Design and Synthesis of Motilin Antagonists Derived from the [1−4] Fragment of Porcine Motilin

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A series of cyclic peptides having the general structure H-Phe-c[-N$^{\epsilon}$-Lys-X-NH-$(CH_2)_n$-CO-] were designed on the basis of structure−activity relationship studies of motilin. All were motilin antagonists. The cyclic peptides, in which X is a 3-*tert*-butyl-substituted tyrosine residue (H-Phe-c[-N$^{\epsilon}$-Lys-Tyr(3-tBu)-βAla-] (**3**), H-Phe-c[-N$^{\epsilon}$-Lys-Tyr(3-tBu)-Gly-] (**6**), H-Phe-c[-N$^{\epsilon}$-Lys-Tyr(3-tBu)-Abu-] (**7**), and H-Phe-c[-N$^{\epsilon}$-Lys-Tyr(3-tBu)-Ahx-] (**8**)) showed potent motilin receptor antagonistic activity in the rabbit smooth muscle ($pA_2 > 7$). The 3-*tert*-butyl Tyr was found to be the moiety responsible for enhanced binding to the motilin receptor, while the size of the ring had little importance.

Introduction

Motilin is a 22 amino acid gastrointestinal (GI) peptide that stimulates contractile activity of gastrointestinal smooth muscle. In man and in dog motilin appears to be involved in the regulation of the fasting motility pattern.[1] Motilin is also able to stimulate gastric emptying, and the finding that erythromycin is a motilin agonist[2] has led to the development of potent erythromycin derivatives, which have been named motilides[3] and which may be useful for the treatment of hypomotility disorders such as gastroparesis.[4]

Recently, it was suggested that motilin may also be involved in the production of some GI symptoms, such as early satiety, abdominal distention, nausea, vomiting, and anorexia,[5] suggesting that motilin antagonists may have clinical applications as well. Moreover, motilin antagonists would be useful tools for studying the physiological role of motilin and its mechanism of action. The first motilin antagonist that was described was an analogue of the 1−14 fragment of porcine motilin, namely, [1−14] Phe^3, Leu^{13} porcine motilin.[6] Soon thereafter, it was shown that the corresponding full length molecule had higher affinity for the motilin receptor and therefore was also a more potent antagonist.[7] Unfortunately both these molecules also have agonist properties. Moreover, they have a short half-life, limiting their usefulness in vivo. Recently, a non-peptide motilin receptor antagonist was identified through electronic screening of a database against a 3D pharmacophore for motilin.[8] This molecule was effective in inhibiting motilin-induced contractility in segments of the rabbit duodenum.[8] However, in vivo assay data have not been reported.

We recently found that [1−4] Tyr^3 porcine motilin (H-Phe-Val-Tyr-Ile-NH_2) has an affinity of 11 μM (IC_{50}) for the motilin receptor (MTL-R) but is devoid of contractile effects up to 100 μM (unpublished data). On the basis of this result, the tetrapeptide was thought to be either a metabolically unstable agonist, even in a contraction assay, or an antagonist. However, metabolic inactivation of the motilin tetrapeptide was not observed in the assay system (unpublished data). On the basis of this result, the compound was hypothesized to be a motilin receptor antagonist. We have therefore focused on the design and synthesis of more potent motilin antagonists via enhancement of the binding affinity of this tetrapeptide.

It was anticipated that appropriate conformational restriction of the peptide, through cyclization, would result in the formation of improved motilin receptor antagonists,[9−11] and three cyclization modes for this peptide were considered: (a) N-terminus to C-terminus, (b) N-terminus to side chain, and (c) side chain to C-terminus (Figure 1). Several ^{1}H NMR studies of motilin[12−15] showed that Phe^1 has considerable flexibility. This residue is very important for binding because its substitution by alanine in [1−14] Leu^{13} motilin causes the binding affinity to drop more than 100-fold.[16] The next residue, Val^2, does not seem to be part of the motilin pharmacophore, which involves residues 1, 4, and 7.[16,17] Residue 7 is a Tyr, and our fragment contained a tyrosine in position 3, the same position that introduced antagonist properties to Leu^{13} porcine motilin when substituted with Phe.[6,7] On the basis of these considerations, we hypothesized that a cyclic peptide, connecting the side chain of the second residue with the C-terminus of the peptide (mode C), would bind tightly to MTL-R. Also, a peptide, designed with this mode, was thought to have almost the same size ring as erythromycin. Therefore, we focused our effort on the design and synthesis of cyclic peptides bearing a modified Tyr with an aim of enhancing the binding affinity.[16−18] Herein, we report the syntheses and biological activities of the cyclic motilin antagonists, H-Phe-c[-N$^{\epsilon}$-Lys-X-NH-$(CH_2)_n$-CO-] (Table 1).

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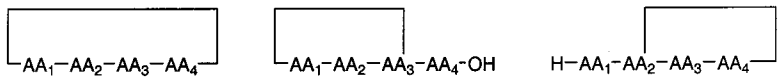


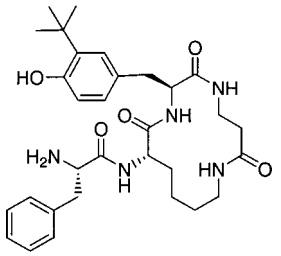
(a) N-terminus to C-terminus (b) N-terminus to side-chain (c) side-chain to C-terminus.

Figure 1. Various modes of cyclization.

Table 1. Structures, Contractile Potency, and Binding Affinity of Cyclic Peptides

	compound	binding affinity IC_{50} (nM)	contractile potency[a] EC_{50} (nM)	contractile potency[a] pA_2[b]
1	H-Phe-c[-N$^{\epsilon}$-Lys-Tyr-βAla-]	25 000 and 42 000[c]	>100 000	4.05 ± 0.07 (n = 6)
2	H-Phe-c[-N$^{\epsilon}$-Lys-Tyr(O-secBu)-βAla-]	480[d]		
3	H-Phe-c[-N$^{\epsilon}$-Lys-Tyr(3-tBu)-βAla-]	10 ± 4 (n = 3)[e]	>100 000	7.37 ± 0.24 (n = 10)
4	H-Phe-c[-N$^{\epsilon}$-Lys-Tyr(O-tBu)-βAla-]	7500[d]	>100 000	3.92 ± 0.38 (n = 6)
5	H-Phe-c[-N$^{\epsilon}$-Lys-Tyr(3,5-di-tBu)-βAla-]	460[d]	>10 000	
6	H-Phe-c[-N$^{\epsilon}$-Lys-Tyr(3-tBu)-Gly-]	7 and 33[c]	>100 000	7.33 ± 0.06 (n = 6)
7	H-Phe-c[-N$^{\epsilon}$-Lys-Tyr(3-tBu)-Abu[f]-]	33 and 59[c]	>100 000	7.46 ± 0.17 (n = 6)
8	H-Phe-c[-N$^{\epsilon}$-Lys-Tyr(3-tBu)-Ahx[g]-]	17 and 65[c]	>100 000	7.08 ± 0.08 (n = 6)
9	H-D-Phe-c[-N$^{\epsilon}$-Lys-Tyr(3-tBu)-βAla-]	1800[d]	>100 000	5.70 ± 0.11 (n = 6)
10	H-Phe-c[-N$^{\epsilon}$- D-Lys-Tyr(3-tBu)-βAla-]	2100[d]	>100 000	5.66 ± 0.08 (n = 6)
11	H-Phe-c[-N$^{\epsilon}$-Lys-D-Tyr(3-tBu)-βAla-]	3100[d]	>100 000	5.27 ± 0.15 (n = 6)

[a] Contractile potency data are presented as the mean ± SEM with the number of repetitions from independent assays (n) listed in parentheses. [b] pA_2 is the negative logarithm of the molar concentration of antagonist causing a 2-fold shift to the right of the concentration–response curve for Motilin. [c] Binding affinity data are based on two determinations. [d] Binding affinity data are based on one determination. [e] Binding affinity data are presented as the mean ± SEM with the number of repetitions from independent assays (n) listed in parentheses. [f] Abu: 4-aminobutyric acid. [g] Ahx: 6-aminohexanoic acid.



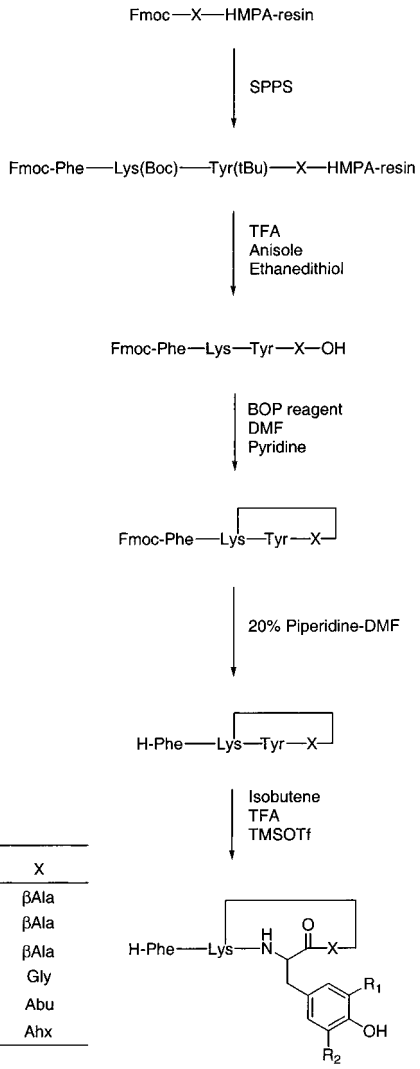
3 (GM-109)

Chemistry

The peptides were synthesized by a solid-phase peptide synthesis (SPPS) methodology (Scheme 1),[19,20] according to an N^{α}-Fmoc (N^{α}-9-fluorenylmethyloxycarbonyl) group strategy[21] on a HMPA resin (*p*-hydroxymethylphenylacetic acid substituted resin). The deprotection of the N^{α}-Fmoc group was conducted with 20% piperidine in *N*,*N*-dimethylformamide (DMF) solution prior to coupling with the next protected amino acid. All amino acids were coupled as a pentafluorophenyl (Pfp) esters in the presence of *N*-hydroxybenzotriazole (HOBt) or as symmetrical anhydrides in the presence of diisopropylcarbodiimide (DIC) and HOBt. The completion of the couplings was verified by the Kaiser test.[22] The peptides were simultaneously deprotected and cleaved from the resin by treatment with trifluoroacetic acid (TFA)/anisole/ethanedithiol (94:5:1; v/v) for 1–3 h at 0 °C. Crude Fmoc peptides were cyclized with BOP reagent, followed by deprotection of the Fmoc moiety. Alkylation of Tyr was carried out with isobutene or 1-butene in the presence of TFA and trimethylsilyl triflate (TMSOTf).

The cyclic peptides were purified by preparative reversed-phase (RP) HPLC. The purified peptides were analyzed by analytical RP-HPLC and characterized by Fourier transform (FT) NMR and high-resolution FAB-MS (see Experimental Section and Supporting Information).

Scheme 1. Synthesis of Cyclic Peptide



No.	R_1	R_2	X
1	H	H	βAla
3	tBu	H	βAla
5	tBu	tBu	βAla
6	tBu	H	Gly
7	tBu	H	Abu
8	tBu	H	Ahx

Results and Discussion

The synthesized cyclic tetrapeptides were tested for binding affinity to the motilin receptor (MTL-R) and for smooth muscle contractile activity in the rabbit smooth muscle, and the data are summarized in Table 1.

All peptides showed binding affinity, but those with an alkyl-substituted Tyr residue (compounds **2**−**11**) were more potent than the one compound with the nonsubstituted Tyr (compound **1**). This result suggested that the alkyl substituent on Tyr^3 contributes to a hydrophobic interaction with the MTL-R. The 3-*tert*-butyl-substituted Tyr derivatives (compounds **3** and **6**−**8**) especially showed good binding affinity, and all were at least 400 times more potent than **1**. The most potent compound, **3**, bound to MTL-R with an IC_{50} of 10 ± 4 nM.

The table also shows that cyclic peptides with varying ring sizes (compounds **3** and **6**−**8**) had similar affinity with an IC_{50} in the range 7−65 nM. The data indicate that the size of the ring is not important in determining the affinity for the MTL-R, although it was important in the macrolides.[4] The molecule was initially designed so that the peptide backbone, via cyclization, would mimic the macrolide ring of erythromycin. The results, however, suggest that the peptide ring may play a role different from that of the macrolide ring.

In contrast, replacement in compound **3** of any of the first three residues with the D isomer (compounds **9**−**11**) strongly reduced binding affinity, suggesting that the stereochemistry of the compound was important. In motilin, changing Phe^1 to its D isomer did not reduce the affinity drastically.[16] The stereoconformation of these compounds seemed to be more important than in motilin itself.

Importantly, the antagonistic activity of these compounds in the contractile assay correlated with their binding affinity, and no contractile activity was observed even at high concentration (>100 μM). Thus, 3-*tert*-butyl-substituted Tyr derivatives (compounds **3** and **6**−**8**) showed good antagonistic activities, with pA_2 values of 7.37 ± 0.24, 7.33 ± 0.06, 7.46 ± 0.17, and 7.08 ± 0.08, respectively in the rabbit duodenum contractile assay. Compound **3** (GM-109), which has the highest binding affinity (pK_i = 7.99 ± 0.04),[23] showed antagonism in an in vivo assay in dog.[24] It has also been reported that compound **3** (GM-109) antagonizes motilin-induced calcium signaling in TE671 cells (a cell line of human cerebellar origin)[25] and in cultured myocytes from the human colon.[26] In these systems, compound **3** (GM-109) did not show any agonist activity. Also, compound **3** (GM-109) had no effect on the contractions induced by substance P, acetylcoline ACh, KCl, and prostaglandin $F_{2\alpha}$ in the rabbit duodenum contractile assay.[23]

In conclusion, we designed and synthesized potent cyclic peptide motilin antagonists based on the structure H-Phe-c[-N$^\epsilon$-Lys-Tyr-βAla-]. Substitution of Tyr with 3-*tert*-butyl-Tyr strongly increases potency. A change of cycle length by replacing βAla^4 with Gly, Abu, or Ahx has little effect. Replacement of residues 1−3 with their D isomers reduced the potency. These antagonists should be useful in the investigation of the physiological role of motilin and may find application in diseases associated with increased motilin secretion.

Experimental Section

Materials. N^α-Fmoc amino acids and Pfp esters were purchased from MilliGen/Biosearch (Burlington, MA) and Watanabe Chemical (Japan). HMPA resin (PEPSYN KA, 0.2 mmol/g) was purchased from MilliGen/Biosearch (Burlington, MA). TFA, TMSOTf, 1,3-diisopropylcarbodiimide (DIC), and HOBt were purchased from Watanabe Chemical. 4-(Dimethylamino)pyridine (DMAP), piperidine, triethylamine, 1-butene, isobutene, ethanedithiol, and anisole were purchased from Tokyo Chemical Industry (Japan). Methanol (MeOH), acetic acid (AcOH), diethyl ether, and $NaHCO_3$ were from Junsei Chemical (Japan). Pd/C was purchased from Kawaken Fine Chemicals (Japan). DMF was purchased from Kokusan Chemical (Japan). HPLC grade acetonitrile and water were from Kanto Chemical (Japan) and Junsei Chemical (Japan).

Solid-Phase Peptide Synthesis of H-Phe-c[-N$^\epsilon$-Lys-Tyr-βAla-] (1). HMPA resin (PEPSYN KA, 0.2 mmol/g, 50 g) was placed in DMF and allowed to swell overnight. N^α-Fmoc-βAla-OH (8 equiv) was dissolved in DMF, and DIC (4 equiv) was added to the solution. DMAP (1 equiv) was dissolved in DMF. The amino acid/DIC solution and DMAP solution were added to the resin in DMF, and this was shaken slowly and left overnight at room temperature.

The resin was washed twice with DMF, twice with MeOH, twice with AcOH, twice with MeOH, and three times with DMF. The following N^α-Fmoc amino acid Pfp esters were sequentially coupled to a growing peptide chain: N^α-Fmoc-Tyr(tBu)-OPfp, N^α-Fmoc-Lys(Boc)-OPfp, N^α-Fmoc-Phe-OPfp. Each coupling cycle consisted of the following: (1) treating three times with 20% piperidine in DMF, (2) washing six times with DMF, (3) coupling the amino acid active ester, and (4) washing six times with DMF. All the N^α-Fmoc amino acid Pfp esters (2.5 equiv) were coupled to a growing peptide chain using HOBt (2.5 equiv) in DMF. All the coupling reactions were shaken on a manual shaker for 3−12 h at room temperature. After coupling of the last N^α-Fmoc amino acid, the peptide resin was washed with MeOH and dried in vacuo to yield the protected Fmoc-Phe-Lys(Boc)-Tyr(tBu)-βAla-HMPA resin (58.6 g).

The protected peptide resin was treated with TFA/anisole/ethanedithiol (94:5:1; v/v) for 3 h at 0 °C. The solution was filtered, concentrated under the reduced pressure, and added dropwise to excess cold diethyl ether. The precipitated peptide was filtered, washed with diethyl ether, and dried. The peptide was dissolved in DMF (3 L) and pyridine (3 L), BOP reagent (28 g) was added, and the mixture was left for 24 h at room temperature. The solvent was removed under reduced pressure, and water was added to the residue. The precipitate was collected by filtration, washed with water, and dried. The Fmoc cyclic peptide was treated with 20% piperidine in DMF for 0.5 h at room temperature, and the solvent was removed under reduced pressure.

The crude peptide was purified by preparative HPLC with a linear gradient of 0−60% of 0.1% TFA in acetonitrile against 0.1% aqueous TFA over 60 min at 10 mL/min. The lyophilized product was a white amorphous solid (6.7 g). Overall yield: 86%. ^{1}H NMR (DMSO-d_6): δ 1.00−1.66 (6H, m, 3 × CH_2), 2.21 (2H, m, CH_2), 2.71−2.97 (4H, m, 2 × CH_2), 3.10−3.65 (4H, m, 2 × CH_2), 3.93 (1H, m, CH), 4.27 (1H, m, CH), 4.44 (1H, m, CH), 6.62 (2H, d, J = 7.6 Hz, aromatic-H), 6.95 (2H, d, J = 7.9 Hz, aromatic-H), 7.11−7.30 (5H, m, aromatic-H), 7.39 (1H, m, NH), 7.64 (1H, m, NH), 8.39 (1H, m, NH), 8.51 (1H, m, NH). FAB-MS m/z: calcd 510.2716, found 510.2722 $(MH)^+$.

H-Phe-c[-N$^\epsilon$-Lys-Tyr(O-secBu)-βAla-] (2). H-Phe-c[-N$^\epsilon$-Lys-Tyr-βAla-] (1) (500 mg) was dissolved in TFA (10 mL), and TMSOTf (0.1 mL) was added. 1-Butene gas was added for 20 min at −10 °C, and the mixture was left for 18 h at room temperature. The reaction solution was added dropwise to excess cold diethyl ether, and the precipitated peptide was collected by centrifugation and dried. The crude peptide was purified by preparative HPLC with a linear gradient of 0−60% of 0.1% TFA in acetonitrile against 0.1% aqueous TFA over 60 min at 10 mL/min. The lyophilized product was a white

amorphous solid. H-Phe-c[-N$^{\epsilon}$-Lys-Tyr(O-secBu)-βAla-] (**2**) (21.7 mg, yield 4%). ^{1}H NMR (DMSO-d_6): δ 0.81 (3H, m, CH_3), 1.07 (3H, t, J = 5.6 Hz, CH_3), 1.12−1.36 (4H, m, 2 × CH_2), 1.37−1.67 (4H, m, 2 × CH_2), 2.23 (2H, m, CH_2), 2.66−2.95 (4H, m, 2 × CH_2), 3.20−3.55 (4H, m, 2 × CH_2), 3.96 (1H, m, CH), 4.14 (1H, m, CH), 4.30 (1H, m, CH), 4.46 (1H, m, CH), 6.74 (2H, d, J = 8.3 Hz, aromatic-H), 7.06 (2H, d, J = 8.3 Hz, aromatic-H), 7.16−7.26 (5H, m, aromatic-H), 7.40 (1H, brt, J = 5.4 Hz, NH), 7.67 (1H, m, NH), 8.49 (1H, d, J = 9.2 Hz, NH), 8.61 (1H, d, J = 17.5 Hz, NH). FAB-MS *m*/*z*: calcd 566.3342, found 566.3352 (MH)$^+$.

H-Phe-c[-N$^{\epsilon}$-Lys-Tyr(3-tBu)-βAla-] (3) and H-Phe-c[-N$^{\epsilon}$-Lys-Tyr(3,5-di-tBu)-βAla-] (5). H-Phe-c[-N$^{\epsilon}$-Lys-Tyr-βAla-] (**1**) (170 mg) was dissolved in TFA (10 mL), and TMSOTf (0.1 mL) was added. Isobutene gas was added for 20 min at −10 °C, and the mixture was left for 12 h at room temperature. The reaction solution was added dropwise to excess cold diethyl ether, and the precipitated peptide was collected by centrifugation and dried. The crude peptide was purified by preparative HPLC with a linear gradient of 0−60% of 0.1% TFA in acetonitrile against 0.1% aqueous TFA over 60 min at 10 mL/min. The lyophilized products were white amorphous solids. H-Phe-c[-N$^{\epsilon}$-Lys-Tyr(3-tBu)-βAla-] (**3**) (85.5 mg, yield 46%). ^{1}H NMR (DMSO-d_6): δ 1.14 (2H, m, CH_2), 1.29 (9H, s, 3 × CH_3), 1.30, 1.40 (2H, 2m, CH_2), 1.54 (2H, m, CH_2), 2.19 (2H, m, CH_2), 2.67 (1H, dd, J = 7.9, 13.9 Hz, CH_2), 2.84 (2H, m, CH_2), 2.94 (1H, dd, J = 5.5, 14.0 Hz, CH_2), 3.26−3.44 (4H, m, 2 × CH_2), 4.00 (1H, brt, J = 5.6 Hz, CH), 4.29 (1H, dt, J = 5.0, 7.6 Hz, CH), 4.42 (1H, dt, J = 7.1, 7.6 Hz, CH), 6.63 (1H, d, J = 7.9 Hz, aromatic-H), 6.78 (1H, dd, J = 2.0, 8.0 Hz, aromatic-H), 6.94 (1H, d, J = 2.0 Hz, aromatic-H), 7.16 (2H, m, aromatic-H), 7.23 (3H, m, aromatic-H), 7.38 (1H, t, J = 6.0 Hz, NH), 7.63 (1H, dd, J = 4.5, 7.5 Hz, NH), 8.38 (1H, d, J = 8.9 Hz, NH), 8.57 (1H, d, J = 7.6 Hz, NH). FAB-MS *m*/*z*: calcd 566.3342, found 566.3328 (MH)$^+$.

H-Phe-c[-N$^{\epsilon}$-Lys-Tyr(3,5-di-tBu)-βAla-] (**5**) (41 mg, yield 20%). ^{1}H NMR (DMSO-d_6): δ 1.10−1.67 (6H, m, 3 × CH_2), 1.34 (18H, s, 6 × CH_3), 2.24 (2H, m, CH_2), 2.42−2.58 (2H, m, CH_2), 2.63−3.02 (6H, m, 3 × CH_2), 4.03 (1H, m, CH), 4.34 (1H, m, CH), 4.43 (1H, m, CH), 6.92 (2H, s, aromatic-H), 7.20 (5H, m, aromatic-H), 7.43 (1H, m, NH), 7.61 (1H, m, NH), 8.65 (1H, d, J = 9.1 Hz, NH), 8.58 (1H, d, J = 8.0 Hz, NH). FAB-MS *m*/*z*: calcd 622.3968, found 622.3961 (MH)$^+$.

H-Phe-c[-N$^{\epsilon}$-Lys-Tyr(O-tBu)-βAla-] (4). *Z*-Lys-Tyr(tBu)-βAla-HMPA resin was synthesized by the same procedure as that for **1** but using N^{α}-Fmoc-βAla-OH, N^{α}-Fmoc-Tyr(O-tBu)-OH, and Z-Lys(Fmoc)-OH. The peptide resin was treated with MeOH (270 mL) and triethylamine (30 mL) for 18 h at room temperature. After filtration, the solution was evaporated to dryness and treated with MeOH/H_2O/Et_3N (2:2:1; v/v) for 24 h at room temperature. After evaporation, the residue was dissolved in DMF (200 mL) and pyridine (200 mL). BOP reagent (2 g) was added to the solution, and the mixture was left for 18 h at room temperature. After evaporation, water was added and the precipitate was filtered, washed once with 5% $NaHCO_3$ (aq) and twice with water. The dried solid was dissolved in DMF (20 mL) and hydrogenated with Pd/C (200 mg) for 3 h. The solution was filtered and evaporated to dryness. The residue was dissolved in DMF (5 mL) and coupled with N^{α}-Fmoc-Phe-OPfp (1.11 g) using HOBt (306 mg) for 18 h at room temperature. After evaporation, water was added and the precipitate was filtered, washed once with water, and washed twice with dry ether. The dried solid was dissolved in DMF (8 mL), treated with piperidine (2 mL) for 1 h, and evaporated to dryness. The crude peptide was purified by preparative HPLC with a linear gradient of 0−60% of 0.1% TFA in acetonitrile against 0.1% aqueous TFA over 60 min at 10 mL/min. The lyophilized product was a white amorphous solid. H-Phe-c[-N$^{\epsilon}$-Lys-Tyr(O-tBu)-βAla-] (**4**) (43 mg). ^{1}H NMR (CD_3OD): δ 1.15−1.28 (2H, m, CH_2), 1.25 (9H, s, 3 × CH_3), 1.40, 1.53 (2H, 2m, CH_2), 1.68 (2H, m, CH_2), 2.37 (2H, m, CH_2), 2.84 (2H, m, CH_2), 3.06 (4H, m, 2 × CH_2), 3.41−3.72 (2H, m, CH_2), 3.97 (1H, dd, J = 5.6, 8.6 Hz, CH), 4.34 (1H, dd, J = 6.9, 6.9 Hz, CH), 4.63 (1H, dd, J = 6.6, 14.5 Hz, CH), 6.89 (2H, d, J = 8.3 Hz aromatic-H), 7.17 (2H, d, J = 8.3 Hz, aromatic-H), 7.20−7.31 (5H, m, aromatic-H). FAB-MS *m*/*z*: calcd 566.3342, found 566.3343 (MH)$^+$.

H-Phe-c[-N$^{\epsilon}$-Lys-Tyr(3-tBu)-Gly-] (6). H-Phe-c[-N$^{\epsilon}$-Lys-Tyr-Gly-] was synthesized by the same procedure as that for **1** but using N^{α}-Fmoc-Gly-OH, N^{α}-Fmoc-Tyr(tBu)-OPfp, N^{α}-Fmoc-Lys(Boc)-OPfp, and N^{α}-Fmoc-Phe-OPfp. H-Phe-c[-N$^{\epsilon}$-Lys-Tyr(3-tBu)-Gly-] was synthesized by the same procedure as that for **3** from H-Phe-c[-N$^{\epsilon}$-Lys-Tyr-Gly-] (878 mg). H-Phe-c[-N$^{\epsilon}$-Lys-Tyr(3-tBu)-Gly-] (6) (288 mg, yield 29%). ^{1}H NMR (DMSO-d_6): δ 0.99−1.65 (6H, m, 3 × CH_2), 1.32 (9H, s, 3 × CH_3), 2.67−2.98 (6H, m, 3 × CH_2), 3.18−3.34 (2H, m, CH_2), 3.82 (1H, dd, J = 6.1, 15.4 Hz, CH), 4.43 (2H, m, 2 × CH), 6.67 (1H, d, J = 8.3 Hz, aromatic-H), 6.86 (1H, d, J = 7.6 Hz, aromatic-H), 7.02 (1H, s, aromatic-H), 7.20 (5H, s, aromatic-H), 8.27 (1H, m, NH), 8.55 (1H, d, J = 7.3 Hz, NH), 8.67 (1H, m, NH), 8.90 (1H, d, J = 7.9 Hz, NH). FAB-MS *m*/*z*: calcd 552.3186, found 552.3180 (MH)$^+$.

H-Phe-c[-N$^{\epsilon}$-Lys-Tyr(3-tBu)-Abu-] (7). H-Phe-c[-N$^{\epsilon}$-Lys-Tyr-Abu-] was synthesized by the same procedure as that for **1** but using N^{α}-Fmoc-Abu-OH, N^{α}-Fmoc-Tyr(tBu)-OPfp, N^{α}-Fmoc-Lys(Boc)-OPfp, and N^{α}-Fmoc-Phe-OPfp. H-Phe-c[-N$^{\epsilon}$-Lys-Tyr(3-tBu)-Abu-] was synthesized by the same procedure as that for **3** from H-Phe-c[-N$^{\epsilon}$-Lys-Tyr-Abu-] (747 mg). H-Phe-c[-N$^{\epsilon}$-Lys-Tyr(3-tBu)-Abu-] (**7**) (300 mg, yield 25%). ^{1}H NMR (DMSO-d_6): δ 1.07 (2H, m, CH_2), 1.24−1.38 (2H, m, CH_2), 1.31 (9H, s, 3 × CH_3), 1.54 (2H, m, CH_2), 1.87 (2H, m, CH_2), 2.22 (2H, m, CH_2), 2.64−2.94 (6H, m, 3 × CH_2), 3.31 (2H, m, CH_2), 4.07 (1H, m, CH), 4.29 (1H, brd, J = 5.6 Hz, CH), 4.43 (1H, m, CH), 6.66 (1H, d, J = 6.6 Hz, aromatic-H), 6.86 (1H, brd, J = 6.3 Hz, aromatic-H), 7.03 (1H, s, aromatic-H), 7.19 (5H, s, aromatic-H), 7.58 (2H, m, 2 × NH), 8.45 (2H, m, 2 × NH). FAB-MS *m*/*z*: calcd 580.3499, found 580.3495 (MH)$^+$.

H-Phe-c[-N$^{\epsilon}$-Lys-Tyr(3-tBu)-Ahx-] (8). H-Phe-c[-N$^{\epsilon}$-Lys-Tyr-Ahx-] was synthesized by the same procedure as that for **1** but using N^{α}-Fmoc-Ahx-OH, N^{α}-Fmoc-Tyr(tBu)-OPfp, N^{α}-Fmoc-Lys(Boc)-OPfp, and N^{α}-Fmoc-Phe-OPfp. H-Phe-c[-N$^{\epsilon}$-Lys-Tyr(3-tBu)-Ahx-] was synthesized by the same procedure as that for **3** from H-Phe-c[-N$^{\epsilon}$-Lys-Tyr-Ahx-] (712 mg). H-Phe-c[-N$^{\epsilon}$-Lys-Tyr(3-tBu)-Ahx-] (**8**) (428 mg, yield 34%). ^{1}H NMR (DMSO-d_6): δ 1.02−1.69 (12H, m, 6 × CH_2), 1.30 (9H, s, 3 × CH_3), 2.00 (2H, m, CH_2), 2.60−3.23 (8H, m, 4 × CH_2), 4.09 (1H, m, CH), 4.39 (2H, m, 2 × CH), 6.64 (1H, d, J = 8.3 Hz, aromatic-H), 6.82 (1H, d, J = 7.9 Hz, aromatic-H), 6.97 (1H, brs, aromatic-H), 7.20 (5H, brs, aromatic-H), 7.54 (1H, m, NH), 7.72 (1H, m, NH), 8.28 (1H, m, NH), 8.50 (1H, brd, J = 6.9 Hz, NH). FAB-MS *m*/*z*: calcd 608.3812, found 608.3806 (MH)$^+$.

H-D-Phe-c[-N$^{\epsilon}$-Lys-Tyr(3-tBu)-βAla-] (9). H-D-Phe-c[-N$^{\epsilon}$-Lys-Tyr-βAla-] was synthesized by the same procedure as that for **1** but using N^{α}-Fmoc-βAla-OH, N^{α}-Fmoc-Tyr(tBu)-OPfp, N^{α}-Fmoc-Lys(Boc)-OPfp, and N^{α}-Fmoc-D-Phe-OPfp (697 mg, yield 74%). H-D-Phe-c[-N$^{\epsilon}$-Lys-Tyr(3-tBu)-βAla-] was synthesized by the same procedure as that for **3** from H-D-Phe-c[-N$^{\epsilon}$-Lys-Tyr-βAla-] (878 mg). H-D-Phe-c[-N$^{\epsilon}$-Lys-Tyr(3-tBu)-βAla-] (**9**) (111 mg, yield 34%). ^{1}H NMR (DMSO-d_6): δ 0.74−1.07 (2H, m, CH_2), 1.12−1.40 (4H, m, 2 × CH_2), 1.31 (9H, s, 3 × CH_3), 2.20 (2H, m, CH_2), 2.60−2.85 (4H, m, 2 × CH_2), 2.95 (2H, d, J = 6.9 Hz, CH_2), 3.26−3.44 (2H, m, CH_2), 4.15 (1H, m, CH), 4.27 (1H, m, CH), 4.38 (1H, m, CH), 6.63 (1H, d, J = 7.9 Hz, aromatic-H), 6.76 (1H, brd, J = 7.6 Hz, aromatic-H), 6.92 (1H, brs, aromatic-H), 7.20−7.40 (6H, m, NH + aromatic-H), 7.57 (1H, m, NH), 8.38 (2H, d, J = 8.3 Hz, 2 × NH). FAB-MS *m*/*z*: calcd 566.3342, found 566.3341 (MH)$^+$.

H-Phe-c[-N$^{\epsilon}$-D-Lys-Tyr(3-tBu)-βAla-] (10). H-Phe-c[-N$^{\epsilon}$-D-Lys-Tyr-βAla-] was synthesized by the same procedure as that for **1** but using N^{α}-Fmoc-βAla-OH, N^{α}-Fmoc-Tyr(tBu)-OPfp, N^{α}-Fmoc-D-Lys(Boc)-OPfp, and N^{α}-Fmoc-Phe-OPfp (796 mg, yield 85%). H-Phe-c[-N$^{\epsilon}$-D-Lys-Tyr(3-tBu)-βAla-] was synthesized by the same procedure as that for **3** from H-Phe-c[-N$^{\epsilon}$-D-Lys-Tyr-βAla-]. H-Phe-c[-N$^{\epsilon}$-D-Lys-Tyr(3-tBu)-βAla-] (**10**) (100 mg, yield 31%). ^{1}H NMR (DMSO-d_6): δ 0.92 (2H, m, CH_2), 1.10−1.53 (4H, m, 2 × CH_2), 1.30 (9H, s, 3 × CH_3), 2.16 (2H,

m, CH_2), 2.55−3.14 (6H, m, 3 × CH_2), 3.28−3.58 (2H, m, CH_2), 4.02 (1H, m, CH), 4.12 (1H, m, CH), 4.23 (1H, dt, J = 7.3, 7.3 Hz, CH), 6.62 (1H, d, J = 7.9 Hz, aromatic-H), 6.76 (1H, brd, J = 7.6 Hz, aromatic-H), 6.89 (1H, brs, aromatic-H), 7.22−7.40 (6H, m, NH + aromatic-H), 7.49 (1H, brd, J = 7.3 Hz, NH), 7.75 (1H, d, J = 7.8 Hz, NH), 8.27 (3H, m, NH_2 + NH). FAB-MS *m/z*: calcd 566.3342, found 566.3332 $(MH)^+$.

H-Phe-c[-N$^\epsilon$-Lys-D-Tyr(3-tBu)-β-Ala-] (11). H-Phe-c[-N$^\epsilon$-Lys-D-Tyr-β-Ala-] was synthesized by the same procedure as that for **1** but using N^α-Fmoc-β-Ala-OH, N^α-Fmoc-D-Tyr(tBu)-OPfp, N^α-Fmoc- Lys(Boc)-OPfp, and N^α-Fmoc-Phe-OPfp (472 mg, yield 50%). H-Phe-c[-N$^\epsilon$-Lys-D-Tyr(3-tBu)-β-Ala-] was synthesized by the same procedure as that for **3** from H-Phe-c[-N$^\epsilon$-Lys-D-Tyr-β-Ala-]. H-Phe-c[-N$^\epsilon$-Lys-D-Tyr(3-tBu)-β-Ala-] (**11**) (78 mg, yield 24%). ^{1}H NMR (DMSO-d_6): δ 1.17 (2H, m, CH_2), 1.28 (9H, s, 3 × CH_3), 1.37 (2H, m, CH_2), 1.61 (2H, m, CH_2), 2.22 (2H, m, CH_2), 2.83 (4H, m, 2 × CH_2), 3.22 (2H, brdd, J = 3.6, 14.5 Hz, CH_2), 3.50 (2H, m, CH_2), 4.18 (3H, m, 3 × CH), 6.58 (1H, d, J = 7.9 Hz, aromatic-H), 6.70 (1H, brd, J = 8.9 Hz, aromatic-H), 6.87 (1H, brs, aromatic-H), 7.33 (5H, m, aromatic-H), 7.42 (1H, m, NH), 7.66 (1H, m, NH), 7.89 (1H, d, J = 8.6 Hz, NH), 8.62 (1H, d, J = 7.3 Hz, NH). FAB-MS *m/z*: calcd 566.3342, found 566.3343 $(MH)^+$.

Peptide Purification. The crude peptides were purified by RP-HPLC using a Waters semiprep system with C18 YMC-Pack S-343-15 column (YMC, Japan, 15 μm, 120 Å, 20 mm × 250 mm). The peptide fractions that were purified by preparative HPLC were lyophilized, and the powder was kept at −20 °C until biological assay.

Peptide Analysis. The purity of the final products was confirmed by RP-HPLC using a Hitachi D-7000 HPLC system with an analytical column (YMC-Pack A-302, 4.6 mm × 150 mm, 120 Å, 5 μm particle size, YMC, Japan). The gradient for analytical RP-HPLC was as follows: (A) 100:0 to 30:70 (0.1% aqueous TFA/0.1% TFA in acetonitrile) over 25 min at 1 mL/min; (B) 100:0 to 30:70 (0.1% aqueous TFA/MeOH) over 25 min at 1 mL/min (see Supporting Information).

The molecular weight of the compounds was determined by FAB-MS (VG70-250SEQ, VG Analytical, UK). The values are expressed for MH^+.

Receptor Binding Assay. The motilin receptor binding assay was performed according to the procedure introduced by Bormans et al.[27] with a slight modification. After exsanguination, the upper part of the rabbit intestine (about 50 cm) was rapidly removed and rinsed with ice-cold 0.9% saline. The smooth muscle tissue was dissected from connective tissue and mucosa, finely minced, and homogenized in 50 mM Tris-HCl buffer (pH 7.4) at 0 °C using tapered tissue grinders (Wheaten, Milliville, NJ) at 2000 rpm for 30 s. The homogenate was centrifuged at 1500 × *g* for 5 min and was washed twice with a fresh buffer. The final pellet was resuspended in 50 mL of 50 mM Tris-HCl buffer (pH 8.0, containing 10 mM $MgCl_2$, 1.5% bovine serum albumin) for binding studies.

The homogenate (about 1.0 mg protein/assay) was incubated at 25 °C for 120 min with 25 pM ^{125}I-pMTL (specific activity, 33−66 kBq/pmol). The final volume was 1 mL. After incubation, a total of 2 mL of cold buffer was added to stop the reaction, and bound and free reagents were separated by centrifugation at 1500 × *g* for 5 min. The pellet was washed with cold buffer, and its radioactivity was determined with a γ counter (ARC-300, Aroka, Tokyo, Japan). Displacement curves were obtained by adding increasing amounts of the peptides to the incubation sample, and the concentration displacing 50% of the label (IC_{50}) was determined by interpolation.

Contraction Assay. Male Japanese white rabbits (about 3 kg) were used. The animals were anesthetized with thiopental sodium (30 mg/kg, iv) and exsanguinated. After laparotomy the upper part of the small intestine was rapidly removed and placed in ice-cold modified Krebs' solution composed of the following (in mM): NaCl 120.0, KCl 4.7, $CaCl_2$ 2.4, KH_2PO_4 1.0, $MgSO_4$ 1.2, $NaHCO_3$ 24.5, and glucose 5.6 (pH 7.4). The duodenum was washed, freed from mesenteric attachment, and cut along the longitudinal axis to obtain muscle strips approximately 10 mm long and 3 mm wide. The preparation was mounted in an organ bath containing 10 mL of modified Krebs' solution kept at 28 °C to prevent excessive spontaneous contractions.[28] The solution was gassed with a mixture of 95% O_2 and 5% CO_2. The longitudinal strips were initially loaded with a 1.0 g weight, and contractile activity was measured by means of isotonic transducers (ME-4012, Medical Electronics Co., Tokyo) and recorded on an ink-writing recorder (type 3066, Yanagisawa-Denki, Tokyo). Before the experiments, each strip was subjected to repeated stimulation with 100 μM ACh until a reproducible response was obtained. The contractile potency of each compound was expressed as a percentage of that induced by 100 μM ACh. The dose giving 50% of the response (EC_{50}) was determined by interpolation.

A commutative concentration-dependent contractile curve for pMTL was established by adding increasing concentrations of pMTL. The effect of each antagonist on the contractile activity of pMTL was further studied by incubating the strip for 15 min with different concentrations of each antagonist and then recording a cumulative concentration−response curve in the presence of each antagonist. The pA_2 value was obtained by regression analysis of Schild plots, as has been described previously.[29]

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Supporting Information Available: Table of HPLC analytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

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