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A Study on Endocrine Disorder in Patients with Chronic Renal Failure. I. Synthesis and Biological Activity of Human Gastrin I[1)]

TAKASHI ABIKO,* IKUKO ONODERA, and HIROSHI SEKINO

Kidney Center, Sendai Insurance Hospital, Tsutsumimachi 3-16-1, Sendai, 980, Japan

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A heptadecapeptide amide, H–Pyr–Gly–Pro–Trp–Leu–Glu–Glu–Glu–Glu–Glu–Ala–Tyr–Gly–Trp–Met–Asp–Phe–NH_2, corresponding to the entire amino acid sequence of human gastric I, was synthesized by the solution method. This peptide inhibited lactate dehydrogenase activity by 87.6% at a concentration of 300 pg/ml.

This peptide was tested for suppression of PHA-induced lymphocyte proliferation, but showed no significant activity.

Keywords——human gastrin I; chronic renal failure; hypergastrinemia; trichloroethyloxycarbonylhydrazide; inhibitory activity towards lactate dehydrogenase

The kidney is well established as a major site of accumulation and degradation of many polypeptide hormones[2–4)] including gastrin.[5)] Gastrin is the most effective activator of gastric acid secretion known, though it can also stimulate pepsin and intrinsic factor release from gastric mucosa. Hypergastrinemia has been found in patients with chronic real failure;[6,7)] the plasma gastrin level decreased and returned to normal only when kidney function improved and blood urea nitrogen and creatinine returned to normal.[8)]

On the other hand, uremic sera have been found to have adverse actions on a variety of biological systems *in vitro*; these toxic effects include reduced activity of serum LDH,[9)] and the stimulation of lymphocytes from normal donors was inhibited by sera from a large percentage of patients with chronic renal failure.[10)]

However, these toxic substances in uremic sera have not been identified chemically or biologically,[11)] but some authors have suggested a peptide nature.[12)] These substances may exert inhibitory effects on glycolytic enzymes[12)] and lymphocyte proliferation.[13)]

In cases of uremia, there is retention in the blood of hormonal polypetides, which are normally metabolized and excreted by the kidneys.[14)] Among extrarenal hormones, gastrin is one of the most frequently found peptide hormones related to the degree of renal impairment.[7)]

In 1964, Anderson *et al.*[15,16)] reported the synthesis of human gastrin I. We now wish to report our synthetic data, obtained by solution methods. We have synthesized human gastrin I and examined it for inhibitory activity towards LDH and for effect on DNA synthesis in lymphocytes stimulated by PHA.

In the present study, protecting groups of amino acid derivatives, Z–Pyr, Asp(OBzl) and Glu(OBzl) were removed by hydrogen fluoride treatment.[17)] The protecting groups survive mostly intact during TFA treatment for the removal of the Boc group, employed as a temporary α-amino protecting group. Met residue was protected as the corresponding sulfoxide in order to prevent partial oxidation and S-alkylation during the synthesis.[18)] In order to suppress nitrosation at the indole moiety of Trp[19)] during the azide coupling procedure, Trp residues were incorporated into the protected peptide amide by the HOBT–DCC procedure.[20)] The hydroxy group of Tyr was not protected. First, the C-terminal tetrapeptide amide, Boc–Trp–Met(O)–Asp(OBzl)–Phe–NH_2 (III), was synthesized stepwise by the HOBT–DCC procedure[20)] starting from H–Phe–NH_2. Next, in order to prepare the peptide hydrazide containing Glu(OBzl), Boc–Gly–OH was condensed with Troc–$NHNH_2$ by means of DCC in the presence of HOBT[21)] to give Boc–Gly–NHNH–Troc (IV). The protected nonapeptide Troc–hydrazide, Boc–Leu–Glu(OBzl)–Glu(OBzl)–Glu(OBzl)–Glu(OBzl)–Glu(OBzl)–Ala–Tyr–Gly–

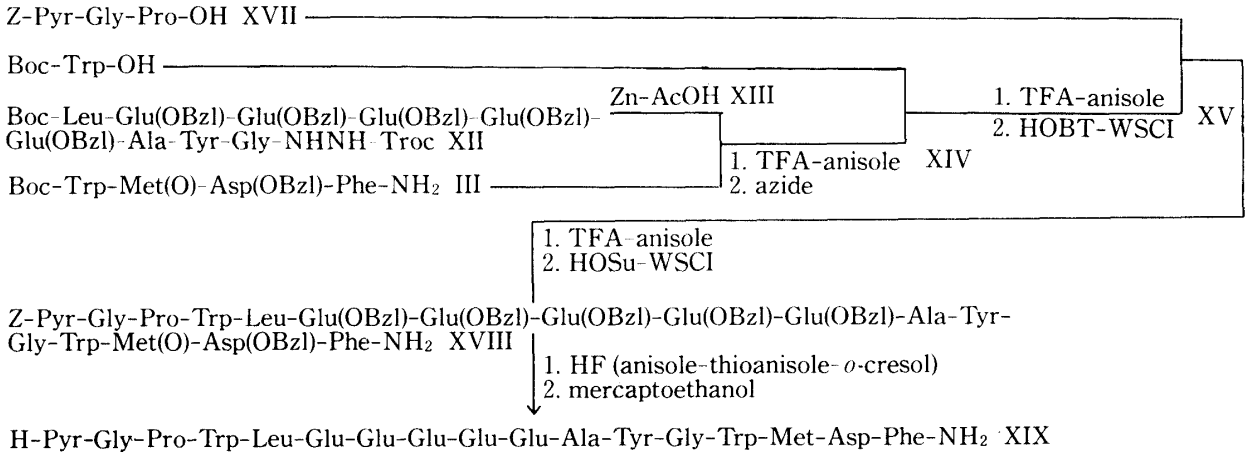

Fig. 1. Synthetic Scheme for the Human Gastrin I

NHNH–Troc (XII), was also synthesized stepwise by the HOBT–DCC procedure[20] starting from Boc–Gly–NHNH–Troc. After that, the Troc group of XII was removed by treatment with Zn[22] in AcOH and DMF. The last trace of metal contamination was removed by treatment with EDTA. Thus, the Glu(OBzl) residues were incorporated into the hydrazide fragment as shown in Fig. 1. The protected tetrapeptide amide III was converted to the corresponding amine by TFA–anisole treatment and was subjected to coupling with the azide derived from Boc–Leu–Glu(OBzl)–Glu(OBzl)–Glu(OBzl)–Glu(OBzl)–Glu(OBzl)–Ala–Tyr–Gly–$NHNH_2$ (XIII) by treatment with 6 N HCl in dioxane, followed by isoamylnitrite,[23] to afford the protected tridecapeptide amide, Boc–Leu–Glu(OBzl)–Glu(OBzl)–Glu(OBzl)–Glu(OBzl)–Glu(OBzl)–Ala–Tyr–Gly–Trp–Met(O)–Asp(OBzl)–Phe–NH_2 (XIV). XIV was purified by silica gel column chromatography with BuOH: $CHCl_3$: DMF (2: 1: 1). The homogeneity of the peptide was assessed by paper chromatography using two different solvent systems, by amino acid analysis of 4 N MSA hydrolysate and by elemental analysis. The tridecapeptide amide XIV was treated with TFA–anisole and the product was condensed with Boc–Trp–OH by the HOBT–DCC procedure to afford Boc–Trp–Leu–Glu(OBzl)–Glu(OBzl)–Glu(OBzl)–Glu(OBzl)–Glu(OBzl)–Ala–Tyr–Gly–Trp–Met(O)–Asp(OBzl)–Phe–NH_2 (XV). The protected tripeptide, Z–Pyr–Gly–Pro–OH (XVII), was synthesized in a stepwise manner by the MA procedure[24] starting from H–Pro–OH. The tetradecapeptide amide XV was treated with TFA-anisole and the product was condensed with the tripeptide XVII by the HOSu–DCC procedure[25] to minimize undesirable racemization, to afford Z–Pyr–Gly–Pro–Trp–Leu–Glu(OBzl)–Glu(OBzl)–Glu(OBzl)–Glu(OBzl)–Glu(OBzl)–Ala–Tyr–Gly–Trp–Met(O)–Asp(OBzl)–Phe–NH_2 (XVIII). XVIII was purified by silica gel column chromatography with BuOH: $CHCl_3$: DMF (2: 1: 1). The homogeneity of the peptide was assessed by amino acid analysis of the 4 N MSA hydrolysate and by elemental analysis. The protected heptadecapeptide amide XVIII was treated with hydrogen fluoride in the presence of anisole–thioanisole–*o*-cresol (1: 1: 1, V/V)[26] to suppress side reaction of H–Tyr–OH,[27] to remove all protecting groups. The free peptide amide, precipitated by adding dry ether, was converted to the corresponding acetate by treatment with Amberlite CG-4B (acetate form). The resulting product was dissolved in H_2O and the solution was incubated in the presence of mercaptoethanol[28] at 45°C for 48 h to reduce sulfoxide on the methionine side chain. The reduced peptide was passed through a column of Sephadex G-25 using 1% AcOH as a solvent to remove the thiol reagent. The product obtained from the main peak was further purified by column chromatography on DEAE–Sephadex A-25 using gradient elution with 0.15 M NH_4OAc buffer. The heptadecapeptide amide (XIX) thus obtained and the authentic human gastrin I were identical on the basis of TLC, paper chromatography and paper electrophoresis. The amino acid compositions in the 4 N MSA hydrolysate of XIX were in good agreement with the theoretical values.

Table I. Inhibitory Effect of the Synthetic Human Gastrin I on LDH Activity

Peptides	Dose (pg/ml)	Inhibitory activity (%)
Human gastrin I[a]	100	0
	150	18.6±6
	200	35.8±7
	250	68.7±7
	300	86.2±6
Synthetic human gastrin I	100	0
	150	17.4±7
	200	36.7±5
	250	67.2±8
	300	87.6±7

a) The human gastrin I was purchased from Calbiochem-Behring Corp., California, USA.

Table II. Effect of the Synthetic Human Gastrin I on Normal Lymphocytes Proliferation

Peptides	Dose (pg/ml)	^{3}H-Thymidine incorporation (cpm)
——	—	37461±2564[a]
Human gastrin I[b]	100	38196±2241
	300	38585±2616
Synthetic human gastrin I	100	36274±2984
	300	39909±3147

a) Normal lymphocytes.
b) The human gastrin I was purchased from Calbiochem-Behring Corp., California, USA.

The synthetic human gastrin I was tested quantitatively for inhibitory activity towards LDH and for effect on DNA synthesis in lymphocytes stimulated by PHA. The results of these biological examinations are given in Tables I and II.

The synthetic human gastrin I inhibited the LDH activity by about 87.6% at a concentration of 300 pg/ml, but this peptide possessed virtually no inhibitory activity on DNA synthesis in lymphocytes stimulated by PHA at the same concentration.

Experimental

Melting points are uncorrected. Rotations were measured with an Atago Polax machine (cell length: 10 cm). The hydrolyses of peptides for amino acid analysis were carried out in 4 N MSA containning 0.2% 3-(2-aminoethyl)indole.[22] Amino acid compositions of 4 N MSA hydrolysates were determined with a JEOL JLC-8AH amino acid analyzer (one-column system). Evaporation of solvents was carried out in a rotary evaporator under reduced pressure at 35 to 45°C. Boc groups of the protected peptides were removed by TFA-anisole treatment. The resulting amino components were chromatographed on filter paper, Toyo Roshi No. 51, at room temperature. Rf^a values refer to the Partidge system[29] and Rf^b values refer to BuOH–pyridine–AcOH–H_2O (30: 20: 6: 24).[30] TLC was performed on silica gel (Kieselgel 60, Merck) plates and Rf values refer to the following solvent systems: Rf^c Partridge system and Rf^d BuOH–pyridine–AcOH–H_2O (30: 20: 6: 24). Authentic human gastrin I was purchased from Calbiochem-Behring Corp., California, USA, and 4 N MSA containing 0.2% 3-(2-aminoethyl)indole was purchased from Pierce Chemical Co., USA.

Boc-Asp(OBzl)-Phe-NH_2 (I)——HOBT (1.5 g) and WSCI (1.7 g) were added to a solution of Boc–Asp-(OBzl)–OH (3.5 g), H–Phe–NH_2 (1.6 g) and NMM (1.1 ml) in DMF (15 ml) with stirring at 0°C. The mixture was stirred for 12 h at 4°C. The reaction mixture was extracted with EtOAc and the extract was washed successively with 1 N citric acid, H_2O, 1 N $NaHCO_3$ and H_2O, dried over $MgSO_4$ and then concentrated *in vacuo*. The residue was recrystallized from EtOAc and ether; yield 3.4 g (80%), mp 129°C, $[\alpha]_D^{28}$ −70.3° (c=1.0, DMF), Rf^a 0.80, Rf^b 0.92, single ninhydrin-positive spot. *Anal.* Calcd for $C_{25}H_{31}N_3O_6$: C, 63.95; H, 6.66; N, 8.95. Found: C, 64.01; H, 6.57; N, 9.00.

Boc-Met(O)-Asp(OBzl)-Phe-NH_2 (II)——I (2.4 g) was treated with TFA (6 ml)–anisole (1.2 ml) at room temperature for 30 min, then excess TFA was removed by evaporation. The residue was washed with dry ether and then dried over KOH pellets *in vacuo* and the deprotected peptide amide was dissolved in DMF

(15 ml). To this ice-chilled solution, NMM (0.6 ml), Boc–Met(O)–OH (1.4 g), HOBT (744 mg) and WSCI (854 mg) were successively added. After being stirred overnight at 4°C, the mixture was extracted with EtOAc and the extract was washed successively with 1 N citric acid, H_2O, 1 N $NaHCO_3$ and H_2O, dried over $MgSO_4$ and concentrated *in vacuo*. *n*-Hexane was added to the residue and the precipitate formed was filtered off *in vacuo*. The product was reprecipitated from MeOH and ether; yield 2.1 g (66%), mp 168—170°C $[\alpha]_D^{29}$ −11.4° (c=1.0, DMF), Rf^a 0.83, Rf^b 0.92, single ninhydrin–positive spot. *Anal.* Calcd for $C_{30}H_{40}N_4O_8S$: C, 57.13; H, 6.39; N, 11.10. Found: C, 57.25; H, 6.45; N, 10.87.

Boc-Trp-Met(O)-Asp(OBzl)-Phe-NH$_2$ (III)——This compound was prepared from II (631 mg), HOBT (149 mg), Boc–Trp–OH (324 mg) and WSCI (171 mg) essentially as described for the preparation of II. The reaction mixture was poured into ice-chilled 1 N $NaHCO_3$ with stirring. The precipitate thus formed was washed succesively with 1 N $NaHCO_3$, H_2O, 1 N citric acid and H_2O. The dried product was recrystallized from hot EtOAc; yield 513 mg (64%), mp 173—176°C, $[\alpha]_D^{28}$ −35.6° (c=1.0, DMF), Rf^a 0.81, Rf^b 0.91, single ninhydrin- and Ehrlich-positive spot. *Anal.* Calcd for $C_{41}H_{50}N_6O_9S$: C, 61.33; H, 6.28; N, 10.74. Found: C, 61.32; H, 6.35; N, 10.24.

Boc-Gly-NHNH-Troc (IV)——HOBT (3.7 g) and WSCI (4.3 g) were added to a mixture of Boc–Gly–OH (4.4 g) and Troc–$NHNH_2$ (5.7 g) in THF (30 ml) and the solution was stirred for 24 h. The mixture was concentrated and the residue was extracted with EtOAc. The extract was washed with 1 N $NaHCO_3$, H_2O, 1 N citric acid and H_2O, dried over $MgSO_4$ and then evaporated to dryness. The residue was precipitated from EtOAc and petroleum ether; yield 8.5 g (oily material) (92%), Rf^a 0.69, Rf^b 0.87, single ninhydrin–positive spot. *Anal.* Calcd for $C_{10}H_{16}Cl_3N_3O_5$: C, 32.94; H, 4.42; N, 11.53. Found: C, 33.23; H, 4.78; N, 11.82.

Boc-Tyr-Gly-NHNH-Troc (V)——This compound was prepared from IV (3.7 g), Boc–Tyr–OH (3 g), HOBT (1.6 g) and WSCI (1.8 g) essentially as described for the preparation of II. The product was reprecipitated from EtOAc and *n*-hexane; yield 6 g (90%), mp 56—60°C, $[\alpha]_D^{29}$ −34.8° (c=1.0, DMF), Rf^a 0.73, Rf^b 0.83, single ninhydrin–positive spot. *Anal.* Calcd for $C_{19}H_{25}Cl_3N_4O_7$: C, 43.24; H, 4.78; N, 10.62. Found: C, 42.91; H, 4.82; N, 10.54.

Boc-Ala-Tyr-Gly-NHNH-Troc (VI)——This compound was prepared from V (2.6 g), Boc–Ala–OH (1.1 g), HOBT (744 mg) and WSCI (854 mg) essentially as described for the preparation of II. The product was recrystallized from EtOAc and *n*-hexane; yield 2.6 g (87%), mp 75—82°C, $[\alpha]_D^{28}$ −21.3° (c=1.0, DMF), Rf^a 0.86, Rf^b 0.91, single ninhydrin-positive spot. *Anal.* Calcd for $C_{22}H_{30}Cl_3N_5O_8$: C, 44.12; H, 5.05; N, 11.70. Found: C, 43.86; H, 5.09; N, 11.48.

Boc-Glu(OBzl)-Ala-Tyr-Gly-NHNH-Troc (VII)——This compound was prepared from VI (2 g), Boc–Glu(OBzl)–OH (1.9 g), HOBT (495 mg) and WSCI (570 mg) essentially as described for the preparation of II. The product was reprecipitated from EtOAc and ether; 1.8 g (60%), mp 78—83°C, $[\alpha]_D^{29}$ −40.6° (c=1.0, DMF), Rf^a 0.76, Rf^b 0.89, single ninhydrin–positive spot. *Anal.* Calcd for $C_{41}H_{49}Cl_3N_6O_{11}$: C, 54.22; H, 5.44; N, 9.25. Found: C, 54.03; H, 5.81; N, 9.42.

Boc-Glu(OBzl)-Glu(OBzl)-Ala-Tyr-Gly-NHNH-Troc (VIII)——This compound was prepared from VII (1.5 g), Boc–Glu(OBzl)–OH (952 mg), HOBT (248 mg) and WSCI (285 mg) essentially as described for the preparation of II. The product was reprecipitated from EtOAc and ether; yield 1.5 g (79%), mp 73—78°C, $[\alpha]_D^{28}$ −25.2° (c=1.0, DMF), Rf^a 0.76, Rf^b 0.92, single ninhydrin-positive spot. *Anal.* Calcd for $C_{53}H_{62}Cl_3N_7O_{14}$: C, 56.46; H, 5.54; N, 8.70. Found: C, 56.81; H, 5.67; N, 8.59.

Boc-Glu(OBzl)-Glu(OBzl)-Glu(OBzl)-Ala-Tyr-Gly-NHNH-Troc (IX)——This compound was prepared from VIII (1.4 g), Boc–Glu(OBzl)–OH (713 mg), HOBT (186 mg) and WSCI (213 mg) essentially as described for the preparation of II. The product was reprecipitated from EtOAc and *n*-hexane; yield 1.4 g (82%), np 81—86°C, $[\alpha]_D^{29}$ −61.7° (c=1.0, DMF), Rf^a 0.73, Rf^b 0.89, single ninhydrin–positive spot. *Anal.* Calcd for $C_{65}H_{75}Cl_3N_8O_{17}$: C, 57.97; H, 5.61; N, 8.23. Found: C, 57.61; H, 5.41; N, 8.74.

Boc-Glu(OBzl)-Glu(OBzl)-Glu(OBzl)-Glu(OBzl)-Ala-Tyr-Gly-NHNH-Troc (X)——This compound was prepared from IX (1.2 g), Boc–Glu(OBzl)–OH (519 mg), HOBT (135 mg) and WSCI (155 mg) essentially as described for the preparation of II. The product was reprecipitated from EtOAc and ether; yield 1.1 g (79%), mp 79—83°C, $[\alpha]_D^{30}$ −15.2° (c=1.0, DMF), Rf^a 0.71, Rf^b 0.80, single ninhydrin–positive spot. *Anal.* Calcd for $C_{77}H_{88}Cl_3N_9O_{20}$: C, 59.60; H, 5.67; N, 8.05. Found: C, 59.40; H, 5.36; N, 8.32.

Boc-Glu(OBzl)-Glu(OBzl)-Glu(OBzl)-Glu(OBzl)-Glu(OBzl)-Ala-Tyr-Gly-NHNH-Troc (XI)——This compound was prepared from X (869 mg), Boc–Glu(OBzl)–OH (317 mg), HOBT (83 mg) and WSCI (95 mg) essentially as described for the preparation of II. The product was reprecipitated from EtOAc and ether; yield 658 mg (66%), mp 74—80°C, $[\alpha]_D^{30}$ −5.0° (c=1.0, DMF), Rf^a 0.73, Rf^b 0.86, single ninhydrin–positive spot. *Anal.* Calcd for $C_{89}H_{101}Cl_3N_{10}O_{23}$: C, 59.88; H, 5.70; N, 7.85. Found: C, 59.44; H, 5.48; N, 8.23.

Boc-Leu-Glu(OBzl)-Glu(OBzl)-Glu(OBzl)-Glu(OBzl)-Glu(OBzl)-Ala-Tyr-Gly-NHNH-Troc (XII)——This compound was prepared from XI (595 mg), Boc–Leu–OH (91 mg), HOBT (50 mg) and WSCI (57 mg) essentially as described for the preparation of II. The product was reprecipitated from MeOH and ether; yield 516 mg (80%), mp 83—91°C, $[\alpha]_D^{30}$ −12.1° (c=1.0, DMF), Rf^a 0.87, Rf^b 0.89, single ninhydrin–positive spot. *Anal.* Calcd for $C_{95}H_{112}Cl_3N_{11}O_{24}\cdot 2H_2O$: C, 58.81; H, 6.03; N, 6.40. Found: C, 58.64; H, 6.32; N, 6.15.

Boc-Leu-Glu(OBzl)-Glu(OBzl)-Glu(OBzl)-Glu(OBzl)-Glu(OBzl)-Ala-Tyr-Gly-NHNH$_2$ (XIII)——XII (418 mg) in a mixture of AcOH (3 ml) and DMF (3 ml) was treated with Zn dust (452 mg) at room temperature for 3 h. The solution was filtered, the filtrate was concentrated *in vacuo* and the residue was treated with

1% EDTA. The resulting gelatinous mass was washed batchwisely with 1 N $NaHCO_3$ and H_2O and then recrystallized from MeOH; yield 296 mg (90%), mp 104—112°C, $[\alpha]_D^{29}$ −61.4° (c=1.0, DMF). *Anal.* Calcd for $C_{85}H_{105}N_{11}O_{22}$: C, 62.53; H, 6.48; N, 9.44. Found: C, 62.22; H, 6.37; N, 9.02.

Boc-Leu-Glu(OBzl)-Glu(OBzl)-Glu(OBzl)-Glu(OBzl)-Glu(OBzl)-Ala-Tyr-Gly-Trp-Met(O)-Asp(OBzl)-Phe-NH_2 (XIV)——III (160 mg) was treated with TFA (2 ml)–anisole (0.4 ml) as usual and dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* and dissolved in DMF (3 ml) containing NMM (0.02 ml). The azide[23] (prepared from 460 mg of XIII with 0.4 ml of 6 N HCl in dioxane and 0.16 ml of isoamylnitrite at −60°C) in DMF (2 ml)–DMSO (2 ml) and NMM (0.6 ml) were added to the above ice-chilled solution and the mixture was stirred for 48 h at 4°C. Then the mixture was poured into ice-chilled 1 N $NaHCO_3$ with stirring. Next, 50% NH_4OAc was added dropwise with stirring to form a precipitate. The precipitate was collected and washed successively with 1 N $NaHCO_3$, H_2O, 1 N citric acid and H_2O. The product was further purified by column chromatography on silica gel (2.1 × 45 cm), equilibrated and eluted with BuOH–$CHCl_3$–DMF (2: 1: 1). The desired fractions (4 ml each, tube Nos. 41—46) were combined and the solvent was removed by evaporation. Ether was added to the residue to give a precipitate. The product was recrystallized from EtOAc; yield 324 mg (70%), mp 141—149°C, $[\alpha]_D^{26}$ −28.4° (c=1.0, DMF), Rf^a 0.84, Rf^b 0.89, single ninhydrin- and Ehrlich-positive spot. *Anal.* Calcd for $C_{121}H_{143}N_{15}O_{29}2H_2O$: C, 62.12; H, 6.33; N, 8.98. Found: C, 61.92; H, 6.51; N, 8.65. Amino acid ratios in 4 N MSA hydrolysate: Gly 1.13, Leu 1.06, Ala 1.00, Tyr 0.89, Met+Met(O) 0.82, Trp 0.81, Phe 1.12, Glu 4.78, Asp 0.94 (recovery of Ala 80%).

Boc-Trp-Leu-Glu(OBzl)-Glu(OBzl)-Glu(OBzl)-Glu(OBzl)-Glu(OBzl)-Ala-Tyr-Gly-Trp-Met(O)-Asp(OBzl)-Phe-NH_2 (XV)——This compound was prepared from XIV (230 mg), Boc–Trp–OH (33 mg), HOBT (15 mg) and WSCI (18 mg) essentially as described for the preparation of II. The reaction mixture was poured into ice-chilled 1 N $NaHCO_3$ with stirring. The precipitate thus formed was washed successively with 1 N $NaHCO_3$, H_2O, 1 N citric acid and H_2O. The dried product was recrystallized from hot MeOH; yield 211 mg (85%), mp 112—115°C, $[\alpha]_D^{28}$ −13.1° (c=1.0, DMF), Rf^a 0.86, Rf^b 0.92, single ninhydrin- and Ehrlich-positive spot. *Anal.* Calcd for $C_{132}H_{153}N_{17}O_{30}S$: C, 63.68; H, 6.19; N, 9.56. Found: C, 64.01; H, 5.85; N, 9.26.

Boc-Gly-Pro-OH (XVI)——H–Pro–OH (600 mg) was dissolved in THF (5 ml). To this ice-chilled solution, a solution of the mixed anhydride[24] (prepared from 1.0 g of Boc–Gly–OH with 0.54 ml of ethylchloroarbonate and 0.5 ml of NMM at −10°C) in THF (5 ml)–acetonitrile (5 ml) was added. The solution was stirred at 4°C for 6 h, then concentrated and the residue was diluted with EtOAc. The solution was washed successively with 1 N citric acid and H_2O, dried over $MgSO_4$ and concentrated. The residue was reprecipitated from EtOAc and *n*-hexane; yield 1.2 g (oily material) (86%), $[\alpha]_D^{28}$ −67.1° (c=1.0, DMF), Rf^a 0.06, Rf^b 0.22, single ninhydrin-positive spot. *Anal.* Calcd for $C_{12}H_{20}N_2O_5$: C, 52.93; H, 7.40; N, 10.29. Found: C, 53.27; H, 7.61; N, 9.88.

Z-Pyr-Gly-Pro-OH CHA Salt (XVII)——XVI (908 mg) was treated with TFA (4 ml)–anisole (0.8 ml) in the usual manner and the deprotected peptide was dissolved in THF (5 ml) containing NMM (0.4 ml). To this ice-chilled solution, a solution of the mixed anhydride (prepared from 966 mg of Z–Pyr–OH with 0.35 ml of ethylchlorocarbonate and 0.4 ml of NMM at −10°C)/in THF (5 ml)–acetonitrile (5 ml) was added. The mixture was stirred at 4°C for 6 h, then concentrated, and the residue was diluted with EtOAc. The solution was washed as described above and then precipitated from EtOAc and *n*-hexane. The oily product was dissolved in MeOH (5 ml)–ether (5 ml) and CHA (1.5 ml) was added. The resulting solid was recrystallized from MeOH and ether; yield 964 mg (57%), mp 45—49°C, $[\alpha]_D^{29}$ −69.3° (c=1.0, DMF). For paper chromatography, the Z group of this peptide was not deblocked: Rf^a 0.63, Rf^b 0.71, single chloride–tolidine-positive spot. *Anal.* Calcd for $C_{20}H_{23}N_4O_5 \cdot C_6H_{13}N$: C, 62.63; H, 7.28; N, 14.05. Found: C, 62.46; H, 7.35; N, 14.29.

Z-Pyr-Gly-Pro-Trp-Leu-Glu(OBzl)-Glu(OBzl)-Glu(OBzl)-Glu(OBzl)-Glu(OBzl)-Ala-Tyr-Gly-Trp-Met(O)-Asp(OBzl)-Phe-NH_2 (XVIII)——XV (125 mg) was treated with TFA (2 ml)–anisole (0.4 ml) as described. above. To an ice-chilled solution of the resulting tetradecapeptide amide trifluoroacetate in DMF (3 ml), XVII (26 mg), HOSu (7 mg) and WSCI (10 mg) were added, followed by addition of NMM (0.01 ml) to keep the solution slightly alkaline. After 36 h at 4°C, the reaction mixture was poured into 1 N $NaHCO_3$ with stirring. The precipitate thus formed was washed successively with 1 N $NaHCO_3$, H_2O, 1 N HCl and H_2O. The dried product was further purified by column chromatography on silica gel (2.1 × 48 cm), equilibrated and eluted with BuOH–$CHCl_3$–DMF (2: 1: 1). The desired fractions (4 ml each, tube Nos. 46—49) were combined and concentrated *in vacuo*. Ether was added to the residue to give a precipitate. The product was recrystallized from hot MeOH; yield 109 mg (78%), mp 167—175°C, $[\alpha]_D^{27}$ −22.4° (c=1.0, DMF). *Anal.* Calcd for $C_{147}H_{166}N_{20}O_{34}S$: C, 63.30; H, 6.00; N, 10.04. Found: C, 62.96; H, 5.79; N, 10.32. Amino acid ratios in 4 N MSA hydrolysate: Gly 2.19, Leu 1.09, Ala 1.00, Tyr 0.86, Trp 1.72, Phe 1.03, Met+Met(O) 0.78, Pro 0.92, Glu 5.76, Asp 0.88 (recovery of Ala 81%).

H-Pyr-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2 (XIX)——The protected heptadecapeptide amide (80 mg) was treated with HF (approximately 4 ml) in the presence of anisole–thioanisole–*o*-cresol (1: 1: 1 v/v 0.5 ml) in an ice-bath for 1 h. After removal of the HF dry ether was added and the resulting powder was dissolved in H_2O (5 ml). The solution was treated with Amberlite CG-4B (acetate form approximately 2 g) for 30 min filtered by suction and evaporated to dryness *in vacuo*. The residue was dissolved in H_2O (2 ml) and the solution was incubated in the presence of mercaptoethanol

(0.1 ml) under N_2 gas at 45°C for 48 h. Then in order to remove mercaptoethanol the solution was applied to a column of Sephadex G-25 (2.8×91 cm) which was eluted with 1% AcOH. Individual fractions (5 ml each) were collected and the absorbancy at 280 nm was determined. The fractions corresponding to the main peak (tube Nos. 58—64) were collected and the solvent was removed by lyophilization. Next the Sephadex-purified sample was dissolved in H_2O (3 ml) and the solution was applied to a column of DEAE-Sephadex A-25 (2.8×60 cm), which was eluted first with H_2O (100 ml) and then with a gradient formed from 0.15 M NH_4OAc buffer at pH 6.10 (300 ml) through a mixing chamber containing H_2O (300 ml). Individual fractions (5 ml each) were collected and the absorbancy at 280 nm was determined. After elution of two minor peaks, the main peak was eluted. Fractions corresponding to this peak (tube Nos. 68—76) were combined and the solvent and NH_4OAc were removed by repeated lyophilization to give a fluffy white powder; yield 18 mg (29%), mp 225—234°C, $[\alpha]_D^{28}$ −65.0° (c=0.4, H_2O), Rf^a 0.15, Rf^b 0.20, Rf^c 0.16, Rf^d 0.65, single chlorine–tolidine- and Ehrlich-positive spot. Amino acid ratios in 4 N MSA hydrolysate: Phe 1.06 Leu 1.12, Ala 1.00, Gly 2.18, Trp 1.71, Tyr 0.86, Pro 0.92, Met 0.80, Glu 5.73, Asp 0.85 (recovery of Ala 83%).

Paper Electrophoresis——The synthesized human gastrin I was subjected to paper electrophoresis. Electrophoresis was carried out on Toyo Roshi No. 51 paper (10×40 cm) using phosphate buffer pH 9.01, at 600 V for 90 min, and the paper was stained with Ehrlich reagent. The synthetic human gastrin I and the authentic human gastrin I showed identical mobilities.

Inhibitory Activity of the Synthetic Human Gastrin I on LDH (EC 1.1.1.27)——The enzyme assay was performed using a Gilford 2400 recording spectrophotometer equipped with a temperature-controlled cuvette compartment held at 32°C by circulating constant-temperature water. The assay mixture (2.9 ml) contained pyruvate (0.6 mM) NADH (0.18 mM) the synthetic human gastrin I (100—300 pg/ml) and phosphate buffer (50 mM pH 7.5). LDH (Boehringer Mannheim GmbH Lot. 107034 200 mU) was added to the mixture prewarmed at 32+0.5°C for 4—5 min and the change in absorbance at 340 nm was measured for 3 min after mixing (Table I).

Inhibition Test of the Synthetic Human Gastrin I on DNA Synthesis in Lymphocytes Stimulated by PHA——Venous blood from normal subjects was withdrawn into heparinized syringes and sedimented at room temperature. Lymphocytes were separated from whole blood on a Ficoll–Isopaque gradient as described by Harris *et al.*[31] for T-cell transformation. The cells were cultured in 0.2 ml of RPMI 1640 (Gibco) with 10% FCS (Dainippon Pharmaceutical Co.) in microplates (12×8 wells) and 0.02 ml (final 10 μg/ml) of PHA-P (Difco) was added with 0.02 ml (final concentration 100—300 pg/ml) of the synthetic human gastrin I. Triplicate cultures of each combination (1×10^5 cells per well) were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 64 h. Eight h before harvesting 0.25 μCi per well of ^{3}H-thymidine was added per culture. The amount of thymidine incorporation into DNA was measured in an LKB-1216 liquid scintillation counter (Table II).

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References and Notes

1) The amino acid residues are of the L-configuration. The abbreviations used to denote amino acid derivatives and peptides are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochim. Biophys. Acta*, **263**, 205 (1972). Other abbreviations: DMF, dimethylformamide; WSCI, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; DCC, dicyclohexylcarbodiimide; TFA, trifluoroacetic acid; HOBT, *N*-hydroxybenzotriazole; DNA, deoxyribonucleic acid; MA, mixed anhydride; FCS, fetal calf serum; PHA, phytohemagglutinin; THF, tetrahydrofuran; HF, hydrogen fluoride; RPMI, Rosewell Park Memorial Institute; EDTA, ethylenediaminetetraacetic acid; DMSO, dimethylsulfoxide; LDA, lactate dehydrogenase; NMM, *N*-methylmorpholine; Z, benzyloxycarbonyl; Boc, *tert*-butoxycarbonyl; OBzl, benzyl ester; AcOH, acetic acid; EtOAc, ethyl acetate; HOSu, *N*-hydroxysuccinimide; TLC, thin-layer chromatography; NHNH-Troc, trichloroethyloxycarbonylhydrazide; MSA, methanesulfonic acid.
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