Identification of Arginine Analogues as Antagonists and Agonists for the Melanocortin-4 Receptor

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In the present study, conducted to explore potent and small molecular melanocortin-4 (MC4) receptor ligands, we found that tripeptide 3a, containing a D-Phe-Arg-2-Nal (Nal; naphthylalanine) sequence, exhibited a moderate affinity for the MC4 receptor. Structural optimization led to the identification of a compound with a high affinity for the MC4 receptor, namely, tripeptide 3e, which showed a 70-fold higher affinity for the MC4 receptor than the lead compound 3a. Moreover, in an effort to further reduce the peptidic characters of tripeptide 3e, we found that dipeptide 3g exhibited a relatively high affinity for the MC4 receptor. Furthermore, in these analogues, the substituted position (1′ *vs.* 2′) of the naphthyl ring of Nal residue at position 7 was found to be important for the differentiation of agonist and antagonist activity. The synthesis and structure–activity relationships of the arginine analogues as MC4 receptor ligands were described in this paper.

Key words melanocortin-4 receptor; antagonist; agonist; depression; anxiety

Five subtypes of melanocortin receptors (MC1—MC5 receptors) belonging to the superfamily of G-protein-coupled receptors (GPCRs) have been cloned and characterized. When stimulated by agonist ligands, the melanocortin receptors (MCRs) activate the cyclic adenosine monophosphate (cAMP) signal transduction pathway. The naturally occurring agonists of MCRs are melanocyte-stimulating hormones (α-MSH; Fig. 1, β-MSH and γ-MSH) and adrenocorticotropic hormone (ACTH), which are derived from proopiomelanocortin (POMC) by enzymatic processing. In addition, two endogenous antagonistic peptides, agouti and agouti-related protein (AGRP), have also been identified. The MCRs mediate a variety of physiological responses that include skin pigmentation (MC1 receptor), steroidogenesis (MC2 receptor), feeding behavior (MC3 and MC4 receptors), and exocrine gland secretion (MC5 receptor). Consequently, the melanocortin system has become an attractive therapeutic target for drug development.[1—4)]

Over the last 30 years, significant progress has been made in the design of peptidic ligands as potential therapeutic agents for the treatment of melanocortin-mediated diseases. Among the first significant research findings on α-MSH was the discovery of [Nle^4, D-Phe^7]α-MSH (NDP-MSH; Fig. 1).[5)] Inverting Phe at position 7 of α-MSH to the D-isomer, in addition to replacing Met at position 4 with Nle, resulted in the formation of the analogue, NDP-MSH, that exhibited an enhanced potency for the MCRs and enzymatic stability.[5)] Further studies on melanocortins indicate that the tetrapeptide His-Phe-Arg-Trp sequence is the minimal core sequence essential for the activation of the MCRs.[1)] The tetrapeptide His-Phe-Arg-Trp sequence has thus been employed as a promising template for structure–activity relationship (SAR) studies of MCR ligands.[6—9)] Moreover, cyclic peptide analogues with reduced conformational freedom were designed to increase the activity and *in vivo* stability of the peptide ligands. Thus, both MT-II and SHU-9119 (Fig. 1), which were designed based on the cyclization of the tetrapeptide His-D-Phe-Arg-Trp sequence, were discovered as cyclic peptides with potent activities on the MCRs.[10,11)]

Among the MCRs, numerous studies have suggested that the MC4 receptor is involved in the regulation of feeding behavior and energy homeostasis.[12—18)] It has also been reportedly involved in the regulation of sexual functions[19,20)] and protection against tumor-induced decreases in body weight.[21,22)] In addition, the MC4 receptor has been a focus of interest for its possible relationship to stress and the regulation of emotional behavior in relation to conditions such as depression and anxiety.[23—29)] These findings till date indicate that the MC4 receptor could be a promising target for the development of drugs for the above-mentioned conditions, and numerous ligands of the MC4 receptor have been reported.[30—56)]

Stress is considered to play a pivotal role in both anxiety and depression. Indeed, dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis, which is caused by continuous exposure to stress, has been reported in patients with major

α-MSH Ac-Ser^1-Trp^2-Ser^3-Met^4-Glu^5-His^6-Phe^7-Arg^8-Trp^9-Gly^{10}-Lys^{11}-pro^{12}-Val^{13}-NH_2

NDP-MSH Ac-Ser^1-Trp^2-Ser^3-Nle^4-Glu^5-His^6-*D*-Phe^7-Arg^8-Trp^9-Gly^{10}-Lys^{11}-pro^{12}-Val^{13}-NH_2

MT-II Ac-Nle-c[Asp-His^6-*D*-Phe^7-Arg^8-Trp^9-Gly-Lys]-NH_2

SHU9119 Ac-Nle-c[Asp-His^6-*D*-Nal^7-Arg^8-Trp^9-Gly-Lys]-NH_2

1 ; Ac-His^6-*D*-Phe^7-Arg^8-Trp^9-NH_2

2 ; Ac-*D*-Phe^7-Arg^8-Trp^9-NH_2

Fig. 1. Endogenous and Synthetic Peptidyl Ligands of the MC4 Receptor

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depressive disorders. Stress responses are initiated by the release of corticotropin-releasing factor (CRF). In addition of CRF system activation, several lines of evidence suggest that melanocortins mediate important behavioral and biochemical responses to stress and, consequently, stress-induced disorders.

We previously reported that a tripeptide MC4 receptor antagonist **3e** (MCL0020) exhibited antidepressant, anxiolytic, and anti-stress effects in a variety of animal models (Table 1).[26] These findings suggest that blockade of the MC4 receptor may be a useful approach for treating subjects with depressive and anxiety disorders. This hypothesis is supported by the demonstration in various rodent modes of depression and anxiety that other MC4 receptor antagonists also exert antidepressant and anxiolytic effects.[27,28]

In the present study, to identify small molecular MC4 receptor ligands, we focused our attention on a tripeptide sequence, D-Phe-Arg-Trp, and modified both the D-Phe and Trp residues to increase the affinity for the MC4 receptor. Moreover, to reduce the peptidic character and molecular weight, the effects of the removing the N- or C-terminus of the tripeptides on the binding affinity for the receptor were explored. The synthesis, SARs and biological evaluation of the arginine analogues as MC4 receptor ligands are described in this study.

Chemistry All the compounds were prepared from Boc-Arg(Z)$_2$-OH **4** according to the general synthetic methods (Methods A and B) described as Charts 1 and 2. The reaction of **4** with H-2-Nal-NH$_2$·HCl **11a** in the presence of HOBt and EDC yielded Boc-Arg(Z)$_2$-2-Nal-NH$_2$ **5a**. After the removal of the Boc group of **5a** by TFA, condensation with Boc-D-2-Nal-OH **12** in the presence of HOBt and EDC yielded Boc-D-2-Nal-Arg(Z)$_2$-2-Nal-NH$_2$ **6a-1**. The removal of the Boc group of **6a-1** by TFA, followed by the treatment with Ac$_2$O and pyridine, yielded Ac-D-2-Nal-Arg(Z)$_2$-2-Nal-NH$_2$ **7a-1**. Compound **3e** was obtained by the removal of the Z groups of **7a-1** using hydrogenolysis with Pd(OH)$_2$ as a catalyst. Compounds **3a**—**d** and **3f**—**h** were also obtained from **4** by using similar synthetic methods to those of **3e** (Method A, Chart 1).

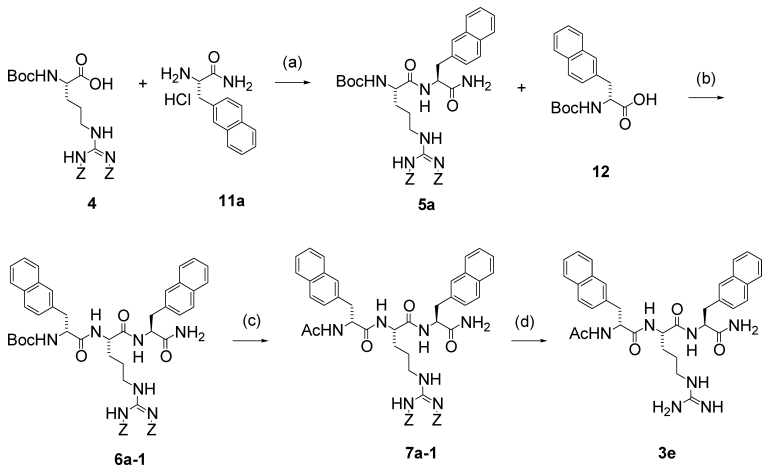
Compounds **9** and **10** were synthesized from **5a** using a synthetic method similar to Method A. The removal of the Boc group of **5a** under acidic conditions, followed by condensation with 2-naphthyl-(CH$_2$)$_2$-CO$_2$H **15** in the presence of HOBt and EDC, yielded 2-naphthyl-(CH$_2$)$_2$-Arg(Z)$_2$-2-Nal-NH$_2$ **8-1**. Compound **9** was obtained by the removal of the Z groups of **8-1** using hydrogenolysis. Compound **10** was also obtained from **5a** by the same synthetic method as that used for **9**. Furthermore, compound **20** was prepared from Boc-Arg(Z)$_2$-NH-(CH$_2$)$_2$-2-naphthyl **5c** using a synthetic method similar to that used for **9** (Method B, Chart 2).

Results and Discussion

The affinities of all the compounds for the MC4 receptor were evaluated based on their binding affinity to the membranes of COS-1 cells expressing the human MC4 receptor; the affinities were calculated from the inhibition curve of [^{125}I]NDP-α-MSH binding,[27] and the IC$_{50}$ values are shown in Table 1.

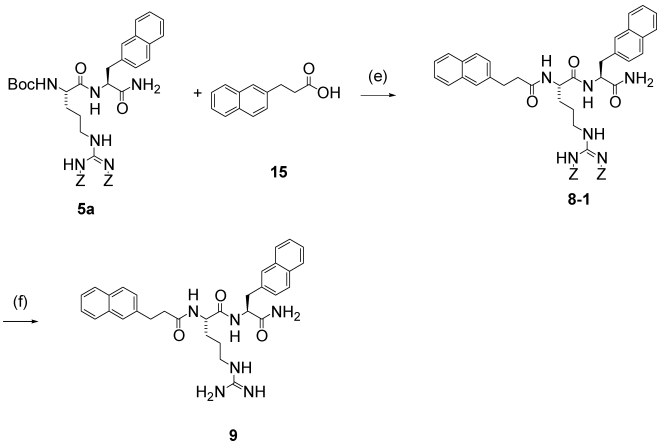
All endogenous agonists for the MCRs contain a tetrapeptide sequence, His-Phe-Arg-Trp, which has been identified as the minimal peptide sequence crucial for MCRs activation. Further studies have shown that the inversion of the chirality of Phe to D-Phe results in an increase in the binding affinity for the MC4 receptor, as described above. Moreover, the His residue in tetrapeptide **1** (Fig. 1), which showed a moderate affinity (IC$_{50}$=1153 nM) for the MC4 receptor, can be deleted without much loss in binding affinity (tripeptide **2**; IC$_{50}$=2081 nM, Fig. 1).[8] Therefore, in the present study, to explore potent and small molecular MC4 receptor ligands, we focused our attention on mimicking the tripeptide, D-Phe-Arg-Trp sequence.

It has been suggested that the chemically reactive indol in the Trp residue could be replaced with a non-reactive naphthyl moiety in the design of peptide melanocortin receptor ligands.[6] In our attempt to identify further potent tripeptide analogues, we initially anticipated that 2-Nal could be used as an alternative to the Trp in the tripeptide, Ac-D-Phe-Arg-Trp-NH$_2$ **2**. We prepared the tripeptides **3a** (containing 2-Nal) and **3b** (containing D-2-Nal), as shown in Chart 1, and evaluated their affinity for the MC4 receptor. The resultant **3a** and **3b** were found to exhibit a similar affinity (IC$_{50}$=



Reagents and conditions: (a) *N*-methyl morpholine, EDC-HCl, HOBt-H$_2$O, DMF, rt (81%); (b) **5a-1**, TFA, CH$_2$Cl$_2$, rt; then **12**, EDC-HCl , HOBt-H$_2$O, DMF, rt (38%); (c) **6a-1**, TFA, CH$_2$Cl$_2$, rt; then Ac$_2$O, pyridine, CHCl$_3$, rt (74%); (d) H$_2$, Pd(OH)$_2$/C, MeOH, rt (95%).

Chart 1. Synthesis of Tripeptide **3e** (General Method of Synthesis for **3a**—**h**, Method A)



Reagents and conditions: (e) **5a**, TFA, CH_2Cl_2, rt; then **15**, EDC-HCl, HOBt-H_2O, DMF, rt (58%); (f) H_2, $Pd(OH)_2$/C, MeOH, rt (96%).

Chart 2. Synthesis of Dipeptide **9** (General Method of Synthesis for Dipeptides **9** and **10**, and **20**, Method B)

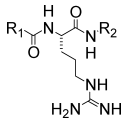
1070 and 2170 nM, respectively) for the MC4 receptor, compared to the corresponding tripeptide **2**. These findings suggest that Trp can be replaced with 2-Nal or D-2-Nal without any significant decrease in binding affinity for the MC4 receptor in these tripeptide analogues. Therefore, both 2-Nal and D-2-Nal were employed in further research efforts to design tripeptides for MC4 receptor ligands.

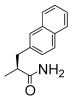
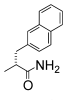
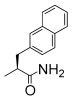
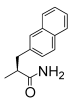
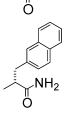
We next considered that the D-Phe in these tripeptides could be replaced with D-Nal, based on previous SAR observations of cyclic peptides, MT-II and SHU-9119 (Fig. 1).[10,11] We prepared **3c**—**f** as shown in Chart 1 and evaluated the affinity for the MC4 receptor. The results indicated that both **3e** and **3f** containing D-2-Nal showed a much higher affinity (IC_{50}=15.4 and 36.5 nM, respectively) for the MC4 receptor than the D-Phe analogues, **3a** and **3b**. In contrast, both **3c** and **3d** containing D-1-Nal exhibited a slightly higher affinity (IC_{50}=327 and 690 nM, respectively) for the MC4 receptor than the corresponding **3a** and **3b**. These findings suggest that, in the case of these tripeptide analogues, the substitution of D-2-Nal at position 7 (α-MSH numbering; Fig. 1) resulted in a drastic increase in the binding affinity for the MC4 receptor.

To better understand the minimal structure fragment required for binding affinity for the MC4 receptor, we initially investigated the effect of removing the N-terminus from **3c** and **3e** on the binding affinity for the receptor. We prepared dipeptides **9** and **10**, as shown in Chart 2, and evaluated the binding affinity for the MC4 receptor. The results indicated that the resultant **9** and **10** showed a much lower affinity (IC_{50}=543 and 3940 nM, respectively) for the MC4 receptor than the corresponding tripeptides **3c** and **3e**. Next, we also investigated the effect of removing the C-terminus from **3a** and **3e** on binding affinity for the MC4 receptor. The results indicated that the dipeptide **3g** showed a relatively high affinity (IC_{50}=117 nM) for the MC4 receptor. This finding suggests that dipeptide analogues, as exemplified by **3g**, might serve as new templates for the design of potent MC4 ligands with reduced peptidic character.

As an additional structural conversion to further reduce the peptidic character and molecular weight, we prepared **20**, which was simplified by removing the N-terminus from the

Table 1. Binding Affinity for the MC4 Receptor and cAMP Stimulation



Compound	R^1	R^2	IC_{50} (nM)	cAMP formation[a)]
3a			1070	
3b			2170	
3c			327	2.8
3d			690	1.9
3e			15.4	0.95
3f			36.5	0.99
3g			117	
3h			7230	
9			543.0	
10			3940	
20			1610	

a) The fold increase *vs.* basal level at a ligand concentration of 10 μM.

relatively high-affinity dipeptide **3g**, and evaluated the affinity for the MC4 receptor. Surprisingly, compound **20** exhibited a moderate affinity for the MC4 receptor (IC_{50}= 1610 nM), and the affinity was almost the same as that of tetrapeptide **1** and tripeptide **2**. Thus, analogues like **20**, which have a significantly reduced peptidic character, might also be useful as new frameworks for MC4 ligands.

We previously reported that **3e** was selective for the MC4 receptor and acted as an antagonist at the MC4 receptor. **3e** was also found to exhibit antidepressant and anxiolytic activities in various rodent models.[26] To further investigate the *in vitro* profiles of the compounds described above, the effects of several compounds on cAMP formation at a ligand concentration of 10 μM in MC4 receptor expressing COS-1 cells were evaluated (Table 1). The results indicated that the tripeptides **3c** and **3d** containing D-1-Nal at position 7 (α-MSH numbering) increased cAMP formation, suggesting

that these compounds act as agonists at the MC4 receptor. In contrast, the tripeptides **3e** and **3f** containing D-2-Nal at position 7 did not influence the stimulation of cAMP, suggesting that these compounds act as antagonists at the MC4 receptor. These findings suggest that, in the case of these tripeptide analogues, the substituted position (1′ *vs.* 2′) of the naphthyl ring of Nal residue at position 7 appears to be important for the determining of agonist and antagonist activity at the MC4 receptor; these results were supported by SAR research on the cyclic peptides, MT-II and SHU-9119.[10,11]

Conclusion

We have reported on the synthesis and SARs of a new series of tripeptides that act as MC4 receptor ligands. The lead compound **3a**, obtained by replacing a Trp residue of tripeptide **2** with 2-Nal, showed a moderate affinity for the MC4 receptor. Structural conversion led to the identification of a compound with a high affinity for the MC4 receptor, namely, **3e** (IC_{50}=15.4 nM), which showed a 70-fold higher affinity for the MC4 receptor than the lead compound **3a**. We also found a dipeptide analogue, **3g**, with a reduced peptidic character that showed a relatively high affinity (IC_{50}=117 nM) for the MC4 receptor. Furthermore, in the case of these tripeptide analogues, the substituted position (1′ *vs.* 2′) of the naphthyl ring of Nal residue at position 7 was found to be important for the differentiation of agonist and antagonist activity at the MC4 receptor. These arginine analogues that have been shown to have a high affinity for the MC4 receptor may be excellent tools for investigating the involvement of the MC4 receptor in the development of stress-related disorders, such as depression and anxiety, and for elucidating the actions mediated by the MC4 receptor.

Experimental

Melting points were determined on a Yanaco MP-500D melting point apparatus and are uncorrected. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were obtained using a Varian Gemini 2000 (200 MHz) or Varian Unity Inova 300 (300 MHz). Chemical shifts are reported in parts per million relative to tetramethylsilane (TMS) as an internal standard. Mass spectra (MS) were obtained on Micromass Platform LC (ESI). High resolution spectra were recorded on a Micromass Q-TOF2 instrument. Elemental analyses were performed by a Perkin–Elmer 2400 or a Yanaco MT-6. Silica gel C-200 (100—200 mesh, Wako Pure Chemical) and Chromatorex NH (100—200 mesh, Fuji Silysia Chemical Ltd.) were used for column chromatography, using the solvent systems (volume ratios) indicated below.

General Method for the Synthesis of 3a—3h (Method A). H-2-Nal-NH$_2$ Hydrochloride (11a) A mixture of Boc-2-Nal-OH (5.00 g, 15.9 mmol), methyl iodide (5.00 ml, 80.3 mmol) and $NaHCO_3$ (2.66 g, 31.7 mmol) in DMF (25 ml) was stirred at room temperature for 72 h. The reaction mixture was concentrated *in vacuo* and partitioned between EtOAc and H_2O. The separated organic phase was washed with 5% aq. $KHSO_4$, saturated aq. $NaHCO_3$ and brine, dried over Na_2SO_4, filtered, and concentrated *in vacuo*. A solution of the above residue in MeOH (50 ml) was saturated by NH_3 with ice-cooling. The reaction mixture was standed at room temperature for 24 h, and concentrated *in vacuo*. To the residue was added a solution of 4 mol/l HCl in 1,4-dioxane (80 ml), and the reaction mixture was stirred at room temperature for 2 h. After concentration of the reaction mixture, the residual solid was dissolved in MeOH (50 ml) followed by filtration, and the filtrate was concentrated *in vacuo*. To the residual solid was added IPA (20 ml) and refluxed for 10 min. The resulting suspension was stirred at room temperature for 15 h, and the precipitate was collected by filtration to obtain **11a** (3.77 g, 95%) as a solid. ^{1}H-NMR (200 MHz, DMSO-d_6) δ: 3.13—3.41 (2H, m), 4.08 (1H, t, J=6.7 Hz), 7.38—7.61 (4H, m), 7.73—7.96 (4H, m), 8.08 (1H, s), 8.36 (1H, br s). MS (ESI, Pos) *m/z*: 215 $(M+H)^+$. *Anal*. Calcd for $C_{13}H_{14}N_2O\cdot$HCl: C, 62.28; H, 6.03; N, 11.17. Found: C, 62.24; H, 6.01; N, 11.10.

Boc-Arg(Z)$_2$-2-Nal-NH$_2$ (5a) To a mixture of Boc-Arg(Z)$_2$-OH **4** (2.16 g, 3.98 mmol), H-2-Nal-NH$_2$ hydrochloride **11a** (1.00 g, 3.99 mmol), *N*-methyl morpholine (0.42 g, 4.2 mmol) and HOBt·H_2O (0.92 g, 6.0 mmol) in DMF (20 ml) was added EDC·HCl (0.96 g, 5.0 mmol) with ice-cooling, and the reaction mixture was stirred at room temperature for 72 h. The mixture was partitioned between EtOAc and H_2O. The separated organic phase was washed with 5% aq. $KHSO_4$, saturated aq. $NaHCO_3$ and brine, dried over Na_2SO_4, filtered, concentrated *in vacuo*. The residue was crystallized from AcOEt, and the precipitate was collected by filtration to give **5a** (2.4 g, 81%) as a solid. ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 1.05—1.70 (13H, m), 3.01—3.32 (2H, m), 3.38—3.77 (2H, m), 3.99—4.13 (1H, m), 4.50—4.64 (1H, m), 5.02—5.23 (5H, m), 5.55 (1H, d, J=7.3 Hz), 6.24 (1H, br s), 6.89 (1H, d, J=8.1 Hz), 7.17—7.52 (14H, m), 7.57—7.76 (3H, m), 9.00—9.35 (2H, m). MS (ESI, Pos) *m/z*: 739 $(M+H)^+$. *Anal*. Calcd for $C_{40}H_{46}N_6O_8\cdot 0.5H_2O$: C, 64.24; H, 6.33; N, 11.24. Found: C, 63.87; H, 6.22; N, 11.14. *Rf* 0.60 ($CHCl_3$: MeOH=9 : 1).

Boc-D-2-Nal-Arg(Z)$_2$-2-Nal-NH$_2$ (6a-1) To a suspension of Boc-Arg(Z)$_2$-2-Nal-NH$_2$ **5a** (500 mg, 0.676 mmol) in CH_2Cl_2 (5 ml) was added TFA (5 ml) and the reaction mixture was stirred at room temperature for 2 h. After concentration of the reaction mixture, the residue was partitioned between $CHCl_3$ and saturated aq. $NaHCO_3$. The separated organic phase was dried over Na_2SO_4, filtered, concentrated *in vacuo* to obtain crude H-Arg(Z)$_2$-2-Nal-NH$_2$. To a mixture of the crude H-Arg(Z)$_2$-2-Nal-NH$_2$, Boc-D-2-Nal-OH **12** (235 mg, 0.745 mmol) and HOBt·H_2O (156 mg, 1.02 mmol) in DMF (15 ml) was added EDC·HCl (162 mg, 0.846 mmol) with ice-cooling, and the reaction mixture was stirred at room temperature for 15 h. The mixture was partitioned between EtOAc and H_2O. The separated organic phase was washed with 5% aq. $KHSO_4$, saturated aq. $NaHCO_3$ and brine, dried over Na_2SO_4, filtered, concentrated *in vacuo*. The residue was chromatographed on Silica gel C-200 ($CHCl_3$/MeOH 50 : 1), and crystallized from EtOAc to give **6a-1** (227 mg, 38%) as a solid. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.14—0.26 (1H, m), 0.53—0.66 (1H, m), 1.11—1.22 (2H, m), 1.47 (9H, s), 2.36—2.62 (2H, m), 2.82—3.01 (3H, m), 3.51—3.63 (1H, m), 4.07—4.30 (2H, m), 4.76—4.83 (1H, m), 5.01 (2H, s), 5.03—5.40 (4H, m), 6.76 (1H, br s), 7.04 (1H, d like, J=8.5 Hz), 7.18—7.64 (25H, m), 9.14 (1H, br s), 9.42 (1H, br s). MS (ESI, Pos) *m/z*: 936 $(M+H)^+$. *Anal*. Calcd for $C_{53}H_{57}N_7O_9\cdot 0.3H_2O$: C, 67.61; H, 6.17; N, 10.41. Found: C, 67.40; H, 6.15; N, 10.34. *Rf* 0.46 ($CHCl_3$: MeOH=9 : 1).

Ac-D-2-Nal-Arg(Z)$_2$-2-Nal-NH$_2$ (7a-1) To a suspension of Boc-D-2-Nal-Arg(Z)$_2$-2-Nal-NH$_2$ **6a-1** (200 mg, 0.214 mmol) in CH_2Cl_2 (3 ml) was added TFA (3 ml) and the reaction mixture was stirred at room temperature for 1 h. After concentration of the reaction mixture, the residue was partitioned between $CHCl_3$ and saturated aq. $NaHCO_3$. The separated organic phase was dried over Na_2SO_4, filtered, concentrated *in vacuo* to obtain crude H-D-2-Nal-Arg(Z)$_2$-2-Nal-NH$_2$. To a solution of the crude H-D-2-Nal-Arg(Z)$_2$-2-Nal-NH$_2$ in $CHCl_3$ (2 ml) was added a mixture of Ac_2O (24 mg, 0.235 mmol) and pyridine (19 mg, 0.235 mmol) in $CHCl_3$ (1 ml), and the reaction mixture was stirred at room temperature for 2 h. After concentration of the reaction mixture, the residue was partitioned between EtOAc and 5% aq. $KHSO_4$. The separated organic phase was washed with saturated aq. $NaHCO_3$ and brine, dried over Na_2SO_4, filtered, concentrated *in vacuo*. The residue was crystallized from EtOAc to obtain **7a-1** (139 mg, 74%) as a solid. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.13—0.28 (1H, m), 0.57—0.69 (1H, m), 0.86—1.02 (1H, m), 1.22—1.40 (1H, m), 2.02 (3H, s), 2.42—2.78 (2H, m), 3.02—3.21 (3H, m), 3.58—3.68 (2H, m), 4.31—4.42 (1H, m), 4.63—4.77 (1H, m), 5.03 (2H, s), 5.09 (1H, d, J=12.1 Hz), 5.47 (1H, d, J=12.1 Hz), 7.03 (1H, dd, J=1.7, 6.7 Hz), 7.17—7.76 (27H, m), 7.99—8.04 (1H, m), 9.27 (1H, br s), 9.53 (1H, br s). MS (ESI, Pos) *m/z*: 900 $(M+Na)^+$. *Anal*. Calcd for $C_{50}H_{51}N_7O_8\cdot 0.4H_2O$: C, 67.84; H, 5.90; N, 11.08. Found: C, 67.67; H, 5.83; N, 11.06. *Rf*: 0.57 ($CHCl_3$: MeOH=9 : 1).

Ac-D-2-Nal-Arg-2-Nal-NH$_2$ Hydrochloride (3e) A suspension of Ac-D-2-Nal-Arg(Z)$_2$-2-Nal-NH$_2$ **7a-1** (120 mg, 0.137 mmol) and Pd(OH)$_2$/C (20 wt% Pd on carbon, wet) (100 mg) in a mixture of MeOH (20 ml) and THF (10 ml) was stirred under hydrogen atmosphere for 15 h. The mixture was filtered through Celite and the filtrate was concentrated *in vacuo*. To the residue was added 4 M HCl in 1,4-dioxane (0.10 ml), and the mixture was concentrated *in vacuo*. To the residual solid was added IPE, and the resulting suspension was stirred at room temperature for 1 h, and the precipitate was collected by filtration to obtain **3e** (85 mg, 95%) as a solid. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 0.96—1.08 (2H, m), 1.17—1.59 (2H, m), 1.82 (3H, s), 2.70—2.79 (2H, m), 2.95—3.31 (4H, m), 3.88—4.02 (1H, m), 4.38—4.47 (1H, m), 4.53—4.72 (1H, m), 7.00—7.19 (2H, m), 7.37—7.51 (9H, m), 7.66—7.99 (10H, m), 8.41—8.49 (2H, m). MS (ESI, Pos) *m/z*: 610 $(M+H)^+$. HR-MS *m/z*: 610.3148 (Calcd for 610.3142). *Rf*: 0.73 (*n*-BuOH : AcOH : H_2O=4 : 1 : 1).

Synthesis of 3a. Boc-D-Phe-Arg(Z)$_2$-2-Nal-NH$_2$ (6a-3) Compound **6a-3** was obtained as a solid from Boc-Arg(Z)$_2$-2-Nal-NH$_2$ **5a** and Boc-D-Phe-OH **14** by similar synthetic method of **6a-1**. ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 1.02—1.38 (13H, m), 2.67—3.24 (5H, m), 3.51—3.63 (1H, m), 4.02—4.22 (2H, m), 4.70—5.35 (7H, m), 6.70 (1H, br s), 6.92—7.71 (24H, m), 9.21 (1H, br s), 9.43 (1H, br s). MS (ESI, Pos) *m/z*: 886 $(M+H)^+$. *Anal*. Calcd for $C_{49}H_{55}N_7O_9$: C, 66.42; H, 6.26; N, 11.07. Found: C, 66.22; H, 6.26; N, 11.05. *Rf*: 0.41 ($CHCl_3$: MeOH = 9 : 1).

Ac-D-Phe-Arg(Z)$_2$-2-Nal-NH$_2$ (7a-3) Compound **7a-3** was obtained as a solid from Boc-D-Phe-Arg(Z)$_2$-2-Nal-NH$_2$ **6a-3** by similar synthetic method of **7a-1**. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.51—0.70 (1H, m), 1.04—1.22 (2H, m), 1.42—1.60 (1H, m), 1.98 (3H, s), 2.88—2.99 (2H, m), 3.08—3.39 (3H, m), 3.58—3.66 (1H, m), 3.69—3.90 (1H, m), 4.21—4.31 (1H, m), 4.63—4.78 (1H, m), 5.04—5.18 (3H, m), 5.38 (1H, d, *J* = 12.0 Hz), 6.98—7.50 (17H, m), 7.58—7.94 (10H, m), 9.31 (1H, br s), 9.52 (1H, br s). MS (ESI, Pos) *m/z*: 828 $(M+H)^+$. *Anal*. Calcd for $C_{46}H_{49}N_7O_8 \cdot 0.5H_2O$: C, 66.01; H, 6.02; N, 11.71. Found: C, 65.99; H, 5.96; N, 11.67. *Rf*: 0.46 ($CHCl_3$: MeOH = 9 : 1).

Ac-D-Phe-Arg-2-Nal-NH$_2$ Hydrochloride (3a) Compound **3a** was obtained as a solid from Ac-D-Phe-Arg(Z)$_2$-2-Nal-NH$_2$ **7a-3** by similar synthetic method of **3e**. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 1.02—1.19 (2H, m), 1.22—1.58 (2H, m), 1.83 (3H, s), 2.79—2.94 (4H, m), 2.99—3.11 (1H, m), 3.19—3.32 (1H, m), 3.92—4.01 (1H, m), 4.39—4.52 (1H, m), 7.02—7.29 (11H, m), 7.38—7.51 (4H, m), 7.69—7.99 (5H, m), 8.43 (2H, dd, *J* = 6.6, 8.1 Hz). MS (ESI, Pos). *m/z*: 560 $(M+H)^+$. HR-MS *m/z*: 560.2991 (Calcd for 560.2985). *Rf*: 0.45 (*n*-BuOH : AcOH : H_2O = 4 : 1 : 1).

Synthesis of 3c. Boc-D-1-Nal-Arg(Z)$_2$-2-Nal-NH$_2$ (6a-2) Compound **6a-2** was obtained as a solid from Boc-Arg(Z)$_2$-2-Nal-NH$_2$ **5a** and Boc-D-1-Nal-OH **13** by similar synthetic method of **6a-1**. ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 0.90—1.29 (4H, m), 1.47 (9H, s), 2.40—2.90 (2H, m), 3.27—3.38 (2H, m), 3.49—3.62 (1H, m), 3.99—4.12 (1H, m), 4.40—4.52 (1H, m), 4.75—4.92 (2H, m), 4.97—5.30 (6H, m), 6.68—7.73 (1H, m), 6.98—7.82 (26H, m), 9.01—9.13 (1H, m), 9.29—9.38 (1H, m). MS (ESI, Pos) *m/z*: 936 $(M+H)^+$. *Anal*. Calcd for $C_{53}H_{57}N_7O_9$: C, 68.01; H, 6.14; N, 10.47. Found: C, 67.79; H, 6.12; N, 10.40. *Rf*: 0.73 ($CHCl_3$: MeOH = 9 : 1).

Ac-D-1-Nal-Arg(Z)$_2$-2-Nal-NH$_2$ (7a-2) Compound **7a-2** was obtained as a solid from Boc-D-1-Nal-Arg(Z)$_2$-2-Nal-NH$_2$ **6a-2** by similar synthetic method of **7a-1**. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.03—0.21 (1H, m), 0.69—0.89 (1H, m), 0.91—1.05 (1H, m), 1.21—1.38 (1H, m), 2.05 (3H, s), 2.71—2.98 (2H, m), 3.06—3.21 (1H, m), 3.46 (2H, d, *J* = 8.1 Hz), 3.58—3.78 (2H, m), 4.50—4.62 (1H, m), 4.65—4.79 (1H, m), 5.09—5.30 (4H, m), 7.12—7.42 (17H, m), 7.45—8.74 (10H, m), 7.78—7.88 (1H, m), 7.94—8.00 (1H, m), 9.24 (1H, br s), 9.52 (1H, br s). MS (ESI, Pos) *m/z*: 878 $(M+H)^+$. *Anal*. Calcd for $C_{50}H_{51}N_7O_8$: C, 68.40; H, 5.85; N, 11.17. Found: C, 68.07; H, 5.87; N, 11.06. *Rf*: 0.54 ($CHCl_3$: MeOH = 9 : 1).

Ac-D-1-Nal-Arg-2-Nal-NH$_2$ Hydrochloride (3c) Compound **3c** was obtained as a solid from Ac-D-1-Nal-Arg(Z)$_2$-2-Nal-NH$_2$ **7a-2** by similar synthetic method of **3e**. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 0.91—1.04 (2H, m), 1.12—1.51 (2H, m), 1.82 (3H, s), 2.76—2.84 (2H, m), 2.99—3.11 (1H, m), 3.21—3.48 (3H, m), 3.91—4.00 (1H, m), 4.37—4.48 (1H, m), 4.51—4.61 (1H, m), 7.15—7.21 (2H, m), 7.35—7.59 (10H, m), 7.68—7.94 (9H, m), 8.12 (1H, d, *J* = 6.5 Hz), 8.32 (1H, d, *J* = 6.5 Hz), 8.51 (1H, d, *J* = 6.5 Hz). MS (ESI, Pos) *m/z*: 610 $(M+H)^+$. HR-MS *m/z*: 610.3152 (Calcd for 610.3142). *Rf*: 0.55 (*n*-BuOH : AcOH : H_2O = 4 : 1 : 1).

Synthesis of 3f. H-D-2-Nal-NH$_2$ Hydrochloride (11b) Compound **11b** was obtained as a solid from Boc-D-2-Nal-OH by similar synthetic method of **11a**. ^{1}H-NMR was accordance to that of **11a**. MS (ESI, Pos) *m/z*: 215 $(M+H)^+$. *Anal*. Calcd for $C_{13}H_{14}N_2O \cdot HCl$: C, 62.28; H, 6.03; N, 11.17. Found: C, 62.21; H, 6.01; N, 11.11.

Boc-Arg(Z)$_2$-D-2-Nal-NH$_2$ (5b) Compound **5b** was obtained as a solid from Boc-Arg(Z)$_2$-OH **4**, H-D-2-Nal-NH$_2$ hydrochloride **11b** by similar synthetic method of **5a**. ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 1.01—1.32 (4H, m), 1.38 (9H, s), 2.76—2.92 (1H, m), 3.24—3.67 (2H, m), 3.92—4.08 (1H, m), 4.89—5.38 (6H, m), 6.87 (1H, br s), 7.03—7.51 (16H, m), 7.59—7.72 (4H, m), 9.10—9.40 (2H, m). MS (ESI, Pos) *m/z*: 739 $(MH)^+$. *Anal*. Calcd for $C_{40}H_{46}N_6O_8$: C, 65.03; H, 6.28; N, 11.37. Found: C, 64.93; H, 6.28; N, 11.34. *Rf*: 0.65 ($CHCl_3$: MeOH = 9 : 1).

Boc-D-2-Nal-Arg(Z)$_2$-D-2-Nal-NH$_2$ (6b-1) Compound **6b-1** was obtained as a solid from Boc-Arg(Z)$_2$-D-2-Nal-NH$_2$ **5b** and Boc-D-2-Nal-OH **12** by similar synthetic method of **6a-1**. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.29—0.43 (1H, m), 0.33—0.78 (1H, m), 1.14—1.25 (2H, m), 1.39 (9H, s), 2.71—3.11 (4H, m), 3.43—3.55 (1H, m), 4.09—4.35 (2H, m), 4.54—4.63 (1H, m), 5.00—5.32 (4H, m), 6.61 (1H, br s), 6.77 (1H, br s), 7.16—7.50 (22H, m), 7.59—7.72 (5H, m), 9.19 (1H, br s), 9.39 (1H, br s). MS (ESI, Pos) *m/z*: 936 $(M+H)^+$. *Anal*. Calcd for $C_{53}H_{57}N_7O_9$: C, 68.00; H, 6.14; N, 10.47. Found: C, 67.61; H, 6.16; N, 10.41. *Rf*: 0.70 ($CHCl_3$: MeOH = 9 : 1).

Ac-D-2-Nal-Arg(Z)$_2$-D-2-Nal-NH$_2$ (7b-1) Compound **7b-1** was obtained as a solid from Boc-D-2-Nal-Arg(Z)$_2$-D-2-Nal-NH$_2$ **6b-3** by similar synthetic method of **7a-1**. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.33—0.48 (1H, m), 0.65—0.84 (1H, m), 1.13—1.32 (2H, m), 1.90 (3H, s), 2.64—2.88 (2H, m), 3.00—3.11 (3H, m), 3.46 (1H, dd, *J* = 4.8, 9.0 Hz), 4.12—4.22 (1H, m), 4.58—4.69 (2H, m), 5.05 (2H, dd, *J* = 11.2, 12.1 Hz), 5.19 (2H, dd, *J* = 6.5, 12.1 Hz), 5.39 (1H, br s), 6.24 (1H, d, *J* = 5.9 Hz), 6.67 (1H, br s), 6.93 (1H, d, *J* = 7.5 Hz), 7.05—7.52 (20H, m), 7.57—7.74 (9H, m), 9.21 (1H, br s), 9.42 (1H, br s). MS (FAB, Pos) *m/z*: 900 $(M+H)^+$. *Rf*: 0.61 ($CHCl_3$: MeOH = 9 : 1).

Ac-D-2-Nal-Arg-D-2-Nal-NH$_2$ Hydrochloride (3f) Compound **3f** was obtained as a solid from Ac-D-2-Nal-Arg(Z)$_2$-D-2-Nal-NH$_2$ **7b-1** by similar synthetic method of **3e**. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 0.83—0.98 (2H, m), 1.07—1.49 (2H, m), 1.77 (3H, s), 2.59—2.69 (2H, m), 2.92—3.38 (4H, m), 4.09—4.22 (1H, m), 4.45—4.63 (1H, m), 6.80—7.49 (14H, m), 7.70 (1H, d, *J* = 8.9 Hz), 7.79—7.89 (10H, m), 8.23 (1H, d, *J* = 8.1 Hz), 8.32 (1H, d, *J* = 7.5 Hz). MS (ESI, Pos) *m/z*: 610 $(M+H)^+$. HR-MS *m/z*: 610.3154 (Calcd for 610.3142). *Rf*: 0.55 (*n*-BuOH : AcOH : H_2O = 4 : 1 : 1).

Synthesis of 3b. Boc-D-Phe-Arg(Z)$_2$-D-2-Nal-NH$_2$ (6b-3) Compound **6b-3** was obtained as a solid from Boc-Arg(Z)$_2$-D-2-Nal-NH$_2$ **5b** and Boc-D-Phe-OH **14** by similar synthetic method of **6a-1**. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.68—0.78 (1H, m), 1.01—1.48 (12H, m), 2.92—3.01 (3H, m), 3.22—3.33 (1H, m), 3.41—3.60 (2H, m), 4.14—4.30 (2H, m), 4.68—4.81 (1H, m), 4.97 (1H, d, *J* = 7.0 Hz), 5.03—5.32 (3H, m), 6.71 (1H, br s), 7.02—7.51 (23H, m), 7.62—7.77 (3H, m), 9.22 (1H, br s), 9.41 (1H, br s). MS (ESI, Pos) *m/z*: 886 $(M+H)^+$. *Anal*. Calcd for $C_{49}H_{55}N_7O_9 \cdot 0.2H_2O$: C, 66.16; H, 6.28; N, 11.02. Found: C, 65.88; H, 6.29; N, 11.08. *Rf*: 0.61 ($CHCl_3$: MeOH = 9 : 1).

Ac-D-Phe-Arg(Z)$_2$-D-2-Nal-NH$_2$ (7b-3) Compound **7b-3** was obtained as a solid from Boc-D-Phe-Arg(Z)$_2$-2-Nal-NH$_2$ **6b-3** by similar synthetic method of **7a-1**. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.64—0.79 (1H, m), 1.01—1.49 (3H, m), 1.85 (3H, s), 2.93 (2H, d, *J* = 7.3 Hz), 2.98—3.08 (1H, m), 3.21—3.38 (1H, m), 3.40—3.56 (2H, m), 4.16—4.24 (1H, m), 4.38—4.48 (1H, m), 4.69—4.81 (1H, m), 5.10—5.31 (5H, m), 6.76 (1H, br s), 6.76—6.82 (1H, m), 7.04—7.41 (20H, m), 7.67—7.75 (4H, m), 9.26 (1H, br s), 9.43 (1H, br s). MS (ESI, Pos) *m/z*: 828 $(M+H)^+$. *Anal*. Calcd for $C_{46}H_{49}N_7O_8 \cdot 0.3H_2O$: C, 66.30; H, 6.00; N, 11.70. Found: C, 66.14; H, 5.98; N, 11.73. *Rf*: 0.47 ($CHCl_3$: MeOH = 9 : 1).

Ac-D-Phe-Arg-D-2-Nal-NH$_2$ AcOH (3b) Compound **3b** was obtained as a solid from Ac-D-Phe-Arg(Z)$_2$-D-2-Nal-NH$_2$ **7b-3** by similar synthetic method of **3e**. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 0.91—1.22 (3H, m), 1.32—1.50 (1H, m), 1.74 (3H, s), 1.76 (3H, s), 2.70—2.99 (4H, m), 3.15—3.48 (2H, m), 4.02—4.17 (1H, m), 4.39—4.57 (2H, m), 7.11—7.27 (7H, m), 7.30—7.43 (5H, m), 7.65—7.83 (6H, m), 8.22—8.41 (3H, m), 8.89 (1H, br s). MS (ESI, Pos) *m/z*: 560 $(M+H)^+$. HR-MS *m/z*: 560.2977 (Calcd for 560.2985). *Rf*: 0.42 (*n*-BuOH : AcOH : H_2O = 4 : 1 : 1).

Synthesis of 3d. Boc-D-1-Nal-Arg(Z)$_2$-D-2-Nal-NH$_2$ (6b-2) Compound **6b-2** was obtained as a solid from Boc-Arg(Z)$_2$-D-2-Nal-NH$_2$ **5b** and Boc-D-1-Nal-OH **13** by similar synthetic method of **6a-1**. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.25—0.42 (1H, m), 0.70—0.87 (1H, m), 0.93—1.24 (2H, m), 1.41 (9H, s), 2.58—2.72 (1H, m), 2.90—3.05 (2H, m), 3.26—3.51 (2H, m), 4.11—4.22 (1H, m), 4.42—4.58 (3H, m), 5.02—5.32 (5H, m), 6.51—6.68 (2H, m), 7.10—7.72 (25H, m), 7.83—7.98 (1H, m), 9.10 (1H, br s), 9.29 (1H, br s). MS (ESI, Pos) *m/z*: 936 $(M+H)^+$. *Anal*. Calcd for $C_{53}H_{57}N_7O_9 \cdot 0.3H_2O$: C, 67.61; H, 6.17; N, 10.41. Found: C, 67.42; H, 6.17; N, 10.33. *Rf*: 0.68 ($CHCl_3$: MeOH = 9 : 1).

Ac-D-1-Nal-Arg(Z)$_2$-D-2-Nal-NH$_2$ (7b-2) Compound **7b-2** was obtained as a solid from Boc-D-1-Nal-Arg(Z)$_2$-D-2-Nal-NH$_2$ **6b-2** by similar synthetic method of **7a-1**. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.31—0.46 (1H, m), 0.72—0.89 (1H, m), 1.02—1.19 (2H, m), 1.91 (3H, s), 2.63—2.78 (2H, m), 3.23—3.51 (3H, m), 4.08—4.18 (1H, m), 4.51—4.70 (2H, m), 5.03—5.31 (5H, m), 6.31 (1H, d, *J* = 6.2 Hz), 6.58 (1H, br s), 6.63—6.74 (1H, m), 7.10—7.77 (24H, m), 8.00 (1H, d, *J* = 7.9 Hz), 9.17 (1H, br s), 9.32 (1H, br s). MS (ESI, Pos) *m/z*: 890 $(M+Na)^+$. *Anal*. Calcd for $C_{50}H_{51}N_7O_8 \cdot 0.3H_2O$: C, 67.98; H, 5.98; N, 11.10. Found: C, 67.79; H, 5.88; N, 11.04. *Rf*: 0.60 ($CHCl_3$: MeOH = 9 : 1).

Ac-D-1-Nal-Arg-D-2-Nal-NH$_2$ Hydrochloride (3d) Compound **3d** was obtained as a solid from Ac-D-1-Nal-Arg(Z)$_2$-D-2-Nal-NH$_2$ **7b-2** by similar synthetic method of **3e**. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 0.75—0.99 (2H, m), 1.02—1.29 (2H, m), 1.80 (3H, s), 2.60—3.10 (3H, m), 3.17—3.38 (2H, m), 4.03—4.21 (1H, m), 4.38—4.62 (2H, m), 6.90—7.63 (12H, m), 7.65—7.96 (7H, m), 8.04—8.29 (4H, m), 8.42 (1H, d, *J* = 7.3 Hz). MS (ESI, Pos)

m/z: 610 $(M+H)^+$. HR-MS *m/z*: 610.3139 (Calcd for 610.3142). *Rf*: 0.46 (*n*-BuOH : AcOH : H_2O=4 : 1 : 1).

Synthesis of 3g. Boc-Arg(Z)$_2$-NH-(CH$_2$)$_2$-2-naphthyl (5c) Compound **5c** was obtained as a solid from Boc-Arg(Z)$_2$-OH **4** and 2-naphthyl-(CH$_2$)$_2$-NH$_2$ **17** by similar synthetic method of **5a**. ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 1.20—1.49 (11H, m), 1.55—1.69 (2H, m), 2.71 (2H, d, *J*=6.9 Hz), 3.03—3.62 (3H, m), 3.78—3.99 (1H, m), 4.10—4.24 (1H, m), 5.09 (2H, dd, *J*=7.5, 12.0 Hz), 5.18 (2H, s), 6.61—7.73 (1H, m), 7.14 (2H, dd, *J*=1.8, 6.6 Hz), 7.25—7.48 (13H, m), 7.60—7.78 (3H, m), 9.02—9.25 (2H, m). MS (ESI, Pos) *m/z*: 696 $(M+H)^+$. *Anal*. Calcd for $C_{39}H_{45}N_5O_7$: C, 67.32; H, 6.52; N, 10.07. Found: C, 67.22; H, 6.53; N, 10.06.

Boc-D-2-Nal-Arg(Z)$_2$-NH-(CH$_2$)$_2$-2-naphthyl (6c-1) Compound **6c-1** was obtained as a solid from Boc-Arg(Z)$_2$-NH-(CH$_2$)$_2$-2-naphthyl **5c** and Boc-D-2-Nal-OH **12** by similar synthetic method of **6a-1**. ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 0.30—0.45 (1H, m), 0.62—0.78 (1H, m), 1.02—1.21 (1H, m), 1.39 (9H, s), 1.62—1.73 (1H, m), 2.35—3.20 (6H, m), 3.25—3.46 (1H, m), 4.36—4.63 (2H, m), 4.96—5.38 (6H, m), 6.83—6.96 (1H, m), 7.05 (2H, dd, *J*=1.6, 6.8 Hz), 7.17—7.48 (18H, m), 7.52—7.73 (5H, m), 9.03 (1H, br s), 9.32 (1H, br s). MS (ESI, Pos) *m/z*: 915 $(M+Na)^+$. *Anal*. Calcd for $C_{52}H_{56}N_6O_8$: C, 69.94; H, 6.32; N, 9.41. Found: C, 69.75; H, 6.38; N, 9.33. *Rf*: 0.44 (hexane : EtOAc=1 : 1).

Ac-D-2-Nal-Arg(Z)$_2$-NH-(CH$_2$)$_2$-2-naphthyl (7c-1) Compound **7c-1** was obtained as a solid from Boc-D-2-Nal-Arg(Z)$_2$-NH-(CH$_2$)$_2$-2-naphthyl **6c-1** by similar synthetic method of **7a-1**. ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 0.23—0.37 (1H, m), 0.61—0.77 (1H, m), 1.06—1.20 (1H, m), 1.57—1.70 (1H, m), 1.91 (3H, s), 2.37—2.76 (4H, m), 2.91—3.03 (2H, m), 3.08 (1H, dd, *J*=5.0, 8.2 Hz), 3.39—3.43 (1H, m), 4.52—4.61 (1H, m), 4.63—4.72 (1H, m), 5.02 (1H, dd, *J*=9.0, 12.0 Hz), 5.14 (1H, d, *J*=12.1 Hz), 5.31 (1H, d, *J*=12.1 Hz), 6.21 (1H, d, *J*=7.2 Hz), 6.84 (1H, t, *J*=5.8 Hz), 7.05 (1H, dd, *J*=1.7, 6.7 Hz), 7.19—7.43 (18H, m), 7.52—7.69 (7H, m), 9.02 (1H, br s), 9.29 (1H, br s). MS (ESI, Pos) *m/z*: 835 $(M+H)^+$. *Anal*. Calcd for $C_{49}H_{50}N_6O_7$: C, 70.49; H, 6.04; N, 10.07. Found: C, 70.16; H, 6.06; N, 9.92. *Rf*: 0.59 ($CHCl_3$: MeOH=9 : 1).

Ac-D-2-Nal-Arg-NH-(CH$_2$)$_2$-2-Naphthyl Hydrochloride (3g) Compound **3g** was obtained as a solid from Ac-D-2-Nal-Arg(Z)$_2$-NH-(CH$_2$)$_2$-2-naphthyl **7c-1** by similar synthetic method of **3e**. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 1.04—1.20 (2H, m), 1.24—1.43 (1H, m), 1.50—1.68 (1H, m), 1.78 (3H, s), 2.79—3.01 (6H, m), 3.10 (1H, dd, *J*=5.9, 8.2 Hz), 3.30—3.42 (2H, m), 4.08—4.18 (1H, m), 4.56—4.66 (1H, m), 6.80—7.30 (5H, m), 7.37 (1H, dd, *J*=1.6, 6.8 Hz), 7.40—7.52 (6H, m), 7.72 (1H, d, *J*=12.7 Hz), 7.79—7.90 (5H, m), 8.07 (1H, t, *J*=6.8 Hz), 8.30—8.38 (2H, m). MS (ESI, Pos) *m/z*: 567 $(M+H)^+$. HR-MS *m/z*: 567.3067 (Calcd for 567.3084). *Rf*: 0.63 (*n*-BuOH : AcOH : H_2O=4 : 1 : 1).

Synthesis of 3h. Boc-D-Phe-Arg(Z)$_2$-NH-(CH$_2$)$_2$-2-naphthyl (6c-3) Compound **6c-3** was obtained as a solid from Boc-Arg(Z)$_2$-NH-(CH$_2$)$_2$-2-naphthyl **5c** and Boc-D-Phe-OH **14** by similar synthetic method of **6a-1**. ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 1.01—1.26 (2H, m), 1.38 (9H, s), 1.51—1.75 (2H, m), 2.72 (2H, q, *J*=7.4 Hz), 2.92 (2H, d, *J*=7.3 Hz), 3.09—3.60 (4H, m), 4.22—4.41 (1H, m), 4.43—5.58 (1H, m), 4.99—5.21 (5H, m), 6.85—7.49 (21H, m), 7.58—7.76 (3H, m), 9.16 (1H, br s), 9.35 (1H, br s). MS (ESI, Pos) *m/z*: 843 $(M+H)^+$. *Anal*. Calcd for $C_{48}H_{54}N_6O_8$: C, 68.39; H, 6.46; N, 9.97. Found: C, 68.11; H, 6.49; N, 10.02. *Rf*: 0.39 (hexane : EtOAc=1 : 1).

Ac-D-Phe-Arg(Z)$_2$-NH-(CH$_2$)$_2$-2-naphthyl (7c-3) Compound **7c-3** was obtained as a solid from Boc-D-Phe-Arg(Z)$_2$-NH-(CH$_2$)$_2$-2-naphthyl **6c-3** by similar synthetic method of **7a-1**. ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 1.08—1.49 (2H, m), 1.52—1.63 (2H, m), 1.88 (3H, s), 2.72 (2H, q, *J*=6.6 Hz), 2.94 (2H, t like, *J*=7.4 Hz), 3.11—3.55 (4H, m), 4.41—4.64 (2H, m), 5.08—5.24 (4H, m), 6.00 (1H, d, *J*=7.5 Hz), 6.80—7.44 (21H, m), 7.59—7.78 (3H, m), 9.16 (1H, br s), 9.32 (1H, br s). MS (ESI, Pos) *m/z*: 785 $(M+H)^+$. *Anal*. Calcd for $C_{45}H_{48}N_6O_7$: C, 68.86; H, 6.16; N, 10.71. Found: C, 68.67; H, 6.19; N, 10.65. *Rf*: 0.55 ($CHCl_3$: MeOH=9 : 1).

Ac-D-Phe-Arg-NH-(CH$_2$)$_2$-2-naphthyl Hydrochloride (3h) Compound **3h** was obtained as a solid from Ac-D-Phe-Arg(Z)$_2$-NH-(CH$_2$)$_2$-2-naphthyl **7c-3** by similar synthetic method of **3e**. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 1.06—1.22 (2H, m), 1.24—1.42 (1H, m), 1.50—1.74 (1H, m), 1.79 (3H, s), 2.69—3.03 (6H, m), 3.30—3.45 (2H, m), 4.04—4.17 (1H, m), 4.43—4.52 (1H, m), 6.90—7.25 (9H, m), 7.38 (1H, dd, *J*=1.7, 6.7 Hz), 7.40—7.45 (2H, m), 7.60 (1H, t, *J*=5.9 Hz), 7.70 (1H, s), 7.79—7.89 (3H, m), 8.04 (1H, t, *J*=5.9 Hz), 8.20—8.29 (2H, m). MS (ESI, Pos) *m/z*: 517 $(M+H)^+$. HR-MS *m/z*: 517.2919 (Calcd for 517.2927). *Rf*: 0.55 (*n*-BuOH : AcOH : H_2O=4 : 1 : 1).

The Synthesis of 9, 10 and 20 (Method B). 2-Naphthyl-(CH$_2$)$_2$-CO-Arg(Z)$_2$-2-Nal-NH$_2$ (8-1) Compound **8-1** was obtained as a solid from Boc-Arg(Z)$_2$-2-Nal-NH$_2$ **5a** and 2-naphthyl-(CH$_2$)$_2$-CO$_2$H **15** by similar synthetic method of **6a-1**. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 1.31—1.57 (4H, m), 2.37—2.46 (2H, m), 2.82—3.02 (3H, m), 3.16 (1H, dd, *J*=5.0, 9.0 Hz), 3.67 (1H, br s), 4.14—4.23 (1H, m), 4.48—4.58 (1H, m), 5.03 (2H, s), 5.19 (2H, s), 7.12 (1H, s), 7.21—7.46 (17H, m), 7.61 (1H, s), 7.66 (1H, s), 7.69—7.83 (6H, m), 7.90 (1H, d, *J*=8.2 Hz), 8.01 (1H, d, *J*=8.2 Hz), 9.13 (2H, br s). MS (ESI, Pos) *m/z*: 821 $(M+H)^+$. *Anal*. Calcd for $C_{48}H_{48}N_6O_7 \cdot 1.6H_2O$: C, 67.85; H, 6.07; N, 9.89. Found: C, 67.54; H, 5.74; N, 9.75. *Rf*: 0.36 ($CHCl_3$: MeOH=20 : 1).

2-Naphthyl-(CH$_2$)$_2$-CO-Arg-2-Nal-NH$_2$ (9) Compound **9** was obtained as a solid from 2-naphthyl-(CH$_2$)$_2$-CO-Arg(Z)$_2$-2-Nal-NH$_2$ **8-1** by similar synthetic method of **3e**. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 1.18—1.59 (4H, m), 2.58—2.66 (2H, m), 2.79—3.04 (5H, m), 3.08—3.31 (1H, m), 4.10—4.22 (1H, m), 4.49—4.56 (1H, m), 6.80—6.90 (1H, m), 6.95—7.84 (21H, m). MS (ESI, Pos) *m/z*: 553 $(M+H)^+$. HR-MS *m/z*: 553.2919 (Calcd for 553.2927). *Rf*: 0.74 (*n*-BuOH : AcOH : H_2O=4 : 1 : 1).

Synthesis of 10. 1-Naphthyl-(CH$_2$)$_2$-CO-Arg(Z)$_2$-2-Nal-NH$_2$ (8-2) Compound **8-2** was obtained as a solid from Boc-Arg(Z)$_2$-2-Nal-NH$_2$ **5a** and 1-naphthyl-(CH$_2$)$_2$-CO$_2$H **16** by similar synthetic method of **6a-1**. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 1.30—1.56 (4H, m), 2.39—2.47 (2H, m), 2.97 (1H, dd, *J*=5.3, 9.0 Hz), 3.09—3.22 (2H, m), 3.59—3.82 (2H, m), 4.16—4.24 (1H, m), 4.47—4.57 (1H, m), 5.00 (2H, s), 5.20 (2H, s), 7.12 (1H, s), 7.20—7.52 (17H, m), 7.65 (1H, s), 7.68—7.95 (6H, m), 8.00 (1H, d, *J*=8.4 Hz), 9.15 (2H, br s). MS (ESI, Pos) *m/z*: 821 $(M+H)^+$. *Anal*. Calcd for $C_{48}H_{48}N_6O_7 \cdot 0.4H_2O$: C, 69.62; H, 5.94; N, 10.15. Found: C, 69.46; H, 5.94; N, 10.25. *Rf*: 0.25 ($CHCl_3$: MeOH=20 : 1).

1-Naphthyl-(CH$_2$)$_2$-CO-Arg-2-Nal-NH$_2$ (10) Compound **10** was obtained as a solid from 1-naphthyl-(CH$_2$)$_2$-CO-Arg(Z)$_2$-2-Nal-NH$_2$ **8-2** by similar synthetic method of **3e**. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 1.22—1.61 (4H, m), 2.78—3.30 (8H, m), 4.16—4.28 (1H, m), 4.49—4.58 (1H, m), 6.80—7.60 (14H, m), 7.69 (1H, s), 7.72—7.94 (6H, m), 8.07 (1H, d, *J*=7.8 Hz). MS (ESI, Pos) *m/z*: 553 $(M+H)^+$. HR-MS *m/z*: 553.2914 (Calcd for 553.2927). *Rf*: 0.74 (*n*-BuOH : AcOH : H_2O=4 : 1 : 1).

The Synthesis of 20. 2-Naphthyl-(CH$_2$)$_2$-CO-Arg(Z)$_2$-NH-(CH$_2$)$_2$-2-naphthyl (19) Compound **19** was obtained as a solid from Boc-Arg(Z)$_2$-NH-(CH$_2$)$_2$-2-naphthyl **5c** and 2-naphthyl-(CH$_2$)$_2$-CO$_2$H **15** by similar synthetic method of **6a-1**. ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 1.01—1.43 (2H, m), 1.51—1.72 (2H, m), 2.38—2.46 (2H, m), 2.63—2.78 (2H, m), 2.96—3.33 (6H, m), 4.43—4.58 (1H, m), 4.99—5.12 (4H, m), 6.52—6.72 (1H, m), 7.02—7.42 (19H, m), 7.58—7.77 (6H, m), 9.15 (1H, br s), 9.34 (1H, br s). MS (ESI, Pos) *m/z*: 788 $(M+H)^+$. *Anal*. Calcd for $C_{47}H_{47}N_5O_6$: C, 72.57; H, 6.09; N, 9.00. Found: C, 72.35; H, 6.13; N, 9.03.

2-Naphthyl-(CH$_2$)$_2$-CO-Arg-NH-(CH$_2$)$_2$-2-naphthyl Hydrochloride (20) Compound **20** was obtained as a solid from 2-naphthyl-(CH$_2$)$_2$-CO-Arg(Z)$_2$-NH-(CH$_2$)$_2$-2-naphthyl **19** by similar synthetic method of **3e**. ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 1.24—1.51 (3H, m), 1.54—1.63 (1H, m), 2.56 (1H, dd, *J*=2.8, 5.8 Hz), 2.85 (2H, d, *J*=7.2 Hz), 2.93—3.03 (3H, m), 3.29—3.41 (2H, m), 3.50—3.71 (2H, m), 4.19—4.27 (1H, m), 6.80—7.25 (5H, m), 7.33—7.50 (6H, m), 7.59—7.72 (3H, m), 7.77—7.90 (5H, m), 8.03 (1H, t, *J*=5.5 Hz), 8.10 (1H, d, *J*=8.2 Hz). MS (ESI, Pos) *m/z*: 510 $(M+H)^+$. HR-MS *m/z*: 510.2859 (Calcd for 510.2869). *Rf*: 0.77 (*n*-BuOH : AcOH : H_2O=4 : 1 : 1).

Binding Test. Material [^{125}I][Nle^4,D-Phe^7]α-Melanocyte stimulating hormone ([Nle^4,D-Phe^7]α-MSH) (specific radioactivity: 81.4 TBq/mmol) was purchased from Amersham International (Buckinghamshire, England). COS-1 cells were purchased from American Type Culture Collection (Rocksville, MD, U.S.A.). [Nle^4,D-Phe^7]α-MSH was purchased from Peninsula Laboratories (Belmont, CA, U.S.A.). All other chemicals used in this study were obtained commercially, and all were of the highest purity available.

[^{125}I][Nle^4,D-Phe^7]α-MSH Binding to Recombinant the MC4 Receptor COS-1 cells expressing the MC4 receptor, prepared according to the method reported previously,[26] were washed with phosphate buffered saline, scraped and pelleted by centrifugation. Cell pellets were homogenized with 50 mM Tris–HCl buffer (pH 7.4) containing 2 mM EDTA, 10 mM $CaCl_2$, and 100 μM phenylmethylsulfonylfluoride, and centrifuged at 48000×g for 20 min at 4 °C. The pellet was washed twice with the buffer, and the final pellet was suspended in an assay buffer (50 mM Tris–HCl buffer (pH 7.4) containing 2 mM EDTA, 10 mM $CaCl_2$, 100 μM phenylmethylsulfonylfluoride and 0.1% bovine serum albumin (BSA)), and served as crude membrane preparation for binding studies. Binding assays of [^{125}I][Nle^4,D-Phe^7]α-MSH were performed according to Chaki *et al.* (2003).[27] Membranes were incubated with [^{125}I][Nle^4,D-Phe^7]α-MSH (0.2 nM) for 120 min at 25 °C, and the reaction was terminated by rapid filtration over a GF/C filter presoaked with 0.5%

BSA, after which the filters were washed three times with the buffer. Radioactivity was quantified in a γ-counter. Nonspecific binding was determined in the presence of 1 mM [Nle^4,D-Phe^7]α-MSH. Specific binding was determined by subtracting nonspecific from total binding. In the competition assay, concentration of the test compound that caused 50% inhibition of the specific binding (IC_{50} value) was determined from each concentration-response curve.

Determination of cAMP COS-1 cells transiently expressing the MC4 receptor and grown in a six-well plate were used. The culture medium was removed, the cells were washed with phosphate buffered saline, and 1 ml of DMEM containing 1 mM isobutylmethylxanthine, a phosphodiesterase inhibitor, was added. The cells were incubated with 10 μM concentration of the test compound for 15 min at 37 °C. The culture medium was then aspirated and the cells were washed with phosphate buffered saline. Two milliliters of ice-cold 65% ethanol were added, and the cells were scraped from the wells. The supernatant was collected by centrifugation at 15000 rpm for 15 min at 4 °C. cAMP formed in the cells was determined using a commercially available cAMP EIA system.

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