Synthesis and Biological Evaluation of Bombesin Constrained Analogues†

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Analogues of bombesin which incorporate dipeptide or turn mimetics have been synthesized. One of them (compound **11**) containing a seven-membered lactam ring revealed a good affinity for GRP/BN receptors on rat pancreatic acini (K_i value of 1.7 ± 0.4 nM) and on Swiss 3T3 cells (K_i value of 1.0 ± 0.2 nM). On the basis of this observation, antagonists containing the same dipeptide mimic were obtained by modification of the C-terminal part of the bombesin analogues. The most potent constrained compounds (**15** and **17**) were able to antagonize 1 nM bombesin-stimulated amylase secretion from rat pancreatic acini with high potency (K_i = 21 ± 3 and 3.3 ± 1.0 nM, respectively) and 10^{-7} M bombesin-stimulated [^{3}H]thymidine incorporation into Swiss 3T3 cells (K_i = 7.8 ± 2.0 and 0.5 ± 0.1 nM, respectively).

Introduction

The amphibian tetra-decapeptide bombesin (BN),[1] mammalian-related gastrin-releasing peptides (GRP),[2] and neuromedin B (NMB)[3] have been shown to stimulate a wide variety of biological responses in the CNS and the gastrointestinal tract, and they have been proposed to play the role of growth factor in human small-cell lung carcinoma systems in vitro[4,5] and in vivo.[6] These latter observations suggest that GRP receptor antagonists may have clinical utility as inhibitors of the physiological response to GRP in human diseases.

A large number of GRP receptor antagonists have been reported,[7] including analogues of bombesin resulting from side-chain modification strategies, analogues of bombesin with modified peptide bonds,[8,9] and bombesin analogues with a modified C-terminal part. Recently, attention has been focused on the conformation of these analogues and their possible role in the interaction with the receptors. Earlier studies showed the importance of Trp[8] and His[12] for the biological activity of BN and GRP. Coy et al.[8] and Leban et al.[10] suggested that bombesin agonists at the GRP receptor adopt a folded antiparallel β-sheet with a γ-turn at Val[10]-Gly[11]-His[12]-Leu[13]. Horwell et al. proposed a new binding conformation of Ac-BN(7−14) consisting of three consecutive γ-turns followed by a bend and finishing with two γ-turns at the NMB and GRP receptors.[11]

We report here the synthesis and biological activity of conformationally restricted bombesin analogues containing nonpeptide moieties. Some of these bombesin analogues exhibited high affinity toward BN receptors, indicating that the presence of a turn in the molecule is favorable for interaction with the receptor.

In the design of peptidomimetics, incorporation of nonpeptide scaffolds into bioactive molecules has been the focus of extensive research over the last 10 years.[12] Conformationally restrained surrogates have been extensively used in the design and synthesis of enzyme inhibitors and antagonists of peptide hormone receptors.[13] The first example of a nonpeptide β-turn mimetic incorporated into a peptide hormone was described by Freidinger et al. to produce a potent LH-RH agonist.[14] We have substituted the dipeptide Val-Gly in the potent (D)Phe-Gln-Trp-Ala-Val-Gly-His-Leu-Leu-NH_2 bombesin agonist by several turn mimetics and extended our study to other constrained dipeptide surrogates. We explored the use of ring (five-, six-, and seven-membered) templates as conformational constraints in these bombesin analogues.

Chemistry

Compounds **1**−**13** were all prepared stepwise on solid support MBHA resin and compounds **18** and **19** on a hydroxymethylpolystyrene resin (Novabiochem, Switzerland) with a procedure derived from those described by Floyd et al.[15] Compounds **14**−**17** were synthesized in solution by condensation of two fragments under conditions where no racemization could occur: activation of the N-terminal fragment at the carboxylic group branched on the seven-membered lactam and acylation by the H-His-Leu modified dipeptides. All amino acids were coupled as their Nα-Boc derivatives with BOP[16] as activating reagent.

H-(D)Phe-Gln-Trp-Ala-Val-Gly-His-Leu-Leu-NH_2 (compound **1**), a potent agonist of the BN/GRP receptor,[17,18] was synthesized as reference for binding studies. The dipeptide Val-Gly was successively replaced by 2-aminobenzoic acid (2-ABA) (compound **2**), 3-aminobenzoic acid (3-ABA) (compound **3**), 4-aminobenzoic acid (4-ABA) (compound **4**), (2*S*,4*S*)-4-amino-1-benzoylpyrrolidine-2-carboxylic acid (compound **6**), five-membered lactam rings (compounds **7**, **9**, and **10**), (*R*,*S*)-3-amino-*N*-1-

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† Abbreviations: BN, bombesin; GRP, gastrin-releasing peptide; BOP [(benzotriazolyl)oxy]tris(dimethylaminophosphonium)hexafluorophosphate; DME, ethylene glycol dimethyl ether; DMF, dimethylformamide; DIEA, *N*-diisopropylethylamine; pHPPA, *p*-hydroxy-3-phenylpropionyl. Other abbreviations used are those recommended by the IUPAC−IUB Commission (*Eur. J. Biochem*. **1984**, *138*, 9−37).

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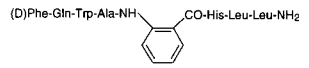
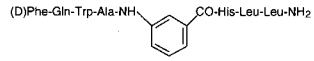
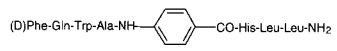
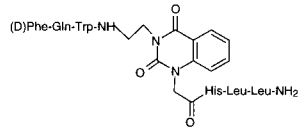
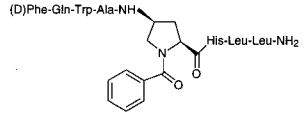
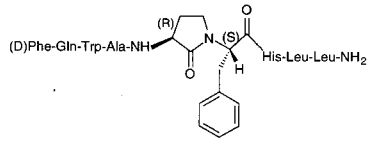
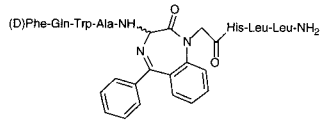
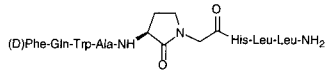
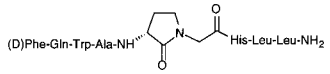
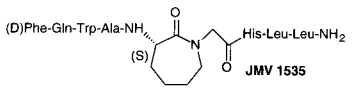
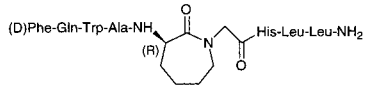
Table 1. Affinity of Compounds **1**−**12** for BN/GRP Receptors on Rat Pancreatic Acini

	Compounds	Binding Ki (nM)
1	(D)Phe-Gln-Trp-Ala-Val-Gly-His-Leu-Leu-NH_2	3.2 ± 1
2		650 ± 60
3		300 ± 35
4		2250 ± 200
5		$>10^{-6}$
6		$>10^{-6}$
7		$>10^{-6}$
8		500 ± 80
9		225 ± 45
10		$>10^{6}$
11	 JMV 1535	1.7 ± 0.4
12		

carboxymethyl-2-oxo-5-phenyl-1,4-benzodiazepine (compound **8**), and seven-membered lactam rings (compounds **11**−**19**); in compound **5**, 3-(2-aminoethyl)-1-carboxymethylquinazoline-2,4-dione was introduced in place of the tripeptide Ala-Val-Gly.

Results and Discussion

All the dipeptide mimetics were first introduced in the sequence H-(D)Phe-Gln-Trp-Ala-X-His-Leu-Leu-NH_2 (except in compound **5**, where the mimetic was supposed to replace Ala-Val-Gly). These bombesin analogues were tested for their capacity to bind GRP/BN receptors on rat pancreatic acini (see Table 1). They were found to have poor affinity for these receptors except for compound **11** (JMV 1535) which exhibited a K_i value of 1.7 ± 0.4 nM (as potent as the BN analogue **1**), whereas its analogue **12**, containing the diastereoisomer (3*R*)-seven-membered lactam ring, presented weak affinity. Compound **11** also showed high affinity for the BN/GRP receptors on Swiss 3T3 cells (K_i = 1.0 ± 0.2 nM). Compound **11** behaved as an agonist at the BN/GRP receptors, being about 7 times less potent than the BN analogue **1** in stimulating amylase secretion on rat pancreatic acini (EC_{50} = 0.22 ± 0.17 nM) and about 4 times more potent than compound **1** in stimulating [^{3}H]-thymidine accumulation in Swiss 3T3 cells (EC_{50} = 0.02 ± 0.01 nM) with full efficacy. Replacement of the N-terminal (D)Phe residue by pHPPA (*p*-hydroxy-3-phenylpropionyl) in **11** resulted in compound **13** which presented the same affinity in recognizing the BN/GRP receptors in rat pancreatic acini (K_i = 1.8 ± 0.6 nM) and was about 4 times more potent in stimulating amylase secretion (EC_{50} = 0.05 ± 0.04 nM). Compound **13** had almost the same affinity as compound **11** on Swiss 3T3 cells (Table 2). These results led us to focus our attention on this dipeptide mimic. According to the strategy developed for the design of bombesin receptor antagonists,[19] we synthesized bombesin analogues with a modified C-terminal part and incorporating this dipeptide mimic.

To obtain GRP/BN receptors antagonists in bombesin analogues incorporating the diazepin-2-one moiety, we replaced the C-terminal tripeptide amide His-Leu-Leu-NH_2 by substitutions which are known to yield potent BN receptor antagonists. The tripeptide was replaced by His-Leu-OMe[20] (compounds **14** and **15**), by histidyl-amino-2-hydroxy-1-isobutylhexane (compounds **16** and **17**),[19] or by His-Leu-NHOH (compounds **18** and **19**).[21] As shown in Table 2, all these compounds were tested for their capacity to displace (3-[^{125}I]iodotyrosyl^{15})GRP from rat pancreatic acini and Swiss 3T3 cells. All the bombesin analogues **14**−**19** were able to interact with the GRP/BN receptors on rat pancreatic acini and Swiss 3T3 cells with K_i values ranging from 0.8 to 127 nM, the most potent compounds being **15** and **17**, with affinities for the rat pancreatic GRP/BN receptors of 3.1 ± 2.1 and 3.8 ± 2.9 nM, respectively. These two compounds also showed high affinity for the GRP/BN receptors on Swiss 3T3 cells (K_i = 1.2 ± 0.6 nM for **15** and 0.8 ± 0.4 nM for **17**) (Table 2). None of the compounds **14**−**19** exhibited agonist activity on rat pancreatic acini or Swiss 3T3 cells up to a concentration of 10^{-6} M. The most interesting compounds (e.g. **14**−**17** and **19**) were tested for their antagonist properties versus bombesin. The most potent compounds (**15** and **17**) were able to antagonize 1 nM bombesin-stimulated amylase secretion from rat pancreatic acini with high potency (K_i = 21 ± 3 nM for **15** and 3.3 ± 1.0 nM for **17**). Similarly, they antagonized the action of 10^{-7} M bombesin-stimulated [^{3}H]thymidine incorporation into Swiss 3T3 cells (K_i = 7.8 ± 2.0 nM for **15** and 0.5 ± 0.1 nM for **17**). Once again, replacement of the N-terminal (D)Phe residue by pHPPA resulted in compounds with enhanced potency (Table 2). The results observed for compounds **16** and **17** confirmed those that were obtained with JMV 641,[22] indicating the high antagonist potency of bombesin analogues with a hydroxy-containing moiety in their C-terminal part. The results of this study confirm that the dipeptide mimic *N*-carbonyl-(methyl)-3*S*-aminoazepin-2-one efficiently replaces the dipeptide Val-Gly in (D)Phe^6-bombesin(6−14) analogues. These results also confirm that the approach for the synthesis of BN/GRP receptor antagonists can be combined with the incorporation of dipeptide mimics. An extended solution structure study of compound **11** by ^{1}H NMR did not allow us to assess the presence of a

Table 2. Biological Activities of Compounds **11**−**19**

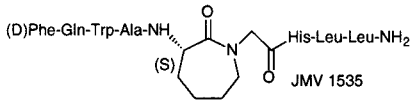
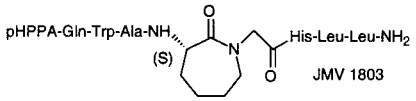
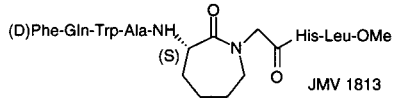
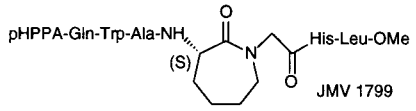
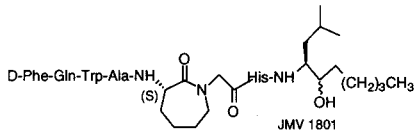
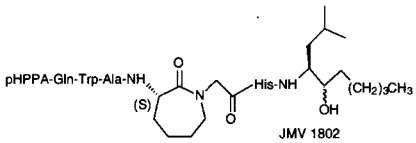
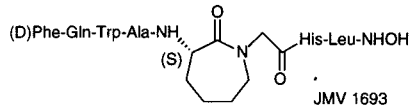
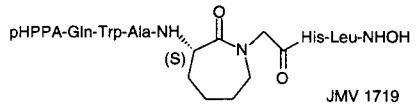
	Compounds	Rat Pancreatic Acini			Swiss 3T3 Cells		
		Binding	Amylase Release		Binding	Proliferation	
		Ki (nM)	EC50 (nM)	Ki (nM)	Ki (nM)	EC50 (nM)	Ki (nM)
1	(D)Phe-Gln-Trp-Ala-Val-Gly-His-Leu-Leu-NH_2	3.2 ± 1.0	0.03 ± 0.01		6.3 ± 2.5	0.08 ± 0.02	
11	 JMV 1535	1.7 ± 0.4	0.22 ± 0.17		1.0 ± 0.2	0.02 ± 0.01	
13	 JMV 1803	1.8 ± 0.6	0.05 ± 0.04		2.0 ± 0.2	0.2 ± 0.1	
14	 JMV 1813	5.2 ± 1.4		240 ± 200	2.0 ± 0.2		86 ± 20
15	 JMV 1799	3.1 ± 2.1		21 ± 3	1.2 ± 0.6		7.8 ± 2.0
16	 JMV 1801	9 ± 5		72 ± 6	2.0 ± 0.2		20 ± 10
17	 JMV 1802	3.8 ± 2.9		3.3 ± 1.0	0.8 ± 0.4		0.5 ± 0.1
18	 JMV 1693	127 ± 60		640 ± 320	53 ± 10		$>10^{-6}$
19	 JMV 1719	13 ± 3		160 ± 100	11 ± 2		9.5 ± 4.0

Table 3. ^{1}H NMR Characteristics of Compound **11** in DMSO-d_6[a]

residues	NH	α	β	γ	δ	ε	other
(D)Phe	8.06	4.08	3.06−2.91				7.33, 7.26(Ar)
Gln	8.53	4.33	1.78−1.63	1.96			7.19−6.76(NH_2)
Trp	8.27	4.57	3.16−2.92				10.77(NH), 7.32(H7), 7.05(H6), 7.68(H4), 7.17(H2), 6.97(H5)
Ala	8.22	4.35	1.22				
lactam	7.84	4.55	1.74−1.39	1.84	1.64−1.52	3.58−3.15	
CH_2		4.07−3.96					
His	8.24	4.61	3.09−2.91				8.82(H2), 7.32(H4)
Leu	8.10	4.26	1.48	1.60	0.88−0.84		
Leu	7.98	4.23	1.46	1.59	0.88−0.82		7.23−6.95(NH_2)

[a] Proton chemical shifts are in ppm.

turn around this mimic since no significant NOEs were observed. Chemical shifts of compound **11** are presented in Table 3. However, compound **11**, bearing a constrained structure within its sequence and which is a potent agonist at the BN/GRP receptors, should be a useful compound to study the molecular interactions of BN with its receptors.

Experimental Section

Melting points were taken on a Buchi apparatus in open capillary tubes. Optical rotation values were determined with a Perkin-Elmer 141 polarimeter at 20 °C. Ascending TLC was performed on precoated plates of silica gel 60 F_{254} (Merck) with different solvent systems (A: AcOEt:hexane, 3:7; B: AcOEt:hexane, 1:5), peptide derivatives were located with Charring reagent or ninhydrin. L,D-Amino acids and derivatives were from Bachem, Novabiochem, or Propeptide. All reagents were of analytical grade.

Compounds **1**−**13** were synthesized manually and stepwise on a MBHA solid support (0.8 mmol/g); compounds **18** and **19** were synthesized on a hydroxamate resin prepared from a hydroxymethyl resin (Novabiochem) with a Floyd derived procedure.[15] Three equivalents of Boc-amino acid and BOP were used; DIEA was added until the apparent pH on moisted pH paper was around 9. The acylation step was monitored by

the Kaiser test; deprotection of the temporary Boc-N-protection was accomplished with a mixture of TFA:DCM (40:60) (v:v) for 3 min and then for 27 min; EDT was added to this mixture (2%) as soon as a Trp residue was present in the sequence. The N-terminal Boc protection was removed under the same conditions before release of the peptide from the resin. This cleavage was performed with anhydrous HF at 0 °C for 1 h in the presence of 10% anisole; compounds **18** and **19** were cleaved by anhydrous HF at room temperature. After evaporation of HF, compounds were precipitated with diethyl ether and filtered. They were separated from the resin by solubilization in a mixture of CH_3CN:H_2O:TFA (50:50:0.1) (v:v:v) and lyophilized. Then they were purified by RP-HPLC.

Compounds **14**−**17** were prepared in solution by condensation of two fragments. The N-terminal fragment was coupled by activation of the azepin-2-one carboxylic function to the C-terminal fragment. BOP reagent was used for the synthesis of the fragments and for fragment coupling steps. These last steps of fragment couplings were monitored by analytical RP-HPLC to assess the completion of the reaction, and the crude peptides were directly loaded in preparative RP-HPLC to be purified. In all cases, crude peptides were purified using preparative RP-HPLC to a purity >95% (based on HPLC with detection at 214 nm). Compounds were all characterized by analytical HPLC, capillary electrophoresis, and electrospray mass spectrometry (Table 3). RP-HPLC analysis was performed on a Beckman apparatus composed of a 507e autosampler, a 168 detector and a 126 solvent module pump with system gold Nouveau software using UV detection at 214 and 254 nm on a Waters Deltapak 4.6- × 150-mm 5-μm C-18 column, which was eluted at 1 mL/min. The elution conditions were standardized and were as follow: the column was equilibrated with water containing 0.1% TFA; after injection the sample was eluted over a period of 50 min by a gradient of 0−100% acetonitrile containing 0.1 TFA. Preparative RP-HPLC was performed on a Waters Delta Prep 4000 equipped with a 486 tunable absorbance detector and a Waters Deltapak 40- × 100-mm, 15-μm column. Detection was monitored at 214 nm, flow rate was 50 mL/min, and compounds were eluted with a gradient mode from water containing 0.1% TFA to acetonitrile containing 0.1% TFA with a slope depending on the compound. Fractions were manually collected and the pure ones were gathered and lyophilized.

Samples were analyzed on a Platform II (Micromass, Manchester, U.K.) quadrupole mass spectrometer fitted with an electrospray interface. The mass spectrometer was calibrated in the positive-ion ESI mode using a mixture of NaI and CsI. Data were acquired in the scan mode from *m*/*z* 200 to 2800 in 4 s; 15 scans were summed to produce the final spectrum. The ESI capillary was set at a potential of 3.6 kV, the source was heated at 60 °C, and the cone voltage was optimized from 30 to 90 V depending on the sample under study. Nitrogen was used as both drying and nebulizing gas. Samples were dissolved in acetonitrile:water (50:50) (v:v) and infused into the ESI source at a flow rate of 10 μL/min. Capillary zone electrophoresis was performed on a PACE 5000 Beckman instrument using an uncoated fused silica capillary (75 μm × 50 cm, 100 × 800 μm aperture) at 23 °C in a 65 mM sodium tetraborate buffer, pH 7; the sample was introduced by pressure injection and submitted to 15 kV. NMR spectrum was recorded on a 360 MHz Bruker AMX spectrometer in DMSO-d_6. Physicochemical characteristics of the synthesized compounds are reported in Table 4.

(*R*)-3-[(*tert*-Butyloxycarbonyl)amino]-2-oxopyrrolidine-(*S*)-1-phenyl-2-propionic acid in compound **7**, (*S*)-3-[(*tert*-butyloxycarbonyl)amino]-2-oxo-1-pyrrolidineacetic acid in compound **9** and (*R*)-3-[(*tert*-butyloxycarbonyl)amino]-2-oxo-1-pyrrolidineacetic acid in compound **10** were obtained according to Freidinger and al.[23] *N*-[(Benzyloxycarbonyl)methyl]-3*S*-[(*tert*-butyloxycarbonyl)amino]azepin-2-one (in compounds **11** and **13**−**19**) and its enantiomer (compound **12**) were synthesized as described by Amblard et al.[24] Briefly, *N*-[(benzyloxycarbonyl)methyl]-3*S*-[(*tert*-butyloxycarbonyl)amino]azepin-2-one was prepared by cyclization of Boc-L-lysine in the presence of sodium bicarbonate as "insoluble base" with BOP as coupling reagent. Alkylation of the nitrogen amide with benzyl bromoacetate followed by removal of the benzyl protecting group by hydrogenolysis afforded *N*-[(benzyloxycarbonyl)methyl]-3*S*-[(*tert*-butyloxycarbonyl)amino]azepin-2-one. 3-(2-Aminoethyl)-1-carboxymethylquinazoline-2,4-dione (in compound **5**) was synthesized according to Gouilleux et al.,[25] 4-amino-1-benzoylpyrrolidine-2-carboxylic acid (in compound **6**) was synthesized according to Curran et al.,[26] and (*R*,*S*)-3-amino-*N*-1-carboxymethyl-2-oxo-5-phenyl-1,4-benzodiazepine (in compound **8**) was from Neosystem (France).

Table 4. Physicochemical Characteristics of Compounds **1**−**19**

compd	yield (%)	formula	MH^+	HPLC[b]	CZE[d]
1	52	$C_{53}H_{76}N_{14}O_9$	1069	23.43[c]	ND
2	45	$C_{53}H_{69}N_{13}O_9$	1032	32.0	ND
3	48	$C_{53}H_{69}N_{13}O_9$	1032	35.23	ND
4	42	$C_{53}H_{69}N_{13}O_9$	1032	35.03	ND
5	23	$C_{55}H_{70}N_{14}O_{10}$	1087	19.12	6.62
6	31	$C_{58}H_{76}N_{14}O_{10}$	1129	19.09	6.27
7	23	$C_{59}H_{78}N_{14}O_{10}$	1143	21.03	7.11
8	42	$C_{63}H_{77}N_{15}O_{10}$	1204	22.68	5.90
9	45	$C_{52}H_{72}N_{14}O_{10}$	1053	30.99	ND
10	37	$C_{52}H_{72}N_{14}O_{10}$	1053	18.44	5.66
11	76	$C_{54}H_{76}N_{14}O_{10}$	1081	22.40	7.06
12	44	$C_{54}H_{76}N_{14}O_{10}$	1081	18.23	7.12
13	72	$C_{54}H_{75}N_{13}O_{11}$	1082	21.02	5.56
14	72[a]	$C_{49}H_{66}N_{12}O_{10}$	983	20.14	5.55
15	68[a]	$C_{52}H_{74}N_{12}O_9$	1011	21.77	2.95*
16	69[a]	$C_{49}H_{65}N_{11}O_{11}$	984	21.63	5.37
17	67[a]	$C_{52}H_{73}N_{11}O_{10}$	1012	24.06	5.42
18	14[a]	$C_{48}H_{65}N_{13}O_{10}$	984	16.87	6.11
19	23[a]	$C_{48}H_{64}N_{12}O_{11}$	985	18.09	6.08

[a] Yield from fragment condensation. [b] RP-HPLC retention time as described in the Experimental Section, monitoring at 214 nm. [c] Monitored at 279 nm. [d] Capillary zone electrophoresis retention time as described in the Experimental Section, except for compound **15** which was analyzed at 30 kV. ND: not determined.

Boc-Leu-$(CH_2)_3$-CH_3. Magnesium (1.95 g, 80 mmol) and bromobutane (8.61 mL, 80 mmol) were dissolved in anhydrous THF (30 mL). To start the reaction, dibromoethane was added. The mixture was heated in refluxing THF. When all the magnesium was dissolved, Boc-Leu-N(OCH_3)CH_3[27] (5.49 g, 20 mmol) in THF (80 mL) was added dropwise. The reaction mixture was stirred for 2 h at 0 °C; 1 N HCl (200 mL) was added followed by ethyl acetate (100 mL). The organic layer was washed with 1 M potassium hydrogen sulfate, water, dried (Na_2SO_4) and concentrated in vacuo. The expected product was purified by silica gel column chromatography with ethyl acetate:hexane (1:5) as eluent: yield 70% (3.8 g); TLC R_{fA} 0.6, R_{fB} 0.28; mp 51−53 °C; $[\alpha]^{20}_D$ −45 (*c* 1.2, DMF). Anal. ($C_{15}H_{29}N_1O_3$) C, H, N.

Boc-LeuΨ(C*HOH)-$(CH_2)_3$-CH_3. To a solution of Boc-Leu-$(CH_2)_3$-CH_3 (1.5 g, 5.53 mmol) in methanol (20 mL) was added $NaBH_4$ (0.21 g, 5.53 mmol). After 60 min, the reaction mixture was concentrated in vacuo to about 3 mL. The expected product was precipitated with a saturated bicarbonate solution (100 mL). It was collected by filtration, washed with water and hexane and dried over KOH pellets. The compound was obtained and used as a mixture of diaastereoisomers containing 50% of **R* and 50% of **S* as determined by HPLC: yield 73% (1.11 g); TLC R_{fB} 0.6; mp 95−96 °C. Anal. ($C_{15}H_{31}N_1O_3$) C, H, N.

Biological Evaluation. All compounds were dissolved in dimethyl sulfoxide (Merck Art. 2951) and stored at −20 °C. Dilutions were made in the incubation medium, and the maximal final concentration did not contain more than 1% DMSO.

Materials. HEPES, D-glucose, calcium chloride, soybean trypsin inhibitor, bacitracin, sodium chloride, potassium chloride, magnesium chloride, sodium pyruvate, and sodium fumarate were from Sigma Chemical Co., St. Louis, MO; purified collagenase (0.88 PZ U/mg) was from Serva Feinbiochemica GmbH & Co., Heidelberg; glutamine, DMEM, and the

essential vitamin mixture were from Gibco Life Technologies Ltd., Scotland; Phadebas amylase test reagent was from Pharmacia France S.A.; bovine plasma albumin (fraction V, pH 7) was from Euromedex, Schiltigheim; (3-[^{125}I]iodotyrosyl^{15})-GRP (2000 Ci/mmol) was from Amersham France S.A.

The buffer used for rat pancreatic acini preparation contained 25.5 mM HEPES (pH 7.4), 98 mM NaCl, 6 mM KCl, 2.5 mM NaH_2PO_4, 5 mM sodium pyruvate, 5 mM sodium glutamate, 2 mM glutamine, 11.5 mM glucose, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 0.01% (w:v) trypsin inhibitor, 1% (v:v) amino acid mixture, and 1% (v:v) essential vitamin mixture.

The standard incubation medium for the amylase release test was the buffer described previously supplemented with 1% (w/v) bovine serum albumin (BSA). The binding medium (pH 7.4) was Krebs−Henseleit buffer (pH 7.4) from Sigma Chemical Co., St. Louis, MO, supplemented with 1% (w:v) bovine plasma albumin. For washing, Krebs−Henseleit buffer was supplemented with 4% (w:v) bovine plasma albumin.

Experiments on Dispersed Rat Pancreatic Acini. Tissue preparation: Male Wistar rats (200−300 g) were obtained from the pharmacological breeding center of Montpellier University. Dispersed acini from rat pancreas were prepared by using the modification[28] of the method described previously.[29]

Binding of (3-[^{125}I]iodotyrosyl^{15})GRP to rat pancreatic acini: Briefly, samples (0.5 mL, containing about 1 mg/mL protein) were incubated for 60 min at 37 °C in the presence of 20 pM (3-[^{125}I]iodotyrosyl^{15})GRP and various concentrations of BN analogues. After centrifugation at 10000*g* and two washings, the radioactivity associated with the acinar pellet was measured. Nonspecific binding was determined in the presence of 10 μM unlabeled BN and was always less than 25% of the total binding. These results were expressed as a percentage of the specific binding (mean ± SD from three independent experiments performed in duplicate).

Amylase release test: Dispersed acini were suspended in 0.5 mL of standard incubation containing about 1 mg of protein/mL. Samples were incubated for 30 min at 37 °C and amylase release was measured as described previously.[30,31] Amylase activity was determined by the method of Ceska[32] using the Phadebas reagent. Amylase release was calculated as the percentage of maximal amylase activity obtained with optimal concentration of the reference BN. Incubations were performed in duplicate and mean values were used for calculations.

Effect of peptides on bombesin-stimulated amylase release: Antagonist activity was determined as described previously.[33] Various concentrations of the analogues to be tested were incubated with 1 nM BN, a concentration that causes maximal stimulation.

Experiments on Swiss 3T3 Cells. Cell culture: Swiss 3T3 cells were a gift from Dr. E. Rozengurt (Imperial College, U.K.). Cells were maintained at 37 °C in a humidified atmosphere containing 10% CO_2 by serial passage in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1 nM glutamine, and 1% penicillin/streptomycin. Before each experiment, cells were seeded into 24-well plates at a concentration of 10^5 cells/well and incubated for 24 h at 37 °C in maintenance medium supplemented with 0.2% BSA without fetal bovine.

Binding of (3-[^{125}I]iodotyrosyl^{15})GRP to Swiss 3T3 cells: After incubation for 24 h, cells were incubated with 1 mL of DMEM containing 0.2% BSA with 20 pM [^{125}I]GRP (Amersham, U.K.) and with various concentration of BN or other compounds for 1 h at 22 °C. The cells were then washed twice with cold phosphate-buffered saline (PBS) supplemented with 0.2% BSA and solubilized with 1 M NaOH, and associated radioactivity was determined. Nonspecific binding was determined in the presence of 10 μM BN. Nonspecific binding was <15% of total binding in all experiments performed in duplicate in at least three separate trials.

Assays of [^{3}H]thymidine incorporation: After incubation for 24 h, the culture medium was removed and DMEM containing 0.2% BSA was added. Cells were incubated for an additional 24 h at 37 °C and then treated with various concentrations of BN or analogues or BN plus analogues. After incubation for 24 h at 37 °C, 0.5 μCi of [^{3}H]thymidine (Amersham, U.K.) was added to each well. After an additional 2-h incubation, cells were washed twice with cold PBS and incubated in 5% trichloroacetic acid for 30 min; then the cells were washed twice with 95% ethanol. Cells were solubilized in 1 M NaOH; samples were removed from the plates, neutralized with 1 M HCl, placed in vials, and assayed for [^{3}H]-thymidine incorporation as described previously.[34]

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