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Studies on Peptides. CXXXVII.[1,2] Conventional Solution Synthesis of Porcine Hypothalamic Growth Hormone Releasing Factor (pGRF)

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A tetratetracontapeptide amide corresponding to the entire amino acid sequence of porcine hypothalamic growth hormone releasing factor (pGRF) was synthesized by a conventional solution method, by assembling nine peptide fragments followed by deprotection with 1 M trifluoromethanesulfonic acid–thioanisole in trifluoroacetic acid. The synthetic peptide was as active as synthetic human GRF–44–NH_2 in an *in vitro* assay. A new substituted hydrazine, 2,2,2-trichloro-*tert*-butoxycarbonyl hydrazine, was employed for the preparation of a hydrazide containing Glu(OBzl). In the deprotecting step, Me_2Se was employed to facilitate acidolytic cleavage of protecting groups, as well as reduction of Met(O).

Keywords——porcine growth hormone releasing factor synthesis; substituted hydrazine; reduction of Met(O); trifluoromethanesulfonic acid deprotection; thioanisole-mediated deprotection; *in vivo* GRF activity

Following the structural elucidation of human pancreatic tumorous peptides with high intrinsic growth hormone releasing activity,[3,4] growth hormone releasing factor (GRF) has been isolated from various mammalian hypothalamic tissues and chemically characterized.[5-9]

We wish to report the solution synthesis of a tetratetracontapeptide amide corresponding to the entire amino acid sequence of porcine GRF (pGRF), the structure of which was determined by Böhlen *et al.*[6] in 1983. This factor possesses the same sequence as human GRF (hGRF), except for replacement of three residues at positions 34, 38 and 42, *i.e.*, Ser, Arg and Ala of hGRF with Arg, Gln and Val, respectively.

The methods we employed here are essentially the same as employed for our previous synthesis of hGRF–44–NH_2.[10] Amino acid derivatives bearing protecting groups removable by 1 M TFMSA–thioanisole in TFA[11] were employed, *i.e.*, Arg(Mts),[12] Glu(OBzl), Asp(OBzl) and Lys(Z). Met was protected as its sulfoxide.[13] Eight peptide hydrazides and the C-terminal dodecapeptide amide were selected as building blocks to construct the peptide backbone of pGRF by the azide procedure[14] (Fig. 1). Of these, six fragments, [2], [5], [6], [7], [8] and [9], are available from our previous synthesis of hGRF,[10] and fragment [3] from our previous synthesis of hGRF–37–NH_2.[15] Fragment [4] (position 23—24) was prepared by the usual hydrazinolysis of Z(OMe)–Leu–Gln–OBzl obtained by the mixed anhydride procedure.[16] The C-terminal fragment [1], which covers the area of species variation between hGRF and pGRF, was synthesized according to the scheme illustrated in Fig. 2. Starting with Z(OMe)–Arg(Mts)–Leu–NH_2,[10] the protected pentapeptide,

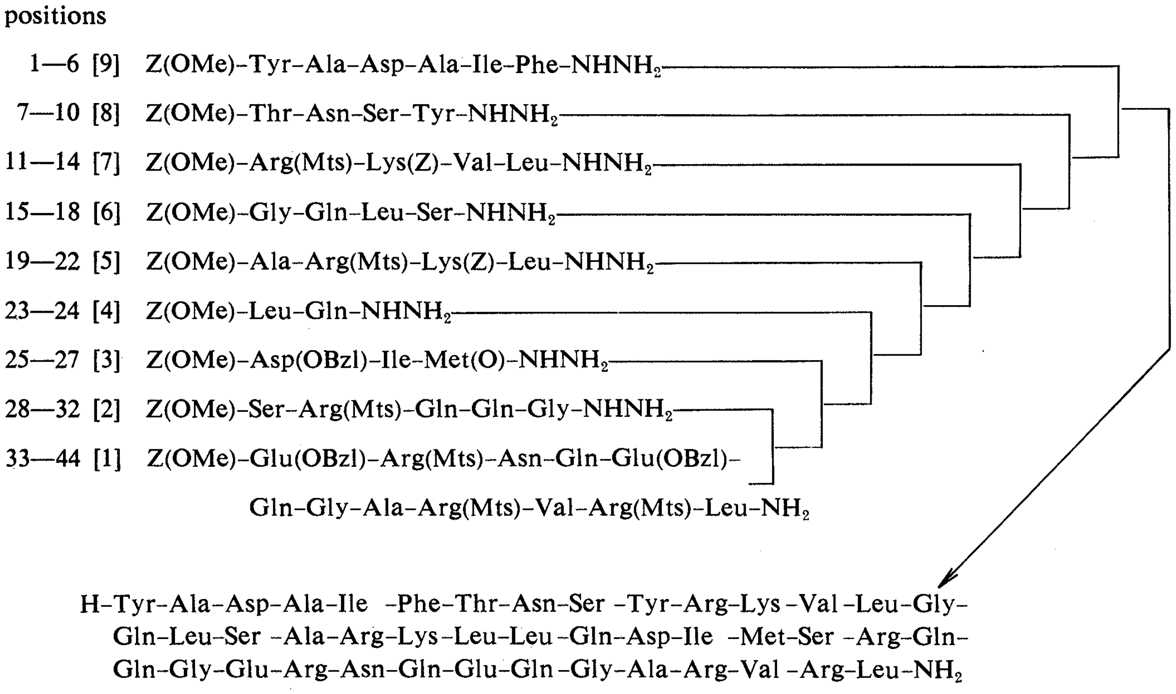

Fig. 1. Synthetic Route to pGRF

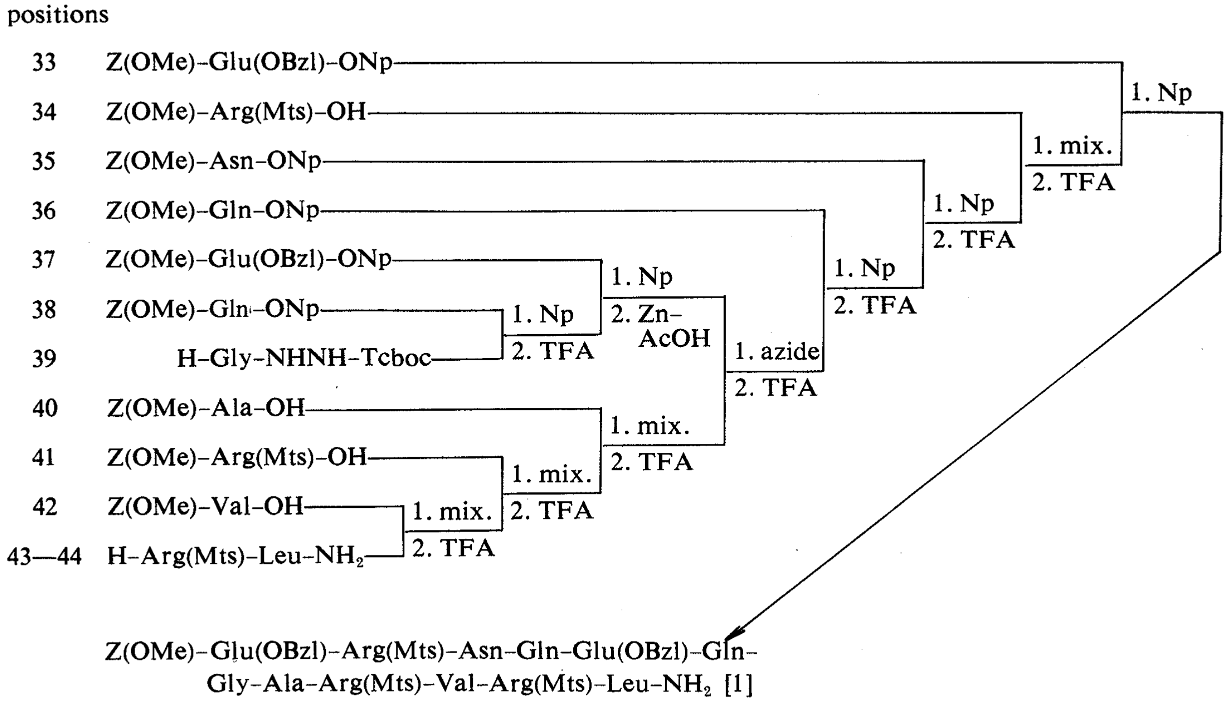

Fig. 2. Synthetic Scheme for the Protected Dodecapeptide Amide Z(OMe)-(pGRF 33—44)-NH_2 [1]

Z(OMe)–Ala–Arg(Mts)–Val–Arg(Mts)–Leu–NH_2, was prepared in a stepwise manner by the mixed anhydride procedure. Next, Z(OMe)–Glu(OBzl)–Gln–Gly–NHNH–Tcboc was prepared by the succesive Np procedure[17] with the aid of Tcboc–$NHNH_2$,[18] the protecting group of which was found to be cleaved by Zn in AcOH[19] more easily than Troc–$NHNH_2$.[20] This peptide, after removal of the Tcboc group, was condensed with a TFA-treated sample of the above pentapeptide amide. The resulting octapeptide chain was elongated to [1] in a

Table I. Amino Acid Ratios in 6 N HCl Hydrolysates of Synthetic pGRF and Its Protected Intermediates

	Protected intermediates								Synthetic pGRF
	28—44	25—44	23—44	19—44	15—44	11—44	7—44	1—44	
Asp	0.90	1.96	2.03	2.19	2.20	2.13	3.06	3.87	3.98 (4)
Thr							0.84	0.83	0.96 (1)
Ser	0.74	0.83	0.85	0.94	1.68	1.70	2.45	2.56	2.69 (3)
Glu	5.97	6.13	7.60	8.10	9.11	8.86	8.83	8.82	8.40 (8)
Gly	1.88	1.99	2.14	2.30	3.17	3.09	3.16	3.23	3.15 (3)
Ala	1.03	1.00	1.10	2.10	2.12	2.10	2.12	3.85	4.01 (4)
Val	0.95	0.98	1.07	1.12	1.08	1.93	1.91	1.90	1.83 (2)
Met		0.88	0.74	0.85	0.92	0.91	0.86	0.82	0.83 (1)
Ile		0.94	0.89	0.96	1.00	0.99	0.99	1.74	1.86 (2)
Leu	1.00	1.00	2.00	3.00	4.00	5.00	5.00	5.00	5.00 (5)
Tyr							0.81	1.66	1.66 (2)
Phe								0.75	0.89 (1)
Lys				1.01	1.06	2.17	2.02	2.03	1.92 (2)
Arg	3.75	4.16	4.54	5.79	5.75	6.55	6.50	6.46	6.14 (6)
Rec. (%)	80	73	69	65	74	77	78	92	67

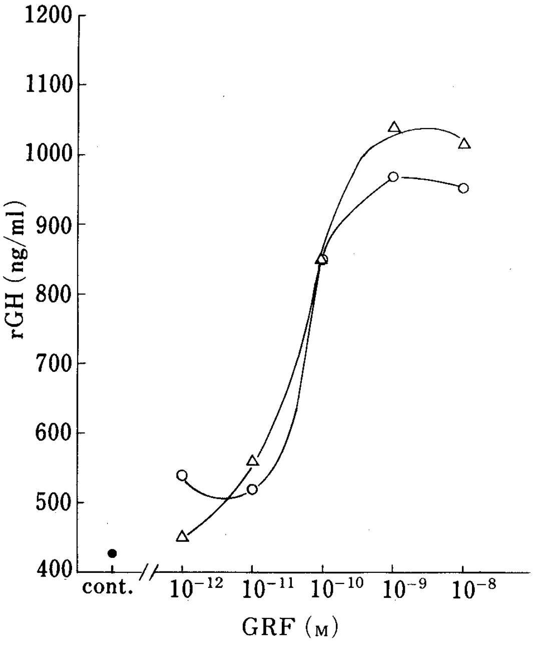

Fig. 3. The *in Vitro* Assay of Synthetic pGRF-44-NH_2

○, hGRF-44-NH_2; △, pGRF-44-NH_2.

stepwise manner by successive addition of appropriate amino acid residues *via* the Np ester or the mixed anhydride.

Nine fragments were assembled successively by the azide procedure according to the route shown in Fig. 1. The products obtained from the coupling reactions of fragments up to [4] were purified by precipitation from DMF or DMSO–DMF with appropriate solvents, such as MeOH, AcOEt or ether, and the other products by gel-filtration on Sephadex LH-60 using DMF as an eluant. Throughout this synthesis, Leu was used as a diagnostic amino acid. Each intermediate was subjected to acid hydrolysis and the recovery of Leu was compared with those of newly added amino acids in order to ascertain satisfactory incorporation, after each condensation, as shown in Table I. The homogeneity of every intermediate was further ascertained by elemental analysis and thin layer chromatography (TLC).

In the final step, the protected pGRF was treated with 1 M TFMSA–thioanisole in TFA in the presence of *m*-cresol and dimethylselenide[21] in an ice-bath for 90 min to remove all the protecting groups employed. Dimethylselenide has proven to have an ability to accelerate the cleavage of protecting groups and in addition to reduce Met(O) more effectively than thioanisole under these acidic conditions.[21] The deprotected peptide, after being converted to the corresponding acetate with Amberlite IRA-400 (acetate form), was treated with dil. ammonia at pH 8.0 to reverse a possible N→O shift[22] at the Ser and Thr residues and then incubated with dithiothreitol to ensure the complete reduction of Met(O). The reduced product was purified by gel-filtration on Sephadex G-25 followed by reverse-phase high-performance liquid chromatography (HPLC) on a Zorbax (Shimadzu) BP-ODS column using gradient elution with acetonitrile (30 to 40%) in 0.3% TFA. The product thus purified was converted to the corresponding acetate by using Amberlite IRA-400. Homogeneity of the synthetic pGRF was ascertained by analytical HPLC and amino acid analyses, after 6 N HCl hydrolysis and enzymatic digestion.

When tested in an *in vitro* system according to Guillemin *et al.*,[23] our synthetic pGRF (0.1—0.5 μg) stimulated the secretion of immunoreactive growth hormone in rats (Fig. 3) and its potency was estimated to be equivalent to that of our synthetic hGRF–44–NH_2.

Experimental

General experimental methods employed in this investigation are essentially the same as described in our hGRF synthesis.[10] An azide was prepared according to Honzl and Rudinger[13] with isoamyl nitrite and a mixed anhydride was prepared according to Vaughan and Osato[16c] with isobutyl chloroformate. Products were purified by precipitation (A) using appropriate solvents or by gel-filtration (B) on Sephadex LH-60. Solvents used for (A): A-1, MeOH–ether; A-2, MeOH–2-propanol; A-3, DMF–EtOH; A-4, DMF–MeOH; A-5, DMSO–DMF (2:1)–MeOH;

TABLE II. Physical Constants and Analytical Data of Fragment [1] and Its Intermediates

Compounds	Puri. proc.	Rf_1	Yield (%)	mp (°C)	$[\alpha]_D^{25}$ (in DMSO)	Formula	Analysis (%) Calcd (Found)		
							C	H	N
Z(OMe)–(42—44)–NH_2	A-1	0.65	94	155—158	−11.7°	$C_{35}H_{53}N_7O_8S$	57.43	7.30	13.40
							(57.15	7.16	13.32)
Z(OMe)–(41—44)–NH_2	A-2	0.63	69	198—200	−10.0°	$C_{50}H_{75}N_{11}O_{11}S_2$	56.10	7.06	14.40
							(56.05	7.11	13.69)
Z(OMe)–(40—44)–NH_2	A-3	0.77	66	195—198	−18.0°	$C_{53}H_{80}N_{12}O_{12}S_2$	55.77	7.07	14.73
							(55.53	7.09	14.61)
Z(OMe)–(38—39)–	A-1	0.50	81	105—110	−2.0°	$C_{21}H_{28}Cl_3N_5O_8$	43.12	4.83	11.98
NHNH–Tcboc							(42.83	4.93	11.73)
Z(OMe)–(37—39)–	A-1	0.62	95	136—140	−2.2°	$C_{33}H_{41}Cl_3N_6O_{11}$	49.29	5.14	10.45
NHNH–Tcboc							(49.02	5.39	10.71)
Z(OMe)–(37—39)–$NHNH_2$	A-3	0.53	63	145—150	−0.6°	$C_{28}H_{36}N_6O_9\cdot$	53.58	6.26	13.39
						$1.5H_2O$	(53.74	5.92	13.21)
Z(OMe)–(37—44)–NH_2	A-4	0.55	76	222—225	−18.0°	$C_{72}H_{104}N_{16}O_8S_2\cdot$	55.30	6.83	14.33
						H_2O	(55.21	6.79	14.39)
Z(OMe)–(36—44)–NH_2	A-5	0.79	95	260	−50.0°	$C_{77}H_{112}N_{18}O_{20}S_2\cdot$	54.08	6.84	14.75
				(dec.)		$2H_2O$	(53.99	6.75	14.85)
Z(OMe)–(35—44)–NH_2	A-6	0.46	87	260	−16.8°	$C_{81}H_{118}N_{20}O_{22}S_2\cdot$	53.86	6.70	15.51
				(dec.)		H_2O	(53.85	6.66	15.29)
Z(OMe)–(34—44)–NH_2	A-7	0.50	99	260	−18.3°	$C_{96}H_{140}N_{24}O_{25}S_3\cdot$	52.88	6.75	15.42
				(dec.)		$3H_2O$	(53.15	6.86	14.94)
Z(OMe)–(33—44)–NH_2	A-8	0.52	99	260	−16.7°	$C_{108}H_{153}N_{25}O_{28}S_3\cdot$	54.46	6.64	14.00
				(dec.)		$2H_2O$	(54.25	6.52	14.77)

TABLE III. Physical Constants and Analytical Data of Fragment [4] and Its Intermediate

Compounds	Puri. proc.	Rf_1	Yield (%)	mp (°C)	$[\alpha]_D^{25}$ (in DMSO)	Formula	Analysis (%) Calcd (Found) C	H	N
Z(OMe)–(23—24)–OBzl	A-1	0.89	63	150—153	−9.5°	$C_{27}H_{35}N_3O_7$	63.14 (63.14	6.87 6.84	8.18 8.01)
Z(OMe)–(23—24)–NHNH$_2$	A-1	0.50	80	208—210	−5.7°	$C_{20}H_{31}N_5O_6$	54.90 (54.73	7.14 7.17	16.01 16.04)

TABLE IV. Physical Constants and Analytical Data of Protected pGRF and Its Intermediates

Compounds	Puri. proc.	Rf_1	Yield (%)	$[\alpha]_D^{25}$ (in DMSO)	Formula	Analysis (%) Calcd (Found) C	H	N
Z(OMe)–(28—44)–NH$_2$	A-6	0.43	70	−14.3°	$C_{138}H_{199}N_{35}O_{38}S_4 \cdot 5H_2O$	52.21 (52.39	6.64 6.46	15.44 15.43)
Z(OMe)–(25—44)–NH$_2$	A-6	0.45	74	−10.8°	$C_{160}H_{230}N_{38}O_{44}S_5 \cdot 5H_2O$	52.79 (52.50	6.65 6.68	14.62 14.43)
Z(OMe)–(23—44)–NH$_2$	A-6	0.40	84	−25.0°	$C_{171}H_{249}N_{41}O_{47}S_5 \cdot 15H_2O$	50.56 (50.80	6.92 6.87	14.14 14.08)
Z(OMe)–(19—44)–NH$_2$	B	0.43	76	−9.5°	$C_{209}H_{305}N_{49}O_{55}S_6 \cdot 6H_2O$	53.58 (53.23	6.82 6.85	14.65 14.96)
Z(OMe)–(15—44)–NH$_2$	B	0.41	87	−20.0°	$C_{225}H_{332}N_{54}O_{61}S_6 \cdot 7H_2O$	53.11 (52.80	6.85 6.73	14.87 15.40)
Z(OMe)–(11—44)–NH$_2$	B	0.57	69	−14.5°	$C_{265}H_{392}N_{62}O_{69}S_7 \cdot 10H_2O$	53.45 (53.46	6.97 6.80	14.58 14.86)
Z(OMe)–(7—44)–NH$_2$	B	0.47	90	−13.3°	$C_{285}H_{419}N_{67}O_{77}S_7 \cdot 8H_2O$	53.61 (53.35	6.87 6.82	14.70 15.16)
Z(OMe)–(1—44)–NH$_2$	B	0.45	82	−16.0°	$C_{319}H_{463}N_{73}O_{86}S_7 \cdot 10H_2O$	53.95 (53.76	6.80 6.68	14.40 14.73)

A-6, DMSO–DMF (1 : 1)–MeOH; A-7, DMSO–DMF (1 : 2)–AcOEt; A-8, DMSO–DMF (1 : 1)–AcOEt. Procedure employed for (B): individual fractions (10 ml each) were examined by ultraviolet (UV) absorption measurement at 280 nm and the fractions corresponding to the front peak were combined. The solvent was removed by evaporation and the residue was treated with ether to afford a powder.

Analytical HPLC was conducted with a Shimadzu LC-4A model equipped with a Cosmosil (Nakarai Chem. Co.) 5C$_{18}$P (4.6 × 150 mm) column using linear gradient elution with acetonitrile (30 to 40%, 20 min) in 0.3% TFA at a flow rate of 1.0 ml/min.

TLC was performed on silica gel (Kieselgel 60 F$_{254}$, Merck). *Rf* values refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH–H_2O (8 : 3 : 1), Rf_2 *n*-BuOH–AcOH–pyridine–H_2O (4 : 1 : 1 : 2).

Synthesis of Fragment [1]——Fragment [1] was prepared according to the scheme shown in Fig. 2. Purification procedures, physical constants and analytical data are shown in Table II.

Synthesis of Fragment [4]——This fragment was prepared as described in the text. Data obtained for characterization are listed in Table III.

Synthesis of Protected pGRF——Nine fragments were condensed successively by the azide procedure. Purification procedures, physical constants and analytical data are listed in Table IV. All protected peptides decomposed at over 260 °C.

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–Arg–Asn–Gln–Glu–Gln–Gly–Ala–Arg–Val–Arg–Leu–NH$_2$, pGRF-44-NH$_2$——Z(OMe)–(pGRF 1—44)–NH$_2$ (100 mg, 14.4 μmol) was treated with 1 M TFMSA–thioanisole in TFA (3.5 ml) in the presence of *m*-cresol (91 μl) and Me$_2$Se (66 μl) in an ice-bath for 90 min, then dry ether was added. The resulting powder was collected by centrifugation, dried over KOH pellets *in vacuo* for 30 min and dissolved in

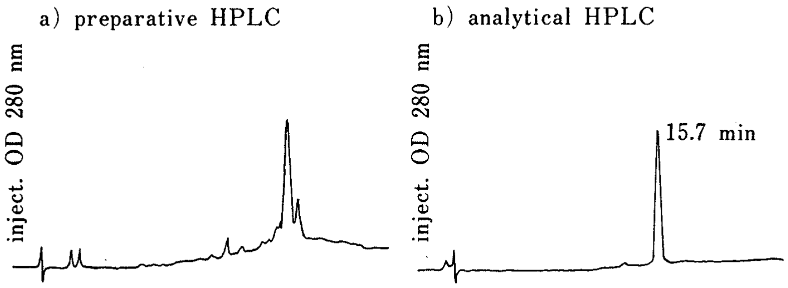

Fig. 4. HPLC of Synthetic pGRF

50% AcOH (10 ml). The solution, after being stirred with Amberlite IRA-400 (acetate form, *ca.* 1 g) for 30 min, was filtered. The pH of the filtrate was adjusted to 8.0 with dil. NH_4OH and after 30 min to pH 6.5 with 1 N AcOH. The solution was incubated with dithiothreitol (111 mg) at 40 °C for 8 h and then lyophilized. The product was purified by gel-filtration on Sephadex G-25 (1.8 × 90 cm) using 1 N AcOH as an eluant. The fractions (4 ml each) corresponding to the front main peak (tube Nos. 23—40, determined by UV absorption measurement at 280 nm) were combined and the solvent was removed by lyophilization to give a fluffy powder; yield 60 mg (82%).

Subsequent purification was performed by reverse-phase HPLC on a Zorbax (Shimadzu) BP-ODS column (7.9 × 250 mm). The above gel-filtered sample (10 mg) was applied to a column, which was eluted with acetonitrile (gradient of concentration from 30% to 40% within 20 min) in 0.3% TFA at a flow rate of 3.0 ml/min. The eluate corresponding to the main peak (retention time 22.6 min, Fig. 4-a) was collected and the solvent was removed by evaporation *in vacuo*. The rest of the sample (50 mg) was similarly purified. The product thus obtained was converted to the corresponding acetate by Amberlite IRA-400 (acetate form) and finally lyophilized to give a fluffy white powder; 12 mg (20%), total yield from the protected pGRF was 16%. $[\alpha]_D^{16} -56.7°$ ($c=0.6$, 1 N AcOH), Rf_2 0.32. A single peak in analytical HPLC: retention time 15.7 min (Fig. 4-b). Amino acid ratios after aminopeptidase M digestion (Merck Art. 24645, Lot. No. 2513445): Asp 2.07 (2), Gln (6) + Thr (1) 6.24 (calcd as Thr), Ser 3.08 (3), Glu 2.49 (2), Gly 2.86 (3), Ala 3.94 (4), Val 2.14 (2), Met 0.98 (1), Ile 1.93 (2), Leu 5.00 (5), Tyr 2.09 (2), Phe 1.09 (1), Lys 2.31 (2), Arg 5.64 (6), Asn (2) N.D. (Recovery of Leu 78%). *Anal.* Calcd for $C_{219}H_{365}N_{73}O_{66}S \cdot 9CH_3COOH \cdot 29H_2O$: C, 46.15; H, 7.44; N, 16.58. Found: C, 45.84; H, 6.89; N, 16.98.

References and Notes

1) Part CXXXVI: N. Fujii, A. Otaka, S. Funakoshi, M. Nomizu, K. Akaji, H. Yajima, I. Yamamoto, K. Torizuka, K. Kitagawa, T. Akita, K. Ando, T. Kawamoto, Y. Shimonishi, and T. Takao, *Chem. Pharm. Bull.*, **34**, 613 (1986).

2) Amino acids and peptides and their derivatives mentioned in this investigation are of the L-configuration. The following abbreviations are used: Z = benzyloxycarbonyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, Mts = mesitylene-2-sulfonyl, Bzl = benzyl, Troc = 2,2,2-trichloroethyloxycarbonyl, Tcboc = 2,2,2-trichloro-*tert*-butoxycarbonyl, Np = *p*-nitrophenyl, DMF = *N*, *N*-dimethylformamide, DMSO = dimethylsulfoxide, TFMSA = trifluoromethanesulfonic acid, TFA = trifluoroacetic acid.

3) R. Guillemin, P. Brazeau, P. Böhlen, and F. Esch, *Science*, **218**, 585 (1982); P. Esch, P. Böhlen, N. Ling, P. Brazeau, W. Wehrenberg, and R. Guillemin, *J. Biol. Chem.*, **258**, 1806 (1983).

4) J. Rivier, J. Spiess, M. Thorner, and W. Vale, *Nature* (London), **300**, 276 (1982); J. Spiess, J. Rivier, M. Thorner, and W. Vale, *Biochemistry*, **21**, 6037 (1982).

5) J. Spiess, J. Rivier, and W. Vale, *Nature* (London), **303**, 532 (1983).

6) P. Böhlen, F. Esch, P. Brazeau, N. Ling, and R. Guillemin, *Biochem. Biophys. Res. Commun.*, **116**, 726 (1983).

7) F. Esch, P. Böhlen, N. Ling, P. Brazeau, and R. Guillemin, *Biochem. Biophys. Res. Commun.*, **117**, 772 (1983).

8) P. Brazeau, P. Böhlen, F. Esch, N. Ling, W. B. Wehrenberg, and R. Guillemin, *Biochem. Biophys. Res. Commun.*, **125**, 606 (1984).

9) N. Ling, F. Esch, P. Böhlen, P. Brazeau, W. Wehrenberg, and R. Guillemin, *Proc. Natl. Acid. Sci. U.S.A.*, **81**, 4302 (1984); P. Brazeau, P. Böhlen, F. Esch, N. Ling, W. B. Wehrenberg, and R. Guillemin, *Biochem. Biophys. Res. Commun.*, **125**, 606 (1984); P. Böhlen, W. B. Wehrenberg, F. Esch, N. Ling, P. Brazeau, and R. Guillemin, *ibid.*, **125**, 1005 (1984).

10) H. Yajima, N. Fujii, M. Shimokura, K. Akaji, S. Kiyama, and M. Nomizu, *Chem. Pharm. Bull.*, **31**, 800 (1983); N. Fujii, M. Shimokura, M. Nomizu, H. Yajima, F. Shono, M. Tsuda, and A. Yoshitake, *ibid.*, **32**, 520 (1984).

11) H. Yajima, N. Fujii, H. Ogawa, and H. Kawatani, *J. Chem. Soc., Chem. Commun.*, **1974**, 107; H. Yajima and N. Fujii, *J. Am. Chem. Soc.*, **103**, 5867 (1981); Y. Kiso, S. Nakamura, K. Ito, K. Ukawa, K. Katagawa, T. Akita, and H. Moritoki, *J. Chem. Soc., Chem. Commun.*, **1979**, 971.

12) H. Yajima, M. Takeyama, J. Kanaki, and K. Mitani, *J. Chem. Soc., Chem. Commun.*, **1978**, 482.

13) B. Iselin, *Hlev. Chim. Acta*, **44**, 61 (1961); N. Fujii, T. Sasaki, S. Funakoshi, H. Irie, and H. Yajima, *Chem. Pharm. Bull.*, **26**, 650 (1978).

14) J. Honzl and J. Rudinger, *Collect. Czech. Chem. Commun.*, **26**, 2333 (1961).

15) N. Fujii, M. Shimokura, H. Yajima, F. Shono, M. Tsuda, and A. Yoshitake, *Chem. Pharm. Bull.*, **32**, 1200 (1984).

16) *a*) Th. Wieland and H. Bernhard, *Justus Liebigs Ann. Chem.*, **572**, 190 (1951); *b*) R. A. Boissonnas, *Helv. Chim. Acta*, **34**, 874 (1951); *c*) J. R. Vaughan, Jr. and R. L. Osato, *J. Am. Chem. Soc.*, **74**, 676 (1952).

17) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).

18) M. Shimokura, S. Hosoi, K. Okamoto, Y. Fujiwara, M. Yoshida, and Y. Kiso, "Peptide Chemistry 1984," ed. by N. Izumiya, Protein Res. Found., Osaka, 1985, p. 235.

19) R. B. Woodward, K. Heusler, J. Gosteli, P. Neigeli, W. Oppolzewr, R. Ramage, S. Ranganathan, and H. Vorbruggen, *J. Am. Chem. Soc.*, **88**, 852 (1966).

20) H. Yajima and Y. Kiso, *Chem. Pharm. Bull.*, **19**, 420 (1971).

21) Y. Kiso, T. Fujisaki, M. Shimokura, K. Okamoto, M. Kaimoto, and S. Uemura, "Peptide Chemistry 1984," ed. by N. Izumiya, Protein Res. Found., Osaka, 1985, p. 289.

22) 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.

23) P. Brazeau, N. Ling, F. Esch, R. Benoit, and R. Guillemin, *Regulatory Peptides*, **1**, 255 (1981).