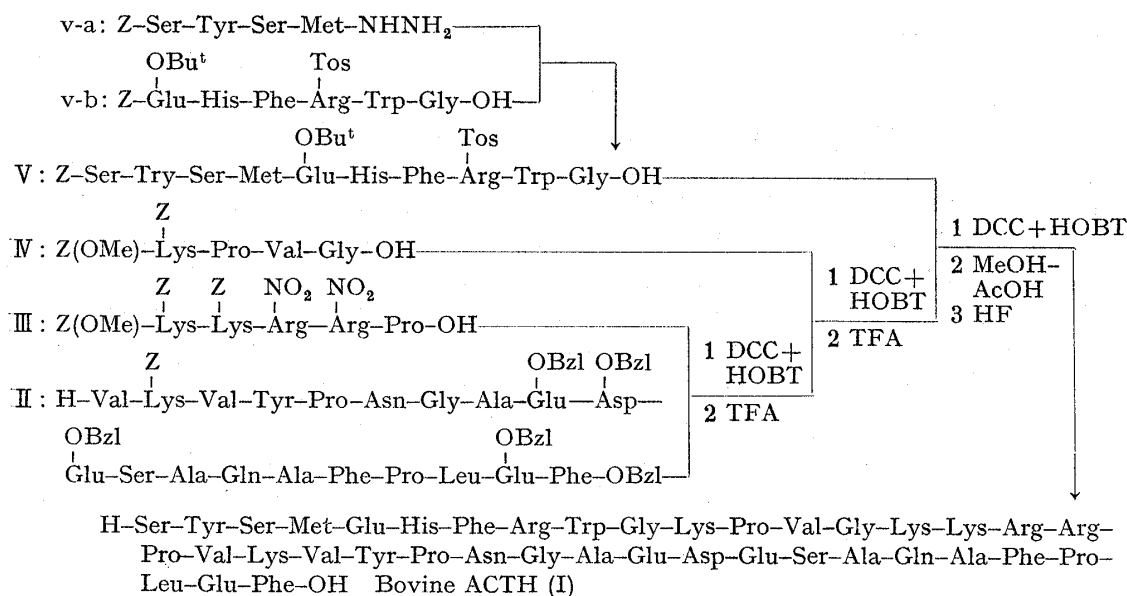


Fig. 1. Synthetic Route to Bovine ACTH

H-Gln-Ala-Phe-Pro-OH<sup>7)</sup> by the modified azide procedure<sup>10)</sup> and the resulting Z-Ser-Ala-Gln-Ala-Phe-Pro-OH, after the catalytic hydrogenation, was condensed with Z(OMe)-Glu(OBzl)-ONP to give the above stated protected heptapeptide. This was then condensed with H-Leu-Glu(OBzl)-Phe-OBzl<sup>7)</sup> by dicyclohexylcarbodiimide (DCC) in the presence of N-hydroxybenzotriazole (HOBT)<sup>11)</sup> to give Z(OMe)-Glu(OBzl)-Ser-Ala-Gln-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl. This peptide was previously prepared by the reaction of Z(OMe)-Glu(OBzl)-ONP with the nonapeptide unit which was obtained after the DCC plus HOBT condensation of Z(OMe)-Ser-Ala-Gln-Ala-Phe-Pro-OH and H-Leu-Glu(OBzl)-Phe-OBzl. Chain elongation of this decapeptide to II was performed as described previously.<sup>7)</sup>

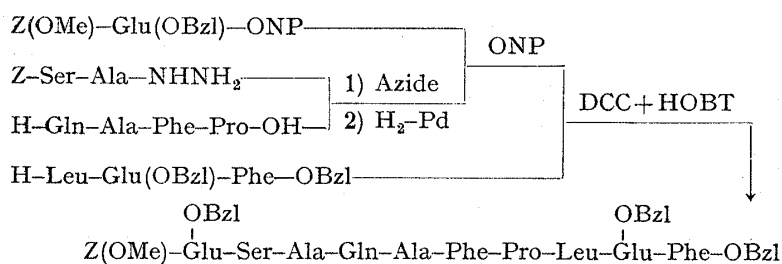


Fig. 2. Alternative Synthetic Scheme of the C-Terminal Protected Decapeptide Ester, Z(OMe)-(bovine ACTH 30-39)-OBzl

As performed in the synthesis of porcine ACTH, the DCC plus HOBT procedure was also employed for the condensation of II and Z(OMe)-Lys(Z)-Lys(Z)-Arg(NO<sub>2</sub>)-Arg(NO<sub>2</sub>)-Pro-OH (III) and the resulting protected pentacosapeptide ester, Z(OMe)-Lys(Z)-Lys(Z)-Arg(NO<sub>2</sub>)-Arg(NO<sub>2</sub>)-Pro-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Ser-Ala-Gln-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl, [abbreviated as Z(OMe)-(bovine ACTH 15-39)-OBzl], was purified by column chromatography on silica using the solvent system of chloroform-methanol-water (8:3:1) as described previously.<sup>8)</sup> Z(OMe)-(bovine ACTH 15-39)-OBzl, after treatment with TFA, followed by conversion to the corresponding hydrochloride and subsequent neutralization with triethylamine, was similarly condensed with Z(OMe)-Lys(Z)-Pro-Val-Gly-OH (IV) by DCC in the presence of HOBT. Purification of the

10) J. Honzl and J. Rudinger, *Coll. Czech. Chem. Commun.*, **26**, 1872 (1974).11) W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).

resulting protected nonacosapeptide ester, Z(OMe)-Lys(Z)-Pro-Val-Gly-Lys(Z)-Lys(Z)-Arg(NO<sub>2</sub>)-Arg(NO<sub>2</sub>)-Pro-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Ser-Ala-Gln-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl, [abbreviated as Z(OMe)-(bovine ACTH 11-39)-OBzl], was also achieved by column chromatography on silica. The DCC plus HOBT procedure was further extended for the final coupling reaction of the TFA treated sample of Z(OMe)-(bovine ACTH 11-39)-OBzl with Z-Ser-Tyr-Ser-Met-Glu(OBu<sup>t</sup>)-His-Phe-Arg(Tos)-Trp-Gly-OH (V) derived from the azide condensation of Z-Ser-Tyr-Ser-Met-NHNH<sub>2</sub> (V-a) with the hydrogenated sample of Z-Glu(OBu<sup>t</sup>)-His-Phe-Arg(Tos)-Trp-Gly-OH (V-b). However, it should be mentioned that as pointed out by Rink and Riniker,<sup>12)</sup> the N<sup>im</sup> of His residue at position 6 in the product was presumably masked with the dicyclohexylamidino moiety during the above DCC plus HOBT condensation reaction. According to the literature,<sup>12)</sup> the crude product was heated in a mixture of dimethylformamide and methanol containing acetic acid at 65° for 4 hr to remove the masking group of the His residue and then purified by column chromatography on silica. Homogeneity of the resulting protected nonatriacontapeptide ester, Z-Ser-Tyr-Ser-Met-Glu(OBu<sup>t</sup>)-His-Phe-Arg(Tos)-Trp-Gly-Lys(Z)-Pro-Val-Gly-Lys(Z)-Lys(Z)-Arg(NO<sub>2</sub>)-Arg(NO<sub>2</sub>)-Pro-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Ser-Ala-Gln-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl [abbreviated as Z-(bovine ACTH 1-39)-OBzl], was examined by three criteria; thin-layer chromatography (TLC), amino acid analysis and elemental analysis. However, it was not easy to identify the presence of the free His residue in such a protected peptide.

Deprotection and purification procedures employed for the synthesis of porcine ACTH were also valid for our present purpose. According to Sakakibara *et al.*,<sup>13)</sup> all of protecting groups: Z, Tos, Bu<sup>t</sup>, NO<sub>2</sub> and Bzl groups, were deblocked from Z-(bovine ACTH 1-39)-OBzl by hydrogen fluoride. Anisole containing 2% ethanedithiol was added to prevent the possible alkylation reaction during this treatment. The product, after conversion to the corresponding acetate by Amberlite IR-4B, was incubated in methanol containing acetic acid at 60° for 8 hr to secure the removal of the dicyclohexylamidino moiety from the N<sup>im</sup> of the His residue mentioned above. Met-sulfoxide formed partially during the above deblocking process was reduced by addition of a small amount of dithiothreitol to this incubated solution.

The crude product was purified first by column chromatography on Sephadex G-25. Minor impurities were removed in this step. The product of the front peak was then submitted to further purification by column chromatography on CM-Sephadex. Gradient elution with 0.3M ammonium acetate buffer at pH 6.9 was employed and eluates were examined by the ultraviolet (UV) absorbancy at 280 mμ. The fluffy powder obtained after lyophilization of the main peak, exhibited a single spot on TLC in two different solvent systems and behaved as a single component in the field of disc electrophoresis at two different pH values. Hydrolysate by 3N *p*-toluenesulfonic acid<sup>14)</sup> contained the constituent amino acids in ratios predicted by theory. By this hydrolysis, the presence of one mole of Trp could be confirmed. By increasing the amount of aminopeptidase (AP-M),<sup>15)</sup> it was possible to digest the synthetic nonatriacontapeptide completely. With this experiment, it became possible to identify the presence of one mole of His in an unprotected form as well as the presence of one mole of Gln. The Gln content could also be estimated from the difference of Glu recoveries between acid and enzymatic hydrolysates. In this amino acid analysis, the Asn peak was overlapped with that of Ser. However from the difference of Asp recoveries between acid and enzymatic hydrolysates, nearly quantitative amount of Asn could be estimated.

12) H. Rink and B. Riniker, *Helv. Chim. Acta*, **57**, 831 (1974).

13) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Japan*, **40**, 2164 (1967).

14) T.Y. Liu and Y.H. Chang, *J. Biol. Chem.*, **216**, 2842 (1971).

15) G. Pfeleiderer and P.G. Celliers, *Biochem. Z.*, **339**, 186 (1963), H. Watanabe, H. Ogawa, and H. Yajima, *Chem. Pharm. Bull. (Tokyo)*, **23**, 375 (1975).

Experimental evidences cited above may justify the conclusion that our synthetic peptide which embodies the entire amino acid sequence of bovine ACTH possessed a high degree of homogeneity. The *in vivo* steroidogenic activity of our synthetic bovine ACTH, after comparison to that of synthetic human ACTH (145 IU/mg),<sup>16)</sup> was judged as 93.8 IU/mg. This figure can be considered as potent enough when compared to those of natural source and synthetic analogs.<sup>17)</sup>

Finally, we wish to mention briefly about our preliminary attempt to synthesize bovine ACTH (1965 formula)<sup>4)</sup> by the fragment condensation procedure on a polymer support. Starting with Z(OMe)-Leu-Glu(OBzl)-Phe-resin (peptide content 0.13 mmol/g), following 6 peptide fragments were condensed by N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline according to the procedure described previously.<sup>18)</sup> Successive condensation of Z(OMe)-Glu(OBzl)-Ala-Glu(OBzl)-Asp(OBzl)-Ser-Ala-Gln-Ala-Phe-Pro-OH, Z(OMe)-Asp(OBzl)-Gly-OH, Z(OMe)-Val-Lys(Z)-Val-Tyr-Pro-OH, Z(OMe)-Lys(Z)-Lys(Z)-Arg(NO<sub>2</sub>)-Arg(NO<sub>2</sub>)-Pro-OH (III) and Z(OMe)-Lys(Z)-Pro-Val-Gly-OH (IV) proceeded in the yield of 100%, 100%, 84%, 96% and 74% respectively. However the final condensation of Z-Ser-Tyr-Ser-Met-Glu(OBzl)-His-Phe-Arg(NO<sub>2</sub>)-Trp-Gly-OH gave the coupling yield only in 31%. Despite of the use of DCCplus HOBT or N-hydroxysuccinimide, any improvement in this coupling step has not been achieved. Although, isolation of the final product was thought to be theoretically possible, this approach has been abandoned because of the announcement of the revised sequence of bovine ACTH.

### Experimental

Thin Layer chromatography was performed on silica gel (Kieselgel, G, Merck). *R<sub>f</sub>* values refer to the following solvent systems: *R<sub>f1</sub>* CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O (8: 3: 1), *R<sub>f2</sub>* *n*-butanol-pyridine-AcOH-H<sub>2</sub>O (4: 1: 1: 2), *R<sub>f3</sub>* *n*-BuOH-pyridine-AcOH-H<sub>2</sub>O (30: 20: 6: 24).

**Z-Ser-Ala-OMe**—This compound was prepared differently from previous routes.<sup>19,20)</sup> Z-Ser-OPCP (9.78 g) was added to a solution of H-Ala-OMe (prepared from 2.80 g of the hydrochloride with 5.6 ml of Et<sub>3</sub>N) in dimethyl formamide (DMF) (30 ml) and the solution was stirred at room temperature overnight. After evaporation of the solvent, the residue was extracted with AcOEt, which was washed with 10% citric acid, 5% Na<sub>2</sub>CO<sub>3</sub> and H<sub>2</sub>O-NaCl, dried over Na<sub>2</sub>SO<sub>4</sub> and then evaporated. The residue was triturated with ether and recrystallized from AcOEt and ether; yield 4.80 g (74%), mp 112—113°, [ $\alpha$ ]<sub>D</sub><sup>25</sup> -32.4° (*c*=0.8, MeOH). (lit.<sup>19,20)</sup> mp 113—114°. *R<sub>f1</sub>* 0.65. *Anal.* Calcd. for C<sub>15</sub>H<sub>20</sub>O<sub>6</sub>N<sub>2</sub>: C, 55.55; H, 6.22; N, 8.64. Found: C, 55.82; H, 6.43; N, 8.84.

**Z-Ser-Ala-NHNH<sub>2</sub>**—In the usual manner, Z-Ser-Ala-OMe was converted to the corresponding hydrazide; yield 96%, mp 227—228° (lit.<sup>21)</sup> 222—223°). *R<sub>f1</sub>* 0.58. *Anal.* Calcd. for C<sub>14</sub>H<sub>20</sub>O<sub>5</sub>N<sub>4</sub>: C, 51.84; H, 6.22; N, 17.28. Found: C, 51.83; H, 6.29; N, 17.29.

**Z-Ser-Ala-Gln-Ala-Phe-Pro-OH**—Under cooling with ice-NaCl, isoamylnitrite (2.2 ml) was added to a solution of Z-Ser-Ala-NHNH<sub>2</sub> (4.22 g) in 1.45 N HCl-DMF (30 ml). The solution, after stirring for 5 min, was neutralized with Et<sub>3</sub>N (5.6 ml). This solution was then combined with a solution of H-Gln-Ala-Phe-Pro-OH (5.0 g) and Et<sub>3</sub>N (3.3 ml) in 90% aqueous DMF (50 ml). After the mixture was stirred at 4° for 48 hr, the solvent was evaporated and the residue was treated with ether. The resulting powder was washed with 10% citric acid, H<sub>2</sub>O and AcOEt and then recrystallized from MeOH and AcOEt; yield 5.20 g (64%), mp 184—186°, [ $\alpha$ ]<sub>D</sub><sup>25</sup> -30.4° (*c*=1.0, DMF). *R<sub>f2</sub>* 0.57. Amino acid ratios in an acid hydrolysate: Ser 0.84, Ala 2.08, Glu 1.07, Phe 1.00, Pro 1.17 (average recovery 98%). *Anal.* Calcd. for C<sub>36</sub>H<sub>47</sub>O<sub>11</sub>N<sub>7</sub>: C, 57.36; H, 6.29; N, 13.01. Found: C, 57.05; H, 6.54; N, 12.56.

**Z(OMe)-Glu(OBzl)-Ser-Ala-Gln-Ala-Phe-Pro-OH**—Z(OMe)-Glu(OBzl)-ONP (6.30 g) in DMF (50 ml) was added to a solution of H-Ser-Ala-Gln-Ala-Phe-Pro-OH (5.0 g) and Et<sub>3</sub>N (2.3 ml) in H<sub>2</sub>O (10 ml) and the

16) O. Nishimura, C. Hatanaka, and M. Fujino, *Chem. Pharm. Bull.* (Tokyo), **23**, 1212 (1975)

17) C.H. Li and J.S. Dixon, *Science*, **124**, 934 (1956), see also a review article; H. Yajima and H. Kawatani, "Synthesis of ACTH Active Peptide and Analogs" in "Chemistry and Biochemistry of Amino Acid, Peptides, and Proteins", ed. by B. Weinstein, Macel Dekker, N.Y. 1974, p 39.

18) H. Yajima and H. Kawatani, *Chem. Pharm. Bull.* (Tokyo), **19**, 1905 (1971); H. Kawatani, F. Tamura, and H. Yajima, *ibid.*, **22**, 1879 (1974).

19) J.S. Fruton, *J. Biol. Chem.*, **146**, 463 (1942).

20) Y. Kiso, Y. Kai, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **21**, 2507 (1973).

21) J.H. Harris and J.S. Fruton, *J. Biol. Chem.*, **191**, 143 (1951).

mixture was stirred at room temperature for 48 hr. After evaporation of the solvent *in vacuo*, the residue was treated with ether and the resulting powder was washed successively with 10% citric acid and H<sub>2</sub>O. It was recrystallized from DMF and AcOEt; yield 6.30 g (78%), mp 168—170°,  $[\alpha]_D^{25} -22.6^\circ$  ( $c=0.8$ , DMF).  $Rf_2$  0.83. Amino acid ratios in an acid hydrolysate: Glu 2.17, Ser 0.83, Ala 2.15, Phe 1.00, Pro 1.13 (average recovery 96%). *Anal.* Calcd. for C<sub>45</sub>H<sub>62</sub>O<sub>15</sub>N<sub>8</sub>: C, 58.67; H, 6.23; N, 11.17. Found: C, 58.39; H, 6.53; N, 10.89.

**Z(OMe)-Glu(OBzl)-Ser-Ala-Gln-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl**—An AcOEt solution of H-Leu-Glu(OBzl)-Phe-OBzl (prepared from 7.4 g of Z(OMe)-Leu-Glu(OBzl)-Phe-OBzl by treatment with 14 ml of TFA as stated previously<sup>7)</sup> was washed with 5% Na<sub>2</sub>CO<sub>3</sub> and H<sub>2</sub>O-NaCl, dried over Na<sub>2</sub>SO<sub>4</sub> and then filtered. The filtrate was combined with a solution of Z(OMe)-Glu(OBzl)-Ser-Ala-Gln-Ala-Phe-Pro-OH (7.86 g) in DMF (50 ml). After addition of HOBT (2.65 g) and DCC (2.42 g), the mixture was stirred at room temperature for 72 hr. The solution, after filtration, was evaporated *in vacuo* and the residue was treated with ether. The resulting powder was washed batchwisely with 5% Na<sub>2</sub>CO<sub>3</sub>, 10% citric acid and H<sub>2</sub>O and then precipitated from DMF with ether; yield 11.70 g (95%), mp 189—190° (anhydride, lit.<sup>7)</sup> dihydrate, mp 132—136°,  $[\alpha]_D^{25} -21.7^\circ$  ( $c=0.6$ , DMF).  $Rf_1$  0.68. Amino acid ratios in an acid hydrolysate: Glu 3.20, Ser 0.80, Ala 2.20, Phe 2.00, Pro 1.10, Leu 1.00 (average recovery 89%). *Anal.* Calcd. for C<sub>83</sub>H<sub>101</sub>O<sub>26</sub>N<sub>11</sub>: C, 63.38; H, 6.47; N, 9.80. Found: C, 63.19; H, 6.46; N, 9.99.

**Z(OMe)-Lys(Z)-Lys(Z)-Arg(NO<sub>2</sub>)-Arg(NO<sub>2</sub>)-Pro-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Ser-Ala-Gln-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl, Z(OMe)-(bovine ACTH 15—39)-OBzl**—The protected eicosapeptide ester, Z(OMe)-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Ser-Ala-Gln-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl, (2.42 g) was treated with TFA (4 ml) in the presence of anisole (2 ml) at 0° for 60 min, when dry ether was added. The resulting powder was dissolved in 0.5 N HCl-DMF (3.2 ml) and the solvent was evaporated *in vacuo*. The residue was solidified with ether. The hydrochloride thus obtained was again dissolved in a small amount of DMF and Et<sub>3</sub>N (0.2 ml) was added. Evaporation of the solvent was repeated and the residue was solidified again by treatment with ether. The deblocked peptide ester (II) was collected by filtration, washed with ether and then dissolved in DMF (20 ml). To this solution, Z(OMe)-Lys(Z)-Lys(Z)-Arg(NO<sub>2</sub>)-Arg(NO<sub>2</sub>)-Pro-OH (1.44 g), HOBT (0.33 g) and DCC (0.33 g) were combined and the mixture was stirred at room temperature for 48 hr. The solution, after filtration, was condensed *in vacuo*. Addition of AcOEt to the residue gave the solid, which was washed batchwisely with 5% citric acid, 5% Na<sub>2</sub>CO<sub>3</sub> and H<sub>2</sub>O and then dissolved in a small amount of the lower phase of CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O (8:3:1). The solution was applied to a column of silica (3 × 25 cm), which was eluted with the same solvent system. Fractions containing the substance of  $Rf_1$  0.60 were combined and the solvent was evaporated. Treatment of the residue with H<sub>2</sub>O gave the fine powder, which was precipitated from DMF with AcOEt; yield 2.63 g (87%), mp 173—175°,  $[\alpha]_D^{25} -25.7^\circ$  ( $c=1.0$ , DMF).  $Rf_1$  0.60. Amino acid ratios in an acid hydrolysate: Lys 3.22, Pro 2.84, Val 1.96, Tyr 0.49, Asp 2.11, Gly 1.05, Ala 3.00, Glu 3.90, Ser 0.75, Phe 1.85, Leu 1.00 (average recovery 89%). *Anal.* Calcd. for C<sub>198</sub>H<sub>256</sub>O<sub>51</sub>N<sub>38</sub> · 5H<sub>2</sub>O: C, 58.37; H, 6.58; N, 13.07. Found: C, 58.38; H, 6.50; N, 13.12.

**Z(OMe)-Lys(Z)-Pro-Val-Gly-Lys(Z)-Lys(Z)-Arg(NO<sub>2</sub>)-Arg(NO<sub>2</sub>)-Pro-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Ser-Ala-Gln-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl, Z(OMe)-(bovine ACTH 11—39)-OBzl**—The above protected pentacosapeptide ester, Z(OMe)-(bovine ACTH 15—39)-OBzl, (0.96 g) was treated with TFA (3 ml) in the presence of anisole (1 ml) in the usual manner. The TFA salt precipitated by addition of dry ether was converted, through the corresponding hydrochloride, to the free base as stated above using 0.5 N HCl-DMF (0.5 ml) and Et<sub>3</sub>N (0.07 ml). This was then dissolved in DMF (15 ml), to which Z(OMe)Lys(Z)-Pro-Val-Gly-OH (0.27 g) HOBT (0.1 g) and DCC (0.11 g) were added. The solution, after stirring at room temperature for 48 hr, was evaporated. Treatment of the residue with AcOEt gave the solid, which was washed batchwisely with base and acid as mentioned above and then dissolved in a small amount of the lower phase of CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O (8:3:1). The solution was applied to a column of silica (3 × 15 cm), which was eluted with the same solvent system. The product was isolated as stated above; yield 0.98 g (90%), mp 174—176°,  $[\alpha]_D^{25} -20.0^\circ$  ( $c=1.0$ , DMF).  $Rf_1$  0.56. Amino acid ratios in an acid hydrolysate: Lys 4.27, Pro 3.80, Val 3.07, Gly 2.06, Tyr 0.52, Asp 2.31, Ala 3.00, Glu 4.05, Ser 0.82, Phe 1.97, Leu 0.91 (average recovery 92%). *Anal.* Calcd. for C<sub>224</sub>H<sub>293</sub>O<sub>57</sub>N<sub>43</sub> · 8H<sub>2</sub>O: C, 57.93; H, 6.71; N, 12.97. Found: C, 57.93; H, 6.48; N, 13.30.

**Z-Glu(OBu<sup>t</sup>)-His-Phe-Arg(Tos)-Trp-Gly-OH**—To a solution of Z-Glu(OBu<sup>t</sup>)-His-NHNH<sub>2</sub><sup>22)</sup> (2.39 g) dissolved in DMF (20 ml), 4 N HCl-DMF (3.75 ml) and isoamylnitrite (0.67 ml) were added under cooling with ice-NaCl. After stirring for 5 min, Et<sub>3</sub>N (2.1 ml) was added. This neutralized solution was then combined with a solution of H-Phe-Arg(Tos)-Trp-Gly-OH (5.80 g) and Et<sub>3</sub>N (1.26 ml) in DMF (35 ml) and the mixture was stirred at 4° for 72 hr. After addition of a few drops of AcOH, the solvent was evaporated and the residue was treated with AcOEt. The resulting powder was washed with 3% AcOH and H<sub>2</sub>O and then precipitated from tetrahydrofuran with AcOEt; yield 4.70 g (86%), mp 155—158°,  $[\alpha]_D^{25} -6.9^\circ$  ( $c=0.5$ , DMF).  $Rf_1$  0.46. *Anal.* Calcd. for C<sub>58</sub>H<sub>80</sub>O<sub>13</sub>N<sub>12</sub>S · 2H<sub>2</sub>O: C, 57.51; H, 6.16; N, 13.88. Found: C, 57.40; H, 6.06; N, 14.09.

22) R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, **44**, 1991 (1961); H. Watanabe, H. Ogawa, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **23**, 375 (1975).

**Z-Ser-Tyr-Ser-Met-Glu(OBu<sup>t</sup>)-His-Phe-Arg(Tos)-Trp-Gly-OH(V)**—Z-Glu(OBu<sup>t</sup>)-His-Phe-Arg(Tos)-Trp-Gly-OH (3.60 g) dissolved in a mixture of tetrahydrofuran (15 ml) and AcOH (50 ml) was hydrogenated over a Pd catalyst in the usual manner. The solution was filtered, the filtrate was condensed and the residue was treated with ether to form a fine powder; yield 3.0 g (96%). This powder (1.87 g) was dissolved in DMF (8 ml) containing Et<sub>3</sub>N (0.8 ml). This solution was then combined with a solution of the azide (prepared from 1.20 g of Z-Ser-Tyr-Ser-Met-NHNH<sub>2</sub> with 2 ml of 2.5 N HCl-DMF, 0.33 ml of isoamyl nitrite and 0.7 ml of Et<sub>3</sub>N) in DMF (10 ml) and the mixture was stirred at 4° for 72 hr. After evaporation of the solvent, the residue was treated with 2% AcOH and the resulting powder was precipitated from MeOH with AcOEt and then from DMF with AcOEt; yield 2.10 g (68%), mp 157–161°,  $[\alpha]_D^{25} -11.8^\circ$  ( $c=0.6$ , DMF).  $R_f$  0.77. Anal. Calcd. for C<sub>78</sub>H<sub>98</sub>O<sub>20</sub>N<sub>16</sub>S<sub>2</sub> · 2H<sub>2</sub>O: C, 55.77; H, 6.12; N, 13.34. Found: C, 55.90; H, 5.92; N, 13.24.

**Z-Ser-Tyr-Ser-Met-Glu(OBu<sup>t</sup>)-His-Phe-Arg(Tos)-Trp-Gly-Lys(Z)-Pro-Val-Gly-Lys(Z)-Lys(Z)-Arg(NO<sub>2</sub>)-Arg(NO<sub>2</sub>)-Pro-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Ser-Ala-Gln-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl, Z-(bovine ACTH 1–39)-OBzl**—The above protected nonacosapeptide ester, Z(OMe)-(bovine ACTH 11–39)-OBzl, (0.98 g) was treated with TFA (4 ml) in the presence of anisole (1 ml) in an ice-bath for 60 min. The TFA salt obtained by addition of dry ether, was converted, through the corresponding hydrochloride, to the free base as stated above. This was then dissolved in DMF (15 ml), to which Z-Ser-Tyr-Ser-Met-Glu(OBu<sup>t</sup>)-His-Phe-Arg(Tos)-Trp-Gly-OH (0.48 g), HOBT (0.08 g) and DCC (0.11 g) were added. The solution, after stirring at room temperature for 48 hr, was condensed *in vacuo* and the residue was treated with AcOEt. The resulting powder was washed batchwisely with acid and base as stated above and then dissolved in a mixture of DMF–MeOH–2 N AcOH (5:4:1) (10 ml). The solution, after heating at 65° for 4 hr, was condensed *in vacuo* and the residue was treated with AcOEt. The resulting powder was then dissolved in a small amount of the lower phase of CHCl<sub>3</sub>–MeOH–H<sub>2</sub>O (8:3:1) and the solution was applied to a column of silica (3 × 15 cm), which was eluted with the same solvent system. The desired product was isolated as stated above and finally precipitated from DMF with AcOEt; yield 1.01 g (76%), mp 182–185°,  $[\alpha]_D^{25} -24.8^\circ$  ( $c=0.1$ , DMF).  $R_f$  0.58. Amino acid ratios in a 3 N Tos-OH hydrolysate: Ser 2.47, Tyr 1.59, Met 0.79, Glu 4.60, His 0.73, Phe 3.01, Arg + Arg(Tos) 3.15, Trp 0.78, Gly 2.95, Lys 4.28, Pro 3.86, Val 2.98, Asp 2.24, Ala 2.95, Leu 1.00 (average recovery 97%). Anal. Calcd. for C<sub>293</sub>H<sub>381</sub>O<sub>73</sub>N<sub>49</sub>S<sub>2</sub> · 10H<sub>2</sub>O: C, 57.30; H, 6.58; N, 13.46. Found: C, 57.31; H, 6.45; N, 13.57.

**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-Gln-Ala-Phe-Pro-Leu-Glu-Phe-OH, bovine ACTH**—The above protected nonatriacontapeptide ester, Z(OMe)-(bovine ACTH 1–39)-OBzl, (147 mg) was treated with HF (approximately 5 ml) in the presence of anisole containing 2% ethanedithiol (1 ml) at –5° for 60 min. The excess HF was removed by evaporation and the residue was dissolved in ice cold H<sub>2</sub>O (5 ml), which was treated with Amberlite IR-4B (acetate form, approximately 3 g) for 30 min. The resin was removed by filtration, the filtrate was washed with ether and then lyophilized. The residue was dissolved in MeOH–2 N AcOH (4:1, v/v, 50 ml) and the solution, after addition of dithiothreitol (30 mg), was incubated at 60° for 8 hr. The solvent was evaporated, the residue was dissolved in a small amount of 3% AcOH and the solution was applied to a column of Sephadex G-25 (2.6 × 60 cm), which was eluted with 3% AcOH. Individual fractions (3.6 ml each) were collected and absorbancy at 280 mμ was determined. The fractions corresponding to the front peak (tube No 30–50) were combined and the solvent was removed by lyophilization to give a white fluffy powder; yield 102 mg (deblocking step 93%). The product was dissolved in a small amount of H<sub>2</sub>O and the solution was applied to a column of CM-Sephadex (2.3 × 1.5 cm), which was eluted first with H<sub>2</sub>O (50 ml) and then with pH 6.9, 0.3 M NH<sub>4</sub>OAc buffer, through a mixing flask containing H<sub>2</sub>O (100 ml). Individual fractions (4 ml each) were collected and absorbancy at 280 mμ was determined. A main peak present in the gradient eluates (tube No 34–46) were combined and the solvent was condensed to approximately 10 ml. This solution was then applied to a column of Sephadex G-25 (2.6 × 60 cm), which was eluted with 3% AcOH. The desired fractions were collected and the solvent was removed by lyophilization to give a fluffy powder; yield 23 mg (over all yield 21%);  $[\alpha]_D^{25} -77.7^\circ$  ( $c=0.3$ , 1% AcOH).  $R_f$  0.46,  $R_f$  0.75. Amino acid ratios in 3 N Tos-OH hydrolysate: Ser 2.56, Tyr 1.84, Met 0.67, Glu 5.37, His 0.73, Phe 2.61, Arg 2.77, Trp 0.84, Gly 2.72, Lys 4.04, Pro 3.85, Val 3.06, Asp 2.07, Ala 3.35, Leu 1.00 (average recovery 90%). Amino acid ratios in AP-M digest (peptide 1 μmol/AP-M 3U, theory is given in parenthesis): Ser + Asn 3.30 (4 Calcd. as Ser), Tyr 2.26 (2), Met 0.59 (1), Glu 3.41 (4), His 0.78 (1), Phe 3.36 (3), Arg 2.50 (3), Trp 0.94 (1), Gly 3.01 (3), Lys 3.31 (4), Pro 3.48 (4), Val 2.88 (3), Ala 3.05 (3), Asp 0.60 (1), Gln 0.61 (1), Leu 1.00 (1) (average recovery 80%). Disc Electrophoretic mobility on 15% polyacrylamide gel (0.5 × 6.0 cm, 5 mA/tube) at pH 8.3 (0.38 M glycine-tris buffer) was 1.8 cm after 55 min from the origin to the anode and at pH 4.0 (0.3 M glycine-AcOH buffer) was 2.8 cm after 130 min to the cathode. Anal. Calcd. for C<sub>207</sub>H<sub>309</sub>O<sub>57</sub>N<sub>37</sub>S · 7CH<sub>3</sub>COOH · 10H<sub>2</sub>O: C, 50.74; H, 7.07; N, 15.26. Found: C, 51.09; H, 6.77; N, 14.78.