Design and Synthesis of N-terminal Cyclic Motilin Partial Peptides: A Novel Pure Motilin Antagonist

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Motilin antagonist was designed and synthesized on the basis of the structure–activity relationship analysis of porcine motilin that we reported recently. The drug design was performed on a specific concept to reduce a flexibility of peptide conformation of porcine motilin partial peptide by its cyclization. The cyclic peptide was synthesized using Boc (*tert*-butyloxycarbonyl) solid phase methodology, followed by cyclization using the azide procedure, and tested for the binding activity to motilin receptor and smooth muscle contractile activity. The cyclic peptides 3, 4, and 5 showed antagonistic property on contraction assay (pA_2 [the negative logarithm of molar concentration of antagonist causing a 2-hold shift to the right of the concentration–response curve for motilin]: 4.5, 4.34, and 4.04, respectively, in rabbit duodenum) and no contractile activity even at high concentration.

Key words motilin; antagonist; cyclic peptide

Motilin, a 22 amino acid gastrointestinal peptide isolated from endocrine cells in gastrointestinal mucosa of various species,[2,3)] stimulates gastrointestinal contractile activity. However, its biological function has not been fully identified.

The physiological relevance of motilin to some gastrointestinal symptoms was suggested: early satiety, abdominal distention, nausea, vomiting, and anorexia.[3)] Therefore, finding of a pure motilin agonist and antagonist is desired for the biological investigation of the peptide and for treatment of patients with its associated diseases.

The potent motilin agonist, including motilin analogues[4—6)] and erythromycin derivatives,[7—9)] was developed as a therapeutic agent in the US, while no therapeutic motilin antagonist has been developed because those existing motilin antagonists[10—13)] reportedly have agonistic contractile activity at high concentration.

We recently reported the structure–activity relationship analysis of porcine motilin (pMTL) which suggested the importance of Phe[1], Ile[4], and Tyr[7] for its pharmacological action.[14)] On the basis of this result, pMTL (1—7)-NH_2 which includes these three important amino acids, was expected to be an effective motilin agonist, although its activity had not been reported. Therefore, we synthesized pMTL (1—7)-NH_2[15)] and tested the binding activity for motilin receptor and contractile activity at the beginning of this study.

PMTL (1—7)-NH_2 was found to bind to motilin receptor with 4.0 μM of IC_{50}, however, its contractile activity was very weak (EC_{50}: 135μM). Based on this interesting phenomenon, the following two possibilities were considered: 1) the compound has agonistic activity but was metabolically converted into inactive form during *ex-vivo* assay, and 2) the compound has an antagonistic effect. The metabolic inactivation was not thought to be realistic, however because no decomposition of motilin analogues was reported in this assay. From this, we hypothesized that the compound would be a motilin antagonist.

In this report, we designed and synthesized the pMTL (1—7) derivatives in order to demonstrate this hypothesis and enhance the antagonistic effects of pMTL (1—7)-NH_2. A specific concept in the drug design is to reduce the flexibility of peptide conformation by its cyclization, because biological profiles of some cyclized peptides were reportedly altered from original agonist to antagonist by cyclization.[16—18)] Herein, we report the synthesis and the biological activity of

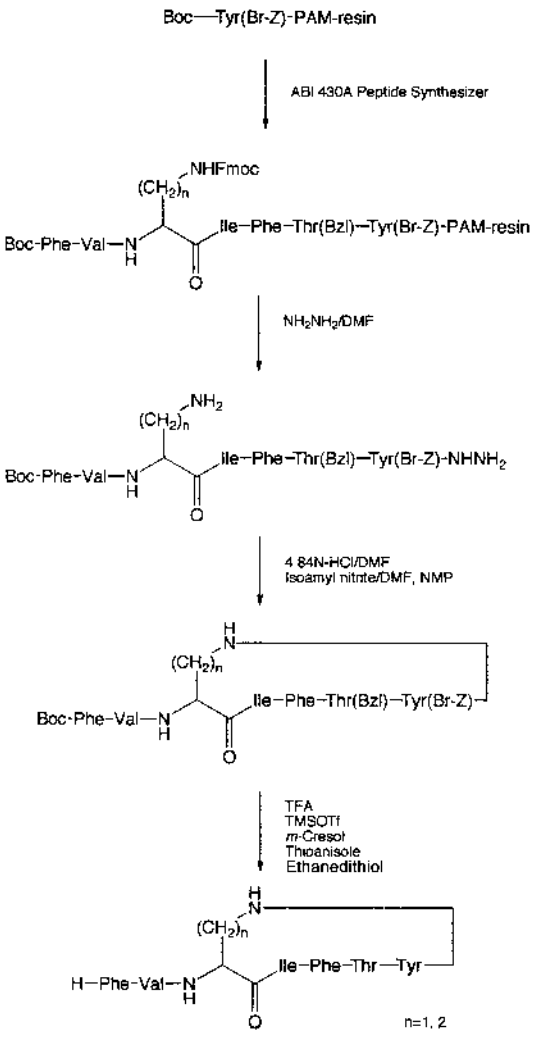

Chart 1. Synthesis of Cyclic Peptide

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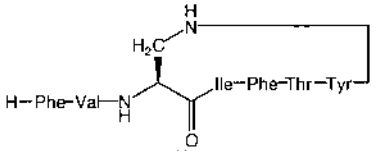
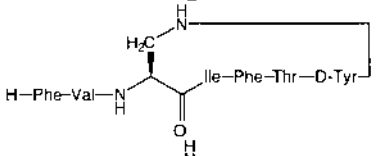
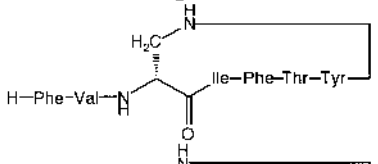
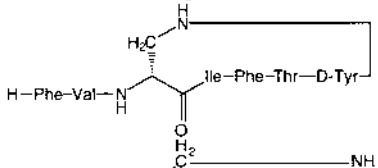
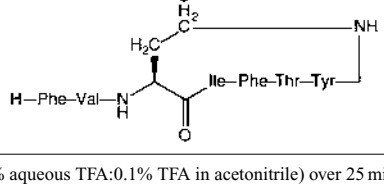
Table 1. Structures and Analytical Properties

No.	Structures	HPLC t_R (min), (purity, %)[a]	FAB-MS (MH^+), (Calcd)
	pMTL (1-7)-NH_2	15.54 (99)	885.4865 (885.4874)
1		17.72 (95)	857.4565 (857.4561)
2		16.46 (97)	857.4579 (857.4561)
3		17.03 (95)	857.4553 (857.4561)
4		16.74 (98)	857.4567 (857.4561)
5		16.89 (99)	871.4718 (871.4717)

a) 100 : 0 to 30 : 70 (0.1% aqueous TFA:0.1% TFA in acetonitrile) over 25 min, 1.0 ml/min.

the cyclic analogues of pMTL (1—7), which were identified as pure motilin antagonists.

Design of Cyclic Peptide Several ^{1}H-NMR studies of motilin[19—23] pointed out a wide nonclassical turn in residues 1—6 and close location of Pro^3 to Tyr^7. Connection between the third residue and Tyr^7 was thought to be most reasonable to cyclize, because the third position of pMTL could be replaced without reduction of affinity, and Pro^3 made no contribution to the pharmacological action in pMTL partial peptide.[14] From the structure of pMTL already reported,[19—23] distance between the third residue and Tyr^7 was similar to that of the side chain of α,β-diaminopropionic acid (Dap) or α,γ-diaminobutylic acid (Dab) on the third residue connecting Tyr^7.

Therefore, we designed the cyclic pMTL (1—7) derivatives, replacing Pro^3 with Dap or Dab, and connecting the side chain of this amino acid and the C-terminus of Tyr^7 (Table 1).

Chemistry The peptides were synthesized (Chart 1) by a solid phase methodology,[24,25] according to an N^{α}-Boc (N^{α}-*tert*-butyloxycarbonyl) group strategy on a PAM-resin (4-hydroxymethylphenylacetamidomethyl substituted polystyrene resin). Deprotection of the N^{α}-Boc group was conducted with 33—50% trifluoroacetic acid (TFA) in dichloromethane (DCM) solution prior to coupling with the next protected amino acid. A preformed N^{α}-Boc amino acid symmetric anhydride was utilized as the acylating species. The procedure for generating an amino acid symmetric anhydride consists of reacting 0.5 eq of dicyclohexylcarbodiimide (DCC) with 1.0 eq of N^{α}-Boc amino acid in DCM. Completion of the couplings was verified by the Kaiser test.[26] The peptides were simultaneously deprotected of 9-fluorenylmethyloxycarbonyl (Fmoc) group and cleaved from the resin as peptide hydrazide by treatment with hydrazine in dimethylformamide (DMF).[27,28] The peptide hydrazide was cyclized by an azide procedure,[27,28] and finally deprotected by treatments with TFA/trimethylsilyl triflate (TMSOTf)/*m*-cresol/thioanisole/ethanedithiol (100 : 31.6 : 8.57 : 19.2 : 3.28; v/v) for 0.5—1 h at 0 °C.

Crude peptides were purified by preparative reversed-phase (RP)-HPLC. The purified peptides were analyzed by analytical RP-HPLC, and characterized by amino acid analysis and high resolution FAB-MS (Table 1).

Results and Discussion

The synthesized cyclic peptides of pMTL (1—7) derivatives were tested for their binding activity to motilin receptor and the smooth muscle contractile activity in rabbit smooth muscle (Table 2).

Every 16-member ring cyclic peptide (peptides **1**—**4**) bound to motilin receptor with 2.3—7.3 μM of IC_{50}, which was comparable to linear partial peptide pMTL (1—7)-NH_2. These data suggested that the third residue could be replaced and Dab was suitable as third residue to cyclize without reducing the affinity to the motilin receptor. Binding activity of peptide **5**, possessing a 17-member ring, was 16 μM of IC_{50}, suggesting that the altered ring size may influence its effect.

For the contractile activity, all cyclic peptides were inactive except for peptide **2**; this peptide possesses D-Tyr as the seventh residue and has very weak contractile activity at 300 μM. This result strongly suggested the importance of interaction between Tyr^7 and motilin receptor for the agonistic con-

tractile activity.

The cyclic peptides **3**, **4**, and **5** showed antagonistic property on contractile assay. pA_2 value was 4.5, 4.34, and 4.04, respectively, in the rabbit duodenum contraction assay. Importantly, these peptides showed no contractile activity even at high concentration (300 μM).

We developed a hypothetical model (Fig. 1) based on these results to understand the binding mode of motilin agonist and antagonist. In this model, the expected binding mode for motilin receptor was estimated as follows: the agonistic partial peptide bound to motilin receptor *via* three point interaction (Phe[1], Ile[4], Tyr[7]) causing contractile activity (Fig. 1, left). The antagonistic cyclic peptide bound to the receptor *via* interaction of Phe[1] and Ile[4], and the antagonistic property resulted from the absence of interaction of Tyr[7] (Fig. 1, right).

Conclusion

The cyclic peptides **3**, **4**, and **5**, designed from the motilin partial peptide pMTL (1—7) showed pure motilin antagonistic activity. This is the first report of synthesis of a pure motilin antagonist. For the antagonistic action, the absence of Tyr[7] interaction with motilin receptor was expected to be critical.

Experimental

Materials N^{α}-Boc amino acid was purchased from either Applied Biosystems (ABI) or Watanabe Chemical. Boc-L-Tyr(2-Br-Z)PAM-resin and Boc-D-Tyr(2-Br-Z)PAM-resin were purchased from Novabiochem. All reagents used in the ABI 430A peptide synthesizer were purchased from ABI. TFA, TMSOTf, 1,3-diisopropylcarbodiimide (DIC), and *N*-hydroxybenzotriazole (HOBt) were purchased from Watanabe Chemical. *m*-Cresol, NH_4F, 2-mercaptoethanol, ethanedithiol and thioanisole were purchased from Tokyo Chemical Industry, and DMF, DCM and *N*-methyl-2-pyrrolidinone (NMP) were from Kokusan Chemical. Methanol and acetic acid were purchased from Junsei Chemical. HPLC grade acetonitrile, and water were from Kanto Chemical or Junsei Chemical.

Table 2. Structures, Binding Activity, and Contractile Activity

No.	Binding activity[a] IC_{50} (μM)	Contractile activity[b]	
		EC_{50} (μM)	pA_2[c]
pMTL (1—7)-NH_2	4.0	135±44 (n=6)	—
1	3.8	>300	—
2	2.3	>300[d]	—
3	3.9	>300	4.5 (n=1)
4	7.3	>300	4.34±0.22 (n=3)
5	16	>300	4.04±0.07 (n=3)

a) Binding activity data are presented from one or two determination(s). *b*) Contractile activity data are presented as the mean±S.D. with the number of repetitions from independent assays (n) listed in parentheses. *c*) pA_2: the negative logarithm of molar concentration of antagonist causing a 2-fold shift to the right of the concentration–response curve for motilin *d*) Weak agonistic activity was detected at 300 μM.

Solid Phase Peptide Synthesis The peptides were synthesized by solid phase peptide chemistry.[24,25] The syntheses were carried out on an ABI Model 430A peptide synthesizer, but the coupling of Boc-Dap(Fmoc)-OH was performed by manual shaker.

Boc-D-Tyr(2-Br-Z)-PAM-resin (0.5 mmol/g; 1 g) was used to prepare the peptide: Boc-Phe-Val-D-Dap(Fmoc)-Ile-Phe-Thr(Bzl)-D-Tyr(2-Br-Z)-PAM-resin, by stepwise coupling of the amino acids Boc-Thr(Bzl)-OH, Boc-Phe-OH, Boc-Ile-OH, Boc-D-Dap(Fmoc)-OH, Boc-Val-OH, Boc-Phe-OH. Each coupling cycle consisted of the following: 1) treated with 33% TFA in DCM for 80 s and with 50% TFA in DCM for 18.5 min, 2) washed three times with DCM, 3) treated twice with 10% diisopropylethylamine (DIEA) in DMF for 1 min, 4) washed five times with DMF, 5) coupled with the amino acid, 6) washed three times with DMF, 7) treated with 10% DIEA in DMF for 45 s, 8) washed with DMF, 9) washed three times with DCM, 10) coupled with the amino acid (double coupling), 11) washed with DMF, and 12) washed five times with DCM. The first cycle included a preliminary wash with DCM. Each amino acid was double-coupled with 4 eq for each coupling reaction. The amino acid was activated as the HOBt ester. After the coupling cycle of Boc-Ile-OH, the peptide resin was transferred to a reaction vessel equipped with a filter disk and a stopcock and suspended in DMF. The resin was treated twice with 20% TFA in DCM, treated twice with 10% DIEA in DMF, and washed six times with DMF. N^{α}-Boc-N^{β}-Fmoc-α,β-D-diaminopropionic acid (4 eq) was then added followed by HOBt (4 eq) and DIC (4 eq) in NMP. The coupling reaction mixture was shaken on a manual shaker for 2.5 h at room temperature. The resin was then washed six times with DMF. The coupling of Boc-Phe-OH and Boc-Val-OH was again performed on an ABI Model 430A peptide synthesizer. The peptide resin was washed with methanol and dried *in vacuo* to yield the protected Boc-Phe-Val-D-Dap(Fmoc)-Ile-Phe-Thr(Bzl)-D-Tyr(2-Br-Z)-PAM-resin (1.478 g).

The protected peptide-resin was treated with 10% hydrazine in NMP for 24 h at room temperature. The solution was filtered and concentrated *in vacuo*. The residue was treated with cold water to precipitate the peptide hydrazide, and the hydrazide was then filtered and dried. The peptide hydrazide (522 mg) dissolved in NMP (16 ml) and DMF (8 ml) was cooled to 30 °C followed by addition of 4.84 N HCl/DMF (0.32 ml) and isoamyl nitrite (0.119 ml). The reaction mixture was allowed to stand at 0 °C and cooled to −30 °C, then added to pyridine (200 ml) and NMP (200 ml) kept at −20 °C and stirred at 0 °C for 25 h. The solution was concentrated *in vacuo*. The residue was treated with cold water to precipitate the protected cyclic peptide. The peptide was filtered and dried.

Deprotection of the peptide was performed with TFA/TMSOTf/*m*-cresol/thioanisole/ethanedithiol (100 : 31.6 : 8.57 : 19.2 : 3.28; v/v) for 0.5 h at 0 °C. The solution was filtered and added dropwise to excess cold diethyl ether. The precipitated peptide was filtered, washed with diethyl ether, and dried. The residue was treated with 2-mercaptoethanol (0.2 ml) and 1 N NH_4F (0.2 ml) in MeOH (2 ml) and H_2O (2 ml) at 0 °C. The solution was adjusted to pH 8.0 with triethylamine and stirred for 0.5 h at 0 °C, then acidified to pH 5.0 with AcOH, and concentrated *in vacuo*. The crude peptide was purified by preparative HPLC with a linear gradient of 0—60% 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. Overall yield: 24%.

A similar protocol was carried out for the syntheses of other peptides in this manuscript.

Peptide Purification The crude peptides were purified by RP-HPLC

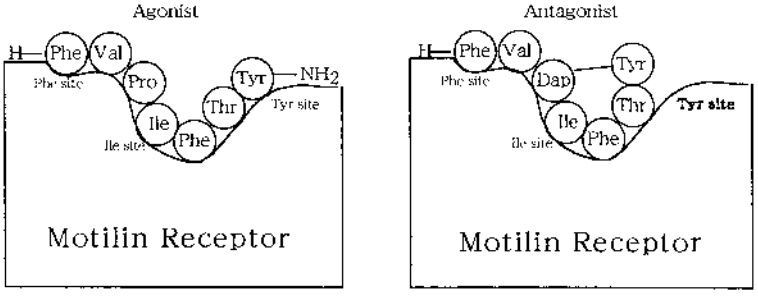


Fig. 1. Hypothetical Model of the Binding Mode of Motilin Agonist and Antagonist

using a Waters semi-prep system with C18 YMC-Pack S-343-15 (YMC, Japan), 15 μm, 120 Å, 20×250 mm, eluting with a linear acetonitrile gradient (0—60%) in water containing a constant concentration of TFA (0.1%, v/v) over 60 min at a flow rate of 10 ml/min. The peptide fractions which were purified by analytical HPLC were lyophilized, and the powder was kept at −20 °C until their 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, 4.6×150 mm, 120 Å, 5 μm particle size, YMC, Japan). The gradient for analytical RP-HPLC was as follows: 100 : 0 to 30 : 70 (0.1% aqueous TFA/0.1% TFA in acetonitrile) over 25 min at 1 ml/min.

The composition of the peptide was verified by quantitative amino acid analysis using Pico-Tag methodology (Waters) and phenyl isothiocyanate (PITC) as amino acid derivatization reagents. Lyophilized samples of peptides (50—1000 pmol) were placed in heat-treated borosilicated tubes (50×4 mm), sealed, and hydrolyzed with 200 μl of 6 N HCl containing 1% phenol in the Pico-Tag workstation for 1 h at 150 °C. A Pico-Tag column (15×3.9 mm) was employed to separate the PITC-amino acid derivatives. Composition of the compounds was as expected.

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

Receptor Binding Assay Binding assay for motilin receptor was performed according to the procedure introduced by Bormans *et al.*[29] with a slight modification. After exsanguination, the upper part of intestine (about 50 cm) of the rabbit was rapidly removed and rinsed with ice-cold 0.9% saline. The smooth muscle tissue was dissected free 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, U.S.A.) 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 protein concentration was determined by the method of Lowry *et al.*[30] using bovine serum albumin as the standard.

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, adding 2 ml of cold buffer stopped the reaction. Bound and free reagents were separated by centrifugation at 1500×***g*** for 5 min. The pellet was washed with a cold buffer, and its radioactivity was determined with a gamma counter (ARC-300, Aroka, Tokyo, Japan). The concentration displacing 50% of the label is expressed (IC_{50}).

Contraction Assay Male Japanese-white rabbits (about 3 kg) were used. The animals were anesthetized with thiopental sodium (30 mg/kg, i.v.) and exsanguinated. The upper part of the small intestine was rapidly removed after laparotomy and placed in an ice-cold modified Krebs' solution composed of (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.[31] 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 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 is expressed (EC_{50}).

The commutative concentration-dependent contractile curve for pMTL was established by adding an increasing concentration of pMTL. The effect of each antagonist on contractile activity of pMTL was further studied by incubating the strip for 15 min with a different concentration 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.[32]

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References and Notes

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