Articles

Potent Gastrin-Releasing Peptide (GRP) Antagonists Derived from GRP(19-27) with a C-Terminal DProΨ[CH_2NH]Phe-NH_2 and N-Terminal Aromatic Residues

Johann J. Leban,*,† Antonio Landavazo,† John D. McDermed,† Emanuel J. Diliberto, Jr.,‡ Marilyn Jansen,‡ Beth Stockstill,§ and Frederick C. Kull, Jr.§

Organic Chemistry Division, Cellular & Molecular Pharmacology Section, Division of Pharmacology, and Division of Cell Biology, Burroughs Wellcome Company, 3030 Cornwallis Road, Research Triangle Park, North Carolina 27709

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We have previously reported that octapeptides with a -DProΨ[CH_2NH]Phe-NH_2 C-terminus are potent GRP antagonists and have greatly enhanced in vivo stability. Now we report the detailed syntheses of such peptides and additional attempts to further increase metabolic stability. Replacement of the -DProΨ[CH_2NH]Phe-NH_2 with a "-DPro-statine"-Phe-NH_2 led to less potent antagonistic activity. The introduction of ThiAla and BzthAla, to replace His and Trp, respectively, did not increase activity. A series of analogs having different aromatic residues at the N-terminal, other than 3-phenylpropionic acid, are equally potent. These residues show increased activity when hydrophilic substitutions are added to the aromatic ring. Replacement of the C-terminal Phe by DPhe and D2Nal is tolerated. Even though none of these peptides have higher activity than the original lead peptide, they are potentially more metabolically stable.

Introduction

Gastrin-releasing peptide (GRP) is a 27-amino-acid residue neuroendocrine hormone. GRP helps to regulate gastrin secretion.[1,2] Additionally, GRP may be involved in the pathophysiology of small-cell lung carcinoma (SCLC) as suggested by the following observations: immunochemical cross-reactive material is found in SCLC[3,4] and in the sera of SCLC patients;[5] GRP is both made by and growth promoting for some cultured SCLC cell lines;[6,7] antibodies[6] inhibit the growth of human SCLC xenografts in nude mice; antagonists[1,8] inhibit the growth of SCLC in vitro and in vivo;[9] and the inhibition of growth of a HT-29 colon cancer xenograft in nude mice by a GRP antagonist was also demonstrated.[10] GRP is also mitogenic for cultured Swiss 3T3 cells.[11,12] Specific binding and growth effects of structurally related peptides were shown on mouse colon cancer cells in vitro.[13]

We decided to develop GRP antagonists using their ability to inhibit [^{3}H]thymidine incorporation into quiescent Swiss 3T3 cells. We believed that such an assay better reflects the potential antiproliferative effects of these agents than either the GRP-binding inhibition or amylase-release inhibition assays.

The first antagonist against GRP was derived from the sequence of BN as developed by Coy et al.[8] Bombesin is an amphibian peptide that is structurally similar to GRP. Our goal was to apply this principle to the C-terminal octapeptide sequence of GRP, since it is known that the complete peptide is not needed for maximum activity.[14] We first found that an octapeptide C-terminal GRP analog having DPro adjacent to the C-terminal amino acid amide was antagonistic with activities of 40 nM in the mitogenic inhibition assay. By combining this principle with Coy's reduced bond modification, specific analogs were found with nanomolar activities in the mitogenic inhibition assays.[15]

The present study was aimed toward further improvement of this analog by modification of the N-terminal aromatic residue and introduction of unnatural amino acids.

Results and Discussion

Chemical Results. We synthesized these peptides by an improved version of the solid-phase method[16] using an Applied Biosystems, Inc., 430A peptide synthesizer. Boc-protected amino acids were coupled to the resin by a modified program to suit the BOP-coupling procedure.[17]

The DProΨ[CH_2NH]Phe unit was introduced on the solid phase by Sasaki's reductive alkylation procedure with Boc-Dprolinal.[18] We obtained Boc-Dprolinal by Swern oxidation of commercially available Boc-Dprolinol.[19] Alternatively, we showed that the peptide could be made by coupling Boc-DProΨ[CH_2NH]Phe directly to the resin. Boc-DProΨ[CH_2NH]Phe can be obtained, according to Martinez et al.,[20] by reductive alkylation of phenylalanine methyl ester with Boc-Dprolinal and alkaline hydrolysis of the product. All peptides were cleaved from the resin by liquid HF and purified by preparative HPLC. Amino acid analysis and FAB-MS data are displayed in Table 1.

Peptide 1 was characterized extensively by ^{1}H NMR and capillary zone electrophoresis (data not shown). Nevertheless, His levels in its amino acid analysis were still low. Therefore, we conclude that our method of hydrolysis yields a low His recovery, consistently observed in our amino acid analysis data.

The appropriate "DPro-statine" was obtained by a Reformatsky reaction of Boc-Dprolinal with ethyl bromoacetate followed by an alkaline hydrolysis of the ethyl ester. This method yields two stereoisomers of "Boc-DPro-statine" (3-(1-(*tert*-butyloxycarbonyl)-2-pyrrolidinyl)-3-

* Address correspondence to this author.
† Organic Chemistry Division.
‡ Cellular & Molecular Pharmacology Section, Division of Pharmacology.
§ Division of Cell Biology.
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Table 1. Analytical Results of Analogs

structure	MH^+ (FAB-MS)	AAA
1	1081.7	Ala (1.96), His (1.50), Val (1.00)
2	1082	Ala (1.02), His (2.06), Val (1.00)
3	1067.1	Ala (1.00), His (3.17)
4	1155.7	Ala (2.01), His (1.05), Phe (1.29), Val (1.00)
5	1139.5	Ala (1.86), His (0.97), Phe (1.22), Val (1.00)
6	1139.6	Ala (2.02), His (1.91), Phe (1.38), Val (1.00)
7	1099.1	Ala (2.04), His (1.71), Val (1.00)
8	1098	Ala (1.92), His (0.59), Val (1.00)
9	1095	Ala (2.68), His (1.38), Val (1.00)
10	1095.9	Ala (1.83), His (1.82), Val (1.00)
11	1081.2	Ala (2.07), His (1.51), Val (1.00)
12	1081.1	Ala (2.05), His (1.44), Val (1.00)
13	1098	Ala (2.04), His (1.96), Val (1.00)
14	1111.9	Ala (1.84), His (1.76), Val (1.00)
15	1171.8	Ala (1.86), His (1.71), Val (1.00)
16	1149.8	Ala (2.17), His (1.50), Val (1.00)
17	1135.4	Ala (2.10), His (1.63), Val (1.00)
18	1135.4	Ala (2.10), His (1.60), Val (1.00)
19	1135.5	Ala (2.24), His (1.68), Val (1.00)
20	1095	Ala (2.00), His (1.55), Val (1.00)
21	1109.5	Ala (2.07), His (1.63), Val (1.00)
22	1103.9	Ala (2.06), His (2.12), Val (1.00)
23	1104.5	Ala (1.82), His (1.32), Val (1.00)
24	1082.2	N/A
25	1146.6	Ala (1.97), His (1.61), Val (1.00)
26	1202.1	Ala (2.25), His (1.58), Val (1.00)
27	1089.8	Ala (1.85), His (1.48), Val (1.00)
28	1093.1	Ala (1.99), His (1.44), Val (1.00)
29	1132.4	Ala (1.96), His (1.42), Val (1.00)
30	1081.9	Ala (2.19), His (1.85), Val (1.00)
31	1097.7	Ala (1.97), His (1.53), Val (1.00)
32	1063.5	N/A

hydroxypropionic acid) in a 1:2 ratio. The absolute stereochemistry was not determined, and both compounds were used separately to synthesize **5** and **6**.

Biological Results. Our biological results are presented in summary form in Table 2. We have previously reported that the introduction of a DProΨPhe group led to potent GRP antagonists.[15] Our initial studies were performed using mitogenesis antagonism that was prone to error, as reported.[15] The GRP-binding assays were performed using intact 3T3 cells and also with isolated 3T3 cell membranes. We observed that the binding affinities in intact 3T3 cells were generally higher than those in 3T3 membranes. This may be explained by partial denaturation of receptors. However, greater variation of the assay data is expected with intact cells (binding, mitogenic stimulation), due to well-to-well variation and culture-to-culture differences, than with the binding assay in 3T3 membranes. Trends were consistent regardless of the assay leading to the following conclusions.

Peptide **1** was previously shown to be not only a highly active GRP antagonist but also quite active in vivo.[21] We intended to further improve metabolic stability by introducing more groups that enhance enzymatic stability.

Replacement of DAla with sarcosine (**2**) gave a slightly weaker analog, which prompted us to retain DAla in this position. In Coy's original report on GRP antagonists, both $\Psi[CH_2NH]^{9,10}$-bombesin and $\Psi[CH_2NH]^{13,14}$-bombesin were reported to have some antagonistic properties.[22] Therefore, we made **3** with $\Psi[CH_2NH]^{9,10}$ and $\Psi[CH_2NH]^{13,14}$ together, but this was inactive.

The statine analog **4**, [Sta[26],Phe[27]]GRP, was almost as potent as our lead, **1**. When we introduced a DPro-statine (**5**), the activity dropped somewhat. It is interesting to note that the stereochemistry of the hydroxyl group in the Pro-statine pair (**5** and **6**) seemed to make a difference in binding activity, which is in contrast to the reported [Sta[13],des-Met[14]]bombesin analogs.[21] There exist only a few possibilities to replace the natural sequence of the C-terminal octapeptide derived from GRP and bombesin.[23] Isosteric replacements of His[20] and His[25] with ThiAla and Trp[21] with BzthAla had not been explored yet. Replacement of Trp[21] and His[20] (**7** and **8**) with these isosteres retained some activity but did not improve on **1**. Replacement of His[25] with ThiAla was performed only on a DPro$\Psi[CH_2NH]$Nle analog, **32**. Also, analogs without an aromatic residue at the C-terminal are less active. We concluded that such a replacement would not be tolerated. It is not surprising that replacement of His[25] leads to lower active analogs. First, the thiophene side chain is albeit isosteric but electronically different than the imidazole ring of His. Also, His[25] is known to be less susceptible to replacement than His[20]. Still, replacement of His[20] with ThiAla did not give good results either. BzthAla is isosteric and electronically quite similar to the indole of Trp, and the loss of activity further reflects on the importance of the indole as a pharmacophore.

We have performed a ^1H NMR study in the membrane-mimetic solvent methanol (A. Aulabaugh, submitted for publication) and concluded that the peptide adopts a helical conformation from residues 2 to 7 consisting of a type I or III β-turn. The N-terminal of the peptide is disordered, and we attempted to introduce residues to restrict this part of the molecule by introducing aromatic groups with an optically active center and hoped to improve on activity. Analogs **27** and **28** were prepared to show the effect of restricting rotation of the aromatic ring. This small series of optically active phenylpropionic and phenylacetic acid analogs showed, in both cases, a preference for the *R* isomers (**9–12**).

Another approach to enhance metabolic stability is the introduction of hydrophobic residues at the N-terminal.[24] While this technique is strictly empirical by trial and error, some groups, such as phenothiazinyl and 2-quinonyl, were previously applied successfully.[25,26] Introduction of hydroxy and methoxy groups in the aromatic ring led to improved analogs (**13–15**). None of the trifluoromethyl-group substitutions, in the acetic acid analogs, led to any improvements (**17–19**). However, by comparing **16** and **18**, there is a 15-fold increase in 3T3 membrane activity and a 1000-fold increase in mitogenic activity when we used a phenyl-substituted propionic acid as opposed to a phenyl-substituted acetic acid at the N-terminal. When considering other aromatic acids for novel analogs, we generally used only substituted propionic acids. In only one instance this observation was proved false (**20** and **21**).

Analog **22** is more active than **23** and **24**, probably due to the increased basicity of the latter analogs. Analog **22** was also as active as our lead, **1**. Analogs **25** and **26** showed a 100-fold decrease in both mitogenic and 3T3 membrane activity when compared to **22**.

Finally, we explored the possibility of replacing the C-terminal Phe with D-amino acids to reduce potential C-terminal deamidation (**29** and **30**). The replacement of Phe with the more hydrophilic Tyr is displayed in **31**. All three residues (DPhe, D2Nal, and Tyr) were all tolerated.

In conclusion, we have modified our lead peptide, **1**, with more unnatural amino acids and a series of N-terminal aromatic blocking groups. Although all three assays do not agree, it seems that **22** and **28** are slightly more active

than our lead. Several others are at least as potent as our lead and have the potential for better bioavailability. These compounds are good candidates for further work in more elaborate in vitro studies using SCLC cell lines and in vivo tumor xenograft experiments in nude mice. We are presently pursuing such studies.

Experimental Section

Materials. The following items were obtained from Advanced Chemtech, Inc., Louisville, KY: *p*-methylbenzhydrylamine·HCl resin (MBHA, substitution ranges from 0.56 to 0.94 meq/g), trifluoroacetic acid, Boc-Dprolinol, and most Boc-protected amino acids. Bachem, Inc., Torrance, CA, supplied Boc-protected L-histidine-im(CBZ), β-thienylalanine, 3-(2-naphthyl)-D-alanine, and L-phenylalanine methyl ester hydrochloride. Methoxinine (*O*-methylhomoserine) was obtained from Biohellas S. A., Greece, and Boc-protected using di-*tert*-butyl dicarbonate (Fluka). Fluka Chemical Corp., Ronkonkoma, NY, supplied 3-(4-hydroxyphenyl)propionic acid, *N*-hydroxysuccinimide, and both (*R*)- and (*S*)-3-phenylbutyric acid. Aldrich Chemical Co., Milwaukee, WI, supplied 3-phenylpropionic acid (hydrocinnamic acid), 4-methylmorpholine, 1-methylimidazole, sodium cyanoborohydride, diisopropylethylamine, DMSO, 2.0 M oxalyl chloride in methylene chloride, ethyl bromoacetate, and zinc powder. The BOP-coupling reagent was obtained from Richelieu Biotechnologies, Inc., St-Hyacinthe, QC, Canada.

Preparation of Boc-Dprolinal. The following procedure is a variation of the reaction performed by Mancuso et al.[19] Into a 500-mL three-necked flask, oxalyl chloride solution (25 mL, 50 mmol) was dissolved in dry CH_2Cl_2 (60 mL) at −60 °C under N_2. DMSO (10 mL, 140 mmol) was dissolved in dry CH_2Cl_2 (25 mL) and added dropwise to the −60-°C solution by an addition funnel, and the solution was then stirred a further 5 min. Boc-Dprolinol (10 g, 50 mmol) was dissolved in dry CH_2Cl_2 (60 mL) and chilled to −60 °C in a 500-mL round-bottomed flask under N_2. To facilitate combination of the contents of both cold flasks, the N_2 line was set up in series to accommodate both flasks. The oxalyl chloride/DMSO mixture was gradually added to the alcohol solution by a double-tipped needle. By manually controlling the N_2 flow rate, the contents were transferred dropwise from one flask to the other over 30 min. Upon complete addition, this mixture was stirred for 20 min. Lastly, triethylamine (20 mL, 140 mmol) was added slowly at −60 °C. The mixture was then allowed to warm to room temperature.

The mixture was washed with water (50 mL), and the aqueous layer was extracted with CH_2Cl_2 (3 × 70 mL). The organics were successively washed with 5% $NaHCO_3$, 1 N HCl, and water (50 mL each). The combined aqueous washings were extracted with CH_2Cl_2 (50 mL), which was then combined with the other organics. The organics were dried (Na_2SO_4), and the solvent was removed by rotary evaporation. The residue was dried on a vacuum pump for 3 h.

According to ^{1}H NMR, the characteristic -OH peak for Boc-Dprolinol (δ = 4.5 ppm) was no longer visible. A sharp singlet at δ = 9.4 ppm denoted the presence of the aldehydic proton of Boc-Dprolinal. TLC (R_f = 0.77 with 5% MeOH/CH_2Cl_2 on silica) indicated no substantial side products, so this was used without further purification.

Preparation of DProΨ[CH_2NH]Phe-MBHA Resin. The reduced bond was introduced into the resin in a variation to Sasaki's technique.[18] MBHA resin (10 g, 0.97 meq/g, 9.7 mmol) was placed in a manual peptide shaker (Milligen). The resin was washed twice (3 and 5 min) with 10% *N*,*N*-diisopropylethylamine (DIEA) in CH_2Cl_2 while shaking. The resin was next rinsed twice with CH_2Cl_2. Complete neutralization of the HCl on the resin was indicated by a qualitative ninhydrin test observed as a very deep blue color.[27]

The resin was next treated with a Boc-amino acid (norleucine, phenylalanine), benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP), and 4-methylmorpholine (2 equiv each), all dissolved in DMF (50 mL). This mixture was shaken for 1 h and then rinsed twice with CH_2Cl_2. Coupling was verified by the ninhydrin test (colorless).[27]

Cleavage of the Boc group on norleucine was undertaken by shaking the resin in the presence of 50% TFA in CH_2Cl_2 twice (5 and 20 min). After rinsing the resin once with CH_2Cl_2, the excess TFA was neutralized with 10% DIEA/CH_2Cl_2 solution (3 and 5 min) with shaking. Following two CH_2Cl_2 rinses, the resin was again qualitatively tested with ninhydrin (very deep blue).[27]

The resin was next shaken for 2 h with Boc-Dprolinal (2 equiv) in 2% glacial HOAc/DMF (50 mL). Over the entire 2 h, sodium cyanoborohydride (3 equiv) was added slowly and gradually. After rinsing twice with CH_2Cl_2, coupling was checked with ninhydrin. Occasionally, this first coupling is incomplete and must be repeated. The resin was now ready for standard solid-phase peptide synthesis.

Alternative Preparation of Boc-DProΨ[CH_2NH]Phe-MBHA Resin. 1. Preparation of Boc-DProΨ[CH_2NH]Phe-methyl Ester. A solution of L-phenylalanine methyl ester hydrochloride in MeOH was treated with triethylamine (1 equiv). The mixture was concentrated in vacuo, taken up in ether, and filtered through Celite. The filtrate was concentrated in vacuo and dissolved in MeOH and then treated with Boc-Dprolinal (1 equiv) followed by type 4A molecular sieves (10 g). This mixture was stirred for 1 h and filtered through Celite. The filtrate was transferred to a Parr bottle, diluted with MeOH, treated with PtO_2 (250 mg), and hydrogenated over 18 h. The mixture was then filtered through Celite and concentrated in vacuo. Silica gel chromatography, with hexane/EtOAc eluent, gave a 78% yield of Boc-DProΨ[CH_2NH]Phe-methyl ester. Elemental analysis: calcd, 66.27, C; 8.34, H; 7.73, N; found, 65.35, C; 8.46, H; 7.66, N. ^{1}H NMR ($CDCl_3$): δ 7.10–7.30 (m, 5H), 3.62 (s, 3H), 3.40–3.55 (m, 1H), 3.15–3.40 (m, 3H), 2.70–3.00 (m, 3H), 2.30–2.60 (br m, 1H), 1.74 (m, 4H), 1.43 (s, 9H). Mass spectrum (CI): *m*/*e* 363 (M + 1, 100), 307 (M − tBu + 1, 60%), 263 (M − tBu-CO_2 + 1, 60). R_f = 0.35 with 2:1 hexane/EtOAc on silica.

2. Preparation of Boc-DProΨ[CH_2NH]Phe-OH. A solution of Boc-DProΨ[CH_2NH]Phe-methyl ester in THF was combined with a solution of NaOH (0.6 g, 15 mmol) in water (10 mL), and MeOH (15 mL) was added. The mixture was stirred for 30 min and then partially concentrated at room temperature. The mixture was diluted with water, washed with ether, and cooled to 0 °C. HCl was added until the pH was approximately 6.5, and the resulting suspension was filtered. The solid was washed with water and ether and dried in a vacuum oven to afford a 60% yield of a colorless solid (mp = 196.0–197.5 °C). Elemental analysis: calcd, 65.49, C; 8.10, H; 8.04, N; found, 65.53, C; 8.13, H; 8.07, N. ^{1}H NMR (DMSO): δ 7.25 (m, 5H), 3.60–3.80 (m, 1H), 3.32 (m, 1H), 3.12 (m, 2H), 2.70–3.00 (m, 3H), 2.30–2.50 (m, 1H), 1.70 (m, 4H), 1.36 (s, 9H). Mass spectrum (CI): *m*/*e* 349 (M + 1, 80), 293 (M − tBu + 1, 100), 249 (M − CO_2-tBu + 1, 50). R_f = 0.35 with 8:1:1 MeCN/H_2O/MeOH on silica.

3. Preparation of Boc-DProΨ[CH_2NH]Phe-MBHA. An amount of MBHA resin was shaken for 3 and 5 min in 10% DIEA to remove the HCl from the resin. The resin was then shaken with Boc-DProΨ[CH_2NH]Phe-OH, BOP reagent, and 4-methylmorpholine (2 equiv of each) in *N*-methylpyrrolidone for 2 h. Upon completion of this coupling, the resin was treated with 10% Ac_2O in CH_2Cl_2 for 20 min to cap all unreacted amino groups.

A duplicate synthesis of *N*-((3-phenyl)propionyl)-HisTrpAlaValDAlaHisDProΨ[CH_2NH]Phe-NH_2 (1) using the Boc-DProΨ[CH_2NH]Phe-MBHA described above was performed. This peptide was equivalent to the original peptide, as defined by FAB-MS (MH^+ (new) = 1081.6; MH^+ (original) = 1081.7).

Preparation of Ethyl 3-(1-(*tert*-Butoxycarbonyl)-2-pyrrolindinyl)-3-hydroxypropionate. Freshly activated zinc powder (0.79 g, 12 mmol) and benzene (50 mL) were placed into a 250-mL two-necked round-bottomed flask under N_2. This flask was attached to a Dean–Stark apparatus, and benzene (25 mL) was distilled into the trap. Under reflux, a solution of Boc-Dprolinal (1.9 g, 9.5 mmol) and ethyl bromoacetate (2.0 g, 12 mmol) was added dropwise. A crystal of iodine was added to initiate the reaction after half of the solution was added. After complete addition, the mixture was refluxed for 3 h, cooled, and carefully washed with 0.5 N HCl. The aqueous solution was extracted with ether (25 mL), and the combined organic solution was washed successively with water (30 mL) and saturated $NaHCO_3$ (30 mL) and then dried (Na_2SO_4) and concentrated under vacuum. A yellow oil was chromatographed on silica (with hexane/EtOAc) to isolate a colorless oil (1.12 g, 41%). Elemental analysis: calcd, 58.52, C; 8.77, H; 4.87, N; found, 58.62, C; 8.79,

Table 2. Peptide Structures and Potencies for Mitogenesis of GRP-Induced Antagonism and Competitive Inhibition of GRP Binding[a]

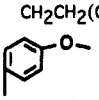
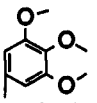
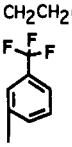
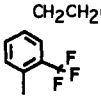
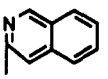
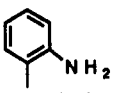
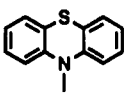
	peptide	antagonism of mitogenesis	inhibition of GRP binding, IC_{50} (M)	
			S 3T3 cells	S 3T3 membrane
1	 $CH_2CH_2(CO)$HisTrpAlaValDAlaHisDProΨPhe-NH_2	$1.0 \times 10^{-8} \pm 0.3 \times 10^{-9}$, $n = 3$	1.0×10^{-12}	$2.2 \times 10^{-10} \pm 0.8 \times 10^{-10}$, $n = 5$
2	 $CH_2CH_2(CO)$HisTrpAlaValSarHisDProΨPhe-NH_2	$4.3 \times 10^{-10} \pm 0.8 \times 10^{-10}$	1.0×10^{-10}	2.1×10^{-8}
3	 $CH_2CH_2(CO)$HisTrpAlaΨValDAlaHisDProΨPhe-NH_2	$4.7 \times 10^{-8} \pm 1.6 \times 10^{-8}$	nt	$>1.0 \times 10^{-8}$
4	 $CH_2CH_2(CO)$HisTrpAlaValDAlaHisStaPhe-NH_2	$6.0 \times 10^{-9} \pm 4.0 \times 10^{-9}$	1.0×10^{-10}	7.3×10^{-8}
5	$PhCH_2CH_2(CO)$HisTrpAlaValDAlaHis[D  $CH(OH)CH_2(CO)$]Phe-NH_2	na	6.0×10^{-8}	1.4×10^{-7}
6	$PhCH_2CH_2(CO)$HisTrpAlaValDAlaHis  $CH(OH)CH_2(CO)$Phe-NH_2	$2.6 \times 10^{-8} \pm 1.0 \times 10^{-8}$	5.0×10^{-8}	3.1×10^{-8}
7	 $CH_2CH_2(CO)$His(BzthAla)AlaValDAlaHisDProΨPhe-NH_2	$4.3 \times 10^{-7} \pm 1.0 \times 10^{-7}$	nt	7.5×10^{-10}
8	 $CH_2CH_2(CO)$ThiAlaTrpAlaValDAlaHisDProΨPhe-NH_2	$5.0 \times 10^{-8} \pm 1.5 \times 10^{-8}$	1.0×10^{-8}	5.6×10^{-8}
9	 (R) $CH_3CHCH_2(CO)$HisTrpAlaValDAlaHisDProΨPhe-NH_2	$1.7 \times 10^{-8} \pm 0.5 \times 10^{-8}$	nt	$4.9 \times 10^{-10} \pm 0.15 \times 10^{-10}$, $n = 4$
10	 (S) $CH_3CHCH_2(CO)$HisTrpAlaValDAlaHisDProΨPhe-NH_2	$1.6 \times 10^{-10} \pm 0.9 \times 10^{-10}$	1.0×10^{-8}	2.1×10^{-10}
11	 (R, -) $CH_3CH(CO)$HisTrpAlaValDAlaHisDProΨPhe-NH_2	$9.0 \times 10^{-10} \pm 1.0 \times 10^{-10}$	3.0×10^{-8}	9.5×10^{-10}
12	 (S, +) $CH_3CH(CO)$HisTrpAlaValDAlaHisDProΨPhe-NH_2	$7.0 \times 10^{-8} \pm 0.9 \times 10^{-8}$	1.0×10^{-9}	1.3×10^{-9}
13	 $CH_2CH_2(CO)$HisTrpAlaValDAlaHisDProΨPhe-NH_2	$1.6 \times 10^{-10} \pm 0.2 \times 10^{-10}$, $n = 4$	5.0×10^{-11}	$2.3 \times 10^{-10} \pm 0.08 \times 10^{-10}$, $n = 3$
14	 $CH_2CH_2(CO)$HisTrpAlaValDAlaHisDProΨPhe-NH_2	$3.0 \times 10^{-10} \pm 0.1 \times 10^{-10}$	1.0×10^{-8}	$4.7 \times 10^{-10} \pm 0.22 \times 10^{-10}$, $n = 3$
15	 $CH_2CH_2(CO)$HisTrpAlaValDAlaHisDProΨPhe-NH_2	$3.0 \times 10^{-10} \pm 0.8 \times 10^{-10}$	1.0×10^{-12}	4.6×10^{-10}
16	 $CH_2CH_2(CO)$HisTrpAlaValDAlaHisDProΨPhe-NH_2	$3.5 \times 10^{-10} \pm 0.1 \times 10^{-10}$	1.0×10^{-8}	1.6×10^{-10}
17	 $CH_2(CO)$HisTrpAlaValDAlaHisDProΨPhe-NH_2	$3.3 \times 10^{-7} \pm 1.4 \times 10^{-7}$	nt	1.2×10^{-8}

Table 2. (Continued)

	peptide	antagonism of mitogenesis	inhibition of GRP binding, IC_{50} (M) S 3T3 cells	S 3T3 membrane
18	 CH_2(CO)HisTrpAlaVal<u>D</u>AlaHis<u>D</u>ProΨPhe-NH_2	$2.6 \times 10^{-7} \pm 1.0 \times 10^{-7}$	nt	8.4×10^{-8}
19	 CH_2(CO)HisTrpAlaVal<u>D</u>AlaHis<u>D</u>ProΨPhe-NH_2	$2.0 \times 10^{-7} \pm 0.7 \times 10^{-7}$	nt	3.7×10^{-9}
20	 $(CH_3)_2C$(CO)HisTrpAlaVal<u>D</u>AlaHis<u>D</u>ProΨPhe-NH_2	$3.4 \times 10^{-10} \pm 0.5 \times 10^{-10}$, $n = 3$	3.0×10^{-12}	$1.8 \times 10^{-10} \pm 0.14 \times 10^{-10}$, $n = 3$
21	 $(CH_3)_2CCH_2$(CO)HisTrpAlaVal<u>D</u>AlaHis<u>D</u>ProΨPhe-NH_2	$1.8 \times 10^{-8} \pm 0.5 \times 10^{-8}$	1.0×10^{-8}	1.2×10^{-9}
22	 (CO)HisTrpAlaVal<u>D</u>AlaHis<u>D</u>ProΨPhe-NH_2	$3.0 \times 10^{-10} \pm 1.0 \times 10^{-10}$	1.0×10^{-12}	5.0×10^{-11}
23	 (CO)HisTrpAlaVal<u>D</u>AlaHis<u>D</u>ProΨPhe-NH_2	$4.0 \times 10^{-8} \pm 1.0 \times 10^{-8}$	nt	$5.7 \times 10^{-10} \pm 0.35 \times 10^{-10}$, $n = 3$
24	 CH_2(CO)HisTrpAlaVal<u>D</u>AlaHis<u>D</u>ProΨPhe-NH_2	$1.3 \times 10^{-8} \pm 0.7 \times 10^{-8}$	1.0×10^{-10}	2.0×10^{-8}
25	 D-CH_2(H2N)CH(CO)HisTrpAlaVal<u>D</u>AlaHis<u>D</u>ProΨPhe-NH_2	$4.5 \times 10^{-8} \pm 0.8 \times 10^{-8}$	nt	7.7×10^{-8}
26	 CH_2CH_2(CO)HisTrpAlaVal<u>D</u>AlaHis<u>D</u>ProΨPhe-NH_2	$2.9 \times 10^{-8} \pm 0.7 \times 10^{-8}$	nt	1.9×10^{-8}
27	 (CO)HisTrpAlaVal<u>D</u>AlaHis<u>D</u>ProΨPhe-NH_2	$4.0 \times 10^{-8} \pm 1.0 \times 10^{-8}$	3.0×10^{-10}	7.9×10^{-11}
28	 (CO)HisTrpAlaVal<u>D</u>AlaHis<u>D</u>ProΨPhe-NH_2	$2.0 \times 10^{-8} \pm 0.6 \times 10^{-8}$	3.0×10^{-11}	$4.9 \times 10^{-11} \pm 0.02 \times 10^{-11}$, $n = 2$
29	 CH_2CH_2(CO)HisTrpAlaVal<u>D</u>AlaHis<u>D</u>ProΨ<u>D</u>2Nal-NH_2	$4.0 \times 10^{-8} \pm 1.5 \times 10^{-8}$	5.0×10^{-9}	$2.0 \times 10^{-8} \pm 0.95 \times 10^{-10}$, $n = 3$
30	 CH_2CH_2(CO)HisTrpAlaVal<u>D</u>AlaHis<u>D</u>ProΨ<u>D</u>Phe-NH_2	nt	nt	3.8×10^{-11}
31	 CH_2CH_2(CO)HisTrpAlaVal<u>D</u>AlaHis<u>D</u>ProΨTyr-NH_2	$1.0 \times 10^{-8} \pm 4.0 \times 10^{-8}$	nt	3.3×10^{-10}
32	 CH_2CH_2(CO)HisTrpAlaVal<u>D</u>AlaThiAla<u>D</u>ProΨNle-NH_2	$3.0 \times 10^{-7} \pm 1.6 \times 10^{-7}$	nt	nt

[a] The activities were assessed in triplicate on quiescent Swiss 3T3 cells as described in ref 25: antagonism of mitogenesis, the capacity to inhibit 6 nM bombesin-induced [^{3}H]thymidine incorporation, and competitive inhibition of binding, the capacity to inhibit the binding of 50 pM [^{125}I]GRP for intact cells and 10 pM [^{125}I]GRP for membranes. All results are one experiment unless n (number of trials) is shown. Values indicate IC_{50}s ± SEM and are representative of replicate trials. For binding, the SEMs were <8% of the mean; for mitogenicity, n = the number of trials and SEM refers to the mean of the replicate trials. The abbreviations are Ψ = [CH_2NH], na = no activity observed at 10^{-4} M, and nt = not tested.

H; 4.85 N. Mass spectral analysis yielded results at 288 (M + 1, 20), 232 (M - tBu + 1, 40), and 188 (M - CO_2-tBu + 1, 100). ^{1}H NMR ($CDCl_3$): δ 4.15 (br m, 3H), 3.92 (m, 1H), 3.48 (m, 1H), 3.26 (m, 1H), 2.40 (m, 2H), 1.65–2.00 (br m, 4H), 1.45 (s, 9H), 1.25 (t, 3H). TLC: R_f = 0.5 with 1:1 hexane/EtOAc on silica.

Preparation of 3-(1-(*tert*-Butoxycarbonyl)-2-pyrrolidinyl)-3-hydroxypropionic Acid. A mixture of ethyl 3-(1-(*tert*-butoxycarbonyl)-2-pyrrolidinyl)-3-hydroxypropionate (3.06 g, 10.6 mmol) and NaOH (0.80 g, 20 mmol) in H_2O:THF:methanol (3:3:1, 35 mL) was stirred at 25 °C for 2 h. The mixture was concentrated gently (30 °C) under vacuum. The aqueous solution was acidified with 1.0 N HCl to pH = 5 and extracted with EtOAc. The organic washings were dried ($MgSO_4$), filtered, and vacuum-concentrated. This residue was recrysallized from hexane/EtOAc yielding 1.19 g (47%) of a colorless solid (A, mp = 122–4 °C). The mother liquor was concentrated down to a viscous, pale yellow oil (B). Elemental analysis: calcd, 55.58, C; 8.16, H; 5.40, N; found, 55.39, C; 8.23, H; 5.37, N. Mass spectral analysis: 260 (M + 1, 60), 204 (M - tBu + 1, 100), 160 (M - 100 + 1, 70). ^{1}H NMR (DMSO): δ 4.09 (m, 1H), 3.57 (br s, 1 H), 3.31 (m, 1H), 3.15 (m, 1H), 2.10–2.40 (m, 2H), 1.60–2.00 (m, 4H), 1.38 (s, 9H). TLC: R_f = 0.30 with 10% MeOH/1:1 hexane/EtOAc on silica. HPLC: one major peak on a NOVA-PAK C_{18} column (4-μm packing, 5- × 100-mm column) with 50% methanol/H_2O/0.1% TCA/0.1% triethylamine (A = 93:7 ratio, B = 1:2 ratio observed on extended HPLC). The two products were determined to be different stereoisomers at the hydroxy position. Both stereoisomers were used in individual peptides; the more active of the two was **6**.

Peptide Synthesis and Purification. The peptides were synthesized using an improved version of the solid-phase method described by R. B. Merrifield,[16] using an Applied Biosystems Inc. (ABI) Model 430A peptide synthesizer.

The appropriate resin (0.5 mmol), e.g., Boc-DProΨ[CH_2NH]-Phe-MBHA, was loaded in the synthesizer, and a standard deprotection (TFA/DCM)–neutralization (DIEA/DCM) program (ABI) was performed.

Boc-protected amino acids were coupled to the resin using a modified program to suit the BOP-coupling procedure.[17] This protocol involved the use of manually filled ABI cartridges containing 1 mmol each of the Boc-amino acid and BOP. These contents were dissolved in DMF and transferred to the activator chamber of the instrument. There, 1 M 1-methylimidazole (or 4-methylmorpholine) (1 mL) in DMF was added, forming the active ester. This active ester was transferred through the concentrator chamber directly to the reaction vessel for coupling to the resin. A small amount of DMF was added from the top of this vessel to aid in mixing of the resin during the coupling time of 1 h. The resin was filtered, and a series of DMF and DCM washes were performed.

After the peptide was assembled on the resin, the peptide was deblocked and cleaved from the resin with liquid HF containing 10% anisole for 1 h at 0 °C, in a variation of the method described by Sakakibara et al.[28] The peptide and the resin were washed with EtOAc. The peptide was then extracted with an aqueous 1% acetic acid solution and freeze-dried to obtain the dry, solid peptide.

The peptides were then purified by reverse-phase HPLC using a Vydac C_{18} (218TP1022) column on a Waters Delta-Prep 3000 system equipped with a Gilson Model 116 UV detector. Purification was achieved by equilibrating the column with 0.1% TFA/water and developing with a linear gradient of CH_3CN from 10% to 40% in 20 min at 20 mL/min. Samples were collected manually and checked for purity on a Spectra-Physics analytical HPLC system (including SP8700, SP8440, SP8780, and SP4200) utilizing a Vydac C_{18} (218TP54) column. A flow rate of 1.5 mL/min was employed using a 0.1% TFA/water:0.1% TFA/CH_3CN gradient from 10% to 60% ACN over 10 min.

The composition of each analog was assessed using fast atom bombardment (FAB)–mass spectroscopy and amino acid analysis (see Table 1). The sample of interest was dissolved in glycerol prior to analysis by FAB-MS. FAB–mass spectra were obtained on a VG Analytical (Manchester, U.K.) 70SQ mass spectrometer of EBQQ geometry using a VG Analytical 11-250J data system for data acquisition. The mass spectrometer was operated at a 7-kV accelerating potential and a resolution of 1000 (10% valley definition). The FAB gun used in the experiments was an Ion Tech (Teddington, U.K.) FAB11N operating at a 7-kV potential and a 1-mA current. Xenon was used as the bombardment gas at a source pressure of 1 × 10^{-5} mbar. Amino acid analysis was performed by the following procedure. Approximately 1–10 nmol of peptide was placed into an acid-washed Pyrex tube. The peptide was hydrolyzed under a reduced N_2 atmosphere with 6 M HCl and 1% phenol for 1 h at 150 °C. The peptide was then dried with a 2:2:1 (vol/vol) mixture of ethanol/water/triethylamine and derivatized with a 7:1:1:1 solution of ethanol/water/triethylamine/phenyl isothiocyanate. The derivatized amino acids were then analyzed by reverse-phase HPLC. Neither of these characterization techniques showed major impurities in our analogs.

Biological Methods. Mitogenicity Assay. Swiss 3T3 cells were a gift from E. Rozengurt (passage unknown). All studies were performed on density arrested, quiescent cells as described previously.[15,29]

Briefly, mitogenicity assays were performed by overlaying peptide solution in triplicate on cell monolayers in 96-well trays (Linbro). Cell culture was resumed for 18 h, and then, cultures were pulsed for 2 h with 0.5 μCi [CH_3-^{3}H]thymidine (6.7 Ci/mmol, New England Nuclear) per well. Inhibition of bombesin-induced mitogenicity was performed similarly, where test peptide solutions, generally $\log_5$ dilutions, were added in the presence of 6 nM bombesin. The monolayers were washed three times with ice-cold phosphate-buffered saline, fixed with cold 40% methanol/12% acetic acid, rinsed with phosphate-buffered saline, solubilized with detergent, and mixed with scintillant (Ready Safe, Beckman Instruments), and the radioactivity was determined by liquid scintillation counting (LKB, 30% efficiency).

Binding (S3T3 Cells). Binding was carried out similarly to published studies.[30–32] Peptides were serially diluted in maintenance medium and overlayed in triplicate on quiescent monolayers in 24-well trays in a total volume of 300 μL containing approximately 10^5 cells and [^{125}I]GRP (50 pM). Binding was carried out at 37 °C for 30 min, whereupon monolayers were washed three times with chilled maintenance medium and solubilized with detergent and the radioactivity was determined (LKB, 55% efficiency).

Binding (S3T3 Membrane). Swiss 3T3 membranes were prepared using the method of Naldini et al.[32] For the binding assay, aliquots of the membrane suspension (10 μg of protein) were added to polypropylene tubes in triplicate with binding medium containing HEPES–KOH, pH 6.8 (20 mM), EGTA (1 mM), $MgCl_2$ (5 mM), bovine serum albumin (0.5 mg/mL) with protease inhibitors aprotinin (10 μg/mL), leupeptin (50 μg/mL), pepstatin (5 μg/mL), and bacitracin (200 μg/mL), and PMSF (1 mM) in a final volume of 1 mL and incubated with [^{125}I]GRP-(1-27) (10 pM, 2000 Ci/mmol:porcine:Amersham Searle) in a shaking 37-°C water bath. Nonspecific binding was defined as [^{125}I]GRP(1-27) binding in the presence of unlabeled GRP(1-27) (10 nM, porcine:Peninsula). Specific binding was calculated by subtracting nonspecific binding from total binding. The binding reaction was terminated after 60 min by suction filtration (Brandel) and the mixture washed with ice-cold buffer containing HEPES–KOH pH 6.8 (20 mM) and bovine serum albumin (0.5 mg/mL) through Whatman GF/B glass fiber filters that had been pretreated overnight with 0.3% polyethyleneimine (Sigma). The filters were rapidly washed four times, removed to tubes, and counted in a γ counter (LKB Wallac 1272). Half-maximum inhibition of specific binding of [^{125}I]GRP(1-27) was determined in the absence or presence of increasing concentrations of unlabeled peptide. The data was calculated using HILLPLOT analysis.

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