Synthesis of Corticotropin Peptides. XIV. The Synthesis of Two Octadecapeptides Corresponding to the Amino Acid Sequence 22—39 of Porcine and Human Corticotropins*

Kunio WATANABE and Ken INOUYE

Shionogi Research Laboratory, Shionogi & Co., Ltd., Fukushima-ku, Osaka 553

(Received June 29, 1976)

The syntheses are described of two octadecapeptides, H-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Leu-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe-OH (Ip) and H-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe-OH (Ih), corresponding to a tryptic fragment (positions 22—39) of porcine corticotropin (α_p-ACTH) and that of human hormone (α_h-ACTH), respectively. The porcine peptide and the corresponding human peptide are prepared simultaneously by an identical synthetic procedure except for the introduction of amino acid residue in position 31. Synthetic peptide Ip is compared with the authentic α_p-ACTH (22—39), which has been isolated from a tryptic hydrolysate of natural α_p-ACTH, in terms of chemical and physicochemical properties to establish their identity. These data as well as those obtained with human peptide Ih prove the satisfactory synthesis of the two octadecapeptides.

In corticotropin (ACTH) lysine 21 is the only basic amino acid residue in the C-terminal half of the hormone molecule (Fig. 1). Thus, the tryptic hydrolysis of ACTH yields a peptide fragment comprising the amino acid residues 22—39 of the hormone;[7] H-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Leu-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe-OH (Ip) from porcine ACTH (α_p-ACTH)[8] and H-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe-OH (Ih) from human ACTH (α_h-ACTH).[8] The present paper describes the synthesis of octadecapeptides Ip and Ih, which has been performed as a step of our total synthesis of α_p-ACTH and α_h-ACTH.[9] The porcine peptide and the corresponding human peptide were prepared in parallel and there was employed an identical synthetic procedure except for the introduction of the amino acid residue in position 31, which makes the only structural difference between the two mammalian hormones.

	1	2	3	4	5	6	7	8
α_p-ACTH:	H-Ser-	Tyr-	Ser-	Met-	Glu-	His-	Phe-	Arg-
α_h-ACTH:	H-Ser-	Tyr-	Ser-	Met-	Glu-	His-	Phe-	Arg-

	9	10	11	12	13	14	15	16	17
α_p-ACTH:	Trp-	Gly-	Lys-	Pro-	Val-	Gly-	Lys-	Lys-	Arg-
α_h-ACTH:	Trp-	Gly-	Lys-	Pro-	Val-	Gly-	Lys-	Lys-	Arg-

	18	19	20	21	22	23	24	25	26
α_p-ACTH:	Arg-	Pro-	Val-	Lys-	Val-	Tyr-	Pro-	Asn-	Gly-
α_h-ACTH:	Arg-	Pro-	Val-	Lys-	Val-	Tyr-	Pro-	Asn-	Gly-

	27	28	29	30	31	32	33	34	35
α_p-ACTH:	Ala-	Glu-	Asp-	Glu-	**Leu**-	Ala-	Glu-	Ala-	Phe-
α_h-ACTH:	Ala-	Glu-	Asp-	Glu-	**Ser**-	Ala-	Glu-	Ala-	Phe-

	36	37	38	39
α_p-ACTH:	Pro-	Leu-	Glu-	Phe-OH
α_h-ACTH:	Pro-	Leu-	Glu-	Phe-OH

Fig. 1. Primary structures of porcine corticotropin (α_p-ACTH)[1-4] and human corticotropin (α_h-ACTH).[4-6]

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

Z-Ala-Glu(OBu^t)-Ala-Phe-Pro-Leu-Glu(OBu^t)-Phe-OBu^t (VIII)[10]

↓ H_2/Pd

↓ Z-Leu-ONp or Z-Ser-N_3

Z-R-Ala-Glu(OBu^t)-Ala-Phe-Pro-Leu-Glu(OBu^t)-Phe-OBu^t (IXp: R=Leu, IXh: R=Ser)

↓ H_2/Pd

↓ Z-Glu(OBu^t)-ONp

Z-Glu(OBu^t)-R-Ala-Glu(OBu^t)-Ala-Phe-Pro-Leu-Glu(OBu^t)-Phe-OBu^t (Xp: R=Leu, Xh: R=Ser)

↓ H_2/Pd

↓ Z-Asp(OBu^t)-ONp

Z-Asp(OBu^t)-Glu(OBu^t)-R-Ala-Glu(OBu^t)-Ala-Phe-Pro-Leu-Glu(OBu^t)-Phe-OBu^t (XIp: R=Leu, XIh: R=Ser)

↓ H_2/Pd

↓ Z-Glu(OBu^t)-ONp

Z-Glu(OBu^t)-Asp(OBu^t)-Glu(OBu^t)-R-Ala-Glu(OBu^t)-Ala-Phe-Pro-Leu-Glu(OBu^t)-Phe-OBu^t (XIIp: R=Leu, XIIh: R=Ser)

Fig. 2. Synthesis of amino acid sequence 28—39 of α_p-ACTH and α_h-ACTH.

Protected dodecapeptides XIIp and XIIh, corresponding to positions 28—39 of α_p-ACTH and α_h-ACTH, respectively, were synthesized as shown in Fig. 2, in which the carboxyl groups were protected as their *t*-butyl esters and the α-amino function was temporarily blocked by a benzyloxycarbonyl group in combination with the subsequent deblocking by catalytic hydrogenolysis. The coupling reactions to lead to the production of porcine peptide XIIp were carried out in a step-by-step manner by the *p*-nitrophenyl ester method throughout, following the strategy employed by Schwyzer and Sieber[10] in their synthesis of the originally proposed amino acid sequence of porcine ACTH.[1,2] This was also applied to our present synthesis of human peptide XIIh with an exception of serine 31 which was introduc-

ed by the azide procedure rather than the active ester method. Among the intermediate compounds involved in the synthesis of XIIp and XIIh, compounds II, III, IV and VII (for structures see Experimental) were obtained in crystalline form. Compounds VIII and IXh separated out of the medium during the coupling reaction. They were amorphous but easily purified by reprecipitation. In the other cases the coupling product had to be chromatographed on a silica gel column for purification, in which methanol-chloroform systems were successfully employed as solvent.

A further extension of the peptide chain at the N-terminal of XIIh produced a tridecapeptide Z–Ala–Glu(OBu^t)–Asp(OBu^t)–Glu(OBu^t)–Ser–Ala–Glu(OBu^t)–Ala–Phe–Pro–Leu–Glu(OBu^t)–Phe–OBu^t, but its low solubility did not allow us to perform chromatographic purification. Therefore, we synthesized a hexapeptide derivative corresponding to the amino acid residues in positions 22—27 and combined it with dodecapeptides XIIp and XIIh to obtain the desired octadecapeptides. The synthesis of this hexapeptide, Z–Val–Tyr–Pro–Asn–Gly–Ala–$NHNH_2$ (XIX), was carried out as illustrated in Fig. 3. Starting with the dicyclohexylcarbodiimide (DCC)-mediated coupling of Z–Ala–OH with *t*-butyl carbazate, the step-by-step lengthening of peptide chain yielded a tetrapeptide *t*-butoxycarbonyl (Boc)-hydrazide (XVI). The benzyloxycarbonyl (Z) group of XVI was removed by catalytic hydrogenolysis and the resulting N^{α}-free compound was coupled with Z–Val–Tyr–N_3, derived from the corresponding hydrazide[11,12] by the treatment with an alkyl nitrite in an anhydrous acid solution,[13] to give a crystalline hexapeptide derivative (XVIII) in a moderate yield. The subsequent treatment

Z–Ala–OH
↓ Boc–$NHNH_2$, DCC
Z–Ala–NHNH–Boc (XIII)
↓ H_2/Pd
↓ Z–Gly–OH, DCC
Z–Gly–Ala–NHNH–Boc (XIV)
↓ H_2/Pd
↓ Z–Asn–ONp
Z–Asn–Gly–Ala–NHNH–Boc (XV)
↓ H_2/Pd
↓ Z–Pro–ONp
Z–Pro–Asn–Gly–Ala–NHNH–Boc (XVI)
↓ H_2/Pd
↓ Z–Val–Tyr–$NHNH_2$[11,12]
Z–Val–Tyr–Pro–Asn–Gly–Ala–NHNH–Boc (XVIII)
↓ HCl/AcOH (+anisole)
Z–Val–Tyr–Pro–Asn–Gly–Ala–$NHNH_2$·HCl (XIX)

XVI $\xrightarrow{\text{HCl/AcOH}}$ Z–Pro–Asn–Gly–Ala–$NHNH_2$·HCl (XVII)

Fig. 3. Synthesis of amino acid sequence 22—27 of ACTH.

of XVIII with hydrogen chloride in acetic acid to remove the Boc group gave the hydrazide hydrochloride (XIX).

Hydrazide XIX obtained above was treated with an alkyl nitrite,[13] and the resulting azide was allowed to react with the N^{α}-free dodecapeptides, derived from XIIp and XIIh by catalytic hydrogenolysis, to give the protected octadecapeptides (XXIp and XXIh). The partially purified preparations of XXIp and XXIh were submitted to catalytic hydrogenolysis and the products were purified on silica gel columns with ethyl acetate–acetic acid–water (4: 1: 1) as solvent to give the N^{α}-free octadecapeptides H–Val–Tyr–Pro–Asn–Gly–Ala–Glu(OBu^t)–Asp(OBu^t)–Glu(OBu^t)–Leu–Ala–Glu(OBu^t)–Ala–Phe–Pro–Leu–Glu(OBu^t)–Phe–OBu^t (XXIIp) and H–Val–Tyr–Pro–Asn–Gly–Ala–Glu(OBu^t)–Asp(OBu^t)–Glu(OBu^t)–Ser–Ala–Glu(OBu^t)–Ala–Phe–Pro–Leu–Glu(OBu^t)–Phe–OBu^t (XXIIh). Compounds XXIIp and XXIIh have proved to serve as intermediates for the total synthesis of α_p-ACTH and α_h-ACTH, respectively.[9]

Octadecapeptide XXIIh was also synthesized by an alternative route, in which dodecapeptide XIIh was acylated consecutively with Z–Pro–Asn–Gly–Ala–N_3, derived from XVII (Fig. 3), and Z–Val–Tyr–N_3 in combination with the removal of the N^{α}-Z group by catalytic hydrogenolysis. The azides were prepared from the corresponding hydrazides by means of the alkyl nitrite procedure.[13]

Partially protected peptides XXIIp and XXIIh obtained above were deprotected with trifluoroacetic acid in the presence of 2-mercaptoethanol followed by treatment with Amberlite CG-400 (acetate form) to liberate the free octadecapeptides α_p-ACTH(22—39) (Ip) and α_h-ACTH(22—39) (Ih), respectively. The crude preparations of Ip and Ih were purified by partition chromatography on a column of Sephadex LH-20 with 1-butanol–acetic acid–water (4: 1: 2) as solvent. The synthetic octadecapeptides thus obtained were found to be homogeneous in TLC and their acid hydrolysates contained the constituent amino acids in the ratios predicted by theory except for tyrosine, whose recovery was 70—80% as compared with the other amino acids.

In order to obtain the authentic sample of α_p-ACTH-(22—39), a purified preparation of natural α_h-ACTH[9] was submitted to tryptic hydrolysis at pH 8.2 and 37 °C for 60 min. The desired octadecapeptide was isolated from the hydrolysate by chromatography on a column of silica gel with ethyl acetate–acetic acid–water (4: 1: 1) as solvent. This was further purified by partition chromatography on a Sephadex LH-20 column with 1-butanol–acetic acid–water (4: 1: 2) as solvent. Thin-layer chromatography and amino acid analysis revealed the homogeneity of the preparation obtained above, although the tyrosine content was found to be considerably lower than the theoretical value. The low tyrosine content was also observed with the synthetic peptides as described above.[14] The synthetic porcine peptide (Ip) was found to be identical with this authentic octadecapeptide in TLC and in optical rotation within the precision of measurement. It is known that asparagine 25 is the only site of alkaline deamidation in the

ACTH molecule; the Asn–Gly bond (positions 25—26) being transformed into the Asp-α/β-Gly bond by way of the formation of a succinimide intermediate.[15] Upon treatment with 0.1 M ammonia at 37 °C overnight, the authentic α_p-ACTH(22—39) and synthetic peptide Ip yielded an identical product which was clearly distinguished from the intact peptide in TLC with 1-butanol–acetic acid–water (4: 1: 2) as solvent. Thus, the identity between the synthetic peptide and the authentic sample from natural origin has now been established on a sound basis. This would also prove the satisfactory synthesis of human peptide Ih, since peptide Ih was prepared in parallel with Ip using the identical synthetic procedure and conditions except for the introduction of serine 31 in place of leucine 31 in Ip.

Human peptide Ih was found to be more soluble in aqueous acetic acid than porcine peptide Ip and also found to be distinguishable from Ip in TLC with 1-butanol–acetic acid–water (4: 1: 2) as solvent (Ip, R_f= 0.27; Ih, R_f=0.22). These observations may be explained as a reflection of the structural difference between Ip and Ih.

Experimental

Thin-layer chromatography (TLC) was performed on silica gel plates (Kieselgel GF_{254} or precoated Kieselgel $60F_{254}$, Merck) with the following solvent systems: A, chloroform–methanol (8: 2); B, chloroform–methanol–acetic acid (95: 5: 3); C, chloroform–methanol–acetic acid (90: 10: 3); D, ethyl acetate–acetic acid–water (4: 1: 1); E, 1-butanol–acetic acid–water (4: 1: 2).

Z-Glu(OBu^t)-Phe-OBu^t (II). Z-Glu(OBu^t)-OH [derived from the dicyclohexylamine salt (14.3 g, 27.6 mmol) in the usual manner][16] and H-Phe-OBu^t (6.1 g, 27.6 mmol)[17] were coupled with dicyclohexylcarbodiimide (DCC; 5.7 g, 27.6 mmol) in ethyl acetate–dichloromethane (1: 1) to give II, which was crystallized from ether–petroleum ether; yield 12.4 g (83%), mp 132—134 °C, $[\alpha]_D^{22}$ −17.5±0.3° (*c* 2.0, methanol), −14.1±0.3° (*c* 2.0, 95% ethanol). TLC: a single component (sulfuric acid) in system B. Found: C, 66.43; H, 7.42; N, 5.41%. Lit, mp 131.5—132.5 °C, $[\alpha]_D$ −14.0±0.5° (*c* 2, 95% ethanol);[10] mp 131.0—131.5 °C, $[\alpha]_D^{22}$ −11.2° [*c* 0.99, *N,N*-dimethylformamide (DMF)].[18]

Z-Leu-Glu(OBu^t)-Phe-OBu^t (III). Compound II (11.5 g, 21.2 mmol) was hydrogenolyzed over palladium in methanol containing acetic acid to give the N^α-free dipeptide ester, which was coupled with Z-Leu-ONp (8.2 g, 21.2 mmol) in DMF. The product was crystallized from ethyl acetate–ether; yield 13.1 g (94%), mp 109—110 °C, $[\alpha]_D^{22}$ −25.5±0.4° (*c* 2.0, 95% ethanol). TLC: a single component (sulfuric acid) in system C. Found: C, 65.98; H, 7.82; N, 6.52%. Lit, mp 116—117.5°C, $[\alpha]_D$ −26.3±0.6° (*c* 2.0, 95% ethanol);[10] mp 113—115 °C, $[\alpha]_D^{22}$ −14.3° (*c* 1.04, DMF).[18]

Z-Pro-Leu-Glu(OBu^t)-Phe-OBu^t (IV). Compound III (7.60 g, 11.6 mmol) was hydrogenolyzed to give the N^α-free tripeptide ester, which was coupled with Z-Pro-ONp (4.30 g, 11.6 mmol) in DMF. The product was crystallized from ether; yield 8.00 g (92%), mp 160—161°C, $[\alpha]_D^{23}$ −58.7±0.5° (*c* 2.0, 95% ethanol). TLC: a single component (sulfuric acid) in system C. Found: C, 65.68; H, 7.79; N, 7.38%. Lit, mp 152—153 °C, $[\alpha]_D$ −56.8±0.6° (*c* 2, 95% ethanol).[10]

Z-Phe-Pro-Leu-Glu(OBu^t)-Phe-OBu^t (V). Compound IV (12.4 g, 16.5 mmol) was hydrogenolyzed to give the N^α-free tetrapeptide ester, which was coupled with Z-Phe-ONp (6.94 g, 16.5 mmol) in DMF. The crude product was purified on a column of silica gel (100 g, Kieselgel H, Merck) with 2% methanol in chloroform as solvent; yield 14.75 g (97.5%), $[\alpha]_D^{23}$ −54.8±1.0° (*c* 1.0, methanol). TLC: a single component (sulfuric acid) in system C. Found: C, 66.76; H, 7.53; N, 7.87%. Lit, $[\alpha]_D$ not given.[10]

Z-Ala-Phe-Pro-Leu-Glu(OBu^t)-Phe-OBu^t (VI). Compound V (14.1 g, 15.7 mmol) was hydrogenolyzed to give the N^α-free pentapeptide ester, which was coupled with Z-Ala-ONp (5.40 g, 15.7 mmol) in DMF. The crude product was purified on a silica gel column with 1% methanol in chloroform as solvent; yield 11.9 g (77%), $[\alpha]_D^{22}$ −67.9±1.1° (*c* 1.0, methanol). TLC: a single component (sulfuric acid) in system C. Found: C, 64.74; H, 7.55; N, 8.60%. Lit, $[\alpha]_D$ not given;[10] $[\alpha]_D^{22}$ −38.6° (*c* 1.01, DMF).[18]

Z-Glu(OBu^t)-Ala-Phe-Pro-Leu-Glu(OBu^t)-Phe-OBu^t (VII). Compound VI (5.80 g, 5.9 mmol) was hydrogenolyzed to give the N^α-free hexapeptide ester, which was coupled with Z-Glu(OBu^t)-ONp (2.74 g, 5.9 mmol) in DMF. The product was crystallized from ethyl acetate–ether; yield 5.30 g (78%), mp 178—180 °C, $[\alpha]_D^{24}$ −64.8±1.1° (*c* 1.0 methanol). TLC: a single component (sulfuric acid) in system C. Found: C, 64.01; H, 7.73; N, 8.51%. Lit, mp 172—173 °C, $[\alpha]_D$ not given;[10] mp 168—170 °C decomp., $[\alpha]_D^{21}$ −34.4° (*c* 1.03, DMF).[18]

Z-Ala-Glu(OBu^t)-Ala-Phe-Pro-Leu-Glu(OBu^t)-Phe-OBu^t (VIII). Compound VII (4.0 g, 3.64 mmol) was hydrogenolyzed to give the N^α-free heptapeptide ester, which was coupled with Z-Ala-ONp (1.25 g, 3.64 mmol) in ethyl acetate. The product which had separated was filtered off and reprecipitated from ethyl acetate–ether; yield 4.02 g (90%), mp 145—147 °C, $[\alpha]_D^{24}$ −63.8±1.0° (*c* 1.0, methanol). TLC: a single component (sulfuric acid) in system C. Found: C, 62.82; H, 7.68; N, 9.30%. Lit, no data given;[10] mp 185—186 °C decomp., $[\alpha]_D^{23}$ −34.9° (*c* 1.05, DMF).[18]

Z-Leu-Ala-Glu(OBu^t)-Ala-Phe-Pro-Leu-Glu(OBu^t)-Phe-OBu^t (IXp). Compound VIII (2.45 g, 2.0 mmol) was hydrogenolyzed to give the N^α-free octapeptide ester, which was coupled with Z-Leu-ONp (0.85 g, 3.3 mmol) in DMF–ethyl acetate (1: 2). The crude product was purified on a silica gel column (70 g) with 3% methanol in chloroform as solvent; yield 2.40 g (90%), mp 125—130 °C, $[\alpha]_D^{23}$ −60.0 ±1.0° (*c* 1.0, methanol). TLC: a single component (sulfuric acid) in system C. Found: C, 63.17; H, 7.73; N, 9.29%. Lit, no data given.[10]

Z-Ser-Ala-Glu(OBu^t)-Ala-Phe-Pro-Leu-Glu(OBu^t)-Phe-OBu^t (IXh). The N^α-octapeptide ester, derived from VIII (2.65 g, 2.2 mmol) as above, and Z-Ser-N_3 [derived from the corresponding hydrazide (0.84 g, 3.3 mmol) by the treatment with nitrous acid in the usual manner] were coupled in ethyl acetate at 4 °C overnight. The product which had separated was filtered off and reprecipitated from ethyl acetate–ether; yield 2.60 g (90%), mp 187—189 °C, $[\alpha]_D^{24}$ −55.9 ±1.0° (*c* 1.0, methanol). TLC: a single component (sulfuric acid) in system C. Found: C, 61.99; H, 7.63; N, 9.45%. Lit, no data given;[15] mp 181—183 °C decomp., $[\alpha]_D^{23}$ −32.9° (*c* 1.04, DMF).[18]

Z-Glu(OBu^t)-Leu-Ala-Glu(OBu^t)-Ala-Phe-Pro-Leu-Glu(OBu^t)-Phe-OBu^t (Xp). Compound IXp (2.37 g, 1.8 mmol) was hydrogenolyzed over palladium in acetic acid to give the N^α-free nonapeptide ester. This was then coupled with Z-Glu(OBu^t)-ONp (0.82 g, 1.8 mmol) in DMF in the presence of triethylamine (0.25 ml, 1.8 mmol) at 4 °C overnight. The crude product was purified on a silica gel column (70 g) with 3% methanol in chloroform as solvent; yield 2.32 g (86%), mp 185—190 °C, $[\alpha]_D^{25}$ −57.4±0.9° (*c* 1.0, methanol).

TLC: single component (sulfuric acid) in system C.

Found: C, 62.71; H, 7.79; N, 9.03%. Calcd for $C_{80}H_{118}N_{10}O_{19}$: C, 63.05; H, 7.81; N, 9.19%.

Z-Glu(OBu^t)-Ser-Ala-Glu(OBu^t)-Ala-Phe-Pro-Leu-Glu(OBu^t)-Phe-OBu^t (Xh). Compound IXh (2.62 g, 2.0 mmol) was hydrogenolyzed to give the N^α-free nonapeptide ester, which was coupled with Z-Glu(OBu^t)-ONp (0.92 g, 2.0 mmol) in DMF. The crude product was purified on a silica gel column with 3—5% methanol in chloroform as solvent; yield 2.60 g (90%), mp 135—137 °C, $[\alpha]_D^{24}$ −48.9 ±0.9° (*c* 1.0, methanol). TLC: a single component (sulfuric acid) in system C. Found: C, 61.20; H, 7.48; N, 9.45%. Lit, no data given;[15] mp 189—190 °C decomp., $[\alpha]_D^{23}$ −29.7° (*c* 0.98, DMF).[18]

Z-Asp(OBu^t)-Glu(OBu^t)-Leu-Ala-Glu(OBu^t)-Ala-Phe-Pro-Leu-Glu(OBu^t)-Phe-OBu^t (XIp). Compound Xp (2.00 g, 1.32 mmol) was hydrogenolyzed over palladium in acetic acid to give the N^α-free decapeptide ester. This was then coupled with Z-Asp(OBu^t)-ONp (0.59 g, 1.32 mmol) in DMF in the presence of triethylamine (0.20 ml, 1.43 mmol) at 4 °C for 2.5 days. The crude product was purified on a silica gel column (70 g) with 3% methanol in chloroform as solvent; yield 2.10 g (94%), mp 224—225 °C decomp., $[\alpha]_D^{26}$ −48.9±0.9° (*c* 1.0, methanol).

Found: C, 61.91; H, 7.67; N, 8.84%. Calcd for $C_{83}H_{131}H_{11}O_{22}$: C, 62.35; H, 7.79; N, 9.09%.

Z-Asp(OBu^t)-Glu(OBu^t)-Ser-Ala-Glu(OBu^t)-Ala-Phe-Pro-Leu-Glu(OBu^t)-Phe-OBu^t (XIh). Compound Xh (2.80 g, 1.87 mmol) was hydrogenolyzed to give the N^α-free decapeptide ester, which was coupled with Z-Asp(OBu^t)-ONp (0.84 g, 1.87 mmol) in DMF. The crude product was purified on a silica gel column with 5% methanol in chloroform as solvent; yield 2.98 g (96%), mp 193—195 °C decomp., $[\alpha]_D^{28}$ −48.1±0.5° (*c* 1.0, methanol). TLC: a single component (sulfuric acid) in system C. Found: C, 60.80; H, 7.65; N, 8.94%. Lit, no data given;[15] mp 194—195 °C decomp., $[\alpha]_D^{28}$ −29.8° (*c* 1.05, DMF).[18]

Z-Glu(OBu^t)-Asp(OBu^t)-Glu(OBu^t)-Leu-Ala-Glu(OBu^t)-Ala-Phe-Pro-Leu-Glu(OBu^t)-Phe-OBu^t (XIIp). Compound XIp (2.00 g, 1.18 mmol) was hydrogenolyzed over palladium in acetic acid to give the N^α-free undecapeptide ester. This was then coupled with Z-Glu(OBu^t)-ONp (0.55 g, 1.20 mmol) in DMF in the presence of triethylamine (0.20 ml, 1.43 mmol) at 4 °C for 2.5 days. The crude product was purified on a silica gel column (50 g) with 3% methanol in chloroform as solvent; yield 2.10 g (95%), mp 230 °C decomp., $[\alpha]_D^{26}$ −41.2±0.8° (*c* 1.0, methanol). Amino acid ratios in acid hydrolysate (theoretical values are given in parentheses): Asp 0.92 (1), Glu 3.89 (4), Pro 0.92 (1), Ala 1.95 (2), Leu 2.00 (2), Phe 1.82 (2).

Found: C, 61.55; H, 7.82; N, 8.91%. Calcd for $C_{97}H_{146}N_{12}O_{25}$: C, 61.96; H, 7.83; N, 8.94%.

Z-Glu(OBu^t)-Asp(OBu^t)-Glu(OBu^t)-Ser-Ala-Glu(OBu^t)-Ala-Phe-Pro-Leu-Glu(OBu^t)-Phe-OBu^t (XIIh). Compound XIh (2.10 g, 1.26 mmol) was hydrogenolyzed to give the N^α-free undecapeptide ester, which was coupled with Z-Glu(OBu^t)-ONp (0.58 g, 1.27 mmol) in DMF. The crude product was purified on a silica gel column with 4% methanol in chloroform as solvent; yield 2.10 g (94%), mp 220—221 °C decomp., $[\alpha]_D^{24.5}$ −26.9±0.7° (*c* 1.0, DMF). TLC: a single component (sulfuric acid) in system C. Amino acid ratios in acid hydrolysate: Asp 1.00 (1), Ser 0.91 (1), Glu 4.11 (4), Pro 1.07 (1), Ala 2.04 (2), Leu 1.00 (1), Phe 1.89 (2). Found: C, 60.77; H, 7.55; N, 8.97%. Lit, no data given;[15] mp 215—216 °C decomp., $[\alpha]_D^{23}$ −27.2° (*c* 1.07, DMF).[18]

Z-Ala-NHNH-Boc (XIII). Z-Ala-OH (11.15 g, 50 mmol) and *t*-butyl carbazate (6.60 g, 50 mmol) were dissolved in ethyl acetate (100 ml) and to this was added DCC (10.35 g, 50 mmol) at 0 °C with ethyl acetate as solvent. The mixture was stirred at 4 °C overnight followed by evaporation *in vacuo*. The residue was submitted to a silica gel column (150 g) with 2% methanol in chloroform as solvent. The fractions (17 g/tube) were examined by TLC in system C and those containing the desired compound as a single component (tubes 39—70) were combined and evaporated *in vacuo* to give a residue which was crystallized from ether-petroleum ether; yield 15.9 g (94%), mp 93—95 °C, $[\alpha]_D^{28}$ −48.2±0.9° (*c* 1.0, methanol).

Found: C, 55.84; H, 6.60; N, 12.34%. Calcd for $C_{16}H_{23}N_3O_5$: C, 56.96; H, 6.87; N, 12.46%.

Z-Gly-Ala-NHNH-Boc (XIV). Compound XIII (3.37 g, 10 mmol) was hydrogenolyzed over palladium in methanol. The resulting N^α-free compound and Z-Gly-OH (2.09 g, 10 mmol) were coupled with DCC (2.06 g, 10 mmol) in ethyl acetate (40 ml) at 4 °C overnight. The reaction mixture was worked up in the usual manner to isolate the crystalline product, which was recrystallized from aqueous methanol; yield 4.10 g (99%), mp 110—112 °C, $[\alpha]_D^{28}$ −45.8 ±0.9° (*c* 1.0, methanol).

Found: C, 52.60; H, 7.00; N, 13.38%. Calcd for $C_{18}H_{26}N_4O_6\cdot H_2O$: C, 52.42; H, 6.84; N, 13.59%.

Z-Asn-Gly-Ala-NHNH-Boc (XV). Compound XIV (3.94 g, 9.6 mmol) was hydrogenolyzed over palladium in methanol. The resulting N^α-free dipeptide derivative was then coupled with Z-Asn-ONp (3.87 g, 10 mmol) in DMF (10 ml) at 4 °C overnight. The product isolated as amorphous solid was reprecipitated from methanol-ether; yield 4.50 g (92%), mp 146—149 °C decomp., $[\alpha]_D^{28}$ −24.6±0.7° (*c* 1.0, methanol).

Found: C, 52.13; H, 6.52; N, 15.89%. Calcd for $C_{22}H_{32}N_6O_8$: C, 51.96; H, 6.34; N, 16.53%.

Z-Pro-Asn-Gly-Ala-NHNH-Boc (XVI). Compound XV (4.30 g, 8.5 mmol) was hydrogenolyzed over palladium in methanol containing acetic acid to give the N^α-free tripeptide derivative, which was coupled with Z-Pro-ONp (3.13 g, 8.5 mmol) in DMF. The product, dissolved in water-saturated 1-butanol-ethyl acetate (1: 1), was washed with 1M acetic acid followed by evaporation *in vacuo*. The residue was precipitated from ethyl acetate-ether (2: 1) and then from methanol-ethyl acetate (1: 3) to give XVI in pure form; yield 4.40 g (83%), mp 145—147 °C, $[\alpha]_D^{24.5}$ −68.1±1.1° (*c* 1.0, methanol). TLC: a single component (sufuric acid) in system A.

Found: C, 51.52; H, 6.63; N, 15.53%. Calcd for $C_{27}H_{39}N_7O_9\cdot H_2O$: C, 52.00; H, 6.63; N, 15.72%.

Z-Pro-Asn-Gly-Ala-NHNH$_2$·HCl (XVII). Compound XVI (4.70 g) was treated with 1M hydrogen chloride in acetic acid (50 ml) at room temperature for 60 min followed by evaporation *in vacuo*. The residue was triturated with ethyl acetate-ether (1: 1) and the resulting precipitates were filtered off and reprecipitated from ethanol (30 ml) two times; yield 2.75 g (65%), mp 145—147 °C decomp., $[\alpha]_D^{24}$ −63.9 ±1.5° (*c* 0.7, methanol).

Found: C, 47.83; H, 6.15; N, 17.28; Cl, 5.71%. Calcd for $C_{22}H_{31}N_7O_7\cdot HCl\cdot H_2O$: C, 47.18; H, 6.12; N, 17.51; Cl, 6.33%.

Z-Val-Tyr-Pro-Asn-Gly-Ala-NHNH-Boc (XVIII). Compound XVI (2.33 g, 3.7 mmol) was hydrogenolyzed over palladium in methanol to give H-Pro-Asn-Gly-Ala-NHNH-Boc (1.96 g) as amorphous solid.

A solution of Z-Val-Tyr-NHNH$_2$ (1.76 g, 4.1 mmol)[11,12] in DMF (18 ml) was chilled to −15—−20 °C and 3.66 M hydrogen chloride in dioxane (4.5 ml) was introduced. To this was added dropwise isopentyl nitrite (0.59 ml, 4.5 mmol)

and the mixture was stirred at the same temperature for 10 min. The resulting azide solution was chilled to −40——50 °C and there were added triethylamine (2.9 ml, 20.6 mmol) and the N^{α}-free tetrapeptide derivative obtained above with DMF (20 ml) as solvent. The bath was removed and the temperature was allowed to rise to 4 °C at which the reaction mixture was stirred for 20 h followed by evaporation *in vacuo*. The residue was shaken with a mixture of 1-butanol–ethyl acetate (1: 1, 20 ml) and 1M acetic acid (20 ml). The organic phase separated was further washed with 1M acetic acid three times followed by evaporation *in vacuo*. The residue was then chromatographed on a silica gel column (70 g) with 15% methanol in chloroform as solvent. The fractions (7 g/tube) were examined by TLC (system A) and those containing the desired compound as a single component were combined and evaporated *in vacuo* to give a residue which was crystallized from ethyl acetate; yield 1.8 g (56%), mp 166—167 °C decomp., $[\alpha]_D^{24}$ −66.4±1.0° (*c* 1.0, methanol).

Found: C, 55.89; H, 6.85; N, 14.34%. Calcd for $C_{41}H_{57}N_9O_{12}$: C, 56.74; H, 6.62; N, 14.53%.

Z-Val-Tyr-Pro-Asn-Gly-Ala-NHNH$_2$·HCl (XIX). Compound XVIII (1.80 g) was treated with 1M hydrogen chloride in acetic acid at room temperature for 60 min in the presence of anisole (0.2 ml) as scavenger. The solvent was evaporated *in vacuo* to give a residue which was triturated with ether; yield 1.70 g, mp 143—145 °C. Amino acid ratios in acid hydrolysate: NH_3 0.99 (1), Asp 1.04 (1), Pro 0.89 (1), Gly 1.04 (1), Ala 1.04 (1), Val 1.00 (1), Tyr 0.87 (1).

Z-Pro-Asn-Gly-Ala-Glu(OBu^t)-Asp(OBu^t)-Glu(OBu^t)-Ser-Ala-Glu(OBu^t)-Ala-Phe-Pro-Leu-Glu(OBu^t)-Phe-OBu^t (XX). Compound XIIh (1.20 g, 0.65 mmol) was hydrogenolyzed over palladium in acetic acid to give the N^{α}-free dodecapeptide ester. A solution of XVII (0.56 g, 1.0 mmol) in tetrahydrofuran containing 1M hydrochloric acid (1.5 ml) was chilled in an ice bath and 2 M sodium nitrite (0.55 ml) was added. The mixture was stirred at 0 °C for 4 min. To this were then introduced ice-cold ethyl acetate (20 ml) and ice-cold 50% potassium carbonate (8 ml). The organic phase separated was dried over magnesium sulfate at 0 °C and was combined with a DMF solution of the dodecapeptide ester obtained above. The mixture was concentrated *in vacuo* at a bath temperature of 5—10 °C to remove ethyl acetate and was stirred at 4 °C for 20 h. The product which was precipitated by the addition of ether and petroleum ether was lyophilized from acetic acid. The resulting soft powder was suspended in aqueous ethanol and the insoluble precipitates were filtered off (1.1 g). Reprecipitation from a mixture of methanol (40 ml) and water (20 ml) afforded hexadecapeptide XX; yield 0.88 g (62%), $[\alpha]_D^{24.5}$ −20.6±0.6° (*c* 1.0, DMF). TLC: almost homogeneous (ninhydrin, after pretreatment with hydrobromic acid) in systems A and E. Amino acid ratios in acid hydrolysate: NH_3 0.93 (1), Asp 1.94 (2), Ser 0.95 (1), Pro 1.96 (2), Glu 4.01 (4), Gly 0.98 (1), Ala 3.00 (3), Leu 1.07 (1), Phe 2.15 (2).

H-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBu^t)-Asp(OBu^t)-Glu(OBu^t)-Leu-Ala-Glu(OBu^t)-Ala-Phe-Pro-Leu-Glu(OBu^t)-Phe-OBu^t (XXIIp). Compound XIIp (0.98 g, 0.52 mmol) was hydrogenolyzed over palladium in acetic acid to give the N^{α}-free dodecapeptide ester.

A solution of XIX (0.45 g, 0.57 mmol) in DMF (5 ml) was chilled to −20——30 °C and 3.66 M hydrogen chloride in dioxane (0.47 ml) was introduced. To this was added dropwise isopentyl nitrite (0.082 ml, 0.63 mmol) and the mixture was stirred at the same temperature for 10 min. The azide solution thus obtained was chilled to −40——50 °C and triethylamine (0.4 ml, 2.87 mmol) was added. This was then combined with a DMF solution of the N^{α}-free dodecapeptide obtained above and the mixture was stirred for 24 h, while the temperature was allowed to rise to 4 °C. After the solvent had been removed by evaporation *in vacuo* the residue was triturated and washed with water and lyophilized from acetic acid. The resulting powder was suspended in 50% ethanol and the insoluble precipitates were filtered off, washed and dried to give the protected octadecapeptide (XXIp, 1.09 g, 84%), which was almost homogeneous in TLC in system A; mp 232—233 °C decomp.

Compound XXIp (1.40 g) prepared as described above was hydrogenolyzed over palladium in acetic acid. The resulting N^{α}-free peptide was purified on silica gel columns (150—250 g, Kieselgel 60, Merck) with ethyl acetate–acetic acid–water (4: 1: 1) as solvent. The resulting pure material was lyophilized from acetic acid; yield 1.09 g (67%), $[\alpha]_D^{23.5}$ −37.0 ±0.7° (*c* 1.0, acetic acid). TLC: a single component (ninhydrin) in system D. Amino acid ratios in acid hydrolysate: NH_3 0.90 (1), Asp 1.90 (2), Glu 3.95 (4), Pro 1.96 (2), Gly 1.04 (1), Ala 2.97 (3), Val 0.95 (1), Leu 2.00 (2), Tyr 0.86 (1), Phe 1.90 (2).

H-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBu^t)-Asp(OBu^t)-Glu(OBu^t)-Ser-Ala-Glu(OBu^t)-Ala-Phe-Pro-Leu-Glu(OBu^t)Phe-OBu^t (XXIIh). *a) By (22—23)+(24—39):* Compound XX (0.83 g, 0.38 mmol) was hydrogenolyzed over palladium in acetic acid to give the N^{α}-free hexadecapeptide ester. This was then coupled with Z-Val-Tyr-N_3 [derived from the corresponding hydrazide (0.21 g, 0.5 mmol)[11,12] by the treatment with nitrous acid in the usual manner] in DMF at 4 °C for 2.5 days. The product was repeatedly precipitated from ethanol–water to give a partially purified preparation of the protected octadecapeptide (XXIh, 0.77 g).

Compound XXIh obtained above was hydrogenolyzed over palladium in acetic acid and the resulting N^{α}-free peptide was purified on silica gel columns in the manner described above for XXIIp. The pure material was lyophilized from acetic acid; yield 0.39 g (43%). TLC: a single component (ninhydrin) in system D.

b) By (22—27)+(28—39). Compound XIIh (1.06 g, 0.57 mmol) was hydrogenolyzed over palladium in acetic acid to give the N^{α}-free dodecapeptide ester. This was then coupled with the acyl hexapeptide azide [derived from XIX (0.52 g, 0.65 mmol) in exactly the same manner as described above] in DMF in the presence of triethylamine (0.45 ml, 3.25 mmol) at 4 °C for 24 h. The crude product was lyophilized from acetic acid and the resulting powder was suspended in 50% ethanol and the insoluble material was collected to give XXIh (1.20 g, 87%), which was almost homogeneous in TLC in system A; mp 224—225 °C decomp.

Compound XXIh (2.20 g) prepared as described above was hydrogenolyzed over palladium in acetic acid. The resulting N^{α}-free peptide was purified on silica gel columns as described above; yield 1.71 g (70%), $[\alpha]_D^{23.5}$ −34.3±0.7° (*c* 1.0, acetic acid). TLC: a single component (ninhydrin) in system D. Amino acid ratios in acid hydrolysate: NH_3 0.91 (1), Asp 1.99 (2), Ser 0.87 (1), Glu 4.01 (4), Pro 1.78 (2), Gly 1.04 (1), Ala 3.09 (3), Val 0.99 (1), Leu 1.00 (1), Tyr 0.90 (1), Phe 1.98 (2).

H-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Leu-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe-OH (Ip). *a) Synthetic Preparation:* Compound XXIIp (100 mg) was dissolved in trifluoroacetic acid (1 ml) together with 2-mercaptoethanol (0.1 ml) and the mixture was kept at room temperature for 60 min. The precipitates which formed upon addition of ether were filtered off and dissolved in 3 M acetic acid (5 ml). The aqueous solution was passed through a column (0.9×7 cm) of Amberlite CG-400 (acetate form) with additional portions

of 3 M acetic acid and the eluates combined were lyophilized. The product was then submitted to partition chromatography on a column of Sephadex LH-20 (3.0×70 cm) with 1-butanol–acetic acid–water (4: 1: 2) as solvent. Five-ml fractions were collected and their absorption at 275 nm was measured. The fractions corresponding to a main peak (tubes 36—60) were pooled and evaporated *in vacuo* at a bath temperature of 45 °C. Lyophilization of the residue from acetic acid afforded Ip; yield 60 mg, $[\alpha]_D^{22}$ −41.6±2.0° (*c* 0.4, acetic acid) TLC: a single component (ninhydrin) in system E. Amino acid ratios in acid hydrolysate: NH_3 1.24 (1), Asp 2.00 (2), Glu 3.83 (4), Pro 2.09 (2), Gly 1.12 (1), Ala 2.99 (3), Val 0.93 (1), Leu 2.03 (2), Tyr 0.70 (1), Phe 2.00 (2).

b) Isolation from Natural α_p-ACTH. To a solution of the purified natural α_p-ACTH (*ca.* 15 mg)[9] in 0.2 M ammonium hydrogencarbonate (pH 8.2, 3.75 ml) was added 0.51 ml of 0.2% trypsin (chymotrypsin-free, in 0.001 M hydrochloric acid) and the mixture was incubated at 37 °C for 60 min. After addition of acetic acid (0.25 ml) the mixture was lyophilized. The tryptic hydrolysate thus obtained was submitted to chromatography on a column of silica gel (5 g, Kieselgel H, Merck) with ethyl acetate–acetic acid–water (4: 1: 1) as solvent. One half-ml fractions were collected and their absorption at 275 nm was measured. The fractions corresponding to a peak (tubes 16—22) were combined, evaporated *in vacuo* at a bath temperature of 40 °C and lyophilized from acetic acid (5 mg). This was further purified on a Sephadex LH-20 column (3.0×74 cm) with 1-butanol–acetic acid–water (4: 1: 2) as solvent. Five-ml fractions were collected and their absorption at 275 nm was measured to reveal the presence of a single component. The fractions corresponding to the peak (tubes 25—29) were combined and evaporated *in vacuo*, and the residue was lyophilized from acetic acid; yield 4 mg, $[\alpha]_D^{22}$ −42.7±3.5° (*c* 0.2, acetic acid). TLC: a single component (ninhydrin) in system E. Amino acid ratios in acid hydrolysate: NH_3 1.11 (1), Asp 2.01 (2), Glu 3.90 (4), Pro 1.91 (2), Gly 1.05 (1), Ala 2.95 (3), Val 0.96 (1), Leu 2.00 (2), Tyr 0.81 (1), Phe 1.83 (2).

H–Val–Tyr–Pro–Asn–Gly–Ala–Glu–Asp–Glu–Ser–Ala–Glu–Ala–Phe–Pro–Leu–Glu–Phe–OH (Ih). Compound XXIIh (100 mg) was treated with trifluoroacetic acid (1 ml) at room temperature for 60 min in the presence of 2-mercaptoethanol (0.1 ml). The precipitates which formed upon addition of ether were passed through an Amberlite CG-400 column with 1M acetic acid as solvent, followed by purification on a Sephadex LH-20 column with 1-butanol–acetic acid–water (4: 1: 2) as solvent in exactly the same manner as described above for Ip; yield 70 mg, $[\alpha]_D^{22}$ −41.4±1.6° (*c* 0.5, acetic acid). TLC: a single component (ninhydrin) in system E. Amino acid ratios in acid hydrolysate: NH_3 1.31 (1), Asp 2.03 (2), Ser 0.93 (1), Glu 3.98 (4), Pro 2.20 (2), Gly 1.08 (1), Ala 3.00 (3), Val 0.95 (1), Leu 1.00 (1), Tyr 0.78 (1), Phe 2.03 (2).

References

1) P. H. Bell, *J. Am. Chem. Soc.*, **76**, 5565 (1954).
2) R. G. Shepherd, S. D. Wilson, K. S. Howard, P. H. Bell, D. S. Davies, S. B. Davis, E. A. Eigner, and N. E. Shakespeare, *J. Am. Chem. Soc.*, **78**, 5067 (1956).
3) L. Gráf, S. Bajusz, A. Patthy, E. Barát, and G. Cseh, *Acta Biochim. Biophys. Acad. Sci. Hung.*, **6**, 415 (1971).
4) B. Riniker, P. Sieber, W. Rittel, and H. Zuber, *Nature New Biol.*, **235**, 114 (1972).
5) T. H. Lee, A. B. Lerner, and V. Buettner-Janusch, *J. Biol. Chem.*, **236**, 2970 (1961).
6) H. P. J. Bennett, *Biochem. J.*, **133**, 11 (1973).
7) W. F. White and W. A. Landmann, *J. Am. Chem. Soc.*, **77**, 1711 (1955).
8) Abbreviations for the ACTH preparations accord with the system of terminology proposed by Li [C. H. Li, *Science*, **129**, 970 (1959)].
9) K. Inouye, K. Watanabe, and H. Otsuka, *Bull. Chem. Soc. Jpn.*, **50**, 211 (1977).
10) R. Schwyzer and P. Sieber, *Helv. Chim. Acta.*, **49**, 134 (1966).
11) H. Schwarz, F. M. Bumpus, and I. H. Page, *J. Am. Chem. Soc.*, **79**, 5697 (1957).
12) K. Inouye, Y. Sumitomo, and M. Shin, *Bull. Chem. Soc. Jpn.*, **49**, 3620 (1976).
13) J. Honzl and J. Rudinger, *Coll. Czech. Chem. Commun.*, **26**, 2333 (1961).
14) In this connection, we should mention that in the amino acid analysis of the full-length ACTH preparations the recovery of tyrosine was excellent.[9] Thus, the extent of decomposition of tyrosine during acid hydrolysis seems to depend on the amino acid composition of a peptide to be hydrolyzed, and some amino acid (*e.g.* methionine) may play a role to protect tyrosine from its decomposition.
15) P. Sieber, W. Rittel, and B. Riniker, *Helv. Chim. Acta.*, **55**, 1243 (1972).
16) R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, **44**, 1991 (1961).
17) G. W. Anderson and F. M. Callahan, *J. Am. Chem. Soc.*, **82**, 3359 (1960).
18) O. Nishimura, C. Hatanaka, and M. Fujino, *Chem. Pharm. Bull.*, **23**, 1212 (1975).