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Studies on Peptides. CXVIII.[1,2] Synthesis of a Hybrid Growth Hormone Releasing Factor (GRF)–PHI Heptacosapeptide Amide

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A hybrid heptacosapeptide amide, consisting of growth hormone releasing factor (GRF) tetradecapeptide (1—14) and PHI tridecapeptide (15—27), was synthesized by a fragment condensation procedure, followed by deprotection with 1 M trifluoromethanesulfonic acid–thioanisole in trifluoroacetic acid. The synthetic peptide exhibited *in vivo* GRF activity (*ca.* 1/10 of that of GRF-44–NH_2), but lacked the blood pressure-decreasing activity of the parent PHI.

Keywords——growth hormone releasing factor; PHI; hybrid GRF–PHI; trifluoromethanesulfonic acid deprotection; thioanisole-mediated deprotection; *in vivo* GRF activity; hypotensive activity

In 1982, Guillemin *et al.*[3] and Rivier *et al.*[4] simultaneously reported the structure of the peptide with growth hormone releasing activity. Three forms of growth hormone releasing factor, GRF (1—44)–NH_2, GRF (1—40)–OH and GRF (1—37)–OH, were isolated from human pancreatic islet tumor. These peptides were found to have striking structural homology with PHI, a heptacosapeptide amide isolated from porcine upper intestinal mucosa by Tatemoto and Mutt[5] in 1980 (Fig. 1). Solid phase synthesis of GRF-related peptides led to the preliminary finding that GRF (1—27)–NH_2, a peptide equivalent to PHI in chain length, still retained 10 to 20% of GRF activity,[4a] but deletion of the *N*-terminal Tyr or Tyr–Ala moiety from GRF (1—44)–NH_2 brought about nearly complete inactivation of this molecule,[3a] indicating an important contribution of the *N*-terminal portion of GRF to its intrinsic activity.[3b] Structural homology between GRF and PHI is seen particularly in the *N*-terminal portions as well as in their middle portions, but PHI was reported to have no GRF activity.[3a,4a]

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GRF-44 Y |AD| A I |F T| N S Y R K V |L G Q L S A| R |K| L |L| Q D I M S R Q Q G E S N Q E R G A R A R L*
PHI    H |AD| G V |F T| S D F S R L |L G Q L S A| K |K| Y |L| E S L I *
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A = Ala; D = Asp; E = Glu; F = Phe; G = Gly; H = His; I = Ile; K = Lys; L = Leu; M = Met; N = Asn; Q = Gln; R = Arg; S = Ser; T = Thr; V = Val; Y = Tyr.
* Amidated COOH terminus.

Fig. 1. Sequence Homology of GRF and PHI

Recently, we synthesized both PHI[6] and GRF (1—44)–NH_2[7] by the fragment condensation procedure. Thus, we decided to synthesize hybrid GRF–PHI, using the available peptide fragments, in order to look for changes in their biological spectra, if any. Starting with

the side-chain protected tridecapeptide amide corresponding to positions 15 to 27 of PHI, H–(PHI 15—27)–NH_2,[6] three GRF fragments,[1,7] Z(OMe)–(GRF 11—14)–$NHNH_2$, Z(OMe)–(GRF 7—10)–$NHNH_2$ and Z(OMe)–(GRF 1—6)–$NHNH_2$, were successively condensed by the azide procedure[8] as shown in Fig. 2 to give the protected heptacosapeptide amide, a hybrid form of GRF (1—14)–PHI (15—27)–NH_2. When the identical hexapeptide sequence

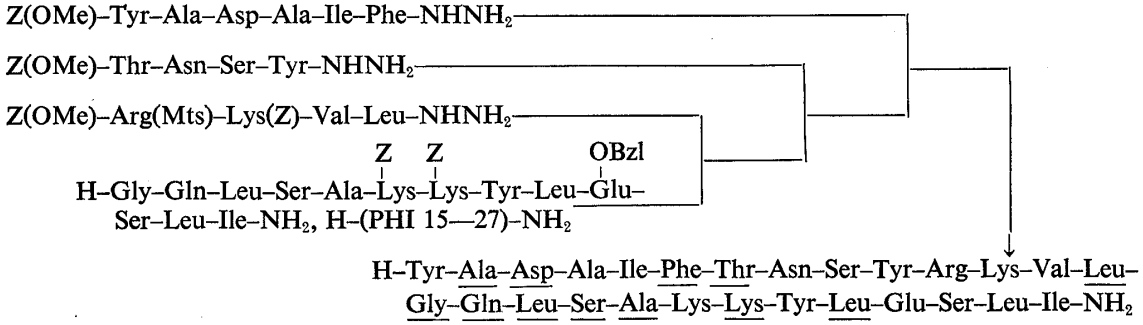

Fig. 2. Synthetic Scheme for Hybrid GRF–PHI-27–NH_2
Identical amino acid residues in GRF and PHI are underlined.

of GRF and PHI, Leu–Gly–Gln–Leu–Ser–Ala (positions 14—19) is taken into account, this hybrid peptide is equivalent to GRF (1—19)–PHI (20—27)–NH_2.

The protected heptacosapeptide amide thus obtained was treated with 1 M TFMSA–thioanisole/TFA[9] in the presence of *m*-cresol to remove all protecting groups employed. Subsequent purification of the deprotected peptide was carried out in essentially the same manner as described in connection with the synthesis of GRF (1—44)–NH_2,[1] *i.e.*, brief dil. ammonia treatment for reversal of the possible N→O shift,[10] 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 synthetic peptide was confirmed by thin-layer chromatography (TLC), HPLC and amino acid analyses, after acid hydrolysis and enzymatic digestion.

When tested in rats according to Guillemin *et al.*,[3a] the *in vivo* GRF potency of our synthetic peptide relative to that of GRF (1—44)–NH_2 (taken as 1) was *ca.* 1/10. This potency is *ca.* one-half of the reported value for GRF (1—27)–NH_2.[4a] This hybrid peptide lacked the hypotensive activity observed in the parent PHI. The result seems to support the view that the N-terminal region of the GRF molecule is important for its intrinsic activity.

Experimental

General experimental procedures employed in this investigation were essentially the same as those described in Part 88[11] of this series. HPLC was conducted with a Waters 204 compact model equipped with a Cosmosil $5C_{18}$ column (1.0 × 25 cm) for preparative purposes and a μBondapak C_{18} column (0.39 × 30 cm) for analytical purposes. *Rf* values on TLC (Kieselgel G, Merck) 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), Rf_3 *n*-BuOH–AcOH–pyridine–H_2O (30 : 6 : 20 : 24). Leucine-aminopeptidase was purchased from Sigma Chemical Co. (Lot. No. 79C-8110).

Z(OMe)–Arg(Mts)–Lys(Z)–Val–Leu–Gly–Gln–Leu–Ser–Ala–Lys(Z)–Lys(Z)–Tyr–Leu–Glu(OBzl)–Ser–Leu–Ile–NH_2, Z(OMe)–[GRF(11—14)–PHI (15—27)]–NH_2——Z(OMe)–(PHI 15—27)–NH_2 (573 mg, 0.29 mmol) was treated with TFA–anisole (2 ml–0.5 ml) in an ice-bath for 60 min, then *n*-hexane was added. The supernatant was removed by decantation and the oily residue was treated with ether to afford a powder. The N^{α}-deprotected peptide thus isolated was dissolved in DMSO–DMF (1 : 1, 5 ml) containing Et_3N (41 μl, 0.29 mmol). The azide [prepared from 441 mg (0.44 mmol) of Z(OMe)–Arg(Mts)–Lys(Z)–Val–Leu–$NHNH_2$] in DMF (5 ml) and Et_3N (61 μl, 0.44 mmol) were added to the above ice-chilled solution and the mixture, after being stirred at 4 °C for 24 h, was concentrated. H_2O (50 ml) was added and the resulting powder was washed with 5% citric acid and H_2O and then purified by gel-filtration on Sephadex LH-60 (3 × 132 cm), using DMF as an eluant. Individual fractions (11 ml each)

were examined by measurement of ultraviolet (UV) absorption at 275 nm. The fractions corresponding to the front main peak (tube Nos. 61—70) were combined and the solvent was removed by evaporation. Treatment of the residue with ether afforded a powder; yield 481 mg (59%), mp 260 °C (dec.), $[\alpha]_D^{18} -10.0°$ ($c=1.3$, DMSO), Rf_1 0.62. *Anal.* Calcd for $C_{138}H_{199}N_{25}O_{34}S$: C, 59.52; H, 7.20; N, 12.58. Found: C, 59.38; H, 7.31; N, 12.67.

Z(OMe)–Thr–Asn–Ser–Tyr–Arg(Mts)–Lys(Z)–Val–Leu–Gly–Gln–Leu–Ser–Ala–Lys(Z)–Lys(Z)–Tyr–Leu–Glu(OBzl)–Ser–Leu–Ile–NH_2, Z(OMe)–[GRF (7—14)–PHI (15—27)]–NH_2——The above protected heptadecapeptide amide (481 mg, 173 μmol) was treated with TFA–anisole (2.5—0.5 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMSO–DMF (1 : 1, 5 ml) containing Et_3N (24 μl, 173 μmol). The azide [prepared from 229 mg (346 μmol) of Z(OMe)–Thr–Asn–Ser–Tyr–$NHNH_2$] in DMF (2 ml) and Et_3N (48 μl, 346 μmol) were added to the above ice-chilled solution and the mixture, after being stirred at 4 °C for 24 h, was diluted with H_2O (100 ml). The resulting powder was washed with H_2O and EtOH and purified by gel-filtration on Sephadex LH-60 as described above; yield 377 mg (67%), mp 280 °C (dec.), $[\alpha]_D^{20} -6.9°$ ($c=0.3$, DMSO), Rf_1 0.71. *Anal.* Calcd for $C_{158}H_{226}N_{30}O_{42}S \cdot 2H_2O$: C, 57.75; H, 7.06; N, 12.79. Found: C, 57.55, H, 6.91; N, 12.67.

Z(OMe)–Tyr–Ala–Asp–Ala–Ile–Phe–Thr–Asn–Ser–Tyr–Arg(Mts)–Lys(Z)–Val–Leu–Gly–Gln–Leu–Ser–Ala–Lys(Z)–Lys(Z)–Tyr–Leu–Glu(OBzl)–Ser–Leu–Ile–NH_2, Z(OMe)–[GRF (1—14)–PHI (15—27)]–NH_2——The above protected heneicosapeptide amide (377 mg, 116 μmol) was treated with TFA–anisole (3 ml–0.3 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMSO–DMF (1 : 1, 2 ml) containing Et_3N (16 μl, 116 μmol). The azide [prepared from 305 mg (348 μmol) of Z(OMe)–Tyr–Ala–Asp–Ala–Ile–Phe–$NHNH_2$] and Et_3N (48 μl, 348 μmol) were added to the above ice-chilled solution and the mixture, after being stirred at 4 °C for 24 h, was diluted with H_2O (100 ml). The resulting powder was washed with H_2O and purified by gel-filtration on Sephadex LH-60 as described above; yield 351 mg (77%), mp 280 °C (dec.), $[\alpha]_D^{20} -10.8°$ ($c=0.7$, DMSO), Rf_1 0.59. *Anal.* Calcd for $C_{192}H_{270}N_{36}O_{51}S \cdot 2H_2O$: C, 58.14; H, 6.96; N, 12.71. Found: C, 58.02; H, 7.08; N, 12.88.

H–Tyr–Ala–Asp–Ala–Ile–Phe–Thr–Asn–Ser–Tyr–Arg–Lys–Val–Leu–Gly–Gln–Leu–Ser–Ala–Lys–Lys–Tyr–Leu–Glu–Ser–Leu–Ile–NH_2——The above protected heptacosapeptide amide (200 mg, 51 μmol) was treated with 1 M TFMSA/TFA (6.1 ml) in the presence of *m*-cresol (0.32 ml, 60 eq) in an ice-bath for 90 min, then dry ether was added. The resulting powder was treated once more under the same conditions. The deprotected peptide isolated by addition of ether was dissolved in H_2O (10 ml) and treated with Amberlite CG 4B (acetate form, approximately 2 g) for 30 min. After filtration, the pH of the solution was adjusted to 8 with 5% NH_4OH, and after 30 min, to 6.5 with 5% AcOH.

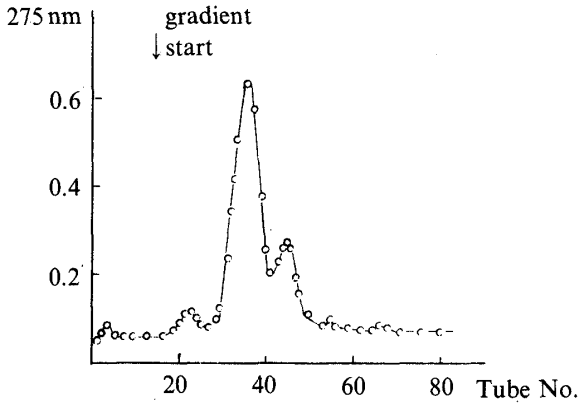

Fig. 3. Purification of H–[GRF (1—14)–PHI (15—27)]–NH_2 by Column Chromatography on CM-Biogel A

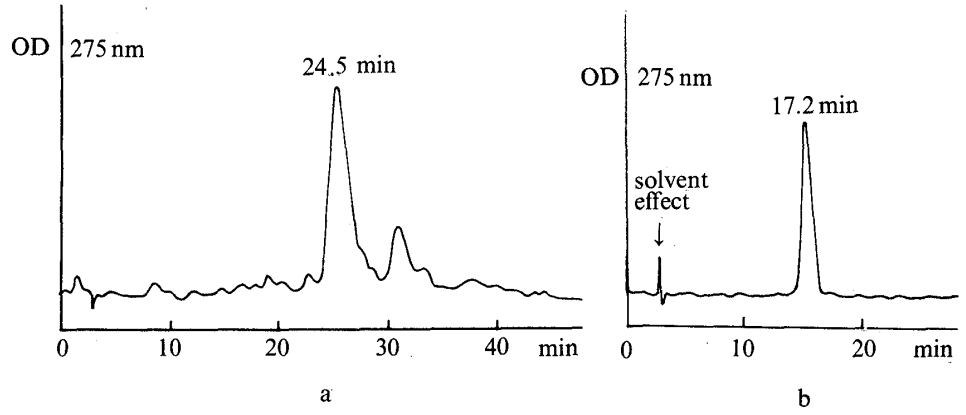

Fig. 4. Purification of CM-Purified GRF–PHI-27–NH_2

a, reverse phase HPLC purification; b, HPLC of purified sample.

Table I. Amino Acid Ratios in 6 N HCl Hydrolysates of Synthetic GRF (1—14)–PHI (15—27)–NH_2 and Its Intermediates

	Protected peptide			Hybrid GRF–PHI-27–NH_2
	GRF (11—14)–PHI (15—27)	GRF (7—14)–PHI (15—27)	GRF (1—14)–PHI (15—27)	
Asp		1.17 (1)	2.09 (2)	2.01 (2)
Thr		1.12 (1)	0.99 (1)	0.97 (1)
Ser	1.85 (2)	3.03 (3)	2.70 (3)	2.74 (3)
Glu	2.16 (2)	2.09 (2)	2.03 (2)	2.10 (2)
Gly	1.14 (1)	1.08 (1)	1.05 (1)	1.04 (1)
Ala	1.17 (1)	1.05 (1)	3.47 (3)	3.28 (3)
Val		0.99 (1)	0.98 (1)	1.05 (1)
Ile	1.00 (1)	1.00 (1)	2.00 (2)	2.00 (2)
Leu	3.18 (3)	4.15 (4)	4.07 (4)	4.11 (4)
Tyr	0.90 (1)	2.10 (2)	2.59 (3)	2.67 (3)
Phe			0.91 (1)	0.97 (1)
Lys	2.12 (2)	3.21 (3)	2.59 (3)	3.07 (3)
Arg		1.08 (1)	1.11 (1)	1.00 (1)
Rec.	89%	72%	86%	92%

Numbers in parentheses are theoretical values.

The solution was lyophilized and the residue was dissolved in 3% AcOH. The solution was applied to a column of Sephadex G-25 (1.8 × 140 cm), which was eluted with the same solvent. Individual fractions (5 ml each) were examined by measurement of UV absorption at 275 nm and the solvent of the desired fractions (tube Nos.39—56) was removed by lyophilization to give a fluffy powder; 109 mg (69%). The partially purified product (21.8 mg) dissolved in H_2O (1 ml) was applied to a column of CM-Biogel A (1 × 14 cm), which was eluted with pH 6.3, 0.2 M $AcONH_4$ (250 ml), through a mixing flask containing pH 4.8, 0.02 M $AcONH_4$ (250 ml). Individual fractions (5 ml each) were examined by measurement of UV absorption at 275 nm. The fractions corresponding to the main peak (tube Nos. 32—40, Fig. 3) were combined and the solvent was removed by lyophilization to give a fluffy powder; yield 15.5 mg (85%).

Next, a part of this sample (3 mg each) was purified by reverse phase HPLC on a Cosmosil $5C_{18}$ column (1.0 × 25.0 cm) using gradient elution with acetonitrile (from 30 to 35% over 40 min) in 0.5% TFA at a flow rate of 3.0 ml per min (Fig. 4a). The eluate corresponding to the main peak (retention time, 24.5 min) was collected. The rest of the sample was similarly purified and the solvent of the combined eluates was removed by evaporation *in vacuo* at 30 °C. The residue was dissolved in pH 7.5, 1 M $AcO \cdot Et_3NH$ buffer (2 ml) and the solvent was desalted by gel-filtration on Sephadex G-25 (2 × 40 cm) using 1 N AcOH as the eluant. The desired fractions were collected and the solvent was removed by lyophilization to give a white fluffy powder; yield 11.7 mg (75%). Total yield from the protected heptacosapeptide amide was 44%. $[\alpha]_D^{24} -60.0°$ ($c=0.5$, 0.2 N AcOH), Rf_2 0.52, Rf_3 0.68. single peak in HPLC (retention time 17.2 min) upon gradient elution with acetonitrile (from 30 to 35% in 20 min) in 0.5% TFA at a flow rate of 1 ml per min. Amino acid ratios in a 6 N HCl hydrolysate are given in Table I. Amino acid ratios in leucine-aminopeptidase digest: Asp 0.99 (1), Thr 0.97 (1), Ser 3.05 (3), Glu 1.13 (1), Gly 1.06 (1), Ala 3.18 (3), Val 1.09 (1), Ile 2.00 (2), Leu 4.27 (4), Tyr 2.58 (3), Phe 0.95 (1), Lys 3.15 (3), Arg 0.88 (1), Asn (1) and Gln (1) were not determined (recovery of Ile 95%). *Anal.* Calcd for $C_{143}H_{228}N_{36}O_{40} \cdot 3CH_3COOH \cdot 10H_2O$: C, 51.84; H, 7.59; N, 14.60. Found: C, 51.82; H, 7.28; N, 14.85.

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References and Notes

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benzyloxycarbonyl; Z(OMe) = *p*-methoxybenzyloxycarbonyl; Mts = mesitylenesulfonyl; Bzl = benzyl; TFA = trifluoroacetic acid; TFMSA = trifluoromethanesulfonic acid; DMF = dimethylformamide; DMSO = dimethylsulfoxide.

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