A New Class of Pseudopeptide Antagonists of the Kinin B_1 Receptor Containing Alkyl Spacers

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Received August 24, 1998

Four previously reported kinin receptor peptide antagonists, including the B_1 receptor-selective peptides desArg[10]-HOE 140 (H-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-OH) and B-9858 (H-Lys-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-D-Igl-Oic-OH), have been modified by replacement of the central tetrapeptide Pro-Hyp-Gly-Xaa with linear alkyl spacers of variable length. The analogue of desArg[10]-HOE 140 containing the 11-aminoundecanoic acid as spacer, MEN 11575 [H-D-Arg-Arg-NH-$(CH_2)_{10}$-CO-Ser-D-Tic-Oic-OH], was found to be slightly more potent than the unmodified peptide ($pA_2 = 7.1$) as a kinin B_1 receptor antagonist in the rat ileum longitudinal smooth muscle assay. Moreover, MEN 11575 is devoid of residual agonist activity at the kinin B_1 receptor (rat ileum) and antagonist activity at the kinin B_2 receptor (guinea pig ileum longitudinal smooth muscle). Both these activities are displayed by the parent peptide desArg[10]-HOE 140. Therefore, despite its greatly simplified chemical structure, MEN 11575 shows an improved pharmacological profile in terms of both potency and selectivity, and it represents a good template for the development of new peptidomimetic kinin B_1 receptor antagonists. We also report an attempt to investigate the conformational role of the flexible, linear spacer of MEN 11575 and to design more constrained analogues, possibly locked in the bioactive conformation, using semirigid spacers based on C^{α}-tetrasubstituted α-amino acids of the family of 1-aminocycloalkane-1-carboxylic acids (Ac_nc).

Introduction

Two types of kinin receptors, termed B_1 and B_2, have been proposed by Regoli and Barabè[1] on the basis of the different rank order of potency of agonists and antagonists. More recently, the human kinin B_1 and B_2 receptors have been cloned,[2] and their genomic organization was defined.[3] The two kinins, bradykinin (BK,[4] H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH) and kallidin (KD, Lys-bradykinin), are generated as a result of the activity of the proteolytic enzymes kallikreins on kininogens. Once released, kinins exert a variety of biological actions of pathophysiological relevance, including pain, vasodilation, increased vascular permeability, smooth muscle contraction, and secretion.[5] Most of these effects are mediated by the kinin B_2 receptor, which exhibits higher affinity for BK and KD. These two kinins can be broken down by activity of carboxypeptidases to desArg[9]-BK and desArg[10]-KD, which in turn display a greater affinity for the B_1 receptor. The expression of this receptor is induced in pathological situations, particularly following tissue damage or the inflammatory process.[6] Consequently, the interest toward the development of kinin B_1 receptor-selective antagonists is constantly increasing.

A first class of peptide antagonists selective for the kinin B_1 receptor was described in the late 1970s by Regoli and co-workers.[1,7] The prototypical compound of this family is an analogue of desArg[10]-KD, with the Phe[9] residue replaced by Leu: desArg[10][Leu[9]]-KD (H-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Leu-OH). This peptide, although fairly potent ($pA_2 = 8.37$ vs desArg[9]-BK in isolated rabbit aorta; $pA_2 = 7.99$ vs desArg[9]-BK in human umbilical vein),[7,8] is rapidly metabolically degraded.[9] A more stable peptide antagonist was reported in 1991, following the observation that the potent kinin B_2 receptor antagonist HOE 140[10] can be converted to a B_1-selective antagonist upon removal of the C-terminal Arg residue: desArg[9]-D-Arg[Hyp[3],Thi[5],D-Tic[7],Oic[8]]-BK (desArg[10]-HOE 140, **1**; Table 1) has an IC_{50} of 1.2×10^{-8} vs desArg[9]-BK in the rabbit aorta.[11]

New antagonists have been obtained by further modifications of both these peptides. Gobeil et al.[9] reported that the modification of desArg[10][Leu[9]]-KD by replacement of Pro[8] by D-β-Nal and Leu[9] by Ile yields an analogue (desArg[10][D-β-Nal[8],Ile[9]]-KD, **2**) which maintains the same antagonist potency as desArg[10][Leu[9]]-KD at the rabbit kinin B_1 receptor ($pA_2 = 8.40$ in the rabbit aorta vs desArg[9]-BK) and is more potent at the human B_1 receptor ($pA_2 = 8.49$ in the human umbilical vein vs desArg[9]-BK). Most importantly, it is also partially resistant to enzymatic degradation.[9] Similarly, Stewart et al.[12] modified **1** by introduction of Igl in the place of Thi in position 5 and D-Igl in the place of D-Tic

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10.1021/jm980495r CCC: $18.00
Published on Web 01/27/1999

Table 1. Structure and Biological Activity of BK Antagonists

no.	structure[a]	pA_2[d] RI[b](B_1)	pA_2[d] GPI[c](B_2)
1	H-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-OH (desArg[10]-HOE 140)	6.8 ± 0.08	5.7 ± 0.13
2	Ac-Lys-Arg-Pro-Pro-Gly-Phe-Ser-D-βNal-Ile-OH	7.2 ± 0.24	6.0 ± 0.20
3	H-Lys-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-D-Igl-Oic-OH (B-9858)	8.1 ± 0.23	5.5 ± 0.22
4	H-D-Arg-Arg-Pro-Hyp-Gly-Igl-Ser-D-Igl-Oic-Arg-OH (B-9430)	7.7 ± 0.16	8.2 ± 0.11
5	H-D-Arg-Arg-Ahx-Ser-D-Tic-Oic-OH	5.7 ± 0.50	na[e]
6	H-D-Arg-Arg-Aoc-Ser-D-Tic-Oic-OH	5.4 ± 0.24	na
7	H-D-Arg-Arg-Aun-Ser-D-Tic-Oic-OH (MEN-11575)	7.1 ± 0.13	na
8	H-D-Arg-Arg-Ado-Ser-D-Tic-Oic-OH	7.0 ± 0.19	5.4 ± 0.25
9	H-Lys-Arg-Aun-Ser-D-Tic-Oic-OH	6.0 ± 0.18	na
10	Ac-Lys-Arg-Aun-Ser-D-Tic-Oic-OH	6.6 ± 0.17	na
11	H-Lys-Lys-Arg-Aun-Ser-D-Tic-Oic-OH	6.1 ± 0.21	na
12	H-D-Arg-Arg-Aun-Ser-D-Igl-Oic-OH	6.3 ± 0.40	6.0 ± 0.22
13	H-Lys-Lys-Arg-Aun-Ser-D-Igl-Oic-OH	6.3 ± 0.38	5.5 ± 0.35
14	H-D-Arg-Arg-Aun-Ser-D-Igl-Oic-Arg-OH	5.0 ± 0.30	6.1 ± 0.11
15	Ac-Lys-Arg-Aun-Ser-D-βNal-Ile-OH	7.0 ± 0.33	5.9 ± 0.11
16	H-D-Arg-Arg-Ac_7c-Ser-D-Tic-Oic-OH	5.9 ± 0.24	na
17	H-D-Arg-Arg-Gly-Ac_7c-Gly-Ser-D-Tic-Oic-OH	5.2 ± 0.22	na
18	H-D-Arg-Arg-βAla-Ac_7c-βAla-Ser-D-Tic-Oic-OH	5.6 ± 0.18	na
19	H-D-Arg-Arg-γ-Abu-Ac_7c-γ-Abu-Ser-D-Tic-Oic-OH	5.7 ± 0.20	≤5
20	H-D-Arg-Arg-Ac_6c-Ser-D-Tic-Oic-OH	5.0 ± 0.20	na
21	H-D-Arg-Arg-Ac_8c-Ser-D-Tic-Oic-OH	5.5 ± 0.13	≤5
22	H-D-Arg-Arg-Ac_9c-Ser-D-Tic-Oic-OH	5.2 ± 0.20	na

[a] Axx = $-NH-(CH_2)_n-CO-$, $n = 5$ Ahx; $n = 7$ Aoc; $n = 10$ Aun; $n = 11$ Ado; Ac_nc = 1-aminocycloalkane-1-carboxylic acids, $n = 6-9$; see ref 4 for other amino acid abbreviations. [b] RI, rat ileum longitudinal smooth muscle. [c] GPI, guinea pig ileum longitudinal smooth muscle. [d] pA_2, negative log of the molar concentration of antagonist which produces an agonist dose ratio of 2. Values are the mean ± SE of the mean of 4−15 experiments. [e] na, no activity up to 10 μM concentration.

in position 8, with a concomitant N-terminal elongation by two Lys residues. The resulting peptide **3** (B-9858) shows a pA_2 of 9.5 for B_1 receptor in the human ileum,[12] and its action is long lasting in vivo.[13] Interestingly, the introduction of the same modifications in the sequence of the B_2 receptor-selective antagonist HOE 140 yields an analogue (**4**, B-9430) showing high antagonist potency at both B_1 and B_2 kinin receptors [p$A_2(B_1) = 7.3$, p$A_2(B_2) = 8.6$ in the human ileum].[12]

Recently, our group reported a new class of B_1-selective pseudopeptide agonists, where the central tetrapeptide Pro-Pro-Gly-Phe of desArg[10]-KD was replaced by a flexible alkyl spacer.[14] The same approach was previously used by Kyle and co-workers[15] to modify the kinin B_2 receptor antagonist HOE 140, on the assumption that the minimal requirements for the interaction of agonist peptides with the kinin B_2 receptor are "an intact C-terminal β-turn structure with appropriate side chains in place and N-terminal amino and guanidine groups". The resulting pseudopeptide H-D-Arg-Arg-NH-$(CH_2)_{11}$-CO-Ser-D-Tic-Oic-Arg-OH, characterized by the presence of the 12-aminododecanoic acid in the place of residues 3−6 of HOE 140, was reported to have a pK_i of 6.4 against [^{3}H]BK in the classical guinea pig ileum B_2 binding assay and to be a functional antagonist against BK-induced contractions in the same tissue (p$A_2 = 5.5$).[15]

We describe here the application of this approach to several first- and second-generation B_1-selective peptide antagonists, by using both flexible and conformationally constrained alkyl spacers, the latter being based on the exploitation of C^α-tetrasubstituted α-amino acids of the family of 1-aminocycloalkane-1-carboxylic acids (Ac_nc).[16]

Chemistry

We previously reported[17] that the C-terminal dipeptide sequence of desArg[10]-HOE 140 (D-Tic-Oic-OH) is particularly prone to a well-known side reaction encountered in solid-phase peptide synthesis: diketopiperazine (DKP) formation by intramolecular aminolysis of the C-terminal dipeptide, with consequent cleavage of the peptide chain from the resin.[18] We also showed that the rate of DKP formation from the resin-bound D-Tic-Oic dipeptide is very high, due to the *N*-alkyl-amino acid structure of both these residues and the D-configuration of the first one. Therefore, it was not possible to suppress DKP formation by increasing the rate of deprotection of the Fmoc residue at the dipeptide stage, using a continuous flow synthetic strategy.[19] However, we obtained good results by the use of a hindered tertiary alcohol linker.[20] Accordingly, all of the peptides characterized by a C-terminal D-Tic-Oic dipeptide described in the present paper have been prepared on the 2-chlorotrityl resin,[21] according to the previously described protocol.[17] Briefly, the 2-chlorotrityl chloride resin was loaded with Fmoc-Oic-OH following the procedure published by Barlos et al.[21c] The amino acid was used in equimolar amount in order to obtain a substitution level in the range 0.3−0.4 mmol/g. Unreacted trityl groups were capped with methanol. Syntheses were performed manually, using a 2.5-fold excess of protected amino acid derivatives and HATU/NMM activation.[22] Each coupling was checked by the Kaiser test[23] and repeated if the test gave a positive result. After cleavage (reagent K, 1.5 h at room temperature) the crude peptide was precipitated by addition of cold diethyl ether, filtered, and lyophilized. Crude products were purified to homogeneity by semipreparative RP-

Table 2. Analytical Data of BK Antagonists

no.	HPLC[a] t_R (min)	MW (ES-MS[a]) calcd[b]	found[c]
1	12.7	1147.4	1147.8
3	8.8	1283.8	1284.0
4	13.6	1338.7	1338.2
5	13.0	840.5	840.5
6	12.0	868.5	868.6
7	14.8	910.6	910.8
8	13.7	924.6	924.5
9	13.2	882.6	882.6
10	13.5	924.7	924.7
11	12.9	1010.7	1010.9
12	14.4	924.6	924.4
13	13.7	1024.7	1024.6
14	12.9	1080.7	1080.6
15	14.5	924.6	924.6
16	13.4	866.5	866.7
17	12.9	980.6	980.6
18	12.9	1008.6	1008.6
19	13.0	1036.6	1036.7
20	12.9	852.5	852.6
21	13.8	880.5	880.8
22	14.3	894.5	894.5

[a] See Experimental Section for HPLC and MS methods. [b] Monoisotopic relative molecular mass. [c] Quasi-molecular ion minus 1 (m/z).

HPLC and characterized by analytical RP-HPLC and mass spectrometry (Table 2).

The Fmoc derivatives of Aoc and Aun, not commercially available, have been prepared by the Fmoc-OSu method.[24] The use of these derivatives in solid-phase peptide synthesis does not give any peculiar problem, under the conditions reported above. The poor water solubility of the Ac_nc residues with large rings ($n \geq 6$) hampers the introduction of N-protecting groups under standard conditions (aqueous/organic solvent mixtures). We extended the methodology proposed by Johnson and co-workers[25] to the Fmoc N-protection of such hydrophobic residues. The use of the lipophilic base tetramethylammonium hydroxide was, nevertheless, not sufficient to completely solubilize the Ac_nc residues in dioxane or acetonitrile, as also reported by Johnson for Ac_5c, but solubilization was easily achieved upon addition of a small amount of water. The yields were acceptable, despite the need of purifying the derivatives by means of a chromatographic technique.

In the past, the synthesis of peptides containing C^{α}-tetrasubstituted amino acids was regarded as difficult because it requires the use of very reactive reagents or harsh reaction conditions. More recently, the development of new coupling reagents enabled the use of these amino acids also in solid-phase peptide synthesis.[26] Accordingly, Fmoc-Ac_nc-OH were easily incorporated into our synthetic peptides using the standard conditions previously optimized for the preparation of desArg^{10}-HOE 140,[14] i.e., 2.5-fold excess of N-protected amino acid and HATU/NMM activation.

Results and Discussion

The biological activity of the pseudopeptides was tested in two functional assays: the rat ileum longitudinal smooth muscle, a kinin B_1 receptor assay, and the guinea pig ileum longitudinal smooth muscle, a preparation endowed only with the kinin B_2 receptor.[27] The results, expressed in terms of pA_2 (the negative log of the molar concentration of antagonist which produces an agonist dose ratio of 2), are listed in Table 1.

In a first series of analogues (compounds **5**−**8**) we introduced the same modification proposed by Kyle et al.[15] and subsequently exploited by us for the preparation of pseudopeptide B_1 receptor agonists,[14] namely, the replacement of the central tetrapeptide by a series of linear alkyl spacers of variable length. We chose as a template the B_1-selective receptor antagonist desArg^{10}-HOE 140 (**1**)[11] in which the tetrapeptide Pro^3-Hyp^4-Gly^5-Thi^6 was replaced by 6-aminohexanoic acid (Ahx, **5**), 8-aminooctanoic acid (Aoc, **6**), 11-aminoundecanoic acid (Aun, **7**), and 12-aminododecanoic acid (Ado, **8**), respectively. All these analogues are devoid of any agonist activity at the B_1 receptor up to 10^{-5} M concentration and behave as competitive antagonists. Interestingly, a first effect related to the introduction of the alkyl spacer into the sequence of desArg^{10}-HOE 140 is the removal of the slight agonist effect displayed by this antagonist.[27] The two analogues containing the shorter spacers, i.e., Ahx (**5**) and Aoc (**6**), are less potent than the unmodified peptide, while those containing Aun (**7**) and Ado (**8**) are slightly more potent. Similarly, the corresponding agonist analogues of desArg^{10}-KD previously described showed a roughly linear correlation between the length of the spacer and their agonist activity at the kinin B_1 receptor.[14] Interestingly, the pseudopeptides **5**−**8** show very weak antagonist activity, if any, at the B_2 receptor, while the reference compound **1** has a clear-cut B_2 receptor antagonist activity ($pA_2 = 5.7$).[11,28] Taken together, these data indicate that the replacement of the central tetrapeptide of desArg^{10}-HOE 140 by a linear alkyl spacer containing 10 (Aun) or 11 (Ado) methylene units improves the pharmacological profile of this B_1 receptor antagonist in terms of both potency and selectivity: in fact, introduction of the spacer enhanced the antagonist potency at the kinin B_1 receptor and abolished both the residual agonist activity at the B_1 receptor and, at least in the case of Aun, the weak antagonist activity at the B_2 receptor. Thus, compound **7** (termed MEN 11575) appears to be a good lead for the rational development of B_1-selective pseudopeptide antagonists.

Subsequently, we studied the structure−activity relationship (SAR) of the two residual peptide portions of MEN 11575 in order to optimize its activity. Initially, we focused our attention on the two positively charged N-terminal residues of desArg^{10}-HOE 140 (D-Arg-Arg). Since literature data indicate that the presence of Lys residue(s) at the N-terminus is favorable to antagonism at the B_1 receptor,[3,9,12] we replaced the N-terminal D-Arg residue in MEN 11575 by Lys (**9**), Ac-Lys (**10**), and Lys-Lys (**11**), respectively. However, these three pseudopeptides are less potent by 0.5−1 log unit at the B_1 receptor in the rat ileum, as compared to the unmodified compound **7**.

Further modifications were performed in the C-terminal portion of MEN 11575, following recent literature reports on new classes of B_1 receptor antagonists,[9,12] as outlined in the Introduction. Accordingly, we synthesized peptides **12** and **13**, bearing a D-Igl residue, as analogues of B-9858 (**4**). More precisely, compound **13** is a true analogue of B-9858, with the central tetrapeptide Pro-Hyp-Gly-Igl replaced by the undecanoic spacer (Aun), while **12** maintains the same N-terminal portion as MEN 11575, i.e., D-Arg-Arg, in

the presence of a D-Igl residue in place of D-Tic. However, both these peptides are less potent as kinin B_1 receptor antagonists as compared to MEN 11575, and they also show a residual B_2 antagonist activity. Similarly, peptide **14** is an analogue of the B_1/B_2 mixed antagonist B-9430, containing Aun in place of the central tetrapeptide. In this case, the modification brings about an almost total loss of kinin B_1 receptor antagonist activity along with a great reduction of activity also at the kinin B_2 receptor. Taken together, these data indicate that the D-Igl modification is not fully compatible with the introduction of an alkyl spacer in the central portion of these peptides.

Peptide **15** is based on the antagonist described by Gobeil et al.[9] (**2**), with the central portion replaced by Aun. In this case the effect of the substitution is more favorable: the resulting pseudopeptide **15** has the same activity as MEN 11575 at the kinin B_1 receptor, although it is also weakly active at the kinin B_2 receptor ($pA_2 = 5.9$).

We subsequently addressed our attention to the alkyl spacer, in an attempt to investigate its role and to design more constrained analogues, possibly locked in the bioactive conformation. As previously observed in the case of the agonists,[14] the higher activity of the peptides containing the longer spacers (cf. **7** and **8** vs **5** and **6**) could be explained by two hypotheses: (a) the optimal distance between the two termini of the ligand requires the longer spacers in an extended conformation; (b) the overall hydrophobicity of the central portion of the ligand plays a crucial role, and the spacers are in a coiled conformation. However, the high flexibility of the alkyl spacers enables their easy interconversion among various conformations. As a consequence, the activity of our pseudopeptide antagonists could result from a combination of both the above-mentioned effects. A first, very simple attempt to design a semirigid spacer was made by the use of C^{α}-tetrasubstituted α-amino acids of the family of 1-aminocycloalkane-1-carboxylic acids (Ac_nc).[16] Accordingly, Ac_7c (1-aminocycloheptane-1-carboxylic acid) was used to replace the flexible Aun spacer either by itself (peptide **16**) or flanked by two residues of Gly (**17**), β-Ala (**18**), or γ-Abu (**19**), which were introduced in order to modulate the overall length of the spacer. The best results in terms of antagonist potency were obtained when Ac_7c was used alone to replace Aun (peptide **16**), even if the potency as kinin B_1 receptor antagonist is lower by more than 1 log unit. This finding would suggest that hydrophobicity, as well as the conformational behavior of the linear alkyl spacer of MEN 11575, plays an important role in the activity of this antagonist. However, the conformationally constrained Ac_7c residue does not mimic effectively the bioactive conformation of the Aun-containing peptide. In addition, the ring size of the Ac_nc residue is not a key determinant for the antagonist activity of these compounds. Indeed, smaller (Ac_6c, peptide **20**) and larger (Ac_8c, **21**, and Ac_9c, **22**) rings roughly maintain the same activity.

In conclusion, we have obtained a new pseudopeptide antagonist selective for the kinin B_1 receptor which, despite the greatly simplified chemical structure, as compared to the reference compound desArg[10]-HOE 140, shows an improved pharmacological profile in terms of both potency and selectivity. This peptide (**7**), termed MEN 11575, is characterized by a linear, flexible alkyl spacer connecting two peptide portions. Preliminary attempts to investigate the role of this spacer indicate that it cannot be effectively mimicked by the conformationally restrained alicyclic C^{α}-tetrasubstituted α-amino acids. In any case, MEN 11575 represents a good template for the development of new peptidomimetic kinin antagonists.

Experimental Section

General. Protected standard amino acid and Fmoc-Ahx-OH were purchased from Novabiochem (Bubendorf, Switzerland); Fmoc-D-Tic-OH, Fmoc-Thi-OH, Fmoc-Oic-OH, Fmoc-βAla-OH, Fmoc-D-βNal-OH, and Fmoc-Hyp(*t*Bu)-OH were from Chem-Impex International (Wood Dale, IL); Fmoc-Ado-OH was from Advanced Chemtech (Louisville, KY), and Fmoc-D-Igl-OH was from Synthetech (Albany, OR). Fmoc-Ile-Novasyn TGA and 2-chlorotrityl chloride resins were purchased from Novabiochem. HATU was from PerSeptive Biosystem (Framingham, MA). Analytical grade CH_3CN, DMF, and MeOH were obtained from Lab-Scan (Dublin, Ireland), and all other reagents were from Aldrich (Milwaukee, WI) and Acros (Geel, Belgium). Peptide **2** (Ac-Lys-Arg-Pro-Pro-Gly-Phe-Ser-D-βNal-Ile-OH) was a kind gift of Prof. D. Regoli (University of Ferrara, Italy).

TLC was performed on silica gel 60-F_{254} aluminum sheets (Merck, Darmstadt, Germany), using the following eluants: 1 = $CHCl_3$/EtOH, 9:1; 2 = 1-BuOH/CH_3COOH/H_2O, 3:1:1; 3 = toluene/EtOH, 7:1; 4 = AcOEt/petroleum ether, 1:3. Melting points (mp) were determined in open capillaries and are uncorrected. ^1H NMR: Varian Gemini 200 MHz. MS analysis was perfomed on a VG Quattro mass spectrometer (Altricham, U.K.) equipped with standard electrospray (ES) ion source. The samples (about 50 μg/mL) were dissolved in a 1:1 mixture of CH_3CN/10 mM ammonium acetate containing 1% acetic acid and introduced by infusion at 5 μL/min using a Harvard Apparatus 11 pump (South Natik, MA). Ionization was obtained using positive ion pneumatically assisted ES, at a nebulizer voltage of 4 kV and a cone voltage of 50−70 V. Nitrogen was used as the nebulization and desolvation gas. The temperature of the ion source was 65 °C. For each sample, either singly or doubly charged quasi-molecular ions were obtained. The experimental and calculated molecular weights in Table 2 are expressed as monoisotopic molecular weights.

Synthesis of Fmoc-Aoc-OH and Fmoc-Aun-OH.[29] The amino acid (1 equiv) was suspended in water and dissolved under reflux, and pH was adjusted to 9 with a 10% Na_2CO_3 solution. A solution of Fmoc-OSu (1 equiv) in dioxane was then added dropwise over 20 min, and the mixture was stirred under reflux for 24 h. The reaction mixture was then diluted with water, acidified to pH 3 with HCl, and extracted twice with CH_2Cl_2. The organic layer was washed with water and dried over Na_2SO_4, and the solvent was removed to dryness.

Fmoc-Aoc-OH: yield 85%; mp 118−119 °C (from AcOEt/petroleum ether); $R_{f1} = 0.50$, $R_{f2} = 0.90$, $R_{f3} = 0.30$, $R_{f4} = 0.05$; ^1H NMR (1×10^{-2} M $CDCl_3$) δ 7.80−7.26 (m, 8H, Fmoc aromatic CH), 4.79 (s, 1H, NH), 4.41 (d, 2H, Fmoc CH_2), 4.22 (t, 1H, Fmoc 9-CH), 3.16 (m, 2H, Aoc αCH_2), 2.35 (t, 2H, Aoc βCH_2), 1.68−1.24 (m, 10H, Aoc ωCH_2).

Fmoc-Aun-OH: yield 92%; mp 117−118 °C (from AcOEt/petroleum ether); $R_{f1} = 0.50$, $R_{f2} = 0.95$, $R_{f3} = 0.30$, $R_{f4} = 0.10$; ^1H NMR (1×10^{-2} M $CDCl_3$) δ 7.80−7.20 (m, 8H, Fmoc aromatic CH), 4.80 (s, 1H, NH), 4.40 (d, 2H, Fmoc CH_2), 4.20 (t, 1H, Fmoc 9-CH), 2.30 (t, 2H, Aun αCH_2), 1.80−1.20 (m, 18H, Aun ωCH_2).

Synthesis of Fmoc-Ac_nc-OH: Typical Procedure. H-Ac_nc-OH (1 equiv) and tetramethylammonium hydroxide (TMAH; 1 equiv) were dissolved under stirring in a 9:1 mixture of dioxane (or CH_3CN)/water. A solution of Fmoc-OSu (1 equiv) in dioxane was then added. After 2 days at room temperature an excess of Fmoc-OSu (0.3 equiv) was added. Stirring was continued for 2 days; then the reaction mixture was concen-

trated under reduced pressure, acidified with 10% $KHSO_4$ solution, and extracted twice with AcOEt. The organic layer was washed with water and dried over Na_2SO_4, and the solvent was removed to dryness. The desired product was purified by means of silica column chromatography.

Fmoc-Ac$_6$c-OH: eluant for column chromatography, $CHCl_3$/EtOH, 99:1; yield 48%; mp 171−172 °C (from Et_2O/petroleum ether); $R_{f1} = 0.75$, $R_{f2} = 0.95$, $R_{f3} = 0.50$, $R_{f4} = 0.30$; ^{1}H NMR (1×10^{-2} M $CDCl_3$) δ 7.77−7.28 (m, 8H, Fmoc aromatic CH), 4.96 (s, 1H, NH), 4.45 (d, 2H, Fmoc CH_2), 4.23 (t, 1H, Fmoc 9-CH), 2.03 and 1.87 (2m, 4H, Ac$_6$c βCH_2), 1.63 and 1.40 (2m, 6H, Ac$_6$c γ and δCH_2).

Fmoc-Ac$_7$c-OH: eluant for column chromatography, petroleum ether/AcOEt/EtOH, 70:25:5; yield 51%; mp 193−194 °C (from AcOEt/petroleum ether); $R_{f1} = 0.80$, $R_{f2} = 0.95$, $R_{f3} = 0.50$, $R_{f4} = 0.35$; ^{1}H NMR (1×10^{-2} M $CDCl_3$) δ 7.77−7.27 (m, 8H, Fmoc aromatic CH), 5.02 (s, 1H, NH), 4.43 (d, 2H, Fmoc CH_2), 4.21 (t, 1H, Fmoc 9-CH), 2.15 and 2.00 (2m, 4H, Ac$_7$c βCH_2), 1.57 (m, 8H, Ac$_7$c γ and δCH_2).

Fmoc-Ac$_8$c-OH: eluant for column chromatography, toluene/EtOH, 26:1; yield 44%; mp 188−189 °C (from AcOEt/petroleum ether); $R_{f1} = 0.85$, $R_{f2} = 0.95$, $R_{f3} = 0.50$, $R_{f4} = 0.35$; ^{1}H NMR (1×10^{-2} M $CDCl_3$) δ 7.77−7.27 (m, 8H, Fmoc aromatic CH), 4.95 (s, 1H, NH), 4.42 (d, 2H, Fmoc CH_2), 4.21 (t, 1H, Fmoc 9-CH), 2.05 (m, 4H, Ac$_8$c βCH_2), 1.54 (m, 10H, Ac$_8$c γ, δ and ϵCH_2).

Fmoc-Ac$_9$c-OH: eluant for column chromatography, toluene/EtOH, 26:1; yield 45%; mp 186−187 °C (from CH_2Cl_2/petroleum ether); $R_{f1} = 0.85$, $R_{f2} = 0.95$, $R_{f3} = 0.50$, $R_{f4} = 0.40$; ^{1}H NMR (1×10^{-2} M $CDCl_3$) δ 7.78−7.27 (m, 8H, Fmoc aromatic CH), 4.90 (s, 1H, NH), 4.44 (d, 2H, Fmoc CH_2), 4.21 (t, 1H, Fmoc 9-CH), 1.97 (m, 4H, Ac$_9$c βCH_2), 1.54 (m, 12H, Ac$_9$c γ, δ and ϵCH_2).

General Procedure for the Solid-Phase Peptide Synthesis (SPPS) with the Fmoc Strategy. (a) Manual Synthesis. All of the peptides containing a C-terminal Oic residue were synthesized manually, in a mechanically agitated reactor, using DMF as solvent. Fmoc-Oic-OH was linked to the 2-chlorotrityl resin following the procedure described by Barlos et al.[21c] The amino acid, in equimolar amount, was dissolved in CH_2Cl_2, and the resin was added together with 1 equiv of DIEA. The reaction mixture was stirred for 5 min, then 1.5 equiv of DIEA in CH_2Cl_2 (1:1) was added, and the mixture was stirred again for 30 min. Subsequently, an excess of methanol was added, to end-cap the remaining trityl group of the resin. The substitution level was measured by UV determination of the 9-fluorenylmethylpiperidine formed after cleavage of the Fmoc group with piperidine. Fmoc deprotection was obtained by treatment with 20% piperidine in DMF for 20 min. Couplings activation was achieved with a 2.5-fold excess of HATU, the same excess of protected amino acids, and a 5-fold excess of NMM for 40−180 min. Completion of each coupling was monitored by the Kaiser test. At the end of the synthesis the resin was washed with DMF, MeOH, CH_2Cl_2, and diethyl ether and dried under vacuum. After cleavage with reagent K (TFA/phenol/thioanisol/H_2O/ethanedithiol, 82.5:5:5:5:2.5, 120 min at room temperature) the crude peptides were precipitated by addition of cold diethyl ether, filtered, dissolved in water, and lyophilized. Crude peptides were analyzed by analytical RP-HPLC on a Beckman System Gold apparatus (SanRamon, CA) under the following conditions: Vydac C_{18} column, 0.46 × 15 cm; eluant A, 0.1% TFA in H_2O; eluant B, 0.1% TFA in CH_3CN; gradient from 5% to 65% B over 20 min; flow 1 mL/min; UV detection at 210 nm.

(b) Automated Synthesis. All other syntheses were performed on a Milligen 9050 peptide synthesizer (Bedford, MA), using the high-flow protocol on a PEG-PS resin. Coupling cycle of 20 min was carried out with 3-fold excess of amino acids and HATU as the activating reagent. Fmoc group removal was obtained by treatment with 2% piperidine and 2% DBU in DMF for 5 min. After completion of the synthesis, the resin was washed and the crude peptide was cleaved and analyzed on RP-HPLC as reported in the previous section.

(c) Purification. Crude peptides were purified by semipreparative RP-HPLC on a Beckman System Gold apparatus, under the following conditions: Vydac C_{18} column, 1 × 25 cm; eluant A, 0.1% TFA in H_2O; eluant B, 0.1% TFA in CH_3CN; gradient from 15% to 45% B over 200 min; flow 4 mL/min; UV detection at 210 nm.

Rat and Guinea Pig Ileum Longitudinal Smooth Muscle Assays. Longitudinal muscle-myenteric plexus preparations of rat or guinea pig ileum were prepared as previously described[27] and put in organ baths filled with oxygenated (95% O_2, 5% CO_2) normal Krebs' solution containing indomethacin, guanethidine (3 μM each), chlorpheniramine, and atropine (1 μM each). Cumulative concentration−response curves to BK or to desArg^9-BK were constructed in the guinea pig or rat ileum, respectively, in the presence of the peptidase inhibitors thiorphan, bestatin, and captopril (1 μM each). We first investigated the possible agonist activity of all the peptides at B_1 and B_2 receptors as follows: Those peptides which did not produce any motor effect (contraction) in the two preparations used, up to 10 μM concentration, were considered inactive as agonists. Those peptides which, at the highest concentration tested (10 μM), produced a contraction lower than 50% of the maximal response produced by the full agonist (BK or desArg^9-BK), were considered as very weak agonists. The latter compounds were expected to produce 50% of the maximal response at concentrations exceeding 10 μM and hence to have pD_2 lower than 5. Those peptides which produced a contraction > 50% of the maximal response at 10 μM or lower concentrations were assayed for their agonist activity in further experiments in which complete concentration−response curves were constructed. For the characterization of the antagonist activity, the compounds under study were administered 15 min before repetition of the agonist curve, at concentrations 1−10 μM, and were tested for their ability to block the contractile response to the agonist. Antagonist potency was expressed in terms of pA_2 (the negative log of the molar concentration of antagonist which produces an agonist dose ratio of 2), while agonist activity was expressed in terms of pD_2 (negative log of the agonist concentration which produces 50% of the maximal effect). All the values reported are mean ± SE of the mean.

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(4) Abbreviations are in accord with the recommendations of the IUPAC−IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.* **1972**, *247*, 977). All amino acids are in the L-configuration unless otherwise specified. Other abbreviations: γ-Abu, 4-aminobutyric acid; Ac$_n$c, 1-aminocycloalkane-1-carboxylic acids; Ado, 12-aminododecanoic acid; Ahx, 6-aminohexanoic acid; Aoc, 8-aminooctanoic acid; Aun, 11-aminoundecanoic acid; BK, bradykinin; Boc, *tert*-butyloxycarbonyl; DIEA, diisopropylethylamine; DMF, dimethylformamide; ES-MS, electrospray ionization mass spectrometry; Fmoc, 9-fluorenylmethoxy-carbonyl; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; Hyp, 4-hydroxy-L-proline; Igl, α-(2-indanyl)-L-glycine; KD, kallidine; βNal, β-3-(2-naphthyl)alanine; NMM, *N*-methylmorpholine; NMP, *N*-methylpyrrolidone; Oic, (3a*S*,7a*S*)-octahydroindole-2-carboxylic acid; PEG-PS, poly(ethylene glycol) polystyrene; RP-HPLC, reverse-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; Thi, β-(2-thienyl)-L-alanine; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid.

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JM980495R