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Synthesis and Biological Evaluation of C-Terminal Hydroxamide Analogues of Bombesin

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Abstract: Bombesin pseudo-peptide analogues containing a hydroxamide function on the C-terminal part of the molecule, e.g. H-D-Phe-Gln-Trp-Ala-Val-Gly-His-Leu-NHOBzl **1** and H-D-Phe-Gln-Trp-Ala-Val-Gly-His-Leu-NHOH **2** were synthesized. These compounds were tested for their ability to recognize the bombesin receptor on rat pancreatic acini and on 3T3 cells, to stimulate (i) amylase secretion from rat pancreatic acini and (ii) accumulation of tritiated thymidine in 3T3 cells. Compounds **1** and **2** were able to recognize bombesin receptors on both models with high affinity ($K_i = 7 \pm 2$ and 5.8 ± 0.9 nM on rat pancreatic acini, and $K_i = 4.1 \pm 1.2$ and 7.7 ± 1.9 nM on 3T3 cells, respectively). Interestingly, compound **1** behaved as a potent agonist in stimulating amylase secretion from rat pancreatic acini and is able to stimulate thymidine accumulation in 3T3 cells, while compound **2** was able to potently antagonize bombesin-stimulated amylase secretion ($K_i = 22 \pm 5$ nM) in rat pancreatic acini and had no proper effect on 3T3 cells; however, it was able to inhibit bombesin-stimulated thymidine accumulation in 3T3 cells with high potency ($K_i = 1.6 \pm 0.6$ nM).

Keywords: antagonist; bombesin; hydroxamide; proliferation; receptors

INTRODUCTION

Bombesin (BN) is a 14 amino acid peptide that was originally isolated from amphibian skin [1]. Gastrin releasing peptide (GRP) is a mammalian 27 amino acid peptide that has very similar biological properties to that of bombesin. Small cell lung carcinoma cell lines (SCLC) express high affinity receptors for GRP and BN, and exhibit a mitogenic response to these peptides, suggesting that they can function as autocrine factors for these cells. On this basis, it has been strongly suggested that bombesin receptor antagonists should be able to suppress the growth of these cells. On the other hand, it has recently been suggested that bombesin receptor antagonists may have an interesting beneficial effect on prostate cancer treatment in addition with LH-RH [2]. As a result, there has been considerable interest in the design and development of competitive BN or GRP receptor antagonists as possible therapeutic agents [3].

The authors have previously reported on a strategy for designing gastrin receptor antagonists, either by suppressing the C-terminal amino acid residue [4], the C-terminal amide function [5] or by chemically modifying a specific peptide bond within the peptide chain [6]. The mechanism of action of gastrin receptor antagonists was tentatively explained by the existence of an activating enzyme system 'associated' with the receptor, responsible

Abbreviations: BN, bombesin; GRP, gastrin releasing peptide; SCLC, small cell lung carcinoma cell lines; BOP, [(benzotriazolyl)oxy]tris(dimethylaminophosphonium)-hexa-fluorophosphate; DME, ethylene glycol dimethylether; DMF, dimethylformamide; DIEA, *N*-diisopropylethylamine; Other abbreviations used were those recommended by IUPAC-IUB Commission [*Eur. J. Biochem. 138*, 9–37 (1984)].

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for the production by hydrolysis of the 'active part' of the peptide hormone released within the receptor [7]. This strategy was successfully applied to the synthesis of various peptide hormone receptor antagonists, being always amidated peptide hormones (i.e. secretin, substance P, etc.). Particularly, this strategy of peptide backbone modification through carbonyl reduction led Coy *et al.* to the design of the first potent bombesin receptor antagonist [Leu^{13}-ψ(CH_2–NH)-Leu^{14}]-bombesin [8]. On the other hand, this pseudo-peptide showed selectivity for the bombesin receptor and was potent in antagonizing Swiss mouse 3T3 cell growth in culture in response to bombesin. Several other bombesin receptor antagonists were developed in various laboratories, mainly by modifying the peptide bond between the two last C-terminal residues at positions 13 and 14, or by suppressing the C-terminal amide function [9]. In this report, the synthesis of a specific and potent bombesin receptor antagonist, in which the C-terminal Leu-NH_2 has been replaced by a hydroxyamide function, is reported. This compound exhibited interesting biological activities and was able to inhibit cell proliferation in the Swiss 3T3 model. The same component with the hydroxyl of hydroxamide function masked with a protecting group acts as an agonist in both the biological systems.

MATERIALS AND METHODS

Chemistry

Melting points were taken on a Buchi apparatus in open capillary tubes. Optical rotation were determined with a Perkin-Elmer 141 polarimeter at 20°C. Ascending TLC was performed on pre-coated plates of silica gel 60 F_{254} (Merck) with the following solvent systems (by volume): AcOEt/hexane: A, (1/5); B, (3/7); C, (5/5); D, (7/3); AcOEt: E; $CHCl_3$/MeOH/AcOH: F, (180/10/5); G, (120/10/5); H, (85/10/5); I, (60/10/5); J, (40/10/5). Peptide derivatives were located with charring reagent or ninhydrine. Column chromatography was performed with silica gel Kieselguhr Merck® G 0.05–0.2 mm. HPLC purifications were run on a Waters delta Prep 4000 instrument on a Delta-pack C18 column (15 μm), 100 Å, 40 × 100 mm, with an UV detection at 220 nm, at a flow rate of 50 ml/min of a mixture of C: acetonitrile/TFA (0.1) and D: H_2O/TFA (0.1%), and HPLC analytical controls were run on a Merck-Hitachi apparatus with a Lichrosorb C18 column (5 μm) 100 Å, 250 × 4.6 mm, 1 ml/min, at 220 nm with a gradient from 0% C to 50% C in 50 min. Mass spectra were recorded on a JEOL JMS DX 300-SX 102 spectrometer in FAB positive mode. Both compounds were also characterized by H^1-NMR studies as described in Table 1. L- and D-aminoacids and derivatives were from Bachem, Novabiochem or Propeptide. All reagents were of analytical grade.

H-D-Phe-Gln-Trp-Ala-Val-Gly-His-Leu-NHOBzl, 1

Boc-Leu-NHOBzl, 1a. Boc-Leu-NCA (1.27 g, 5 mmol) was dissolved in DMF (10 ml) containing HCl, H_2N–OBzl (0.8 g, 5 mmol) and DIEA (0.86 ml, 5 mmol) was added to this solution. The reaction mixture was stirred for 1 h at room temperature. Then, an aqueous saturated sodium bicarbonate solution (50 ml) was added under stirring, followed by ethyl acetate (50 ml). The organic layer was washed with aqueous saturated sodium bicarbonate solution (2 × 50 ml), water, 1 M potassium hydrogen sulphate aqueous solution (2 × 50 ml), water, dried over sodium sulphate and then concentrated *in vacuo* to yield a clear oil: yield 100% (1.75 g); TLC R_f (B) 0.59, (C) 0.88; $[\alpha]_D$-14 (*c* 0.8, DMF).

Boc-His(Boc)-Leu-NHOBzl, 1b. Compound **1a** (1.75 g, 5 mmol) was partially deprotected with trifluoroacetic acid (20 ml). After 30 min at room temperature, the trifluoroacetic acid was removed *in vacuo* by co-evaporation with hexane. The expected TFA salt was crystallized in ether/hexane, 1/10 (100 ml). It was collected, washed with ether and dried *in vacuo* over KOH. Yield 86% (1.51 g). The TFA salt was dissolved in DMF (10 ml) containing Boc-His(Boc)-OH (4.31 mmol), and BOP (1.9 g, 4.31 mmol). DIEA (1.5 ml, 8.62 mmol) was added to this solution and the reaction mixture was stirred overnight at room temperature. Then, an aqueous saturated sodium bicarbonate solution (50 ml) was added under stirring, followed by ethyl acetate (50 ml). The organic layer was washed with an aqueous saturated sodium bicarbonate solution (2 × 50 ml), water, 1 M aqueous potassium hydrogen sulphate solution (2 × 50 ml), water, dried over sodium sulphate and then concentrated *in vacuo* to leave a residue that solidified upon trituration in hexane. It was collected, washed with hexane and dried *in vacuo* over KOH. Yield 89% (2.2 g); TLC R_f (D) 0.47, (A) 0.92; m.p. 90–92°C (dec.); $[\alpha]_D$-21 (*c* 1.6, DMF).

Boc-Gly-OBg, 1c. DBU (2.72 ml, 18.26 mmol) and benzhydrylglycolamide bromide (5 g, 16.43 mmol) were successively added to a solution of Boc-Gly-OH (3.2 g, 18.26 mmol) in acetonitrile (50 ml). The

Table 1 ^{1}H-NMR Characteristics of Compounds **1** and **2** in d_6-DMSO

Residue	NH	a	b	c	d	Other
Compound **1**						
D-Phe	—	4.06	3.06–2.81			7.33, 7.27 (Ar)
Gln	8.53 (7.9)	4.32	1.79–1.62	1.95		7.19–6.76 (NH_2)
Trp	8.27 (7.8)	4.56	3.12–2.91			10.78 (NH), 7.32 (H7), 7.05 (H6), 7.66 (H4), 7.17 (H2), 6.97 (H5)
Ala	8.21 (7.6)	4.40	1.19 (7.0)			
Val	7.74 (8.5)	4.16	1.97	0.85–0.84		
Gly	8.18	3.79–3.70 (5.4–16.4)				
His	8.07 (8.3)	4.61	3.05–2.92			8.89 (H2), 7.29 (H4)
Leu	8.09 (7.8)	4.17	1.47	1.54	0.87–0.83	10.78 (NH)
Compound **2**						
D-Phe	—	4.08	3.05–2.92			7.33, 7.27 (Ar)
Gln	8.53 (7.9)	4.32	1.79–1.63	1.94		7.19–6.75 (NH_2)
Trp	8.28 (7.9)	4.55	3.11–2.93			10.79 (NH), 7.32 (H7), 7.05 (H6), 7.66 (H4), 7.17 (H2), 6.97 (H5)
Ala	8.20 (7.2)	4.40	1.19 (7.0)			
Val	7.74 (8.5)	4.16	1.97	0.85–0.83		
Gly	8.19	3.79–3.70 (5.4–16.4)				
His	8.09 (7.6)	4.62	3.05–2.96			8.83 (H2), 7.30 (H4)
Leu	8.15 (7.6)	4.11	1.46	1.50	0.85–0.80 (6.3)	11.43 (NH), 4.77 (CH_2), 7.38 (Ar)

Proton chemical shifts in ppm and proton–proton coupling constants in Hz in brackets. Spectra were recorded on a 360 MHz Bruker AMX spectrometer.

reaction mixture was refluxed under stirring for 2 h, then cooled. A saturated sodium bicarbonate solution (100 ml) was added under stirring, followed by ethyl acetate (200 ml). The organic layer was washed with a saturated sodium bicarbonate solution (3 × 100 ml), water, 1 M potassium hydrogen sulphate (3 × 100 ml), water, dried over sodium sulphate and then concentrated *in vacuo* to leave a residue that cristallized in an ether/hexane solution (1/5). It was collected and dried *in vacuo* over P_2O_5. Yield 91% (5.96 g); TLC R_f (C) 0.55, (D) 0.79; m.p. 115–120°C (dec.).

Boc-Val-Gly-OBg*, *1d. Compound **1c** (4 g, 10.05 mmol) was partially deprotected with trifluoroacetic acid (20 ml). After 30 min at room temperature, the trifluoroacetic acid was removed *in vacuo* by co-evaporation with hexane. The expected TFA salt was precipitated in ether (100 ml). It was collected, washed with ether and dried *in vacuo* over KOH. Yield 100% (4.1 g). The TFA salt (2 g, 4.85 mmol) was dissolved in DMF (50 ml). Boc-Val-OH (1.05 g, 4.85 mmol), BOP (2.14 g, 4.85 mmol) and DIEA (1.67 ml, 9.7 mmol) were added and the reaction mixture was stirred for 2 h at room temperature. The reaction mixture was treated as described for compound **1b**. Yield 91% (2.19 g); TLC R_f (E) 0.82, (D) 0.55; m.p. 130–135°C; $[\alpha]_D$-8 (*c* 1.2, DMF).

Boc-Ala-Val-Gly-OBg*, *1e. Compound **1d** (1 g, 2.01 mmol) was partially deprotected with trifluoroacetic acid (20 ml). After 30 min at room temperature, the trifluoroacetic acid was removed *in vacuo* by co-evaporation with hexane. The expected TFA salt was dissolved in DMF (50 ml) containing Boc-Ala-OH (0.38 g, 2.01 mmol), BOP (0.88 g, 2.01 mmol) and DIEA (0.7 ml, 4.02 mmol). The mixture was stirred for 2 h at room temperature. Then, a saturated sodium bicarbonate solution (100 ml) was added. The resulting precipitate was filtered, washed with water, 1 M potassium hydrogen sulphate solution, water, hexane, ether and dried *in vacuo* over KOH pellets. Yield 97% (1.11 g); TLC R_f (A) 0.59, (D) 0.22; m.p. 179–181°C; $[\alpha]_D$-19.5 (*c* 1, DMF).

***Fmoc-Trp-Ala-Val-Gly-OBg*, *1f*.** Compound **1e** (1 g, 1.76 mmol) was partially deprotected with trifluoroacetic acid (10 ml) as previously described. Yield 100% (1.02 g). The TFA salt (1.02 g, 1.76 mmol) was dissolved in DMF (50 ml). Fmoc-Trp-OH (0.75 g, 1.76 mmol), BOP (0.78 g, 1.76 mmol) and DIEA (0.6 ml, 3.52 mmol) were added and the reaction mixture was stirred overnight at room temperature. The reaction mixture was treated as described for compound **1e**. Yield 100% (1.54 g); TLC R_f (E) 0.36, (F) 0.58; m.p. 190–192°C; $[\alpha]_D$-19 (*c* 0.9, DMF).

***Fmoc-Gln-Trp-Ala-Val-Gly-OBg*, *1g*.** Compound **1f** (1.54 g, 1.71 mmol) was partially deprotected in a mixture of DMF (17 ml) and diethylamine (1.73 ml, 17.1 mmol) for 1 h at room temperature. The mixture was concentrated *in vacuo*. The residue was dissolved in DMF (50 ml) containing Fmoc-Gln-OH (0.63 g, 1.71 mmol), BOP (0.76 g, 1.71 mmol) and DIEA (0.29 ml, 1.71 mmol). The reaction mixture was stirred overnight at room temperature and treated as described for compound **1e**. Yield 96% (1.65 g); TLC R_f (F) 0.2, (G) 0.53; m.p. 190–195°C; $[\alpha]_D$-9 (*c* 0.7, DMF).

***Boc-D-Phe-Gln-Trp-Ala-Val-Gly-OBg*, *1h*.** Compound **1g** (1.5 g, 1.49 mmol) was partially deprotected in a mixture of DMF (15 ml) and diethylamine (1.5 ml, 14.9 mmol) for 1 h at room temperature. The mixture was concentrated *in vacuo*. The expected amine was precipitated in ether (50 ml). It was collected, washed with ether and dried *in vacuo* over KOH. Yield 100%. It was dissolved in DMF containing Boc-D-PheOH (0.39 g, 1.49 mmol), BOP (0.66 g, 1.49 mmol) and DIEA (0.26 ml, 1.49 mmol). The mixture was stirred overnight at room temperature and treated as described for compound **1e**. Yield 85% (1.3 g); TLC R_f (H) 0.67, (G) 0.39; m.p. 210–212°C; $[\alpha]_D$-18 (*c* 1.4, DMF).

***Boc-D-Phe-Gln-Trp-Ala-Val-Gly-OH*, *1i*.** Compound **1h** (1 g, 0.97 mmol) was dissolved in DMF (40 ml) and partially deprotected by addition of a solution containing potassium carbonate (0.4 g, 2.91 mmol) in water (20 ml) at room temperature. After 1 h, the mixture was concentrated *in vacuo*. The expected acid was precipitated in an aqueous solution of 1 M potassium hydrogen sulphate (100 ml). It was collected, washed with ether and dried *in vacuo* over KOH. Yield 93% (0.73 g); TLC R_f (J) 0.7, (I) 0.2; m.p. 195–197°C; $[\alpha]_D$-26 (*c* 0.9, DMF).

***Boc-D-Phe-Gln-Trp-Ala-Val-Gly-His-Leu-NHOBzl*, *1j*.** Compound **1b** (0.26 g, 0.46 mmol) was partially deprotected with trifluoroacetic acid (10 ml) as previously described. The TFA salt (0.27 g, 0.46 mmol) was dissolved in DMF containing Boc-D-Phe-Gln-Trp-Ala-Val-Gly-OH **1i** (0.37 g, 0.46 mmol), BOP (0.2 g, 0.46 mmol) and DIEA (0. ml, 1.38 mmol). After a night at room temperature, the reaction mixture was concentrated *in vacuo*. The expected compound was precipitated in ethyl acetate (50 ml). It was collected, washed with ethyl acetate and dried *in vacuo* over KOH. Yield 98% (0.53 g); TLC R_f (I) 0.66, (J) 0.82; m.p. 160–162°C; $[\alpha]_D$-16 (*c* 1.3, DMF).

H-D-Phe-Gln-Trp-Ala-Val-Gly-His-Leu-NHOBzl, 1

Compound **1j** (0.4 g, 0.34 mmol) was added to a solution of 2-methyl-indole (452 mg, 3.44 mmol) in TFA (10 ml) and the mixture was stirred for 30 min at room temperature. The expected compound was precipitated upon addition of ether (100 ml). It was collected, thoroughly washed with ether, and dried *in vacuo* over KOH. Yield 100% (0.4 g). It was finally purified by preparative RP-HPLC with a gradient from 0% C to 20% C in 5 min, to 25% C in 5 min, then to 50% C in 50 min and lyophilized. $R_t = 40.5$ min (analytical HPLC conditions given in the Material and Methods section). $M + H^+ = 1061$. ^{1}H-NMR chemical shifts are given in Table 1.

H-D-Phe-Gln-Trp-Ala-Val-Gly-His-Leu-NHOH, 2

Compound **1j** (0.2 g, 0.17 mmol) was dissolved in 95% EtOH (100 ml) at room temperature and hydrogenated in the presence of a 10% Pd/C catalyst. After 2 days, the catalyst was removed by filtration and the filtrate concentrated *in vacuo*. The compound was dried i*n vacuo* over KOH. Yield 95% (0.16 g). It was finally purified by preparative RP-HPLC with a gradient from 0% C to 15% C in 5 min, to 20% C in 5 min, then to 40% C in 20 min and lyophilized. $R_t = 27.9$ min (analytical HPLC conditions given in the Material and Methods section). $M + H^+ = 971$. ^{1}H-NMR chemical shifts are given in Table 1.

PHARMACOLOGY

In Vitro Studies on Rat Pancreatic Acini

All compounds were dissolved in dimethylsulphoxide (Merck Art 2951) and then appropriately diluted. Final solutions used for testing 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, sodium fumarate were from Sigma, St Louis, MO.; purified collagenase (0.88 PZ U/mg) was from Serva Feinbiochemica GmbH, Heidelberg; glutamine, MEM non-essential amino acids (100 ×), 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]iodo-tyrosyl^{15}) gastrin releasing peptide was from Amersham France S.A.

The buffer used for rat pancreatic acini preparation contained 25.5 ml 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, l.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 binding solution (pH 7.4) was Krebs–Henseleit buffer from Sigma (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.

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 using the modification [10] of the method described previously [11].

Binding of (3-(^{125}I)iodotyrosyl^{15}) Gastrin Releasing Peptide to Acini. Briefly, samples (0.5 ml containing about 1 mg/ml protein) were incubated for 60 min at 37°C [12] in the presence of 20 pM (3-[^{125}I]Tyr^{15}) gastrin releasing peptide and various concentrations of bombesin analogs. After centrifugation at 10000 × *g* and two washings, the radioactivity associated with the acinar pellet was measured [13]. Binding in the absence of any unlabeled bombesin–peptide was 7.5% of the total radioactivity present in the sample. Non-specific binding was determined in the presence of 10 μM of unlabeled bombesin and was always less than 25% of the total binding. These results were expressed as a percentage of the specific binding.

Amylase Release Test. Dispersed acini were suspended in 0.5 ml of standard incubation media containing about 1 mg/ml of protein; samples were incubated for 30 min at 37°C and amylase release was measured as described previously [14,15]. Amylase activity was determined by the method of Ceska [16], using the Phadebas reagent. Amylase release was calculated as the percentage of maximal amylase activity obtained with optimal concentration of the reference BN.

Effect of Peptides on Bombesin-stimulated Amylase Release. Antagonist activity was determined as described previously [17]. Various concentrations of analogues to be tested were incubated in the presence of 1 nM BN that causes maximal stimulation of amylase secretion.

In Vitro Studies on SWISS 3T3 Cells. All compounds were dissolved in dimethylsulphoxide (Merck Art 2951) and then appropriately diluted. Final solutions used for testing did not contain more than 1% DMSO.

Cell cultures

Swiss 3T3 cells were a gift from Dr E. Rozengurt (Imperial College, UK). 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 an experiment, cells were seeded into 24-well plates at a concentration of 10^5 cells per 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}) Gastrin Releasing Peptide to Swiss 3T3 Cells. After 24 h incubation time, cells were incubated with 1 ml DMEM containing 0.2% BSA with 20 pM ^{125}I-GRP (2000 Ci/mmol per well) (Amersham, UK) and with various concentration of bombesin or compounds for 1 h at 22°C. The cells were then washed twice with cold phosphate saline buffer (PBS) supplemented with 0.2% BSA, solubilized with 1 M NaOH and associated radioactivity was determined. Non-specific binding was determined in the presence of 10 μM bombesin. Non-specific 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 24 h incubation time, 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 were treated with various concentrations of

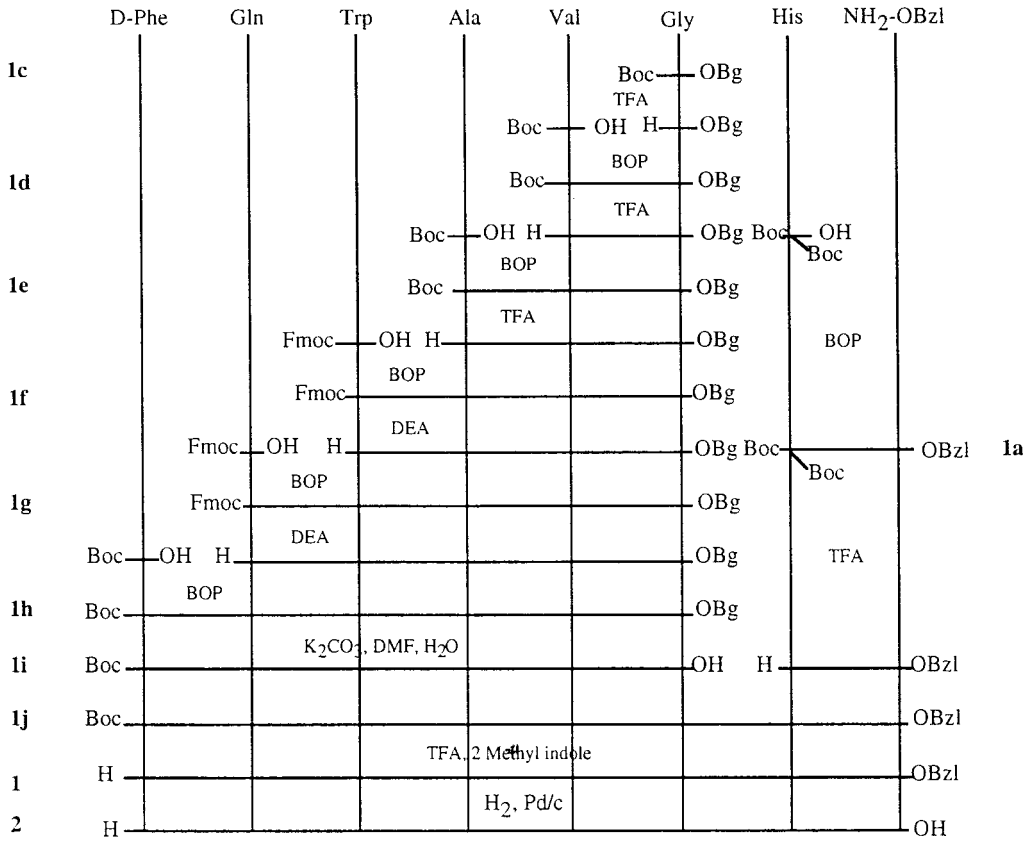

Figure 1 Scheme of the solution synthesis of both pseudo-peptides.

bombesin or compounds or bombesin plus compounds. After 24 h incubation at 37°C, 0.5 mCi [^{3}H]thymidine (Amersham, UK) was added in each well. After an additional 2 h incubation cells were washed twice with cold phosphate buffered saline (PBS), with 5% trichloroacetic acid (TCA) during 30 min and twice with 95% ethanol. Cells were solubilized in 1 M NaOH, samples were removed from the plates, placed in vials and assayed for [^{3}H]thymidine incorporation as described previously [18].

RESULTS

The bombesin analogues **1** and **2** were synthesized in solution, as described in Figure 1. Bombesin was synthesized on solid phase, using a MBHA resin and according to a Boc strategy. They were tested for their biological activities on rat pancreatic acini and on 3T3 cells. Results are gathered in Table 2. Compounds **1** and **2** were able to inhibit binding of ^{125}I-GRP to rat pancreatic with high potency ($K_i = 7 \pm 2$ nM and 5.8 ± 0.9 nM, respectively) (Figure 2). Peptide **1** behaved as a full agonist in stimulating amylase secretion from rat pancreatic acini, inducing a maximal response at about 50 nM that plateaued, and producing half maximal response at 3.5 nM (Figure 3). Peptide **2** was without proper effect on rat pancreatic acini (Figure 3). However, compound **2** dose-dependently inhibited 1 nM bombesin-stimulated amylase release ($K_i = 22 \pm 5$ nM) (Figure 4).

As previously reported, bombesin inhibits binding of ^{125}I-GRP to 3T3 cells. Peptides **1** and **2** were potent in the binding inhibition assay of ^{125}I-GRP on these cells ($K_i = 4.1 \pm 1.2$ nM and 7.7 ± 1.9 nM, respectively) (Figure 5). Bombesin dose-dependently stimulated [^{3}H]thymidine incorporation in Swiss 3T3 at about 100 nM bombesin with a maximal response that was about ten times over the non-stimulated level. Peptide **1** was found to be able to stimulate [^{3}H]thymidine incorporation ($EC_{50} = 100 \pm 10$ nM) while peptide **2** was without any proper effect on 3T3 cells even at high doses (up to 10 μM) (Figure 6). However, compound **2** was able to dose-dependently inhibit 100 nM bombesin-stimulated [^{3}H]thymidine incorporation into serum-starved 3T3 cells with K_i of 1.6 ± 0.5 nM (Figure 7).

Table 2 Biological Activity of Bombesin and Bombesin Analogues **1** and **2** on Rat Pancreatic Acini and in Swiss 3T3 Cells

Compounds	Rat pancreatic acini			3T3 cells		
	Binding	Amylase release		Binding	[^{3}H]Thymidine accumulation	
	K_i (nM)	EC_{50} (nM)	K_i (nM)	K_i (nM)	EC_{50} (nM)	K_i (nM)
Bombesin	1.8 ± 0.9	0.07 ± 0.05		1.6 ± 0.7	5.9 ± 1.1	
1	7 ± 2	3.5 ± 1.2		4.1 ± 1.2	100 ± 10	
2	5.8 ± 0.9		22 ± 0.5	7.7 ± 1.9		1.6 ± 0.6

DISCUSSION

In this work, the authors have synthesized two new octapeptide analogues of bombesin in which the C-terminal methionine amide residue has been replaced by a benzyl protected hydroxylamine (e.g. compound **1**) or by hydroxylamine (e.g. compound **2**) obtained after hydrogenolysis of compound **1**.

Both compounds **1** and **2** were able to recognize the bombesin receptor on rat pancreatic acini with high affinity. Interestingly, compound **1** behaved as a potent bombesin receptor full agonist in stimulating amylase secretion from rat pancreatic acini, about 50 times less potent than bombesin itself. This result was quite unexpected, since suppression of the C-terminal amide function of bombesin or suppression of the C-terminal residue usually resulted in bombesin receptor antagonists [9]. However, it indicates that the C-terminal amide bond is crucial neither for recognizing the bombesin receptor, nor for the biological activity on rat pancreatic acini. It indicates also that the C-terminal methionine amide of bombesin can be mimicked by a benzyl hydroxylamine. Compound **2** behaved as a potent

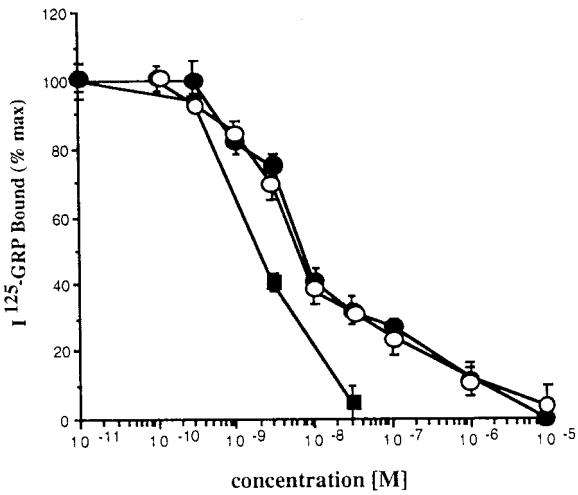

Figure 2 Ability of bombesin (■), peptide **1** (●) and peptide **2** (○) to inhibit binding of ^{125}I-GRP to pancreatic acini. Results are expressed as the percentage of the specific binding obtained with ^{125}I-GRP alone. Non-specific binding was determined in the presence of 10 μM bombesin and was always less than 15% of the total binding. In each experiment, each value was determined in duplicate, and the results given are the means from three separate experiments.

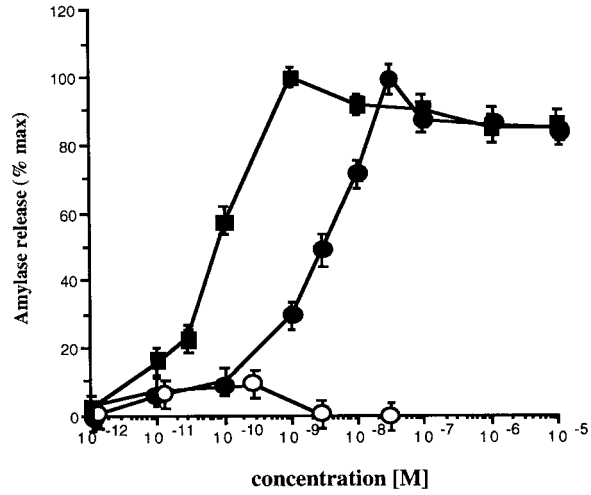

Figure 3 Effects of bombesin (■), peptide **1** (●) and peptide **2** (○) on amylase release from rat pancreatic acini. Dispersed pancreatic acini from rat were prepared and amylase release was measured as the difference of amylase activity at the end of incubation that was released into the extracellular medium with or without secretagogue and expressed as the percentage of maximal stimulation. In each experiment, each value was determined in duplicate, and the results given are the means from three separate experiments.

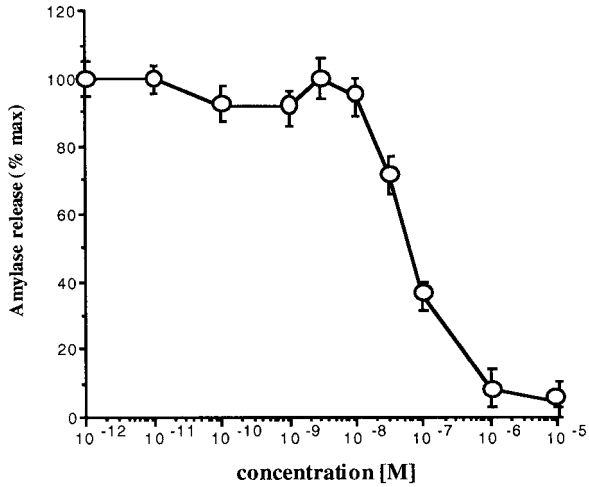

Figure 4 Ability of peptide **2** (○) to inhibit bombesin-stimulated amylase secretion from rat pancreatic acini. Acini were incubated for 30 min at 37°C with various concentrations of peptide **2** plus 1 nM bombesin. Results are expressed as the percentage of the stimulation of amylase secretion obtained without peptide **2**. In each experiment, each value was determined in duplicate, and the results given are the means from three separate experiments.

bombesin receptor antagonist on rat pancreatic acini, its antagonist potency being in accordance with its affinity for the bombesin receptor.

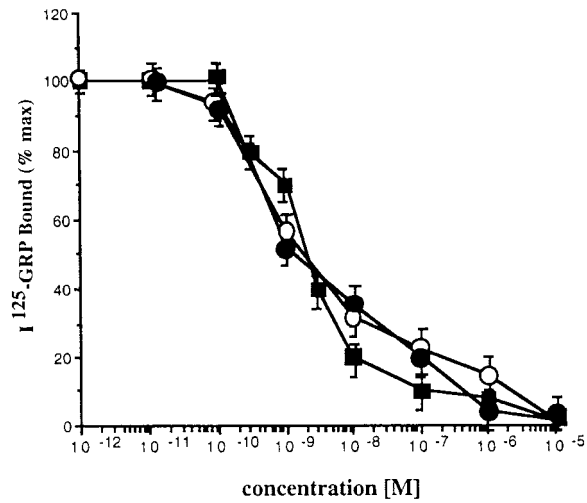

Figure 5 Ability of bombesin (■), peptide **1** (●) and peptide **2** (○) to inhibit binding of ^{125}I-GRP to Swiss 3T3 cells. Results are expressed as the percentage of the specific binding obtained with ^{125}I-GRP alone. Non-specific binding was determined in the presence of 10 μM bombesin and was always less than 15% of the total binding. In each experiment, each value was determined in duplicate, and the results given are the means from three separate experiments.

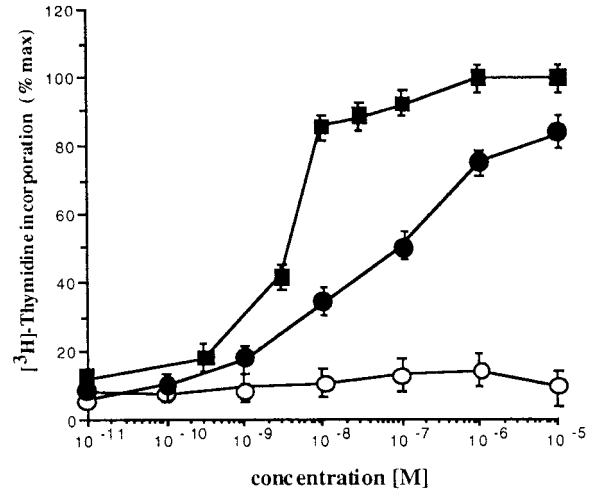

Figure 6 Capacity of bombesin (■), peptide **1** (●) and peptide **2** (○) to induce [^{3}H]thymidine incorporation into quiescent Swiss 3T3 (agonism). Maximal response represented about 10-times the non-stimulated response. In each experiment, each value was determined in triplicate, and the results given are the means from four separate experiments.

On 3T3 cells, both compounds **1** and **2** were able to recognize the bombesin receptor with high affinity. As expected from the results with pancreatic acini, peptide **1** was able to stimulate proliferation of 3T3 cells, as measured by the tritiated thymidine incorporation. Compound **2** was able to potently antagonize the effect of bombesin on thymidine in-

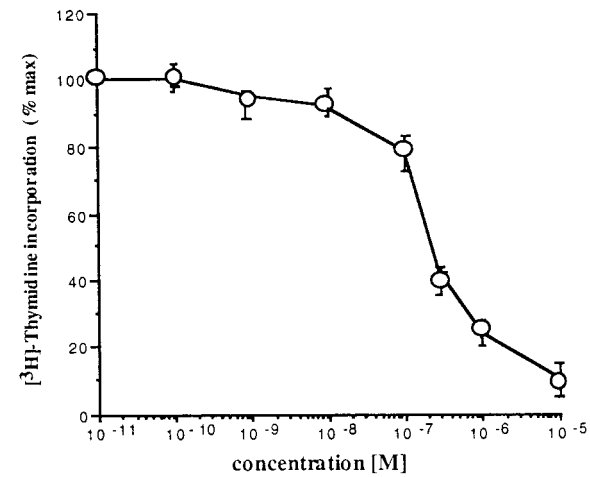

Figure 7 Capacity of peptide **2** (○) to inhibit 100 nM bombesin-stimulated [^{3}H]thymidine incorporation into quiescent Swiss 3T3 cells. In each experiment, each value was determined in triplicate, and the results given are the means from four separate experiments.

corporation into these cells, even at low concentrations. In fact its inhibitory potency was more effective than expected from its binding affinity.

In conclusion, the authors have shown in this paper that the C-terminal amidated residue of bombesin could be mimicked by a *O*-benzyl hydroxylamine function; it was then possible to obtain bombesin analogues without the C-terminal amidated residue with high affinity for the bombesin receptor and exhibiting full agonist activity on rat pancreatic acini and a weaker agonist activity on 3T3 cells. When the protecting group of the hydroxyl function is removed, the obtained compound (peptide **2**) presented potent antagonist properties against bombesin/GRP receptors in both tested models.

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