Synthesis by Conventional Methods of Bovine and Ovine Growth Hormone-(125—133)-nonapeptides

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Two nonapeptides, Arg-Glu-Leu-Glu-Asp-Gly-Thr-Pro-Arg and Arg-Glu-Leu-Glu-Asp-Val-Thr-Pro-Arg, corresponding to the sequence 125—133 of bovine and ovine growth hormones (BGH and OGH) were prepared by conventional methods. A stepwise procedure was applied, active ester and dicyclohexylcarbodi-imide were used in the acylation reactions, and all the protected intermediates were isolated and characterized for homogeneity. Both peptides synthesized by solution method and OGH-(125—133) previously prepared by a solid-phase method showed very low biological activity in the hypophysectomized rat tibia epiphyseal width test.

In a preliminary communication,[1] we described the solid-phase synthesis of fragments corresponding to sequences 125—133, 111—133, and 95—133 of OGH.† The last is homologous with the octatriaconapeptide 96—133 obtained by Yamasaki *et al.*[2] through controlled tryptic digestion of BGH and which is endowed with considerable biological activity both in the tibia test and the body-weight gain test. It has been subsequently reported[3] that the BGH-(125—133)-nonapeptide also shows activity in the tibia test but is inactive in the weight-increase test. Contrary to expectations we found that fragments 125—133 of OGH, varying from the corresponding fragment of BGH only by having glycine in place of valine, had a very low activity in the tibia test when compared with the BGH standard.

We have synthesized by conventional methods a nonapeptide which spans positions 125—133 of the BGH primary structure,[4] with the aim of comparing directly its activity with that of the corresponding fragment of OGH. The above-mentioned nonapeptide will also be used as an intermediate for preparing longer BGH fragments [if possible, the BGH-(96—133)-octatriacontapeptide].

RESULTS AND DISCUSSION

BGH-(125—133)-nonapeptide possesses the aspartylglycine sequence at positions 129—130, and cleavage by HF gives an imide derivative[5-7] which, after subsequent mild alkaline hydrolysis, leads to the formation of a mixture of α- and β-aspartyl-peptides.

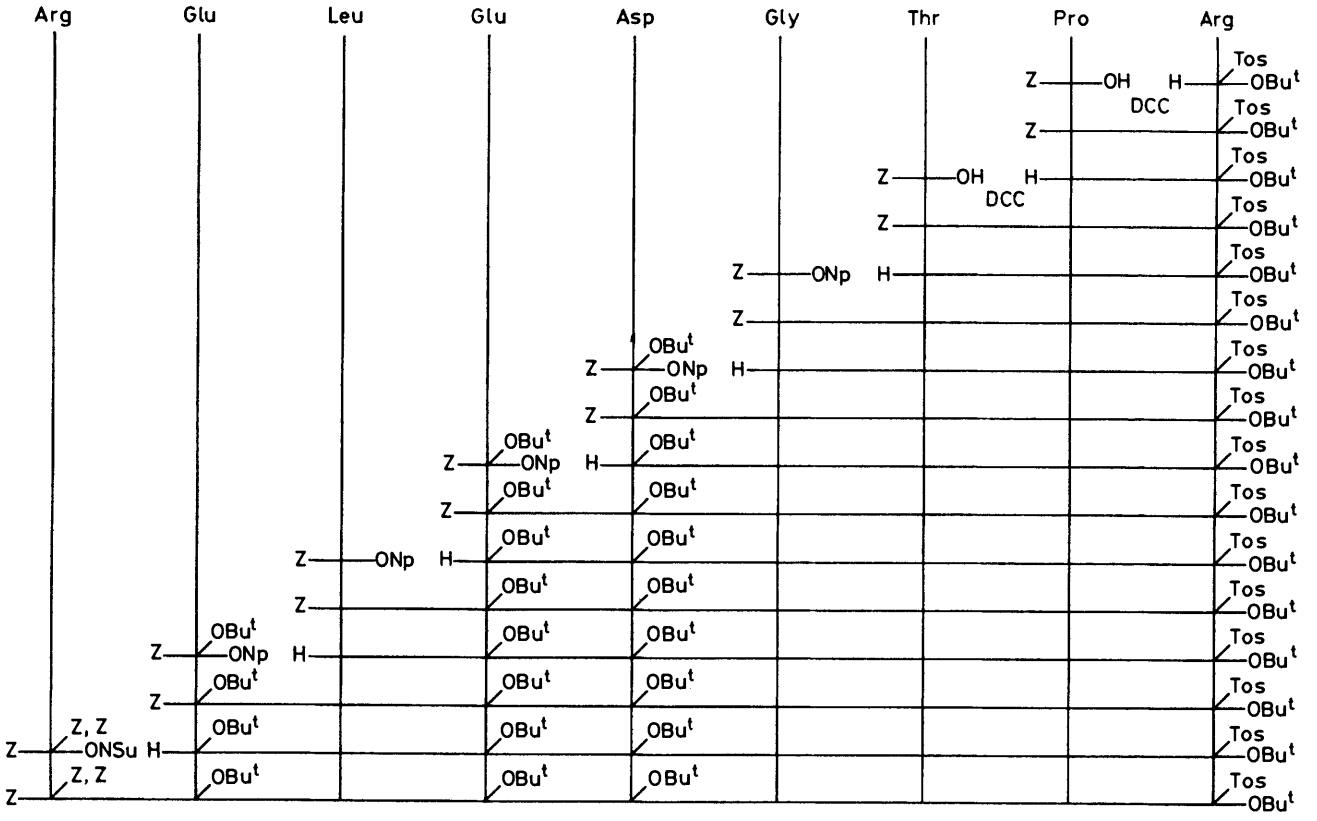
The synthesis of BGH-(125—133)-nonapeptide is summarized in Scheme 1. To protect the guanidino-group of the *C*-terminal arginine we chose the tosyl group. In addition to satisfactorily masking the basic properties of the arginine, this group can be removed with hydrofluoric acid at the end of the synthesis without giving secondary products.[8] We prepared the t-butyl ester from N^G-tosyl-arginine by the Taschner method.[9] The guanidino-group of the *N*-terminal arginine residue was protected by bisbenzyloxycarbonylation.[10] This makes it possible to use active esters[11] in the coupling reactions. All attempts to prepare active esters from the derivatives of tosyl-arginine led to the corresponding lactams.

Choice of the t-butyl group for protection of the carboxy groups in the side chains of aspartic and glutamic acids was dictated mainly by the presence of the aspartylglycine sequence. The considerable steric hindrance exerted by the t-butyl group succeeds in shielding the carbonyl from nucleophilic attack by the amide nitrogen. To minimize acid-catalysed rearrangement, the removal of protecting groups was carried out in two steps, first with trifluoroacetic acid and then with hydrofluoric acid.[3] Paper electrophoresis at pH 6.5 showed only a small amount of the cyclic imide derivative, as identified earlier by Wang *et al.*[3]

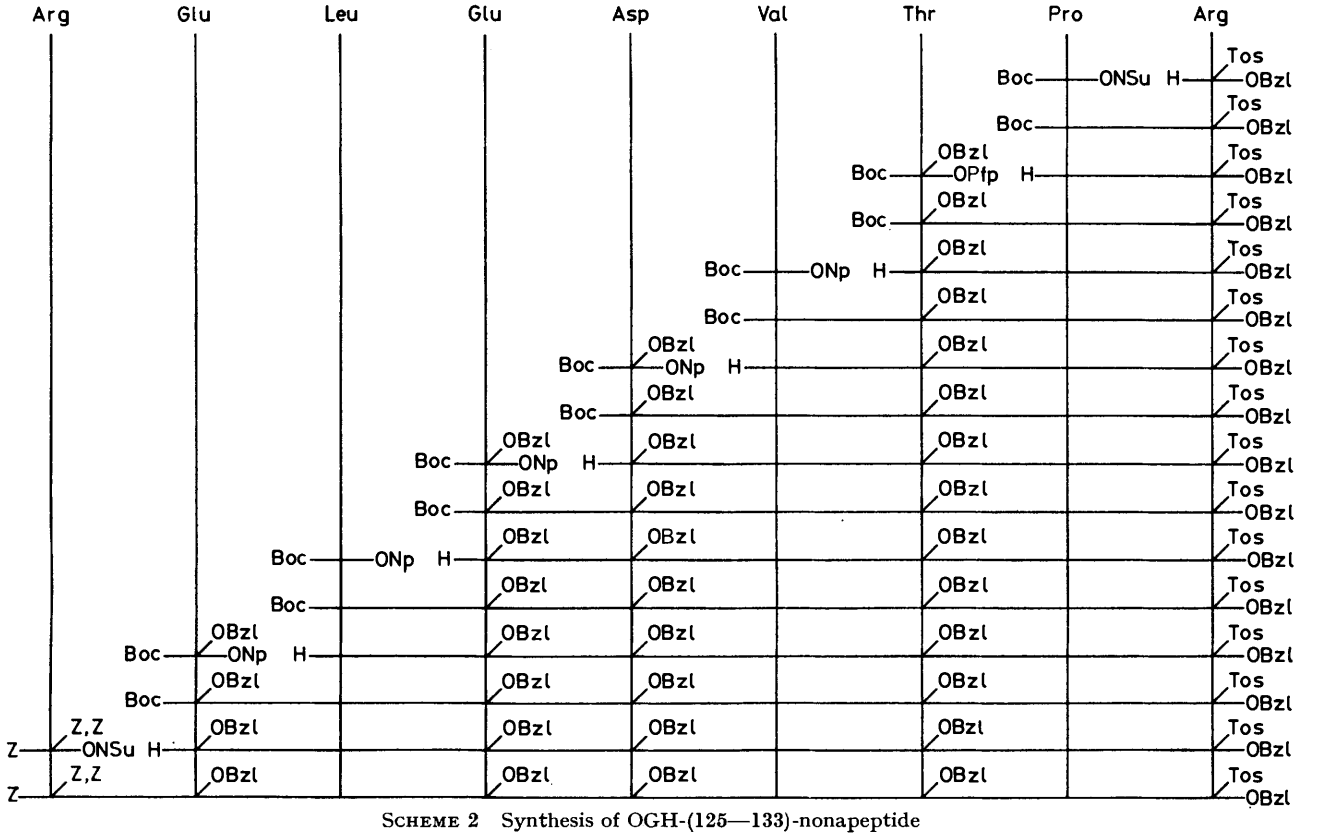
We prepared, again by conventional methods, the nonapeptide corresponding to residues 125—133 of the OGH primary structure.[12] As outlined in Scheme 2, in this synthesis we reversed the use of the protecting groups. t-Butoxycarbonyl was used for temporary protection of the α-amino-group, while benzyloxycarbonyl and benzyl were used for permanent protection of the other functional groups. The tosyl group was reserved for protection of the guanidino-group of the *C*-terminal arginine residue. A small portion of the protected nonapeptide was subjected to acidolysis with HF.

The nonapeptide synthesised according to Scheme 2, in addition to providing a classical synthesis of the OGH 125—133 fragment, is a potential intermediate for a future synthesis by conventional methods of the OGH 93—133 nonatriacontapeptide, planned so as to avoid imide formation of the Asp-Ser sequence (residues 99—100). Indeed Yang and Merrifield[7] reported recently that the Asp-Ser sequence gives a cyclic 'aspartoyl' imide derivative during the HF-cleavage step. The approach which we have taken to minimize aspartimide formation depends on the observation[3,5] that acid-catalysed cyclization is greatly retarded when the β-carboxy group of the aspartyl residue is free. The incorporation of this idea into a suitable synthetic scheme requires the use of a temporary protecting group for aspartic acid that can be removed selectively before exposure to strong anhydrous acid. An alternative pathway towards the synthesis of OGH-(93—133)-nonatriacontapeptide will be explored in which the nitrogen of the Asp-Ser peptide bond is suitably protected in order to prevent the rearrangement during acidolysis by HF.

† Abbreviations used in this paper include: OGH, ovine pituitary growth hormone; BGH, bovine pituitary growth hormone; Z, benzyloxycarbonyl; OBzl, benzyl ester; Bzl, benzyl; Boc, t-butoxycarbonyl; OBu^t, t-butyl ester; Tos, tosyl; DCC, dicyclohexylcarbodi-imide; ONp, *p*-nitrophenyl ester; ONSu, *N*-hydroxysuccinimide ester; OPfp, pentafluorophenyl ester; DMF, dimethylformamide.



SCHEME 1 Synthesis of BGH-(125—133)-nonapeptide



SCHEME 2 Synthesis of OGH-(125—133)-nonapeptide

TABLE 1

Compound	Method of coupling and solvent	Yield (%)	M.p. (°C) (solvent)	R_F A	R_F B	$[\alpha]_D^{20}$	Formula	Analysis * (Found above, Calc. below) C	H	N
Z-Pro-Arg(Tos)-OBut (1)	DCC, CH_2Cl_2	78	60 * (EtOAc–LP ‡)	0.52	0.73	−18.6 (*c* 0.5, EtOH)	$C_{30}H_{41}N_5O_7S$	58.40 (58.00	6.80 6.72	11.50 11.38)
Z-Thr-Pro-Arg(Tos)-OBut (2)	DCC, CH_2Cl_2	75	145—146 † (EtOAc)	0.30	0.68	−30.9 (*c* 1, EtOH)	$C_{34}H_{48}N_6O_9S$	56.80 (56.96	6.75 6.75	12.00 12.27)
Z-Gly-Thr-Pro-Arg(Tos)-OBut (3)	Np ester, DMF	87	98—99 † EtOH–LP ‡	0.07	0.58	−21.6 (*c* 1, EtOH)	$C_{36}H_{51}N_7O_{10}S$	56.00 (55.87	6.50 6.64	12.50 12.67)
Z-Asp(OBut)-Gly-Thr-Pro-Arg(Tos)-OBut (4)	Np ester, DMF	72	113—114 † (EtOAc)		0.60	−18.1 (*c* 1, DMF)	$C_{44}H_{64}N_8O_{13}S$	56.05 (55.91	6.90 6.80	11.10 11.86)
Z-Glu(OBut)-Asp(OBut)-Gly-Thr-Pro-Arg(Tos)-OBut (5)	Np ester, DMF	70	98—100 † (EtOAc)		0.68	−14.0 (*c* 0.5, DMF)	$C_{53}H_{79}N_9O_{16}S$	56.75 (56.33	7.35 7.05	10.95 11.42)
Z-Leu-Glu(OBut)-Asp(OBut)-Gly-Thr-Pro-Arg(Tos)-OBut (6)	Np ester, DMF	79	138—140 † (EtOH)		0.67	−12.5 (*c* 0.5, DMF	$C_{59}H_{90}N_{10}O_{17}S$	57.10 (56.98	7.50 7.30	11.05 11.26)
Z-Glu(OBut)-Leu-Leu-Glu(OBut)-Asp(OBut)-Gly-Thr-Pro-Arg(Tos)-OBut (7)	Np ester, DMF	81	163—165 † (EtOH)		0.70	−15.6 (*c* 0.5, DMF)	$C_{68}H_{106}N_{11}O_{20}S$	57.35 (57.16	7.55 7.48	10.70 10.78)
Z-Arg(Z)-Glu(OBut)-Leu-Leu-Glu(OBut)-Asp(OBut)-Gly-Thr-Pro-Arg(Tos)-OBut (8)	NSu ester, DMF	74	196–198 (decomp.) †		0.81	−23.3 (*c* 1, DMF)	$C_{99}H_{130}N_{15}O_{25}S$	58.45 (58.33	7.15 7.06	11.30 11.38)

* Amorphous. † Crystalline. ‡ Light petroleum.

TABLE 2

Compound	Method of coupling and solvent	Yield (%)	M.p. (°C) (solvent)	R_F A	R_F B	$[\alpha]_D^{20}$	Formula	Analysis (Found above, Calc. below) C	H	N
Boc-Pro-Arg(Tos)-OBz (1)	NSu ester, THF	96	67—69 * (EtOAc–LP †)	0.64	0.85	−35.2 (*c* 1, EtOH)	$C_{30}H_{41}N_5O_7S$	58.30 (58.22	6.90 6.71	11.10 11.37)
Boc-Thr(Bz)-Pro-Arg(Tos)-OBzl (2)	Pfp ester, THF	98	72—75 * (EtoAc–LP ‡)	0.39	0.80	−35.4 (*c* 1, EtOH)	$C_{41}H_{54}N_6O_9S$	61.05 (61.03	7.0 6.74	10.15 10.41)
Boc-Val-Thr(Bzl)-Pro-Arg(Tos)-OBz (3)	Np ester, DMF	95	89—90 * (EtOAc–LP ‡)	0.21	0.61	−41.6 (*c* 1, EtOH)	$C_{46}H_{62}N_7O_{10}S$	61.15 (60.97	7.20 7.01	10.60 10.82)
Boc-Asp(OBzl)-Val-Thr(Bzl)-Pro-Arg(Tos)-OBzl (4)	Np ester, DMF	87	89—90 * (EtOAc–LP ‡)	0.11	0.72	−28.2 (*c* 1, DMF)	$C_{57}H_{74}N_8O_{13}S$	61.30 (61.60	6.90 6.71	9.95 10.08)
Boc-Glu(OBzl)-Asp(OBzl)-Val-Thr(Bzl)-Pro-Arg(Tos)-OBz (5)	Np ester, DMF	82	90—92 † (EtOAc–LP ‡)		0.79	−26.4 (*c* 1, DMF)	$C_{69}H_{87}N_9O_{16}S$	62.35 (62.29	6.65 6.59	9.55 9.47)
Boc-Leu-Glu(OBzl)-Asp(OBzl)-Val-Thr(Bzl)-Pro-Arg(Tos)-OBzl (6)	Np ester, DMF	77	129—131 † (EtOAc–LP) ‡		0.84	−28.3 (*c* 1, DMF)	$C_{75}H_{98}N_{10}O_{17}S$	62.55 (62.40	6.95 6.84	9.30 9.70)
Boc-Glu(OBzl)-Leu-Glu(OBzl)-Asp(OBzl)-Val-Thr(Bzl)-Pro-Arg(Tos)-OBzl (7)	Np ester, DMF	75	196—198 † (EtOAc)		0.87	−24.4 (*c* 1, DMF)	$C_{87}H_{111}N_{11}O_{20}S$	62.80 (62.84	6.70 6.73	9.15 9.26)
Z-Arg(Z)-Glu(OBzl)-Leu-Glu(OBzl)-Asp(OBzl)-Val-Thr(Bzl)-Pro-Arg(Tos)-OBzl (8)	NSu ester, DMF	74	204—206 † (EtOH)		0.90	−22.8 (*c* 1, DMF)	$C_{100}H_{129}N_{15}O_{23}S$	62.60 (62.65	6.70 6.51	10.00 10.06)

* † ‡ See Table 1.

TABLE 3

Growth-promoting activity of OGH-(125—133) (A) synthesized by the solid-phase method, and of OGH-(125—133) (B) and BGH-(125—133) (C) synthesized by conventional methods, assayed by the rat tibia test

Preparation	Daily dose ($\times 10^{-6}$ g)	Number of rats	Mean response ($\times 10^{-6}$ m)	*P*
Saline		15	135.6 ± 2.41	
BGH	18.75	10	177.9 ± 5.07	<0.001
BGH	75	12	231.6 ± 7.02	<0.001
OGH-(125—133) (A)	187.5	7	139.6 ± 1.50	
OGH (125—133) (A)	750	7	144.5 ± 3.10	
OGH-(125—133) (B)	100	8	154.8 ± 5.36	<0.01
OGH-(125—133) (B)	400	9	152.6 ± 3.17	<0.01
BGH-(125—133) (C)	100	10	156.6 ± 3.87	<0.001
BGH-(125—(133) (C)	400	8	145.7 ± 2.49	<0.05

Only the syntheses of new intermediates of amino-acids and the final peptides are described in detail in this report; the physicochemical data for the other compounds are summarized in Tables 1 and 2.

The results of the tibia test bioassay of two synthetic nonapeptides prepared as indicated here and of OGH-(125—133) synthesized by the solid-phase method [1] are summarized in Table 3. The biological activity of the fragment 125—133 of OGH synthesized by the solid-phase method [1] was very low even with the higher daily dose (0.75 mg per rat) and the increases of epiphyseal cartilage width compared with the controls were not significant. The activity of the fragment 125—133 of OGH synthesised by the conventional methods was measurable and showed a significant difference compared with the controls. BGH-(125—133) synthesised by conventional methods showed a slight but measurable activity which however was clearly lower than that reported for the same fragment by Wang *et al.*[3] These investigators found that very low doses of the nonapeptide (0.001—0.002 mg per rat over 4 d; about 15 times the molar dose) produced an equivalent response to BGH but higher levels of the peptide produced no further increase in epiphyseal width, a fact observed also in our experiments (see Table 3). Since the dose–response curve of the nonapeptides (synthesised by us or by others) was not parallel with that from the international standard of BGH, and the precision of the assay was not high, a simple statement of the proportion of growth hormone activity retained in the nonapeptides cannot be made.

The reported data on the biological activity on the nonapeptides, although showing a low level of activity, suggest the existence of active cores responsible for selected effects of the growth hormone molecule. On the other hand, small peptides may be very susceptible to enzymic degradation. The preparation of peptides which may survive longer *in vivo* is envisaged as a future step in the present research but we think that even small peptides may be important at least as useful starting material indicating minimum structural requirements for growth-hormone-like activity.

EXPERIMENTAL

M.p.s were determined with a Büchi–Tottoli apparatus.

All optically active amino-acid residues are of the L-configuration. Optical rotations were measured with a Perkin-Elmer P-141 polarimeter at 589 nm.

Paper electrophoresis was carried out in pyridine acetate buffer (AcOH–pyridine–H_2O 1 : 10 : 100), pH 6.5, using a high-voltage Pherograph apparatus (Mini 65; Hormuth–Vetter, Wiescloch).

T.l.c. was performed on silica gel G (Merck, nach Stahl) with the solvent systems: A, benzene–ethyl acetate–glacial acetic acid–water (10 : 10 : 2 : 1); and B, chloroform–methanol–glacial acetic acid (85 : 10 : 5). Peptide spots were detected by exposure to gaseous HCl for 15 min, followed by spraying with buffered ninhydrin, or by exposure to iodine vapour.

Elemental analyses were made with a Hewlett–Packard 185 apparatus. Analytical samples were dried *in vacuo* over P_2O_5 at 50 °C.

Acidic hydrolyses were performed at 110 °C for 24 h in glass ampoules sealed *in vacuo*. The amino-acid compositions of the hydrolysates were determined with a Beckman automatic 121 amino-acid analyser.

Counter-current distribution was performed on an automatic Contraflow apparatus (S.r.l. Elbe), with 10 ml tubes for each phase.

Catalytic hydrogenolyses were carried out in the presence of 10% palladium–charcoal, using the apparatus described by Greenstein and Winitz.[13]

The standard procedure used to remove the Boc groups was as follows: the protected peptide was treated with 4M HCl in dioxan for 30 min and the mixture was evaporated to dryness *in vacuo* (bath temperature 30 °C). The residue was triturated with ether, filtered off, washed with ether, and dried *in vacuo* (KOH).

N^G-*Tosyl*-L-*arginine Hydrochloride* t-*Butyl Ester*.—A solution of N^α-benzyloxycarbonyl-N^G-tosyl-L-arginine [14] (30 g, 65 mmol) in t-butyl alcohol (160 ml) and anhydrous pyridine (65 ml) was cooled to −5 °C. Phosphoryl chloride (65 ml) was added dropwise with vigorous stirring, the mixture was stirred for 1 h at −5 °C, and then set aside overnight at room temperature. It was then diluted with ethyl acetate (180 ml) and water (180 ml). The organic phase was washed with citric acid, aq. $NaHCO_3$, and water. The ethyl acetate solution was dried (Na_2SO_4) and evaporated to dryness *in vacuo* to yield 29.9 g of an oily residue, homogeneous by t.l.c., R_F 0.84 (system A).

Palladium–charcoal (10%) was added to a solution of the oil in ethanol. The suspension was stirred under hydrogen at normal pressure. The catalyst was removed and the filtrate was concentrated to dryness *in vacuo*. An ether solution of the residue was cooled to 0 °C and neutralized with 1 equiv. of 4M-HCl in dioxan, causing immediate crystallization of the N^G-tosyl-L-arginine hydrochloride t-butyl ester (19.7 g, 72%); m.p. 108—109 °C; homogeneous by t.l.c., R_F 0.78 (Bu^nOH–AcOH–H_2O 4 : 1 : 1); $[\alpha]_D^{24}$ −4.2° (*c* 0.5, ethanol).

A sample of the product was treated with trifluoroacetic acid for 1 h at 20 °C. In t.l.c. the residue gave one spot corresponding to N^G-tosyl arginine, R_F 0.60 (Bu^nOH–AcOH–H_2O 4 : 1 : 1) (Found: C, 48.25; H, 7.0; N, 13.05. Calc. for $C_{17}H_{29}ClN_4O_4S$: C, 48.5; H, 6.95; N, 13.3%).

N-t-*Butoxycarbonyl*-O-*benzyl*-L-*threonine Pentafluorophenyl Ester*.—*N*-t-Butoxycarbonyl-*O*-benzyl-L-threonine [15] (24.13 g, 78 mmol) and pentafluorophenol (14.36 g, 78 mmol) were dissolved in ethyl acetate (250 ml). To the ice-cold solution was added DCC (16.10 g, 78 mmol), and the mixture was stirred at 0 °C, for 2 h. Dicyclohexylurea was then removed by filtration and washed with ethyl acetate, and the filtrate plus the washings evaporated to dryness *in vacuo*, affording a residue which soon crystallized. Two recrystallizations from n-hexane yielded 34.5 g (93%) of the ester, m.p. 90—92 °C; $[\alpha]_D^{24}$ −30.6° (*c* 0.5, ethanol) (Found: C, 55.5; H, 4.55; N, 2.95. Calc. for $C_{22}H_{22}F_5NO_5$: C, 55.6; H, 4.65; N, 2.95%).

L-*Arginyl*-L-*glutamyl*-L-*leucyl*-L-*glutamyl*-L-*aspartyl*-*glycyl*-L-*threonyl*-L-*prolyl*-L-*arginine*.—The protected nonapeptide (1.00 g) was dissolved in trifluoroacetic acid (25 ml) and the mixture stirred for 45 min at room temperature. After removal of the acid *in vacuo*, the residue was washed several times with diethyl ether. The solid was treated with anhydrous HF (20 ml) containing anisole (1 ml) for

30 min at 0 °C, using the equipment described by Sakakibara *et al.*[16] The acid was evaporated off under high vacuum and the residue was triturated several times with ethyl acetate and dried *in vacuo* (KOH).

The crude free nonapeptide was distributed through 250 counter-current transfers in the solvent system n-butanol–pyridine–acetic acid–water (4 : 2 : 1 : 7). The peptide was located by the Sakaguchi reaction,[15] in 0.2 ml samples. The main component (tubes 5—22) was collected and lyophilized to give 0.45 g of amorphous solid. T.l.c. showed the product to be homogeneous, R_F 0.35 (Bu^nOH–pyridine–AcOH–H_2O 32 : 24 : 8 : 20); R_F 0.12 (Bu^nOH–AcOH–EtOAc–H_2O 1 : 1 : 1 : 1). Paper electrophoresis at pH 6.5 showed one major negatively charged component and one small neutral component. $[\alpha]_D^{25}$ −60.7° (*c* 0.5, water). Amino-acid analysis: Asp, 1.01; Thr, 0.92; Glu, 2.03; Pro, 0.99; Gly, 1.00; Leu, 1.04; Arg, 1.98.

L-*Arginyl*-L-*glutamyl*-L-*leucyl*-L-*glutamyl*-L-*aspartyl*-L-*valyl*-L-*threonyl*-L-*prolyl*-L-*arginine*.—The protected nonapeptide (0.7 g) was mixed with 0.5 ml of anisole and 10 ml of distilled HF and stirred for 30 min, at 0 °C. Isolation and purification were carried out as described for the previous nonapeptide. The material in the major Sakaguchi-positive peak (tubes 7—25) was pooled and concentrated under reduced pressure, and the residue was freeze-dried from glacial acetic acid; yield 0.30 g of chromatographically-homogeneous material, R_F 0.37 in Bu^nOH–pyridine–AcOH–H_2O (32 : 24 : 8 : 30); R_F 0.14 in Bu^nOH–AcOH–EtOAc–H_2O (1 : 1 : 1 : 1). Paper electrophoresis at pH 6.5 showed one negatively charged component. $[\alpha]_D^{25}$ −78.3° (*c* 0.5, water). Amino-acid analysis: Asp, 0.97; Thr, 0.98; Glu, 2.07; Pro, 0.97; Val, 1.05; Leu, 1.00; Arg, 1.96.

Bioassay of Fragments.—The biological assay of growth hormone-like activity of the three nonapeptides by the tibia test was carried out by the method of Greenspan *et al.*[17]

Sprague–Dawley female rats (80—90 g), hypophysectomized 14 d previously, were injected intraperitoneally with the standard and the synthesized peptides once daily for 4 d. The reference standard used was bovine growth hormone (NIH-BGH-B17), with relative potency corresponding to 0.92 I.U. mg^{-1}. The results were analysed by the Student *t*-test.

We thank the C.N.R. (Rome) for support.

[7/1894 *Received*, 31*st October*, 1977]

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