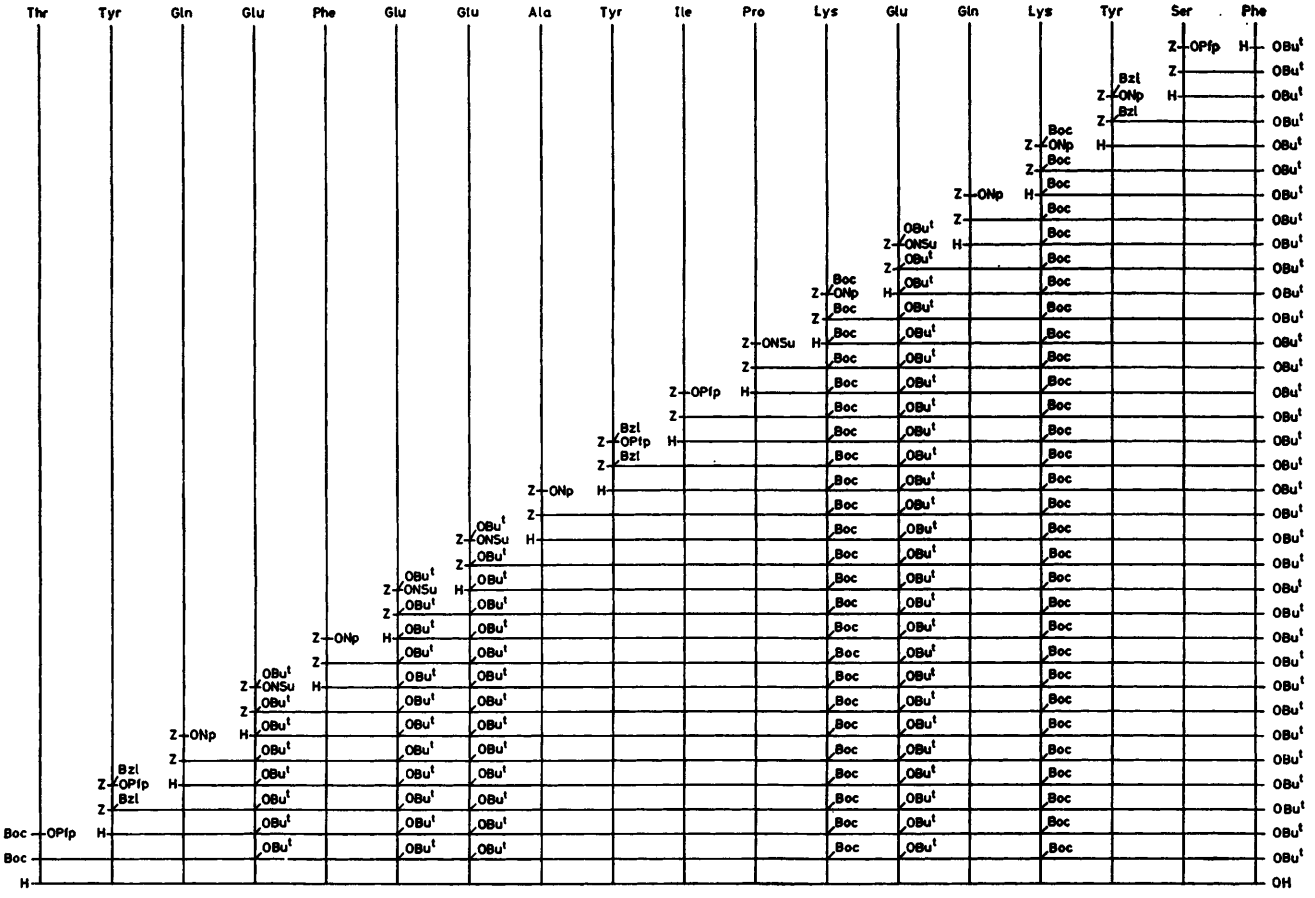
Synthesis of Human Growth Hormone-(27—44)-octadecapeptide and some Smaller Fragment Peptides

By Francesco Chillemi, Department of Organic Chemistry, University of Milan, Milan, Italy

Synthesis in solution of human growth hormone-(27—44)-octadecapeptide and a series of smaller fragments was carried out by a stepwise procedure, starting at the carboxy-terminus and uitilizing active esters in the acylation reactions.

In recent years evidence has accumulated that brain areas contain a family of peptides considered to be neurotransmitters or their precursors. In addition, it has been reported that peptides similar to, if not identical with, pituitary hormones can be synthesized by the cells within certain brain areas. One of these, a peptide with growth hormone-like immunoreactivity, has been detected in the brain by immunohistochemical techniques, and shown to be chromatographically identical with native growth hormone.[1]

The fact that fragments of hypophyseal β-lipotropin [2-4] have endorphin activity and that fragments of ACTH (4—10, 18—39) also are active in the central nervous system,[5,6] suggest that partial sequences of native hormones might have biological effects in the central nervous system. In continuation of our previous studies [7-11] of the biological activities of peptides corresponding to partial sequences of the hGH molecule,† we decided to synthesize other hGH fragments to be tested for possible effects in the central nervous system.



SCHEME 1 Synthesis of hGH-(27—44)-octadecapeptide

This paper reports the synthesis of octadecapeptide corresponding to sequence 27—44 of the hGH and a series of smaller fragments. In preliminary pharmacological experiments [12] the tetradecapeptide hGH 31—44 appears to have antagonist activity on two well-known morphine

† Abbreviations used in this paper include: hGH, human pituitary growth hormone; Z, benzyloxycarbonyl; Bzl, benzyl; Boc, t-butyloxycarbonyl; OBu^t, t-butyl ester; DCC, dicyclohexycarbodi-imide; ONp, *p*-nitrophenyl ester; ONSu, *N*-hydroxysuccinimide; OPfp, pentafluorophenyl ester; DMF, dimethylformamide; THF, tetrahydrofuran.

TABLE 1

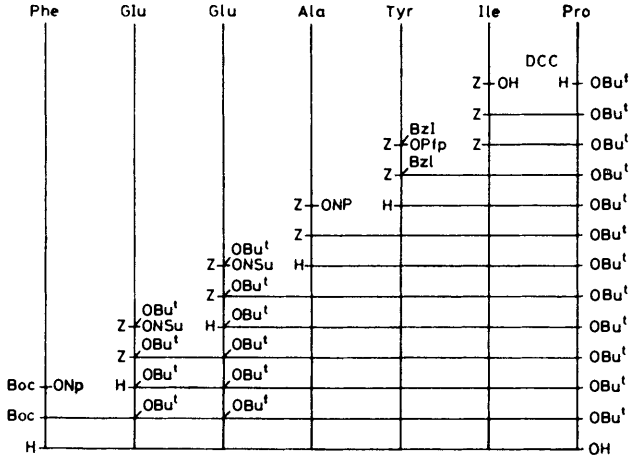
Physicochemical properties of the intermediates of hGH-(27—44)-octadecapeptide

	Method of coupling and solvent	Yield (%)	M.p. (°C) (solvent)	R_F A	R_F B	$[\alpha]_D^{28}$ (c 1.0, 80% AcOH)	Formula	Analysis Found (Calc.) C	H	N
Z-Ser-Phe-OBut	Pfp ester, THF	83	118—119 (EtOAc–light petroleum)	0.80	0.98	+2.0	$C_{34}H_{30}N_2O_6$	65.4 (65.13)	6.5 (6.83)	6.5 (6.33)
Z-Tyr(Bzl)-Ser-Phe-OBut	Np ester, THF	87	153—154 (EtOAc)	0.54	0.92	+4.5	$C_{40}H_{44}N_2O_6$	68.8 (69.04)	6.9 (6.51)	5.9 (6.03)
Z-Lys(Boc)-Tyr-Ser-Phe-OBut	Np ester, DMF	85	119 (EtOAc)	0.15	0.84	−4.9	$C_{44}H_{59}N_5O_{11}$	63.7 (63.36)	6.9 (7.13)	8.65 (8.39)
Z-Gln(Boc)-Lys-Tyr-Ser-Phe-OBut	Np ester, DMF	80	188 (EtOH)	0.12	0.47	−8.0	$C_{49}H_{67}N_7O_{13}$	60.95 (61.17)	7.2 (7.02)	9.85 (10.19)
Z-Glu(OBut)-Gln(Boc)-Lys-Tyr-Ser-OBut	NSu ester, DMF	84	213 (EtOH)	0.13	0.54	−14.7	$C_{55}H_{82}N_8O_{16}$	60.5 (60.71)	7.05 (7.20)	10.0 (9.76)
Z-Lys(Boc)-Glu(OBut)-Gln(Boc)-Lys-Tyr-Ser-Phe-OBut	Np ester, DMF	86	218 (DMF–H_2O)	0.10	0.67	−18.1	$C_{83}H_{102}N_{10}O_{18}$	60.05 (60.24)	7.8 (7.48)	9.9 (10.18)
Z-Pro-Lys(Boc)-Glu(OBut)-Gln-Lys(Boc)-Tyr-Ser-Phe-OBut	NSu ester, DMF	90	215 (MeOH)	0.13	0.65	−32.0	$C_{74}H_{102}N_{11}O_{20}$	60.55 (60.35)	7.2 (7.46)	10.35 (10.46)
Z-Ile-Pro-Lys(Boc)-Glu(OBut)-Gln-Lys(Boc)-Tyr-Ser-Phe-OBut	Pfp ester, DMF	95	204 (MeOH)	0.20	0.71	−40.2	$C_{80}H_{130}N_{12}O_{21}$	60.4 (60.59)	7.5 (7.62)	10.85 (10.60)
Z-Tyr(Bzl)-Ile-Pro-Lys(Boc)-Glu(OBut)-Gln-Lys(Boc)-Ser-Phe-OBut	Pfp ester, DMF	92	195—199 (MeOH)		0.79	−33.0	$C_{86}H_{128}N_{12}O_{22}$	62.4 (62.69)	7.6 (7.40)	10.1 (9.90)
Z-Ala-Tyr-Ile-Pro-Lys(Boc)-Glu(OBut)-Gln-Lys(Boc)-Ser-Phe-OBut	Np ester, DMF	78	198 (MeOH)		0.50	−37.8	$C_{93}H_{134}N_{14}O_{24}$	60.85 (60.71)	7.35 (7.42)	10.5 (10.77)
Z-Glu(OBut)-Ala-Tyr-Ile-Pro-Lys(Boc)-Glu(OBut)-Gln-Lys(Boc)-Tyr-Ser-Phe-OBut	NSu ester, DMF	80	204 (MeOH)		0.52	−40.2	$C_{101}H_{148}N_{16}O_{27}$	60.6 (60.49)	7.55 (7.49)	10.6 (10.47)
Z-Glu(OBut)-Glu(OBut)-Ala-Tyr-Ile-Pro-Lys(Boc)-Glu(OBut)-Gln-Lys(Boc)-Tyr-Ser-Phe-OBut	NSu ester, DMF	82	208 (MeOH)		0.55	−40.0	$C_{110}H_{166}N_{16}O_{30}$	60.45 (60.30)	7.3 (7.55)	10.4 (10.23)
Z-Phe-Glu(OBut)-Glu(OBut)-Ala-Tyr-Ile-Pro-Lys(Boc)-Glu(OBut)-Gln-Lys(Boc)-Tyr-Ser-Phe-OBut	Np ester, DMF	78	214 (MeOH)		0.57	−36.0	$C_{119}H_{172}N_{17}O_{31}$	60.9 (61.13)	7.3 (7.46)	10.35 (10.18)
Z-Glu(OBut)-Phe-Glu(OBut)-Glu-Ala-Tyr-Ile-Pro-Lys(Boc)-Glu(OBut)-Gln-Lys(Boc)-Tyr-Ser-Phe-OBut	NSu ester, DMF	79	240 (MeOH)		0.59	−38.0	$C_{126}H_{188}N_{18}O_{34}$	61.15 (60.93)	7.4 (7.51)	9.8 (9.99)
Z-Gln(OBut)-Glu(OBut)-Phe-Glu(OBut)-Glu-Ala-Tyr-Ile-Pro-Lys(Boc)-Glu(OBut)-Gln-Lys(Boc)-Tyr-Ser-Phe-OBut	Np ester, DMF	85	248 (MeOH)		0.54	−38.1	$C_{132}H_{196}N_{20}O_{36}$	60.45 (60.25)	7.6 (7.45)	10.31 (10.57)
Z-Tyr(Bzl)-Gln(OBut)-Glu(OBut)-Phe-Glu(OBut)-Glu-Ala-Tyr-Ile-Pro-Lys(Boc)-Glu(OBut)-Gln-Lys(Boc)-Tyr-Ser-Phe-OBut	Pfp ester, DMF	88	236 (MeOH)		0.56	−34.6	$C_{142}H_{211}N_{21}O_{38}$	61.4 (61.61)	7.25 (7.32)	10.3 (10.13)
Boc-Thr-Tyr-Gln(OBut)-Glu-Phe-Glu(OBut) - Glu(OBut)-Ala-Tyr-Ile-Pro-Lys(Boc)-Glu(OBut)-Gla(OBut)-Lys(Boc)-Tyr-Ser-Phe-OBut	Pfp ester, DMF	80	240—241 (MeOH)		0.41	—31.1	$C_{142}H_{212}N_{22}O_{40}$	60.3 (60.14)	7.5 (7.33)	10.7 (10.57)

effects.* This peptide, when assayed for lipolytic activity in isolated rabbit fat cells, showed no lipolytic activity in contrast with the reports of Keda *et al.*[13] and Yudaev *et al.*[14]

RESULTS AND DISCUSSION

All the peptides were synthesized by classical methods in solution, using a stepwise approach. The benzyloxycarbonyl group was selected for temporary protection



SCHEME 2 Synthesis of hGH-(31—37)-heptapeptide

of the α-amino-group, while the t-butyloxycarbonyl group was reserved for permanent protection of the ε-amino-group of lysine and the t-butyl ester for the carboxy-groups. The phenol function of tyrosine was protected as the benzyl ether,[15] so that the active ester could be synthesized more easily. After the introduction of tyrosine into the peptide chain this protecting group was removed by hydrogenolysis together with the benzyloxycarbonyl group. The alcoholic hydroxy-groups of the serine and threonine were left free.

The stepwise assembly of the octadecapeptide chain was carried out by using the pentafluorophenyl, the *para*-nitrophenyl, and the *N*-hydroxysuccinimide active esters of the protected amino-acids, in the coupling reactions. The stepwise approach was also used to synthesize the hGH-(31—37)-heptapeptide, for study of the pharmacological activity of the *N*-terminal segment.

The syntheses of hGH-(27—44)-octadecapeptide and GH-(31—37)-heptapeptide are summarized in Schemes 1 and 2, respectively. The physicochemical data for the newly synthetized compounds are given in Tables 1—3.

EXPERIMENTAL

All amino-acid derivatives had the L-configuration and were synthesized by literature procedures. Elemental analyses were made with a Hewlett-Packard 185 apparatus. Analytical samples were dried *in vacuo* over P_2O_5 at 50 °C. M.p.s were determined with a Büchi-Tottoli apparatus and are uncorrected. Optical rotations were measured in a jacketed 1-dm cell in a Perkin-Elmer Model 141 Polarimeter. T.l.c. was performed on silica gel G (Merck, nach Stahl) plates. For protected peptides the following systems were used: (1) benzene–ethyl acetate–glacial acetic acid–water (10 : 10 : 2 : 1); and (B) chloroform–methanol–glacial acetic acid (85 : 10 : 5); the chromatograms were developed with iodine vapour. For free peptides, system (C) was used, butanol–glacial acetic acid–pyridine–water (15 : 3 : 10 : 12). Peptide spots were detected with buffered ninhydrin or by exposure to iodine vapour.

N-Benzyloxycarbonyl-O-benzyl-L-tyrosine Pentafluorophenyl Ester.—This derivative was prepared by the method of Kisfaludy *et al.*[16] It was recrystallized from EtOAc–n-hexane, yield 82%, m.p. 100 °C, $[\alpha]_D^{20}$ −11.8 ° (*c* 1.0, THF) (Found: C, 63.15; H, 3.8; N, 2.5. Calc. for $C_{30}H_{22}F_5NO_5$: C, 63.04; H, 3.88; N, 2.45%).

General Procedure of Coupling by Active Esters.—One equivalent of Et_3N was added to 0.02—0.1M solutions of the peptide (usually in DMF) at 0 °C, followed by addition of one equivalent of the active ester of the protected amino-acid. After 2 h at 0 °C, the mixture was allowed to come to room temperature and held there overnight. The completeness of the acylation reactions was checked by t.l.c. The reaction mixture was then diluted with water (3—4 times the volume of DMF). The precipitate was filtered, washed with water and ether (with light petroleum for the smaller peptides), and dried *in vacuo*. The products were recrystallized from suitable solvents until homogeneous by t.l.c. In some cases (Table 1), THF was used as the reaction solvent

TABLE 2

Physicochemical properties of the intermediates of hGH-(31—37)-heptapeptide

	Method of coupling and solvent	Yield %	M.p. (°C) solvent	R_F A	R_F B	$[\alpha]_D^{20}$ (*c* 1.0, 80% AcOH	Formula	Analysis Found (Calc.) C	H	N
Z-Ile-Pro-OBut	DCC, CH_2Cl_2	95	Oil	0.99	0.98					
Z-Tyr(Bzl)-Ile-Pro-OBut	Pfp ester, DMF	87	130—131 EtOAc	0.98	0.96	−32.9°	$C_{39}H_{48}N_3O_7$	69.45 (69.72)	7.1 (7.35)	6.4 (6.25)
Z-Ala-Tyr-Ile-Pro-OBut	Np ester, DMF	83	209—210 decomp. EtOH–H_2O	0.65	0.81	−73.2°	$C_{36}H_{43}N_4O_8$	64.6 (64.39)	7.55 (7.41)	8.4 (8.58)
Z-Glu(OBut)-Ala-Tyr-Ile-Pro-OBut	NSU ester, DMF	85	220—221 EtOH	0.61	0.80	−64.6°	$C_{44}H_{63}N_5O_{11}$	63.4 (63.06)	7.3 (7.58)	8.6 (8.36)
Z-Glu(OBut)-Glu(OBut)-Ala-Tyr-Ile-Pro-OBut	NSu ester, DMF	80	214—215 EtOH	0.63	0.80	−61.6°	$C_{53}H_{78}N_4O_{14}$	62.05 (62.21)	7.75 (7.68)	7.9 (8.21)
Boc-Phe-Glu(OBut)-Glu(OBut)-Ala-Tyr-Ile-Pro-OBut	Np ester, DMF	85	226—227 EtOH	0.64	0.78	−58.2°	$C_{59}H_{89}N_7O_{15}$	62.6 (62.35)	7.7 (7.89)	8.85 (8.63)

* The details of the pharmacological studies will be published elsewhere by V. R. Olgiati, F. Guidobono, and A. Pecile from the Department of Pharmacology, University of Milan, Italy.

TABLE 3

Physicochemical properties and amino-acid analyses of the free peptides

		R_F (system C)	$[\alpha]_D^{20}$ (*c* 1.0, 50% AcOH)	Amino-acid analysis (6N HCl, 110 °C, 24 h)
hGH 39—44	H-Glu-Gln-Lys-Tyr-Ser-Phe-OH	0.46	− 7.4	$Ser_{0.99}Glu_{2.03}Tyr_{0.98}Phe_{1.01}Lys_{0.99}$
hGH 37—44	H-Pro-Lys-Glu-Gln-Lys-Tyr-Ser-Phe-OH	0.57	−40.8	$Ser_{1.00}Glu_{2.02}Pro_{0.98}Tyr_{1.02}Phe_{0.99}Lys_{2.01}$
hGH 35—44	H-Tyr-Ile-Pro-Lys-Glu-Gln-Lys-Tyr-Ser-Phe-OH	0.54	−42.7	$Ser_{0.98}Glu_{1.99}Pro_{1.00}Ile_{0.99}Tyr_{2.01}Phe_{0.98}Lys_{2.02}$
hGH 33—44	H-Glu-Ala-Tyr-Ile-Pro-Lys-Glu-Gln-Lys-Tyr-Ser-Phe-OH	0.51	−55.2	$Ser_{1.02}Glu_{3.05}Pro_{0.97}Ala_{1.02}Ile_{1.00}Tyr_{1.97}Phe_{1.01}Lys_{1.98}$
hGH 31—44	H-Phe-Glu-Glu-Ala-Tyr-Ile-Pro-Lys-Glu-Gln-Lys-Tyr-Ser-Phe-OH	0.53	−57.1	$Ser_{1.01}Glu_{4.06}Pro_{0.99}Ala_{1.00}Ile_{1.03}Tyr_{2.02}Phe_{2.03}Lys_{1.99}$
hGH 27—44	H-Thr-Tyr-Gln-Glu-Phe-Glu-Glu-Ala-Tyr-Ile-Pro-Lys-Glu-Gln-Lys-Tyr-Ser-Phe-OH	0.55	−53.2	$Thr_{0.96}Ser_{0.99}Glu_{6.08}Pro_{1.00}Ala_{1.01}Ile_{1.02}Tyr_{3.06}Phe_{2.03}Lys_{2.03}$
hGH 31—37	H-Phe-Glu-Glu-Ala-Tyr-Ile-Pro-OH	0.58	−52.0	$Glu_{2.01}Pro_{0.99}Ala_{1.00}Ile_{1.01}Tyr_{0.98}Phe_{1.02}$

This solvent was evaporated *in vacuo*. When the protected peptide was to be hydrogenated in methanol solution (see next section), Et_3N was not added in the coupling reaction. When the coupling reactions involved pentafluorophenyl esters, the reaction times at room temperature were shortened to 4—6 h.

Details of the coupling method, the reaction, and the recrystallization solvent are reported with the yields, and the physicochemical and analytical data in Tables 1 and 2. The yields listed are overall for the coupling and hydrogenolysis reactions and are calculated on the basis of analytically pure material.

General Procedure for Benzyloxycarbonyl Group Removal.—Palladium–charcoal (10%) (1—5 g of catalyst for each mmol of peptide) was added to 0.01—0.05 molar solutions of the protected peptides in 80% acetic acid. The mixture was hydrogenated at atmospheric pressure until carbon dioxide evolution ceased, using the apparatus described by Greenstein and Winitz.[17] The catalyst was filtered off and the filtrate was evaporated *in vacuo*. The residue was triturated with ether, filtered, washed several times with ether, and dried *in vacuo* (KOH). The yields of the hydrogenolysis reactions were quantitative or nearly so.

In the hydrogenolysis of smaller peptides (up to pentapeptides) methanol was used as the solvent instead of 80% acetic acid. For these, the washing with ether was omitted.

General Procedure for the Final Removal of Protecting Groups.—Protected peptide (1 mmol) was dissolved in trifluoroacetic acid–anisole (10 : 1, 150 ml). The solution was allowed to stand at room temperature for 1—1.5 h. Completeness was checked by t.l.c. Most of the trifluoroacetic acid was removed *in vacuo* (bath temperature 20 °C). The residue was triturated with ether, filtered, washed several times with ether, and dried *in vacuo* (KOH).

For the preparation of the first five peptides in Table 3, hydrogenolysis was carried out in the manner described above, before treatment with trifluoroacetic acid–anisole.

The free peptide yields were essentially quantitative. They were sufficiently pure that further purification was unnecessary.

Samples of free peptides were hydrolysed in 6N HCl for 24 h at 110 °C in sealed, evacuated tubes and the amino-acids analysed on the Beckman Model 121M amino-acid analyser. The homogeneity of the free peptides was assessed by t.l.c., by high-voltage electrophoresis, and by isoelectric focusing. Paper electrophoresis was carried out in formic acid–acetic acid–water (15 : 10 : 75) buffer, pH 1.2, using a high-voltage Pherograph apparatus (Mini 65; Hormuth-Vetter, Wiesloch). The details of the isoelectric focusing technique were as previously described.[18-20]

N-Benzyloxycarbonyl-L-isoleucyl-L-proline t-Butyl Ester (*Table* 2).—*N*-Benzyloxycarbonyl-L-isoleucine (13.2 g, 50 mmol) and L-proline t-butyl ester (8.5 g, 50 mmol) were dissolved in methylene chloride (150 ml). DCC (10.3 g, 50 mmol) was added to the ice-cold solution and the mixture was stirred for 2 h at 0 °C, and then left to stand overnight at room temperature. The *NN'*-dicyclohexylurea which separated out was filtered off and washed with methylene chloride. The combined filtrate and washing were evaporated to dryness and the oily residue was dissolved in ethyl acetate (100 ml). The mixture was filtered and the filtrate evaporated *in vacuo* to yield 20 g (95%) of an oily residue, homogeneous by t.l.c.

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