Synthesis and Biological Activity of New Peptide Segments of Gastrin Exhibiting Gastrin Antagonist Property

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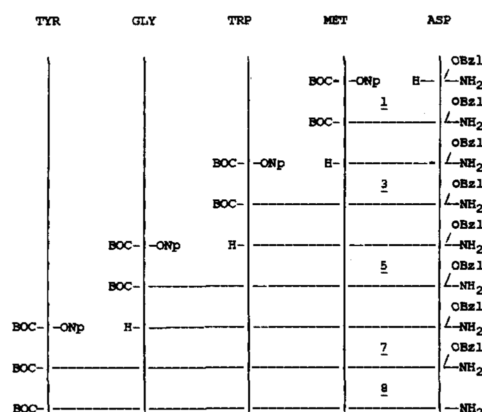
A series of C-terminal peptide segments of gastrin, i.e., (*tert*-butyloxycarbonyl)-L-tryptophyl-L-methionyl-L-aspartic acid amide, (*tert*-butyloxycarbonyl)-glycyl-L-tryptophyl-L-methionyl-L-aspartic acid amide, (*tert*-butyloxycarbonyl)-L-tyrosyl-glycyl-L-tryptophyl-L-methionyl-L-aspartic acid amide, and (benzyloxycarbonyl)-L-glutamyl-L-alanyl-L-tyrosyl-glycyl-L-tryptophyl-L-methionyl-L-aspartic acid amide were prepared and were shown to competitively inhibit the binding of labeled human gastrin to its receptors in an isolated gastric mucosal cell preparation and to antagonize the action of gastrin on gastric acid secretion (ED50 from 1.5 to 7 mg/kg) in vivo in the reperfused rat stomach, determined according to the method of Ghosh and Schild. From these studies, it could be concluded that the C-terminal phenylalanine residue, which is of primary importance for intrinsic biological gastrin-like activity, is not essential for binding to gastrin receptors.

The heptadecapeptide amides gastrin I and II (Figure 1) play an important role in the stimulation of gastric acid secretion. Their pharmacological mode of action, however, is still unknown. Some information concerning structure–activity relationships were available: two sites in the gastrin sequence are involved both in binding to the receptors and in biological activity. (1) The C-terminal tetrapeptide amide was extensively studied by Morley et al.,[1-3] who showed that tryptophan-14 and the C-terminal phenylalanine amide residue are capable of powerful interaction with the receptors. Formylation of the indole nucleus of tryptophan-14 dramatically decreases binding and acid secretion[4] as does the deletion of the C-terminal amide function.[5] The aspartic acid-16 residue, due to its electronic character,[3] is crucial in eliciting acid secretion. (2) The N-terminal extensions of the C-terminal tetrapeptide L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine amide up to the entire human gastrin (HG-17) sequence increases the acid secretory response,[6] in terms of potency and also in binding to the receptors.[7] The N-terminal pentaglutamic acid sequence plays an important role in this amplification. Similar observations were made in connection with cholecystokinin on pancreatic enzyme secretion.[8,9] Hence, we studied the role of the aspartic acid residue in position 32 of cholecystokinin-27–33 (CCK-27–33) and showed that certain replacements of this residue by β-alanine or by L-glutamic acid lead to the dissociation of cholecystokinic, pancreozyminic, and gastrinic activities.[10] These results permitted a rational approach in the design of inhibitors. We recently synthesized[11] the first known and most potent peptidic antagonist of the cholecystokinin receptor[12] (CCK-27–32-NH_2) in which the C-terminal phenylalanine residue of CCK-27–33 was deleted. This peptide CCK-27–32-NH_2, also inhibits gastrin-induced acid stimulation, in the rat in vivo.[13] Because of the similarity between the two hormones, we postulated that this deletion in the gastrin sequence should lead to gastrin receptor antagonists and inhibitors of gastric acid secretion. This paper presents the effects both on gastrin binding to gastric mucosal cells and on gastric acid secretion of various peptides obtained by deletion of the C-terminal phenylalanine residue from the natural sequence of gastrin.

Chemistry. The gastrin-inhibiting peptides **4**, **6**, and **8** were synthesized according to Scheme I. Dipeptide (*tert*-butyloxycarbonyl)-L-methionyl-β-benzyl-L-aspartic acid amide (**1**) was secured through the reaction of (*tert*-butyloxycarbonyl)-L-methionine *p*-nitrophenyl ester[23] with

Scheme I



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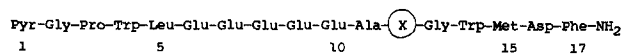
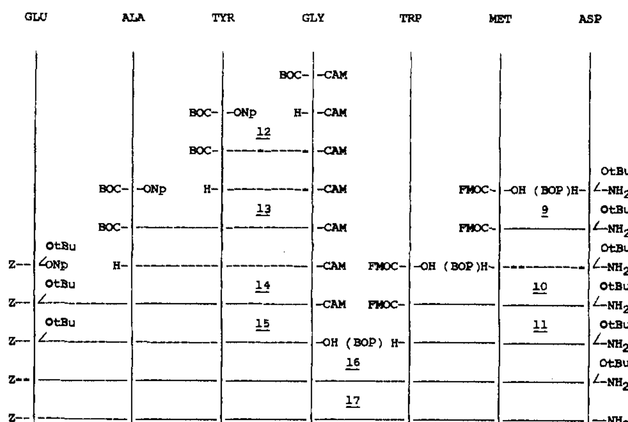


Figure 1. Amino acid sequence of gastrin I and II.

Scheme II



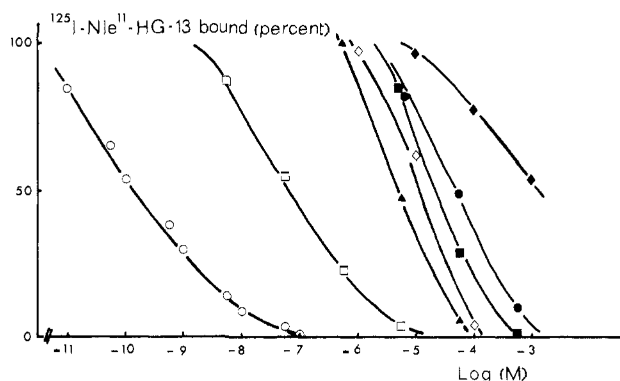


Figure 2. Displacement curves of the ^{125}I-(Nle^{11})-HG-13 binding to its cellular receptors by various gastrin analogues. ^{125}I-(Nle^{11})-HG-13 (20 pM) was incubated for 30 min at 37 °C with 5×10^6 cells/mL in the presence or absence of various peptides. After centrifugation, the radioactivity associated with the cell pellet was counted. Values obtained in the presence of various peptide concentrations were reported to the binding determined in the absence of cold peptide and expressed as percent. (○) (Nle^{11})-HG-13; (□) Boc-β-Ala-Trp-Met-Asp-Phe-NH_2 (pentagastrin); (▲) Z-Glu-Ala-Tyr-Gly-Trp-Met-Asp-NH_2 (17); (◇) Boc-Tyr-Gly-Trp-Met-Asp-NH_2 (8); (■) Boc-Gly-Trp-Met-Asp-NH_2 (6); (●) Boc-Trp-Met-Asp-NH_2 (4); (♦) Boc-Met-Asp-NH_2 (2).

the trifluoroacetate salt of β-benzyl-L-aspartic acid amide in the presence of 1-hydroxybenzotriazole.[17] Partial deprotection of 1 with trifluoroacetic acid and coupling with (*tert*-butyloxycarbonyl)-L-tryptophan *p*-nitrophenyl ester[15] yielded (*tert*-butyloxycarbonyl)-L-tryptophyl-L-methionyl-β-benzyl-L-aspartic acid amide (**3**). By treating **3** with trifluoroacetic acid and then with (*tert*-butyloxycarbonyl)glycine *p*-nitrophenyl ester[16] in the presence of 1-hydroxybenzotriazole,[17] we obtained (*tert*-butyloxycarbonyl)-glycyl-L-tryptophyl-L-methionyl-β-benzyl-L-aspartic acid amide (**5**). This peptide was partially deprotected with trifluoroacetic acid and acylated with (*tert*-butyloxycarbonyl)-L-tyrosine *p*-nitrophenyl ester[18] to yield (*tert*-butyloxycarbonyl)-L-tyrosyl-glycyl-L-tryptophyl-L-methionyl-β-benzyl-L-aspartic acid amide (**7**). Peptides **1**, **3**, **5**, and **7** were chromatographed on a silica gel column and hydrogenolyzed in a mixture of dimethylformamide, *N,N*-diisopropylethylamine, and water in the presence of 10% Pd/$BaSO_4$ as catalyst[19] to afford pure (*tert*-butyloxycarbonyl)-L-methionyl-L-aspartic acid amide (**2**), (*tert*-butyloxycarbonyl)-L-tryptophyl-L-methionyl-L-aspartic acid amide (**4**), (*tert*-butyloxycarbonyl)-glycyl-L-tryptophyl-L-methionyl-L-aspartic acid amide (**6**), and (*tert*-butyloxycarbonyl)-L-tyrosyl-glycyl-L-tryptophyl-L-methionyl-L-aspartic acid amide (**8**) representing N-acylated C-terminal segments of gastrin. Compounds **2**, **4**, **6**, and **8** were dissolved in 0.2 M NH_4OH and lyophilized. The synthesis of the peptide (benzyloxycarbonyl)-L-glutamyl-L-alanyl-L-tyrosyl-glycyl-L-tryptophyl-L-methionyl-L-aspartic acid amide (**17**) representing N-acylated C-terminal heptapeptide segment of gastrin without the phenylalanine residue was performed according to Scheme II by segment condensation.

The dipeptide [[(9-fluorenylmethyl)oxy]carbonyl]-L-methionyl-β-*tert*-butyl-L-aspartic acid amide (**9**) was obtained by the reaction of [[(9-fluorenylmethyl)oxy]carbonyl]-L-methionine with the hydrochloride salt of β-*tert*-butyl-L-aspartic acid amide in the presence of [(benzotriazolyl)oxy]tris(dimethylamino)phosphonium hexafluorophosphate.[14] Removal of the 9-[[(fluorenylmethyl)oxy]carbonyl] N-protecting group by a mixture of diethylamine–dimethylformamide (1:9) and acylation with 9-[[(fluorenylmethyl)oxy]carbonyl]-L-tryptophan *p*-nitrophenyl ester[20] yielded the tripeptide 9-[[(fluorenylmethyl)oxy]carbonyl]-L-tryptophyl-L-methionyl-β-*tert*-butyl-L-aspartic acid amide (**10**). Partial deprotection of **10** by a diethylamine–dimethylformamide mixture gave the tripeptide H-L-tryptophyl-L-methionyl-β-*tert*-butyl-L-aspartic acid amide (**11**). The second fragment was prepared from the trifluoroacetate salt of glycine carbamoylmethyl ester.[21,22] Acylation with (*tert*-butyloxycarbonyl)-L-tyrosine *p*-nitrophenyl ester[18] afforded (*tert*-butyloxycarbonyl)-L-tyrosyl-glycine carbamoylmethyl ester (**12**), which, after deprotection with trifluoroacetic acid and acylation with (*tert*-butyloxycarbonyl)-L-alanine *p*-nitrophenyl ester[16] yielded (*tert*-butyloxycarbonyl)-L-alanyl-L-tyrosyl-glycine carbamoylmethyl ester (**13**). Removal of the amine-protecting group and coupling with (benzyloxycarbonyl)-γ-*tert*-butyl-L-glutamic acid *p*-nitrophenyl ester[23] yielded (benzyloxycarbonyl)-γ-*tert*-butyl-L-glutamyl-L-alanyl-L-tyrosyl-glycine carbamoylmethyl ester (**14**). Compound **14** was then saponified with a slight excess of sodium carbonate in a mixture of dimethylformamide–water (in 1 h)[22] to afford (benzyloxycarbonyl)-γ-*tert*-butyl-L-glutamyl-L-alanyl-L-tryptophyl-glycine **15**. Coupling of the two peptides **11** and **15** with the aid of [(benzotriazolyl)oxy]tris(dimethylamino)phosphonium hexafluorophosphate as coupling reagent yielded (benzyloxycarbonyl)-γ-*tert*-butyl-L-glutamyl-L-alanyl-L-tyrosyl-glycyl-L-tryptophyl-L-methionyl-β-*tert*-butyl-L-aspartic acid amide (**16**), which was purified on a silica gel column. Final deprotection with trifluoroacetic acid containing anisole and thioanisole[24] afforded (benzyloxycarbonyl)-L-

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Table I. Antagonist in Vivo Activity on Reperfused Rat Stomach (Ghosh) and in Vitro Inhibition of Binding of ^{125}I-(Nle^{11})-HG-13 to Its Gastric Mucosal Cell Receptors by C-Terminal Desphenylalanine Fragments[a]

peptides	binding IC50, M	antagonist act.: ED50, mg/kg
Boc-L-Met-L-Asp-NH_2 (2)	2×10^{-3}	
Boc-L-Trp-L-Met-L-Asp-NH_2 (4)	5×10^{-5}	7.5
Boc-Gly-L-Trp-L-Met-L-Asp-NH_2 (6)	3×10^{-5}	5
Boc-L-Tyr-Gly-L-Trp-L-Met-L-Asp-NH_2 (8)	1.5×10^{-5}	2
Z-L-Glu-L-Ala-L-Tyr-Gly-L-Trp-L-Met-L-Asp-NH_2 (17)	5×10^{-6}	1.5

[a] These peptides did not show any agonist activity even at doses as high as 50 mg/kg.

glutamyl-L-alanyl-L-tyrosyl-glycyl-L-tryptophyl-L-methionyl-L-aspartic acid amide (17), representing the C-terminal sequence of gastrin without the phenylalanine residue. Compound 17 was dissolved in 0.2 M NH_4OH and lyophilized. Compounds 2, 4, 6, 8, and 17 were homogeneous by TLC and LC, gave the expected amino acid composition, and were identified by elemental analysis.

Biological Results and Discussion

Peptides 2, 4, 6, 8, and 17 were tested for their ability to inhibit gastrin-induced acid secretion. The effects on the biological activity of the shortening of the desphenylalanine–gastrin analogue sequences are shown in Table I and Figure 2. The "minimal fragment" with gastrin antagonist activity is the C-terminal tripeptide (*tert*-butyloxycarbonyl)-L-tryptophyl-L-methionyl-L-aspartic acid amide (4). The tryptophan residue seems to be of primary importance since the dipeptide (*tert*-butyloxycarbonyl)-L-methionyl-L-aspartic acid amide (2) was devoid of antagonist or agonist activities. Increasing the length of the peptide chain from the N-terminus gave analogues of higher antagonist activity: ED50 = 7.5 mg/kg for (*tert*-butyloxycarbonyl)-L-tryptophyl-L-methionyl-L-aspartic acid amide (4), ED50 = 5 mg/kg for (*tert*-butyloxycarbonyl)-glycyl-L-tryptophyl-L-methionyl-L-aspartic acid amide (6), ED50 = 2 mg/kg for (*tert*-butyloxycarbonyl)-L-tyrosyl-glycyl-L-tryptophyl-L-methionyl-L-aspartic acid amide (8), and ED50 = 1.5 mg/kg for (benzyloxycarbonyl)-L-glutamyl-L-alanyl-L-tyrosyl-glycyl-L-tryptophyl-L-methionyl-L-aspartic acid amide (17). The results of the inhibition of binding of labeled (norleucine-11)-human gastrin 13, (Nle^{11})-HG-13, by these peptides to cell surface gastrin receptors are shown in Figure 2. Displacement curves obtained with the peptides tested were parallel to that obtained with unlabeled (Nle^{11})-HG-13, suggesting a competitive inhibition. The half-maximal binding inhibition was determined for each peptide: IC50 = 5×10^{-5} M for 4, IC50 = 3×10^{-5} M for 6, IC50 = 1.5×10^{-5} M for 8, and IC50 = 5×10^{-6} M for 17. They are in accordance with in vivo inhibitory activities (Table II). These compounds were tested with respect to their agonist activity. None of them, even when tested at concentrations as high as 50 mg/kg, showed stimulation of acid secretion. In the present study, measuring the effects of gastrin antagonists on ^{125}I-(Nle^{11})-HG-13 binding on isolated gastric mucosal cells, we correlated receptor occupancy to regulation of biological event: inhibition of gastrin-induced acid secretion. The smallest active segment that causes inhibition of gastrin-stimulated acid secretion is the tri-

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Table II. Correlation between Receptor Occupancy and in Vivo Antagonist Activity for Different C-Terminal Desphenylalanine Gastrin Fragments

peptides	binding IC50, M	in vivo antagonist act.[a]	ratio act. binding
gastrin	2×10^{-10}		
Boc-L-Met-L-Asp-NH_2 (2)	2×10^{-3}		
Boc-L-Trp-L-Met-L-Asp-NH_2 (4)	5×10^{-5}	1.6×10^{-4}	3
Boc-Gly-L-Trp-L-Met-L-Asp-NH_2 (6)	3×10^{-5}	1×10^{-4}	3
Boc-L-Tyr-Gly-L-Trp-L-Met-L-Asp-NH_2 (8)	1.5×10^{-5}	3.1×10^{-5}	2
Z-L-Glu-L-Ala-L-Tyr-Gly-L-Trp-L-Met-L-Asp-NH_2 (17)	5×10^{-6}	1.8×10^{-5}	4

[a] In vivo antagonist activities were calculated from ED50 values shown in Table I, by estimating at 25 mL the mean blood volume in a 300-g rat and expressed as molarity.

peptide (*tert*-butyloxycarbonyl)-L-tryptophyl-L-methionyl-L-aspartic acid amide (4). Adding a glycine residue to the N-terminus does not affect its binding to gastrin receptors or its antagonist activity, suggesting, as already demonstrated for cholecystokinin,[25] that the glycine residue merely provides the proper spacing between the C-terminal tripeptide portion of the molecule and the remaining N-terminal portion of the peptide. Having in mind that sulfation of the tyrosine residue in gastrin does not modify agonist activity,[26] we added an *N*-acyltyrosine to the sequence. This increased to some extent the binding property to gastrin receptors and also the antagonist activity on gastrin-stimulated acid secretion, suggesting a significant but not essential role for the tyrosine residue. Increasing the length of the peptide chain by adding alanine and glutamic acid residues gave somewhat enhanced inhibition of binding of labeled (Nle^{11})-HG-13 to gastrin receptors, but the in vivo antagonist activity was improved to a lesser extent. These peptides are devoid of agonist activity and inhibit the interaction of gastrin with its cell surface receptors, causing a parallel rightward shift in the dose–response curve for the stimulation and for the binding of ^{125}I-(Nle^{11})-HG-13 to its receptors. In terms of their ability to inhibit binding of ^{125}I-(Nle^{11})-HG-13 to its cell surface receptors, removal of the phenylalanine residue from the C-terminal gastrin segments causes a 5000–50 000 decrease in the apparent affinity of the peptide to its receptors.

Conclusions

Our results indicate that C-terminal fragments of gastrin, without the phenylalanine amide residue, are competitive gastrin receptor antagonists. The present findings show that the C-terminal phenylalanine residue of gastrin is not essential for binding to cell surface gastrin receptors but is crucial for intrinsic biological gastrin-like activity. Previous studies have shown that omission of the C-terminal phenylalanine residue from C-terminal heptapeptide fragments of cholecystokinin leads to a new class of cholecystokinin receptor antagonists.[12] The peptide CCK-27-32-NH_2 was found to antagonize the action of gastrin on acid secretion.[13] Recent results[27] revealed that the N-terminal tridecapeptide fragment 1–13 of gastrin-17 inhibits gastric acid production induced by gastrin-17 or

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pentagastrin. The present study, along with previous reported results, contributes to the concept that fragments of the natural hormone can modulate the action of the parent hormone.

Experimental Section

Capillary melting points were determined on a Buchi apparatus and are reported uncorrected. Thin-layer chromatography (TLC) was carried out on Merck silica gel GF_{254} plates with the following solvent systems: solvent A, chloroform 7, hexane 3; B, ethyl acetate 8, hexane 2; C, ethyl acetate 9, methanol 1, acetic acid 1; D, ethyl acetate 9, methanol 1; E, ethyl acetate; F, ethyl acetate 98, methanol 2; G, ethyl acetate 25, methanol 1. Column chromatography was performed on Merck silica gel, 60–229 mesh, ASTM. Elemental analyses were provided by "Le Service Central de Microanalyse du CNRS" de Montpellier. ^{1}H NMR spectra were recorded by "le Service de RMN du Centre de Pharmacologie-Endocrinologie" de Montpellier on a 360-MHz Brucker instrument. Abreviations used were DMF, dimethylformamide; BOP, [(benzotriazolyl)oxy]tris(dimethylamino)phosphonium hexafluorophosphate; DIEA, *N,N*-diisopropylethylamine; CAM, carbamoylmethyl ester.

Synthesis of Peptides. (*tert*-Butyloxycarbonyl)-L-methionyl-β-benzyl-L-aspartic Acid Amide (1). To a solution of β-benzyl-L-aspartic acid amide trifluoroacetate (1.91 g, 5.7 mmol) in DMF (10 mL) cooled in an ice water bath were added (*tert*-butyloxycarbonyl)-L-methionine *p*-nitrophenyl ester[23] (2.07 g, 5.6 mmol), 1-hydroxybenzotriazole[17] (0.86 g, 5.6 mmol), and *N,N*-diisopropylethylamine (2.06 mL, 12 mmol). After 30 min, the solution was allowed to warm at room temperature and was stirred overnight. The solvent was removed in vacuo, and the residue was dissolved in ethyl acetate (200 mL), washed with a saturated sodium bicarbonate solution (2 × 60 mL), water (2 × 50 mL), a 10% citric acid solution (2 × 50 mL), and water (2 × 50 mL). The organic layer was dried over sodium sulfate and then concentrated in vacuo. The residue, triturated twice with a mixture of ethyl acetate/ether (1:9, v/v) and then with ether, gave a white powder: yield 85% (2.16 g); R_f (A) 0.4, R_f (B) 0.3; mp 109–111 °C; $[\alpha]_D$ −37.5° (c 1, DMF). Anal. ($C_{21}H_{31}N_3O_6S$) C, H, N.

(*tert*-Butyloxycarbonyl)-L-methionyl-L-aspartic Acid Amide (2). Compound 1 (0.500 g, 1.1 mmol) was hydrogenated in a mixture (25 mL) of DMF/water/*N,N*-diisopropylethylamine (10:2:1) in the presence of 10% $Pd/BaSO_4$ (50 mg) as previously described[19], during 12 h. The catalyst was filtered and the residue dissolved in a mixture of ethyl acetate (10 mL) and 5% ammonia solution (10 mL). The aqueous layer was acidified by solid citric acid, extracted twice with ethyl acetate (20 mL), dried over sodium sulfate, and concentrated in vacuo to yield a white powder after trituration with hexane (0.27 g, 70%): R_f (C) 0.6; mp 126–129 °C; $[\alpha]_D$ −27° (c 1, DMF). Anal. ($C_{14}H_{25}N_3O_6S$) C, H, N.

(*tert*-Butyloxycarbonyl)-L-tryptophyl-L-methionyl-β-benzyl-L-aspartic Acid Amide (3). Compound 1 (2 g, 4.4 mmol) was partially deprotected in trifluoroacetic acid (10 mL). After 30 min, ether (150 mL) was added and the precipitate was collected by filtration, washed several times with ether, and dried in vacuo. The trifluoroacetate salt was dissolved in DMF (10 mL) and treated with (*tert*-butyloxycarbonyl)-L-tryptophan *p*-nitrophenyl ester[15] (1.7 g, 4 mmol), 1-hydroxybenzotriazole (0.61 g, 4 mmol), and *N,N*-diisopropylethylamine (1.5 mL, 8.7 mmol). After standing overnight at room temperature, the reaction mixture was treated as described for 1. The crude product was purified on a column of silica gel with a mixture of ethyl acetate/methanol (9:1) as eluent: yield 86% (2.2 g); R_f (A) 0.35; R_f (B) 0.20; mp 178–180 °C; $[\alpha]_D$ −24.8° (c 1, DMF). Anal. ($C_{32}H_{41}N_5O_7S$) C, H, N.

(*tert*-Butyloxycarbonyl)-L-tryptophyl-L-methionyl-L-aspartic acid amide (4) was obtained by hydrogenation of 3 (0.5 g, 0.78 mmol) as described for 2: yield 70% (0.3 g); R_f (C) 0.45; mp 210 °C dec; $[\alpha]_D$ −27.1° (c 1, DMF). Anal. ($C_{25}H_{35}N_5O_7S$) C, H, N.

(*tert*-Butyloxycarbonyl)-glycyl-L-tryptophyl-L-methionyl-β-benzyl-L-aspartic Acid Amide (5). After partial deblocking of 3 (1 g, 1.6 mmol) by TFA (10 mL), acylation was carried out in dimethylformamide (10 mL) with (*tert*-butyloxycarbonyl)-glycine *p*-nitrophenyl ester[16] (0.48 g, 1.6 mmol), 1-hydroxybenzotriazole (0.25 g, 1.6 mmol), and *N,N*-diisopropylethylamine (0.6 mL, 3.3 mmol). After standing overnight at room temperature, the reaction mixture was treated as described in the previous paragraph and the product was purified on a silica gel column with a mixture of ethyl acetate/methanol (9:1) as eluent: yield 86% (1.02 g); R_f (D) 0.55; mp 152–156 °C; $[\alpha]_D$ −16.6° (c 1, DMF). Anal. ($C_{34}H_{44}N_6O_8S$) C, H, N.

(*tert*-Butyloxycarbonyl)-glycyl-L-tryptophyl-L-methionyl-L-aspartic Acid Amide (6). Compound 5 (0.5 g, 0.7 mmol) was hydrogenated to yield 6 (0.27 g, 65%). Anal. ($C_{27}H_{38}N_6O_8S$) C, H, N. The partially deblocked intermediate was dissolved in 0.2 N NH_4OH and lyophilized: R_f (C) 0.40; mp 170 °C dec; $[\alpha]_D$ −18.6 (c 1, DMF).

(*tert*-Butyloxycarbonyl)-L-tyrosyl-glycyl-L-tryptophyl-L-methionyl-β-benzyl-L-aspartic Acid Amide (7). After partial deprotection of 5 (0.71 g, 1.02 mmol) with TFA as previously described, acylation was carried out in dimethylformamide (10 mL) with (*tert*-butyloxycarbonyl)-L-tyrosyl *p*-nitrophenyl ester[18] (0.4 g, 1 mmol) and *N,N*-diisopropylethylamine (0.18 mL, 1.04 mmol). Compound 7 was purified on a silica gel column with a mixture of ethyl acetate–methanol (9:1) as eluent and gave a white powder: yield 72% (0.63 g); R_f (D) 0.40; mp 127–135 °C; $[\alpha]_D$ −10.9° (c, 1, DMF). Anal. ($C_{43}H_{53}N_7O_{10}S$) C, H, N.

(*tert*-Butyloxycarbonyl)-L-tyrosyl-glycyl-L-tryptophyl-L-methionyl-L-aspartic Acid Amide (8). Compound 7 (0.5 g, 0.58 mmol) was hydrogenated to yield 8 (56% 0.25 g): R_f (C) 0.4; mp 210 °C dec; $[\alpha]_D$ −18.7° (c 0.63, DMF). Anal. ($C_{36}H_{47}N_7O_{10}S$) H, N; C: calcd, 55.72; found, 56.15.

[[(9-Fluorenylmethyl)oxy]carbonyl]-L-methionyl-β-*tert*-butyl-L-aspartic Acid Amide (9). To a cooled (0 °C) solution of the hydrochloride salt of β-*tert*-butyl-L-aspartic acid amide (5 g, 22.2 mmol) in dimethylformamide (50 mL) containing [[(9-fluorenylmethyl)oxy]carbonyl]-L-methionine[29] (7.42 g, 20 mmol) and [(benzotriazolyl)oxy]tris(dimethylamino)phosphonium hexafluorophosphate (8.84 g, 20 mmol) was added *N,N*-diisopropylethylamine (7.8 mL, 45 mmol). After stirring overnight at room temperature, the solvent was removed in vacuo and the residue treated as described for compound 1: yield 87% (9.4 g); R_f (E) 0.60; mp 184–185 °C; $[\alpha]_D$ −9.1° (c 1, DMF). Anal. ($C_{27}H_{35}N_3O_6S$) C, H, N.

[[(9-Fluorenylmethyl)oxy]carbonyl]-L-tryptophyl-L-methionyl-β-*tert*-butyl-L-aspartic Acid Amide (10). A sample of compound 9 (5.41 g, 9 mmol) was dissolved in a 9:1 mixture of dimethylformamide and diethylamine (150 mL). After 2 h at room temperature, the solvents were thoroughly removed in vacuo, and the residue was dissolved in dimethylformamide (20 mL) and cooled in an ice water bath. To this solution was added [[(9-fluorenylmethyl)oxy]carbonyl]-L-tryptophan[29] (3.82 g, 9 mmol), [(benzotriazolyl)oxy]tris(dimethylamino)phosphonium hexafluorophosphate (3.98 g, 9 mmol), and *N,N*-diisopropylethylamine (3.4 mL, 20 mmol). After overnight at room temperature, the solvent was concentrated in vacuo and the residue was treated with ethyl acetate. The precipitate was filtered, washed several times with ethyl acetate, a saturated sodium bicarbonate solution, water, a 10% citric acid solution, and water, and dried in vacuo over phosphorus pentoxide: yield 75% (4.9 g); R_f (E) 0.5; mp 175 °C dec; $[\alpha]_D$ −23° (c 1, DMF). Anal. ($C_{39}H_{45}N_5O_7S$) C, H, N.

(*tert*-Butyloxycarbonyl)glycine carbamoylmethyl ester was prepared from the cesium salt of (*tert*-butyloxycarbonyl)-glycine[30] (3.5 g, 20 mmol) and α-chloroacetamide (2.82 g, 29 mmol) in dimethylformamide (100 mL) at 50 °C during 24 h. The solvent was concentrated in vacuo, and the residue dissolved in ethyl acetate, washed with a sodium bicarbonate solution, water, a 10% citric acid solution, and water, dried over sodium sulfate, and concentrated in vacuo. (*tert*-Butyloxycarbonyl)glycine carbamoylmethyl ester crystallized in ether: yield 70% (4.9 g); mp 64–67 °C. Anal. ($C_9H_{16}N_2O_5$) C, H, N.

(*tert*-Butyloxycarbonyl)-L-tyrosyl-glycine Carbamoylmethyl Ester (12). A sample of (*tert*-butyloxycarbonyl)-glycine carbamoylmethyl ester (9.3 mmol, 2.4 g) was dissolved in TFA. After 30 min, ether (150 mL) was added, and the precipitate filtered, rinsed several times with ether, and dried in vacuo. It was dissolved in dimethylformamide (20 mL) in the presence of (*tert*-butyloxycarbonyl)-L-tyrosine *p*-nitrophenyl ester (3.22 g, 8 mmol), cooled in an ice water bath, and treated with *N,N*-diisopropylethylamine (1.3 mL). After standing overnight at room

temperature, the reaction mixture was treated as described for 1: yield 85% (2.77 g); R_f (F) 0.35; mp 113–116 °C; $[\alpha]_D$ -9.2° (c 1, DMF). Anal. ($C_{18}H_{25}N_3O_7$) C, H, N.

***(tert*-Butyloxycarbonyl)-L-alanyl-L-tyrosyl-glycine carbamoylmethyl ester (13)** was prepared from 12 (3.55 g, 9 mmol), which was deprotected with TFA and acylated with (*tert*-butyloxycarbonyl)-L-alanine *p*-nitrophenyl ester (2.63 g, 8.5 mmol) in the presence of *N,N*-diisopropylethylamine (1.7 mL, 10 mmol) as previously described: yield 80% (3.2 g); R_f (F) 0.25; mp 128–132 °C; $[\alpha]_D$ -21.1° (c 1, DMF). Anal. ($C_{21}H_{30}N_4O_8$) C, H, N.

(Benzyloxycarbonyl)-γ-*tert*-butyl-L-glutamyl-L-alanyl-L-tyrosyl-glycine carbamoylmethyl ester (14) was prepared from 13 (0.93 g, 2 mmol), which was deprotected in TFA (5 mL) and acylated with (benzyloxycarbonyl)-γ-*tert*-butyl-L-glutamic acid *p*-nitrophenyl ester (1.08 g, 2.2 mmol) in the presence of *N,N*-diisopropylethylamine (0.38 mL, 2.2 mmol) in DMF (10 mL). Compound 14 crystallized on trituration with a mixture of ethyl acetate and ether (1:1): yield 85% (1.16 g); R_f (G) 0.6; mp 135–137 °C; $[\alpha]_D$ -12° (c 1, DMF). Anal. ($C_{33}H_{43}N_5O_{11}$) C, H, N.

(Benzyloxycarbonyl)-γ-*tert*-butyl-L-glutamyl-L-alanyl-L-tyrosyl-glycine (15). Compound 14 (1.37 g, 2 mmol) was dissolved in a 1:1 mixture of dimethylformamide and water (50 mL) and treated with sodium carbonate (0.32 g, 3 mmol), with vigourous stirring. The solution was maintained clear by addition of one of the two solvents if necessary. After 1 h no more starting material could be detected by TLC. The reaction mixture was neutralized with a 20% citric acid solution (pH 7), and the solvents were removed in vacuo. The residue was dissolved in a 20% sodium carbonate solution (20 mL) and extracted with ethyl acetate (2 × 15 mL). The aqueous layer was cooled and acidified with solid citric acid. A precipitate formed, which was filtered and washed with water: yield 75% (0.95 g); R_f (C) 0.3; mp 184–185 °C; $[\alpha]_D$ -11° (c 1, DMF); ^1H NMR (Me_2SO-d_6) δ 1.18 (d, CH_3 Ala), 1.40 (s, Bu^t), 1.72 and 1.37 (m, CH_2 β Glu), 2.24 (t, CH_2 γ glu), 2.72 and 2.92 (m, CH_2 Tyr), 3.70 (CH α Gly), 4.02, 4.26, and 4.42 (m, 3 CH α), 5.03 (s, $PhCH_2$), 6.63 (d, Tyr), 7.00 (d, Tyr), 7.37 (C_6H_5 + NH), 7.41 (d, NH), 7.91 (NH), 8.02 (NH). Anal. ($C_{31}H_{40}N_4O_{10}$) C, H, N.

(Benzyloxycarbonyl)-γ-*tert*-butyl-L-glutamyl-L-alanyl-L-tyrosyl-glycyl-L-tryptophyl-L-methionyl-β-*tert*-butyl-L-aspartic Acid Amide (16). Compound 10 (1.45 g, 2 mmol) was dissolved in a 9:1 mixture of dimethylformamide and diethylamine (150 mL). After 2 h, the solvents were thoroughly removed in vacuo and the residue dissolved in dimethylformamide (20 mL). Peptide 15 (1.26 g, 2 mmol) was added followed by [(benzotriazolyl)oxy]tris(dimethylamino)phosphonium hexafluorophosphate (0.84 g, 1.9 mmol). The solution was cooled in an ice water bath and treated with *N,N*-diisopropylethylamine (0.69 mL, 4 mmol). After stirring overnight at room temperature, the solvent was removed in vacuo and the residue triturated several times in ethyl acetate. The precipitate was filtered, washed with a saturated sodium bicarbonate solution (2 × 50 mL), water (2 × 50 mL), a 20% citric acid solution (2 × 50 mL), water (2 × 50 mL), and ethyl acetate–ether, and dried in vacuo over phosphorus pentoxide: yield 72% (1.5 g); R_f (C) 0.45; mp 210 °C dec; $[\alpha]_D$ -23° (c 1, DMF). Anal. ($C_{55}H_{73}N_9O_{14}S$) H, N; C: calcd, 58.65; found, 59.17.

(Benzyloxycarbonyl)-L-glutamyl-L-alanyl-L-tyrosyl-glycyl-L-tryptophyl-L-methionyl-L-aspartic Acid Amide (17). Compound 16 (1.1 g, 1 mmol) was dissolved in a mixture of TFA, anisole, and thioanisole (10:1:1) (10 mL). After 1 h at room temperature, ether (200 mL) was added and a white powder precipitated. It was filtered, rinsed several times with ether, dried in vacuo, and purified on a column of silica gel with a mixture of ethyl acetate, pyridine, acetic acid, and water, (80:20:6:10) as eluent: yield 72% (0.52 g); R_f 0.4 (ethyl acetate, pyridine, acetic acid, water (80:20:6:10)); mp 210 °C dec; $[\alpha]_D$ -20° (c 1, DMF). Anal. ($C_{47}H_{57}N_9O_{14}S$) H, N; C: calcd, 55.79; found, 56.21.

Biological Tests. Gastric acid secretion was determined in vivo in the reperfused rat stomach according to the method of Ghosh and Shild. The gastric pouch of an anesthesized rat (urethane ip) was continuously washed at 30 °C with a propionate–succinate solution. The cumulative pH was recorded with time and used as an index of acid secretion. Synthetic (Leu[15])-human gastrin I (gift from Professor E. Wunsch, Max Plank Institute, Munchen) was dissolved in 0.9% NaCl and bolus injected intravenously. The amount of secreted H^+ was determined by the pH difference between stimulated and basal recorded traces. Eighty picamoles of gastrin was employed as stimulant. The inhibitory effect of synthetic peptides was measured after simultaneous bolus injection of the compounds in water alkaline solution and of gastrin. The amount of secreted H^+ in presence of various doses of the peptides was reported to the amount of secreted H^+ after gastrin alone and expressed as percent of inhibition. The mean H^+ secretion after gastrin injection was 203 ± 28 μmol of H^+/nmol of peptide (n = 17).

Binding Studies. Isolation of gastric cells was carried out by the collagenase/EDTA procedure previously described.[28] Fundic mucosa was scraped, and tissues were chopped into small cubes and then dispersed in medium A (132 mM NaCl, 5.4 mM KCl, 5 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 25 mM Hepes, 0.2% glucose, 0.2% bovine serum albumin, 0.02% phenol red, pH 7.4) (gassed O_2/CO_2) containing 0.30 mg/mL of collagenase. After 15 min of incubation at 37 °C, tissue fragments were allowed to settle and the medium was discarded. The fragments were washed in Ca^{2+}-free medium A containing 2 mM EDTA and then incubated in the same medium for 10 min. The fragments were tranferred to medium A containing fresh 0.30 mg/mL of collagenase and incubated for 15 min at 37 °C with continuous gassing (O_2/CO_2). The cell suspension was centrifuged for 15 min at 200*g* and then washed twice with medium A. This procedure gave about 5×10^7 cells/g of wet mucosa with 95% viability (trypan blue exclusion). The mixed population contained 45% parietal cells. (Nle[11])-HG-13 was iodinated following a modification of the already described chloramine T procedure.[28] After purification of DE-52 ion-exchange chromatography, the monoiodinated peptide was obtained with full biological activity. Specific gastrin binding was determined by incubation in medium B (Earle's balanced salt medium without bicarbonate and containing 10 mM Hepes and 0.2% BSA, pH 7.4) of 20 pM labeled (Nle[11])-HG-13 (≃40 000 cpm/mL) for 30 min at 37 °C with 5×10^6 cells/mL ± various concentrations of peptides or unlabeled (Nle[11])-HG-13. Nonsaturable binding was determined as the amount of radioactivity associated with cells in presence of 1×10^{-6} M of cold (Nle[11])-HG-13.

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