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Studies on Peptides. CXX.[1,2] Synthesis of Growth Hormone Releasing Factor (GRF-37–NH_2) and N^{α}-Biotinyl-GRF-44–NH_2

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The amide form of the heptatriacontapeptide corresponding to the entire amino acid sequence of one of the human pancreatic tumor-derived peptides, termed growth hormone releasing factor (hpGRF), was synthesized in a conventional manner using a thioanisole-mediated deprotection procedure. In *in vivo* assay, this peptide (hpGRF-37–NH_2) was as active as the synthetic tetratetracontapeptide amide (hpGRF-44–NH_2). However, in *in vitro* assay, the potency of synthetic hpGRF-37–NH_2 was only 15% of that of synthetic hpGRF-44–NH_2. In addition, N^{α}-biotinyl-GRF-44–NH_2 was prepared for histochemical receptor studies. This GRF-derivative exhibited approximately 60% of the activity of hpGRF-44–NH_2.

Keywords——GRF-37–NH_2 synthesis; *in vivo* activity of GRF-37–NH_2; *in vitro* activity of GRF-37–NH_2; N^{α}-biotinyl-GRF-44–NH_2; thioanisole-mediated deprotection; trifluoromethanesulfonic acid deprotection

hpGRF-37–OH is the smallest of the human pancreatic tumor-derived peptides[3,4] termed growth hormone releasing factor (hpGRF) or somatocrinin.[3c] Solid phase synthesis of this peptide and its amide were preliminarily reported by Guillemin *et al.*[3d]

Following the solution syntheses of two larger forms of GRFs (hpGRF-44–NH_2[5] and hpGRF-40–OH[1]), we next synthesized the amide form of hpGRF-37 in a conventional manner. In the case of small biologically active peptides, peptide amides generally exhibit much higher activity than the peptides with the free C-terminus, and so we also synthesized N^{α}-biotinyl-hpGRF-44–NH_2 as a useful derivative for future histochemical studies.

Synthesis of hpGRF-37–NH_2 was carried out according to the scheme illustrated in Fig. 1. Eight peptide fragments and two amino acid Np esters served to construct this entire peptide backbone. Of these, fragments [2], [4], [5], [6], [7] and [8] were the same fragments as used for our previous synthesis of hpGRF-44–NH_2.[5] In the previous synthesis, Z(OMe)–Leu–Gln–Asp(OBzl)–Ile–Met(O)–$NHNH_2$ was selected as one unit. This hydrazide is less soluble in DMF, compared to the other hydrazides employed. Because of a technical problem which we encountered, we decided to introduce the corresponding segment in three steps, *i.e.*, the azide condensation of Z(OMe)–Asp(OBzl)–Ile–Met(O)–$NHNH_2$, then successive Np introductions[6] of the Gln and Leu residues.

The necessary C-terminal pentapeptide, Z(OMe)–Glu(OBzl)–Ser–Asn–Gln–Glu(OBzl)–NH_2 [1], was prepared according to the scheme illustrated in Fig. 2. First, Z(OMe)–Glu(OBzl)–OH was amidated by the Np ester procedure. To identify the product, the protecting groups were removed by TFA treatment followed by hydrogenolysis. Physical constants (mp, rotation) and infrared (IR) spectra of the resulting amino acid agreed well with those of an authentic sample of isoglutamine. The N^{α}-protecting group of Z(OMe)–

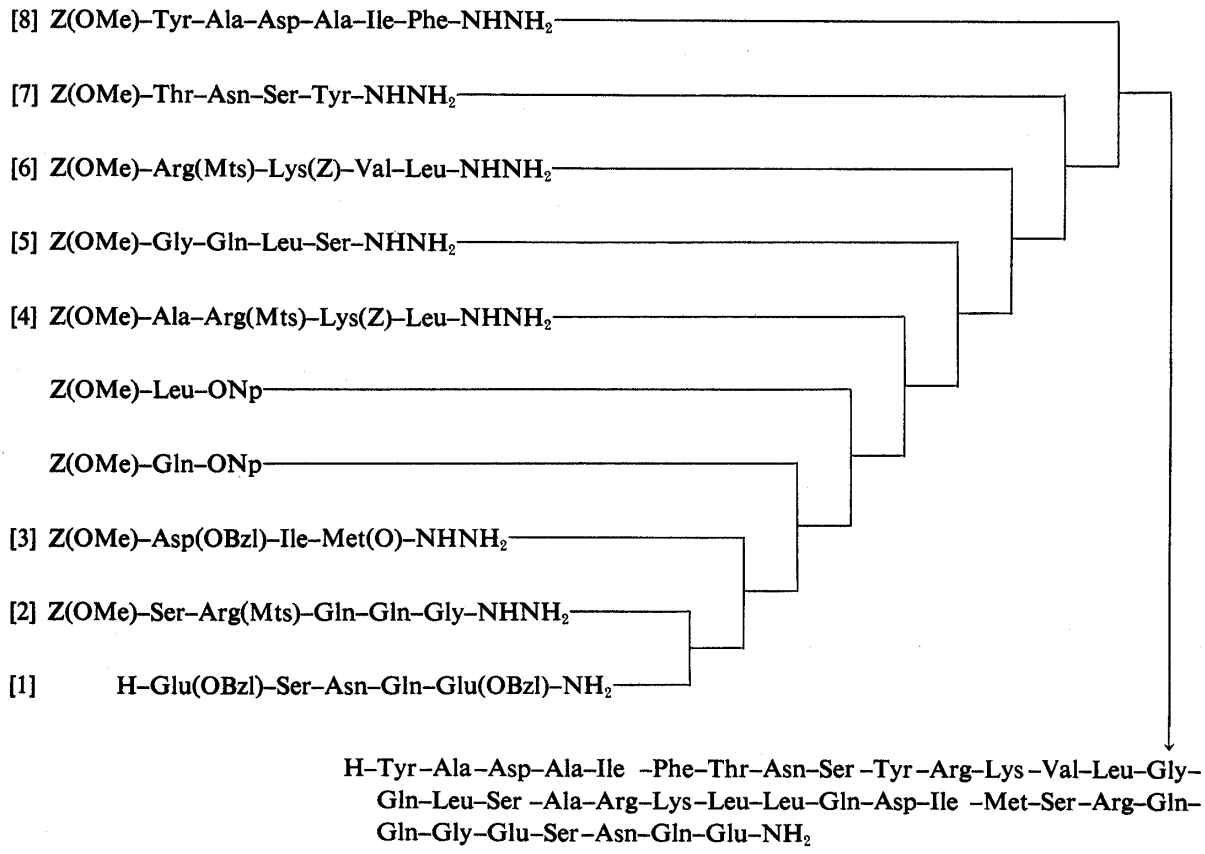

Fig. 1. Synthetic Route to hpGRF-37–NH_2

Glu(OBzl)–NH_2 was removed by TFA treatment in the presence of anisole as usual, then the chain was elongated in a stepwise manner by successive Np introductions of the appropriate amino acid residues, except for the Ser residue, which was introduced by the azide procedure.[7] The purity of the resulting pentapeptide amide [1] was confirmed by thin layer chromatography (TLC), elemental analysis and amino acid analysis.

Another necessary fragment, Z(OMe)–Asp(OBzl)–Ile–Met(O)–$NHNH_2$ [3], was derived from the corresponding Troc-derivative[5b] by treatment with Zn–AcOH.[8]

Eight peptide fragments thus prepared and two amino acid derivatives were successively assembled according to the route illustrated in Fig. 1. Throughout this synthesis, Gly was selected as a diagnostic amino acid in acid hydrolysis. By comparison of the recovery of Gly with those of newly incorporated amino acids after each condensation reaction, satisfactory incorporation of each fragment was confirmed as shown in Table I.

Deprotection of the protected heptatriacontapeptide amide and subsequent purification of the deprotected product were carried out in essentially the same manner as described in the synthesis of hpGRF-44–NH_2, *i.e.*, treatment with 1 M TFMSA–thioanisole/TFA,[9] conversion to the corresponding acetate by Amberlite CG-4B treatment, dil. ammonia treatment for conversion of possible N→O shift,[10] reduction of a small amount of remaining Met(O) with DTT, gel-filtration on Sephadex G-25, ion-exchange chromatography on CM-Biogel A, and finally preparative high performance liquid chromatography (HPLC). The purity of the final product was confirmed by TLC, HPLC, disk isoelectrophoresis, and amino acid analyses after acid hydrolysis and leucine aminopeptidase digestion. The content of isoglutamine was estimated as glutamic acid due to α-deamination by the enzyme.

No significant difference in *in vivo* activity[3a] was observed between hpGRF-37–NH_2 and hpGRF-44–NH_2 as shown in Fig. 3. However, in *in vitro* assay,[3a] the relative potency of

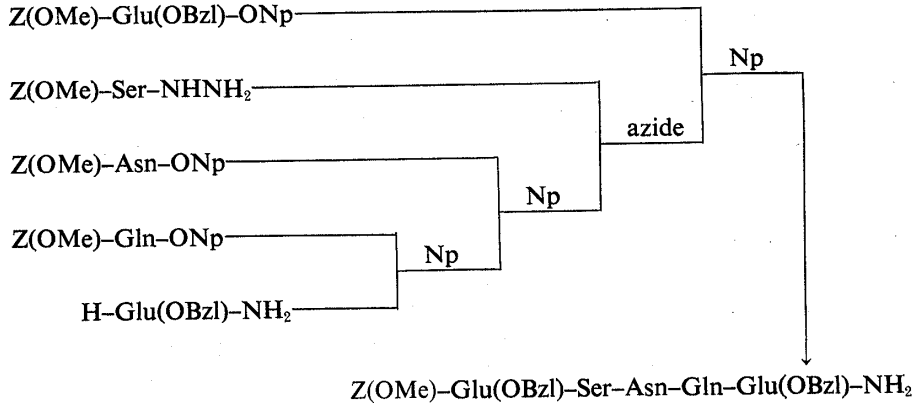

Fig. 2. Synthetic Scheme for the Protected Pentapeptide Amide, Z(OMe)–(hpGRF-33—37)–NH_2

TABLE I. Amino Acid Ratios in 6 N HCl Hydrolysates of hpGRF-37–NH_2 and Its Intermediates

	Protected peptide									Synthetic GRF-37–NH_2
	28—37	25—37	24—37	23—37	19—37	15—37	11—37	7—37	1—37	
Asp	1.00 (1)	2.00 (2)	1.98 (2)	2.01 (2)	2.07 (2)	1.86 (2)	1.89 (2)	2.86 (3)	3.74 (4)	3.92 (4)
Thr								0.95 (1)	0.85 (1)	0.97 (1)
Ser	1.84 (2)	1.81 (2)	1.82 (2)	1.84 (2)	1.78 (2)	2.53 (3)	2.47 (3)	3.39 (4)	3.24 (4)	3.76 (4)
Glu	4.91 (5)	4.97 (5)	5.90 (6)	6.03 (6)	6.10 (6)	6.75 (7)	6.51 (7)	6.54 (7)	6.46 (7)	6.89 (7)
Gly	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	2.00 (2)	2.00 (2)	2.00 (2)	2.00 (2)	2.00 (2)
Ala					1.20 (1)	1.00 (1)	0.93 (1)	0.95 (1)	3.02 (3)	3.06 (3)
Val							0.98 (1)	0.97 (1)	1.08 (1)	0.83 (1)
Met[a)]		0.65 (1)	0.67 (1)	0.68 (1)	0.62 (1)	0.54 (1)	0.55 (1)	0.64 (1)	0.64 (1)	0.86 (1)
Ile		1.01 (1)	1.00 (1)	1.00 (1)	1.04 (1)	0.95 (1)	0.92 (1)	0.93 (1)	1.84 (2)	1.75 (2)
Leu				1.06 (1)	2.10 (2)	2.93 (3)	3.88 (4)	3.90 (4)	3.70 (4)	3.82 (4)
Tyr								0.89 (1)	1.59 (2)	1.87 (2)
Phe									0.84 (1)	0.85 (1)
Lys					1.12 (1)	0.96 (1)	1.82 (2)	1.96 (2)	1.98 (2)	1.92 (2)
Arg	0.97 (1)	0.98 (1)	0.98 (1)	0.86 (1)	2.23 (2)	1.90 (2)	3.00 (3)	3.03 (3)	2.93 (3)	2.83 (3)
Rec.	88%	87%	82%	75%	89%	89%	74%	83%	89%	73%

a) Met + Met(O).

synthetic hpGRF-37–NH_2 to synthetic hpGRF-44–NH_2 (taken as 1) was only 0.15 (Fig. 3b). This *in vitro* potency seems to be equivalent to that reported for natural hpGRF-37–OH,[3c] indicating that the C-terminal amide has no significant role in *in vitro* GRF activity, contrary to our expectation.

Biotinyl peptide hormones are known to be useful derivatives for histochemical receptor studies.[11,12] Thus, we decided to synthesize N^{α}-biotinyl-hpGRF-44–NH_2. The available protected hpGRF-44–NH_2[5] was treated with TFA, then the biotinyl moiety was introduced by the use of biotinyl-*N*-hydroxysuccinimide ester.[13] Deprotection with 1 M TFMSA–thioanisole/TFA was performed in the presence of dimethylsulfide with care to prevent S-alkylation at the biotinyl moiety, and the product, after treatments with Amberlite CG-4B, DTT and dil. ammonia, was purified by ion-exchange chromatography on CM-Biogel A.

In *in vivo* assay, N^{α}-biotinyl-hpGRF-44–NH_2 was found to have approximately 60% of

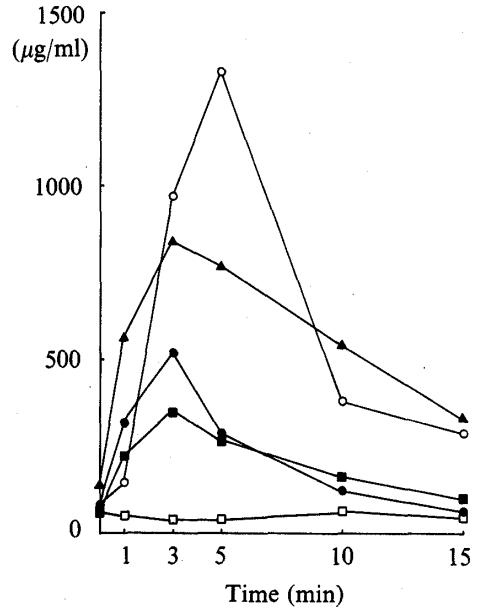

Fig. 3a. *In Vivo* Assay of Synthetic GRF Peptides

○—○, GRF-44–NH_2 (0.5 μg); ●—●, GRF-44–NH_2 (0.1 μg); ■—■, GRF-37–NH_2 (0.1 μg); ▲—▲, biotinyl-GRF-44–NH_2 (0.5 μg); □—□, control (saline).

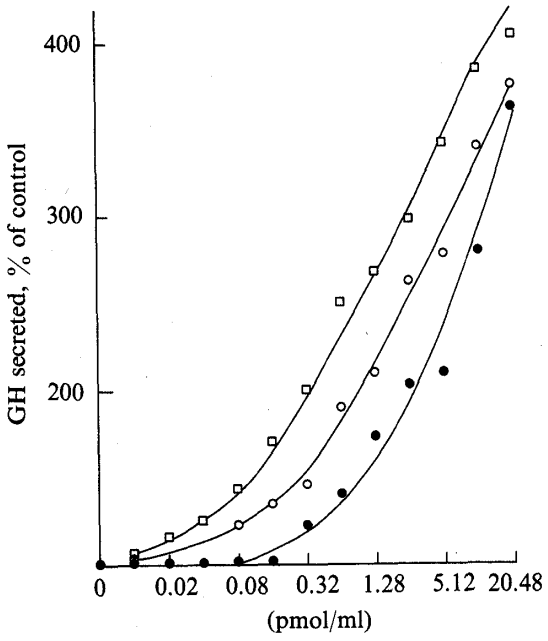

Fig. 3b. *In Vitro* Assay of Synthetic GRF Peptides

●—●, GRF-37–NH_2; ○—○, GRF-40–OH; □—□, GRF-44–NH_2 ($n=3$).

the activity of synthetic hpGRF-44–NH_2 (Fig. 3a). This result indicated that the α-amino function of hpGRF is not essential for its intrinsic growth hormone releasing activity, though inactivation of hpGRF by deletion of the N-terminal Tyr residue was reported by Guillemin *et al.*[3a] This biotinyl-hpGRF-44–NH_2, which retains unmodified biological function and presumably immunological functions as well, should be a useful compound for future histochemical studies on GRF.

Experimental

General experimental methods employed in this investigation were essentially the same as described in Part LXXXVIII[14] of this series. *Rf* values in TLC performed on silica gel (Kieselgel G, Merck) refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH–H_2O (8:3:1), Rf_2 $CHCl_3$–MeOH (10:0.5), Rf_3 $CHCl_3$–MeOH–AcOH (9:1:0.5), Rf_4 *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2), Rf_5 *n*-BuOH–AcOH–pyridine–H_2O (30:20:6:24). HPLC was conducted with a Waters 204 compact model. Leucine aminopeptidase was purchased from Sigma Chemical Co. (Lot 62F-8000).

Z(OMe)–Glu(OBzl)–NH_2——An aqueous 28% NH_4OH solution (2.58 ml, 38 mmol) was added to an ice-chilled solution of Z(OMe)–Glu(OBzl)–ONp (10.0 g, 19.1 mmol) in DMF (20 ml) and the mixture, after being stirred for 60 min, was neutralized with AcOH and concentrated. Trituration of the residue with 5% $NaHCO_3$ afforded a powder, which was washed with 5% $NaHCO_3$ and H_2O and precipitated from DMF with EtOH; yield 5.50 g (72%), mp 147—149 °C, $[\alpha]_D^{20}+4.0°$ ($c=1.2$, DMF), Rf_2 0.31, Rf_3 0.66. IR ν_{max}^{Nujol} cm^{-1}: 1725 (CO ester), 1645 (CO amide); the active ester band at 1770 of the starting material had disappeared. *Anal.* Calcd for $C_{21}H_{24}N_2O_6$: C, 62.99; H, 6.04; N, 7.00. Found: C, 63.01; H, 6.09; N, 6.94. A part of the product was treated with TFA, then hydrogenated over a Pd catalyst as usual to afford isoglutamine, the properties of which (mp, $[\alpha]_D$, IR) were identical with those of an authentic sample of isoglutamine (Ajinomoto).

Z(OMe)–Gln–Glu(OBzl)–NH_2——Z(OMe)–Glu(OBzl)–NH_2 (5.0 g, 12.5 mmol) was treated with TFA–anisole (10.8 ml–2.7 ml) in an ice-bath for 60 min, then 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–DMSO (1:1, 50 ml), together with Et_3N (3.5 ml, 25.0 mmol) and Z(OMe)–Gln–ONp (5.39 g, 12.5 mmol). After being stirred at room temperature overnight, the mixture was poured into 5% citric acid (200 ml). The resulting powder was washed with 5% citric acid, 5%

$NaHCO_3$ and H_2O and precipitated from DMF with EtOH; yield 6.10 g (92%), mp 187—188 °C, $[\alpha]_D^{20}$ +5.6° (c=0.9, DMSO), Rf_1 0.63. *Anal.* Calcd for $C_{26}H_{32}N_4O_8$: C, 59.08; H, 6.10; N, 10.60. Found: C, 58.82; H, 6.11; N, 10.50.

Z(OMe)–Asn–Gln–Glu(OBzl)–NH_2——Z(OMe)–Gln–Glu(OBzl)–NH_2 (6.10 g, 11.5 mmol) was treated with TFA–anisole (10 ml–2.5 ml) in an ice-bath for 60 min, then ether was added. The resulting powder was dried over KOH pellets *in vacuo* for 3 h and dissolved in DMF–DMSO (1 : 1, 50 ml), together with Et_3N (3.2 ml, 23.0 mmol) and Z(OMe)–Asn–ONp (5.30 g, 12.7 mmol). After being stirred overnight, the solution was neutralized with AcOH and concentrated. Treatment of the residue with 5% citric acid afforded a powder, which was purified by the washing procedure as described above followed by precipitation twice from DMF–DMSO (1 : 1) with EtOH; yield 6.25 g (84%), mp 242—245 °C, $[\alpha]_D^{20}$ −2.9° (c=1.0, DMSO), Rf_1 0.54, Rf_4 0.72. *Anal.* Calcd for $C_{30}H_{38}N_6O_{10}$: C, 56.06; H, 5.96; N, 13.08. Found: C, 55.89; H, 5.82; N, 13.18.

Z(OMe)–Ser–Asn–Gln–Glu(OBzl)–NH_2——Z(OMe)–Asn–Gln–Glu(OBzl)–NH_2 (6.25 g, 9.7 mmol) was treated with TFA–anisole (12.6 ml–3.2 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMSO–DMF (1 : 1, 30 ml) containing Et_3N (1.4 ml, 9.7 mmol). The azide [prepared from 3.58 g (12.6 mmol) of Z(OMe)–Ser–$NHNH_2$] in DMF (5 ml) and Et_3N (2.1 ml, 15.2 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4 °C overnight, while the product precipitated from the solution. H_2O (100 ml) was added and the solid, collected by filtration, was precipitated from DMF with EtOH; yield 5.0 g (70%), mp 216—218 °C, $[\alpha]_D^{20}$ −11.9° (c=1.3, DMSO), Rf_1 0.50, Rf_4 0.62. *Anal.* Calcd for $C_{33}H_{43}N_7O_{12}$: C, 54.31; H, 5.94; N, 13.44. Found: C, 54.16; H, 5.88; N, 13.14.

Z(OMe)–Glu(OBzl)–Ser–Asn–Gln–Glu(OBzl)–NH_2 [1]——Z(OMe)–Ser–Asn–Gln–Glu(OBzl)–NH_2 (5.0 g, 6.9 mmol) was treated with TFA–anisole (8.9 ml–2.2 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMSO–DMF (1 : 1, 20 ml), together with Et_3N (1.9 ml, 13.7 mmol) and Z(OMe)–Glu(OBzl)–ONp (3.94 g, 7.5 mmol). The solution was stirred overnight, then H_2O (80 ml) was added. The resulting solid was washed with H_2O and precipitated twice from DMSO–DMF (1 : 1) with MeOH; yield 4.75 g (73%), mp 246—249 °C, $[\alpha]_D^{20}$ −6.3° (c=1.0, DMSO), Rf_4 0.89. Amino acid ratios in 6 N HCl hydrolysate: Asp 1.00, Ser 0.98, Glu 2.95 (recovery of Asp 89%). *Anal.* Calcd for $C_{45}H_{56}N_8O_{15}$: C, 56.95; H, 5.95; N, 11.81. Found: C, 56.48; H, 5.78; N, 11.70.

Z(OMe)–Asp(OBzl)–Ile–Met(O)–$NHNH_2$ [3]——Z(OMe)–Asp(OBzl)–Ile–Met(O)–NHNH–Troc (3.0 g, 3.6 mmol) dissolved in a mixture of DMF (20 ml) and AcOH (2.1 ml) was treated with Zn powder (4.7 g, 20 eq) at room temperature for 2 h. The solution was then filtered, the filtrate was concentrated and the residue was treated with aqueous 20% ethylenediaminetetraacetic acid (EDTA). The resulting powder was washed with 5% $NaHCO_3$ and H_2O and precipitated from DMF with EtOH; yield 1.10 g (46%), mp 208—210 °C, $[\alpha]_D^{20}$ −9.1° (c=1.4, DMSO), Rf_1 0.76, Rf_3 0.06. Amino acid ratios in 6 N HCl hydrolysate: Asp 1.01, Met 0.63, Ile 1.00 (recovery of Ile 84%). *Anal.* Calcd for $C_{31}H_{43}N_5O_9S$: C, 56.26; H, 6.55; N, 10.58. Found: C, 56.00; H, 6.46; N, 10.49.

Z(OMe)–Ser–Arg(Mts)–Gln–Gln–Gly–Glu(OBzl)–Ser–Asn–Gln–Glu(OBzl)–NH_2——Z(OMe)–Glu(OBzl)–Ser–Asn–Gln–Glu(OBzl)–NH_2 (1.0 g, 1.05 mmol) was treated with TFA–anisole (2.3 ml–0.6 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMSO–DMF(1 : 1, 8 ml) containing Et_3N (146 μl, 1.05 mmol). The azide [prepared from 1.28 g (1.37 mmol) of Z(OMe)–Ser–Arg(Mts)–Gln–Gln–Gly–$NHNH_2$] in DMSO–DMF (1 : 1, 8 ml) and Et_3N (228 μl, 1.64 mmol) were added to the above ice-chilled solution and the mixture, after being stirred at 4 °C overnight, was neutralized with AcOH. The solvent was removed by evaporation and the residue was treated with 5% citric acid. The resulting powder was washed with 5% citric acid and H_2O and precipitated from DMSO–DMF (1 : 1) with MeOH; yield 1.73 g (97%), mp 260 °C (dec.), $[\alpha]_D^{20}$ −1.4° (c=0.7, DMSO), Rf_4 0.72. *Anal.* Calcd for $C_{75}H_{102}N_{18}O_{25}S \cdot H_2O$: C, 52.81; H, 6.15; N, 14.78. Found: C, 52.74; H, 6.13; N, 14.92.

Z(OMe)–Asp(OBzl)–Ile–Met(O)–Ser–Arg(Mts)–Gln–Gln–Gly–Glu(OBzl)–Ser–Asn–Gln–Glu(OBzl)–NH_2—— The above protected decapeptide amide (1.73 g, 1.03 mmol) was treated with TFA–anisole (3.5 ml–0.9 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMSO–DMF (1 : 1, 10 ml) containing Et_3N (143 μl, 1.03 mmol). The azide [prepared from 1.02 g (1.54 mmol) of Z(OMe)–Asp(OBzl)–Ile–Met(O)–$NHNH_2$] in DMF (8 ml) and Et_3N (257 μl, 1.85 mmol) were added to the above ice-chilled solution and the mixture, after being stirred at 4 °C overnight, was poured into H_2O (60 ml). The resulting powder was washed with 5% citric acid and H_2O and precipitated from DMSO–DMF (1 : 1) with MeOH; yield 2.04 g (92%), mp 260 °C (dec.), $[\alpha]_D^{20}$ −8.5° (c=0.9, DMSO), Rf_4 0.87. *Anal.* Calcd for $C_{97}H_{133}N_{21}O_{31}S_2$: C, 54.10; H, 6.23; N, 13.66. Found: C, 53.71; H, 6.15; N, 13.22.

Z(OMe)–Gln–Asp(OBzl)–Ile–Met(O)–Ser–Arg(Mts)–Gln–Gln–Gly–Glu(OBzl)–Ser–Asn–Gln–Glu(OBzl)–NH_2——The above protected tridecapeptide amide (2.04 g, 0.95 mmol) was treated with TFA–anisole (4.1 ml–1.0 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMSO–DMF (1 : 1, 20 ml) together with Et_3N (264 μl, 1.89 mmol) and Z(OMe)–Gln–ONp (613 mg, 1.42 mmol). After being stirred overnight, the solution was poured into H_2O (60 ml) and the resulting powder was purified by washing with EtOH, followed by precipitation from DMSO–DMF (1 : 1) with MeOH; yield 2.07 g (96%), mp 260 °C (dec.), $[\alpha]_D^{20}$ −15.6° (c=0.8, DMSO), Rf_4 0.91. *Anal.* Calcd for $C_{102}H_{141}N_{23}O_{33}S_2$: C, 53.69; H, 6.23; N, 14.12. Found: C, 53.42; H, 6.25; N, 13.86.

Z(OMe)–Leu–Gln–Asp(OBzl)–Ile–Met(O)–Ser–Arg(Mts)–Gln–Gln–Gly–Glu(OBzl)–Ser–Asn–Gln–Glu(OBzl)–NH_2——The above protected tetradecapeptide amide (2.07 g, 0.91 mmol) was treated with TFA–anisole (4.3 ml–1.1 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMSO–DMF (1 : 1, 20 ml), together

with Et_3N (252 μl, 1.82 mmol) and Z(OMe)–Leu–ONp (567 mg, 1.36 mmol). After being stirred overnight, the solution was poured into H_2O (60 ml) and the resulting powder was purified by washing with H_2O, followed by precipitation from DMSO with EtOH; yield 2.0 g (92%), mp 260 °C, $[\alpha]_D^{20}$ −9.6° (c=2.1, DMSO), Rf_4 0.82. *Anal.* Calcd for $C_{108}H_{152}N_{24}O_{34}S_2 \cdot H_2O$: C, 53.76; H, 6.43; N, 13.93. Found: C, 53.63; H, 6.29; N, 13.94.

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)–NH_2——The above protected pentadecapeptide amide (2.0 g, 0.84 mmol) was treated with TFA–anisole (4.0 ml–1.0 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMSO–DMF (1 : 1, 15 ml) containing Et_3N (116 μl, 0.84 mmol). The azide [prepared from 1.64 g (1.67 mmol) of Z(OMe)–Ala–Arg(Mts)–Lys(Z)–Leu–$NHNH_2$] in DMF (6 ml) and Et_3N (279 μl, 2.0 mmol) were added to the above ice-chilled solution and the mixture, after being stirred at 4 °C overnight, was poured into H_2O (80 ml). The resulting powder was purified by washing with H_2O, followed by precipitation from DMF–DMSO (1 : 1) with EtOH; yield 1.56 g (59%), mp 270 °C (dec.), $[\alpha]_D^{20}$ −15.2° (c=0.8, DMSO), Rf_1 0.18. *Anal.* Calcd for $C_{146}H_{208}N_{32}O_{42}S_3 \cdot 2H_2O$: C, 54.53; H, 6.65; N, 13.94. Found: C, 54.34; H, 6.41; N, 14.00.

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)–NH_2——The above protected nonadecapeptide amide (1.0 g, 0.32 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, 15 ml) containing Et_3N (44 μl, 0.32 mmol). The azide [prepared from 458 mg (0.79 mmol) of Z(OMe)–Gly–Gln–Leu–Ser–$NHNH_2$] in DMSO–DMF (1 : 1, 10 ml) and Et_3N (131 μl, 0.95 mmol) were added to the above ice-chilled solution and the mixture, after being stirred at 4 °C overnight, was poured into H_2O (80 ml). The resulting powder was purified by washing with H_2O followed by gel-filtration on Sephadex LH-60 (3 × 128 cm) using DMF as an eluant. Each fraction (11 ml) was examined for ultraviolet (UV) absorption at 280 nm. The desired fractions (tube Nos. 42—52) were combined and the solvent was removed by evaporation. Treatment of the residue afforded a powder; yield 843 mg (75%), mp 143—150 °C, $[\alpha]_D^{20}$ −10.0° (c=0.7, DMSO), Rf_1 0.18. *Anal.* Calcd for $C_{162}H_{235}N_{37}O_{48}S_3$: C, 54.58; H, 6.64; N, 14.54. Found: C, 54.33; H, 6.55; N, 14.67.

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)–NH_2——The above protected tricosapeptide amide (843 mg, 0.24 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 (33 μl, 0.24 mmol). The azide [prepared from 714 mg (0.71 mmol) of Z(OMe)–Arg(Mts)–Lys(Z)–Val–Leu–$NHNH_2$] in DMF (3 ml) and Et_3N (118 μl, 0.85 mmol) were added to the above ice-chilled solution and the mixture, after being stirred at 4 °C overnight, was poured into H_2O (50 ml). The resulting powder was purified by gel-filtration on Sephadex LH-60 (3 × 128 cm) with DMF as an eluant as described above. The desired fractions (each fraction 11 ml, tube Nos. 34—50) were combined, the solvent was removed by evaporation and the residue was treated with ether to afford a powder; yield 860 mg (83%), mp 260 °C, (dec.), $[\alpha]_D^{20}$ −8.7° (c=1.2, DMSO), Rf_1 0.27. *Anal.* Calcd for $C_{202}H_{295}N_{45}O_{56}S_4 \cdot 2H_2O$: C, 54.96; H, 6.83; N, 14.28. Found: C, 54.78; H, 7.01; N, 14.10.

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)–NH_2——The above protected heptacosapeptide amide (860 mg, 0.20 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 (27 μl, 0.20 mmol). The azide [prepared from 389 mg (0.59 mmol) of Z(OMe)–Thr–Asn–Ser–Tyr–$NHNH_2$] in DMSO–DMF (1 : 1, 5 ml) and Et_3N (98 μl, 0.71 mmol) were added to the above ice-chilled solution and the mixture, after being stirred overnight, was poured into H_2O (60 ml). The resulting powder was purified by gel-filtration on Sephadex LH-60 (3 × 128 cm) using DMF as an eluant as described above. The desired fractions (each fraction 11 ml, tube Nos. 30—51) were combined, the solvent was evaporated off, and the residue was treated with ether to afford a powder; yield 786 mg (83%), mp 157—162 °C, $[\alpha]_D^{20}$ −8.8° (c=0.8, DMSO), Rf_1 0.13. *Anal.* Calcd for $C_{222}H_{322}N_{50}O_{64}S_4$: C, 55.05; H, 6.70; N, 14.46. Found: C, 54.78; H, 6.73; N, 14.50.

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)–NH_2——The above protected untriacontapeptide (786 mg, 0.16 mmol) was treated with TFA–anisole (5 ml–0.3 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMSO–DMF (1 : 1, 20 ml) containing Et_3N (23 μl, 0.16 mmol). The azide [prepared from 426 mg (0.49 mmol) of Z(OMe)–Tyr–Ala–Asp–Ala–Ile–Phe–$NHNH_2$] in DMSO–DMF (1 : 1, 5 ml) and Et_3N (149 μl, 1.07 mmol) were added to the above ice-chilled solution and the mixture, after being stirred at 4 °C overnight, was poured into 5% citric acid (6 ml). The resulting powder was washed with H_2O and purified by gel-filtration on Sephadex LH-60 (3 × 128 cm) using DMF as an eluant. The desired fractions (each fraction 11 ml, tube Nos. 34—53) were combined, the solvent was evaporated off, and the residue was treated with ether to afford a powder; yield 742 mg (83%), mp 280 °C (dec.), $[\alpha]_D^{20}$ −1.8° (c=0.5, DMSO), Rf_1 0.12. *Anal.* Calcd for $C_{256}H_{366}N_{56}O_{73}S_4$: C, 55.66; H, 6.68; N, 14.20. Found: C, 55.55; H, 6.83; N, 14.22.

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–NH_2——The above protected heptatri-

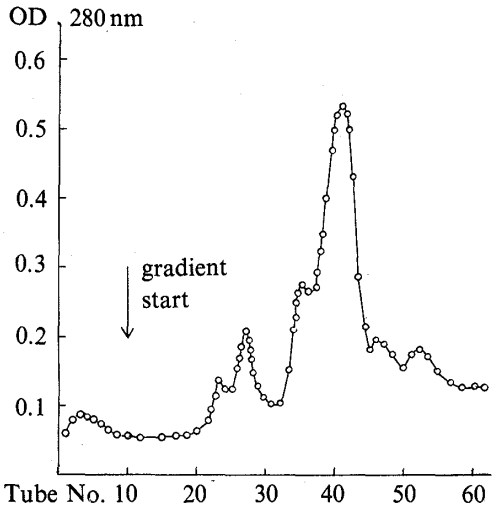

Fig. 4. Purification of Synthetic hpGRF-37–NH_2 by Ion-Exchange Chromatography on CM-Biogel A

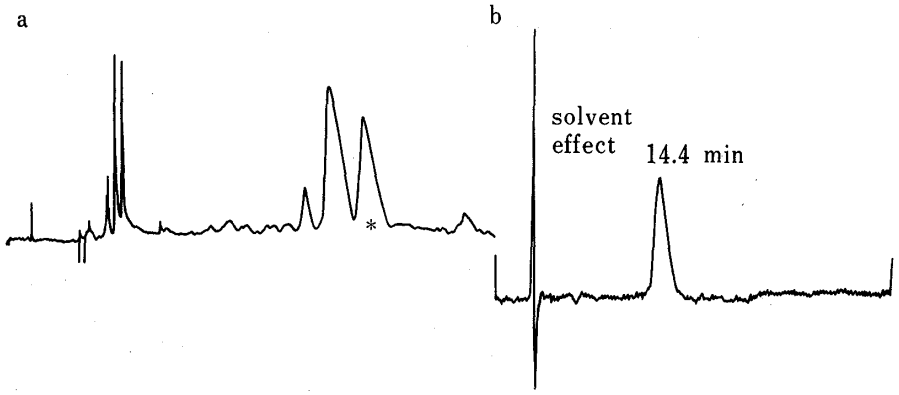

Fig. 5. HPLC of GRF-37–NH_2

a: purification of CM-purified sample.
b: purified sample.
The material obtained from the marked peak (*) gave a low recovery of Arg in the enzymatic digest.

acontapeptide amide (100 mg, 18.1 μmol) was treated with 1 M TFMSA–thioanisole in TFA–anisole (3.8 ml) in the presence of *m*-cresol (85 μl, 45 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 (140 mg), treated with Amberlite CG-4B (acetate form, approximately 1 g) for 30 min with stirring, and then filtered. The pH of the solution was adjusted to 8.0 with 3% NH_4OH and after being stirred for 30 min in an ice bath, the solution was readjusted to pH 6.5 with 1 N AcOH, then lyophilized to give a hygroscopic powder, which was dissolved in H_2O (2 ml) and incubated with dithiothreitol (280 mg, 100 eq) under an argon atmosphere at 37 °C for 12 h.

Then the solution was applied to a column of Sephadex G-25 (1.8 × 138 cm), which was eluted with 0.5 N AcOH. The UV absorption at 280 nm was determined for each fraction (6 ml). The fractions corresponding to the front main peak (tube Nos. 22—32) were combined and the solvent was removed by lyophilization to give a white powder; yield 69.5 mg (86%). The crude product thus obtained was dissolved in pH 4.8, 0.01 M NH_4OAc buffer, and the solution was applied to a column of CM-Biogel A (1.6 × 10.5 cm), which was first eluted with the same buffer (77 ml), and then with a linear gradient formed from the starting buffer (200 ml) and pH 6.5, 0.2 M NH_4OAc buffer (200 ml). The UV absorption of each fraction (7.7 ml) was determined. The fractions corresponding to the main peak (tube Nos. 37—45) were combined and the solvent and ammonium salt were removed by repeated lyophilization to give a fluffy powder; yield 31.7 mg (39% from the protected peptide). Subsequent purification was performed by reverse phase HPLC on a Cosmosil $5C_{18}$ column (1.0 × 25 cm, Nakarai Chemical Co.) using gradient elution with acetonitrile (30—35% in 40 min) in 0.1% TFA at a flow rate of 3 ml per min (Fig. 5a). About 5 mg of the sample was repeatedly applied to the column in each run and the combined eluates corresponding to the main peak (retention time 20 min)

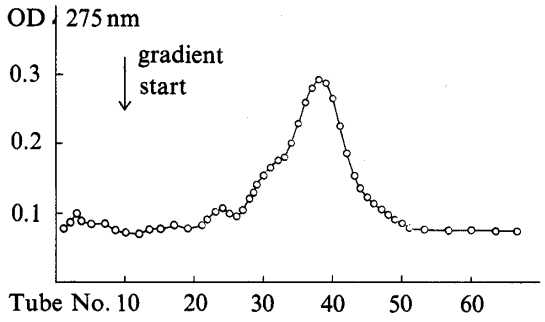

Fig. 6. Purification of Synthetic N^{α}-Biotinyl-hpGRF-44–NH_2 by Ion-Exchange Chromatography on CM-Biogel A

were concentrated by rotary evaporation *in vacuo* at 30 °C. The residue was dissolved in pH 7.5, 1 M Et_3NHOAc buffer (2 ml) and the solution was desalted by gel-filtration on Sephadex G-25 (2 × 40 cm) with 0.5 N AcOH as the eluant. The desired fractions were collected and the solvent was removed by lyophilization to give a white fluffy powder; yield 13.5 mg (17% from the protected peptide). $[\alpha]_D^{20}$ −72.5° (c=0.1, 0.2 N AcOH), Rf_4 0.32, Rf_5 0.19. Amino acid ratios in a 6 N HCl hydrolysate are shown in Table I. Amino acid ratios in leucine aminopeptidase digest: 2Asp 1.94, 4Ser 3.89, 2Glu 1.98, 2Gly 2.00, 3Ala 3.05, 1Val 0.99, 1Met 0.95, 2Ile 1.91, 4Leu 3.96, 2Tyr 1.88, 1Phe 0.99, 2Lys 1.99, 3Arg 2.76, 1Thr, 5Gln, and 2Asn were not determined (recovery of Gly 87%). *Anal.* Calcd for $C_{183}H_{298}N_{56}O_{59}S \cdot 6CH_3COOH \cdot 25H_2O$: C, 46.20; H, 7.40; N, 15.47. Found: C, 45.91; H, 6.82; N, 15.60.

The synthetic peptide exhibited a single peak at a retention time of 14.4 min on HPLC using a μBondapak C_{18} column (0.39 × 30 cm) when subjected to gradient elution with acetonitrile (from 30 to 35% within 20 min) in 0.1% TFA at a flow rate of 1 ml per min (Fig. 5b). It exhibited a single band in disk isoelectrofocusing on 7.5% polyacrylamide gel (0.5 × 7.0 cm) containing Pharmalyte (pH 9.0—11.0): mobility 0.7 cm from the origin toward the cathodic end of the gel, after running at 200V for 4 h.

N^{α}-Biotinyl-(GRF 1-44)–NH_2——Z(OMe)–(GRF 1-44)–NH_2 (100 mg, 14.6 μmol) was treated with TFA–anisole (2 ml–0.5 ml) in an ice-bath for 1 h and the N^{α}-deprotected peptide, isolated by precipitation with ether, was dissolved in DMSO–DMF (1 : 1, 2 ml), together with biotinyl-*N*-hydroxysuccinimide ester (10 mg, 29.2 μmol) and Et_3N (6 μl, 43.8 μmol). After being stirred overnight, the ninhydrin-negative solution was neutralized with AcOH, then concentrated, and the residue was treated with H_2O. The resulting powder was dissolved in DMF (4 ml) and the solution was applied to a column of Sephadex LH-60 (3 × 128 cm), which was eluted with the same solvent. Each fraction (10 ml) was examined for UV absorption at 280 nm. The fractions corresponding to the main peak (tube Nos. 35—46) were combined and the solvent was removed by evaporation. Treatment of the residue with ether afforded a powder; yield 45 mg (45%), mp 280 °C (dec.), $[\alpha]_D^{20}$ +5.1° (c=0.2, DMSO), Rf_1 0.18. Amino acid ratios in 6 N HCl hydrolysate: Asp 4.32, Thr 1.07, Ser 3.66, Glu 7.15, Gly 3.14, Ala 5.79, Val 1.08, Met 0.69, Ile 2.09, Leu 5.00, Tyr 1.95, Phe 1.08, Lys 2.01, Arg 6.02 (recovery of Leu 83%). *Anal.* Calcd for $C_{316}H_{462}N_{74}O_{85}S_8 \cdot 5H_2O$: C, 54.19; H, 6.79; N, 14.80. Found: C, 54.06; H, 6.86; N, 15.02.

The above protected form of N^{α}-biotinyl-(GRF 1-44)–NH_2 (41 mg, 5.93 μmol) was treated with 1 M TFMSA–thioanisole in TFA (1.3 ml) in the presence of *m*-cresol (34 μl) and Me_2S (22 μl) in an ice-bath for 60 min. This treatment was repeated twice more under the same conditions. The deprotected peptide precipitated with ether was dissolved in H_2O (3 ml) containing DTT (100 mg), and treated with Amberlite CG-4B (acetate form, approximately 1 g). After filtration, the solution was adjusted to pH 8.0 with 5% NH_4OH in an ice-bath, and after 30 min to pH 6.5 with 1 N AcOH. Next, the solution was incubated with DTT (100 mg, 110 eq) at 37 °C for 24 h and then applied to a column of Sephadex G-25 (1.8 × 135 cm), which was eluted with 0.5 N AcOH. Individual fractions (5 ml) were examined for UV absorption at 280 nm. Fractions corresponding to the main peak (tube Nos. 30—38) were combined and the solvent was removed by lyophilization to give a powder; yield 29.2 mg (85.7%). To remove traces of impurities, the crude product thus obtained was purified by ion-exchange chromatography on CM-Biogel A (1 × 14 cm), which was first eluted with pH 4.8, 0.02 M NH_4OAc (50 ml) and then with a gradient formed from pH 6.5, 0.2 M NH_4OAc (250 ml) through a mixing flask containing the above starting buffer (250 ml). The UV absorption in each fraction (5 ml) was determined (Fig. 6). The fractions corresponding to the main peak (tube Nos. 33—43) were combined and the solvent and ammonium salt were removed by repeated lyophilization to afford a white fluffy powder; yield 19.6 mg (67%), total yield from the protected peptide 57.5%. $[\alpha]_D^{20}$ −62.6° (c=0.6, 0.2 N AcOH), Rf_4 0.56, Rf_5 0.34. Amino acid ratios in 6 N HCl hydrolysate: 4Asp 3.89, 1Thr 0.94, 4Ser 3.44, 7Glu 6.96, 3Gly 3.06, 5Ala 5.27, 1Val 0.99. 1Met 0.79, 2Ile 1.92, 5Leu 5.00, 2Tyr 1.64, 1Phe 0.96, 2Lys 1.96, 6Arg 5.94 (recovery of Leu 86%). *Anal.* Calcd for $C_{225}H_{372}N_{74}O_{68}S_2 \cdot 8AcOH \cdot 27H_2O$: C, 46.44; H, 7.41; N, 16.63. Found: C, 46.16; H, 6.75; N, 16.87.

References and Notes

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2) Amino acids, peptides and their derivatives are of the L-configuration. The following abbreviations were used: Z = benzyloxycarbonyl, Z (OMe) = *p*-methoxybenzyloxycarbonyl, Bzl = benzyl, Mts = mesitylene-2-sulfonyl, Troc = β,β,β-trichloroethyloxycarbonyl, Np = *p*-nitrophenyl, DMF = dimethylformamide, TFA = trifluoroacetic acid, DMSO = dimethylsulfoxide, TFMSA = trifluoromethanesulfonic acid, DTT = dithiothreitol, Su = *N*-hydroxysuccinimidyl.
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