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Design and Synthesis of Novel Tetra-Peptide Motilin Agonists

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Abstract—A series of novel tetra-peptide motilin agonists, having the general structure H-Phe-Val-X-Ile-NH_2, were designed, on the basis of structure–activity relationship studies of motilin. Peptides, in which X is a side chain substituted tryptophan residue, have agonistic activity. H-Phe-Val-Trp(2′-CH_2CH_2OH)-Ile-NH_2 (**7**), H-Phe-Val-Trp(2′-SCH_3)-Ile-NH_2 (**8**), and H-Phe-Val-Trp(2′-$SCH_2CH_2CH_3$)-Ile-NH_2 (**9**), showed an EC_{50} for contractile activity in the rabbit smooth muscle of 14.1 ± 3.2, 12.9 ± 4.1, and 4.6 ± 1.6 μM, respectively. Interaction of the tryptophan aliphatic side chain with motilin receptor appears to influence the signal transduction via motilin receptor.

Introduction

Motilin, a single-chain peptide of 22 amino acid residues, was isolated from endocrine cells in gastrointestinal (GI) mucosa of various species.[1] Although the biological function of motilin has not been fully elucidated, motilin is known to stimulate GI motility in many species.[1]

The amino acid sequence of porcine motilin (pMTL) was first determined in 1973 (FVPIF TYGEL QRMQE KERNK GQ).[2,3] Human motilin, isolated in 1995,[4] was found to be identical with porcine motilin, but different from canine motilin by five residues at the 7th, 8th, 12th, 13th, and 14th amino acid residues.

Motilin has been suggested to have physiological relevance to a number of gastrointestinal symptoms, including early satiety, abdominal distention, nausea, vomiting, and anorexia.[1] Motilin agonists, such as motilin analogues[5] and erythromycin derivatives,[6–8] and their three-dimensional structure–activity relationship (SAR) study[9] have been reported. Although they have been suggested to be effective in the treatment of such symptoms, the detailed mechanisms of motilin agonistic action, after binding to the motilin receptor (MTL-R), have not yet been explored. The discovery of small molecule motilin agonists would be expected to stimulate new studies on the biological and physiological mechanism of the motilin and perhaps lead to the discovery of new drugs for the treatment of patients with hypomotility syndromes.

The previous studies[10,11] on the SAR of pMTL suggest that Phe^1, Ile^4, and Tyr^7 play an important role in agonism of the motilin receptor. Several ^1H NMR studies of motilin indicate that residues 1–6 adopt a wide nonclassical turn conformation, which brings Pro^3 and Tyr^7 into close proximity with one another.[12–14] Therefore, the Tyr^7 binding site of MTL-R was expected to be occupied by the 3rd residue side chain. In addition, Pro^3 was reported not to contribute to the agonistic effect of the pMTL partial peptide.[10,11] From these considerations, we hypothesized that if the third amino acid (Pro) was replaced with Tyr, even the N-terminal tetra-peptide would have the agonistic effect without Tyr^7. With this idea in mind, we synthesized and evaluated tetra-peptide **1** (H-Phe-Val-Tyr-Ile-NH_2). Peptide **1** was found to bind to MTL-R with an IC_{50} of 11 μM. However, the contractile activity of **1** was found to be extremely weak (EC_{50}: > 100 μM). The low agonistic activity of this peptide could be due to the inadequate interaction with the Tyr^7 binding site of MTL-R. The importance of such an interaction was reported in a previous study of a motilin analogue peptide.[15] In other words, the third amino acid of the N-terminal tetra-peptide may play a critical role for its agonism. Therefore, we have focused our efforts on modifications of **1**, with the aim of conducting an extensive SAR analysis of the N-terminal moiety of motilin, in order to enhance

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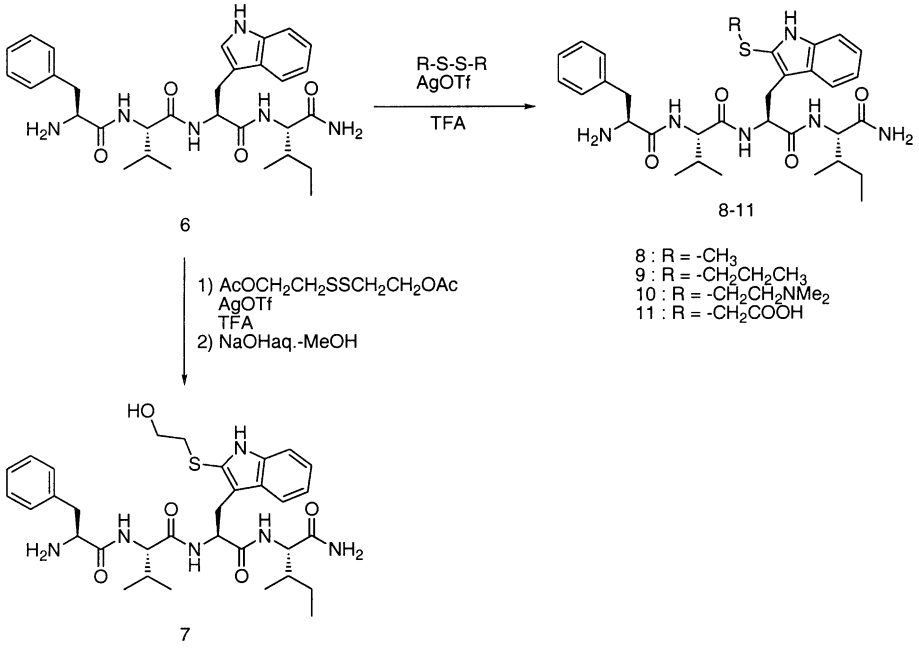

Scheme 1. Modification of Trp on tetra-peptide.

the contractile effect of this type of molecule. Herein, we would like to report the syntheses and biological activities of novel tetra-peptide motilin agonists, H-Phe-Val-X-Ile-NH_2.

Chemistry

The peptides were synthesized by a solid phase methodology,[16,17] according to an N^{α}-Boc (N^{α}-*t*-butyloxycarbonyl) protecting group strategy or N^{α}-Fmoc (N^{α}-9-Fluorenylmethyloxycarbonyl) protecting group strategy on a MBHA-resin (*p*-methylbenzhydrylamine substituted polystyrene resin).[18,19]

In the case of the N^{α}-Boc group strategy, the deprotection of 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 2.0 equivalents of dicyclohexylcarbodiimide (DCC) with 4.0 equivalents of N^{α}-Boc amino acid in DCM.

In the case of the N^{α}-Fmoc group strategy, N^{α}-Fmoc group was removed with 20% piperidine in *N,N*-dimethylformamide (DMF) prior to coupling with the next protected amino acid. All N^{α}-Fmoc amino acids were coupled as their pentafluorophenyl (Pfp) esters, in presence of *N*-hydroxybenzotriazole (HOBt).

The completion of the couplings was verified by the Kaiser test.[20] The peptides were simultaneously deprotected and cleaved from the resin by treatment with TFA/trimethylsilyltriflate (TMSOTf)/*m*-cresol/thioanisole (100:28.6:7.47:16.7; v/v) or TFA/TMSOTf/*m*-cresol/thioanisole/ethanedithiol (100:12:6.5:15:5; v/v) for 1–4 h at 0 °C or room temperature depending on the side-chain protecting groups. Modification of the indole ring of Trp was performed with the corresponding disulfide in the presence of silver triflate (Scheme 1).

Crude peptides were then purified to homogeneity by preparative reversed-phase high-performance liquid chromatography (RP-HPLC) eluting with a linear gradient of 0.1% aqueous TFA in acetonitrile. Peptide fractions found to be homogeneous by analytical RP-HPLC were combined, concentrated, and lyophilized. The peptides were characterized by Fourier transform (FT) NMR and high-resolution fast atom bombardment mass spectrometry (FABMS) (Table 3).

Results and Discussion

The synthesized peptides (Table 1), H-Phe-Val-X-Ile-NH_2 (X = Tyr, Pro, Lys, Glu, Gln, Trp), were tested for binding activity to MTL-R and for the rabbit smooth muscle contractile activity.

As shown in Table 1, **1**–**3** and **6** showed the binding activity to the MTL-R, whereas **4** and **5** did not. Tryptophan derivative **6** was 30 times more potent than tyrosine derivative **1**. The binding activities of the proline and lysine derivatives **2** and **3** were comparable to that of **1**. However, **1**, **2** and **6** showed extremely weak agonistic activity in the rabbit duodenal smooth muscle contractile assay. On the basis of these results, we concluded that **1, 2** and **6** bound to MTL-R, but the signal transduction via MTL-R was incomplete. In order to achieve more complete signal transduction, a series of extensively modified peptides were prepared (Table 2).

Based on the binding activity of the tryptophan containing peptide **6**, we focused our attention on the preparation of peptides with a substituted tryptophan at residue 3.

The 2′-substituted tryptophan derivatives, H-Phe-Val-X-Ile-NH_2 (X = 2′-substituted tryptophan) were tested for binding and contractile activity. The binding activities of **7**, **8**, and **9** were comparable to that of the tryptophan derivative **6**, whereas, those of **10** and **11** were less potent (Table 2). Importantly, **7**, **8**, and **9** were all effective in the contractile assay (EC_{50}: 14.1±3.2, 12.9±4.1, and 4.6±1.6 μM, respectively), demonstrating that the N-terminal tetra-peptide can act as motilin agonist in vitro. As shown in Figure 1, **7** induced contractile activity in the rabbit duodenal smooth muscle in a concentration-dependent manner.

In the earlier study derived for a large series of motilin analogues, Peeters et al. mentioned the correlation between binding affinity and agonistic effect ($pEC_{50} = 1.13 + 0.74\ pIC_{50}$),[10] which led to a difference between pEC_{50} and pIC_{50} of about one log unit. This correlation was also true for motilin partial peptides,[21] and erythromycin derivatives.[22,23] However, **7** and **8** showed the almost two log units difference between pEC_{50} and pIC_{50} in this study. The fact suggested that these peptides bound to MTL-R, but receptor mediate signal transductions by these peptides were weaker than those of other Tyr^7 containing motilin analogues and erythromycin derivatives.

A structural feature, distinguishing the contractile-active peptides, **7**, **8**, and **9** from the contractile-inactive derivative **6**, is the aliphatic side chain on the tryptophan ring. Although the Trp 2′-substituents do not contribute MTL-R binding, they appear to play an important role in inducing receptor mediated signal transduction. In the model proposed by Gouda et al.,[9] ethyl group of an erythromycin derivative was presumed to correspond to Tyr^7 of Motilin. Aliphatic side chain in **7**, **8**, and **9** seemed to play the same role as this ethyl group.

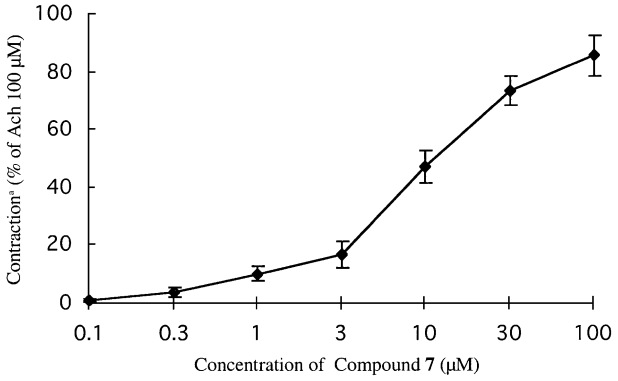

Figure 1. Contractile activity of compound **7** in the rabbit duodenal smooth muscle. [a]Contraction % of 100 μM ACh max. Data are presented as the mean±SEM with the number of repetitions from independent assays ($n = 6$).

Table 1. Structures, binding activity and contractile activity of tetra-peptides

No.	Structures	Binding activity (IC_{50}: μM)	Contractile activity[a]		
			EC_{50} (μM)	Maximum contraction (% of 100 μM ACh max)	*n*
1	H-Phe-Val-Tyr-Ile-NH_2	11	>100	8.7[b]	2
2	H-Phe-Val-Pro-Ile-NH_2	33	>300	17.5±6.0[c]	6
3	H-Phe-Val-Lys-Ile-NH_2	6.4	—	—	—
4	H-Phe-Val-Glu-Ile-NH_2	>100	—	—	—
5	H-Phe-Val-Gln-Ile-NH_2	>100	—	—	—
6	H-Phe-Val-Trp-Ile-NH_2	0.37	>100	14.2±5.1[b]	6

[a]Contractile activity data are presented as the mean±SEM with the number of repetitions from independent assays (*n*).
[b]Maximum contraction (%) at 100 μM.
[c]Maximum contraction (%) at 300 μM.

Table 2. Structures, binding activity and contractile activity of Trp modificated peptides

No.	Structures	Binding activity (IC_{50}: μM)	Contractile activity[a]		
			EC_{50} (μM)	Maximum contraction (% of 100 μM ACh max)	*n*
6	H-Phe-Val-Trp-Ile-NH_2	0.37	>100	14.2±5.1[b]	6
7	H-Phe-Val-Trp(2′-SCH_2CH_2OH)-Ile-NH_2	0.57	14.1±3.2	85.7±6.8[b]	6
8	H-Phe-Val-Trp(2′-SCH_3)-Ile-NH_2	0.42	12.9±4.1	58.2±3.6[c]	5
9	H-Phe-Val-Trp(2′-$SCH_2CH_2CH_3$)-Ile-NH_2	0.23	4.6±1.6	67.7±5.0[c]	3
10	H-Phe-Val-Trp(2′-$SCH_2CH_2NMe_2$)-Ile-NH_2	1.0	>300	2.9±2.9[d]	3
11	H-Phe-Val-Trp(2′-SCH_2COOH)-Ile-NH_2	5.6	>30	1.5±1.5[c]	3

[a]Contractile activity data are presented as the mean±SEM with the number of repetitions from independent assays (*n*).
[b]Maximum contraction (%) at 100 μM.
[c]Maximum contraction (%) at 30 μM.
[d]Maximum contraction (%) at 300 μM.

Table 3. Analytical properties of peptides

No.	HPLC t_R (min), (purity, %)		FAB-MS (MH^+) (calcd)
	t_R[a]	t_R[b]	
1	13.13 (100)	17.71 (100)	540.3175 (540.3181)
2	11.98 (100)	15.91 (100)	474.3082 (474.3080)
3	10.85 (99.1)	15.14 (100)	505.3504 (505.3502)
4	11.85 (99.3)	16.37 (100)[c]	506.2980 (506.2978)
5	11.56 (98.5)	15.72 (100)	505.3141 (505.3138)
6	15.38 (99.6)	21.07 (100)	563.3350 (563.3345)
7	15.04 (100)	21.15 (100)	639.3328 (639.3328)
8	16.25 (96.2)	22.24 (100)	609.3230 (609.3223)
9	18.01 (99.0)	24.45 (100)	637.3529 (637.3536)
10	13.62 (99.4)	19.86 (100)	666.3802 (666.3801)
11	15.20 (95.2)	21.14 (97.9)	653.3125 (653.3121)

[a]100:0 to 30:70 (0.1% aqueous TFA/0.1% TFA in acetonitrile) over 25 min, 1.0 mL/min.
[b]100:0 to 30:70 (0.1% aqueous TFA/MeOH) over 25 min, 1.0 mL/min, unless otherwise noted.
[c]100:0 to 2:98 (0.1% aqueous TFA/MeOH) over 35 min, 1.0 mL/min.

In conclusion, we designed motilin N-terminal tetrapeptide derivatives (H-Phe-Val-X-Ile-NH_2), and explored extensive SAR analyses of the N-terminal moiety of motilin. Peptides **7**, **8**, and **9**, possessing an aliphatic side chain on the tryptophan ring, showed contractile activity in the rabbit smooth muscle. The interaction of the aliphatic side chain with MTL-R appears to exert an influence on the signal transduction via MTL-R.

Information from this report will hopefully stimulate studies on the biological and physiological mechanism of motilin, and assist in developing a remedies for motilin associated diseases.

Experimental

Materials

Fmoc amino acids, and Pfp esters were purchased from MilliGen/Biosearch (Burlington, MA, USA) and Watanabe Chemical (Japan). Boc amino acids were purchased from Applied Biosystems (ABI; Foster City, CA, USA) and Watanabe Chemical (Japan). MBHA-resin was purchased from the Peptide Institute (Japan) and Watanabe Chemical (Japan). All reagents used in ABI 430A peptide synthesizer were purchased from ABI. TFA, TMSOTf, 1,3-diisopropylcarbodiimide (DIC), and HOBt were purchased from Watanabe Chemical (Japan). Di-*n*-propyl disulfide, dithiodiglycolic acid, silver trifluoromethanesulfonate, triethylamine, dithiothreitol, NH_4F, 2-mercaptoethanol, *m*-cresol, ethanedithiol and thioanisole were purchased from Tokyo Chemical Industry (Japan). Dimethyl disulfide was purchased from Nakarai Tesque (Japan). DMF, DCM, and *N*-methyl-2-pyrrolidinone (NMP) were purchased from Kokusan Chemical (Japan). Methanol (MeOH), diethyl ether, *n*-hexane, NaOH, and acetic acid (AcOH) were purchased from Junsei Chemical (Japan). HPLC grade acetonitrile, and water were purchased from Kanto Chemical (Japan) and Junsei Chemical (Japan).

Solid-phase peptide synthesis

The peptides were synthesized by solid phase peptide chemistry.[16,17] The Boc strategy syntheses were carried out on an ABI Model 430A peptide synthesizer. The Fmoc strategy syntheses were performed using a manual shaker.

H-Phe-Val-Tyr-Ile-NH_2 (1) (Boc method). MBHA resin (0.42 mmol/g; 1.2094 g) was used to prepare the peptide: Boc-Phe-Val-Tyr(Br-Z)-Ile-MBHA-resin, by stepwise coupling of the amino acids Boc-Ile-OH, Boc-Tyr(Br-Z)-OH, Boc-Val-OH, Boc-Phe-OH. All the Boc amino acids (4 equiv) were coupled to the growing peptide chain as a symmetric anhydride by using DCC (2 equiv). Boc group was removed with 33–50% TFA in DCM solution prior to coupling with the next protected amino acid. After coupling of the last Boc amino acid, the peptide resin was washed with methanol and dried in vacuo to yield the Boc-Phe-Val-Tyr(Br-Z)-Ile-MBHA-resin (1.5447 g).

The protected peptide resin was treated with TFA/TMSOTf/*m*-cresol/thioanisole (100:28.6:7.47:16.7; v/v) for 1 h at 0 °C and for 2 h at room temperature. The solution was filtered and added drowse to excess cold diethyl ether (600 mL) and *n*-hexane (300 mL). The precipitated peptide was collected by centrifugation, washed with diethyl ether and *n*-hexane (1:1), and dried. The residue was treated with 2-mercaptoethanol (0.4 mL) and 1 N NH_4F (0.4 mL) in MeOH (8 mL) and H_2O (8 mL) at 0 °C. The solution was adjusted to pH 8.0 with triethylamine and then acidified to pH 4.0 with AcOH, and applied to gel chromatography with Sephadex G-10 (Amersham Pharmacia Biotech, Sweden) in 1 N AcOH. The lyophilized crude peptide was purified by RP-HPLC 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 verified by analytical HPLC, were lyophilized. Yield: 0.0758 g (28%).^{1}H NMR (DMSO-d_6) δ 0.82 (12H, m, 4×CH_3), 1.06 (1H, m, CH_2), 1.42 (1H, m, CH_2), 1.70 (1H, m, CH), 1.99 (1H, m, CH), 2.65–3.03 (4H, m, 2×CH_2), 4.12 (2H, m, 2×CH), 4.27 (1H, dd, J = 6.3, 8.6 Hz, CH), 4.52 (1H, dd, J = 4.6, 7.9 Hz, CH), 6.63 (2H, d, J = 8.6 Hz, aromatic–H), 7.06 (2H, d, J = 7.1 Hz, aromatic–H), 7.21 (5H, m, aromatic–H), 7.77 (1H, d, J = 8.9 Hz, NH), 8.18 (1H, d, J = 7.9 Hz, NH), 8.44 (1H, d, J = 8.9 Hz, NH).

H-Phe-Val-Pro-Ile-NH_2 (2). The title compound was synthesized using the same procedure as that for **6**, but using Fmoc-Ile-OPfp, Fmoc-Pro-OPfp, Fmoc-Val-OPfp, Fmoc-Phe-OPfp. Yield: 0.0553 g (24%). ^{1}H NMR (CD_3OD) δ 0.96 (12H, m, 4×CH_3), 1.23 (1H, m, CH_2), 1.60 (1H, m, CH_2), 1.80 (1H, m, CH), 2.00–2.16 (5H, m, CH, 2×CH_2), 3.01, 3.21 (2H, 2dd, J = 5.6, 8.1, 14.2 Hz, CH_2), 3.66 (1H, m, CH_2), 3.83 (1H, m, CH_2), 4.19 (2H, m, 2×CH), 4.49 (2H, m, 2×CH), 7.29 (6H, m, NH, aromatic–H), 7.96 (1H, d, J = 8.6 Hz, NH)

H-Phe-Val-Lys-Ile-NH_2 (3). The title compound was synthesized using the same procedure as that for **1**, but using Boc-Ile-OH, Boc-Lys(Cl-Z)-OH, Boc-Val-OH, Boc-Phe-OH. Yield: 0.10 g (38%). ^{1}H NMR (DMSO-d_6) δ 0.84 (12H, m, 4×CH_3), 1.02–1.70 (9H, m, 4×CH_2, CH), 1.96 (1H, m, CH), 2.75 (2H, t, J = 7.1 Hz, CH_2), 2.90, 3.07 (2H, 2dd, J = 4.3, 7.6, 13.9 Hz, CH_2), 4.17 (2H, m, 2×CH), 4.28 (2H, m, 2×CH), 7.27 (5H, m, aromatic–H), 7.70 (1H, d, J = 8.9 Hz, NH), 8.18 (1H, d, J = 7.6 Hz, NH), 8.54 (1H, d, J = 8.9 Hz, NH).

H-Phe-Val-Glu-Ile-NH_2 (4). The title compound was synthesized in the same way as that for **1**, but using Boc-Ile-OH, Boc-Glu(OBzl)-OH, Boc-Val-OH, Boc-Phe-OH. Yield: 0.069 g (33%).^{1}H NMR (DMSO-d_6) δ 0.86 (12H, m, 4×CH_3), 1.10 (1H, m, CH_2), 1.39 (1H, m, CH_2), 1.63–2.11 (4H, m, 2×CH, CH_2), 2.25 (2H, m, CH_2), 2.92, 3.08 (2H, 2dd, J = 4.8, 7.9, 13.9 Hz, CH_2), 4.09–4.44 (4H, m, 4×CH), 7.30 (5H, m, aromatic–H), 8.21–8.40 (2H, m, 2×NH), 8.52 (1H, t, J = 8.4 Hz, NH).

H-Phe-Val-Gln-Ile-NH_2 (5). The title compound was synthesized in the same way as that for **1**, but using Boc-Ile-OH, Boc-Gln-OH, Boc-Val-OH, Boc-Phe-OH. Boc-Gln-OH was activated as the HOBt ester formed using 4.0 equivalents of DCC in DMF and DCM. Yield: 0.123 g (66%).^{1}H NMR (DMSO-d_6) δ 0.84 (12H, m, 4×CH_3), 1.07 (1H, m, CH_2), 1.38 (1H, m, CH_2), 1.56–2.03 (4H, m, 2×CH, CH_2), 2.12 (2H, t, J = 7.9 Hz, CH_2), 4.14 (2H, m, 2×CH), 4.28 (2H, m, 2×CH), 7.29 (5H, m, aromatic–H), 7.69 (1H, d, J = 8.3 Hz, NH), 8.25 (2H, m, 2×NH) 8.53 (1H, d, J = 8.9 Hz, NH).

H-Phe-Val-Trp-Ile-NH_2 (6) (Fmoc method). MBHA resin (0.79 mmol/g; 3.418 g) was treated twice with 20% piperidine in DMF and washed eight times with DMF. Fmoc-Ile-OPfp (3.5 equiv) was then added followed by HOBt (3.5 equiv). The coupling reaction mixture was shaken on a manual shaker for 3 h at room temperature. The resin was then washed six times with DMF. The following Fmoc amino acid Pfp esters were sequentially coupled to the growing peptide chain: Fmoc-Trp-OPfp, Fmoc-Val-OPfp, Fmoc-Phe-OPfp. All the Fmoc amino acid Pfp esters (3 equiv) were coupled to a growing peptide chain using HOBt (3 equiv) in DMF. All the coupling reactions were shaken on a manual shaker for 3–30 h at room temperature. Fmoc group was removed with 20% piperidine in DMF prior to coupling with the next protected amino acid. After deprotection of the last Fmoc group, the peptide resin was washed with MeOH and dried in vacuo to yield the H-Phe-Val-Trp-Ile-MBHA resin (4.725 g).

The peptide resin was treated with TFA/TMSOTf/*m*-cresol/thioanisole/1,2-ethandithiol (100:12:6.5:15:5; v/v) for 2 h at 0 °C and additional 2 h at room temperature. The solution was filtered and added dropwise to cold diethyl ether (500 mL) and n-hexane (1500 mL). The precipitated peptide was collected by centrifugation, and washed with diethyl ether and *n*-hexane (1:3), and dried. The residue was treated with 2-mercaptoethanol (0.2 mL) in MeOH (10 mL) and H_2O (6 mL) at 0 °C. The solution was adjusted to pH 8.0 with triethylamine for 0.5 h, and then acidified to pH 4.0 with AcOH. 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. Yield: 0.134 g (9%).^{1}H NMR (DMSO-d_6) δ 0.83 (12H, m, 3×CH_2), 1.06 (1H, m, CH_2), 1.37 (1H, m, CH_2), 1.65 (1H, m, CH), 1.98 (1H, m, CH), 2.72–3.24 (4H, m, 2×CH_2), 4.13 (2H, m, 2×CH), 4.29 (1H, m, CH), 4.67 (1H, m, CH), 6.94–7.30 (10H, m, aromatic–H), 7.62 (1H, d, J = 6.9 Hz, NH), 8.25 (2H, m, 2×NH) 8.35 (1H, d, J = 9.2 Hz, NH).

H-Phe-Val-Trp(2′-SCH_2CH_2OH)-Ile-NH_2 (7). 6 (0.191 g, 0.339 mmol) was added to a solution of Bis-(2-acetoxyethyl)disulfide (0.82 g, 3.44 mmol) in TFA (20 mL) under ice-water cooling, followed by trifluoromethanesulfonic acid silver salt (0.8449 g, 3.29 mmol). The solution was stirred for 8 h under ice water cooling, and for 120 h at room temperature. The solvent was removed under reduced pressure, and the residue was dissolved in MeOH (10 mL). The solution was adjusted to pH 9 with 1 N NaOHaq for 0.5 h, filtered, and then acidified to pH 4 with AcOH. The solvent was removed under reduced pressure to give a residue that was purified by preparative RP-HPLC. The lyophilized product was a white amorphous solid. Yield: 0.108 g (50%). ^{1}H NMR (DMSO-d_6) δ 0.80 (12H, m, 4×CH_3), 1.03 (1H, m, CH_2), 1.38 (1H, m, CH_2), 1.66 (1H, m, CH), 1.92 (1H, m, CH), 2.77 (2H, m, CH_2), 2.87 (2H, m, CH_2), 3.04, 3.20 (2H, 2dd, J = 5.9, 8.3, 14.2 Hz, CH_2), 3.47 (2H, m, CH_2), 4.12 (1H, dd, J = 7.3, 8.6 Hz, CH), 4.26 (1H, dd, J = 6.9, 8.2 Hz, CH), 4.68 (1H, dd, J = 6.6, 7.9 Hz, CH), 4.91 (1H, t, J = 5.6 Hz, CH), 6.95 (2H, m, aromatic–H), 7.03 (1H, t, J = 7.4 Hz, aromatic–H), 7.12 (5H, s, aromatic–H), 7.20 (1H, d, J = 7.6 Hz, aromatic–H), 7.66 (1H, d, J = 8.9 Hz, NH), 7.69 (1H, d, J = 7.9 Hz, NH), 8.19 (1H, d, J = 7.9 Hz, NH) 8.40 (1H, d, J = 8.9 Hz, NH).

H-Phe-Val-Trp(2′-SCH_3)-Ile-NH_2 (8). 6 (0.589 g, 1.05 mmol) was added to a solution of dimethyl disulfide (1.028 g, 10.9 mmol) in TFA (20 mL) under ice-water cooling, followed by trifluoromethanesulfonic acid silver salt (1.308 g, 5.09 mmol). The solution was stirred for 0.5 h under ice water cooling, and for 48 h at room temperature. The solution was added dropwise to cold diethyl ether (400 mL) and *n*-hexane (100 mL). The precipitated peptide was collected by centrifugation, and washed with diethyl ether. The residue was treated with dithiothreitol (0.8 g) in MeOH (30 mL), AcOH (30 mL), and H_2O (20 mL) for 1 h. The precipitate was removed by centrifugation. The solvent was removed under reduced pressure to give a residue that was purified by preparative RP-HPLC. The lyophilized product was a white amorphous solid. Yield: 0.322 g (43%). ^{1}H NMR (DMSO-d_6) δ 0.81 (12H, m, 4×CH_3), 1.03 (1H, m, CH_2), 1.34 (1H, m, CH_2), 1.64 (1H, m, CH), 1.93 (1H, m, CH), 2.40 (3H, s, CH_3), 2.87 (2H, m, CH_2), 3.03, 3.24 (2H, 2dd, J = 6.3, 7.6, 13.9 Hz, CH_2), 4.12 (2H, m, 2×CH), 4.23 (1H, dd, J = 6.9, 8.3 Hz, CH), 4.69 (1H, dd, J = 7.1, 14.4 Hz, CH), 6.90–7.06 (4H, m, aromatic–H), 7.17 (5H, m, aromatic–H), 7.60 (1H, d,

$J=8.9$ Hz, NH), 7.67 (1H, d, $J=7.9$ Hz, NH), 8.21 (1H, d, $J=7.9$ Hz, NH), 8.44 (1H, d, $J=8.9$ Hz, NH).

H-Phe-Val-Trp(2′-$SCH_2CH_2CH_3$)-Ile-NH_2 (9). The title compound was synthesized from **6** (0.592 g, 1.05 mmol) and di-*n*-propyl disulfide following the procedure describe for **8**. Yield: 0.369 g (47%). ^{1}H NMR (DMSO-d_6) δ 0.76 (12H, m, 4×CH_3), 0.93 (3H, t, $J=7.3$ Hz, CH_3), 1.05 (1H, m, CH_2), 1.41 (1H, m, CH_2), 1.48 (2H, six, $J=7.3$ Hz, CH_2), 1.64 (1H, m, CH), 1.95 (1H, m, CH), 2.79–2.95 (4H, m, 2×CH_2), 3.04 3.26, (2H, 2dd, $J=6.3$, 8.0, 14.2 Hz, CH_2), 4.12 (2H, m, 2×CH), 4.33 (1H, dd, $J=7.3$, 8.2 Hz, CH), 4.66 (1H, dd, $J=7.3$, 14.9 Hz, CH), 6.90–7.06 (4H, m, aromatic–H), 7.17 (5H, m, aromatic–H), 7.55 (1H, d, $J=8.9$ Hz, NH), 7.66 (1H, d, $J=7.6$ Hz, NH), 8.19 (1H, d, $J=8.2$ Hz, NH), 8.44 (1H, d, $J=8.9$ Hz, NH).

H-Phe-Val-Trp(2′-$SCH_2CH_2NMe_2$)-Ile-NH_2 (10). The title compound was synthesized from **6** (0.592 g, 1.05 mmol) and tetramethyl cystamine[24] following the procedure describe for **8**. Yield: 0.589 g (63%).^{1}H NMR (DMSO-d_6) δ 0.82 (12H, m, 4×CH_3), 1.02(2H, m, CH_2), 1.38 (2H, m, CH_2), 1.65 (1H, m, CH), 1.94 (1H, m, CH), 2.77 (6H, s, 2×CH_3), 2.87 (2H, m, CH_2), 3.00–3.33 (6H, m, 3×CH_2), 4.11 (2H, m, 2×CH), 4.27 (1H, dd, $J=6.8$, 8.4 Hz, CH), 4.73 (1H, dd, $J=6.8$, 7.6 Hz, CH), 6.91–7.27 (9H, m, aromatic–H), 7.69 (1H, d, $J=9.2$ Hz, NH), 7.74 (1H, d, $J=8.2$ Hz, NH), 8.28 (1H, m, NH), 8.45 (1H, d, $J=8.9$ Hz, NH).

H-Phe-Val-Trp(2′-SCH_2COOH)-Ile-NH_2 (11). The title compound was synthesized from **6** (0.584 g, 1.04 mmol) and dithiodiglycolic acid following the procedure describe for **8**. Yield: 0.35 g (42%). ^{1}H NMR (DMSO-d_6) δ 0.79 (12H, m, 4×CH_3), 1.00 (1H, m, CH_2), 1.39 (1H, m, CH_2), 1.66 (1H, m, CH), 1.91 (1H, m, CH), 2.81, 2.93 (2H, 2dd, $J=4.6$, 8.3, 14.2 Hz, CH_2), 3.09, 3.20 (2H, 2dd, $J=5.9$, 8.3, 14.2 Hz, CH_2), 3.63 (2H, d, $J=1.7$ Hz, CH_2), 4.11 (2H, m, 2×CH), 4.25 (1H, dd, $J=6.9$, 8.2 Hz, CH), 4.71 (1H, dd, $J=6.9$, 7.4 Hz, CH), 6.90–7.23 (9H, m, aromatic–H), 7.65 (1H, d, $J=10.2$ Hz, NH), 7.68 (1H, d, $J=8.6$ Hz, NH), 8.12 (1H, d, $J=8.2$ Hz, NH), 8.51 (1H, br, NH).

Peptide purification

The crude peptides were purified by RP-HPLC 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 verified 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 in two different columns and linear gradient solvent systems. One solvent system (A) was 100:0 to 30:70 (0.1% aqueous TFA/0.1% TFA in acetonitrile) over 25 min at 1 mL/min using YMC-Pack A-302 analytical column (4.6×150 mm, 120 Å, 5 μm particle size, YMC, Japan). The other (B) was 100:0 to 30:70, or 100:0 to 2:98 (0.1% aqueous TFA/MeOH) over 25 min, 1.0 mL/min using μ Bondasphere 5μ C18 300 Å (3.9×150 mm, Waters).

FT-NMR was performed on a JEOL JNM-A500, Varian Mercury 300, and JEOL JNM-EX270. Spectra was taken in DMSO-d_6 or MeOH-d_4. The molecular weight of the compounds was determined by FABMS (VG70-250SEQ, VG Analytical, UK). The values are expressed as MH^+ (Table 3).

Receptor binding assay

Binding assay for MTL-R was performed according to the procedure introduced by Bormans et al.[25] 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, USA) 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, the reaction was stopped by adding 2 mL of cold buffer. 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, iv) 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.[26] 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,

Japan) and recorded on an ink-writing recorder (Type 3066, Yanagisawa-Denki, Tokyo, Japan). Before the experiments, each strip was subjected to repeated stimulation with 100 μM acetylcholine (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}).

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