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Porcine Big Gastrin: Sequence, Synthesis, and Immunochemical Studies

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The synthesis of the tetracontapeptide corresponding to a revised sequence for porcine big gastrin is described. Both the synthetic and natural material were identical by radioimmunoassay using L33 antiserum, which is specific for the N-terminal region of the sequence.

INTRODUCTION

Porcine big gastrin is present in hog antral mucosa as two peptides differing only in the presence or absence of sulphation at Tyr-29. Earlier studies (*1, 2*), using a combination of sequencing, synthesis, and radioimmunoassay, resulted in structural elucidation of the N-terminal nonadecapeptide (**1**,R=OH) and hence the entire sequence of pG34 as (**1**).

Glp–Leu–Gly–Leu–Gln–Gly–Pro–Pro–His–Leu–
Val–Ala–Asp–Leu–Ala–Lys–Lys–Gln–Gly-R [1]

— — — — — —^{7}His— — —^{9}Pro- — — — — — — — — — — — [2]

R = Pro–Trp–Met-[Glu]$_5$-Ala–Tyr–Gly–Trp–Met–Asp–Phe-NH_2
Glp-Leu–Gly–Leu–Gln–Gly–Pro–Pro–His–Leu–Val–Ala–Asp(OBu^t)-Leu–
Ala–Lys(Adoc)-Lys(Adoc)–Gln–Gly-OH [3]

Bpoc-Pro–Trp–Met-[Glu(OBu^t)]$_5$-Ala–Tyr(Bu^t)-
Gly–Trp–Met–Asp(OBu^t)-Phe-NH_2 [4]

It was important, therefore, to extend these researches to complete the synthesis of pG34 and compare the synthetic material with natural pG34 by immunochemical analysis using antiserum L33, since the biological activity of pG34, as evidenced by acid secretion in the conscious dog, is determined by the C-terminal tetrapeptide sequence.

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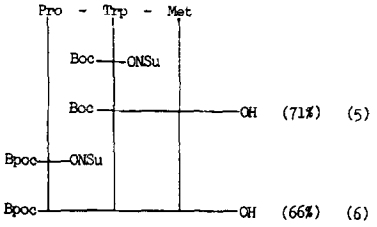
Sequence Studies

The pG34 peptide was first treated with pyrrolidone decarboxylase to remove the cyclized N-terminal residue and then applied to an automated Beckman 890B sequenator that had been modified as described by Hunkapiller and Hood (*3*). The Pth amino acid residues derived from the sequenator were identified by high-pressure liquid chromatography (*4*); and the yields of each amino acid derivative obtained at each cycle are presented in Fig. 1, in which the predominant residue at each cycle is denoted by a triangle. The sequence (**1**) resulting from these studies indicates the errors in the previous (*5*) structure (**2**) to be at positions 7 and 9.

DISCUSSION OF SYNTHETIC METHODS

The strategy of fragment coupling was designed to make use of fragments emanating from our earlier studies (*2, 6*) on the synthesis of gastrointestinal hormones. In particular the required nonadecapeptide (**3**) was a key substrate for the immunological studies leading to the structural assignment of the N-terminal region of porcine G34 (**1**). Fragment (**4**) was synthesized as the N_α-Bpoc[3] protected peptide in order to allow preferential acidolytic cleavage of N_α protection in the presence of the *t*-butyl side chain protecting groups. Since the suitably protected octapeptide (**7**) and tetrapeptide (**9**) required for the construction of (**4**) were available from previous research (*12*), the only new fragment to be synthesized was Bpoc–Pro–Trp–Met–OH (*6*). This was accomplished according to Scheme 1, using *N*-hydroxysuccinimide active ester couplings.



SCHEME 1

After reductive deprotection of the N_α-Z protection in (**7**), the amino component was coupled to the preformed *N*-hydroxysuccinimide active ester of (**6**). Hydrolysis (*7, 12*) of the protected undecapeptide (**8**), using alkaline hydrogen peroxide at pH 10.5 in aqueous trifluoroethanol in the presence of a scavenger (DMS) to avoid oxidation on the tryptophan and methionine residues, afforded the free acid (**8**,R=H). This was coupled to the C-terminal tetrapeptide fragment (**9**) using

[3] Abbreviations used: DMF, dimethylformamide; NMM, *N*-methylmorpholine; DCCI, dicyclohexylcarbodiimide; DMS, dimethylsulfide; TFA, trifluoroacetic acid; HONSu, *N*-hydroxysuccinimide; HOBt, 1-hydroxybenzotriazole; Bpoc, biphenylisopropyloxycarbonyl; Adoc, adamantyloxycarbonyl; HMPA, hexamethylphosphortriamide; NMP, *N*-methylmorpholine; BGI, unsulfated big gastrin; AE, aminoethyl.

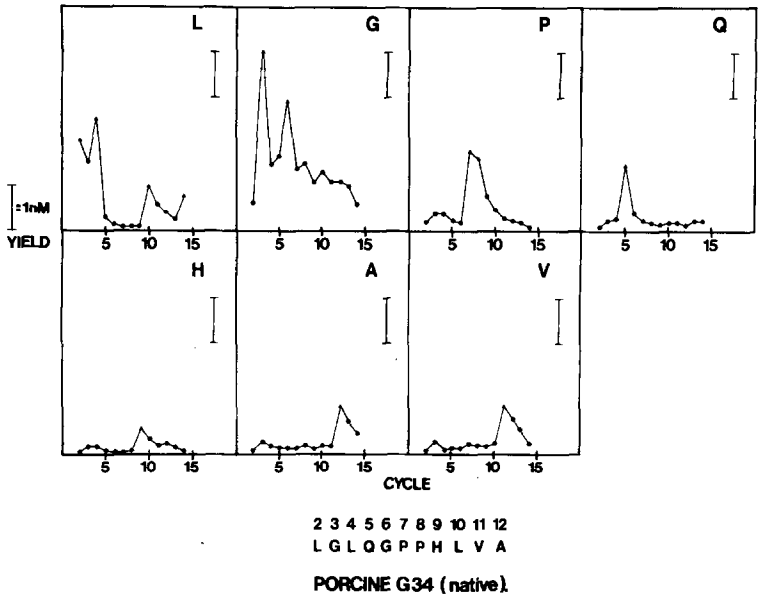


FIG. 1. Identification of residues 2–12 of porcine G34 (native) as Pth derivatives using a modified Beckman 890B sequenator. Aliquots of each cycle were analyzed by high-pressure chromatography on Du Pont Zorbar Cyanopropylsilane with a gradient of methanol–acetonitrile (17:3) in sodium acetate buffer, pH 5.4

$$Z\text{-}[Glu(OBu^t)]_5\text{-}Ala\text{–}Tyr(Bu^t)\text{-}Gly\text{-}OPh \quad [7]$$

$$Bpoc\text{-}Pro\text{–}Trp\text{–}Met\text{-}[Glu(OBu^t)]_5\text{-}Ala\text{–}Tyr(Bu^t)\text{-}Gly\text{-}OR \quad [8]$$

$$H\text{-}Trp\text{–}Met\text{–}Asp(OBu^t)\text{-}Phe\text{-}NH_2 \quad [9]$$

DCCI/HOBt (*8*) to give the desired fragment (**4**) after LH20/DMF purification (*9*). Careful acidolytic cleavage of (**4**) in aqueous trifluoroethanol containing DMS at pH 0.5 afforded the amino component, which was coupled with the nonadecapeptide free acid (**3**) (*2*) in HMPA-NMP using DCCI/HONSu (*10*) in the presence of NMM as base. After initial purification of the protected product by LH20/DMF gel filtration the side chain functionality of the hormone was revealed by acidolytic cleavage of the *t*-butyl protecting groups. This was smoothly effected by TFA in the presence of 1,2-ethanedithiol as a scavenger for the intermediate t-butyl carbonium ions. After chromatography (G50; 0.4% NH_4HCO_3) the peptide was further purified by gradient elution from aminoethyl-cellulose (AE, Whatman). The product was tested by subcutaneous injection into a conscious dog provided with a gastric fistula and was shown to stimulate gastric acid secretion with a time course and potency comparable with that of natural porcine BGI. Comparison of the synthetic product with natural material by high-pressure liquid chromatography showed these to have identical chromatographic properties on Whatman Partisil ODS 10 using a gradient of 10–80% (v/v) methanol containing 0.01 *M* ammonium acetate (pH 7.0).

The alternative sequence corresponding to the earlier structure (2) was synthesized according to the same method as described above for (1) by employing the [7-His, 9-Pro] analog of (3) (*2*) and the amino component derived from (4).

IMMUNOREACTIVITY OF SYNTHETIC pG34

The immunochemical properties of synthetic peptides were studied in a radioimmunoassay system according to published methods using an antiserum previously shown to be specific for the 4–9 region of G34 (*1, 11*). Synthetic porcine G34 and its NH_2-terminal nonadecapeptide were virtually equipotent with natural porcine G34 in inhibiting the binding of ^{125}I-labeled porcine G34 to antiserum (Fig. 2). Thus the synthetic porcine G34 prepared in this study according to the revised sequence (1) is indistinguishable from the natural porcine peptide in its immunochemical properties, whereas the peptide prepared according to the earlier reported sequence (2) was found to be 1000 times less active. On the basis of these results we conclude that the properties of the newly prepared porcine G34 are compatible with the revised but not the original sequence.

EXPERIMENTAL

Melting points are reported uncorrected. Thin-layer chromatograms (silica gel,

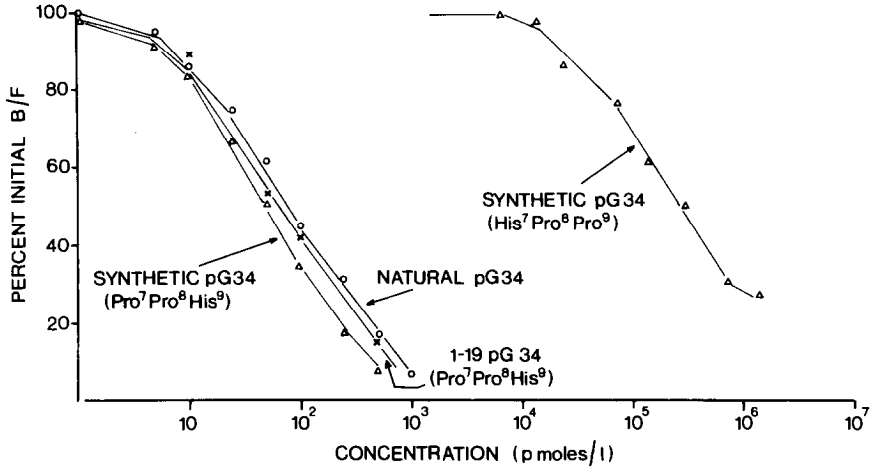


FIG. 2. Inhibition of binding of ^{125}I-labeled natural porcine G34 to antiserum L33 (1:3000) by addition of graded concentrations of unlabeled synthetic porcine G34 prepared in the present study with the revised sequence (Pro^7, Pro^8, His^9) and prepared earlier with the originally reported sequence (His^7, Pro^8, Pro^9), compared with natural porcine G34 and the synthetic NH_2-terminal nonadecapeptide of G34 with the revised structure. Natural porcine G34 was iodinated by the chloramine-T technique, and incubations were performed at 4°C for 48 hr in 2.0 ml sodium barbitone buffer, pH 8.4, 0.02 *M*. Inhibition of binding is expressed as percentage of the ratio of antibody bound to free labeled peptide in the absence of competing concentrations of unlabeled peptide. Antibody bound and free label were separated by the addition of 20 mg of dextran-coated charcoal and centrifuging (2000 g, 10 min).

Merck) were developed with the solvent systems 1, acetonitrile–water (9:1); 2, chloroform–methanol (6:1); 3, *n*-butanol–pyridine–acetic acid–water (60:20:6:24); 4, chloroform–i-propanol (6:1); 5, *n*-butanol–acetic acid–water (3:1:1); 6, ethyl acetate–pyridine–acetic acid–water (120:20:60:11); 7, chloroform–methanol–33% ammonia (19:17:3); 8, chloroform–methanol–acetic acid–water (60:18:2:3); 9, chloroform–methanol (9:1); 10, chloroform–i-propanol (3:1); 11, chloroform–methanol (4:1); 12, *n*-butanol–pyridine–acetic acid–water (70:6:20:6); 13, acetic acid-i-propanol–acetic acid–water (3:1:1:1); 14, *n*-butanol–pyridine–acetic acid–water (3:1:1:1); 15, i-propanol–acetic acid–water (5:1:1); 16, i-butanol–3% ammonia (3:7); 17, amyl alcohol-pyridine-water (35:35:30); 18, ethyl acetate–pyridine–acetic acid–water (60:20:6:11); 19, chloroform–trifluoroethanol (2:1); 20, chloroform–methanol–trifluoroethanol (45:5:10); 21, chloroform–methanol (7:3); 22, chloroform–i-propanol (7:3); 23, chloroform–i-propanol (7:1); 24, chloroform–i-propanol (4:1); 25, *n*-butanol–pyridine–formic acid–water (44:24:2:20). For amino acid analysis, samples were hydrolyzed with redistilled 6 *M* hydrochloric acid for 18 hr and analyzed on a Jeol JLC-5AH instrument. Peptides after gel filtration on Sephadex LH20 were detected by monitoring the optical rotation of the solution using a NPL automatic polarimeter and also the uv absorption at 280 nm using an LKB Uvicord II. Optical rotations were measured using a Bendix-Ericcson ETL-NPL instrument.

*N-t-Butyloxycarbonyl-*L*-tryptophanyl–*L*-methionine* (**5**). L-Methionine (1.86 g, 12.46 m*M*) was dissolved in water (10 ml)/DMF (60 ml), triethylamine (1.74 ml) added, and the solution cooled to 0°C. *N*-t-Butyloxycarbonyl-L-tryptophan-*N*-hydroxysuccinimide ester (5 g, 12.46 m*M*) was added and the reaction stirred at room temperature for 48 hr. The solution was evaporated and the residue taken up in 5% $NaHCO_3$ solution (200 ml) and the solution washed with ethyl acetate (2 × 100 ml). The pH of the solution was adjusted to approximately 2 with solid citric acid and the solution extracted with ethyl acetate (3 × 100 ml). The combined organic phase was washed neutral in the usual manner, dried, and evaporated to an oily residue. This residue was reprecipitated from ethyl acetate by the addition of petroleum ether (60–80°C).

Yield: 3.85 g, 71%; mp 74–75°C; $[\alpha]_D^{24}$ −13.6° (*c* 1, DMF); TLC-21, R_f 0.75; TLC-5, R_f 0.91. Found: C,57.42; H,6.84; N,9.77. $C_{21}H_{29}O_3N_3S$ requires C,57.91; H,6.71; N,9.65.

*N-1-(4-Biphenylyl)-1-methylethoxycarbonyl-*L*-prolyl–*L*-tryptophanyl–*L*-methionine* (**6**). Protected dipeptide (**5**) (6.75 g, 15.5 m*M*) was dissolved in 90% aqueous TFA (25 ml) containing anisole (1.5 ml) and 2-mercaptoethanol (1.5 ml) and the reaction stirred for 90 min at room temperature. The mixture was evaporated and the residual oil dissolved in propan-2-ol and poured into vigorously stirred anhydrous diethyl ether (2 liters).The precipitate was collected by filtration and dried under vacuum.

Yield: 5.9 g, 86%; TLC-8, R_f 0.32, TLC-3, R_f 0.68.

The above dipeptide trifluoroacetate salt (2.24 g, 5.00 m*M*) was dissolved in DMF (40 ml) and cooled to 0°C. *N*-1-(4-Biphenylyl)-1-methylethoxycarbonyl-L-proline-*N*-hydroxysuccinimide ester (2.25 g, 5.00 m*M*) and triethylamine (1.40 ml, 10.00 m*M*) were added and the reaction stirred at 0°C for 72 hr. The solution was

concentrated to approximately 10 ml and applied to a Sephadex LH20 column. The desired material eluted at a V_e/V_t value of 0.48. Finally the tripeptide was reprecipitated from ethyl acetate by the addition of petroleum ether (60–80°C).

Yield: 1.91 g, 57%, mp 66°C; $[\alpha]_D^{24}$ −2.4° (*c* 0.25, DMF); TLC-8, R_f 0.72. Amino acid analysis: $Pro_{1.02}Trp_{0.97}Met_{1.01}$. Found: C,64.04; H,6.76; N,8.63. $C_{37}H_{42}N_4O_6 \cdot H_2O$ requires C,64.51; H,6.44; N,8.16.

*N-1-(4-Biphenylyl)-1-methylethoxycarbonyl-*L*-prolyl-*L*-tryptophanyl-*L*-methionyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-*L*-analyl-O-t-butyl-*L*-tyrosylglycine phenyl ester* (**8**, *R = Ph*). Protected octapeptide (**7**) (0.91 g, 0.61 m*M*) was dissolved in DMF (30 ml) and hydrogenated in the presence of *p*-toluenesulfonic acid (0.12 *g*, 0.61 m*M*) over 10% palladium on charcoal (100 mg) overnight at room temperature and pressure. After filtration the filtrate was evaporated and the residue dissolved in DMF (2 ml) and cooled to 0°C. Protected tripeptide (**6**) (0.41 g, 0.61 m*M*) was dissolved in DMF (1.5 ml) and cooled to 0°C. *N*-Hydroxysuccinimide (0.14 g, 1.21 m*M*) and DCCI (0.15 g, 0.72 m*M*) were added and the reaction stirred for 4 min. The precooled solution of amino component was added followed by NMM (70 μl) and the reaction stirred for 48 hr at room temperature. The solution was applied to a Sephadex LH20 column and the desired material eluted at a V_e/V_t value of 0.38. Finally the product was reprecipitated from DMF by the addition of dry diethyl ether.

Yield: 660 mg, 52%; mp 248–250°C; $[\alpha]_D^{24}$ − 18.5° (*c* 1, DMF); TLC-5, R_f 0.79; TLC-3, R_f 0.73; Amino acid analysis: $Pro_{0.96}Trp_{0.96}Met_{0.96}Glu_{4.96}Ala_{1.03}Tyr_{1.10}Gly_{0.99}$. Found: C,62.43; H,7.57; N,8.30. $C_{106}H_{146}N_{12}O_{25}S \cdot 1\ H_2O$ requires C,62.46; H,7.32; N,8.25.

*N-1-(4-Biphenylyl)-1-methylethoxycarbonyl-*L*-prolyl-*L*-tryptophanyl-*L*-methionyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-*L*-alanyl-O-t-butyl-*L*-tyrosyl-glycine* (**8**, *R = H*). The undecapeptide phenyl ester (**8**, R = Ph) (350 mg, 0.18 m*M*) was dissolved in 90% aqueous trifluoroethanol (5 ml). DMS (0.4 ml) was added and the pH adjusted to 10.2 with 1 *M* NaOH, H_2O_2 (100 vol, 10 μl) was added and the reaction stirred for 30 min when a further aliquot of H_2O_2 (10 μl) was added. After 30 min the pH was adjusted to 4.5 with 10% citric acid solution and the free acid completely precipitated by the addition of water. The precipitate was collected by filtration and washed with a large volume of water then dried.

Yield: 280 mg, 82%; mp 248°C; $[\alpha]_D^{24}$ −45.6° (*c* 0.25, DMF); TLC-5, R_f 0.80; TLC-6, R_f 0.60. Found: C,59.37; H,7.32; N,8.64. $C_{100}H_{142}N_{12}O_{25}\ S \cdot 3\ H_2O$ requires C,60.10; H,7.47; N,8.41.

*N-1-(4-Biphenylyl)-1-methylethoxycarbonyl-*L*-prolyl-*L*-tryptophanyl-*L*-methionyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-γ-t-butyl-*L*-glutamyl-*L*-alanyl-O-t-butyl-*L*-tyrosylglycyl-*L*-tryptophanyl-*L*-methionyl-β-t-butyl-*L*-aspartyl-*L*-phenylalanine amide* (**4**). The preceding undecapeptide (**8**, R = H) (270 mg, 0.14 m*M*) was dissolved in DMF (3.0 ml) and cooled to 0°C. HOBt (37.5 mg, 0.28 m*M*) and DCCI (34.5 mg, 0.17

m*M*) were added and the reaction stirred for 10 min. The C-terminal tetrapeptide amide free base (**9**) (92 mg, 0.14 m*M*) was added and the reaction stirred for 72 hr. The reaction was diluted with HMPA, filtered, and loaded onto a Sephadex LH20 column. The desired material eluted at a V_e/V_t value of 0.37 and was precipitated from DMF by the addition of dry diethyl ether.

Yield: 245 mg, 68%; mp 250–254°C; $[\alpha]_D^{22}$ −58.4° (*c* 0.5, DMF); TLC-5, R_f 0.91; TLC-8, R_f 0.66. Amino acid analysis (*13*): $Asp_{1.02}Glu_{5.05}Pro_{1.06}Gly_{1.11}Ala_{1.10}Met_{2.05}Tyr_{1.11}Phe_{1.00}Trp_{1.94}$. Found: C,60.71; H,7.47; N,9.62. $C_{133}H_{184}N_{18}O_{30}S_2 \cdot 4\ H_2O$ requires C,60.25; H,7.30; N,9.51.

L - *Prolyl* - L - *tryptophanyl* – L - *methionyl* – γ - *t* - *butyl* - L - *glutamyl* – γ - *t* - *butyl* - L - *glutamyl* – γ - *t* - *butyl* - L - *glutamyl* – γ - *t* - *butyl* - L - *glutamyl* – L - *alanyl* – *O* - *t* - *butyl* - L - *tyrosylglycyl* – L - *tryptophanyl* – L - *methionyl* – β - *t* - *butyl* - L - *aspartyl* – L - *phenyl alanine amide hydrochloride*. The pentadecapeptide (**4**) (95 mg, 0.04 m*M*) was dissolved in 90% aqueous trifluoroethanol (2 ml) and DMS added (0.1 ml). The solution was taken to pH 0.5 by the pH stat controlled addition of 0.05 *M* HCl in 90% aqueous trifluoroethanol. After 2 hr acid consumption had ceased. The solution was evaporated, triturated under dry diethyl ether, filtered, and dried.

Yield: 73 mg, 78%; TLC-5, R_f 0.10; TLC-19, R_f 0.28; TLC-20, R_f 0.34.

This material was used directly in the subsequent acylation.

L - Pyroglutamyl - L - *leucylglycyl* – L - *leucyl* – L - *glutaminylglycyl* – L - *prolyl* – L - *prolyl* – L - *histidyl* – L - *leucyl* – L - *valyl* – L - *alanyl* – L - *aspartyl* – L - *leucyl* – L - *alanyl* – L - *lysyl* – L - *lysyl* – L - *glutaminylglycyl* – L - *prolyl* – L - *tryptophanyl* – L - *methionyl* – L - *glutamyl* – L - *glutamyl* – L - *glutamyl* – L - *glutamyl* – L - *glutamyl* – L - *alanyl* – L - *tyrosylglycyl* – L - *tryptophanyl* – L - *methionyl* – L - *aspartyl* – L - *phenylalanine amide* (*1*). The nonadecapeptide free acid (**3**) (93 mg, 39.5 μ*M*) and the above pentadecapeptide hydrochloride (93 mg, 39.5 μ*M*) were dissolved in a mixture (1 : 1) of HMPA and NMP (1.0 ml) and cooled to 0°C. HONSu (9.1 mg, 79.1 μ*M*) and DCCI (8.1 mg, 39.5 μ*M*) were added followed by NMM (47.6 μl, 39.6 μ*M*) and the reaction mixture stirred for 48 hr. After cooling to 0°C, further aliquots of HONSu (4.5 mg) and DCCI (4.0 mg) were added and the reaction allowed to continue for another 48 hr. Diluted with DMF (2 ml) the reaction mixture was loaded onto a column of LH20/DMF. The material eluting at a V_e/V_t value of 0.33 was collected, evaporated to 0.5 ml and the product precipitated with ethyl acetate, washed well with ethyl acetate, and dried under high vacuum.

Yield: 30 mg; 16.2%; TLC-3, R_f 0.80. Amino acid analysis: $Lys_{1.88}His_{0.88}Asp_{2.00}Glu_{8.15}Gly_{4.11}Ala_{3.00}Val_{0.96}Met_{1.80}Leu_{3.92}Tyr_{1.07}Phe_{1.00}Pro_{3.11}Trp_{0.50}$.

The protected tetratriacontapeptide (23 mg, 4.80 μ*M*) was treated with 90% aqueous TFA (4.0 ml) in the presence of 1,2-ethanedithiol (22.5 μl). The solution was kept in the dark under nitrogen for 4.5 hr, then excess diethyl ether was added and the precipitated product was separated by centrifugation, washed with diethyl ether, and dried under high vacuum. This solid was dissolved in 0.4% NH_4HCO_3 and the solution applied to a column of G50 (2.5 × 55 cm) and eluted with the same buffer. The desired material was eluted at a volume of 129.5 ml and the solvent removed by careful evaporation under vacuum.

TLC-25, R_f 0.21. Amino acid analysis: $Lys_{1.87}His_{0.92}Asp_{1.84}Glu_{8.20}Pro_{3.36}Gly_{4.20}Ala_{3.04}Val_{0.96}Met_{1.76}Leu_{4.00}Tyr_{0.96}Phe_{0.96}$.

The material was further purified by gradient elution from aminoethyl-cellulose. A column (1 × 10 cm) was prepared from material precycled as recommended by the makers and equilibrated overnight at 4°C with the starting buffer which was 0.05 *M* triethylamine (redistilled) which had been gassed with CO_2 to constant pH 6.3–6.4 at room temperature. The sample was dissolved in 0.025 *M* triethylamine and the solution gassed to constant pH (6.0–6.1) at room temperature. The conductivity was measured to confirm that it was lower than the starting buffer. The sample was then applied to the column, followed by starting buffer pumped overnight at 8 ml/hr through a magnetically stirred closing mixing flask volume 350 ml, monitoring the effluent at 280 m and 206 nm using a LKB Uvicord III spectrophotometer.

The reservoir was then changed to 0.5 *M* triethylamine which had been gassed at room temperature with CO_2 to constant pH (7.3–7.4) and fractions collected at 10-min intervals. A major peak, biologically active, emerged in a position characteristic for porcine BG in this system between fractions 84–102; two minor peaks, constituting not more than 10% of the major peak, emerged respectively before and after the latter. The fractions comprising the major peak were pooled. The BG content was calculated from the absorption at 280 nm and the molar extinction coefficient (12261).

Amino acid analysis of this material gave: $Lys_{2.23}His_{1.01}Asp_{1.95}Glu_{7.71}Pro_{3.31}Gly_{3.95}Ala_{3.13}Val_{0.99}Met_{1.77}Leu_{3.98}Tyr_{0.98}Phe_{1.00}$.

The preparation was also compared with natural porcine BG by hplc, using a column 250 × 4 mm packed with Whatman Partisil ODS 10 through which was pumped at 1.7 ml/min and approximately 100 bars a gradient of 10–80% (v/v) methanol containing 0.01 *M* ammonium acetate, pH 7.0. Synthetic porcine BG (100 μg) was mixed with natural porcine BGI (100 μg) and applied to the column (stopped-flow septum injection). The effluent was recorded at 280 nm with a Cecil 212 UV monitor. The mixture of synthetic and natural BG emerged at a position in the gradient corresponding to approximately 40% MeOH as a single symmetrical peak, and no other components of significance were observed.

L - *Pyroglutamyl* - L - *leucylglycyl* – L - *leucyl* – L - *glutaminylglycyl* – L - *histidyl* – L - *prolyl* – L - *prolyl* – L - *leucyl* – L - *valyl* – L - *alanyl* – L - *aspartyl* – L - *leucyl* – L - *alanyl* – L - *lysyl* – L - *lysyl* – L - *glutaminylglycyl* – L - *prolyl* – L - *tryptophanyl* – L - *methionyl* – L - *glutamyl* – L - *glutamyl* – L - *glutamyl* – L - *glutamyl* – L - *glutamyl* – L - *alanyl* – L - *tyrosylglycyl* – L - *tryptophanyl* – L - *methionyl*–L-*aspartyl*–L-*phenylalanine amide* (**2**). The coupling reaction was effected using the same conditions stated for the above method from the [7-His,9-Pro] analog of (**3**) (93 mg) and the pentadecapeptide hydrochloride (93 mg) derived from (**4**). The protected tetracontapeptide (65 mg, 35%) was isolated using LH20/DMF gel filtration.

Amino acid analysis: $Lys_{2.11}His_{1.01}Asp_{2.00}Glu_{8.24}Pro_{3.50}Gly_{3.66}Ala_{2.84}Val_{0.79}Met_{2.19}Leu_{3.48}Tyr_{1.10}Phe_{1.0}$.

The protected peptide (41 mg) was deprotected as in the preparation of (**1**). The crude product was first purified by chromatography on Sephadex G50 (0.4%

NH_4HCO_3) then the relevant fractions lyophilized to give material which was then subjected to AE-cellulose chromatography eluting with a gradient of 0.05 to 0.5 *M* triethylammonium carbonate at a rate of 12.0 ml/hr. The major peak was lyophilized to give (2) (12 mg, 37%).

Amino acid analysis: $Lys_{2.00}His_{0.88}Asp_{2.15}Glu_{8.12}Pro_{2.61}Gly_{3.94}Ala_{3.46}Val_{1.08}Met_{1.90}Leu_{3.82}Tyr_{1.03}Phe_{1.05}$.

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