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Studies on Peptides. CXIX.[1,2] Synthesis of Growth Hormone Releasing Factor (hpGRF-40–OH)

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The tetracontapeptide corresponding to the entire amino acid sequence of growth hormone releasing factor (hpGRF-40–OH) was synthesized by fragment condensation in solution, followed by deprotection with 1 M trifluoromethanesulfonic acid–thioanisole in trifluoroacetic acid. In *in vivo* assay, synthetic hpGRF-40–OH was as active as synthetic hpGRF-44–NH_2, but in *in vitro* assay, the potency of synthetic hpGRF-40–OH was only 36% of that of synthetic hpGRF-44–NH_2.

Keywords——human pancreatic tumor-derived GRF; hpGRF-40–OH solution synthesis; trifluoromethanesulfonic acid deprotection; thioanisole-mediated deprotection; *in vivo* assay of hpGRF-40–OH; *in vitro* assay of hpGRF-40–OH

hpGRF-40–OH is a tetracontapeptide isolated, together with hpGRF-44–NH_2 and hpGRF-37–OH, from human pancreatic islet tumor by Guillemin *et al.*[3-5] in 1982. Isolation of a peptide of the same size was simultaneously reported by Rivier *et al.*[6,7] Solid phase synthesis of this peptide was described in the preliminary papers.[3,6]

Following the synthesis of hpGRF-44–NH_2,[8-10] we wish to report the solution synthesis of hpGRF-40–OH, *via* the route illustrated in Fig. 1.

The C-terminal octapeptide, Z(OMe)–Glu(OBzl)–Ser–Asn–Gln–Glu(OBzl)–Arg(Mts)–Gly–Ala–OBzl [1], was now synthesized by starting from Z(OMe)–Gly–Ala–OBzl, as shown in Fig. 2. For chain elongation, N^{α}-deprotection with TFA was performed in the presence of anisole, prior to each condensation reaction as usual. The amino acid residues, Arg(Mts),[11] Glu(OBzl), Gln, Asn, Ser and Glu(OBzl), were successively introduced in a stepwise manner by the mixed anhydride[12] or the Np[13] or the azide[14] procedure. The resulting fragment [1] was purified by precipitation from DMF with EtOH and its purity was ascertained by thin layer chromatography (TLC), acid hydrolysis and elemental analysis.

Seven peptide fragments, [2] to [8] used for our previous synthesis of hpGRF-44–NH_2, were successively condensed by the azide procedure onto the TFA-treated sample of the above protected octapeptide ester [1]. The protected intermediates, obtained after condensations of fragments [2] to [4], were purified by precipitation from DMSO with MeOH and the other intermediates, including the protected GRF-40–OH, were purified by gel-filtration on Sephadex LH-60. Throughout this synthesis, Gly was taken as a diagnostic amino acid in acid hydrolysis. By comparison of the recovery of Gly with those of newly incorporated amino acids, satisfactory condensation of each fragment was confirmed (Table I).

The protected tetracontapeptide ester thus obtained was treated with 1 M TFMSA–thioanisole in TFA[15] in an ice-bath for 60 min to remove all protecting groups employed. This treatment was repeated 3 times under similar conditions to ensure complete depro-

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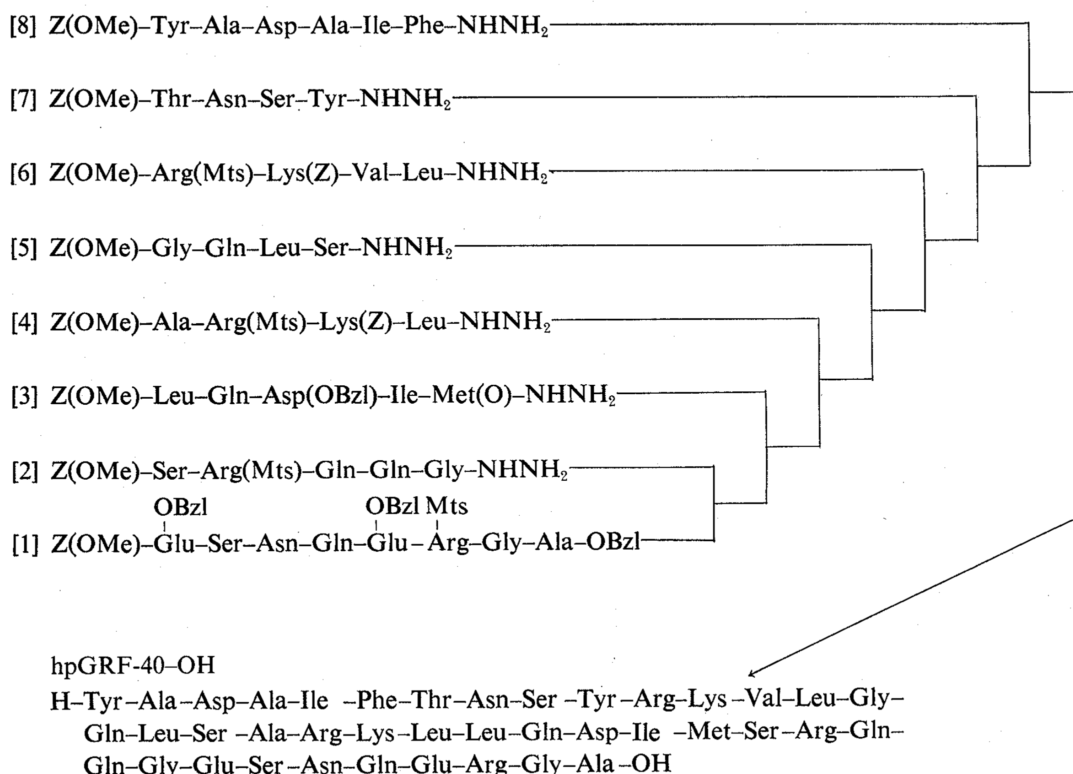

Fig. 1. Synthetic Route to hpGRF-40-OH

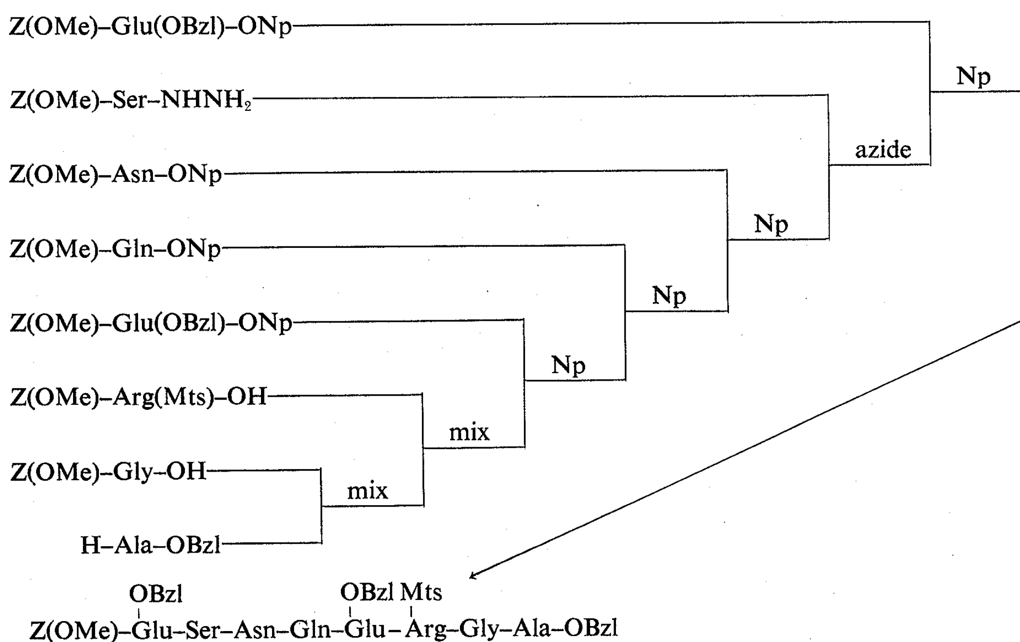

Fig. 2. Synthetic Scheme for the C-Terminal Octapeptide Ester, Z(OMe)-(hpGRF 33—40)-OBzl [1]

tection. The deprotected peptide was converted to the corresponding acetate by treatment with Amberlite CG-4B, treated with dil. ammonia to reverse N→O shift,[16] and then briefly incubated with dithiothreitol to reduce the Met(O) residue. Subsequent purification was carried out in essentially the same manner as described in the synthesis of GRF-44-NH_2, *i.e.*, by ion-exchange chromatography on CM-Biogel A and then preparative high performance

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Table I. Amino Acid Ratios in 6 N HCl Hydrolysates of Synthetic hpGRF-40–OH and Its Intermediates

	Protected peptide							Synthetic hpGRF-40–OH
	28—40	23—40	19—40	15—40	11—40	7—40	1—40	
Asp	0.99 (1)	1.90 (2)	1.89 (2)	1.96 (2)	1.96 (2)	2.92 (3)	3.97 (4)	3.96 (4)
Thr						0.97 (1)	0.95 (1)	0.98 (1)
Ser	1.78 (2)	1.76 (2)	1.78 (2)	2.56 (3)	2.62 (3)	3.46 (4)	3.54 (4)	3.56 (4)
Glu	4.88 (5)	5.77 (6)	5.89 (6)	6.88 (7)	6.98 (7)	6.71 (7)	6.68 (7)	6.88 (7)
Gly	2.00 (2)	2.00 (2)	2.00 (2)	3.00 (3)	3.00 (3)	3.00 (3)	3.00 (3)	3.00 (3)
Ala	0.96 (1)	0.97 (1)	1.95 (2)	2.00 (2)	2.00 (2)	1.99 (2)	4.34 (4)	4.19 (4)
Val					0.97 (1)	0.94 (1)	0.90 (1)	0.98 (1)
Met[a]		0.61 (1)	0.60 (1)	0.54 (1)	0.62 (1)	0.75 (1)	0.64 (1)	0.90 (1)
Ile		0.90 (1)	0.81 (1)	0.97 (1)	0.93 (1)	0.94 (1)	1.94 (2)	1.94 (2)
Leu		0.92 (1)	1.81 (2)	2.96 (3)	3.87 (4)	3.86 (4)	3.80 (4)	3.91 (4)
Tyr						0.95 (1)	2.14 (2)	1.95 (2)
Phe							1.05 (1)	1.04 (1)
Lys			0.88 (1)	0.96 (1)	1.83 (2)	1.88 (2)	1.98 (2)	1.95 (2)
Arg	1.91 (2)	1.98 (2)	2.94 (3)	3.00 (3)	4.12 (4)	4.00 (4)	3.68 (4)	3.68 (4)
Recovery	85%	83%	96%	75%	73%	85%	86%	77%

a) Met+Met(O).

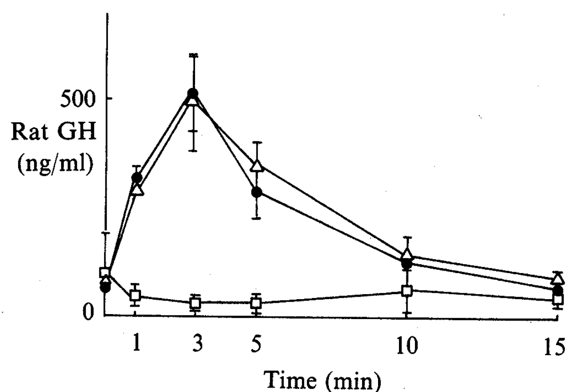

Fig. 3. *In Vivo* Assay of Synthetic hpGRF-40–OH

△—△, GRF (1—40)–OH (100 ng); ●—●, GRF (1—44)–NH_2 (100 ng); □—□, control (saline).

liquid chromatography (HPLC). The purity of the synthetic GRF-40–OH was assessed by TLC, HPLC, isoelectrofocusing (pH 6.5—9) and amino acid analyses after acid hydrolysis and enzymatic digestion.

When tested in anesthetized rats,[3a,4] our synthetic hpGRF-40–OH was as active as our synthetic hpGRF-44–NH_2, as shown in Fig. 3. However, in an *in vitro* system,[3a,5] the relative potency of synthetic hpGRF-40–OH to synthetic hpGRF-44–NH_2 (taken as 1) was only 0.36. The *in vivo* and *in vitro* activities of our synthetic hpGRF-40–OH are consistent with those reported for natural hpGRF.[4,5]

Experimental

General experimental methods employed in this work were essentially the same as described in Part LXXXVIII[17] of this series. HPLC was performed with a Waters 204 compact model. TLC was performed on silica gel (Kiesel gel G, Merck) and *Rf* values refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH–H_2O (8 : 3 : 1), Rf_2 $CHCl_3$–MeOH–AcOH (9 : 1 : 0.5), Rf_3 $CHCl_3$–MeOH (10 : 0.5), Rf_4 *n*-BuOH–AcOH–pyridine–H_2O (4 : 1 : 1 : 2), Rf_5 *n*-BuOH–AcOH–pyridine–H_2O (30 : 20 : 6 : 24). Leucine aminopeptidase was purchased from Sigma Chemical Co. (Lot. 62F-8000).

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Z(OMe)–Gly–Ala–OBzl——A mixed anhydride [prepared from 3.57 g (14.9 mmol) of Z(OMe)–Gly–OH] in DMF (30 ml) was added to an ice-chilled solution of H–Ala–OBzl [prepared from 5.0 g (14.9 mmol) of the tosylate] in DMF (30 ml) and the mixture was stirred in an ice-bath for 3 h. The solvent was removed by evaporation and the residue was dissolved in AcOEt. The organic phase was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O–NaCl, dried over Na_2SO_4 and evaporated. Trituration of the residue afforded a powder, which was recrystallized from THF and ether; yield 4.35 g (73%), mp 70—71 °C, $[\alpha]_D^{20}$ −20.3° (*c* = 1.3, DMF), *Rf*$_1$ 0.75, *Rf*$_2$ 0.89, *Rf*$_3$ 0.42. *Anal.* Calcd for $C_{21}H_{24}N_2O_6$: C, 62.99; H, 6.04; N, 7.00. Found: C, 62.74; H, 6.18; N, 6.88.

Z(OMe)–Arg(Mts)–Gly–Ala–OBzl——Z(OMe)–Gly–Ala–OBzl (7.70 g, 19.2 mmol) was treated with TFA–anisole (16.6 ml–4.2 ml) in an ice-bath for 60 min, then the TFA was removed by evaporation. The residue was washed with *n*-hexane, dried over KOH pellets *in vacuo* for 3 h and dissolved in DMF (30 ml) containing Et_3N (2.7 ml, 19.2 mmol). A mixed anhydride [prepared from 13.10 g (21.2 mmol) of Z(OMe)–Arg(Mts)–OH·CHA] in DMF (50 ml) was added to the above ice-chilled solution and the mixture was stirred in an ice-bath for 3 h. After evaporation of the solvent, the residue was purified by the same extraction procedure as described above followed by column chromatography on silica (4.3 × 30 cm) using $CHCl_3$–MeOH (10 : 0.5) as an eluant. The product was finally recrystallized from AcOEt and ether; yield 12.20 g (87%), mp 81—83 °C, $[\alpha]_D^{20}$ −5.8° (*c* = 2.1, DMF), *Rf*$_1$ 0.64, *Rf*$_2$ 0.51, *Rf*$_3$ 0.22. *Anal.* Calcd for $C_{36}H_{46}N_6O_9S$: C, 58.52; H, 6.28; N, 11.38. Found: C, 58.31; H, 6.25; N, 11.22.

Z(OMe)–Glu(OBzl)–Arg(Mts)–Gly–Ala–OBzl——Z(OMe)–Arg(Mts)–Gly–Ala–OBzl (5.0 g, 6.8 mmol) was treated with TFA–anisole (8.8 ml–2.2 ml) and the N^α-deprotected peptide isolated as stated above was dissolved in DMF (20 ml) together with Et_3N (1.9 ml, 13.5 mmol) and Z(OMe)–Glu(OBzl)–ONp (3.5 g, 6.8 mmol). After being stirred for 14 h, the solution was neutralized with AcOH and the solvent was removed by evaporation. Trituration of the residue with ether and 5% citric acid afforded a powder, which was washed with 5% $NaHCO_3$, 5% citric acid and H_2O and precipitated from DMF with 2-propanol; yield 4.50 g (69%), mp 100—101 °C, $[\alpha]_D^{20}$ −5.4° (*c* = 1.8, DMF), *Rf*$_1$ 0.73, *Rf*$_2$ 0.60, *Rf*$_3$ 0.27. *Anal.* Calcd for $C_{48}H_{59}N_7O_{12}S$: C, 60.17; H, 6.21; N, 10.23. Found: C, 59.97; H, 6.04; N, 10.09.

Z(OMe)–Gln–Glu(OBzl)–Arg(Mts)–Gly–Ala–OBzl——Z(OMe)–Glu(OBzl)–Arg(Mts)–Gly–Ala–OBzl (5.50 g, 5.7 mmol) was treated with TFA–anisole (9.9 ml–2.5 ml) as stated above, and then dry ether was added. The resulting powder was washed with ether, dried over KOH pellets *in vacuo* for 3 h and dissolved in DMF (20 ml) together with Et_3N (1.6 ml, 11.4 mmol) and Z(OMe)–Gln–ONp (2.48 g, 5.7 mmol). After being stirred for 14 h, the solution was neutralized with AcOH and concentrated. The residue was triturated with ether and 5% citric acid and the resulting powder was purified by washing with base and acid as stated above, and precipitated from DMF with 2-propanol: yield 5.45 g (87%), mp 126—127 °C, $[\alpha]_D^{20}$ −6.0° (*c* = 1.3, DMF), *Rf*$_1$ 0.60, *Rf*$_2$ 0.41. *Anal.* Calcd for $C_{53}H_{67}N_9O_{14}S$: C, 58.60; H, 6.22; N, 11.61. Found: C, 58.40; H, 6.28; N, 11.48.

Z(OMe)–Asn–Gln–Glu(OBzl)–Arg(Mts)–Gly–Ala–OBzl——Z(OMe)–Gln–Glu(OBzl)–Arg(Mts)–Gly–Ala–OBzl (5.45 g, 5.0 mmol) was treated with TFA–anisole (10.8 ml–2.7 ml) and the N^α-deprotected peptide isolated as stated above was dissolved in DMF (30 ml) together with Et_3N (1.4 ml, 10.0 mmol) and Z(OMe)–Asn–ONp (2.51 g, 6.0 mmol). After being stirred for 14 h, the solution was neutralized with AcOH and concentrated. The residue was triturated with ether and 5% citric acid and the resulting powder was purified by washing with base and acid as stated above and precipitated from DMF with EtOH; yield 5.21 g (86%), mp 197—199 °C, $[\alpha]_D^{20}$ −1.7° (*c* = 0.6, DMSO), *Rf*$_1$ 0.69. Amino acid ratios in 6 N HCl hydrolysate: Asp 1.02, Glu 2.02, Gly 1.00, Ala 0.99, Arg 0.96 (recovery of Gly 75%). *Anal.* Calcd for $C_{57}H_{73}N_{11}O_{16}S$: C, 57.03; H, 6.13; N, 12.84. Found: C, 57.11; H, 6.13; N, 13.03.

Z(OMe)–Ser–Asn–Gln–Glu(OBzl)–Arg(Mts)–Gly–Ala–OBzl——Z(OMe)–Asn–Gln–Glu(OBzl)–Arg(Mts)–Gly–Ala–OBzl (3.50 g, 2.9 mmol) was treated with TFA–anisole (6.3 ml–1.6 ml) and the N^α-deprotected peptide isolated as stated above was dissolved in DMF–DMSO (1 : 1, 20 ml) containing Et_3N (0.4 ml, 2.9 mmol). The azide [prepared from 1.24 g (4.4 mmol) of Z(OMe)–Ser–$NHNH_2$] in DMF (2 ml) and Et_3N (0.7 ml, 5.3 mmol) were added to the above ice-chilled solution and the mixture, after being stirred at 4 °C for 14 h, was concentrated. The residue was triturated with AcOEt and 5% citric acid and the resulting powder was purified by washing with base and acid as stated above, followed by precipitation from DMF with EtOH; yield 3.40 g (90%), mp 194—197 °C, $[\alpha]_D^{20}$ −9.7° (*c* = 2.7, DMSO), *Rf*$_1$ 0.55. Amino acid ratios in 6 N HCl hydrolysate: Asp 0.97, Ser 0.88, Glu 1.95, Gly 1.00, Ala 0.93, Arg 0.89 (recovery of Gly 83%). *Anal.* Calcd for $C_{60}H_{78}N_{12}O_{18}S$: C, 55.97; H, 6.11; N, 13.06. Found: C, 56.01; H, 6.13; N, 13.10.

Z(OMe)–Glu(OBzl)–Ser–Asn–Gln–Glu(OBzl)–Arg(Mts)–Gly–Ala–OBzl——The above heptapeptide ester (3.40 g, 2.6 mmol) was treated with TFA–anisole (6.9 ml–1.7 ml) and the N^α-deprotected peptide isolated as stated above was dissolved in DMF–DMSO (1 : 1, 20 ml) together with Et_3N (0.7 ml, 5.2 mmol) and Z(OMe)–Glu(OBzl)–ONp (1.52 g, 2.9 mmol). After being stirred for 14 h, the solution was neutralized with AcOH and concentrated. The residue was triturated with ether and 5% citric acid. The resulting powder was washed with base and acid as stated above and precipitated from DMF with EtOH; yield 3.35 g (84%), mp 207—210 °C, $[\alpha]_D^{20}$ −1.9° (*c* = 0.5, DMSO), *Rf*$_1$ 0.70. Amino acid ratios in 6 N HCl hydrolysate: Asp 1.01, Ser 0.94, Glu 3.01, Gly 1.00, Ala 1.02, Arg 0.99 (recovery of Gly 85%). *Anal.* Calcd for $C_{72}H_{91}N_{13}O_{21}S$: C, 57.39; H, 6.09; N, 12.09. Found: C, 57.22; H, 6.07; N, 11.90.

Z(OMe)–Ser–Arg(Mts)–Gln–Gln–Gly–Glu(OBzl)–Ser–Asn–Gln–Glu(OBzl)–Arg(Mts)–Gly–Ala–OBzl——The above protected octapeptide ester (1.0 g, 0.66 mmol) was treated with TFA–anisole (2.0 ml–0.5 ml) and the N^α-

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deprotected peptide isolated as stated above was dissolved in DMSO–DMF (1 : 1, 10 ml) containing Et_3N (93 μl, 0.66 mmol). The azide [prepared from 931 mg (1.0 mmol) of Z(OMe)–Ser–Arg(Mts)–Gln–Gln–Gly–$NHNH_2$] in DMSO–DMF (1 : 1, 15 ml) and Et_3N (167 μl, 1.19 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4 °C overnight. After addition of a few drops of AcOH, the solvent was removed by evaporation and the residue was triturated with AcOEt and 5% citric acid. The resulting powder was washed with base and acid as described above and precipitated from DMSO with MeOH; yield 1.40 g (94%), mp 245 °C (dec.), $[\alpha]_D^{20}$ +2.1 ° (c=0.5, DMSO), Rf_4 0.71. *Anal.* Calcd for $C_{102}H_{137}N_{23}O_{31}S_2 \cdot H_2O$: C, 54.12; H, 6.19; N, 14.23. Found: C, 54.15; H, 6.22; N, 13.95.

Z(OMe)–Leu–Gln–Asp(OBzl)–Ile–Met(O)–Ser–Arg(Mts)–Gln–Gln–Gly–Glu(OBzl)–Ser–Asn–Gln–Glu(OBzl)–Arg(Mts)–Gly–Ala–OBzl——The above protected tridecapeptide ester (1.40 g, 0.62 mmol) was treated with TFA–anisole (2.7 ml–0.7 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMSO–DMF (1 : 1, 10 ml) containing Et_3N (87 μl, 0.62 mmol). The azide [prepared from 1.13 g (1.25 mmol) of Z(OMe)–Leu–Gln–Asp(OBzl)–Ile–Met(O)–$NHNH_2$] in DMSO–DMF (1 : 1, 30 ml) and Et_3N (209 μl, 1.50 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4 °C overnight. After addition of a few drops of AcOH, the solvent was removed by evaporation and the residue was treated with 5% citric acid. The resulting powder was purified by the procedure as described above followed by precipitation from DMSO with MeOH; yield 1.56 g (85%), mp 265 °C (dec.), $[\alpha]_D^{20}$ −9.3 ° (c=1.5, DMSO), Rf_4 0.90. *Anal.* Calcd for $C_{135}H_{189}N_{29}O_{40}S_3 \cdot H_2O$: C, 54.59; H, 6.41; N, 13.68. Found: C, 54.37; H, 6.38; N, 13.86.

Z(OMe)–Ala–Arg(Mts)–Lys(Z)–Leu–Leu–Gln–Asp(OBzl)–Ile–Met(O)–Ser–Arg(Mts)–Gln–Gln–Gly–Glu-(OBzl)–Ser–Asn–Gln–Glu(OBzl)–Arg(Mts)–Gly–Ala–OBzl——The above protected octadecapeptide ester (1.56 g, 0.53 mmol) was treated with TFA–anisole (3.4 ml–0.9 ml) and the N^{α}-deprotected peptide isolated as described above was dissolved in DMSO–DMF (1 : 1, 15 ml) containing Et_3N (73 μl, 0.53 mmol). The azide [prepared from 1.03 g (1.05 mmol) of Z(OMe)–Ala–Arg(Mts)–Lys(Z)–Leu–$NHNH_2$] in DMF (5 ml) and Et_3N (176 μl, 1.26 mmol) were added to the above ice-chilled solution and the mixture, after being stirred at 4 °C overnight, was neutralized with AcOH and concentrated. Trituration of the residue with 5% citric acid and ether afforded a powder, which was purified by the washing procedure as described above, followed by precipitation twice from DMSO with MeOH; yield 1.14 g (58%), mp 270 °C (dec.), $[\alpha]_D^{20}$ −14.0 ° (c=1.4, DMSO), Rf_1 0.36. *Anal.* Calcd for $C_{173}H_{243}N_{37}O_{48}S_4$: C, 55.60; H, 6.55; N, 13.87. Found: C, 55.52; H, 6.69; N, 14.03.

Z(OMe)–Gly–Gln–Leu–Ser–Ala–Arg(Mts)–Lys(Z)–Leu–Leu–Gln–Asp(OBzl)–Ile–Met(O)–Ser–Arg(Mts)–Gln–Gln–Gly–Glu(OBzl)–Ser–Asn–Gln–Glu(OBzl)–Arg(Mts)–Gly–Ala–OBzl——The above protected docosapeptide ester (1.14 g, 0.31 mmol) was treated with TFA–anisole (2.4 ml–0.6 ml) and the N^{α}-deprotected peptide isolated as described above was dissolved in DMSO–DMF (1 : 1, 10 ml) containing Et_3N (42 μl, 0.31 mmol). The azide [prepared from 0.44 g (0.76 mmol) of Z(OMe)–Gly–Gln–Leu–Ser–$NHNH_2$] in DMF–DMSO (1 : 1, 20 ml) and Et_3N (127 μl, 0.92 mmol) were added and the mixture, after being stirred at 4 °C overnight, was neutralized with AcOH and concentrated. The residue was treated with H_2O. The resulting powder was purified by gel-filtration on Sephadex LH-60 (3 × 128 cm) with DMF. The ultraviolet (UV) absorption at 280 nm was determined in each fraction (11 ml) and the desired fractions containing a substance of Rf_1 0.40 were combined. The solvent was removed by evaporation and the residue was treated with ether to afford a powder; yield 1.08 g (86%), mp 260 °C (dec.), $[\alpha]_D^{20}$ −24.3 ° (c=0.7, DMSO), Rf_1 0.40, Rf_4 0.84. *Anal.* Calcd for $C_{189}H_{270}N_{42}O_{54}S_4 \cdot 7H_2O$: C, 53.42; H, 6.74; N, 13.85. Found: C, 53.28; H, 6.71; N, 14.09.

Z(OMe)–Arg(Mts)–Lys(Z)–Val–Leu–Gly–Gln–Leu–Ser–Ala–Arg(Mts)–Lys(Z)–Leu–Leu–Gln–Asp(OBzl)–Ile–Met(O)–Ser–Arg(Mts)–Gln–Gln–Gly–Glu(OBzl)–Ser–Asn–Gln–Glu(OBzl)–Arg(Mts)–Gly–Ala–OBzl——The above protected hexacosapeptide ester (1.08 g, 0.26 mmol) was treated with TFA–anisole (2.0 ml–0.5 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMSO–DMF (1 : 1, 10 ml) containing Et_3N (36 μl, 0.26 mmol). The azide [prepared from 0.79 g (0.79 mmol) of Z(OMe)–Arg(Mts)–Lys(Z)–Val–Leu–$NHNH_2$] in DMF (2 ml) and Et_3N (131 μl, 0.94 mmol) were added to the above ice-chilled solution and the mixture, after being stirred at 4 °C overnight, was neutralized with AcOH and concentrated. The residue was treated with H_2O and the resulting powder was purified by gel-filtration on Sephadex LH-60 as described above followed by precipitation from DMF with ether; yield 0.98 g (76%), mp 280 °C (dec.), $[\alpha]_D^{20}$ −12.9 ° (c=1.2, DMSO), Rf_1 0.47. *Anal.* Calcd for $C_{229}H_{330}N_{50}O_{62}S_5 \cdot 4H_2O$: C, 54.92; H, 6.80; N, 13.99. Found: C, 54.63; H, 6.86; N, 14.23.

Z(OMe)–Thr–Asn–Ser–Tyr–Arg(Mts)–Lys(Z)–Val–Leu–Gly–Gln–Leu–Ser–Ala–Arg(Mts)–Lys(Z)–Leu–Leu–Gln–Asp(OBzl)–Ile–Met(O)–Ser–Arg(Mts)–Gln–Gln–Gly–Glu(OBzl)–Ser–Asn–Gln–Glu(OBzl)–Arg(Mts)–Gly–Ala–OBzl——The above protected triacontapeptide ester (980 mg, 0.20 mmol) was treated with TFA–anisole (5.0 ml–0.5 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMSO–DMF (1 : 1, 10 ml) containing Et_3N (28 μl, 0.2 mmol). The azide [prepared from 395 mg (0.60 mmol) of Z(OMe)–Thr–Asn–Ser–Tyr–$NHNH_2$] in DMSO–DMF (1 : 1, 5 ml) and Et_3N (100 μl, 0.72 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4 °C overnight. After addition of a few drops of AcOH, the solution was concentrated and the residue was treated with 5% citric acid. The resulting powder was purified by gel-filtration on Sephadex LH-60 as described above followed by precipitation from DMF with ether; yield 806 mg (75%), mp 260 °C (dec.), $[\alpha]_D^{20}$ −12.2 ° (c=0.9, DMSO), Rf_1 0.55. *Anal.* Calcd for $C_{249}H_{357}N_{55}O_{70}S_5 \cdot 4H_2O$: C, 54.64; H, 6.72; N, 14.08. Found: C, 54.46;

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H, 6.70; N, 14.17.

Z(OMe)–Tyr–Ala–Asp–Ala–Ile–Phe–Thr–Asn–Ser–Tyr–Arg(Mts)–Lys(Z)–Val–Leu–Gly–Gln–Leu–Ser–Ala–Arg(Mts)–Lys(Z)–Leu–Leu–Gln–Asp(OBzl)–Ile–Met(O)–Ser–Arg(Mts)–Gln–Gln–Gly–Glu(OBzl)–Ser–Asn–Gln–Glu(OBzl)–Arg(Mts)–Gly–Ala–OBzl——The above protected tetratriacontapeptide ester (806 mg, 0.15 mmol) was treated with TFA–anisole (5 ml–0.4 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMSO–DMF (1 : 1, 10 ml) containing Et_3N (21 μl, 0.15 mmol). The azide [prepared from 392 mg (0.45 mmol) of Z(OMe)–Tyr–Ala–Asp–Ala–Ile–Phe–$NHNH_2$] in DMSO–DMF (1 : 1, 5 ml) and Et_3N (137 μl, 0.98 mmol) were added to the above ice-chilled solution and the mixture, after being stirred at 4 °C overnight, was neutralized with AcOH and concentrated. Treatment of the residue with H_2O afforded a powder, which was purified by gel-filtration as described above followed by precipitation from DMF with ether; yield 740 mg (82%), mp 162—166 °C, $[\alpha]_D^{20}$ $-9.1°$ (c=1.1, DMSO), Rf_1 0.25. *Anal.* Calcd for $C_{283}H_{401}N_{61}O_{79}S_5$: C, 55.88; H, 6.65; N, 14.05. Found: C, 55.40; H, 6.64; N, 13.96.

H–Tyr–Ala–Asp–Ala–Ile–Phe–Thr–Asn–Ser–Tyr–Arg–Lys–Val–Leu–Gly–Gln–Leu–Ser–Ala–Arg–Lys–Leu–Leu–Gln–Asp–Ile–Met–Ser–Arg–Gln–Gln–Gly–Glu–Ser–Asn–Gln–Glu–Arg–Gly–Ala–OH——The above protected tetracontapeptide ester (100 mg, 16.5 μmol) was treated with 1 M TFMSA–thioanisole in TFA (3.6 ml) in the presence of *m*-cresol (94 μl, 55 eq) in an ice-bath for 60 min and dry ether was added. The resulting powder was collected by centrifugation and dried over KOH pellets *in vacuo* for 30 min. This treatment was repeated twice more to ensure complete deprotection. The deprotected peptide thus obtained was dissolved in H_2O (2 ml) containing dithiothreitol (126 mg) and treated with Amberlite CG-4B (acetate form, approximately 1 g) for 30 min with stirring. The solution was filtered. The pH of the filtrate was adjusted to 8.0 with 3% NH_4OH and, after being stirred for 30 min in an ice-bath, the filtrate was readjusted to 6.5 with 1 N AcOH. The solution was lyophilized to give a hygroscopic powder, which was incubated with dithiothreitol (252 mg, 100 eq) in H_2O (2 ml) under an argon atmosphere at 37 °C for 12 h. The solution was applied to a column of Sephadex G-25 (1.8 × 68 cm), which was eluted with 0.5 N AcOH. The UV absorption at 280 nm was determined in each fraction (3 ml each). The fractions corresponding to the front main peak (tube Nos. 10—17) were combined and the solvent was removed by lyophilization to give a white powder; yield 71.9 mg (93%).

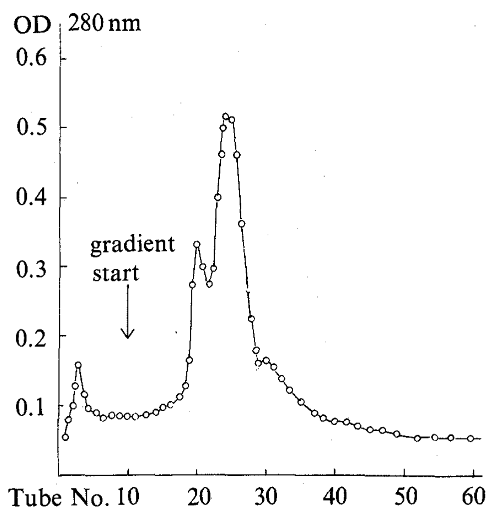

Fig. 4. Purification of Crude hpGRF-40–OH by Ion-Exchange Chromatography on CM-Biogel A

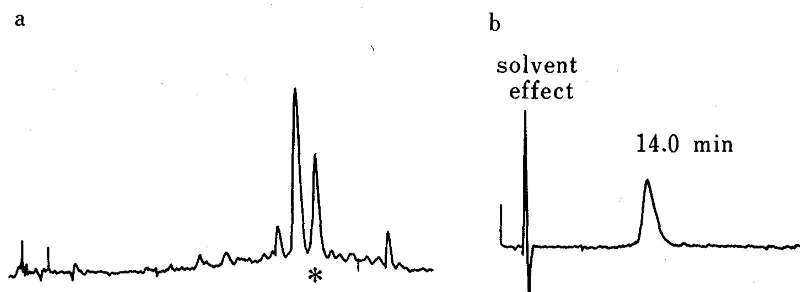

Fig. 5. HPLC of hpGRF-40–OH

a: purification of CM-purified hpGRF-40–OH.
b: purified hpGRF-40–OH.
The marked peak (*) seems to be due to a mixture of partially deprotected Arg(Mts)-derivatives, because enzymatic digestion gave a low recovery of Arg; 3.25 (theory 4).

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The crude product (37.6 mg) was dissolved in pH 4.8, 0.01 M NH_4OAc buffer and the solution was applied to a column of CM-Biogel A (1 × 12.5 cm), which was first eluted with the same buffer and then with a linear gradient formed from the starting buffer (150 ml) and pH 6.5, 0.2 M NH_4OAc buffer (150 ml). The UV absorption in each fraction (4.8 ml each) was determined (Fig. 4). The fractions corresponding to the main peak (tube Nos. 22—29) were combined and the solvent and ammonium salt were removed by repeated lyophilization to give a white fluffy powder. The rest of the crude sample (34.3 mg) was similarly purified; total yield 34.2 mg (44% from the protected peptide).

Next, a part of the CM-purified sample (5 mg each) was purified by reverse phase HPLC on a Cosmosil $5C_{18}$ column (1.0 × 25.0 cm, Nakarai Chemical Co.) using gradient elution with acetonitrile (from 30 to 35% in 40 min) in 0.1% TFA at a flow rate of 2.5 ml/min (Fig. 5a). The rest of the sample was similarly purified and the combined eluates corresponding to the main peak (retention time 28.03 min) were concentrated *in vacuo* at 30 °C. The residue was dissolved in pH 7.5, 1 M $Et_3N \cdot AcOH$ buffer (2 ml) and the solution was desalted by gel-filtration on Sephadex G-25 (2 × 40 cm), using 0.5 N AcOH as the eluant.

The desired fractions (tube Nos. 9—15) were collected and the solvent was removed by lyophilization to give a white fluffy powder; yield 18.6 mg (24% from the protected tetracontapeptide), $[\alpha]_D^{20}$ −64.6 ° (c=0.4, 0.2 N AcOH), Rf_4 0.32, Rf_5 0.16. The synthetic peptide exhibited a single peak on HPLC using a μBondapak C_{18} column (0.39 × 30 cm) at a retention time of 14.0 min, when eluted with a gradient of acetonitrile (30 to 35% in 20 min) in 0.1% TFA at a flow rate of 1.0 ml per min (Fig. 5b), and a single band in disk isoelectrofocusing on 7.5% polyacrylamide gel (0.5 × 6.7 cm) containing Pharmalyte (pH 6.5—9.0): mobility 5.9 cm from the origin toward the cathodic end of the gel, after running at 200 V for 4 h. Amino acid ratios in 6 N HCl hydrolysate are shown in Table I. Amino acid ratios in leucine-aminopeptidase digest: 2Asp 1.98, 4Ser 3.93, 2Glu 2.01, 3Gly 3.00, 4Ala 4.13, 1Val 1.01, 1Met 0.97, 2Ile 1.95, 4Leu 3.99, 2Tyr 2.07, 1Phe 1.14, 2Lys 2.04, 4Arg 3.76, 2Asn, 1Thr and 5Gln were not determined (recovery of Gly 74%). *Anal.* Calcd for $C_{194}H_{317}N_{61}O_{63}S \cdot 6CH_3COOH \cdot 20H_2O$: C, 46.99; H, 7.29; N, 16.23. Found: C, 46.68; H, 6.93; N, 16.51.

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References and Notes

1) Part CXVIII: N. Fujii, W. Lee, M. Shimokura, and H. Yajima, *Chem. Pharm. Bull.*, **32**, 739 (1984).
2) Amino acids and peptide derivatives mentioned in this investigation are of the L-configuration. The following abbreviations are used: Z=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, Bzl=benzyl, Mts=mesitylene-2-sulfonyl, Np=*p*-nitrophenyl, CHA=cyclohexylamine, TFA=trifluoroacetic acid, DMF=dimethylformamide, DMSO=dimethylsulfoxide, TFMSA=trifluoromethanesulfonic acid.
3) *a*) R. Guillemin, P. Brazeau, P. Böhlen, F. Esch, N. Ling, and W. B. Wehrenberg, *Science*, **218**, 585 (1982); *b*) F. S. Esch, P.Böhlen, N. C. Ling, P. E. Brazeau, W. B. Wehrenberg, M. O. Thorner, M. J. Cronin, and R. Guillemin, *Biochem. Biophys. Res. Commun.*, **109**, 152 (1982); *c*) F. S. Esch, P. Böhlen, N. C. Ling, P. E. Brazeau, W. B. Wehrenberg, and R. Guillemin, *J. Biol. Chem.*, **258**, 1806 (1983).
4) W. B. Wehrenberg, N. Ling, P. Brazeau, F. Esch, P. Böhlen, A. Baird, S. Ying, and R. Guillemin, *Biochem. Biophys. Res. Commun.*, **109**, 382 (1982).
5) P. Brazeau, N. Ling, P. Böhlen, F. Esch, S.-Y. Ying, and R. Guillemin, *Proc. Natl. Acad. Sci. U.S.A.*, **79**, 7909 (1982).
6) J. Rivier, J. Spiess, M. Thorner, and W. Vale, *Nature* (London), **300**, 276 (1982).
7) J. Spiess, J. Rivier, M. Thorner, and W. Vale, *Biochemistry*, **21**, 6037 (1982).
8) H. Yajima, N. Fujii, M. Shimokura, K. Akaji, S. Kiyama, and M. Nomizu, *Chem. Pharm. Bull.*, **31**, 1800 (1983).
9) N. Fujii, M. Shimokura, K. Akaji, S. Kiyama, and H. Yajima, *Chem. Pharm. Bull.*, **32**, 510 (1984).
10) N. Fujii, M. Shimokura, M. Nomizu, H. Yajima, F. Shono, and A. Yoshitake, *Chem. Pharm. Bull.*, **32**, 520 (1984).
11) H. Yajima, M. Takeyama, J. Kanaki, and K. Mitani, *J. Chem. Soc., Chem. Commun.*, **1978**, 482.
12) Th. Wieland and H. Bernhard, *Justus Liebigs Ann. Chem.*, **572**, 190 (1951); R. A. Boissonnas, *Helv. Chim. Acta*, **34**, 874 (1951); J. R. Vaughan, Jr. and R. L. Osato, *J. Am. Chem. Soc.*, **74**, 676 (1952).
13) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).
14) J. Honzl and J. Rudinger, *Collect. Czech. Chem. Commun.*, **26**, 2333 (1961).
15) H. Yajima and N. Fujii, *J. Am. Chem. Soc.*, **103**, 5867 (1981).
16) S. Sakakibara, "Chemistry and Biochemistry of Amino Acids, Peptides and Proteins," Vol. 1, ed. by B. Weinstein, Academic Press, Inc., New York, 1971, p. 51.
17) N. Fujii and H. Yajima, *J. Chem. Soc., Perkin Trans. 1*, **1981**, 789.

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