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# Studies on Peptides. CXL.[1,2] Synthesis of Human Gastrin-Releasing Polypeptide (hGRP)

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Human gastrin-releasing polypeptide (hGRP), which consists of 27 amino acid residues with a C-terminal amide, was synthesized by a conventional solution method, by assembling six peptide fragments followed by deprotection with 1 M trifluoromethanesulfonic acid–thioanisole in trifluoroacetic acid. Met(O) employed was reduced by treatment with phenylthiotrimethylsilane in the presence of trimethylsilyl trifluoromethanesulfonate before deprotection. The synthetic peptide was as active as synthetic porcine GRP, in terms of immunoreactive gastrin release in rats.

**Keywords**——human gastrin-releasing polypeptide synthesis; $N^{in}$-mesitylenesulfonyltryptophan; trifluoromethanesulfonic acid deprotection; methionine sulfoxide reduction; phenylthiotrimethylsilane; trimethylsilyl trifluoromethanesulfonate; immunoreactive gastrin release

Following our preliminary communication of the first synthesis of human gastrin-releasing polypeptide (hGRP),[3] we wish to present a detailed account of the total synthesis of this newly found gastrointestinal polypeptide by a conventional solution method.

In 1984, Spindel *et al.*[4] elucidated the structure of hGRP by the cDNA sequence analysis of its precursor. Independently, Orloff *et al.*[5] isolated hGRP as a bombesin-like polypeptide from acid extracts of liver tissue containing a metastatic human bronchial carcinoid tumor and determined the sequence of the first 11 residues. So far, peptides related in structure to hGRP have been isolated from porcine[6] and canine[7] small intestines and avian proventriculus[8] and have been chemically characterized. GRPs, including hGRP are C-amidated peptides consisting of 27 amino acid residues, as predicted from studies of the intercellular processing of other C-amidated peptides.[4] The C-terminal portion of mammalian GRPs so far sequenced is identical with the sequence of amphibian tetradecapeptide, bombesin.[9] However their N-terminal portions differ from each other by substitution of several amino acids at positions 1, 3, 4, 5, 7, 8, 9, 10, 12, 14 and 19, as shown in Fig. 1.

Our synthetic route to hGRP is illustrated in Fig. 2. In contrast to our previous syntheses of porcine[10] and chicken GRPs,[11] we employed a new Trp-derivative, Trp(Mts),[12] to suppress indole-alkylation[13] during TFA-deprotection of the Z(OMe) group.[14] Met(O)[15] employed was reduced, before deprotection, with a newly found reducing reagent, phenylthiotrimethylsilane.[16] In combination with the TFA-labile Z(OMe) group as a temporary $N^{\alpha}$-protection, amino acid derivatives bearing protecting groups removable by 1 M TFMSA–thioanisole in TFA[17] were employed, *i.e.*, Arg(Mts),[18] Lys(Z) and Trp(Mts) mentioned above. The C-terminal heptapeptide amide [1] and five peptide hydrazides, [2] to [6], were selected as building blocks to construct the entire peptide backbone of hGRP. Of these, fragment [1] is an intermediate of our synthesis of neuromedin C,[19] a porcine spinal cord peptide.[20] Fragment [2] is a hydrazide used for our synthesis of galanin,[21] a new

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H-(1)-Pro-(3)-(4)-(5)-Gly-(7)-(8)-(9)-(10)-Leu-(12)-Lys-(14)-
Tyr-Pro-Arg-Gly-(19)-His-Trp-Ala-Val-Gly-His-Leu-Met-$NH_2$

| | 1 | 3 | 4 | 5 | 7 | 8 | 9 | 10 | 12 | 14 | 19 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| human | Val | Leu | Pro | Ala | Gly | Gly | Thr | Val | Thr | Met | Asn |
| porcine | Ala | Val | Ser | Val | Gly | Gly | Thr | Val | Ala | Met | Asn |
| canine | Ala | Val | Pro | Gly | Gln | Gly | Thr | Val | Asp | Met | Asn |
| avian | Ala | Leu | Gln | Pro | Gly | Ser | Pro | Ala | Thr | Ile | Ser |

Fig. 1. Structures of hGRP and Related Peptides

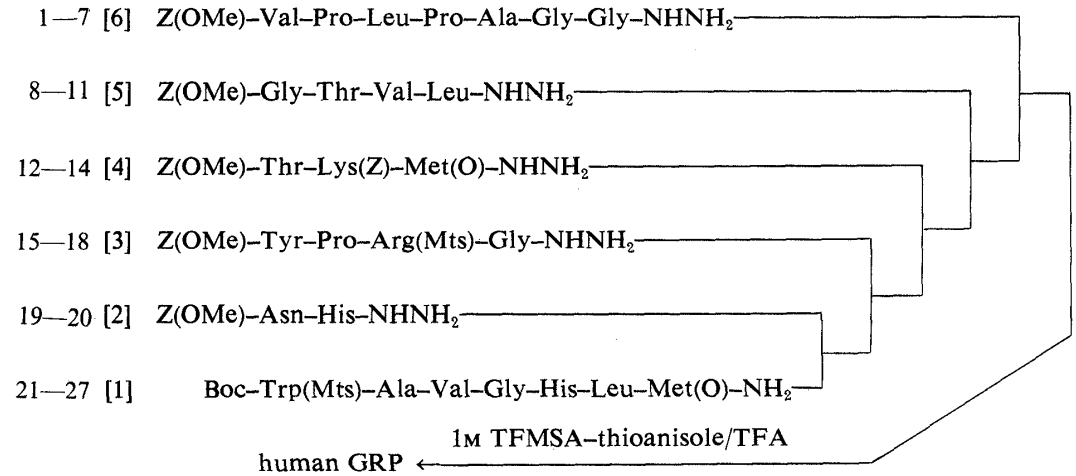


Fig. 2. Synthetic Route to Human GRP

gastrointestinal polypeptide.[22] The corresponding Boc-derivatives of fragments [3] and [5] were used in our previous syntheses of porcine and chicken GRPs. Thus, Z(OMe)-derivatives of fragments [3] and [5] and two hydrazides, fragments [4] and [6], were prepared for the present synthesis.

For the preparation of fragment [3], Z(OMe)–Tyr–Pro–OH prepared by the azide procedure[23] was condensed with a TFA-treated sample of Z(OMe)–Arg(Mts)–Gly–OMe[10] by DCC.[24] The resulting protected tetrapeptide ester was converted to the corresponding hydrazide, Z(OMe)–Tyr–Pro–Arg(Mts)–Gly–$NHNH_2$ [3] by the usual hydrazine treatment. For the preparation of fragment [5], Z(OMe)–Gly–OH was condensed, *via* the Su ester,[25] with a TFA-treated sample of Z(OMe)–Thr–Val–Leu–OMe[10] and the resulting protected tetrapeptide ester was converted to the corresponding hydrazide, Z(OMe)–Gly–Thr–Val–Leu–$NHNH_2$ [5], as stated above.

Fragment [4], Z(OMe)–Thr–Lys(Z)–Met(O)–$NHNH_2$, was prepared by the azide condensation of Z(OMe)–Thr–$NHNH_2$ with a TFA-treated sample of Z(OMe)–Lys(Z)–Met(O)–OMe,[10] followed by the usual hydrazine treatment of the resulting protected tripeptide ester.

The N-terminal fragment, Z(OMe)–Val–Pro–Leu–Pro–Ala–Gly–Gly–$NHNH_2$ [6], was prepared according to the scheme shown in Fig. 3. Z(OMe)–Ala–Gly–$NHNH_2$ was condensed, *via* the azide, with H–Gly–OMe. The peptide chain of the resulting tripeptide was elongated in a stepwise manner by introduction of the corresponding amino acid residues *via* active esters, such as the Np[26] or the Su ester. The resulting protected heptapeptide ester was converted to [6] by the usual hydrazine treatment. The purity of fragment [6] was confirmed by thin layer chromatography (TLC), amino acid analysis after acid hydrolysis and elemen-

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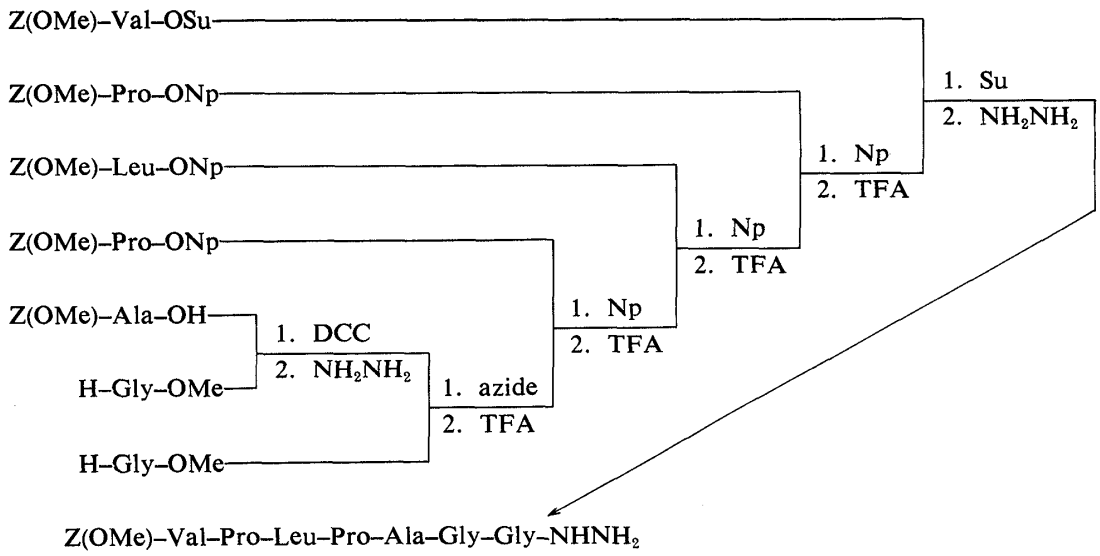


Fig. 3. Synthetic Scheme for the Protected Heptapeptide Hydrazide, Z(OMe)–(hGRP 1—7)–$NHNH_2$ [6]

TABLE I. Amino Acid Ratios in 6 N HCl Hydrolysate of Synthetic hGRP and Its Intermediates

| | Protected peptides | | | | | Syn. hGRP | Residue |
|---|---|---|---|---|---|---|---|
| | 19—27 | 15—27 | 12—27 | 8—27 | 1—27 | | |
| Asp | 1.07 | 1.02 | 1.01 | 0.97 | 0.96 | 0.89 | (1) |
| Thr | | | 1.00 | 2.00 | 1.85 | 1.89 | (2) |
| Pro | | 0.98 | 0.87 | 0.90 | 3.03 | 2.97 | (3) |
| Gly | 1.00 | 2.13 | 2.02 | 3.05 | 4.95 | 4.97 | (5) |
| Ala | 1.10 | 1.06 | 1.03 | 0.95 | 2.18 | 2.18 | (2) |
| Val | 0.99 | 0.98 | 0.98 | 1.93 | 2.66 | 2.66 | (3) |
| Met | 0.92 | 0.89 | 1.73 | 1.67 | 1.64 | 1.91 | (2)[a] |
| Leu | 1.00 | 1.00 | 1.00 | 2.00 | 3.00 | 3.00 | (3)[b] |
| Tyr | | 1.05 | 0.95 | 0.92 | 0.91 | 0.99 | (1) |
| Trp | 0.90 | 0.87 | 0.86 | 0.88 | 0.92 | N.D. | (1)[c] |
| Lys | | | 1.07 | 0.99 | 0.97 | 1.01 | (1) |
| His | 2.00 | 1.92 | 1.97 | 1.88 | 1.86 | 1.92 | (2) |
| Arg | | 1.13 | 0.96 | 0.93 | 1.05 | 0.96 | (1) |
| Rec. % | 70% | 76% | 76% | 81% | 87% | 85% | |

*a*) Met + Met(O). *b*) Diagnostic amino acid. *c*) Determined by 4 M MSA hydrolysis.

tal analysis, as was done with other fragments.

Necessary fragments thus obtained were assembled successively by the azide procedure to minimize racemization. The reactions were performed in DMF or a mixture of DMF and DMSO and the amount of the acyl component was increased from 1.4 to 2.0 equivalents as the chain was elongated. In the present synthesis, by the use of $N^{in}$-protected Trp, the $N^{\alpha}$-TFA deprotection of the Z(OMe) group could be conducted smoothly without any detectable side products on TLC. Protected products were purified by precipitation from DMF with EtOH or by gel-filtration on Sephadex LH-60 using DMF as an eluant. Throughout the synthesis, Leu was selected as a diagnostic amino acid in the acid hydrolysate. Thus, by comparison of the recovery of Leu with those of newly added amino acids, satisfactory condensation of each

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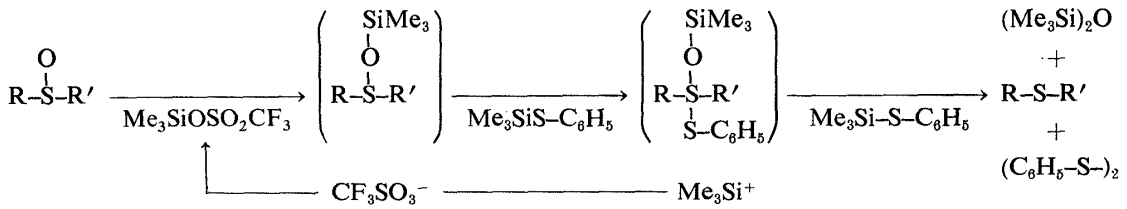


Fig. 4. Reduction of Met(O) with Trimethylsilyl Compounds

TABLE II. Bioassay of Synthetic hGRP

| | | | | | |
|---|---|---|---|---|---|
| 1. | Effect on immunoreactive gastrin release in rat plasma ($n=5$) | | | | |
| | hGRP (γ/kg/h) | 1/4 | 1 | 4 | 16 |
| | IR-gastrin (pg/ml) | 12.2 | 54 | 176 | 240 |
| 2. | Effect on pancreatic secretion in rats ($n=5$) | | | | |
| | γ/kg | Control | 1/4 | 1 | 4 |
| | hGRP | 100 | 162.6 | 265 | 369.5 |
| | pGRP | | | 205 | 276 |
| | cGRP | | | 140 | 262 |

fragment was ascertained as shown in Table I.

The protected hGRP thus obtained was treated with phenylthiotrimethylsilane to reduce the two Met(O) residues. In this step, addition of a small amount of oxygenophilic reagent, trimethylsilyl trifluoromethanesulfonate,[27] was found to accelerate the reduction; the mechanism may be as shown in Fig. 4. Without addition of the latter reagent, we had to treat the protected hGRP with the former reagent twice at 40 °C for 30 min to obtain a product exhibiting a single spot on TLC, while with the combination of the two reagents, treatment at 25 °C for 30 min was sufficient. The progress of the reduction could be monitored by the Met test on TLC and by 4 M MSA hydrolysis.[28] In the latter case, the Met(O) residue in peptides can be determined after hydrolysis, as Met(O), whereas 6 N HCl hydrolysis converts most of Met(O) to Met.

The reduced peptide was exposed to 1 M TFMSA–thioanisole in TFA in the presence of *m*-cresol and EDT[19] in an ice-bath for 3 h to remove all protecting groups employed. The deprotected peptide was treated with dil. ammonia to reverse possible N→O shift[29] at the Thr residue. This treatment also seems to be effective to hydrolyze the O-trimethylsilyl bond at the Thr residue (possibly formed during the above reduction step). For the isolation of Met-containing peptides, rather long incubation with thiol compounds is usually necessary to ensure the complete reduction of Met(O). However, in the present synthesis, we could eliminate this time-consuming treatment. The product was then purified by gel-filtration on Sephadex G-25, followed by high performance liquid chromatography (HPLC) on Cosmosil $5C_{18}$ using gradient elution with acetonitrile (28—38%) in 0.1% TFA.

The synthetic peptide exhibited a sharp single spot on TLC in two different solvent systems and gave a single peak in analytical HPLC. Its homogeneity was further ascertained by disk isoelectrofocusing and amino acid analyses, after acid hydrolysis and enzymatic digestion.

When administered (i.v.) to rats ($n=5$), our synthetic hGRP (1/4 to 16 μg/kg/h) raised the plasma immunoreactive gastrin level (Table II-1). This activity was judged to be of the same magnitude as those of synthetic porcine and chicken GRPs. Our hGRP (1/4 to 4 μg/kg) exhibited a slightly higher activity in pancreatic secretion in rats than the synthetic porcine and chicken GRPs (Table II-2).

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## Experimental

General experimental methods employed here are essentially the same as described in Part LXXXVIII[30] of this series.

**$N^{\alpha}$-Deprotection**——The $N^{\alpha}$-protecting group, Z(OMe) or Boc, was cleaved by TFA (*ca.* 10 ml per 1 g of a peptide) in the presence of anisole (2 eq or more) at ice-bath temperature for 60 min. After evaporation of the TFA *in vacuo* at 15—20 °C, the residue was treated with dry ether. If a powder was obtained, it was collected by filtration, dried over KOH pellets *in vacuo* for 3 h and then used for the condensation reaction. If an oily precipitate was obtained, it was washed with *n*-hexane, dried over KOH pellets *in vacuo* for 3 h and then used for the condensation reaction.

**Condensation Reactions**——The DCC and the active ester condensations were performed at room temperature. Each hydrazide was converted to the corresponding azide by treatment with isoamyl nitrite, and the azide reaction, unless otherwise mentioned, was performed at 4 °C. A mixed anhydride was prepared using isobutyl chloroformate and allowed to react with an amino component in an ice-bath for 5 h.

**Purification**——Unless otherwise mentioned, products were purified by one of the following procedures.

Procedure A: For purification of a protected peptide soluble in AcOEt, the extract was washed with 5% citric acid, 5% $NaHCO_3$ and $H_2O$–NaCl, dried over $Na_2SO_4$ and concentrated. The residue was crystallized or precipitated from appropriate solvents.

Procedure B: For purification of a peptide less soluble in AcOEt, the crude product was triturated with ether and 5% citric acid. The resulting powder was washed with 5% citric acid, 5% $NaHCO_3$, and $H_2O$ and recrystallized or precipitated from appropriate solvents. For purification of peptides containing the His residue, 5% $NaHCO_3$ and $H_2O$ were used for washing.

HPLC was conducted with a Waters 204 compact model. TLC 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 (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–AcOEt–$H_2O$ (1 : 1 : 1 : 1).

**Z(OMe)–Tyr–Pro–OH**——The azide [prepared from 12.0 g (33.39 mmol) of Z(OMe)–Tyr–$NHNH_2$] in DMF (100 ml) and $Et_3N$ (5.5 ml, 40.07 mmol) were added to an ice-chilled solution of H–Pro–OH (6.69 g, 66.78 mmol) and $Et_3N$ (9.3 ml, 66.78 mmol) in $H_2O$ (5 ml) and the mixture, after being stirred for 24 h, was concentrated. The residue was dissolved in 10% $NaHCO_3$ and the aqueous phase, after being washed with AcOEt, was acidified with citric acid. The resulting precipitate was extracted with AcOEt. The extract was washed with $H_2O$–NaCl, dried over $Na_2SO_4$ and concentrated. Trituration of the residue with ether afforded a powder which was recrystallized from MeOH and AcOEt; yield 8.45 g (57%), mp 146—147 °C, $[\alpha]_D^{18}$ −30.9° ($c$ = 1.0, MeOH), $Rf_1$ 0.52. *Anal.* Calcd for $C_{23}H_{26}N_2O_7$: C, 62.43; H, 5.92; N, 6.33. Found: C, 62.29; H, 5.95; N, 6.43.

**Z(OMe)–Tyr–Pro–Arg(Mts)–Gly–OMe**——DCC (0.84 g, 4.06 mmol) was added to a mixture of H–Arg(Mts)–Gly–OMe [prepared from 2.00 g (3.38 mmol) of the Z(OMe)-derivative[10] by TFA treatment followed by neutralization with $Et_3N$ through the HCl salt], Z(OMe)–Tyr–Pro–OH (1.50 g, 3.38 mmol) and HOBt (46 mg, 0.34 mmol) in DMF (10 ml), and the mixture, after being stirred overnight, was filtered. The filtrate was concentrated and the product was purified by procedure B followed by recrystallization from MeOH and AcOEt; yield 2.34 g (81%), mp 109—114 °C, $[\alpha]_D^{18}$ −42.3° ($c$ = 1.0, MeOH), $Rf_1$ 0.60. *Anal.* Calcd for $C_{41}H_{53}N_7O_{11}S \cdot 1/2H_2O$: C, 57.20; H, 6.32; N, 11.39. Found: C, 57.28; H, 6.38; N, 11.27.

**Z(OMe)–Tyr–Pro–Arg(Mts)–Gly–$NHNH_2$, Z(OMe)–(hGRP 15—18)–$NHNH_2$ [3]**——The above protected tetrapeptide ester (2.00 g, 2.35 mmol) in DMF–MeOH (20 ml–10 ml) was treated with 80% hydrazine hydrate (1.43 ml, 10 eq) for 20 h. The solvent was removed by evaporation and the residue was precipitated from DMF with ether; yield 1.40 g (70%), mp 128—132 °C, $[\alpha]_D^{20}$ −35.4° ($c$ = 1.0, MeOH), $Rf_1$ 0.43. Amino acid ratios in 6 N HCl hydrolysate: Pro 0.89, Gly 1.00, Tyr 0.86, Arg 0.98 (recovery of Gly 99%). *Anal.* Calcd for $C_{40}H_{53}N_9O_{10}S \cdot H_2O$: C, 55.22; H, 6.37; N, 14.49. Found: C, 55.24; H, 6.22; N, 14.57.

**Z(OMe)–Thr–Lys(Z)–Met(O)–OMe**——The azide [prepared from 6.78 g (22.8 mmol) of Z(OMe)–Thr–$NHNH_2$] in DMF (50 ml) and $Et_3N$ (2.62 ml, 19.0 mmol) were added to an ice-chilled solution of H–Lys(Z)–Met(O)–OMe [prepared from 11.51 g (19.00 mmol) of the Z(OMe)-derivative[10]] in DMF (50 ml) and the mixture, after being stirred overnight, was concentrated. The product was purified by procedure B followed by precipitation from DMF with AcOEt; yield 11.93 g (89%), mp 114—117 °C, $[\alpha]_D^{20}$ −10.8° ($c$ = 1.0, DMF), $Rf_3$ 0.60. *Anal.* Calcd for $C_{33}H_{46}N_4O_{11}S$: C, 56.07; H, 6.56; N, 7.93. Found: C, 55.95; H, 6.53; N, 7.96.

**Z(OMe)–Thr–Lys(Z)–Met(O)–$NHNH_2$, Z(OMe)–(hGRP 12—14)–$NHNH_2$ [4]**——The above protected tripeptide ester (2.24 g, 3.17 mmol) in DMF (20 ml) was treated with 80% hydrazine hydrate (0.79 ml, 5 eq) overnight. $H_2O$ (50 ml) was added and the resulting powder was precipitated from DMSO with MeOH; yield 2.18 g (97%), mp 119—203 °C, $[\alpha]_D^{20}$ +1.0° ($c$ = 1.0, DMSO), $Rf_1$ 0.41. Amino acid ratios in 6 N HCl hydrolysate: Thr 1.00, Lys 1.00, Met + Met(O) 0.87 (recovery of Thr 99%). *Anal.* Calcd for $C_{32}H_{46}N_6O_{10}S$: C, 54.37; H, 6.56; N, 11.89. Found: C, 54.12; H, 6.44; N, 11.65.

**Z(OMe)–Gly–Thr–Val–Leu–OMe**——A mixture of Z(OMe)–Gly–OSu (4.04 g, 12.0 mmol), $Et_3N$ (3.07 ml,

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22.0 mmol) and a TFA-treated sample of Z(OMe)–Thr–Val–Leu–OMe[10] (5.10 g, 10.0 mmol) in DMF (65 ml) was stirred overnight, then concentrated. The residue was purified by procedure B followed by precipitation from DMF with MeOH; yield 4.00 g (71%), mp 198—199 °C, $[\alpha]_D^{20} -5.4°$ ($c=0.6$, DMF), $Rf_2$ 0.35. *Anal.* Calcd for $C_{27}H_{42}N_4O_9 \cdot 1/2H_2O$: C, 56.33; H, 7.53; N, 9.73. Found: C, 56.54; H, 7.55; N, 9.76.

**Z(OMe)–Gly–Thr–Val–Leu–NHNH$_2$, Z(OMe)–(hGRP 8—11)–NHNH$_2$ [5]**——The above protected tetrapeptide ester (2.30 g, 4.06 mmol) in DMF–MeOH (30 ml–5 ml) was treated with 80% hydrazine hydrate (2.54 ml, 10 eq) overnight. The solvent was removed by evaporation and the residue was precipitated from DMF with EtOH; yield 1.25 g (54%), mp 257—261 °C, $[\alpha]_D^{20} -7.0°$ ($c=0.6$, DMF), $Rf_1$ 0.64. Amino acid ratios in 6 N HCl hydrolysate; Gly 0.99, Thr 0.92, Val 0.95, Leu 1.00 (recovery of Gly 99%). *Anal.* Calcd for $C_{26}H_{42}N_6O_8$: C, 55.11; H, 7.47; N, 14.83. Found: C, 54.82; H, 7.42; N, 14.82.

**Z(OMe)–Ala–Gly–OMe**——The title compound was prepared by the usual DCC procedure and purified by procedure A followed by recrystallization from MeOH and ether; yield 81%, mp 107—109 °C, $[\alpha]_D^{15} -24.0°$ ($c=1.0$, MeOH), $Rf_2$ 0.36. *Anal.* Calcd for $C_{15}H_{20}N_2O_6$: C, 55.55; H, 6.22: N, 8.64. Found: C, 55.68; H, 6.19; N, 8.78.

**Z(OMe)–Ala–Gly–NHNH$_2$**——The above dipeptide ester was converted to the corresponding hydrazide as usual; yield 86%, mp 135—138 °C, $[\alpha]_D^{15} +9.5°$ ($c=1.1$, DMF), $Rf_1$ 0.51. *Anal.* Calcd for $C_{14}H_{20}N_4O_5$: C, 51.84; H, 6.22; N, 17.28. Found: C, 51.60; H, 6.11; N, 17.06.

**Z(OMe)–Ala–Gly–Gly–OMe**——The azide [prepared from 17.99 g (55.5 mmol) of Z(OMe)–Ala–Gly–NHNH$_2$] in DMF–DMSO (2 : 1, 150 ml) and $Et_3N$ (7.65 ml, 55.5 mmol) were added to an ice-chilled solution of H–Gly–OMe [prepared from 10.45 g (83.2 mmol) of the HCl salt] in DMF (100 ml) and the mixture, after being stirred for 24 h, was concentrated. The product was purified by procedure A, followed by precipitation from DMF with AcOEt; yield 16.88 g (80%), mp 115—118 °C, $[\alpha]_D^{20} +4.0°$ ($c=1.0$, DMF), $Rf_3$ 0.51. *Anal.* Calcd for $C_{17}H_{23}N_3O_7$: C, 53.53. H, 6.08; N, 11.02. Found: C, 53.41; H, 6.19; N, 11.10.

**Z(OMe)–Pro–Ala–Gly–Gly–OMe**——A mixture of Z(OMe)–Pro–ONp (3.46 g, 8.65 mmol), $Et_3N$ (1.09 ml, 8.65 mmol) and H–Ala–Gly–Gly–OMe [prepared from 3.00 g (7.87 mmol) of the Z(OMe)-derivative] in DMF (50 ml) was stirred overnight, then the solvent was removed by evaporation. The product was purified by repeated recrystallization from MeOH and ether; yield 3.52 g (94%), mp 153—155 °C, $[\alpha]_D^{18} -47.3°$ ($c=1.0$, MeOH), $Rf_3$ 0.58. *Anal.* Calcd for $C_{22}H_{30}N_4O_8 \cdot 1/2H_2O$: C, 54.20; H, 6.40; H, 11.49. Found: C, 54.35; H, 6.54; N, 11.79.

**Z(OMe)–Leu–Pro–Ala–Gly–Gly–OMe**——A mixture of Z(OMe)–Leu–ONp (2.98 g, 7.15 mmol), $Et_3N$ (0.90 ml, 7.15 mmol) and H–Pro–Ala–Gly–Gly–OMe [prepared from 3.11 g (6.50 mmol) of the Z(OMe)-derivative] in DMF (50 ml) was stirred overnight, then the solvent was removed by evaporation. The product was purified by procedure A followed by recrystallization from MeOH and isopropyl ether; yield 3.48 g (91%), mp 62—66 °C, $[\alpha]_D^{18} -74.3°$ ($c=1.0$, MeOH), $Rf_3$ 0.50. *Anal.* Calcd for $C_{28}H_{41}N_5O_9$: C, 56.84; H, 6.99; N, 11.84. Found: C, 56.93; H, 7.15: N, 11.54.

**Z(OMe)–Pro–Leu–Pro–Ala–Gly–Gly–OMe**——A mixture of Z(OMe)–Pro–ONp (2.25 g, 5.61 mmol), $Et_3N$ (0.70 ml, 5.61 mmol) and H–Leu–Pro–Ala–Gly–Gly–OMe [prepared from 3.02 g, 5.10 mmol) of the Z(OMe)-derivative] in DMF (30 ml) was stirred overnight, then the solvent was evaporated. The residue was extracted with *n*-BuOH. The organic phase was washed with $H_2O$–NaCl, dried over $Na_2SO_4$ and concentrated. The residue was triturated with ether and the resulting powder was precipitated from $CHCl_3$ with ether; yield 3.35 g (95%), mp 80—84 °C, $[\alpha]_D^{18} -116.2°$ ($c=1.0$, MeOH), $Rf_1$ 0.60. *Anal.* Calcd for $C_{33}H_{48}N_6O_{10} \cdot H_2O$: C, 56.08; H, 7.03; N, 11.89. Found: C, 56.24; H, 7.13; N, 11.89.

**Z(OMe)–Val–Pro–Leu–Pro–Ala–Gly–Gly–OMe**——A mixture of Z(OMe)–Val–OSu (10.99 g, 29.0 mmol), $Et_3N$ (2.0 ml, 29.0 mmol) and H–Pro–Leu–Pro–Ala–Gly–Gly–OMe [prepared from 10.00 g, 14.5 mmol) of the Z(OMe)-derivative] in DMF (100 ml) was stirred for 2 d, then the solvent was removed by evaporation. The product was purified by procedure A followed by recrystallization from MeOH and ether; yield 8.71 g (76%), mp 96—101 °C, $[\alpha]_D^{20} -132.9°$ ($c=1.0$, MeOH), $Rf_1$ 0.68. *Anal.* Calcd for $C_{38}H_{57}N_7O_{11} \cdot H_2O$: C, 56.63; H, 7.38; N, 12.17. Found: C, 56.74; H, 7.30; N, 12.15.

**Z(OMe)–Val–Pro–Leu–Pro–Ala–Gly–Gly–NHNH$_2$, Z(OMe)–(hGRP 1—7)–NHNH$_2$ [6]**——The above protected heptapeptide ester (8.71 g, 11.1 mmol) in MeOH (80 ml) was treated with 80% hydrazine hydrate (2.77 ml, 5 eq) overnight. The solvent was removed by evaporation and the residue was recrystallized from MeOH and ether; yield 8.38 g (96%), mp 116—119 °C, $[\alpha]_D^{22} -123.1°$ ($c=1.0$, MeOH), $Rf_1$ 0.61. Amino acid ratios in 6 N HCl hydrolysate: Val 0.96, Pro 2.02, Leu 1.01, Ala 1.09, Gly 2.00 (recovery of Gly 88%). *Anal.* Calcd for $C_{37}H_{57}N_9O_{10} \cdot H_2O$: C, 55.14; H, 7.38; N, 15.64. Found: C, 55.23; H, 7.32; N, 15.20.

**Z(OMe)–Asn–His–Trp(Mts)–Ala–Val–Gly–His–Leu–Met(O)–NH$_2$, Z(OMe)–(hGRP 19—27)–NH$_2$**——The C-terminal fragment [1] (5.51 g, 4.96 mmol) was treated with TFA–anisole (11 ml–2.7 ml) in an ice-bath for 90 min, then dry ether was added. The resulting powder was dried over KOH pellets *in vacuo* for 60 min and dissolved in DMF (30 ml) containing $Et_3N$ (1.37 ml, 9.92 mmol). The azide [prepared from 3.33 g (7.44 mmol) of Z(OMe)–Asn–His–NHNH$_2$] in DMF (30 ml) and $Et_3N$ (1.03 ml, 7.44 mmol) were added to the above ice-chilled solution and the mixture, after being stirred for 48 h, was concentrated. The product was purified by procedure B followed by precipitation from DMF with EtOH; yield 5.27 g (75%), mp 209—213 °C, $[\alpha]_D^{22} -20.1°$ ($c=1.0$, DMF), $Rf_1$ 0.17. *Anal.* Calcd for $C_{66}H_{88}N_{16}O_{16}S_2 \cdot 5H_2O$: C, 52.30; H, 6.52; N, 14.84. Found: C, 52.38; H, 6.24; N, 14.79.

**Z(OMe)–Tyr–Pro–Arg(Mts)–Gly–Asn–His–Trp(Mts)–Ala–Val–Gly–His–Leu–Met(O)–NH$_2$, Z(OMe)–(hGRP**

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**15—27)-$NH_2$**——The azide [prepared from 0.84 g (0.98 mmol) of Z(OMe)-(hGRP 15—18)-$NHNH_2$] in DMF (5 ml) and $Et_3N$ (136 $\mu$l, 0.98 mmol) were added to an ice-chilled solution of H-(hGRP 19—27)-$NH_2$ [prepared from 1.00 g (0.70 mmol) of the Z(OMe)-derivative] in DMF–DMSO (5 : 1, 6 ml) and the mixture, after being stirred for 42 h, was concentrated. The residue was extracted with *n*-BuOH. The organic phase was washed with $H_2O$, dried over $Na_2SO_4$ and concentrated. Treatment of the residue with isopropyl alcohol afforded a powder, which was precipitated from DMF with EtOH; yield 1.30 g (89%), mp 205—209 °C, $[\alpha]_D^{20}$ −24.7° ($c$=0.6, DMF), $Rf_1$ 0.16. *Anal.* Calcd for $C_{97}H_{129}N_{23}O_{23}S_3 \cdot 10H_2O$: C, 51.51; H, 6.64; N, 14.25. Found: C, 51.74; H, 6.38; N, 13.95.

**Z(OMe)-Thr-Lys(Z)-Met(O)-Tyr-Pro-Arg(Mts)-Gly-Asn-His-Trp(Mts)-Ala-Val-Gly-His-Leu-Met(O)-$NH_2$, Z(OMe)-(hGRP 12—27)-$NH_2$**——The azide [prepared from 0.63 g (0.89 mmol) of Z(OMe)-(hGRP 12—14)-$NHNH_2$] in DMF–DMSO (1 : 1, 10 ml) and $Et_3N$ (137 $\mu$l, 0.98 mmol) were added to an ice-chilled solution of H-(hGRP 15—27)-$NH_2$ [prepared from 1.16 g (0.56 mmol) of the Z(OMe)-derivative] in DMF–DMSO (1 : 1, 10 ml) and the mixture, after being stirred at −20 °C for 72 h, was concentrated. A part of the residue (0.70 g) was purified by gel-filtration on Sephadex LH-60 (3 × 139 cm) using DMF as an eluant. Fractions corresponding to the front main peak (tube Nos. 56—80, 10 ml each, determined by ultraviolet (UV) absorption measurement at 280 nm) were combined and the solvent was removed by evaporation. Treatment of the residue with ether afforded a powder. The rest of the product was similarly purified; total yield 1.10 g (69%), mp 206—209 °C, $[\alpha]_D^{20}$ −25.5° ($c$=0.5, DMF), $Rf_1$ 0.22. *Anal.* Calcd for $C_{120}H_{163}N_{27}O_{30}S_4 \cdot 3H_2O$: C, 54.47; H, 6.44; N, 14.29. Found: C, 54.45; H, 6.52; N, 14.31.

**Z(OMe)-Gly-Thr-Val-Leu-Thr-Lys(Z)-Met(O)-Tyr-Pro-Arg(Mts)-Gly-Asn-His-Trp(Mts)-Ala-Val-Gly-His-Leu-Met(O)-$NH_2$, Z(OMe)-(hGRP 8—27)-$NH_2$**——The azide [prepared from 0.37 g (0.66 mmol) of Z(OMe)-(hGRP 8—11)-$NHNH_2$] in DMF–DMSO (2 : 1, 5 ml) and $Et_3N$ (91 $\mu$l, 0.66 mmol) were added to an ice-chilled solution of H-(hGRP 12—27)-$NH_2$ [prepared from 0.85 g (0.33 mmol) of the Z(OMe)-derivative] in DMF–DMSO (2 : 1, 5 ml) and the mixture was stirred for 4 d. $H_2O$ (100 ml) was added and the resulting powder was precipitated from DMF with EtOH; yield 0.88 g (91%), mp 184—188 °C, $[\alpha]_D^{22}$ −14.7° ($c$=0.2, DMF), $Rf_1$ 0.19. *Anal.* Calcd for $C_{137}H_{193}N_{31}O_{35}S_4 \cdot 3H_2O$: C, 54.55; H, 6.64; N, 14.40. Found: C, 54.42; H, 6.80; N, 14.20.

**Z(OMe)-Val-Pro-Leu-Pro-Ala-Gly-Gly-Gly-Thr-Val-Leu-Thr-Lys(Z)-Met(O)-Tyr-Pro-Arg(Mts)-Gly-Asn-His-Trp(Mts)-Ala-Val-Gly-His-Leu-Met(O)-$NH_2$, Protected hGRP**——The azide [prepared from 0.34 g (0.43 mmol) of Z(OMe)-(hGRP 1—7)-$NHNH_2$] in DMF (5 ml) and $Et_3N$ (59 $\mu$l, 0.43 mmol) were added to an ice-chilled solution of H-(hGRP 8—27)-$NH_2$ [prepared from 0.63 g (0.21 mmol) of the Z(OMe)-derivative] in DMF–DMSO (2 : 1, 6 ml) and the mixture, after being stirred for 12 h, was concentrated. The residue was treated with $H_2O$ (100 ml) and the resulting powder was purified by gel-filtration on Sephadex LH-60 (3 × 139 cm) using DMF as an eluant. The fractions corresponding to the front main peak (tube Nos. 53—62, 10 ml each, determined by UV absorption measurement at 280 nm) were combined and the solvent was removed by evaporation. Treatment of the residue with isopropyl alcohol afforded a powder; yield 0.49 g (63%), mp 201—204 °C, $[\alpha]_D^{24}$ +5.9° ($c$=0.5, DMF), $Rf_1$ 0.20. *Anal.* Calcd for $C_{165}H_{238}N_{38}O_{42}S_4 \cdot 4H_2O$: C, 54.65; H, 6.84; N, 14.68. Found: C, 54.89; H, 7.19; N, 14.21.

**Reduction of the Protected hGRP**——Under an argon atmosphere, the protected hGRP obtained above (150 mg) in DMF (3 ml) was treated with phenylthiotrimethylsilane (0.77 ml, 100 eq) and trimethylsilyl trifluoromethanesulfonate (37.6 $\mu$l, 4 eq) at 25 °C for 30 min, while the starting material disappeared and a new spot, $Rf_5$ 0.52, was detected on TLC. The solution, after being neutralized with $Et_3N$, was concentrated and the residue was treated with ether to form a powder; yield 134 mg (90%), mp 175—178 °C, $[\alpha]_D^{24}$ −16.2° ($c$=0.5, DMF), $Rf_5$ 0.52. Met and Leu (diagnostic amino acid) ratios in 4 M MSA hydrolysate: 1.72 (2) : 3.00 (3). The amount of Met(O) found by an amino acid analyzer was negligible. When this reduction was performed without trimethylsilyl trifluoromethanesulfonate, treatment with the former silane (60 eq, 40 °C, 30 min) had to be repeated to obtain reduced product which gave a single spot on TLC.

**H-Val-Pro-Leu-Pro-Ala-Gly-Gly-Gly-Thr-Val-Leu-Thr-Lys-Met-Tyr-Pro-Arg-Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-$NH_2$, hGRP**——The above reduced peptide (117 mg, 33.2 $\mu$mol) was treated with 1 M TFMSA–thioanisole in TFA (7 ml) in the presence of *m*-cresol (278 $\mu$l, 80 eq) and EDT (111 $\mu$l, 40 eq) in an ice-bath for 180 min, then dry ether was added. The resulting powder was dissolved in $H_2O$ (8 ml). The pH of the ice-chilled solution was adjusted to 8.0 with 5% $NH_4OH$ and after 30 min, to 5.0 with 1 N AcOH. The solution was applied to a column of Sephadex G-25 (2.3 × 131 cm), which was eluted with 1 N AcOH. The fractions corresponding to the front main peak (tube Nos. 32—51, 6 ml each, determined by UV absorption measurement at 280 nm) were combined and the solvent was removed by lyophilization to give a crude deprotected peptide; yield 97 mg (96%).

A part of the above sample (45 mg) was dissolved in 0.1% TFA (4.5 ml) and applied to an HPLC column of Cosmosil $5C_{18}$ (10 × 250 mm), which was eluted with MeCN (gradient 25—35%, 80 min) in 0.1% TFA at a flow rate of 2.0 ml/min. The eluate corresponding to the main peak (retention time 39.3 min; the elution pattern of the test run is shown in Fig. 5-a) was concentrated and the residue was passed through a column of Sephadex G-25 (1.8 × 57 cm) using 1 N AcOH as an eluant. The desired fractions (tube Nos. 9—15, 5 ml each) were collected and the solvent was removed by lyophilization to give a fluffy powder; yield 14.0 mg. The rest of the sample was similarly purified by HPLC; total yield of the HPLC step, 30 mg (31%). Overall yield from the reduced peptide was 30%. $[\alpha]_D^{20}$ −99.9° ($c$=0.1, 1 N AcOH), $Rf_4$ 0.44, $Rf_5$ 0.05. A single peak was obtained in analytical HPLC on a Cosmosil $5C_{18}$ column (4 × 150 mm); retention time 13.0 min on gradient elution with MeCN (28—38%, 30 min) in 0.1% TFA (Fig. 5-b). A

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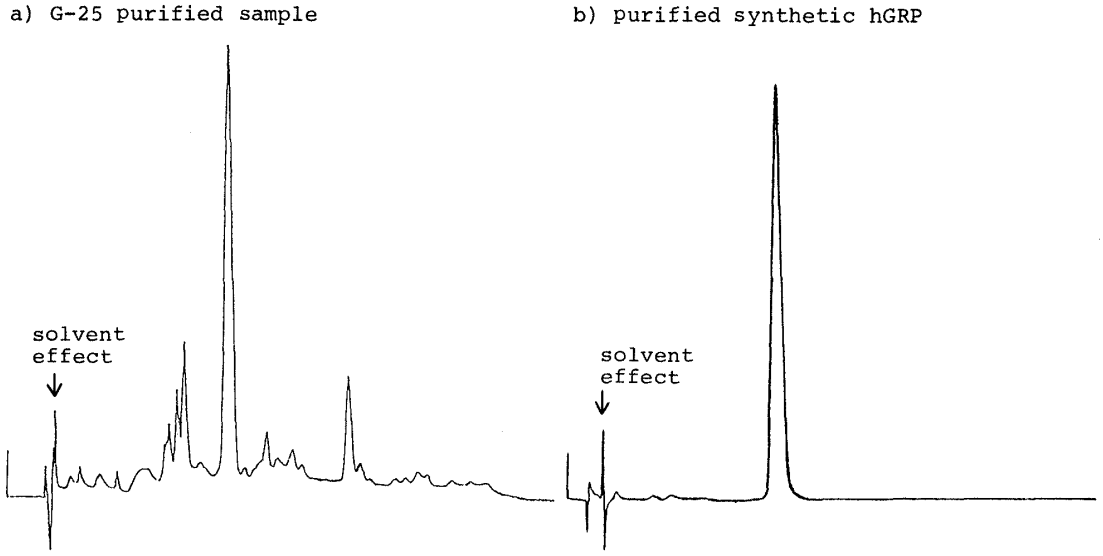


Fig. 5. HPLC of Synthetic hGRP


Fig. 6. Disk Isoelectrofocusing of Synthetic hGRP

single band was obtained in disk isoelectrofocusing on 7.5% polyacrylamide gel (0.5 × 7.0 cm) containing Pharmalyte (pH 3.0—10.0): mobility 6.4 cm (stained with Coomassie Brilliant Blue G-250, Sigma) from the origin toward the cathodic end of the gel, after running at 200 V for 5 h (Fig. 6). Amino acid ratios in 6 N HCl hydrolysate are shown in Table I. Amino acid ratios in papain plus leucineaminopeptidase digestion: Thr 2.05, Pro 2.12, Gly 4.51, Ala 2.05, Val 2.48, Met 1.53, Leu 2.49, Tyr 0.88, Lys 1.00, His 1.58, Arg 1.04, Trp 1.04. (recovery of Lys 94%).

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2) Amino acids and peptide derivatives mentioned in this communication are of the L-configuration. The following abbreviations are used: Z = benzyloxycarbonyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, Boc = *tert*-butoxycarbonyl, Mts = mesitylenesulfonyl, DCC = dicyclohexylcarbodiimide, Np = *p*-nitrophenyl, Su = *N*-hydroxysuccinimidyl, HOBt = *N*-hydroxybenzotriazole, TFA = trifluoroacetic acid, TFMSA = trifluoromethanesulfonic acid, MSA = methanesulfonic acid, DMF = dimethylformamide, DMSO = dimethylsulfoxide, EDT = ethanedithiol, cDNA = complementary deoxyribonucleic acid.
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