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Studies on Peptides. CXVII.[1,2] Solution Synthesis of the Tetratetracontapeptide Amide Corresponding to the Entire Amino Acid Sequence of Growth Hormone Releasing Factor, Somatocrinin

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The tetratetracontapeptide corresponding to the entire amino acid sequence of growth hormone releasing peptide derived from human pancreas islet tumor was synthesized by assembling eight peptide fragments in a conventional manner, followed by thioanisole-mediated deprotection with 1 M trifluoromethanesulfonic acid in TFA. Our synthetic peptide significantly stimulated the release of immunoreactive growth hormone *in vivo*.

Keywords——human pancreatic growth hormone releasing peptide; somatocrinin synthesis; N^G-mesitylenesulfonylarginine; reduction of methionine sulfoxide; thioanisole-mediated deprotection; trifluoromethanesulfonic acid deprotection; immunoreactive growth hormone; rat growth hormone

As described in the preceding paper, in 1982 two groups[3,4] at the Salk Institute reported independently and simultaneously the structure of growth hormone releasing factor (GRF), termed somatocrinin,[5] derived from human pancreatic islet tumor. The three forms of natural peptide so far known, GRF-44–NH_2, GRF-40–OH and GRF-37–OH, were soon synthesized by the solid phase method.[3,4] Because of our great interest in obtaining this releasing factor for clinical testing, we undertook an alternative synthesis of the largest form of GRF, the tetratetracontapeptide amide, H–(hpGRF 1—44)–NH_2, by the solution method. As reported in the preceding paper,[1] the protected docosapeptide amide corresponding to positions 23 to 44 of our target, Z(OMe)–(hpGRF 23—44)–NH_2, was synthesized by assembling three peptide fragments, Z(OMe)–(hpGRF 33—44)–NH_2 [1], Z(OMe)–(hpGRF 28—32)–$NHNH_2$ [2] and Z(OMe)–(hpGRF 23—27)–$NHNH_2$ [3].

We wish to report in this paper that we succeeded in synthesizing the biologically active tetratetracontapeptide amide by further chain elongation of the above intermediate followed by the thioanisole-mediated deprotection of all protecting groups employed with TFMSA.[6] As shown in Fig. 1, five hydrazides, [4] to [8], have now been synthesized using amino acid derivatives bearing protecting groups removable by the above reagent, *i.e.*, Lys(Z) and Arg(Mts).[7] Of these, fragment [5], Z(OMe)–Gly–Gln–Leu–Ser–$NHNH_2$, is identical with that used for our previous synthesis of PHI,[8] a gastrointestinal peptide newly found by Tatemoto and Mutt[9] in 1980.

First, fragment [4], Z(OMe)–Ala–Arg(Mts)–Lys(Z)–Leu–$NHNH_2$, was synthesized in a stepwise manner starting with the known dipeptide, Z(OMe)–Lys(Z)–Leu–OMe,[10] as shown in Fig. 2. Prior to every condensation reaction, the Z(OMe) group was removed by TFA in the presence of anisole as usual. A mixed anhydride procedure[11] was convenient to condense Z(OMe)–Arg(Mts)–OH onto the TFA-treated sample of the above dipeptide. Next, in order

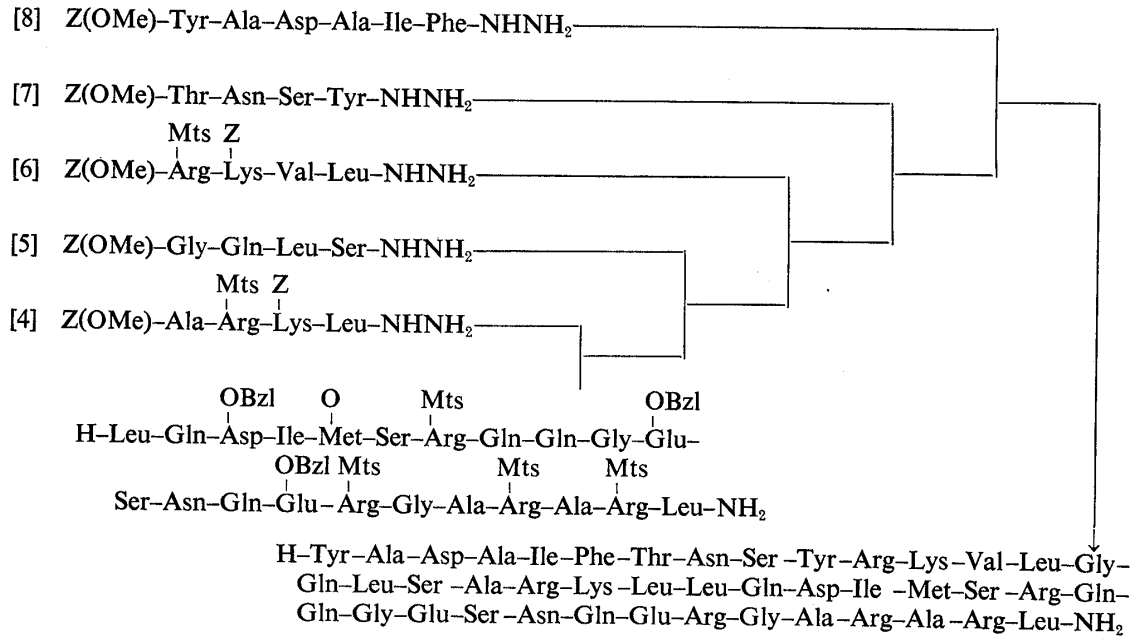

Fig. 1. Synthetic Route to hpGRF

to elongate the resulting tripeptide ester, a thiazolidine-2-thione derivative of alanine was employed. This new amide-forming reagent was recently introduced by us[12] and independently by Nagao *et al.*[13] The protected tetrapeptide ester, Z(OMe)–Ala–Arg(Mts)–Lys(Z)–Leu–OMe, obtained in 90% yield by this new method, was then converted to the corresponding hydrazide [4] by the usual hydrazine treatment.

Fragment [6], Z(OMe)–Arg(Mts)–Lys(Z)–Val–Leu–$NHNH_2$ was also prepared in a stepwise manner starting with the known dipeptide, Z(OMe)–Val–Leu–OMe,[14] as shown in Fig. 3. The mixed anhydride procedure was used to condense Z(OMe)–Lys(Z)–OH and Z(OMe)–Arg(Mts)–OH successively to yield the protected tetrapeptide ester, Z(OMe)–Arg(Mts)–Lys(Z)–Val–Leu–OMe, which was converted to the corresponding hydrazide [6] by the usual hydrazine treatment.

Fragment [7], Z(OMe)–Thr–Asn–Ser–Tyr–$NHNH_2$, was also prepared in a stepwise manner as shown in Fig. 4. The Ser and Thr residues were introduced by the azide procedure[15] and the Asn residue by the Np ester procedure.[16] The resulting protected tetrapeptide ester, Z(OMe)–Thr–Asn–Ser–Tyr–OMe, was smoothly converted to the corresponding hydrazide [7] as described above.

Fragment [8], Z(OMe)–Tyr–Ala–Asp–Ala–Ile–Phe–$NHNH_2$, corresponding to the N-terminal segment of GRF, was prepared starting with the known dipeptide, Z(OMe)–Ile–Phe–OMe,[17] as shown in Fig. 5. The mixed anhydride procedure was used to condense Z(OMe)–Ala–OH and Boc–Asp(OBzl)–OH onto the above dipeptide successively to give the protected tetrapeptide ester, Boc–Asp(OBzl)–Ala–Ile–Phe–OMe. The base-catalyzed succinimide formation of the Asp(OBzl) residue seems to be sequence-dependent[18] and available information indicated that the situation is favorable for the Asp(OBzl) residue linked to the Ala residue. Thus, the Bzl group was removed from the above tetrapeptide by catalytic hydrogenolysis prior to the next coupling reaction. After removal of the Boc Group by TFA treatment, the resulting tetrapeptide ester, H–Asp–Ala–Ile–Phe–OMe, was condensed with Z(OMe)–Tyr–Ala–$NHNH_2$,[19] *via* the azide, and the resulting protected hexapeptide ester, Z(OMe)–Tyr–Ala–Asp–Ala–Ile–Phe–OMe, was then converted to the corresponding hydrazide [8] by the usual hydrazine treatment.

The five fragments thus prepared were then assembled successively onto the previously

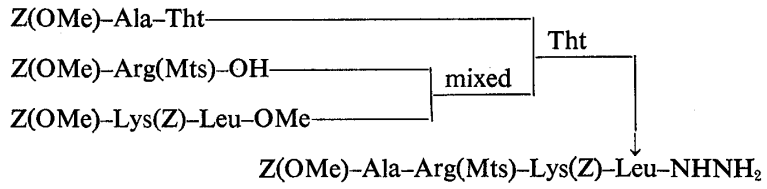

Fig. 2. Synthetic Scheme for the Protected Tetrapeptide Hydrazide, Z(OMe)-(hpGRF 19—22)-$NHNH_2$ [4]

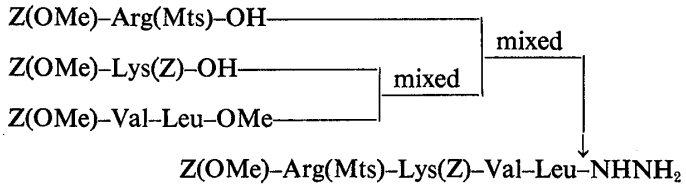

Fig. 3. Synthetic Scheme for the Protected Tetrapeptide Hydrazide, Z(OMe)-(hpGRF 11—14)-$NHNH_2$ [6]

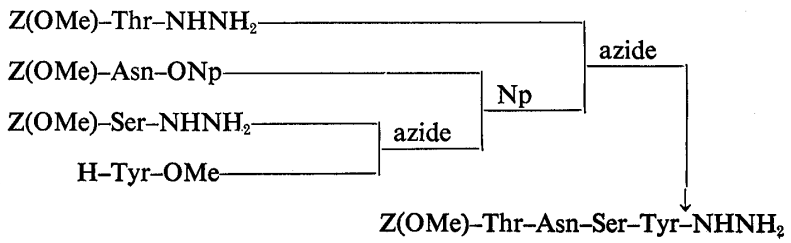

Fig. 4. Synthetic Scheme for the Protected Tetrapeptide Hydrazide, Z(OMe)-(hpGRF 7—10)-$NHNH_2$ [7]

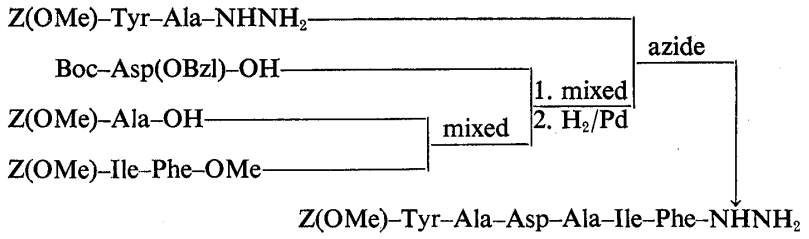

Fig. 5. Synthetic Scheme for the Protected Hexapeptide Hydrazide, Z(OMe)-(hpGRF 1—6)-$NHNH_2$ [8]

prepared docosapeptide, Z(OMe)-(hpGRF 23—44)-NH_2 by the azide procedure, as shown in Fig. 1. The amount of the acyl component was increased from 2 to 3 equivalents as the chain elongation progressed. After ensuring completion of the coupling reaction by ninhydrin testing, each product was isolated and subsequently purified by precipitation from DMSO with MeOH. In the later steps, gel-filtration on Sephadex LH-60 was found to be effective for purifying the protected products, including the protected hpGRF. As described in the preceding paper,[1] Leu was used as a diagnostic amino acid in acid hydrolysis. By comparison of the recovery of Leu with those of newly incorporated amino acids after acid hydrolysis, satisfactory incorporation in each condensation reaction was ascertained as shown in Table I.

Next, deprotection and subsequent purification were carried out according to the following scheme (Fig. 6). The protected tetratetracontapeptide amide, Z(OMe)-(hpGRF 1—44)-NH_2, was treated with 1 M TFMSA in TFA in the presence of thioanisole and *m*-cresol in an

TABLE I. Amino Acid Ratios in 6 N HCl Hydrolysates of Synthetic hpGRF and Its Intermediates

	Protected peptide					Synthetic hpGRF	Residue
	19—44	15—44	11—44	7—44	1—44		
Asp	1.82	1.87	2.05	3.06	4.12	4.10	4
Thr				1.01	0.97	1.01	1
Ser	1.77	2.49	2.80	3.66	3.51	3.66	4
Glu	5.98	6.89	7.14	7.03	7.02	7.18	7
Gly	2.03	3.18	3.15	3.10	3.03	3.05	3
Ala	3.25	3.24	3.10	3.23	5.29	5.19	5
Val			0.99	1.00	0.99	1.01	1
Met[a)]	0.52	0.78	0.31	0.48	0.60	0.88	1
Ile	0.88	0.97	0.97	0.95	2.08	1.96	2
Leu	3.00	4.00	5.00	5.00	5.00	5.00	5
Tyr				0.87	2.11	1.97	2
Phe					1.11	1.04	1
Lys	0.96	0.95	1.99	2.03	2.06	2.02	2
Arg	4.92	5.12	5.81	6.29	6.50	5.83	6
Rec.	91.6%	92.4%	94.7%	72.1%	79.0%	85.0%	

a) Met(O) was not calculated (110 °C, for 24 h).

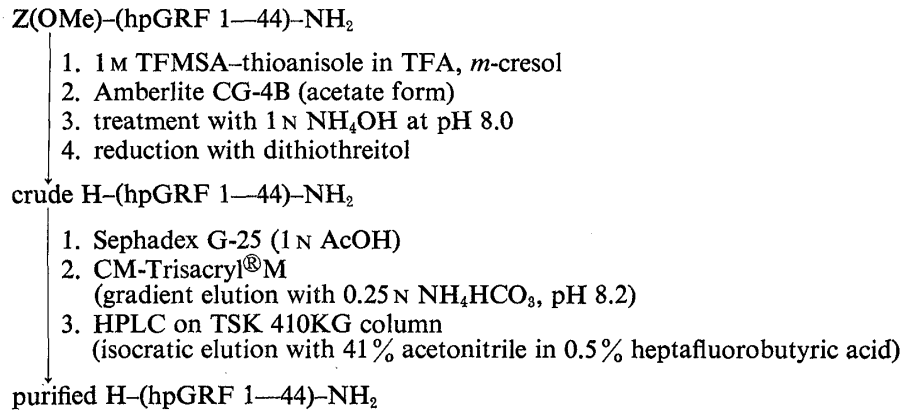

Fig. 6. Deprotection and Purification of H-(hpGRF 1—44)-NH_2

ice-bath for 60 min. The above reagent system has the ability to cleave the Mts group from Arg residue within 60 min at 0 °C, as well as all other protecting groups based on benzyl alcohol, without any marked acid-catalyzed succinimide formation of the Asp residue.[20] After precipitation of the deprotected peptide with ether, this treatment was repeated twice more to ensure complete deprotection. The role of thioanisole was previously discussed.[1] An additional scavenger, *m*-cresol, efficiently suppresses *O*-sulfonylation at the Tyr residue.[7b] The deprotected peptide was converted to the corresponding acetate by treatment with Amberlite CG-4B and then treated with dilute ammonia at pH 8.0 in order to reverse the possible N→O shift[21] at the Ser and Thr residues. As also pointed out in the preceding paper,[1] the thioanisole-mediated deprotection method that we employed has the ability to reduce the Met(O) residue to methionine,[22] besides accelerating the acidolysis of protecting groups.[23] To ensure the complete reduction of the Met(O) residue, the deprotected peptide was incubated with dithiothreitol. After removal of the reducing reagent by gel-filtration on Sephadex G-25, the product was purified by ion-exchange chromatography on CM-Trisacryl®

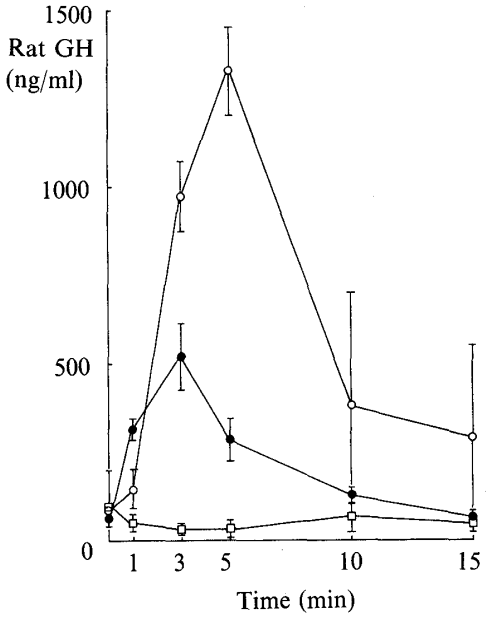

Fig. 7. *In Vivo* Assay of Synthetic H–(hpGRF 1—44)–NH_2

○—○, GRF (1—44)–NH_2 (0.5 μg); ●—●, GRF (1—44)–NH_2 (0.1 μg); □—□, control (saline).

M. When eluted with a gradient up to 0.25 M ammonium bicarbonate, a minor peak was detected in front of the main peak. The minor peak was discarded without examination in detail. The main product obtained at this stage exhibited a single spot on thin layer chromatography (TLC), but showed small side peaks on high performance liquid chromatography (HPLC). Preparative reverse phase HPLC on TSK 410KG column was effective for removing these impurities, one of which (eluted in front of the main peak) seems to be the Met(O) derivative of hpGRF and the other (eluted behind the main peak) seems to be the aminosuccinimide or β-Asp derivative of hpGRF. The former peak became prominent when 30% hydrogen peroxide was added to the solution of the main peak. The enzymic digestion of the product from the latter peak gave a low recovery of Asp in amino acid analysis. The main product thus obtained exhibited a single peak on HPLC and a sharp single spot on TLC in two different solvent systems. It also behaved as a single component on disk-isoelectrofocusing at pH 9—11. Its purity was further confirmed by amino acid analysis after acid hydrolysis and enzymic digestion.

When tested in rats anesthetized with sodium pentobarbital (60 mg/kg) according to Guillemin *et al.*,[5] our synthetic peptide (0.1—0.5 μg) significantly stimulated the secretion of immunoreactive growth hormone (Fig. 7).

Experimental

General experimental procedures used in this investigation were those described in part LXXXVIII[24] of this series. Unless otherwise mentioned, products were purified by procedure A or B as described in the preceding paper.[1]

TLC of the products obtained in this investigation was performed on silica gel (Kieselgel G, Merck). *Rf* values refer to the following solvent systems (v/v): 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–AcOH (9:3:0.5), Rf_4 *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2), Rf_5 *n*-BuOH–AcOH–pyridine–H_2O (30:6:20:24).

Analytical HPLC was conducted with a Waters 204 compact model equipped with a μBondapak C_{18} (0.39 × 30 cm) column using isocratic elution with 42% acetonitrile in 0.5% heptafluorobutyric acid at a flow rate of 1 ml per min. Preparative HPLC was conducted with a Kyowa Seimitsu K-880 model on a TSK 410KG column (2.15 × 30 cm, Toyo Soda Co., Ltd.) using isocratic elution with 41% acetonitrile in 0.5% heptafluorobutyric acid.

Leucine aminopeptidase (Loc. 79C-8110) was purchased from Sigma Chemical Co.

Z(OMe)–Arg(Mts)–Lys(Z)–Leu–OMe——Z(OMe)–Lys(Z)–Leu–OMe (5.0 g, 8.8 mmol) was treated with TFA–anisole (11.3 ml–2.8 ml) and the N^{α}-deprotected peptide isolated as usual was dissolved in DMF (20 ml) containing Et_3N (1.22 ml, 8.8 mmol). A mixed anhydride [prepared from 5.96 g (9.6 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 procedure A, followed by recrystallization from MeOH–ether; yield 5.0 g (63%), mp 128—129 °C, $[\alpha]_D^{20}$ −8.9° (*c*=0.9, DMF), Rf_1 0.84, Rf_2 0.44, Rf_3 0.34. *Anal.* Calcd for $C_{45}H_{63}N_7O_{11}S$: C, 59.39; H, 6.98; N, 10.77. Found: C, 59.13; H, 6.88; N, 10.48.

Z(OMe)–Ala–Arg(Mts)–Lys(Z)–Leu–OMe——The above tripeptide (5.0 g, 5.5 mmol) was treated with TFA–anisole (9.5 ml–2.4 ml) and the N^{α}-deprotected peptide, isolated as usual, was dissolved in DMF (50 ml), together with Et_3N (1.5 ml, 11 mmol) and Z(OMe)–Ala–Tht (1.95 g, 5.5 mmol). After being stirred for 14 h, the solution was concentrated and the residue was purified by procedure A, followed by recrystallization from MeOH and ether; yield 4.73 g (90%), mp 129—130 °C, $[\alpha]_D^{20}$ −12.8° (*c*=1.8, DMF), Rf_1 0.89, Rf_2 0.39, Rf_3 0.25. *Anal.* Calcd for $C_{48}H_{68}N_8O_{12}S$: C, 58.76; H, 6.99; N, 11.42. Found: C, 58.75; H, 6.94; N, 11.15.

Z(OMe)–Ala–Arg(Mts)–Lys(Z)–Leu–NHNH$_2$ [4]——The above protected tetrapeptide ester (4.40 g, 4.5 mmol), dissolved in MeOH (50 ml), was treated with 80% hydrazine hydrate (2.6 ml, 10 eq). The solid, formed on standing for 14 h, was washed with H_2O and precipitated from DMF with EtOH; yield 4.0 g (91%), mp 199—200 °C, $[\alpha]_D^{20}$ −11.2° (*c*=1.2, DMSO), Rf_1 0.64, Rf_2 0.24. Amino acid ratios in 6 N HCl hydrolysate: Ala 0.99, Leu 1.00, Lys 0.91, Arg 0.87 (recovery of Leu 87%). *Anal.* Calcd for $C_{47}H_{68}N_{10}O_{11}S$: C, 57.53; H, 6.99; N, 14.28. Found: C, 57.43; H, 7.12; N, 14.35.

Z(OMe)–Lys(Z)–Val–Leu–OMe——Z(OMe)–Val–Leu–OMe (5.0 g, 12.2 mmol) was treated with TFA–anisole (10.4 ml–2.6 ml) and the N^{α}-deprotected peptide, isolated as usual, was dissolved in DMF (20 ml) containing Et_3N (1.7 ml, 12.2 mmol). A mixed anhydride [prepared from 5.40 g (12.2 mmol) of Z(OMe)–Lys(Z)–OH] in THF (70 ml) was added to the above ice-chilled solution and the mixture was stirred in an ice-bath for 3 h. The solvent was removed by evaporation and the product was purified by procedure B, followed by precipitation from DMF with MeOH; yield 5.10 g (62%), mp 192—193 °C, $[\alpha]_D^{20}$ −4.8° (*c*=0.6, DMF), Rf_1 0.93, Rf_3 0.76. *Anal.* Calcd for $C_{35}H_{50}N_4O_9$: C, 62.67; H, 7.51; N, 8.35. Found: C, 62.69; H, 7.50; N, 8.24.

Z(OMe)–Arg(Mts)–Lys(Z)–Val–Leu–OMe——The above protected tripeptide (5.0 g, 7.5 mmol) was treated with TFA–anisole (9.6 ml–2.4 ml) and the N^{α}-deprotected peptide, isolated as usual, was dissolved in DMF (40 ml) containing Et_3N (1.0 ml, 7.5 mmol). A mixed anhydride [prepared from 5.08 g (8.2 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. The solvent was removed by evaporation and the product was purified by procedure B, followed by precipitation from DMF with AcOEt; yield 5.30 g (71%), mp 180—181 °C, $[\alpha]_D^{20}$ −8.7° (*c*=1.3, DMF), Rf_1 0.84, Rf_2 0.43, Rf_3 0.28. *Anal.* Calcd for $C_{50}H_{72}N_8O_{12}S$: C, 59.50; H, 7.19; N, 11.10. Found: C, 59.29; H, 7.08; N, 10.83.

Z(OMe)–Arg(Mts)–Lys(Z)–Val–Leu–NHNH$_2$ [6]——The above protected tetrapeptide (5.30 g, 5.3 mmol) was dissolved in DMF–MeOH (1 : 1, 50 ml) and treated with 80% hydrazine hydrate (1.6 ml, 5 eq). After being kept standing for 14 h, additional hydrazine (1.6 ml) was added and the solution was kept at 30 °C for a further 14 h. After evaporation of the solvent, the residue was treated with H_2O and the resulting powder was precipitated from DMF with EtOH; yield 4.50 g (85%), mp 204—205 °C, $[\alpha]_D^{20}$ −10.3° (*c*=1.4, DMSO), Rf_1 0.69, Rf_2 0.25. Amino acid ratios in 6 N HCl hydrolysate: Val 0.99, Leu 1.00, Lys 1.04, Arg 1.01 (recovery of Leu 94%). *Anal.* Calcd for $C_{49}H_{72}N_{10}O_{11}S$: C, 58.31; H, 7.19; N, 13.88. Found: C, 58.01; H, 7.19; N, 14.09.

Z(OMe)–Ser–Tyr–OMe——The azide [prepared from 14.70 g (51.8 mmol) of Z(OMe)–Ser–NHNH$_2$] in DMF (100 ml) and Et_3N (8.7 ml, 62.2 mmol) were added to an ice-chilled solution of H–Tyr–OMe [prepared from 10.0 g (43.2 mmol) of the hydrochloride] in DMF (50 ml) and the mixture was stirred at 4 °C overnight. After evaporation of the solvent, the product was purified by procedure A, followed by recrystallization from AcOEt and ether; yield 14.80 g (77%), mp 118—120 °C, $[\alpha]_D^{20}$ +13.3° (*c*=1.7, DMF), Rf_1 0.73. *Anal.* Calcd for $C_{22}H_{26}N_2O_7$: C, 59.18; H, 5.87; N, 6.28. Found: C, 59.16; H, 5.88; N, 6.37.

Z(OMe)–Asn–Ser–Tyr–OMe——Z(OMe)–Ser–Tyr–OMe (4.40 g, 9.9 mmol) was treated with TFA–anisole (8.5 ml–2.1 ml) and the N^{α}-deprotected peptide, isolated as usual, was dissolved in DMF (20 ml), together with Et_3N (2.7 ml, 18.7 mmol) and Z(OMe)–Asn–ONp (4.50 g, 10.8 mmol). After being stirred overnight, the solution was concentrated and the residue was triturated with AcOEt. The resulting powder was precipitated three times from DMF with AcOEt; yield 3.75 g (68%), mp 165—167 °C, $[\alpha]_D^{20}$ −3.2° (*c*=1.5, DMF), Rf_1 0.62. *Anal.* Calcd for $C_{26}H_{32}N_4O_{10}\cdot 1/2H_2O$: C, 54.83; H, 5.84; N, 9.84. Found: C, 55.10; H, 5.79; N, 9.79.

Z(OMe)–Thr–Asn–Ser–Tyr–OMe——The above tripeptide (5.0 g, 8.9 mmol) was treated with TFA–anisole (7.7 ml, 1.9 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMF (20 ml) containing Et_3N (1.2 ml, 8.9 mmol). The azide [prepared from 2.65 g (8.9 mmol) of Z(OMe)–Thr–NHNH$_2$] in DMF (20 ml) and Et_3N (1.5 ml, 10.7 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4 °C overnight. After evaporation of the solvent, the residue was dissolved in *n*-BuOH. The organic phase was washed with 5% citric acid and H_2O–NaCl, dried over $MgSO_4$ and concentrated. Trituration of the residue with ether afforded a powder, which was precipitated from DMF with AcOEt; yield 4.05 g (69%), mp 115—120 °C, $[\alpha]_D^{20}$ −11.1° (*c*=1.2, DMF) Rf_1 0.46. *Anal.* Calcd for $C_{30}H_{39}N_5O_{12}\cdot H_2O$: C, 53.01; H, 6.08; N, 10.31. Found: C, 53.06; H, 6.06; N, 10.30.

Z(OMe)–Thr–Asn–Ser–Tyr–NHNH$_2$ [7]——The above protected tetrapeptide (4.05 g, 6.12 mmol) in DMF–MeOH (1 : 1, 60 ml) was treated with 80% hydrazine hydrate (1.9 ml, 5 eq) overnight. The resulting solid was collected by filtration and precipitated from DMSO with MeOH; yield 2.85 g (70%), mp 223—225 °C, $[\alpha]_D^{20}$ −15.3°

($c=1.7$, DMSO), Rf_3 0.13. Amino acid ratios in 6 N HCl hydrolysate: Asp 1.00, Thr 0.94, Ser 0.87, Tyr 0.90 (recovery of Asp 100%). *Anal.* Calcd for $C_{29}H_{39}N_7O_{11}\cdot 1/2H_2O$: C, 51.93; H, 6.01; N, 14.62. Found: C, 51.99; H, 5.97; N, 14.48.

Z(OMe)–Ala–Ile–Phe–OMe——Z(OMe)–Ile–Phe–OMe (5.0 g, 11.0 mmol) was treated with TFA–anisole (9,5 ml–2.4 ml) and the N^{α}-deprotected peptide isolated as usual was dissolved in DMF (20 ml) containing Et_3N (1.5 ml, 11.0 mmol). A mixed anhydride [prepared from 2.77 g (11.0 mmol) of Z(OMe)–Ala–OH] in DMF (50 ml) was added to the above ice-chilled solution and the mixture was stirred in an ice-bath for 3 h. The solvent was evaporated off and the product was purified by procedure B, followed by precipitation from DMF with AcOEt; yield 4.0 g (69%), mp 207—208 °C, $[\alpha]_D^{20}$ −1.8° ($c=0.6$, DMF), Rf_1 0.87, Rf_2 0.77. *Anal.* Calcd for $C_{28}H_{37}N_3O_7$: C, 63.74; H, 7.07; N, 7.97. Found: C, 63.90; H, 7.24; N, 7.83.

Boc–Asp(OBzl)–Ala–Ile–Phe–OMe——The above tripeptide (5.0 g, 9.5 mmol) was treated with TFA–anisole (10 ml–3 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMF (20 ml) containing Et_3N (1.3 ml, 9.5 mmol). A mixed anhydride [prepared from 3.06 g (9.5 mmol) of Boc–Asp(OBzl)–OH] in DMF (30 ml) was added to the above ice-chilled solution and the mixture was stirred in an ice-bath for 3 h. The solvent was removed by evaporation and the product was purified by procedure B, followed by recrystallization from THF and ether; yield 5.60 g (88%), mp 154—155 °C, $[\alpha]_D^{20}$ −17.5° ($c=0.9$, DMF), Rf_1 0.92, Rf_2 0.84, Rf_3 0.33. *Anal.* Calcd for $C_{35}H_{48}N_4O_9$: C, 62.85; H, 7.23; N, 8.38. Found: C, 62.89; H, 7.33; N, 8.48.

Z(OMe)–Tyr–Ala–Asp–Ala–Ile–Phe–OMe——Boc–Asp(OBzl)–Ala–Ile–Phe–OMe (3.46 g, 5.2 mmol) in DMF (50 ml) was hydrogenated over a Pd catalyst for 10 h. After filtration, the filtrate was concentrated and the residue was treated with ether. The resulting powder (Rf_1 0.64) was treated with TFA–anisole (6.7 ml–1.7 ml) and the N^{α}-deprotected peptide, isolated as usual, was dissolved in DMF (20 ml) containing Et_3N (1.5 ml, 10.4 mmol). The azide [prepared from 2.68 g (6.2 mmol) of Z(OMe)–Tyr–Ala–$NHNH_2$] in DMF (5 ml) and Et_3N (1.0 ml, 7.5 mmol) were added to the above ice-chilled solution, and the mixture was stirred at 4 °C for 14 h. After addition of a few drops of AcOH, the solvent was removed by evaporation and the product was purified by procedure B, followed by precipitation from DMF with MeOH; yield 3.25 g (71%), mp 215—216 °C, $[\alpha]_D^{20}$ −20.4° ($c=0.9$, DMSO), Rf_1 0.42, Rf_2 0.42. *Anal.* Calcd for $C_{44}H_{56}N_6O_3$: C, 60.26; H, 6.44; N, 9.58. Found: C, 59.77; H, 6.50; N, 9.67.

Z-(OMe)–Tyr–Ala–Asp–Ala–Ile–Phe–$NHNH_2$[8]——The above protected hexapeptide (2.0 g, 2.3 mmol) in DMF–MeOH (2 : 1, 30 ml) was treated with 80% hydrazine hydrate (1.4 ml, 10 eq) for 24 h. The solvent was evaporated off, the residue was treated with H_2O, and the resulting powder was precipitated from DMF with MeOH; yield 1.75 g (88%), mp 222—224 °C, $[\alpha]_D^{20}$ −14.8° ($c=0.5$, DMSO), Rf_1 0.28. Amino acid ratios in 6 N HCl hydrolysate: Asp 1.13, Ala 2.27, Ile 1.03, Tyr 1.09, Phe 1.00 (recovery of Phe 90%). *Anal.* Calcd for $C_{43}H_{56}N_8O_{12}$: C, 58.89; H, 6.44; N, 12.78. Found: C, 58.73; H, 6.41; N, 12.51.

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–Arg(Mts)–Ala–Arg–(Mts)–Leu–NH_2, Z(OMe)–(hpGRF 19—44)–NH_2——The protected docosapeptide amide, Z(OMe)–(hpGRF 23—44)–NH_2 (5.20 g, 1.40 mmol) was treated with TFA–anisole (12 ml–4 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMSO–DMF (1 : 1, 50 ml) containing Et_3N (0.20 ml, 1.40 mmol). The azide [prepared from 2.74 g (2.79 mmol) of Z(OMe)–(hpGRF 19—22)–$NHNH_2$] in DMF (20 ml) and Et_3N (0.39 ml, 2.79 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 36 h. H_2O (150 ml) was added to the above ninhydrin-negative solution and the resulting powder was purified by procedure B, followed by precipitation from DMSO with MeOH; yield 5.24 g (83%), mp 270 °C (dec.), $[\alpha]_D^{20}$ −6.0° ($c=0.8$, DMSO), Rf_1 0.39. *Anal.* Calcd for $C_{205}H_{298}N_{48}O_{55}S_6\cdot 12H_2O$: C, 52.12; H, 6.87; N, 14.24. Found: C, 52.36; H, 6.72; N, 13.90.

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–Arg(Mts)–Ala–Arg(Mts)–Leu–NH_2, Z(OMe)–(hpGRF 15—44)–NH_2——The above protected hexacosapeptide amide, Z(OMe)–(hpGRF 19—44)–NH_2 (5.13 g, 1.14 mmol) was treated with TFA–anisole (12 ml–4 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMSO–DMF (50 ml) containing Et_3N (0.16 ml, 1.14 mmol). The azide [prepared from 1.33 g (2.28 mmol) of Z(OMe)–(hpGRF 15—18)–$NHNH_2$] in DMF (10 ml) and Et_3N (0.32 ml, 2.28 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 24 h. Additional azide [prepared from 0.33 g (0.57 mmol) of the hydrazide] in DMF (3 ml) and Et_3N (0.08 ml, 0.57 mmol) were then added and stirring was continued for an additional 24 h, until the solution became ninhydrin-negative. Addition of H_2O (150 ml) afforded a powder, which was purified by procedure B, followed by precipitation twice from DMSO with MeOH; yield 4.28 g (77%), mp 270 °C (dec.), $[\alpha]_D^{20}$ +5.3° ($c=0.2$, DMSO), Rf_1 0.47. *Anal.* Calcd for $C_{221}H_{325}N_{53}O_{61}S_6\cdot 6H_2O$: C, 53.08; H, 6.79; N, 14.85. Found: C, 52.80; H, 6.72; N, 14.58.

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–Arg(Mts)–Ala–Arg(Mts)–Leu–NH_2, Z(OMe)–(hpGRF 11—44)–NH_2——The above protected triacontapeptide amide, Z-(OMe)–(hpGRF 15—44)–NH_2, (1.01 g, 0.21 mmol) was treated with TFA–anisole (5 ml–1 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMSO–DMF (1 : 1, 10 ml) containing Et_3N (29 μl, 0.21 mmol). The azide [prepared from 0.63 g (0.62 mmol) of Z(OMe)–(hpGRF 11—14)–$NHNH_2$] in DMF (5 ml) and Et_3N (86 μl, 0.62 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4 °C overnight.

After addition of H_2O (50 ml), the resulting powder was purified by procedure B, followed by gel-filtration on Sephadex LH-60 (3 × 128 cm), using DMF as an eluant. Individual fractions (10 ml each) were examined by measurement of ultraviolet (UV) absorption at 280 nm, and the fractions corresponding to the front peak (tube Nos. 38—68) were combined. The solvent was removed by evaporation and the residue was treated with ether to afford a powder; yield 796 mg (68%), mp 280 °C (dec.), $[\alpha]_D^{20}$ −9.2° (c=0.8, DMSO), Rf_1 0.49. *Anal.* Calcd for $C_{261}H_{385}N_{61}O_{69}S_7$: C, 54.94; H, 6.80; N, 14.98. Found: C, 54.76; H, 6.88; N, 14.69.

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–Arg(Mts)–Ala–Arg(Mts)–Leu–NH_2, Z(OMe)–(hpGRF 7—44)–NH_2——The above protected tetratriacontapeptide amide, Z(OMe)–(hpGRF 11—44)–NH_2 (666 mg, 117 μmol), was treated with TFA–anisole (4 ml–0.7 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMSO–DMF (1 : 2, 10 ml) containing Et_3N (24 μl, 117 μmol). The azide [prepared from 309 mg (467 μmol) of Z(OMe)–(hpGRF 7—10)–$NHNH_2$] in DMF (5 ml) and Et_3N (65 μl, 467 μmol) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 24 h. The solvent was removed by evaporation and the residue was treated with H_2O to form a powder, which was purified by procedure B, followed by gel filtration on Sephadex LH-60 (3 × 128 cm) using DMF as an eluant. Individual fractions (11 ml each) were examined by measurement of UV absorption at 280 nm, and the fractions corresponding to the main peak (tube Nos. 37—44) were combined. The solvent was removed by evaporation and the residue was treated with ether to afford a powder; yield 669 mg (93%), mp 270 °C (dec.), $[\alpha]_D^{20}$ −2.7° (c=0.4, DMSO), Rf_1 0.47, Rf_4 0.74. *Anal.* Calcd for $C_{281}H_{412}N_{66}O_{77}S_7$: C, 54.69; H, 6.73; N, 14.98. Found: C, 54.56; H, 6.73; N, 15.06.

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–Arg(Mts)–Ala–Arg(Mts)–Leu–NH_2, Protected hpGRF 1—44——The above protected octatriacontapeptide amide, Z(OMe)–(hpGRF 7—44)–NH_2, (669 mg, 108 μmol) was treated with TFA–anisole (5 ml–1 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMSO–DMF (1 : 2, 6 ml) containing 10% Et_3N–DMF (15 μl, 108 μmol). The azide [prepared from 238 mg (271 μmol) of Z(OMe)–Tyr–Ala–Asp–Ala–Ile–Phe–$NHNH_2$] in DMF (2 ml) and Et_3N (38 μl, 271 μmol) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 24 h. The solvent was removed by evaporation and the residue was treated with H_2O. The resulting powder was purified by procedure B, followed by gel-filtration on Sephadex LH-60 (3 × 128 cm) using DMF as an eluant as described above. The desired fractions (tube Nos. 41—55) were combined, the solvent was evaporated off and the residue was treated with ether to afford a powder; yield 583 mg (79%), mp 167—170 °C, $[\alpha]_D^{20}$ −8.5° (c=0.1, DMSO), Rf_1 0.44. *Anal.* Calcd for $C_{315}H_{456}N_{72}O_{86}S_7 \cdot 4H_2O$: C, 54.64; H, 6.76; N, 14.57. Found: C, 54.60; N, 6.49; N, 14.50.

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–Arg–Ala–Arg–Leu–NH_2, H–(hpGRF 1—44)–NH_2——The above protected tetratetracontapeptide amide (200 mg, 29 μmol) was treated with 1 M TFMSA–thioanisole in TFA (7 ml) in the presence of *m*-cresol (183 μl, 60 eq) in an ice-bath for 60 min, then 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 (4 ml) containing dithiothreitol (100 mg), treated with Amberlite CG-4B (acetate form, approximately 2 g) for 30 min with stirring, and then filtered. The pH of the filtrate was adjusted to 8.0 with 1 N NH_4OH and, after the solution had been stirred for 30 min in an ice-bath, readjusted to 6.5 with 1 N AcOH. The solution was lyophilized to give a hygroscopic powder, which was dissolved in H_2O (3 ml), then incubated with dithiothreitol (200 mg, 50 eq) under an argon gas atmosphere at 37 °C for 24 h. Then the solution was applied to a column of Sephadex G-25 (2.6 × 93 cm), which was eluted with 1 N AcOH. The UV absorption at 275 nm was determined for each fraction (6 ml). The fractions corresponding to the front main peak (tube Nos. 44—59) were combined and the solvent was removed by lyophilization to give a white fluffy powder; yield 146 mg (99%).

The above crude product (102 mg) was dissolved in pH 8.0, 0.01 M NH_4HCO_3 buffer, and the solution was applied to a column of CM-Trisacryl ®M (3 × 9 cm), which was eluted first with the same buffer, and then with a linear gradient formed from the starting buffer (500 ml) and pH 8.2, 0.25 M NH_4HCO_3 buffer (500 ml). The UV absorption in each fraction (6 ml) was determined (Fig. 8). The fractions corresponding to the main peak (tube Nos. 86—111) were combined and the solvent and ammonium salt were removed by repeated lyophilization to give a white fluffy powder; yield 82 mg (80%).

Subsequent purification was performed by reverse phase HPLC on a TSK 410KG column (2.15 × 30 cm). A part of the CM-purified sample (45 mg) was applied to the above column, which was eluted with 41% acetonitrile in 0.5% heptafluorobutyric acid under isocratic conditions at a flow rate of 14 ml/min. The eluate corresponding to the main peak (retention time, 52 min, Fig. 9a) was collected. The rest of the sample (36 mg) was similarly purified and after addition of β-mercaptoethanol (1 ml), the solvent was removed from the combined eluate by rotary evaporation *in vacuo*. The residue was dissolved in pH 7.5, 1 M $CH_3COO \cdot Et_3NH$ buffer (2 ml) and the solution was desalted by the gel-filtration on Sephadex G-25 (2 × 40 cm), using 1 N AcOH as eluant. The desired fractions were collected and the

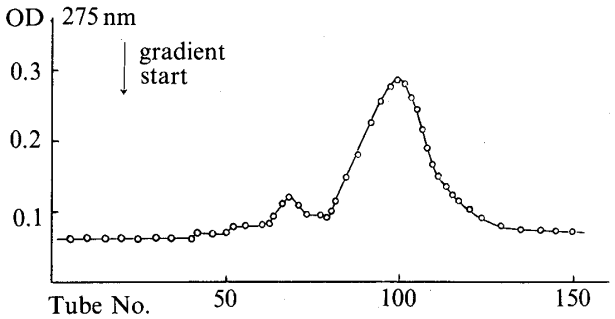

Fig. 8. Purification of Synthetic H–(hpGRF 1—44)–NH_2 by Ion-Exchange Chromatography on CM-Trisacryl® M

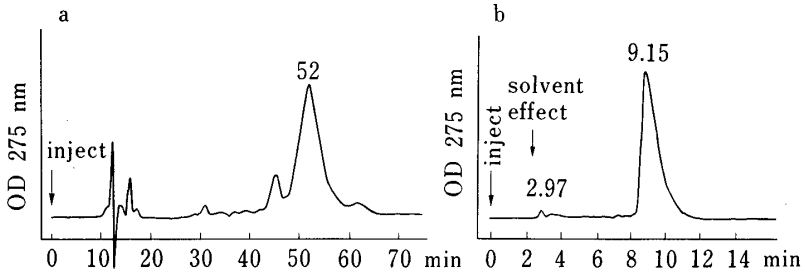

Fig. 9. HPLC of H–(hpGRF 1—44)–NH_2

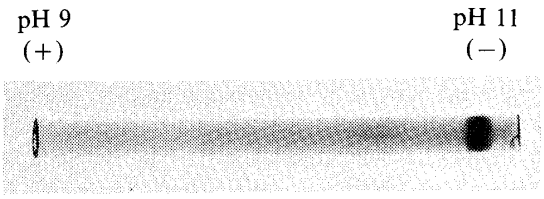

Fig. 10. Disk Isoelectrofocusing of Synthetic H–(hpGRF 1—44)–NH_2

solvent was removed by lyophilization to give a white fluffy powder; yield 46 mg (57%), total yield from the protected hpGRF (1—44)–NH_2 45%; $[\alpha]_D^{20}$ −60.0° (c = 0.2 in 0.2 N AcOH), Rf_4 0.30, Rf_5 0.36. The synthetic peptide exhibited a single peak on HPLC at a retention time of 9.15 min (Fig. 9b) and a single band in disk isoelectrofocusing on 7.5% polyacrylamide gel (0.5 × 6.0 cm) containing Pharmalyte (pH 9—11): [mobility, 5.4 cm from the origin toward the cathodic end of the gel after running at 200 V for 4 h, stained with Coomassie Brilliant Blue G-250 (Sigma)] (Fig. 10). Amino acid ratios in 6 N HCl hydrolysates are shown in Table I. Amino acid ratios after leucine aminopeptidase digestion; Asp 2.07 (2), Thr 0.94 (1), Ser 3.99 (4), Glu 2.20 (2), Gly 3.06 (3), Ala 5.27 (5), Val 1.13 (1), Met 0.85 (1), Ile 2.02 (2), Leu 5.00 (5), Tyr 2.00 (2), Phe 1.10 (1), Lys 2.07 (2), Arg 6.03 (6), Asn (2) and Gln (5) were not determined (recovery of Leu 78%). *Anal.* Calcd for $C_{215}H_{358}N_{72}O_{66}S \cdot 9AcOH \cdot 21H_2O$: C, 46.96; H, 7.38; N, 16.93. Found: C, 46.67; H, 7.02; N, 17.31.

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