Influence of Lipophilicity on the Biological Activity of Cyclic Pseudopeptide NK-2 Receptor Antagonists[1]

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A series of cyclic pseudopeptides of the formula cyclo(LeuΨ[CH_2NH]Xaa-Gln-Trp-Phe-βAla), where Xaa represents the residue of an α-amino acid, has been synthesized in order to establish the role of the Xaa side chain for tachykinin NK-2 receptor antagonist activity. Syntheses have been carried out in solid phase with either Fmoc or Boc strategy. The antagonist potency on NK-2 receptors in the hamster isolated trachea (HT) and the rabbit isolated pulmonary artery (RPA) bioassays increases with Xaa lipophilicity; cyclo(LeuΨ[CH_2NH]Cha-Gln-Trp-Phe-βAla) and cyclo(LeuΨ[CH_2NH]Asp(NHBzl)-Gln-Trp-Phe-βAla) resulted in being the two most active antagonists (pA_2 = 9.06 and 9.26 on HT, respectively). A significant linear correlation was found between pA_2 values determined in HT and RPA bioassays and capacity factors measured in reversed phase HPLC. The comparison between the biological activities of cyclic hexapeptides containing or not containing the aminomethylene moiety proved the crucial role of the pseudopeptide bond for determining high antagonist potency at the NK-2 receptor.

Introduction

Neurokinin A (NKA) is, among the three natural mammalian tachykinins, the most potent and selective agonist at the NK-2 receptor. Since antagonists of NKA might be useful in the therapy of asthma, inflammation, and excessive intestinal motility, this field has aroused a great interest. A limited number of NK-2 receptor antagonists, either peptide[2–6] or nonpeptide[7] in nature, has been developed in the last few years. Among the different approaches used to determine the structural requirements for interaction with the NK-2 receptor, that based on the use of conformational constraints led to the development of rigid NKA analogs, such as cyclic peptides and lactams.[8] One of the most interesting structural types of NKA antagonists is represented by the family of cyclic hexapeptides exemplified by L659,-877 (cyclo(Leu-Met-Gln-Trp-Phe-Gly)). This was the first potent antagonist with nanomolar affinity at NK-2 receptors to be developed.[8] Recently Harbeson et al.[9] explored the introduction of the aminomethylene moiety in the place of each amide bond in the NKA (4-10) peptide backbone. In this study, the introduction of the LeuΨ[$CH_2N(CH_2)_2CH_3$]Phe moiety in the place of the C-terminal Leu-Met dipeptide fragment led to a pseudopeptide showing affinity for the NK-2 receptor comparable to that of the natural ligand NKA (IC_{50} = 1 nM on hamster urinary bladder). The same approach has been used for L659,877, by substituting the Leu-Met dipeptide with the pseudodipeptide moiety LeuΨ[CH_2NCH_3]Leu, to give a potent and selective NK-2 receptor antagonist, cyclo(LeuΨ[CH_2NCH_3]Leu-Gln-Trp-Phe-Gly) (MDL29,913, pA_2 = 8.6 on hamster trachea).[10]

With these examples in mind, we aimed to verify thoroughly the role of the amino acid in the Met position, previously considered to be uninfluential for the expression of biological activity.[10] We replaced the Met residue with basic, acidic, aromatic, or aliphatic residues. In order to improve the selectivity for the NK-2 receptor, we substituted also Gly with βAla, an approach which has been employed in our laboratories to improve selectivity for the NK-2 receptor in the case of linear peptide derivatives.[11] Therefore a series of pseudopeptides of the formula cyclo(LeuΨ[CH_2NH]Xaa-Gln-Trp-Phe-βAla) was synthesized. The antagonist activity at the NK-2 receptor has been determined on the rabbit isolated pulmonary artery (RPA) and the hamster isolated trachea (HT) preparations, two bioassays endowed with two putative NK-2 receptor subtypes,[12–14] with the aim of determining the influence of increasing hydrophobicity on the selectivity of antagonists. The introduction of lipophilic side chains caused a considerable increase in potency, which appears to be linearly related to the lipophilicity of the molecule. The importance of the aminomethylene moiety for receptor interaction was also investigated by synthesizing two analogs without this modification.

Peptide synthesis was carried out in solid phase, with either Fmoc or Boc strategies. In order to increase the antagonist activity and/or the metabolic stability of the compounds, new amino acid derivatives, such as β-amides of Boc-protected aspartic acid, were also synthesized.

Results

Chemistry. The synthesis of the linear precursors H-LeuΨ[CH_2NR]Xaa-Gln-Trp-Phe-βAla-OH (peptides **1–11**, Table 1) was carried out in solid phase with the methodology described by Sasaki and Coy.[15] For peptides **1–8**, Fmoc strategy on *p*-(benzyloxy)benzyl alcohol resin was used. Formation of the aminomethylene moiety in solid phase with Fmoc chemistry has been recently described, the preferred reducing agent for the conversion of intermediate Fmoc-AA-N(OCH_3)CH_3 to the corresponding aldehyde being $LiAlH_4$.[16] In agreement with the literature data, a very low extent of

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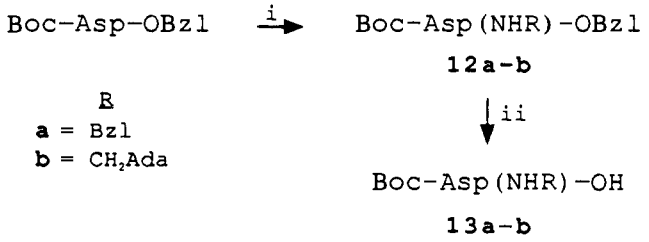
Table 1. Structures of Linear Pseudopeptides

compd	sequence
1	H-LeuΨ[CH_2NH]**Met**-Gln-Trp-Phe-βAla-OH
2	H-LeuΨ[CH_2NH]**Leu**-Gln-Trp-Phe-βAla-OH
3	H-LeuΨ[CH_2NH]**Phe**-Gln-Trp-Phe-βAla-OH
4	H-LeuΨ[CH_2NH]**Nal**-Gln-Trp-Phe-βAla-OH
5	H-LeuΨ[CH_2NH]**Asp(OBzl)**-Gln-Trp-Phe-βAla-OH
6	H-LeuΨ[CH_2NH]**Asn**-Gln-Trp-Phe-βAla-OH
7	H-LeuΨ[CH_2NH]**Cha**-Gln-Trp-Phe-βAla-OH
8	H-LeuΨ[CH_2NH]**Lys(Z)**-Gln-Trp-Phe-βAla-OH
9	H-LeuΨ[CH_2NH]**Asp(NHCH$_2$Ada)**-Gln-Trp-Phe-βAla-OH
10	H-LeuΨ[CH_2NH]**Asp(NHBzl)**-Gln-Trp-Phe-βAla-OH
11	H-LeuΨ[CH_2NCH_3]**Cha**-Gln-Trp-Phe-βAla-OH

Scheme 1[a]



[a] Reagents: (i) H_2N-R, PyBOP, DIEA; (ii) H_2, Pd/C, ethanol.

racemization was observed in the reductive alkylation step. The presence of diastereoisomers in the isolated products, **1**−**11**, was checked using two different HPLC systems. A peak very close to that of the desired compound was present in nearly every crude product for peptides **1**−**10**, but its amount was invariably less than 2% of the main compound. Only for peptide **8** (Xaa = Lys(Z)) the area of this peak represented 4% of the main peak. This byproduct was isolated via semipreparative HPLC, and its molecular weight was found to be identical to that of the main compound.

Compounds **9** and **10** were prepared according to the Boc strategy on PAM resin, using the amino acid derivatives Boc-Asp(NHBzl)-OH and Boc-Asp(NHCH$_2$-Ada)-OH, respectively, as the source of the Xaa residue. A schematic description of the synthesis of these two intermediates is given in Scheme 1. A minute amount of diastereomeric compounds could be detected also in the products of these two syntheses.

Compound **11** was prepared following two different procedures. According to the first, the pseudodipeptide moiety Boc-LeuΨ[CH_2NCH_3]Cha-OH (**18**, Scheme 2) was synthesized and coupled to the peptide in solid phase. The synthesis of the pseudodipeptide moiety **18** was performed by reductive alkylation with formaldehyde of the intermediate Boc-LeuΨ[CH_2NH]Cha-OBzl (**16**); the crude product required two successive purification steps to give pure compound **17** in 26% overall yield. In addition, coupling of pseudodipeptide **18** by standard methodology, although repeated twice and controlled by the Kaiser test, whose response was negative, was not complete. The HPLC profile of crude product showed in fact a major impurity, which when isolated revealed a molecular weight corresponding to that of the tetrapeptide H-Gln-Trp-Phe-βAla-OH by FAB-MS analysis. According to the second procedure, N-methylation of Fmoc-Cha-OH with the methodology described by Freidinger et al.[17] was followed by addition of the N-methylated amino acid to the peptide chain and standard reductive alkylation with Fmoc-Leu-H. A better overall yield (20%) was obtained with this precedure.

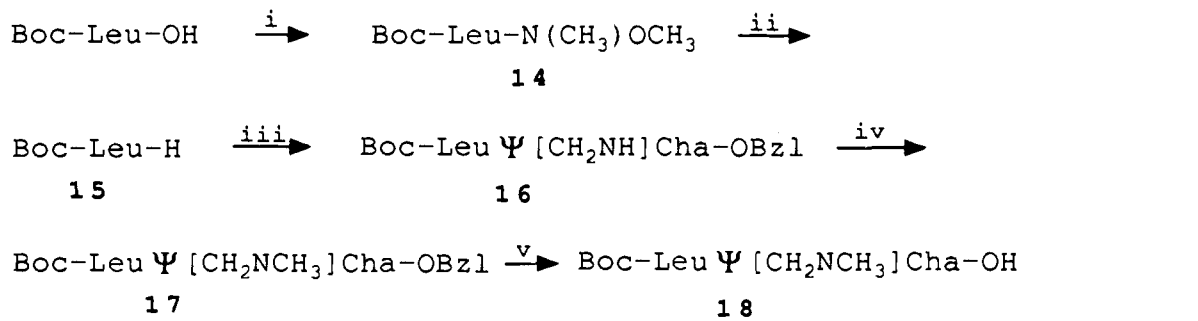
Table 2. Structures, Capacity Factors (k'), and Antagonist Activities (expressed as pA_2 values ± SEM) at NK-2 Tachykinin Receptors in the Rabbit Pulmonary Artery (RPA) and Hamster Trachea (HT) Bioassays of the Cyclic Pseudopeptides Cyclo(LeuΨ[CH_2NR]Xaa-Gln-Trp-Phe-βAla)[a]

				pA_2	
compd	R	Xaa	k'	RPA	HT
19	H	Met	9.6	6.65 ± 0.10	7.89 ± 0.19
20	H	Leu	9.9	6.95 ± 0.09	7.80 ± 0.11
21	H	Phe	10.5	7.10 ± 0.13	7.73 ± 0.10
22	H	Nal	12.0	7.37 ± 0.05	8.79 ± 0.07
23	H	Asp(OBzl)	11.8	7.27 ± 0.04	8.66 ± 0.12
24	H	Asp(NHBzl)	11.1	7.81 ± 0.06	9.26 ± 0.05
25	H	Cha	11.6	7.41 ± 0.05[b]	9.06 ± 0.04
26	CH_3	Cha	13.1	6.93 ± 0.04	8.42 ± 0.16
27	H	Asp(NHCH$_2$Ada)	14.5	6.89 ± 0.10	8.39 ± 0.10
28	H	Asn	7.7	6.16 ± 0.07	6.96 ± 0.02
29	H	Lys(Z)	11.9	6.89 ± 0.11	8.17 ± 0.05
30	H	Lys	6.8	na[c]	6.51 ± 0.10
31	H	Asp	7.9	5.97 ± 0.07	7.17 ± 0.04

[a] Values of the capacity factor (k') were determined in analytical HPLC by eluting a 1 mg/mL methanolic solution of pure peptide on a reversed phase C18 analytical column with solvent system 1 (solvent A, 0.1% TFA in distilled water; solvent B, 0.1% TFA in acetonitrile; 1 mL/min; linear gradient from 20% B to 80% B in 20 min). Each pA_2 value in the table is mean pA_2 (negative logarithm of antagonist dissociation constant) ± SEM of 9−12 determinations. Neurokinin A was used as the agonist. Peptidase inhibitors (thiorphan, captopril, and bestatin, 1 mM each) were added to the bath 15 min before the agonist. na: not active up to 5 μM. [b] Partial agonist activity at 3−10 μM. [c] Active as agonist (pD_2 = 5.96 ± 0.12).

Structures of cyclic pseudopeptides are reported in Table 2. Cyclizations were performed, using millimolar solutions of the peptides in DMF, with BOP and DIEA activation, at room temperature. Good yields were obtained for all peptides, and no byproducts due to the

Scheme 2[a]



[a] Reagents: (i) HN(CH_3)OCH_3·HCl, DMAP, DCC; (ii) $LiAlH_4$, 0 °C; (iii) H-Cha-OBzl, $NaBH_3CN$, methanol, 0 °C; (iv) $(CH_2O)_n$, $NaBH_3CN$, toluene, reflux; (v) H_2, Pd/C, ethanol.

Table 3. Structures and Antagonist Activities (expressed as pA_2 values ± SEM) at NK-2 Tachykinin Receptors in the Rabbit Pulmonary Artery (RPA) and Hamster Trachea (HT) Bioassays of Cyclic Peptides[a]

compd	sequence	pA_2 RPA	pA_2 HT
32	cyclo(Leu-Cha-Gln-Trp-Phe-βAla)	5.84 ± 0.25	7.50 ± 0.08
33	cyclo(Leu-Asp(NHCH$_2$Ada)-Gln-Trp-Phe-βAla)	5.10 ± 0.10	7.00 ± 0.24

[a] Each pA_2 value in the table is mean pA_2 (negative logarithm of antagonist dissociation constant) ± SEM of 9–12 determinations. Neurokinin A was used as the agonist. Peptidase inhibitors (thiorphan, captopril, and bestatin, 1 mM each) were added to the bath 15 min before the agonist.

acylation of the aminomethylene moiety were found. However, poor results were obtained in the preparation of peptide **26**, which in these conditions underwent complete degradation. In fact, immediately after the addition of the phosphonium salt, the starting compound disappeared and several byproducts were detected via HPLC. Similar results were obtained at lower temperature or with DPPA activation. An alternative procedure with 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDCI) and DMAP as activating agents[18] had to be adopted. Although in low yield, the desired product was obtained.

Biology. The biological activity of compounds **19**–**33** (Tables 2 and 3) as antagonists at the NK-2 receptors was tested in the two bioassays RPA and HT, expressing two different NK-2 receptor putative subtypes.[14] Several studies have been carried out on the characterization of these two putative subtypes with different agonists and antagonists. In particular, the discovery of the linear pseudopeptide MDL28,564 played a key role in the differentiation of the two receptors, since this compound acted as an agonist on the RPA (pD_2 = 6.86) and as an antagonist on the HT (pA_2 = 6.21).[20] Compound **30**, in which the Xaa position is occupied by a Lys residue, displayed a similar behavior (pD_2 = 5.96 on the RPA, pA_2 = 6.51 on the HT; Table 2). Interestingly, the ability to stimulate the NK-2 receptor appeared when the ε-amino function of Lys was free; the presence of this amino group or both the two amino groups of Lys and of the aminomethylene moiety resulted in the selective stimulation of the NK-2 receptor expressed in the RPA.

As far as the antagonist activity of our compounds for the two putative NK-2 subtypes is concerned, we found that increasing lipophilicity of the Xaa residue produced an increase of antagonist potency in both preparations. In fact, pA_2 values varied from 6.51 (compound **30**, Xaa = Lys) to 9.26 (compound **24**, Xaa = Asp(NHBzl)) on the HT preparation and from 5.97 (compound **31**, Xaa = Asp) to 7.81 (compound **24**, Xaa = Asp(NHBzl)) in the RPA preparation.

Peptide **26**, the only one containing a tertiary amine function, showed an antagonist potency (pA_2 = 6.93 on RPA and 8.42 on HT) devoid of partial agonist activity, although lower than that shown by the parent compound **25** (pA_2 = 7.41 on RPA and 9.06 on HT).

Peptides **32** and **33** (Table 3), not containing the aminomethylene moiety, still act as antagonists either on RPA preparation (pA_2 = 5.84 and 5.10, respectively) or on HT preparation (pA_2 = 7.5 and 7.0, respectively).

Chromatographic and Correlation Analysis. It is currently accepted that retention times and capacity factors in reversed phase chromatography provide an accurate estimate of the relative lipophilic character in homogeneous series of organic compounds.[19] Therefore, as cyclic pseudopeptides **19**–**31** possessed the same chemical structure, Xaa residue excepted, the influence of the Xaa residue on lipophilicity can be deduced by comparing the k' values obtained in the same elution conditions. The purified compounds were dissolved in methanol (1 mg/mL) and eluted with solvent system 1. The resulting k' values are reported in Table 2.

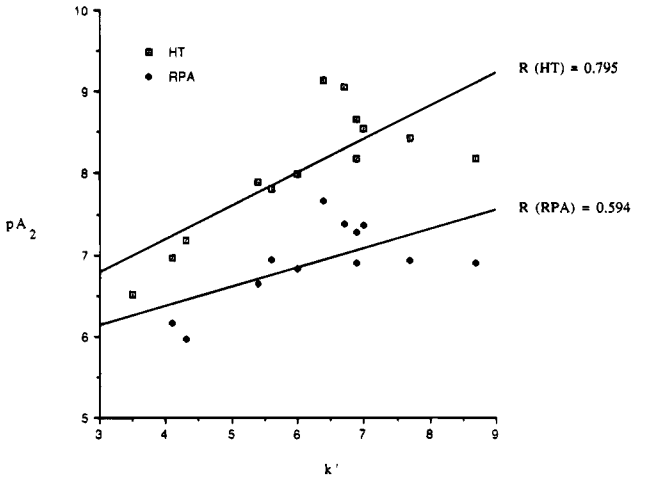


Figure 1. Plot of pA2 values on HT and RPA bioassays versus capacity factors of cyclic pseudopeptides (data from Table 2). Correlation coefficients (R) are indicated for the linear correlations found.

The measured k' values were related with the pA_2 values determined in the two different bioassays, HT and RPA (Figure 1). A significant linear relationship was found, the correlation coefficients (R) being 0.795 for HT ($p < 0.01$) and 0.594 for RPA ($p < 0.05$).[21]

Discussion

The present study aimed at establishing the structural requirements for the optimization of biological activity in a series of cyclic pseudopeptides displaying potent NK-2 receptor antagonist properties. We focused on the Xaa amino acid residue in the general structure cyclo(LeuΨ[CH$_2$NH]Xaa-Gln-Trp-Phe-βAla) and investigated the effect of lipophilicity of the molecule resulting from changes in the said residue on the antagonist potency, as measured in *in vitro* preparations containing the NK-2 receptor. Values of k' in reversed phase HPLC have been used as an estimate of the relative lipophilicity of the compounds.

The pA_2 values determined in the two different bioassays, HT and RPA, were related with the measured k' values in order to verify the existence of a correlation between the antagonist activity on the receptor and the lipophilicity of the molecules (Figure 1). The linear relationships found indicate that a lipophilic pocket of the NK-2 receptors is involved in the interaction with the cyclic pseudopeptides. This conclusion is in agreement with the decrease in antagonist potency resulting from the presence of a less lipophilic side chain, as with

the Asn residue (peptide **28**, $pA_2 = 6.16$ on RPA and $pA_2 = 6.96$ on HT). A similar effect was observed with the introduction of an Asp residue (peptide **31**, $pA_2 = 5.97$ on RPA and $pA_2 = 7.17$ on HT), while for Xaa = Lys the fall in activity on HT was even higher (peptide **30**, $pA_2 = 6.51$ on HT). On the other hand, the behavior of compound **24**, which deviates significantly from a linear dependence of potency versus lipophilicity because of a higher antagonist activity than expected on the basis of the k' value, might be ascribed to the reduced conformational flexibility of the amide bond in respect to the ester bond. Another explanation might be the ability of the NH group to act as a hydrogen bond donor toward a hypothetical heteroatom of the receptor. Compounds **26** and **27** show a lower increment in antagonist activity with lipophilicity and consequently deviate from the overall pattern found within the series. Compound **26** contains a tertiary amine function that can influence the chromatographic behavior in a peculiar way. For peptide **27**, steric factors associated with the bulkiness of the corresponding Xaa residue might well be responsible for the observed deviation.

The highest potency was obtained for Xaa = Cha (compound **25**, $pA_2 = 7.37$ on RPA and $pA_2 = 9.06$ on HT) and Xaa = Asp(NHBzl) (compound **24**, $pA_2 = 7.81$ on RPA and $pA_2 = 9.26$ on HT). The latter showed selectivity toward the NK-2 receptor expressed in HT tissue comparable to that found for pseudopeptides **23** and **25**.[22] Peptide **26** was synthesized with the aim of reducing the partial agonist activity of compound **25**. In fact it has been reported[9] that alkylation of the aminomethylene group in linear pseudopeptide analogs of NKA abolished the ability to stimulate the NK-2 receptor. We found the same effect in our cyclic analog because, while compound **25** showed partial agonist activity on RPA at 3–10 μM, compound **26** acts as a competitive antagonist with no detectable agonist activity. However, the alkylation of the aminomethylene moiety produced a decrease in antagonist activity in both RPA and HT.

Finally, it is worth noting that compounds **32** and **33** (Table 3) showed a lower antagonist activity on the RPA and HT than did their pseudopeptide analogs **25** and **27**, respectively. The decrease (50 times) in potency indicated that the aminomethylene moiety was favorable for effective interaction with NK-2 receptors.

In summary, for the series of compounds described in this study, the increasing lipophilicity of the Xaa residue was accompanied by a linearly related increment in NK-2 receptor antagonist activity. It can be deduced, therefore, that the different lipophilicity of the variable amino acid was a major parameter for the observed changes in biological activity. Apparently, the NK-2 receptor contains a lipophilic pocket which interacts with a hydrophobic portion of the ligand. In the presence of either small or polar groups, the interaction is not favored and a too bulky chain does not fit adequately in the cavity. This conclusion may be of importance for the design of further NK-2 receptor antagonists.

Furthermore, compound **24** (Xaa = Asp(NHBzl)) represents a potent antagonist with high selectivity toward the NK-2 subtype expressed on HT and might be a useful tool for further elucidating the hypothesized heterogeneity of this receptor.

Experimental Section

Materials. Boc- and Fmoc-protected aminoacids were purchased from Bachem and Novabiochem; DCC, HOBt, BOP, and PyBOP were from Novabiochem. DCM, DMF, IsOH (Merck), TFA, DIEA, TEA (Janssen), and Pip (Carlo Erba) were all of analytical grade and used without further purification.

Methods. Analytical HPLC characterization of the peptides was performed on a Beckman System Gold apparatus, using a Waters Delta-Pack C18 (100 Å, 150 × 3.9 mm i.d.) column. Two solvent systems were used. System 1: 0.1% TFA in distilled water (solvent A); 0.1% TFA in acetonitrile (solvent B); linear gradient from 20% B to 80% B in 20 min, flow rate 1 mL/min; detection at 210 nm. System 2: 0.01 M $(NH_4)_2HPO_4$ and 0.1 M $NaClO_4 \cdot H_2O$ in distilled water (solvent A); 0.1 M $NaClO_4 \cdot H_2O$ in 5% distilled water in acetonitrile (solvent B); linear gradient from 20% B to 80% B in 20 min, flow rate 1.3 mL/min; detection at 210 nm.

Purifications on a semipreparative scale were performed on a Waters Delta Prep 3000 system, using a Waters Delta-Pack C18 (100 Å, 300 × 19 mm i.d.) column, with the indicated linear gradients of acetonitrile (0.1% TFA) in distilled water (0.1% TFA), flow rate 20 mL/min, detection at 240 nm. The crude product was dissolved in 1–2 mL of TFA and the solution diluted with 2 mL of mobile phase and then filtered through a PTFE filter (0.45 μm) and directly injected. The appropriate fractions were collected; the resultant solution was evaporated under vacuum and lyophilized. Purified compounds showed an HPLC purity of at least 98%.

NMR spectra of intermediates were recorded at 200 MHz at room temperature on a Varian Gemini 200 apparatus.

Mass spectra of intermediates were recorded on a HP59881 mass spectrometer, using either positive ion thermospray ionization or methane chemical ionization. For thermospray spectra, samples were dissolved in methanol and flow injected in a 0.1 M ammonium acetate/methanol 60/40 (v/v) mobile phase, flow rate 1 mL/min. The ion source temperature was 276 °C, and the vaporization temperature was 90 °C. For chemical ionization spectra, samples were introduced by a direct insertion probe which was ballistically heated to 250 °C. The ion source temperature was 200 °C, and the methane pressure was 1 ton.

FAB-MS spectra were recorded on a VG Quattro mass spectrometer equipped with a FAB ion source. Ionization was achieved using a cesium gun (8 keV, 2.3 A), and thioglycerol was used as the matrix compound.

AAA were carried out by HPLC (Hewlett-Packard 1090 M) after hydrolysis of peptide samples for 24 h at 110 °C in boiling hydrochloric acid containing 2.5% thioglycolic acid and precolumn derivatization with *o*-phthalaldehyde. Pseudodipeptide moieties were not detected in these conditions.

Synthesis of Intermediates. Fmoc-(N-Me)Cha-OH (36). (4*S*)-3[[(9-Fluorenylmethyl)oxy]carbonyl]-4-(cyclohexyl)methyl-5-oxooxazolidine (39) was synthesized starting from Fmoc-Cha-OH (1 g, 2.5 mmol), which was suspended in toluene and treated with 0.5 g of paraformaldehyde and 50 mg of $TsOH \cdot H_2O$. The mixture was heated to reflux for 1 h and then cooled and washed with 1 N $NaHCO_3$ (2 × 25 mL). The organic layer was dried with $MgSO_4$ and then evaporated under vacuum. The resulting oil was dissolved in 3 mL of DCM, and 70 mL of petroleum ether was added. A crystalline solid slowly separated. Yield of pure compound **39**: 780 mg (78%). MS: $[M + H]^+$, 405 *m/e* (calcd, 404.5). ^{1}H-NMR ($CDCl_3$): δ 7.85–7.25 (6H, m), 5.40 (1H, m), 5.1 (1H, d), 4.65 (2H, m), 4.25 (1H, t), 4.2–3.8 (1H, m), 1.8–0.7 (13H, m).

Compound **39** (700 mg, 1.7 mmol) was dissolved in 10 mL of $CHCL_3$, and 1 mL of Et_3SiH was added. The solution was stirred at room temperature for 5 h, and then it was evaporated to dryness. The resulting oil was washed three times with DCM and evaporated. Crude product was purified by column chromatography on silica gel eluted with DCM/CH_3-OH (10/1, $R_f = 0.5$), obtaining 390 mg (53%) of compound **36** as a viscous oil. FAB-MS: $[M + H]^+$, 409 *m/e* (calcd, 407.5).

^{1}H-NMR ($CDCl_3$): δ 7.85–7.25 (6H, m, Fmoc), 4.8 (1H, s), 4.6 (1H, m), 4.45 (2H, m), 4.25 (1H, t), 2.9 (3H, s), 2.1–1.0 (13H, m).

Boc-Leu-H (15). Boc-Leu-OH·H_2O (2 g, 8 mmol) was dissolved in 40 mL of DCM, and 0.90 g (9.2 mmol) of $HN(CH_3)$-OCH_3·HCl, 0.048 g (3.9 mmol) of DMAP, and 1.65 g (8 mmol) of DCC were added. The solution was stirred overnight at room temperature, and then DCU was filtered off and the solution evaporated to dryness. The residue was dissolved in diethyl ether and washed with a saturated aqueous solution of NaCl, a saturated aqueous solution of $NaHCO_3$ (3 × 10 mL), a 10% aqueous solution of citric acid (3 × 10 mL), and a saturated aqueous solution of NaCl. The organic layer was dried over $MgSO_4$ and evaporated to dryness. The crude product was purified by column chromatography on silica gel eluted with DCM/ethyl acetate (10/1, R_f = 0.35), obtaining 1.8 g (85%) of Boc-Leu-N(CH_3)OCH_3 (14) as a viscous oil. MS: $[M + H]^+$, 275 *m/e* (calcd, 275). ^{1}H-NMR ($CDCl_3$): δ 5.0 (1H, d), 4.2 (1H, m), 3.75 (3H, s), 3.16 (3H, s), 1.9–1.1 (12H), 1.0 (3H, d), 0.81 (3H, d).

Compound 14 (1 g, 3.6 mmol) was dissolved in anhydrous diethyl ether and the solution cooled to 0 °C, and then 0.4 g (0.010 mmol) of $LiAlH_4$ was added in small portions. After 4 h, 5 mL of a 10% aqueous solution of $KHSO_4$ was added dropwise and the aqueous layer extracted three times with 50 mL of diethyl ether. The organic fractions were collected and washed with 3 N HCl (3 × 30 mL), a saturated aqueous solution of $NaHCO_3$ (3 × 30 mL), and a saturated aqueous solution of NaCl (3 × 30 mL). The solution was then dried over $MgSO_4$ and evaporated to dryness, to obtain 0.6 g (85%) of Boc-leucinal, which was used without further purification. MS: $[M + H]^+$, 216 *m/e* (calcd, 215.2). ^{1}H-NMR ($CDCl_3$): δ 9.57 (1H, s), 4.9 (1H, d), 4.2 (1H, m), 1.8–1.1 (12H), 0.91 (6H, d).

Fmoc-Leu-H (41). Fmoc-Leu-OH (1.8 g, 5 mmol) was dissolved in 120 mL of DCM, and 0.88 mL (5 mmol) of DIEA and 2.24 g (5 mmol) of BOP were added. After 20 min, 0.54 g (5.5 mmol) of $HN(CH_3)OCH_3$·HCl and 0.97 mL (10 mmol) of DIEA dissolved in 30 mL of DCM were added and the mixture was stirred for 3 h. The resulting solution was diluted with 100 mL of DCM and washed with 3 N HCl (3 × 30 mL) and then with a saturated aqueous solution of $NaHCO_3$ (3 × 30 mL) and a saturated aqueous solution of NaCl (3 × 30 mL). The organic layer was dried over $MgSO_4$ and evaporated to dryness. The crude product was purified by column chromatography on silica gel eluted with *n*-hexane/ethyl acetate (5/2, R_f = 0.4), obtaining 1.6 g (80%) of Fmoc-Leu-N(CH_3)OCH_3 (**40**) as a viscous oil. MS: $[M + H]^+$, 396 *m/e* (calcd, 395.4). ^{1}H-NMR ($CDCl_3$): δ 9.59 (1H, s), 7.8–7.29 (8H, m), 5.2 (1H, d), 4.6–4.2 (4H), 1.9–1.1 (3H), 0.96 (6H, d).

Compound **40** was reduced following the procedure described for 14, starting from 0.70 g (1.8 mmol) of **40** to obtain 0.51 g (85%) of 41 as a viscous oil. MS: $[M + H]^+$, 339 *m/e* (calcd, 338.4). ^{1}H-NMR ($CDCl_3$): δ 9.59 (1H, s), 7.8–7.29 (8H, m), 5.2 (1H, d), 4.6–4.2 (4H), 1.9–1.1 (3H), 0.96 (6H, d).

Boc-Asp(NHBzl)-OH (13a). Boc-Asp-OBzl (1 g, 3 mmol) was dissolved in 150 mL of dioxane, and 1.92 g (3.7 mmol) of PyBOP and 1.28 mL (7.2 mmol) of DIEA were added. To the resultant solution was added 0.32 g (3 mmol) of benzylamine, and the mixture was stirred at room temperature for 3 h. After evaporation to dryness, the mixture was purified by column chromatography on silica gel eluted with petroleum ether/ethyl acetate (1/1, R_f = 0.30), to obtain 0.86 g (70%) of Boc-Asp-(NHBzl)-OBzl (**12a**) as a viscous oil. MS: $[M + H]^+$, 381 *m/e* (calcd, 381.5). ^{1}H-NMR ($CDCl_3$): δ 7.28 (5H, s), 7.35 (5H, s), 5.94 (1H, d), 5.81 (1H, t), 5.22 (2H, s), 4.61 (1H, m), 2.9–2.65 (2H, m), 1.46 (9H, s).

Compound **12a** (0.8 g, 2.1 mmol) was dissolved in 80 mL of 95% ethanol and the solution added to a suspension of 300 mg of Pd/C (1/10) in 20 mL of 95% ethanol previously saturated with hydrogen. The mixture was allowed to stand for 2 h in a hydrogen atmosphere and then was filtered and the solution evaporated to dryness, to obtain 0.54 g (91%) of **13a** as a viscous oil. MS: $[M + H]^+$, 323 *m/e* (calcd, 323.4). ^{1}H-NMR ($CDCl_3$): δ 7.49 (5H, s), 5.90 (1H, d), 6.30 (1H, t), 5.22 (2H, s), 4.73 (1H, m), 3.1–2.65 (2H, m), 1.46 (9H, s).

Boc-Asp(NHCH$_2$Ada)-OH (13b). Boc-Asp-OBzl (1 g, 3 mmol) was dissolved in 150 mL dioxane, and 1.92 g (3.7 mmol) of PyBOP and 1.28 mL (7.2 mmol) of DIEA were added. To the resultant solution was added 0.51 g (3 mmol) of (2-adamantyl)methylamine, and the mixture was stirred at room temperature for 3 h. After evaporation to dryness, the mixture was purified by column chromatography on silica gel eluted with cyclohexane/ethyl acetate (3/2, R_f = 0.4), to obtain 1.34 g (92%) of Boc-Asp(NHCH$_2$Ada)-OBzl (**12b**). MS: $[M + H]^+$, 470 *m/e* (calcd, 469.6). ^{1}H-NMR ($CDCl_3$): δ 7.34 (5H, s), 5.80 (1H, d), 5.54 (1H, t), 5.18 (2H, s), 4.53 (1H, m), 3.0–2.65 (4H, d), 1.5–2.0 (15H, m), 1.43 (9H, s).

Compound **12b** (1.24 g, 2.6 mmol) was dissolved in 100 mL of 95% ethanol and the solution added to a suspension of 500 mg of Pd/C (1/10) in 20 mL of 95% ethanol previously saturated with hydrogen. The mixture was allowed to stand for 2 h under hydrogen, and then it was filtered and the solution evaporated to dryness, to obtain 1.0 g of **13b** (99%) as a viscous oil. MS: $[M + H]^+$, 382 *m/e* (calcd, 381.5). ^{1}H-NMR ($CDCl_3$): δ 5.90 (1H, d), 6.75 (1H, t), 4.35 (1H, m), 3.2–2.8 (4H, d), 1.5–2.0 (15H, m), 1.43 (9H, s).

Boc-LeuΨ[CH$_2$NH]Cha-OBzl (16). Boc-Cha-OH·DCHA (2 g, 4.7 mmol) was suspended in 5 mL of ethyl acetate, and 15 mL of 2 M $KHSO_4$ in water was added. The organic layer was washed twice with water and evaporated to dryness, obtaining 1.2 g of an oily residue that was dissolved in 50 mL of DCM. Then 1 mL of benzyl alcohol was added and the solution cooled to 0 °C. DMAP (50 mg, 0.41 mmol) and DCC (1 M) in DCM (4.8 mL, 4.8 mmol) were added and the suspension was stirred for 10 min at 0 °C and then for 2 h at room temperature. The resultant mixture was filtered and the solution evaporated. The crude product was purified by column chromatography on silica gel eluted with *n*-hexane/ethyl acetate (4/1, R_f = 0.4), obtaining 1.4 g (87%) of Boc-Cha-OBzl (**42**) as a viscous oil. MS: $[M + H]^+$, 362 *m/e* (calcd, 362.5). ^{1}H-NMR ($CDCl_3$): δ 7.35 (5H, s), 5.15 (2H, m), 4.5 (1H, d), 3.65 (1H, m), 3.42 (1H, t), 2.8–2.3 (2H, m), 1.8–1.1 (25H), 0.88 (6H, d).

Compound **42** (1.3 g, 3.6 mmol) was dissolved in 40 mL of TFA/DCM (1/1) and stirred for 1 h. The solution was evaporated to dryness and washed three times with DCM. The resultant TFA salt of H-Cha-OBzl was used directly without further purification by dissolving the residue in 40 mL of 1% CH_3COOH in CH_3OH. The solution was cooled to 0 °C, and 1.55 g (5.6 mmol) of Boc-Leu-H was added. A solution of 0.52 g (8.2 mmol) of $NaBH_3CN$ in the same solvent was added dropwise over a period of 40 min. After 1 h, the mixture was washed with saturated aqueous $NaHCO_3$ and the organic layer dried over $MgSO_4$ and evaporated to dryness. The crude product was purified by column chromatography on silica gel eluted with petroleum ether/ethyl acetate (5/1, R_f = 0.25) to yield 1.1 g (43%) of **16**. FAB-MS: $[M + H]^+$, 462 *m/e* (calcd, 461.7). ^{1}H-NMR ($CDCl_3$): δ 7.35 (5H, s), 5.15 (2H, m), 4.5 (1H, d), 3.65 (1H, m), 3.42 (1H, t), 2.8–2.3 (2H, m), 1.8–1.1 (25H), 0.88 (6H, d).

Boc-LeuΨ[CH$_2$NCH$_3$]Cha-OH (18). Compound **16** (1.1 g, 2.4 mmol) was dissolved in 80 mL of 1% CH_3COOH in methanol, and 0.122 g (1.3 mmol) of trioxymethylene was added. A solution of 0.166 g (2.6 mmol) of $NaBH_3CN$ in 80 mL of the same solvent was added dropwise in 40 min. After this time, the resultant suspension was maintained at reflux for 2 h and then cooled to 0 °C and 120 mL of saturated aqueous $NaHCO_3$ was added dropwise. The mixture was extracted five times with ethyl acetate, and the organic layers were collected, dried over $MgSO_4$, and evaporated to dryness. The resultant crude product (1.1 g) was purified two times by column chromatography on silica gel eluted with cyclohexane/ethyl acetate (10/1, R_f = 0.3), to yield 0.3 g (26%) of Boc-LeuΨ[CH$_2$NCH$_3$]Cha-OBzl (**17**) as a viscous oil. FAB-MS: $[M + H]^+$, 475 *m/e* (calcd, 475). ^{1}H-NMR ($CDCl_3$): δ 7.35 (5H, s), 5.15 (2H, m), 4.4 (1H, d), 3.65 (1H, m), 3.35 (1H, t), 2.8–2.3 (2H, m), 2.3 (3H, s), 1.8–1.1 (25H), 0.90 (6H, d).

Compound **17** (0.19 g, 0.4 mmol) was dissolved in 100 mL of 95% ethanol and the solution added to a suspension of 0.09 g of Pd/C (1/10) in 20 mL of 95% ethanol previously saturated with hydrogen. The mixture was allowed to stand for 2 h in

a hydrogen atmosphere, and then it was filtered and the solution evaporated to dryness, to obtain 0.16 g (99%) of **18** as a viscous oil. FAB-MS: $[M + H]^+$, 385 *m/e* (calcd, 385.5). ^{1}H-NMR ($CDCl_3$): δ 4.4 (1H, d), 3.9 (1H, m), 3.5 (1H, m), 3.1–2.5 (2H, m), 2.65 (3H, s), 1.9–1.1 (25H), 0.90 (6H, d).

Synthesis of Peptides. Linear sequences were synthesized on a Labortec SP640 apparatus following the standard protocols supplied by the manufacturer, for either Boc or Fmoc strategy. A detailed description of the protocols has been previously reported.[23]

Synthesis of the Intermediate H-Gln-Trp-Phe-βAla-resin (34) on *p*-(Benzyloxy)benzyl Alcohol Resin. Following the procedure described by Grandas et al.,[24] 4.86 g (15.6 mmol) of Fmoc-βAla-OH was dissolved in 20 mL of anhydrous (molecular sieves) DMA. To this solution was added 1.581 g of HOBt·H_2O (11.7 mmol) dissolved in 5 mL of DMA. The resultant solution was cooled to 0 °C, and a cold solution of 3.21 g of DCC (15.6 mmol) was added. The resulting mixture was allowed to stand for 10 min, and then it was added to a suspension of 1.3 g (1 mequiv) of *p*-(benzyloxy)benzyl alcohol resin (Wang resin, Novabiochem, 0.78 mequiv/g) in 10–20 mL of DMA. The suspension was gently shaken for 18 h, and then the reaction mixture was filtered off and the resin washed three times with 10 mL of DMF, DCM, IsOH, and DMF. The resultant substitution level was 0.41 mequiv/g, as determined by spectrophotometric measurement of the Fm group.

Fmoc-βAla-resin (0.5 g, 0.41 mequiv/g, 0.205 mequiv) was washed with DMF and deprotected with a 20% solution of Pip in DMF (v/v). The resin was washed again with DMF; then HOBt·H_2O (0.094 g, 0.61 mmol) and Fmoc-Phe-OH (0.31 g, 0.81 mmol) dissolved in 10 mL of DCM/DMF (2/1, v/v) were added. The resin suspension was shaken for 2 min, and then a 1 M solution of DCC (0.82 mL, 0.82 mmol) in DCM was added. The coupling reaction was carried out for 90 min at room temperature. After this time, the resin was washed and the extent of acylation checked by the qualitative Kaiser test. After getting a negative response, the Fmoc group was removed by Pip. The following residues were subsequently added, Fmoc-Trp-OH (0.35 g, 0.82 mmol), Fmoc-Gln-OH (0.30 g, 0.82 mmol, double coupling). The Fmoc group was finally removed by Pip to yield intermediate **34**.

Synthesis of the Intermediate H-Gln-Trp(Boc)-Phe-βAla-resin (35) on Hydroxymethyl Resin. Fmoc-βAla-OH (1.24 g, 4 mmol) was dissolved in 5 mL of DCM and 2 mL of DMF, the solution was cooled to 0 °C, and 2 mL of 1 M DCC in DCM (2 mmol) was added. After 10 min, the urea was filtered off and the solution added to 1 g (1 mequiv) of hydroxymethyl resin (HO-CH_2-Ph-polymer, Bachem, 1.0 mequiv/g) previously washed with DCM. A solution of DMAP (0.122 g, 1 mmol) in 2 mL of DMF was added and the suspension shaken for 2 h. The resulting substitution level was 0.38 mequiv/g. The coupling was repeated by adding a solution of 0.93 g of Fmoc-βAla-OH and 0.61 g of HOBt (4 mmol) in 12 mL of DCM/DMF (2/1, v/v) to the resin and, after 2 min of preactivation time, 3 mL of 1 M DCC in DCM. The suspension was shaken for 2 h. The final substitution level resulted 0.67 mequiv/g.

Fmoc-βAla-resin (0.5 g, 0.67 mequiv/g, 0.335 mequiv) was washed with DMF and then deprotected, and HOBt·H_2O (0.20 g, 1.3 mmol) and Fmoc-Phe-OH (0.39 g, 1 mmol) dissolved in 10 mL of DCM/DMF (2/1, v/v) were added. The resin suspension was shaken for 2 min; then a 1 M solution of DCC (1 mL, 1 mmol) in DCM was added. The coupling reaction was carried out for 90 min at room temperature. After this time, the resin was washed and the extent of acylation checked by the qualitative Kaiser test. After getting a negative response, the Fmoc group was removed by Pip. The following residues were subsequently added, Fmoc-Trp(Boc)-OH (0.54 g, 1 mmol), Fmoc-Gln-OH (0.37 g, 1 mmol). The Fmoc group was finally removed by Pip to yield intermediate **35**.

H-LeuΨ[CH_2NH]Leu-Gln-Trp-Phe-βAla-OH (2). To 0.5 g (0.205 mequiv) of intermediate **34** was added Fmoc-Leu-OH (0.29 g, 0.8 mmol) using the HOBt/DCC activation procedure. After Fmoc deprotection, a solution of Fmoc-Leu-H (0.17 g, 0.5 mmol) in 5 mL of acetic acid 1%/DMF (v/v) was added and $NaBH_3CN$ (70 mg, 1.1 mmol), dissolved in 5 mL of CH_3COOH 1%/DMF, was added dropwise to the shaken resin during a period of 40 min. The suspension was shaken for an additional 4 h and then washed and checked by the Kaiser test. A double reductive alkylation was necessary in order to obtain a completely negative Kaiser test. After the last coupling, the peptide–resin was deprotected, washed, and dried under vacuum, yielding 0.7 g of dried product. This was placed in a 100 mL round bottomed flask, and 10 mL of TFA 50%/DCM (v/v), 1 mL of *p*-cresol, and 1 mL of 1,2-ethanedithiol were added. The resultant resin suspension was stirred for 2 h at room temperature. After this time, the resin was filtered off and rinsed three times with 5 mL of TFA 50%/DCM. The resultant solution was diluted with 150 mL of cold diethyl ether and 50 mL of cold petroleum ether. The white solid that separated was filtered, washed three times with cold ether, dissolved in water, and lyophilized. Yield of crude product: 202 mg. The peptide was purified via semipreparative HPLC, with a linear gradient from 15% B to 30% B in 60 min (0.25% B/min), 100 mg of crude product giving 17 mg (21%) of peptide **2**. FAB-MS: $[M + H]^+$, 763 *m/e* (calcd, 763). AAA: Glx 1.15 (1), Trp 1.12 (1), Phe 1.00 (1), βAla 1.09 (1). *k'* (system 1) = 3.9 min; *k'* (system 2) = 7.9 min.

H-LeuΨ[CH_2NH]Met-Gln-Trp-Phe-βAla-OH (1). To 0.5 g (0.205 mequiv) of intermediate **34** were successively coupled Fmoc-Met-OH (0.30 g, 0.8 mmol) and Fmoc-Leu-H (0.17 g, 0.5 mmol), as described for peptide **2**. The cleavage yielded 230 mg of crude product, which was purified in the same conditions used for peptide **2**, giving 42 mg (26%) of peptide 1. FAB-MS: $[M + H]^+$, 779 *m/e* (calcd, 779.5). AAA: Glx 1.18 (1), Trp 1.13 (1), Phe 1.00 (1), βAla 1.15 (1). *k'* (system 1) = 3.7 min; *k'* (system 2) = 8.1 min.

H-LeuΨ[CH_2NH]Phe-Gln-Trp-Phe-βAla-OH (3). To 0.5 g (0.205 mequiv) of intermediate **34** were successively added Fmoc-Phe-OH (0.31 g, 0.8 mmol) and Fmoc-Leu-H (0.17 g, 0.5 mmol), as described for peptide **2**. The cleavage yielded 250 mg of crude product, whose HPLC purification with a linear gradient from 20% B to 35% B in 40 min (0.25% B/min) gave 94 mg (46%) of peptide **3**. FAB-MS: $[M + H]^+$, 800 *m/e* (calcd, 800.2). AAA: Glx 1.20 (1), Trp 1.14 (1), Phe 1.00 (1), βAla 1.10 (1). *k'* (system 1) = 5.3 min; *k'* (system 2) = 8.9 min.

H-LeuΨ[CH_2NH]Nal-Gln-Trp-Phe-βAla-OH (4). To 0.5 g (0.205 mequiv) of intermediate **34** were coupled Fmoc-Nal-OH (0.36 g, 0.8 mmol, double coupling) and Fmoc-Leu-H (0.17 g, 0.5 mmol), as described for peptide **2**. The cleavage yielded 189 mg of crude product, which was purified in the same conditions used for peptide **3**, giving 43 mg (25%) of peptide 4. FAB-MS: $[M + H]^+$, 848 *m/e* (calcd, 847.5). AAA: Glx 1.21 (1), Trp 1.12 (1), Phe 1.00 (1), βAla 1.00 (1). *k'* (system 1) = 5.5 min; *k'* (system 2) = 9.9 min.

H-LeuΨ[CH_2NH]Asp(OBzl)-Gln-Trp-Phe-βAla-OH (5). To 0.5 g (0.205 mequiv) of intermediate **34** were coupled Fmoc-Asp(OBzl)-OH (0.36 g, 0.8 mmol) and Fmoc-Leu-H (0.17 g, 0.5 mmol), as described for peptide **2**. The cleavage yielded 206 mg of crude product, which was purified in the same conditions used for peptide **3**, giving 82 mg (47%) of peptide **5**. FAB-MS: $[M + H]^+$, 855 *m/e* (calcd, 854.8). AAA: Glx 1.09 (1), Trp 1.16 (1), Phe 1.00 (1), βAla 1.18 (1). *k'* (system 1) = 5.5 min; *k'* (system 2) = 9.6 min.

H-LeuΨ[CH_2NH]Cha-Gln-Trp-Phe-βAla-OH (7). To 0.5 g (0.205 mequiv) of intermediate **34** were coupled Fmoc-Cha-OH (0.32 g, 0.8 mmol, double coupling) and Fmoc-Leu-H (0.17 g, 0.5 mmol), as described for peptide **2**. The cleavage yielded 195 mg of crude product, which was purified in the same conditions used for peptide **3**, giving 19 mg (11%) of peptide **7**. FAB-MS: $[M + H]^+$, 803 *m/e* (calcd, 803.2). AAA: Glx 1.16 (1), Trp 0.89 (1), Phe 1.00 (1), βAla 1.15 (1). *k'* (system 1) = 4.9 min; *k'* (system 2) = 9.5 min.

H-LeuΨ[CH_2NCH_3]Cha-Gln-Trp-Phe-βAla-OH (11). The synthesis was performed with two different procedures: (i) Intermediate **34** (0.5 g, 0.205 mequiv) was deprotected, and then Boc-LeuΨ[CH_2NCH_3]Leu-OH (**18**, 0.24 g, 0.6 mmol) was coupled with HOBt/DCC activation. In the presence of a slightly colored Kaiser test, an equimolar amount of the pseudodipeptide (0.079 mg, 0.2 mmol) was used for a double coupling. Cleavage yielded 140 mg of crude product, whose HPLC purification with a linear gradient from 25% B

to 37.5% B in 50 min (0.25% B/min) gave 22 mg (13%) of peptide 11. (ii) To 0.5 g (0.205 mequiv) of intermediate **34** were successively coupled Fmoc-(N-Me)Cha-OH (**36**, 0.25 g, 0.6 mmol, double coupling) and Fmoc-Leu-H (0.17 g, 5 mmol), as described for peptide **2**. Cleavage yielded 105 mg of crude product, whose purification in the conditions described above gave 33 mg (20%) of peptide 11. FAB-MS: $[M + H]^+$, 817 *m/e* (calcd, 816.8). AAA: Glx 0.95 (1), Trp 0.99 (1), Phe 1.00 (1), βAla 1.06 (1). *k'* (system 1) = 7.3 min; *k'* (system 2) = 12.1 min.

H-LeuΨ[CH_2NH]Asn-Gln-Trp-Phe-βAla-OH (6). To 0.5 g (0.205 mequiv) of intermediate **34** were coupled Fmoc-Asn-OH (0.29 g, 0.8 mmol) and Fmoc-Leu-H (0.17 g, 0.5 mmol), as described for peptide **2**. The cleavage yielded 185 mg of crude product, which was purified in the same conditions used for peptide **2**, giving 22 mg (14%) of peptide **6**. FAB-MS: $[M + H]^+$, 764 *m/e* (calcd, 764.5). AAA: Glx 0.94 (1), Trp 1.02 (1), Phe 1.00 (1), βAla 0.98 (1). *k'* (system 1) = 2.7 min; *k'* (system 2) = 6.5 min.

H-LeuΨ[CH_2NH]Lys(Z)-Gln-Trp-Phe-βAla-OH (8). To 0.5 g (0.205 mequiv) of intermediate **34** were coupled Fmoc-Lys(Z)-OH (0.41 g, 0.8 mmol) and Fmoc-Leu-H (0.17 g, 0.5 mmol), as described for peptide **2**. The cleavage yielded 210 mg of crude product, which was purified via HPLC a first time with a linear gradient from 25% B to 40% B in 60 min (0.25% B/min) to yield 105 mg of 95% product. A second purification step was performed with a linear gradient from 25% B to 45% B in 80 min (0.15% B/min). Two fractions were isolated, the first one at *k'* = 38–41 min (F1, 20 mg, 10%) and the second one at *k'* = 50–55 min (F2, 1 mg). FAB-MS: $[M + H]^+$, 913 *m/e* (calcd, 912.5) for both F1 and F2. AAA (F1): Glx 1.10 (1), Trp 0.89 (1), Phe 1.00 (1), βAla 1.06 (1). *k'* (system 1) = 4.9 min and *k'* (system 2) = 9.6 min for F1.

H-LeuΨ[CH_2NH]Asp($NHCH_2$Ada)-Gln-Trp-Phe-βAla-OH (9). To 0.5 g (0.19 mequiv) of intermediate **35** was coupled Boc-Asp($NHCH_2$Ada)-OH (0.38 g, 0.8 mmol) with the HOBt/DCC activation procedure. After deprotection with 50% TFA in DCM (v/v), the resin was washed, neutralized with 10% TEA in $CHCl_3$ (v/v), and washed again. A solution of Fmoc-Leu-H (0.28 g, 0.8 mmol) in 5 mL of 1% acetic acid in DMF (v/v) was added, and $NaBH_3CN$ (70 mg, 1.1 mmol) dissolved in 5 mL of 1% CH_3COOH in DMF was added dropwise to the shaken resin during a period of 40 min. The suspension was shaken for additional 4 h and then washed and checked by the Kaiser test. A double reductive alkylation was necessary in order to obtain a negative Kaiser test. After the last coupling, the peptide–resin was deprotected, washed, and dried under vacuum, to give 800 mg of dry product. For the cleavage, the peptide–resin was placed in the reaction vessel of a liquid HF apparatus containing 1 mL of anisole and 0.5 mL of dimethyl sulfide as scavengers. HF (10 mL) was added, and the cleavage reaction proceeded for 1 h at 0 °C. After evaporation of HF under vacuum, the resin was washed with diethyl ether and the peptide was extracted with acetic acid, diluted with water, and lyophilized to give 420 mg of crude peptide **9**. HPLC purification with a linear gradient from 27% B to 37% B in 40 min (0.25% B/min) gave 160 mg (53%) of peptide **9**. FAB-MS: $[M + H]^+$, 912 *m/e* (calcd, 912.3). AAA: Glx 0.88 (1), Trp 0.94 (1), Phe 1.00 (1), βAla 1.08 (1). *k'* (system 1) = 7.2 min; *k'* (system 2) = 13.1 min.

H-LeuΨ[CH_2NH]Asp(NHBzl)-Gln-Trp-Phe-βAla-OH (10). To 0.5 g (0.19 mequiv) of intermediate **35** were coupled Boc-Asp(NHBzl)-OH (0.36 g) and Fmoc-Leu-H (0.28 g), as described for peptide **9**. The cleavage yielded 360 g of crude product, which was purified in the same conditions used for peptide **3**, giving 99 mg (35%) of peptide **10**. FAB-MS: $[M + H]^+$, 855 *m/e* (calcd, 854.5). AAA: Glx 0.86 (1), Trp 0.84 (1), Phe 1.00 (1), βAla 0.98 (1). *k'* (system 1) = 6.0 min; *k'* (system 2) = 10.8 min.

H-Leu-Cha-Gln-Trp-Phe-βAla-OH (37). To 0.5 g (0.205 mequiv) of intermediate **34** were coupled Fmoc-Cha-OH (0.32 g, 0.8 mmol, double coupling) and Fmoc-Leu-OH (0.29 g, 0.8 mmol), as described for peptide **2**. The cleavage yielded 0.125 g of crude product, whose HPLC purification with a linear gradient from 25% B to 35% B in 40 min (0.25% B/min) gave 86 mg (52%) of peptide **37**. FAB-MS: $[M + H]^+$, 818 *m/e* (calcd, 817.2). AAA: Leu: 0.91 (1), Cha 1.20 (1), Glx 0.87 (1), Trp 0.91 (1), Phe 1.00 (1), βAla 0.91 (1). *k'* (system 1) = 5.7 min.

H-Leu-Asp($NHCH_2$Ada)-Gln-Trp-Phe-βAla-OH (38). To 0.5 g (0.19 mmol) of intermediate **35** were coupled Boc-Asp($NHCH_2$Ada)-OH (0.38 g, 0.8 mmol) and Fmoc-Leu-OH (0.28 g, 0.8 mmol), as described for peptide **9**. The cleavage yielded 0.35 g of crude product, whose HPLC purification with a linear gradient from 30% B to 36% B in 30 min (0.2% B/min) gave 160 mg (51%) of peptide **38**. FAB-MS: $[M + H]^+$, 927 *m/e* (calcd, 926.5). AAA: Leu 1.00 (1), Asp($NHCH_2$Ada) 0.85 (1), Glx 0.99 (1), Trp 1.05 (1), Phe 1.00 (1), βAla 1.03 (1). *k'* (system 1) = 7.0 min.

Cyclo(LeuΨ[CH_2NH]Leu-Gln-Trp-Phe-βAla) (20). The cyclization reaction was carried out according to the following procedure: A 2.5 mM solution of pure linear compound **2** (35 mg, 0.046 mmol) in DMF (19 mL) was cooled to 0 °C, and then 1.2 equiv of BOP (24 mg, 0.054 mmol) and 2.4 equiv of DIEA (0.019 mL, 0.11 mmol) were added. The reaction was tested by HPLC (system 1). After 1 h, no starting compound was present and only one product was detected in the reaction mixture. The solution was evaporated to dryness under vacuum and then dissolved in 2 mL of DMF and purified directly by semipreparative HPLC in the same conditions used for peptide **11**, giving 16 mg (47%) of peptide **20**. FAB-MS: $[M + H]^+$, 745 *m/e* (calcd, 744.9). AAA: Glx 0.92 (1), Trp 0.96 (1), Phe 1.00 (1), βAla 1.10 (1).

Cyclo(LeuΨ[CH_2NH]Met-Gln-Trp-Phe-βAla) (19). Cyclization of 30 mg (0.038 mmol) of **1** was performed as for peptide **20**, and HPLC purification with a linear gradient from 20% B to 32% B in 30 min (0.4% B/min) gave 18 mg (60%) of peptide **19**. FAB-MS: $[M + H]^+$, 763 *m/e* (calcd, 763.4). AAA: Glx 0.94 (1), Trp 0.93 (1), Phe 1.00 (1), βAla 1.03 (1).

Cyclo(LeuΨ[CH_2NH]Phe-Gln-Trp-Phe-βAla) (21). Cyclization of 63 mg (0.079 mmol) of **3** was performed as for peptide **20**, and HPLC purification with a linear gradient from 20% B to 40% B in 40 min (0.5% B/min) gave 33 mg (54%) of peptide **21**. FAB-MS: $[M + H]^+$, 790 *m/e* (calcd, 789.9). AAA: Glx 1.03 (1), Trp 0.89 (1), Phe 1.00 (1), βAla 1.05 (1).

Cyclo(LeuΨ[CH_2NH]Nal-Gln-Trp-Phe-βAla) (22). Cyclization of 53 mg (0.062 mmol) of **4** was performed as for peptide **20**, and HPLC purification with a linear gradient from 30% B to 40% B in 40 min (0.25% B/min) gave 28 mg (53%) of peptide **22**. FAB-MS: $[M + H]^+$, 830 *m/e* (calcd, 830.0). AAA: Glx 0.96 (1), Trp 0.90 (1), Phe 1.00 (1), βAla 1.08 (1).

Cyclo(LeuΨ[CH_2NH]Asp(OBzl)-Gln-Trp-Phe-βAla) (23). Cyclization of 73 mg (0.085 mmol) of **5** was performed as for peptide **20**, and HPLC purification in the same conditions used for peptide **20** gave 38 mg (52%) of peptide **23**. FAB-MS: $[M + H]^+$, 837 *m/e* (calcd, 836.9). AAA: Glx 0.95 (1), Trp 0.96 (1), Phe 1.00 (1), βAla 1.03 (1).

Cyclo(LeuΨ[CH_2NH]Asp(NHBzl)-Gln-Trp-Phe-βAla) (24). Cyclization of 31 mg (0.036 mmol) of **10** was performed as for peptide **20**, and HPLC purification in the same conditions used for peptide **20** gave 13 mg (42%) of peptide **24**. FAB-MS: $[M + H]^+$, 836 (calcd, 836.5). AAA: Glx 1.06 (1), Trp 0.91 (1), Phe 1.00 (1), βAla 1.06 (1).

Cyclo(LeuΨ[CH_2NH]Cha-Gln-Trp-Phe-βAla) (25). Cyclization of 24 mg (0.030 mmol) of **7** was performed as for peptide **20**, and HPLC purification with a linear gradient from 25% B to 40% B in 30 min (0.5% B/min) gave 13 mg (54%) of peptide **25**. FAB-MS: $[M + H]^+$, 785 *m/e* (calcd, 785.4). AAA: Glx 0.92 (1), Trp 0.86 (1), Phe 1.00 (1), βAla 1.12 (1).

Cyclo(LeuΨ[CH_2NH]Asp($NHCH_2$Ada)-Gln-Trp-Phe-βAla) (27). Cyclization of 45 mg (0.048 mmol) of **9** was performed as for peptide **20**, and HPLC purification in the same conditions used for peptide **25** gave 21 mg (47%) of peptide **27**. FAB-MS: $[M + H]^+$, 894 *m/e* (calcd, 894.0). AAA: Glx 0.92 (1), Trp 0.90 (1), Phe 1.00 (1), βAla 1.12 (1).

Cyclo(LeuΨ[CH_2NH]Asn-Gln-Trp-Phe-βAla) (28). Cyclization of 25 mg (0.033 mmol) of **6** was performed as for peptide **20**, and HPLC purification in the same conditions used for peptide **3** gave 11 mg (44%) of peptide **28**. FAB-MS: $[M + H]^+$, 747 *m/e* (calcd, 746.4). AAA: Glx 0.92 (1), Trp 0.88 (1), Phe 1.00 (1), βAla 1.06 (1).

Cyclo(LeuΨ[CH_2NH]Lys(Z)-Gln-Trp-Phe-βAla) (29). Cyclization of 31 mg (0.038 mmol) of **8** was performed as for

peptide **20**, and HPLC purification in the same conditions used for peptide **20** gave 19 mg (61%) of peptide **29**. FAB-MS: $[M + H]^+$, 895 *m/e* (calcd, 895.1). AAA: Glx 0.89 (1), Trp 0.85 (1), Phe 1.00 (1), βAla 1.13 (1).

Cyclo(LeuΨ[CH_2NH]Asp-Gln-Trp-Phe-βAla) (31). Compound **23** (30 mg, 0.036 mmol) was dissolved in 3 mL of 95% ethanol and the solution added to a suspension of 15 mg of Pd/C (1/10) in 2 mL of 95% ethanol previously saturated with hydrogen. The mixture was allowed to stand for 2 h in a hydrogen atmosphere and then filtered and the solution evaporated to dryness and lyophilized. The resultant pure peptide **31** (22 mg, 82%) was not further purified. FAB-MS: $[M + H]^+$, 747 *m/e* (calcd, 746.8). AAA: Glx 0.99 (1), Trp 0.92 (1), Phe 1.00 (1), βAla 1.03 (1).

Cyclo(LeuΨ[CH_2NH]Lys-Gln-Trp-Phe-βAla) (30). Compound **29** (10 mg, 0.011 mmol) was hydrogenated as for compound **31**, obtaining 6 mg (60%) of pure peptide **30**. FAB-MS: $[M + H]^+$, 761 *m/e* (calcd, 760.8). AAA: Glx 0.90 (1), Trp 0.86 (1), Phe 1.00 (1), βAla 1.06 (1).

Cyclo(LeuΨ[CH_2NCH_3]Cha-Gln-Trp-Phe-βAla) (26). Compound 11 (13 mg, 0.016 mmol) was dissolved in 15 mL of DCM/DMF (1/1, v/v), and 5.9 mg (0.048 mmol) of DMAP and 1.3 μL (0.016 mmol) of NMM were added. The solution was cooled to −15 °C, and 9.2 mg (0.048 mmol) of EDCI dissolved in 10 mL of DCM/DMF (1/1, v/v) was added dropwise. The mixture was allowed to stand for 40 h at 5 °C; then it was evaporated to dryness under vacuum, dissolved in 2 mL of DMF, and purified directly by semipreparative HPLC with a linear gradient from 30% B to 40% B in 40 min (0.25% B/min), obtaining 7 mg (54%) of peptide **26**. FAB-MS: $[M + H]^+$, 799 *m/e* (calcd, 798.9). AAA: Glx 0.93 (1), Trp 0.90 (1), Phe 1.00 (1), βAla 1.05 (1).

Cyclo(Leu-Cha-Gln-Trp-Phe-βAla) (32). Cyclization of 35 mg (0.043 mmol) of **37** was performed as for peptide **20**, and purification with a linear gradient from 30% B to 50% B in 40 min (0.5% B/min) gave 22 mg (63%) of peptide **32**. FAB-MS: $[M + H]^+$, 800 *m/e* (calcd, 799.5). AAA: Leu 0.89 (1), Cha 1.29 (1), Glx 0.92 (1), Trp 0.93 (1), Phe 1.00 (1), βAla 0.91 (1). *k'* (system 1) = 8.3 min.

Cyclo(Leu-Asp(NHCH_2Ada)-Gln-Trp-Phe-βAla) (33). Cyclization of 50 mg (0.054 mmol) of **38** was performed as for peptide **20**, and HPLC purification with a linear gradient from 35% B to 55% B in 40 min (0.5% B/min) gave 34 mg (68%) of peptide **33**. FAB-MS: $[M + H]^+$, 909 *m/e* (calcd, 908.7). AAA: Leu 1.02 (1), Asp(NHCH_2Ada) 0.93 (1), Glx 1.18 (1), Trp 0.85 (1), Phe 1.00 (1), βAla 0.97 (1). *k'* (system 1) = 10.3 min.

Biology. Male albino New Zealand rabbits (2.5−3 kg) and male Syrian golden hamsters (90−130 g) were stunned and bled. Endothelium-denuded strips of RPA and rings of HT were prepared for isometric tension recording in 5 mL baths for isolated organs containing oxygenated (96% O_2 and 4% CO_2) Krebs solution as described previously.[14] NKA was used as an agonist in the RPA and HT because the contractile response to tachykinins is entirely mediated, in these preparations, through NK-2 receptors. The responses to the agonist were concentration related and reproducible after 45−60 min from the first challange. Cumulative dose−response curves to NKA were constructed, and then the stated concentration of antagonist was added. After a 15 min incubation period, the curve to the agonist was repeated. Experiments were performed in the presence of captopril, bestatin, and thiorphan (1 μM each, 15 min before) to reduce peptide degradation.

Regression analysis was performed by means of the least square method; EC_{50} and 95% confidence limits were calculated accordingly. pD_2 values were calculated as −log molar concentration of agonist producing 50% of maximal effect (EC_{50}). At least three different concentrations of each antagonist were investigated. Only one concentration of antagonist was tested in each experiment. Schild plot analysis[25] was performed on at least nine experiments for each antagonist and each preparation. Since Schild plot analysis was compatible with competitive antagonism (slope not significantly different from −1), pA_2 values were calculated using the constrained Schild plot method.[26]

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References

(1) Abbreviations used follow the nomenclature recommended by the IUPAC-IUB Joint Commission on Biochemical Nomenclature (*Eur. J. Biochem.* **1984**, *138*, 9). The "psi-bracket" nomenclature system (i.e., the symbol "Ψ" followed by the structure that replaces the amide group inserted within square brackets) to indicate the replacement of an amide bond has been used according to Spatola, A. F. Peptide backbone modifications. In *Chemistry and Biochemistry of Amino Acids, Peptides and Proteins*; Weinstein, B., Ed.; Dekker: New York, 1983; pp 267−357. Other abbreviations: AAA, amino acid analysis; Ac, acetyl; Ada, 2-adamantyl; Boc, (*tert*-butyloxy)carbonyl; BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; Bzl, benzyl; Cha, β-cyclohexylalanine; DCM, dichloromethane; DCC, *N,N'*-dicyclohexylcarbodiimide; DCU, *N,N'*-dicyclohexylurea; DIEA, diisopropylethylamine; DMA, dimethylacetamide; DMAP, 4-(dimethylamino)pyridine; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; DPPA, diphenyl phosphorazidate; EDCI, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; FAB-MS, fast atom bombardment mass spectrometry; Fm, 9-fluorenylmethyl; Fmoc, [(9-fluorenylmethyl)oxy]carbonyl; HOBt, 1-hydroxybenzotriazole; i.d., internal diameter; IsOH, isopropyl alcohol; *k'*, capacity factor; Me, methyl; Nal, β-(2-naphthyl)alanine; NMM, *N*-methylmorpholine; PIP, piperidine; PyBOP, benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate; TEA, triethylamine; TFA, trifluoroacetic acid; TsOH, *p*-toluenesulfonic acid; Z, (benzyloxy)carbonyl.

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