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N-Terminal Sequence of Porcine Big Gastrin: Sequence, Synthesis, and Immunochemical Studies

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The synthesis of fragments corresponding to the *N*-terminal region of porcine big gastrin is described. Radioimmunoassay using synthetic peptides supports the revised structure for the hormone.

INTRODUCTION

After the isolation (*1*) of porcine big gastrin in sulfated and unsulfated form, the sequence (**1**) was assigned (*2*) to the unsulfated peptide. Continuing our interest in the synthesis of gastrointestinal hormones (*3*) we decided to verify the structure (**1**) by synthesis using a strategy which would provide *N*-terminal fragments suitable for immunochemical studies (*4*). This decision was vindicated in an unexpected and instructive way. Although the natural and synthetic pG34 peptides[3] were found to have equal immunoreactivity with *C*-terminal specific antisera, the synthetic pG34 exhibited drastically reduced immunochemical potency compared with natural porcine big gastrin with an antiserum raised to the natural peptide and specific for the *N*-terminal section of the molecule. Tryptic cleavage of natural pG34 afforded an *N*-terminal fragment having the same potency as intact natural pG34 in inhibiting the binding of ^{125}I-labeled natural pG34 to an antiserum raised against natural pG34 and specific to the *N*-terminal sequence. The error in the assigned sequence (**1**) could be placed in the 1–19 region since the synthetic peptide (**1**, R = OH) did not bind to the antibody.

In order to localize the error further the *N*-terminal dodecapeptide (**3**) was tested and found to have low immunoreactivity. The dodecapeptide (**4**), corresponding to the revised structure (**2**) for pG34, was found to have full immunoreactivity compared with the *N*-terminal tryptic fragment of natural pG34. The deprotected synthetic peptides

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Glp-Leu-Gly-Leu-Gln-Gly-His-Pro-Pro-Leu-Val-Ala-Asp-Leu-Ala-Lys-Lys-Gln-Gly-R (1)
-----------------------Pro-----His---------------------------------------- (2)

R = Pro-Trp-Met-[Glu]5-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH2
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FIG. 1

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[3] Synthesis of this series will be reported in a subsequent publication.

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corresponding to fragments (**10**), (**12**), (**5**), and (**6**)[3] exhibited low immunoreactivity and, in particular, comparison of (**12**) with (**4**) shows the selectivity of the *N*-terminal specific antiserum for the 7–12 region of pG34. In support of the revised structure (**2**) for pG34 the synthetic peptide (**2**, R = OH) exhibited full immunoreactivity with the *N*-terminal specific antisera.

TABLE 1
IMMUNOCHEMICAL POTENCY AND PEPTIDE STRUCTURE

Peptide	Immunochemical potency[a] ($\times 10^3$)
pG34	
Natural	1000
Trypsinized	780
H–Leu–Gly–Leu–Gln–Gly–OH	Nil
(**12**)	Nil
[4-Pro] (**12**) (*3*)	Nil
(**3**)	1.5
(**4**)	730
(**5**)	0.3
(**6**)[b]	Nil
(**1**, R = OH)	3.4
(**2**, R = OH)	1000

[a] Immunochemical potency (*4*) = molar ratios relative to natural pG34ns in inhibiting binding of ^{125}I-pG34 to L33. (Nil represents a value <0.1.)

[b] Synthesis of this series will be reported in a subsequent publication.

Thus the combination of synthesis and immunochemical methods (Table 1) supports the revised structure (**2**) for natural porcine big gastrin.

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Glp-Leu-Gly-Leu-Gln-Gly-His-Pro-Pro-Leu-Val-Ala-OH   (3)
-----------------------Pro-----His---------------   (4)
-------------------------------Ser---------------   (5)
-----------Pro-------------------------------------   (6)
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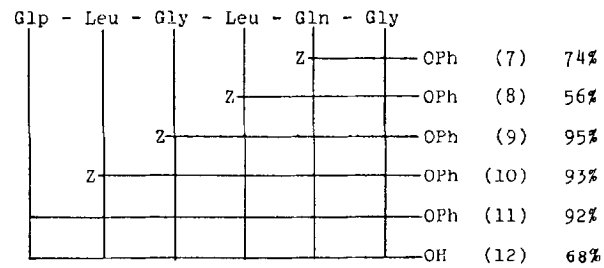
FIG. 2

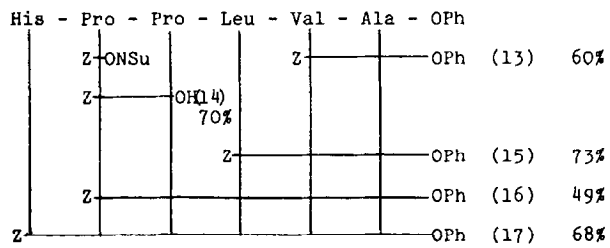
DISCUSSION OF SYNTHETIC METHODS

The 1–6 fragment was synthesized by the standard stepwise strategy starting from H–Gly–OPh using pivalic mixed anhydride couplings until the penultimate Z–LeuONSu and final Glp–OPcp stages as shown in Scheme 1. The intermediate Z-peptides were cleaved by hydrogenolysis (H_2/Pd) prior to coupling.

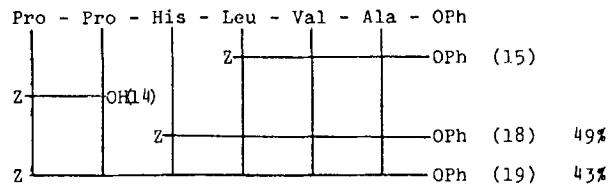
The 7–12 fragments Z–His–Pro–Pro–Leu–Val–Ala–OPh (**17**, Scheme 2a) and Z–Pro–Pro–His–Leu–Val–Ala–OPh (**19**, Scheme 2b) were synthesized from H–Ala–OPh using pivalic mixed anhydride coupling to give Z–Leu–Val–Ala–OPh (**15**). Both proline residues were added as Z–Pro–Pro–OH (**14**) and Z–His–N_3 was used to add the histidine residue.



SCHEME 1

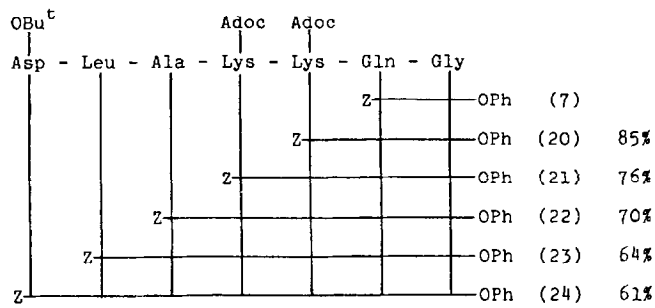


SCHEME 2a



SCHEME 2b

The 13–19 fragment was constructed (Scheme 3) by the pivalic mixed anhydride method until the tetrapeptide stage; then the DCCl/HONSu method (*5*) was employed to add the alanine unit. The final two residues were added as the *N*-hydroxysuccinimide active esters.



SCHEME 3

For immunological studies the dodecapeptides (**3**), (**4**), (**5**), and (**6**)[3] were synthesized from the corresponding hexapeptide acids (**12**) and the [4-Pro] analog (*3*) by the DCCl/HOBt method (*6*) of coupling with the amines corresponding to (**17**),(**19**), and the [3-Ser(Bu^t)] analog (*3*) of (**17**). Cleavage of the intermediate phenyl ester (*7*) function to give the dodecapeptides was effected at pH 10.5 followed by purification by gel filtration on LH20/DMF (*8*). In the case of (**5**) a further deprotection using trifluoroacetic acid was required to cleave the t-butyl ether.

The peptides (**1**, R = OH) and (**2**, R = OH), encompassing the entire sequence of the *N*-terminal tryptic fragment, were synthesized by coupling the peptide acids (**3**) and (**4**) with the amino component derived from removal of the Z-protection of (**24**). After purification of the phenyl esters (**1**, R = OPh) and (**2**, R = OPh) using LH20/DMF gel filtration, the esters were hydrolyzed at pH 10.5 followed by final deprotection using trifluoroacetic acid.

EXPERIMENTAL

Melting points are reported uncorrected. Thin-layer chromatograms (silica gel, Merck) were developed with the following 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). 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 uv absorption at 280 nm using an LKB Uvicord II. Optical rotations were measured using a Bendix–Ericcson ETL-NPL instrument.

N-Benzyloxycarbonyl-L-glutaminylglycine phenyl ester (*7*). *N*-Benzyloxycarbonyl-L-glutamine (49.8 g, 170 m*M*) was dissolved in DMF[4] (340 ml) and cooled to −15°C. NMM (19.08 ml) was added followed by pivaloyl chloride (19.88 ml, 163 m*M*) and the reaction stirred at −10°C for 20 min. A precooled solution of glycine phenyl ester hydrobromide (33 g, 142 m*M*) in DMF (280 ml) was added followed by NMM (15.9 ml). The reaction was stirred overnight at room temperature and evaporated, and the residue triturated in water. After filtration the crude dipeptide derivative was washed neutral in the usual manner and dried. Yield: 52 g, 74%; mp 182°C; tlc-1, R_f 0.69; $[\alpha]_D^{27}$ − 16.75° (c = 1, DMF); amino acid analysis $Glu_{1.03}Gly_{0.97}$.

Anal. Calcd for $C_{21}H_{23}N_3O_6$: C, 61.01; H, 5.61; N, 10.16. Found: C, 61.22; H, 5.60; N, 10.36.

[4] Abbreviations used: DMF, dimethylformamide; NMM, *N*-methylmorpholine; DCCI, dicyclohexylcarbodiimide; DMS, methylsulfide; TFA, trifluoroacetic acid.

N-Benzyloxycarbonyl-L-leucyl-L-glutaminylglycine phenyl ester (**8**). Protected dipeptide (**7**) (8.26 g, 20 m*M*) was dissolved in DMF (80 ml) and hydrogenated in the presence of *p*-toluenesulfonic acid (3.8 g, 20 m*M*) over 10% palladium on charcoal (1.5 g) at room temperature and pressure overnight. After filtration the filtrate was cooled to −10°C.

N-Benzyloxycarbonyl-L-leucine (9.58 g, 36 m*M*) was dissolved in CH_2Cl_2 (180 ml) and cooled to −20°C. NMM (3.96 ml, 36 m*M*) and pivaloyl chloride (4.25 ml, 34.5 m*M*) were added and the reaction mixture was stirred for 20 min. After this time the precooled solution of $Tos^-H_2^+Gln$–Gly–OPh was added followed by NMM (2.2 ml, 20 m*M*). The reaction was stirred overnight at room temperature and then the solvent evaporated to give an oil which was triturated under 5% $NaHCO_3$ when a white solid was produced. The product was filtered and washed neutral in the usual manner, then dried. Yield: 5.8 g, 56%; mp 193–196°C; $[\alpha]_D^{27}$ − 17.9° (*c* = 1, DMF); tlc-2, R_f 0.59; tlc-3, R_f0.86; amino acid analysis $Glu_{0.98}Gly_{1.02}Leu_{1.00}$.

Anal. Calcd for $C_{27}H_{34}N_4O_7$: C, 61.58; H, 6.51; N, 10.64. Found: C, 61.15; H, 6.51; N, 10.30.

N-Benzyloxycarbonyl-glycyl-L-leucyl-L-glutaminylglycine phenyl ester (**9**). Protected tripeptide (**8**) (5.93 g, 11.2 m*M*) was dissolved in DMF (60 ml) and hydrogenated in the presence of *p*-toluenesulfonic acid (2.15 g, 11.2 m*M*) over 10% palladium on charcoal (0.75 g) at room temperature and pressure overnight. After filtration the solution was cooled to −20°C.

N-Benzyloxycarbonyl-glycine (4.23 g, 20.25 m*M*) in CH_2Cl_2 was cooled to −20°C. NMM (2.23 ml, 20.25 m*M*) and pivaloyl chloride (2.4 ml, 19.5 m*M*) were added and the reaction was stirred for 20 min. After this time the above pre-cooled solution of $Tos^-H_2^+Leu$–Glu–Gly–OPh was added followed by NMM (1.35 ml, 11.2 m*M*) and the solution stirred overnight at room temperature. The solution was evaporated and the residue triturated under 5% sodium bicarbonate, filtered, and washed neutral in the usual manner, then dried. Yield: 5.95 g, 95%; $[\alpha]_D^{25}$ − 22.9° (*c* = 1, DMF); mp 188–191°C; tlc-2, R_f 0.55; tlc-4, R_f 0.17; amino acid analysis $Glu_{0.98}Leu_{1.02}Gly_{2.00}$.

Anal. Calcd for $C_{29}H_{37}N_5O_8$: C, 58.97; H, 6.32; N, 11.86. Found: C, 58.96; H, 6.31; N, 11.76.

N-Benzyloxycarbonyl-L-leucylglycyl-L-leucyl-L-glutaminylglycine phenyl ester (**10**). Protected tetrapeptide (**9**) (5.83 g, 10 m*M*) was dissolved in DMF (50 ml) and hydrogenated in the presence of *p*-toluenesulfonic acid (1.9 g, 10 m*M*) over 10% palladium on charcoal (0.7 g) at room temperature and pressure overnight. After filtration the solution was cooled to 0°C.

N-Benzyloxycarbonyl-L-leucine-*N*-hydroxysuccinimide ester (*9*) (3.98 g, 11 m*M*) was added to the above solution followed by NMM (1.1 ml, 10 m*M*) and the reaction mixture stirred for 1 hr at 0°C. After this time the solution was allowed to warm to room temperature and stirred for 48 hr. After evaporation of solvent the residue was triturated with water, washed neutral, and dried. Finally the product was reprecipitated from DMF by the addition of Et_2O. Yield: 6.5 g, 93%; mp 222–225°C; $[\alpha]_D^{25}$ − 24.3° (*c* = 1, DMF); tlc-2, R_f0.64; tlc-3, R_f0.84; amino acid analysis $Leu_{1.00}Glu_{2.00}Gly_{2.00}$.

Anal. Calcd for $C_{35}H_{48}N_6O_9 \cdot 0.5H_2O$: C, 59.56; H, 7.00; N, 11.91. Found: C, 59.68; H, 7.40; N, 11.61.

L-Pyroglutamyl-L-leucylglycyl-L-leucyl-L-glutaminylglycine phenyl ester (*11*). Protected pentapeptide (**10**) (0.35 g, 0.5 m*M*) was dissolved in DMF (10 ml) and hydrogenated in the presence of *p*-toluenesulfonic acid (85 mg, 0.5 m*M*) over 10% palladium on charcoal (40 mg) at room temperature and pressure overnight. After filtration the filtrate was cooled to 0°C.

L-Pyroglutamic acid pentachlorophenyl ester (198 mg, 0.525 m*M*) was added to the above solution followed by 1-hydroxybenzotriazole (*10*) (67.5 mg) and NMM (55 μl). The reaction was stirred overnight at room temperature. The reaction mixture was concentrated to approximately 3 ml and a large excess of diethyl ether added. After filtration the white solid was washed thoroughly with Et_2O, then petroleum ether (40–60°C), and finally dried. Yield: 0·31 g, 92%; mp 215–218°C; $[\alpha]_D^{22}$ − 21.3° (c = 1, DMF); tlc-2, R_f 0.57; tlc-5, R_f 0.70; amino acid analysis $Glu_{1.03}Gly_{0.95}Leu_{1.02}$.

Anal. Calcd for $C_{32}H_{47}N_7O_9 \cdot 1H_2O$: C, 55.57; H, 7.09; N, 14.18. Found: C, 55.21; H, 6.78; N, 13.93.

L-Pyroglutamyl-L-leucylglycyl-L-leucyl-L-glutaminylglycine (*12*). The hexapeptide phenyl ester (**11**) (3.1 g, 4.60 m*M*) was dissolved in 85% aqueous DMF (70 ml) and DMS (15.2 ml). The pH was adjusted to 10.5 with 1 *M* NaOH and H_2O_2 (100 vol, 0.65 ml) added. A pH of 10.5 was maintained during controlled addition of 1 *M* NaOH. After 1 hr an additional aliquot of H_2O_2 (0.3 ml) was added and the reaction stirred for a further 30 min. HCL, 1 *M*, was added until a pH of 6.5 was obtained. The solution was evaporated to a small volume, MeOH (200 ml) added, and the solid material filtered after prolonged stirring and washed with Et_2O. Yield: 1.5 g, 68%; mp 225–229°C (dec.); $[\alpha]_D^{25}$ − 17.4° (c = 1, DMF); tlc-5, R_f 0.49; tlc-6, R_f 0.10; amino acid analysis $Glu_{2.02}Gly_{2.00}Leu_{1.98}$.

Anal. Calcd for $C_{26}H_{43}N_7O_9 \cdot 0.5H_2O$: C, 51.49; H, 7.26; N, 16.16. Found: C, 51.46; H, 7.66; N, 16.14.

N-Benzyloxycarbonyl-L-valyl-L-alanine phenyl ester (*13*). *N*-Benzyloxycarbonyl-L-valine (30.10 g, 120 m*M*) was dissolved in freshly distilled CH_2Cl_2 (250 ml) and triethylamine (16.80 ml, 120 m*M*)) added. The solution was cooled to −15°C, pivaloyl chloride added (13.86 ml, 115 m*M*), and the reaction stirred for 20 min at −10°C.

A precooled solution of L-alanine phenyl ester *p*-toluenesulfonate (33.7 g, 100 m*M*) in DMF (250 ml) was added followed by triethylamine (14.0 ml. 100 m*M*). The reaction mixture was stirred overnight at room temperature. After evaporation of the solvent the residue was triturated with 5% $NaHCO_3$, filtered, and washed neutral in the usual manner, then dried and recrystallized from ethyl acetate–petroleum ether (60–80°C). Yield: 24.0 g, 60%; mp 173–174°C; $[\alpha]_D^{30}$ − 42.1° (c = 1, DMF); tlc-7, R_f 0.90; tlc-8, R_f 0.86; amino acid analysis $Val_{1.04}Ala_{0.96}$.

Anal. Calcd for $C_{22}H_{26}N_2O_5$: C, 66.33; H, 6.53; N, 7.03. Found: C, 66.43; H, 6.44; N, 7.07.

N-Benzyloxycarbonyl-L-prolyl-L-proline (*14*). L-Proline (3.7 g, 32 m*M*) was dissolved in water (20 ml)/DMF (120 ml). The solution was cooled to 0°C and triethylamine added (4.5 ml), followed by *N*-benzyloxycarbonyl-L-proline-*N*-hydroxysuccinimide ester (10.0 g, 32 m*M*), and the solution was stirred at room temperature for 36 hr. *N,N*-Dimethylaminopropylamine (1.0 ml) was added and the reaction stirred for 2 hr. The solution was evaporated and treated with 5% $NaHCO_3$ solution (120 ml). This aqueous solution was washed with diethyl ether, the pH adjusted to 2 with concen-

trated hydrochloric acid, and the solution extracted with ethyl acetate (3 × 100 ml). The combined extracts were washed with brine, dried, and evaporated. The residue was crystallized from ethyl acetate–petroleum ether (60–80°C). Yield: 8.9 g, 80%; mp 187°C (*11*); $[\alpha]_D$ − 65.3° (c = 1, DMF); tlc-2, R_f 0.33; tlc-8, R_f 0.53.

Anal. Calcd for $C_{18}H_{22}N_2O_5 \cdot 0.5H_2O$: C, 60.86; H, 6.53; N, 7.89. Found: C, 60.74; H, 6.23; N, 7.76.

N-Benzyloxycarbonyl-L-leucyl-L-valyl-L-alanine phenyl ester (*15*). Protected dipeptide (14.0 g, 35.1 m*M*) was hydrogenated in DMF (100 ml) in the presence of *p*-toluenesulfonic acid (6.69 g), over 10% palladium on charcoal (1.0 g) at room temperature and pressure overnight. After filtration the filtrate was concentrated to a volume of approximately 60 ml.

N-Benzyloxycarbonyl-L-leucine (12.72 g, 47.4 m*M*) was dissolved in CH_2Cl_2 (100 ml) and cooled to −15°C. NMM (5.31 ml) was added, followed by pivaloyl chloride (5.69 ml), and the reaction stirred at −15°C for 20 min. The precooled solution of the dipeptide *p*-toluenesulfonate was added followed by NMM (3.93 ml). After stirring overnight at room temperature the reaction mixture was concentrated, then poured into water. The resulting crude tripeptide derivative was filtered and the solid washed neutral in the usual manner, then dried. Yield: 13.1 g, 73%; mp 186°C; $[\alpha]_D^{26}$ − 45.1° (c = 1, DMF); tlc-4, R_f 0.75; tlc-8, R_f 0.81; amino acid analysis $Leu_{1.02}Val_{0.99}Ala_{0.99}$.

Anal. Calcd for $C_{28}H_{37}N_3O_6$: C, 65.75; H, 7.24; N, 8.22. Found: C, 65.77; H, 7.40; N, 8.35.

N-Benzyloxycarbonyl-L-prolyl-L-prolyl-L-leucyl-L-valyl-L-alanine phenyl ester (*16*). Protected tripeptide (**15**) (1.0 g, 1.96 m*M*) was dissolved in DMF (25 ml) and hydrogenated in the presence of *p*-toluenesulfonic acid (0.37 g, 1.96 m*M*) over 10% palladium on charcoal (150 mg) at room temperature and pressure overnight. After filtration the filtrate was evaporated.

N-Benzyloxycarbonyl-L-prolyl-L-proline (0.68 g, 1.96 m*M*) was dissolved in DMF (5 ml) and the solution cooled to 0°C. 1-Hydroxybenzotriazole (0.53 g, 3.92 m*M*) and DCCI (0.48 g, 2.35 m*M*) were added and the solution was stirred at 0°C for 10 min. The *p*-toluenesulfonate produced by the above hydrogenation was dissolved in DMF (4 ml) and added to the above solution, followed by NMM (0.22 ml), and the reaction was stirred at room temperature for 48 hr. The solution was filtered and applied to a Sephadex LH20 column. The desired material eluted at a *Ve*/*Vt* value of 0.49. Yield: 0.67 g, 49%; mp 142–143°C; $[\alpha]_D^{26}$ − 80.2° (c = 1, DMF); tlc-2, R_f 0.86; tlc-8, R_f 0.89; amino acid analysis $Pro_{1.96}Leu_{1.03}Val_{1.00}Ala_{1.01}$.

Anal. Calcd for $C_{38}H_{51}N_5O_8 \cdot 2.5H_2O$: C, 63.48; H, 7.85; N, 9.74. Found: C, 63.62; H, 7.39; N, 9.90.

N-Benzyloxycarbonyl-L-histidyl-L-prolyl-L-prolyl-L-leucyl-L-valyl-L-alanine phenyl ester (*17*). Protected pentapeptide (**16**) (1.0 g, 1.41 m*M*) was dissolved in DMF (10 ml) and hydrogenated in the presence of *p*-toluenesulfonic acid (0.27 g, 1.41 m*M*) over 10% palladium on charcoal (100 mg) at room temperature and pressure overnight. After filtration the filtrate was concentrated to approximately 3 ml and cooled to −15°C.

N-Benzyloxycarbonyl-L-histidine hydrazide (0.98 g, 32 m*M*) was suspended in DMF (10 ml) and cooled to −20°C; then 3.8 *M* HCl in dioxane (2.28 ml, 8.7 m*M*) was added and the reaction allowed to warm to −15°C. t-Butyl nitrite (0.48 ml) was added

and the reaction stirred for 10 min at −10°C. The solution of *p*-toluenesulfonate was added, followed by ethyldiisopropylamine (1.14 ml, 10.31 m*M*), and the reaction was stirred for 3.5 days at 0–4°C. After filtration the filtrate was applied to a Sephadex LH20 column. The desired material eluted at a *Ve/Vt* value of 0.49. The solvent was evaporated and the residue taken up in ethyl acetate. The solution was extracted with 2 *M* citric acid, the citric acid solution neutralized to pH 6.5 with solid $NaHCO_3$, and the separated oil taken up in n-butanol. The *n*-butanol solution was washed with brine, dried, and evaporated, and the residue was triturated with dry diethyl ether, then filtered. Yield: 0.8 g, 68%; mp 138–140°C; $[\alpha]_D^{26}$ − 65.1° (*c* = 1, DMF); tlc-4, R_f 0.31; tlc-5, R_f 0.74; amino acid analysis $His_{0.99}\ Pro_{0.02}Ala_{0.94}Val_{1.01}Leu_{1.04}$.

Anal. Calcd for $C_{44}H_{58}N_8O_9 \cdot 2H_2O$: C, 60.12; H, 7.11; N, 12.75. Found: C, 59.48; H, 7.35; N, 13.39.

N-Benzyloxycarbonyl-L-hystidyl-L-leucyl-L-valyl-L-alanine phenyl ester (**18**). The protected tripeptide (**15**) (1.0 g, 2.0 m*M*) was dissolved in DMF (20 ml) and hydrogenated in the presence of *p*-toluenesulfonic acid (0.38 g, 2.0 m*M*) over 10% palladium on charcoal (0.10 g) at room temperature overnight. After filtration the filtrate was evaporated *in vacuo* and the residue dissolved in $CHCl_3$ (15 ml), then cooled to −5°C.

N-Benzyloxycarbonyl-L-histidine hydrazide (0.60 g, 2 m*M*) was dissolved in 0.24 *N* HCl (20 ml) and cooled to 0°C. To this solution was added $NaNO_2$ (0.25 g, 3.60 m*M*) and the reaction mixture was stirred for 5 min at 0°C followed by addition of the above $CHCl_3$ solution of the amino component and Et_3N (0.55 ml, 4.0 m*M*). After stirring the mixture at 0°C for 2 hr $CHCl_3$ (50 ml) was added. An emulsion formed which was passed through Celite and the $CHCl_3$ layer was separated. The Celite was washed with DMF and the combined organic phase concentrated to 10 ml. This solution was subjected to gel filtration using LH20/DMF. The material *Ve/Vt* = 0.51 was obtained by evaporation of the DMF followed by trituration with EtOAc. Yield: 0.64 g, 49%; $[\alpha]_D^{26}$ − 52.40° (*c* = 1, DMF); tlc-9, R_f 0.40; tlc-10, R_f 0.37; amino acid analysis $His_{0.96}Ala_{1.04}Val_{1.03}Leu_{0.95}$.

Anal. Calcd for $C_{34}H_{44}N_6O_7 \cdot H_2O$: C, 61.26; H, 6.90; N, 12.61. Found: C, 60.94; H, 6.62; N, 12.48.

Carbobenzyloxy-L-prolyl-L-prolyl-L-histidyl-L-leucyl-L-valyl-L-alanine phenyl ester (**19**). The protected tetrapeptide (**18**) (1.0 g, 1.54 m*M*) was dissolved in DMF (20 ml) and hydrogenated in the presence of *p*-toluenesulfonic acid (0.58 g, 3.08 m*M*) over 10% palladium on charcoal at room temperature and pressure overnight. After filtration the filtrate was concentrated to 10 ml.

The carbobenzyloxydipeptide acid (**14**) (0.60 g, 1.74 m*M*) was dissolved in DMF (12 ml) and HONSu (0.20 g, 1.74 m*M*) added. After cooling the mixture to 0°C, DCCI (0.35 g, 1.74 m*M*) was added and stirred for 10 min when the above solution of the tetrapeptide component was added followed by NMM (0.34 ml, 3.08 m*M*). The reaction mixture was stirred at 0°C for 2 hr, then at room temperature for 72 hr, whereupon the urea was filtered and the filtrate submitted to gel filtration using LH20/DMF. The product eluted at *Ve/Vt* = 0.47 and was isolated by evaporation of DMF and trituration with Et_2O. Yield: 0.60 g, 42.8%; mp 112–116°C; $[\alpha]_D^{26}$ − 82.9° (*c* = 0.55, DMF); tlc-10, R_f 0.25; tlc-11, R_f 0.52; amino acid analysis $His_{1.00}Pro_{1.93}Ala_{0.97}Val_{1.06}Leu_{1.06}$.

Anal. Calcd for $C_{44}H_{58}N_8O_9 \cdot H_2O$: C, 61.39; H, 6.97; N, 13.02. Found: C, 61.49; H, 7.09; N, 13.27.

L-Pyroglutamyl-L-leucylglycyl-L-leucyl-L-glutaminylglycl-L-prolyl-L-prolyl-L-histidyl-L-leucyl-L-valyl-L-alanine (**4**). The protected hexapeptide (**19**) (0.50 g, 0.61 m*M*) in DMF (20 ml) was hydrogenated in the presence of *p*-toluenesulfonic acid (0.23 g, 1.22 m*M*) over 10% palladium on charcoal (50 mg) at room temperature and pressure overnight. After filtration the filtrate was reduced to 3 ml.

The hexapeptide acid (**12**) (0.36 g, 0.61 m*M*) and 1-hydroxybenzotriazole (0.09 g, 0.67 m*M*) were dissolved in DMF (10 ml) and cooled to 0°C, then DCCI (0.14 g, 0.67 m*M*) was added. After stirring for 10 min the above solution of amino component was added to the reaction mixture followed by NMM (0.13 ml, 1.22 m*M*). The reaction was stirred at 0°C for 2 hr, then for 72 hr at room temperature, then filtered to give a filtrate which was subjected to gel filtration on LH20/DMF. The phenyl ester of (**4**) *Ve/Vt* = 0.41 was obtained by evaporation of DMF followed by trituration with Et_2O. Yield: 0.30 g, 38%; tlc-12, R_f 0.47; tlc-13, R_f 0.51; amino acid analysis $His_{0.96}Glu_{2.02}Pro_{2.17}Gly_{1.95}Ala_{0.97}Val_{0.89}Leu_{3.11}$.

Anal. Calcd for $C_{61}H_{91}N_{15}O_{15} \cdot 3H_2O$: C, 55.16; H, 7.30; N, 15.85. Found: C, 55.06; H, 7.52; N, 16.07.

The phenyl ester (0.30 g, 0.23 m*M*) was dissolved in 90% aqueous DMF (5 ml) containing DMS (0.87 ml, 11.9 m*M*). After adjusting the pH to 10.5, 0.4 *M* NaOH and 100 vol H_2O_2 (23 μl, 0.23 m*M*) were added. The reaction mixture was maintained at pH 10.5 throughout the hydrolysis and after 0.5 hr more H_2O_2 (12.5 μl) was added. After 1 hr tlc examination showed the reaction to be completed. The solution was acidified to pH 6.5 with 0.1 *M* HCl and the solvent removed *in vacuo* to give a residue which was subjected to gel filtration using LH20/DMF. The dodecapeptide free acid *Ve/Vt* = 0.40 was isolated in the usual manner and found to be very hygroscopic. Yield: 0.24 g, 86%; $[\alpha]_D^{26}$ – 58.4° (*c* = 0.5, DMF); tlc-13, R_f 0.26; tlc-14, R_f 0.27; amino acid analysis $His_{0.98}Glu_{2.08}Pro_{2.23}Gly_{2.01}Ala_{0.98}Val_{0.89}Leu_{2.85}$.

Anal. Calcd for $C_{55}H_{87}N_{15}O_{15} \cdot 6H_2O$: requires: C, 50.57; H, 7.58; N, 16.09. Found: C, 50.39; H, 8.10; N, 16.76.

L-Pyroglutamyl-L-leucylglycyl-L-leucyl-L-glutaminylglycyl-L-histidyl-L-prolyl-L-prolyl-L-leucyl-L-valyl-L-alanine (**3**). The dodecapeptide (**3**) was synthesized by the method described in detail for (**4**) from the protected hexapeptide (**17**) (0.60 g, 1.47 m*M*) and the hexapeptide (**12**) (0.46 g, 1.54 m*M*). The intermediate phenyl ester was isolated by LH20/DMF gel filtration at *Ve/Vt* = 0.41. Yield: 807 mg, 43%; mp 184–186°C; $[\alpha]_D^{23}$ – 60.6° (*c* = 1, DMF); tlc-5, R_f 0.53; amino acid analysis $His_{1.00}Glu_{2.07}Pro_{2.04}Gly_{2.00}Ala_{0.99}Val_{0.96}Leu_{2.96}$. Hydrolysis of the phenyl ester was achieved at pH 10.5 using 1 *M* $NaOH/H_2O_2$ as described for (**4**) to give the required dodecapeptide (**3**). Yield: 98 mg, 95%; mp 184–188°C; $[\alpha]_D^{22}$ – 44.5° (*c* = 1, DMF); tlc-5, R_f 0.67; amino acid analysis $His_{1.02}Glu_{1.98}Pro_{2.00}Gly_{2.03}Ala_{1.01}Val_{0.98}Leu_{2.98}$.

L-Pyroglutamyl-L-leucylglycyl-L-leucyl-L-glutaminylglycyl-L-histidyl-L-prolyl-L-seryl-L-leucyl-L-valyl-L-alanine (**5**). The dodecapeptide (**5**) was synthesized by the method described in detail for (**4**) from the [3-Ser(Bu^t)] analog of (**17**) (0.180 g, 0.21 m*M*) and the hexapeptide (**12**) (0.126 g, 0.21 m*M*). The intermediate phenyl ester was isolated by LH20/DMF gel filtration at *Ve/Vt* = 0.41. Yield: 182 mg, 68%; amino acid analysis

$His_{1.00}Ser_{0.89}Glu_{1.98}Pro_{1.29}Gly_{2.00}Ala_{1.03}Val_{1.01}Leu_{2.98}$. Hydrolysis of the phenyl ester was achieved at pH 10.5 as described for (**4**) to give the corresponding acid as a white solid (85 mg, 85%) after LH20/DMF purification. This material (42 mg), tlc-8, R_f 0.41, was treated with 90% TFA (3.5 ml) in the presence of anisole (100 mg) and mercaptoethanol (100 mg) at room temperature for 3 hr. Evaporation of the solvent *in vacuo* followed by Et_2O treatment gave the desired dodecapeptide (**5**) as a white solid (38 mg). Amino acid analysis $His_{0.97}Glu_{1.98}Pro_{1.00}Gly_{1.97}Ala_{1.07}Val_{1.06}Leu_{2.89}Ser_{0.87}$.

N-Benzyloxycarbonyl-N$^{\epsilon}$-adamantyloxycarbonyl-L-lysyl-L-glutaminylglycine phenyl ester (*20*). *N*-Benzyloxycarbonyl-L-glutaminylglycine phenyl ester (12.0 g, 24.2 m*M*) was dissolved in DMF (75 ml) and hydrogenated in the presence of *p*-toluenesulfonic acid (4.6 g, 24.2 m*M*) over 10% palladium on charcoal (1.0 g) at room temperature and pressure overnight. After filtration the filtrate was evaporated to give the *p*-toluenesulfonate salt.

N-Benzyloxycarbonyl-N$^{\epsilon}$-admantyloxycarbonyl-L-lysine (16.68 g, 36.3 m*M*) was dissolved in CH_2Cl_2 (4.0 ml)/DMF (4 ml) and cooled to −15°C. NMM (4.07 ml) and pivaloyl chloride (4.32 ml, 35.1 m*M*) were added and the reaction was stirred for 20 min at −10°C. A precooled solution of *p*-toluenesulfonate in DMF (50 ml) was added followed by NMM (2.71 ml) and the reaction stirred overnight at room temperature. The solvent was evaporated and the residue triturated under 5% $NaHCO_3$, then filtered. The solid material was washed neutral in the usual manner and dried. Yield: 14.85 g, 85%; mp 180°C; $[\alpha]_D^{26}$ − 14.1° (*c* = 1, DMF); tlc-5, R_f 0.72; tlc-3, R_f 0.87; amino acid analysis $Lys_{1.00}Glu_{1.00}Gly_{1.01}$.

Anal. Calcd for $C_{38}H_{49}N_5O_9$: C, 63.40; H, 6.86; N, 9.73. Found: C, 63,65; H, 6.84; N, 9.93.

N-Benzyloxycarbonyl-N$^{\epsilon}$-adamantyloxycarbonyl-L-lysyl-N$^{\epsilon}$-adamantyloxycarbonyl-L-lysyl-L-glutaminylglycine phenyl ester (*21*). Protected tripeptide (14.4 g, 19.98 m*M*) was dissolved in DMF (60 ml) and hydrogenated in the presence of *p*-toluenesulfonic acid (3.8 g) over 10% palladium on charcoal (1.0 g) at room temperature and pressure overnight. After filtration the filtrate was concentrated to approximately 40 ml.

N-Benzyloxycarbonyl-N$^{\epsilon}$-adamantyloxycarbonyl-L-lysine (13.73 g, 29.97 m*M*) was dissolved in CH_2Cl_2 (50 ml) and cooled to −15°C. NMM (3.36 ml) was added followed by pivaloyl chloride (3.54 ml) and the solution stirred at −10°C for 20 min. The above precooled solution of *p*-toluenesulfonate was added followed by NMM (2.24 ml) and the reaction was stirred overnight at room temperature. The solvent was evaporated *in vacuo* and the residue triturated under 5% $NaHCO_3$, filtered, washed neutral in the usual manner, and dried. Yield: 15.5 g, 76%, mp 208°C; $[\alpha]_D^{27}$ − 16.0° (*c* = 1, DMF); tlc-3, R_f 0.92; tlc-5, R_f 0.83; amino acid analysis $Lys_{1.94}Glu_{1.03}Gly_{1.03}$.

Anal. Calcd for $C_{55}H_{74}N_7O_{12} \cdot 0.5H_2O$: C, 63.93; H, 7.31; N, 9.49. Found: C, 63.66; H, 7.50; N, 9.86.

N-Benzyloxycarbonyl-L-alanyl-N$^{\epsilon}$-adamantyloxycarbonyl-L-lysyl-N$^{\epsilon}$-adamantyloxycarbonyl-L-lysyl-L-glutaminylglycine phenyl ester (*22*). Protected tetrapeptide (**21**) (5.0 g, 4.87 m*M*) was dissolved in DMF (75 ml) and hydrogenated in the presence of *p*-toluenesulfonic acid (0.93 g, 4.87 m*M*) over 10% palladium on charcoal (200 mg) at room temperature and pressure for 7 hr. After filtration the filtrate was evaporated and the residue dissolved in DMF (20 ml) and cooled to 0°C. *N*-Benzyloxycarbonyl-L-alanine (1.09 g, 4.88 m*M*), *N*-hydroxysuccinimide (1.34 g, 11.7 m*M*), and DCCI (1.21 g, 5.86

m*M*) were added followed by NMM (0.55 ml). The reaction was stirred for 48 hr at room temperature. After filtration the filtrate was poured into water (1 liter) and extracted with EtOAc (3 × 200 ml). The organic phase was washed neutral in the usual manner, dried, and evaporated, and the residue was recrystallized from EtOAc–petroleum ether (60–80°C). Yield: 3.75 g, 70%; mp 190–192°C; $[\alpha]_D^{26} - 10.4°$ ($c = 1$, DMF); tlc-5, R_f 0.69; tlc-8, R_f 0.59; amino acid analysis $Lys_{1.89}Glu_{1.01}Gly_{1.02}Ala_{1.07}$.

Anal. Calcd for $C_{58}H_{80}N_8O_{13} \cdot 1H_2O$: C, 62.46; H, 7.41; N, 10.05. Found: C, 62.64; H, 7.61; N, 10.38.

N-Benzyloxycarbonyl-L-leucyl-L-alanyl-N$^\epsilon$-adamantyloxycarbonyl-L-lysyl-N$^\epsilon$-adamantyloxycarbonyl-L-lysyl-L-glutaminyl-glycine phenyl ester (*23*). Protected pentapeptide (**22**) (4.5 g, 4.1 m*M*) was dissolved in DMF (75 ml) and hydrogenated in the presence of *p*-toluenesulfonic acid (0.75 g, 4.1 m*M*) over 10% palladium on charcoal (200 mg) at room temperature and pressure overnight. After filtration the filtrate was evaporated and the residue taken up in DMF (10 ml) and cooled to 0°C. *N*-Benzyloxycarbonyl-L-leucine-*N*-hydroxysuccinimide ester (1.49 g, 4.1 m*M*) was added followed by NMM (0.44 ml) and the reaction stirred for 48 hr at room temperature. The solution was loaded directly onto Sephadex LH20 and the desired material was eluted at *Ve/Vt* = 0.46. Finally the product was reprecipitated from DMF by the addition of Et_2O. Yield: 3.2 g, 64%; mp 228–230°C; $[\alpha]_D^{22} - 14.8°$ ($c = 1$, DMF); tlc-3, R_f 0.89; tlc-8, R_f 0.83; amino acid analysis $Leu_{1.03}Ala_{0.98}Lys_{1.94}Glu_{1.03}Gly_{1.02}$.

Anal. Calcd for $C_{64}H_{91}N_9O_{14} \cdot 1.5H_2O$: C, 62.11; H, 7.66; N, 10.19. Found: C, 61.90; H, 7.90; N, 10.57.

N-Benzyloxycarbonyl-β-t-butyl-L-aspartyl-L-leucyl-L-alanyl-N$^\epsilon$-adamantyloxycarbonyl-L-lysyl-N$^\epsilon$-adamantyloxycarbonyl-L-lysyl-L-glutaminylglycine phenyl ester (**24**). Protected hexapeptide (**23**) (2.7 g, 2.23 m*M*) was dissolved in DMF (45 ml) and hydrogenated in the presence of *p*-toluenesulfonic acid (0.42 g, 2.23 m*M*) over 10% palladium on charcoal (150 mg) at room temperature and pressure. After filtration the filtrate was evaporated and the residue taken up in DMF (10 ml) and cooled to 0°C. *N*-Benzyloxycarbonyl-β-t-butyl-L-aspartic acid-*N*-hydroxysuccinimide ester (*12*) (1.17 g, 2.78 m*M*) was added followed by NMM (0.25 ml) and the reaction stirred for 72 hr at room temperature. The solution was loaded directly onto a Sephadex LH20 column and the desired material eluted at a *Ve/Vt* value of 0.39. Finally the product was reprecipitated from DMF by the addition of Et_2O. Yield: 1.88 g, 61%; mp 234–236°C; $[\alpha]_D^{23} = 16.6°$ ($c = 1$, DMF); tlc-8, R_f 0.94; tlc-5, R_f 0.38; amino acid analysis $Asp_{0.94}Leu_{1.06}Ala_{0.98}Lys_{2.05}Glu_{0.99}Gly_{1.02}$.

Anal. Calcd for $C_{72}H_{104}N_{10}O_{17} \cdot 1H_2O$: C, 61.78; H, 7.63; N, 10.01. Found: C, 61.46; H, 7.77; N, 10.16.

L-Pyroglutamyl-L-leucylglycyl-L-leucyl-L-glutaminylglycyl-L-prolyl-L-prolyl-L-histidyl-L-leucyl-L-valyl-L-alanyl-β-t-butyl-L-aspartyl-L-leucyl-L-alanyl-N$^\epsilon$-adamantyloxycarbonyl-L-lysyl-N$^\epsilon$-adamantyloxycarbonyl-L-lysyl-L-glutaminylglycine (**2**, *R = OH*). The protected heptapeptide (**24**) (0.217 g, 0.15 m*M*) in DMF (15 ml) was hydrogenated in the presence of *p*-toluenesulfonic acid (29.6 mg, 0.15 m*M*) and 10% palladium on charcoal (21 mg). After filtration the filtrate was concentrated to 3 ml and cooled to 0°C.

The dodecapeptide acid (**4**) (0.188 g, 0.15 m*M*) was dissolved in DMF (5 ml) and 1-hydroxybenzotriazole (23.0 mg, 0.17 m*M*) was added. After cooling the solution to 0°C,

DCCI (35.0 mg, 0.17 m*M*) was added, then stirring continued for 5 min, whereupon the above cooled solution of amino component was added followed by NMM (17.1 μl, 0.15 m*M*). After stirring at 0°C for 2 hr the reaction was stirred at room temperature for 72 hr, then filtered. The filtrate was subjected to gel filtration using LH20/DMF to afford the protected phenyl ester *Ve/Vt* = 0.37. Yield: 192 mg, 50.5%; mp 205°C (d); $[\alpha]_D^{26}$ − 46.4° (*c* = 1, DMF); tlc-3, R_f 0.75; tlc-15, R_f 0.48; amino acid hydrolysis $Lys_{2.00}His_{1.02}Asp_{1.00}Glu_{3.04}Pro_{2.26}Gly_{2.94}Ala_{1.97}Val_{0.97}Leu_{3.93}$.

Anal. Calcd for $C_{119}H_{183}N_{25}O_{29}\cdot 3H_2O$: C, 57.60; H, 7.62; N, 14.11. Found: C, 57.89; H, 7.99; N, 14.25.

The protected phenyl ester (100 mg, 0.04 m*M*) in 90% aqueous DMF (3 ml) containing DMS (0.15 ml, 2.03 m*M*) was hydrolyzed with 0.1 *M* NaOH at a constant pH of 10.5 in the presence of 100 vols H_2O_2 (4.1 μl, 0.04 m*M*). After 45 min an additional aliquot of H_2O_2 (2 μl, 0.02 m*M*) was added and the hydrolysis was found to be complete after 1.5 hr. The pH was adjusted to 6.5 with 10% citric acid and the solution evaporated *in vacuo* whereupon the residue was purified by LH20/DMF gel filtration to give the protected peptide acid *Ve/Vt* = 0.38 after evaporation of the eluant and trituration with EtOAc. The solid product was washed with i-propanol and dried *in vacuo*. Yield: 77 mg, 97%; mp > 250°C(d); $[\alpha]_D^{26}$ − 36.2° (*c* = 0.5, DMF); amino acid analysis $Lys_{1.93}His_{1.00}Asp_{1.03}Glu_{3.10}Pro_{1.17}Gly_{3.06}Ala_{1.96}Val_{0.93}Leu_{4.20}$.

Anal. Calcd for $C_{113}H_{179}N_{25}O_{29}\cdot 5H_2O$: C, 55.93; H, 7.82; N, 14.10. Found: C, 56.17; H, 7.80; N, 14.08.

Deprotection to give (*2, R = OH*). The above protected peptide acid (20 mg, 8.4 μ*M*), in a centrifuge tube containing anisole (0.41 ml, 4.16 m*M*) and mercaptoethanol (0.41 ml, 5.76 m*M*) under a N_2 atmosphere, was treated with 90% aqueous TFA (4.0 ml). After 3.5 hr anhydrous Et_2O was added and the precipitated material separated by centrifugation, washed with Et_2O, then dissolved in distilled H_2O (0.5 ml). This solution was applied to a column of CM-Sephadex CM50 equilibrated with 0.1 *M* NH_4OAc. The nonadecapeptide (**2**, R = OH) (4.0 mg) was eluted after a volume of 30 ml on a gradient of 0.1 to 0.5 *M* NH_4OAc using a 100-ml mixing vessel. Amino acid analysis $Lys_{1.86}His_{1.01}Asp_{1.00}Glu_{3.13}Pro_{1.98}Gly_{2.90}Ala_{0.93}Val_{0.93}Leu_{4.32}$. This material was submitted to Dr. G. J. Dockray for radioimmunoassay and found to be fully active with respect to porcine G34.

L-Pyroglutamyl-L-leucylglycyl-L-leucyl-L-glutaminylglycyl-L-histidyl-L-prolyl-L-prolyl-L-leucyl-L-valyl-L-alanyl-β-t-butyl-L-aspartyl-L-leucyl-L-alanyl-N$^{\epsilon}$-adamantyloxycarbonyl-L-lysyl-N$^{\epsilon}$-adamantyloxycarbonyl-L-lysyl-L-glutaminylglycine (*1, R = OH*). The nonadecapeptide (**1**, R = OH) was synthesized by the method described in detail for (**2**, R = OH) from the protected heptapeptide (**24**) (100 mg, 0.08 m*M*) and the dodecapeptide acid (**3**) (97 mg, 0.08 m*M*). The intermediate phenyl ester was isolated by LH20/DMF gel filtration at *Ve/Vt* = 0.36. Yield: 82 mg, 42%; mp 230°C; tlc-8, R_f 0.54; tlc-16, R_f 0.38; amino acid analysis $Glu_{3.11}Leu_{4.05}Gly_{3.31}His_{0.74}Pro_{1.90}Val_{0.98}$-$Ala_{2.00}Asp_{1.06}Lys_{1.88}$.

Hydrolysis of the phenyl ester (50 mg, 0.02 m*M*) at pH 10.5 in the presence of H_2O_2 (0.02 m*M*) and DMS (0.10 ml) followed by LH20/DMF gel filtration gave the desired protected peptide acid at *Ve/Vt* = 0.37. Yield: 26 mg, 53%; mp 218°C; tlc-8, R_f 0.35. This material was treated with 90% TFA (2 ml) in the presence of anisole (100 mg) and mercaptoethanol (100 mg). The desired nonadecapeptide (**1**, R = OH) was isolated by

the procedures described for (2, R = OH). Amino acid hydrolysis $Lys_{1.99}His_{0.90}$-$Asp_{1.09}Glu_{3.00}Pro_{2.03}Gly_{3.03}Ala_{1.91}Val_{1.03}Leu_{4.04}$.

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