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Design of cyclic peptides with agonist activity at melanocortin receptor-4

Takenao Odagami,[a] Yuko Tsuda,[b,c,*] Yuji Kogami,[a] Hiroyuki Kouji[a] and Yoshio Okada[b,c]

[a]*Research Center, Pharmaceutical R&D Division, Asahi Kasei Pharma Corporation, Izunokuni 410-2321, Japan*
[b]*Faculty of Pharmaceutical Sciences, Kobe Gakuin University, Kobe 651-2180, Japan*
[c]*The Graduate School of Food and Medicinal Sciences, Kobe Gakuin University, Kobe 651-2180, Japan*

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Abstract—A series of cyclic pentapeptides, *c*(His-D-Phe-Arg-Trp-Z) (Z = ω-amino acid), were prepared and biologically evaluated. The effects of increasing alkyl chain length of ω-amino acid on the functional activities and the receptor binding affinities for human melanocortin receptors (hMC-Rs) were studied. Compound **2** was an agonist for hMC-4R with an EC_{50} value of 15.4 nM, which was 4.7 times more potent than that of α-MSH. Compound **2** also showed a 4.3-fold higher hMC-4R selectivity over hMC-1R, thus providing us with information concerning size and chemical structure of the lactam ring for the development of the agonist with hMC-4R selectivity.

It has been recognized that α-melanocyte-stimulating hormone (α-MSH), an endogenous ligand for the melanocortin receptors, is involved in a wide range of physiological functions: the regulation of skin pigmentation and immune system,[1] the control of steroid production,[2] the central nervous system regulation of body weight,[3,4] and the regulation of secretion from exocrine gland.[5] Five melanocortin receptors, MC-1R, -2R, -3R, -4R, and -5R, were identified.

Because of the importance of MC-4R in feeding behavior, many researchers have focused their attention to discover highly potent and selective MC-4R agonists and antagonists. A lot of nonpeptide agonists and antagonists, such as piperidine-,[6,7] piperazine-,[8,9] or guanidine[10]-based ligands, were developed and evaluated. On the other hand, peptidic MC-4R ligands were also investigated based on the sequence of endogenous MC-R agonists, for example, ACTH and α-, β-, and γ-MSH. They contain the common tetrapeptide sequence, His-Phe-Arg-Trp, which is an essential core for biological activity. Further studies with MC-Rs have shown that Phe position is a critical determinant for activity, when it was replaced with D-Phe or D-Nal(2) [Nal(2) = 2-naphthylalanine], as seen with melanotan II [MT-II, Ac-Nle-*c*(Asp-His-D-Phe-Arg-Trp-Lys)-NH_2][11] and SHU/9119 [Ac-Nle-*c*(Asp-His-D-Nal(2)-Arg-Trp-Lys)-NH_2].[12] In fact, MT-II exhibited high affinity and agonism at MC-4R (IC_{50} = 0.07 nM for binding and EC_{50} = 0.5 nM for cAMP accumulation),[13] while SHU/9119 showed potent antagonism at MC-4R (IC_{50} = 0.04 nM for binding and pA_2 = 9.63 for cAMP accumulation).[13] However, although MT-II and SHU/9119 were potent ligands, they were not selective for MC-4R. Bednarek et al.[14] reported that the cyclic lactam *c*(CO-CH_2-CH_2-CO-His-D-Phe-Arg-Trp-Dab)-NH_2 (Dab = 2,4-diaminobutyric acid) exhibited potent agonism at hMC-4R (IC_{50} = 37 nM for binding, EC_{50} = 4 nM for cAMP accumulation), and was 55-fold more selective over hMC-3R and 1000-fold more selective over hMC-5R. In this communication, we describe the synthesis of *c*(His-D-Phe-Arg-Trp-Z) (Z = ω-amino acid) and their evaluation by intracellular cAMP accumulation and binding assays. The ligand-hMC-Rs interaction was further studied using lactams with various ring sizes.

Bednarek's cyclic lactam *c*(**X**-His-D-Phe-Arg-Trp-**Y**)-NH_2 contained the essential core sequence (His-D-Phe-Arg-Trp) required for high affinity at hMC-Rs and a linker consisting of **X** and **Y**, where **X** is a succinic acid or an ω-aminocarboxylic acid and **Y** is an α, ω-diaminocarboxylic acid or an ω-carboxy-α-amino acid.

Keywords: Cyclic peptide; Agonist; hMC-4R.
* Corresponding author. Tel.: +81 78 974 4798; fax: +81 78 974 5689; e-mail: tsuda@pharm.kobegakuin.ac.jp

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In this study, instead of a linkage through **X** and **Y**, we employed a simpler approach using one ω-amino acid to produce membered lactams (Fig. 1). Namely, the molecule was simplified by removing one amide bond between **X** and **Y**.

Synthesis of cyclic peptides was achieved by a combination of the solid-phase methods and the cyclization reaction in a solution synthesis procedure[15] shown in Scheme 1.

All crude peptides were purified by semi-preparative RP-HPLC [in an acetonitrile/water gradient (10–50% over 40 min) containing 0.05% TFA]. The purity of products was determined by HPLC.[16] Structures were characterized by high-resolution mass spectrometry[16] and amino acid analysis.

The functional activities and binding activities of compounds **1**–**7** at hMC-1R and -4R are summarized in Table 1. The functional activities of the compounds were estimated as an accumulation of cAMP in SaoS2 cells (ATCC HTB-85) expressing MC1/plDNA or MC4/pcDNA3.1. The natural ligand, α-MSH, had agonist activity (EC_{50} = 1.77 and 72.9 nM, at hMC-1R and -4R, respectively). At hMC-4R, both compound **1**, *c*(His-D-Phe-Arg-Trp-Aoc) (Aoc = 8-aminooctanoic acid), and compound **2**, *c*(His-D-Phe-Arg-Trp-Ahp) (Ahp = 7-aminoheptanoic acid), exhibited agonist activity (EC_{50} = 16.4 and 15.4 nM, respectively) meaning that 21- and 20-membered cyclic peptides were about a 5-fold more potent agonist than α-MSH at hMC-4R.

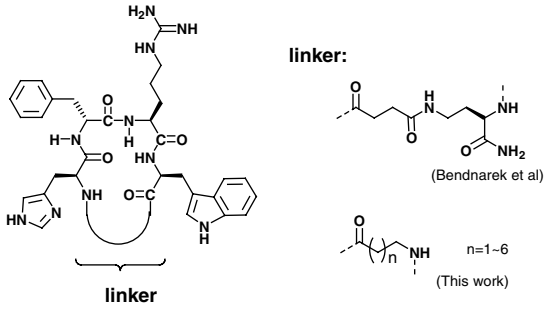
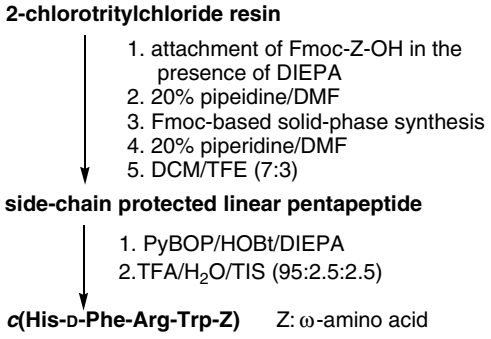


Figure 1. The chemical structures of compounds investigated in this study.



Scheme 1. Outline of synthesis of cyclic peptides.

An approach to shorten the 21-membered lactam bridge of compound **1** by substitution of Aoc with 6-aminohexanoic acid (Ahx) led to compound **3** (19-membered cyclic peptide, EC_{50} = 3120 nM), which was 190-fold less potent than α-MSH at hMC-4R. Compounds **4**–**6**, with a smaller lactam bridge, exhibited a weak agonist activity at hMC-4R in the μM range. Interestingly, at hMC-4R, compounds **1** and **2** acted as a full agonist, while compounds **3**–**6** acted as a partial agonist. On the other hand, at hMC-1R, compounds **1**–**6** exhibited weaker agonist potency than that of α-MSH, being in the range of 10^{-8}–10^{-6} M and acted as a partial agonist. Compound **7** with L-Phe was the weakest agonist at both hMC-1R and -4R. These data coincide well with the fact that the inversion of chirality of the Phe to D-Phe resulted in a dramatic increase of agonist potency at MCRs.[11,12]

The binding affinities of the compounds at MC-4R were measured with a [^{125}I]NDP-α-MSH displacement assay in human recombinant HEK-293 cells expressing the cloned MC-4R. The K_i values were 319 and 244 nM for compounds **1** (21-membered cyclic peptide) and **2** (20-membered cyclic peptide), respectively, while the K_i value of a 18-membered cyclic peptide was more than 10 μM. It means that a suitable ring size is required for the formation and the stabilization of ligand–receptor complexes. Compound **2** was 3.9 times less potent and possessed 6.6-fold lower affinity than Bednarek's cyclic lactam *c*(CO-CH_2-CH_2-CO-His-D-Phe-Arg-Trp-Dab)-NH_2 at MC-R4.This implies that not only proper ring size, but also chemical structure of the linker, is important to produce potent agonists.

Next, we compared the structure of compounds **2** (a relatively potent agonist) and **4** (a weak agonist) using CD[17] and NMR[18] spectroscopy. The CD spectra of compounds **2** and **4** were obtained in trifluoroethanol (TFE) in which all spectra were characterized by the overall negative signal with a shape typically associated with random-coiled polypeptides (Fig. 2). Distilled water was added in order to obtain their CD spectra in the presence of 10%, 20%, 40%, and 50% water in TFE. The overall shapes of the CD spectra for both compounds were not significantly different in the presence of 0–50% water in TFE, suggesting that both compounds **2** and **4** would predominantly take a random-coil conformation.

The 2D NMR spectra were assigned using the standard assignment procedure, and the NOESY spectrum was used to make sequential connections between them. The NOE patterns for compounds **2** and **4** are summarized in Figure 3. In both compounds **2** and **4**, the sequential NOEs, d_{NN} and $d_{\alpha N}$ were observed. For compound **2**, the existence of a $d_{\alpha N}(i, i + 2)$ contact between His and Arg suggests the possibility of a small population of turn-like structure among the predominantly random conformation. Compound **4** showed one $d_{\alpha N}(i, i + 2)$ contact between His and Arg, and the other $d_{\alpha N}(i, i + 2)$ contact between Phe and Trp. This indicates that a higher population of turn-like structures exists in compound **4** compared with compound **2**. A relatively

Table 1. Functional activities and binging affinities of compounds **1**–**7** at hMC-1R and -4R

Compound	Sequence	Ring size	Functional activity/EC_{50} (nM)[a]			Binding affinity at hMC-4R[b]	
			hMC-1R	hMC-4R	hMC-1R/-4R	K_i (nM)	Hill coeff.
α-MSH			1.8	72.9	0.024	—	—
NDP-α-MSH			N.T.	N.T.	—	0.102	0.792
1	*c*(His-D-Phe-Arg-Trp-Aoc)	21	77.4	16.4	4.7	319	0.628
2	*c*(His-D-Phe-Arg-Trp-Ahp)	20	67.0	15.4	4.3	244	0.747
3	*c*(His-D-Phe-Arg-Trp-Ahx)	19	1303.7	3121.2	0.42	670	0.673
4	*c*(His-D-Phe-Arg-Trp-Ava)	18	3301.8	10133.6	0.33	N.D.	N.D.
5	*c*(His-D-Phe-Arg-Trp-Abu)	17	269.9	1578.0	0.17	5780	0.613
6	*c*(His-D-Phe-Arg-Trp-βAla)	16	135.0	1113.1	1.19	N.D.	N.D.
7	*c*(His-L-Phe-Arg-Trp-Aoc)	21	5095.8	9592.4	0.53	4420	0.604

[a] Values are means of three experiments (N.T., not tested).
[b] Values are means of two experiments (N.D., not determined); NDP-α-MSH, [Nle^4, D-Phe^7]-α-MSH.

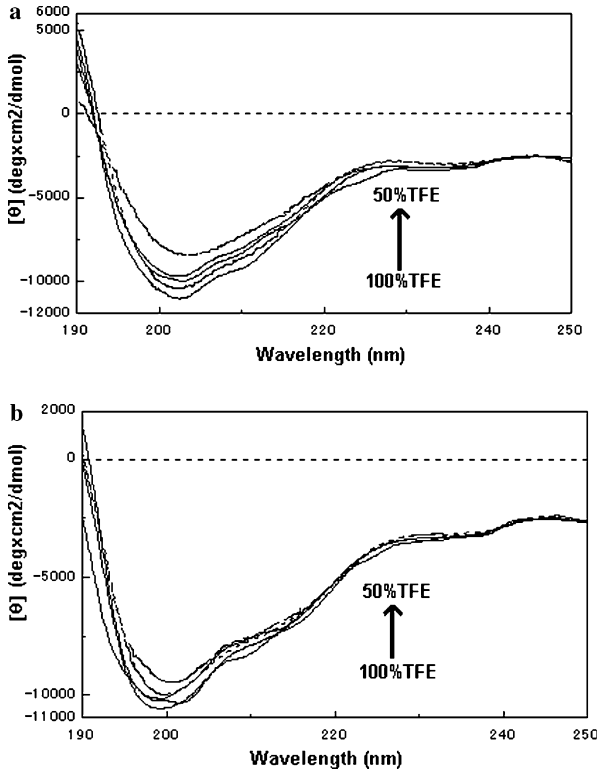


Figure 2. CD spectra for compound **2** at 0.5 mM (a) and compound **4** at 0.5 mM (b) in the presence of 0%, 10%, 20%, 40% and 50% water in TFE, respectively.

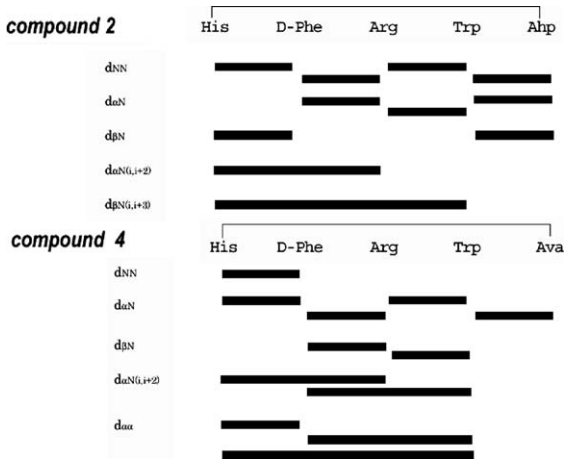


Figure 3. Summary of the NOE patterns for compounds **2** and **4**.

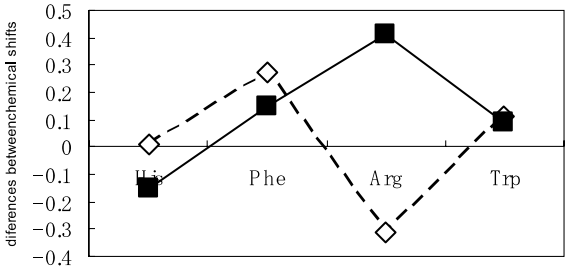


Figure 4. Chemical shift differences in each amino acid residues for NH (opened square) and CαH (closed square): $\Delta\delta NH = \delta NH^{compound\ 2} - \delta NH^{compound\ 4}$, $\Delta\delta C\alpha H = \delta C\alpha H^{compound\ 2} - \delta C\alpha H^{compound\ 4}$.

large difference between chemical shifts of NH and CαH, $\Delta\delta NH$ ($\delta NH^{compound\ 2} - \delta NH^{compound\ 4}$) and $\Delta\delta C\alpha H$ ($\delta C\alpha H^{compound\ 2} - \delta C\alpha H^{compound\ 4}$), was observed on the Arg residue (Fig. 4), suggesting the possibility that the change of lactam ring properties could affect the orientation of the side chain of Arg to induce diverse activity. The MC-Rs belong to the superfamily of seven transmembrane spanning G-protein coupled receptors in which the side chain of Arg has been thought to interact with the Asp residue in the putative transmembrane spanning domain (TM-3) and a Glu residue in TM-2.[19,20] The head-to-tail cyclization using ω-amino acid could generate an agonist due to an alteration in the orientation of the Arg side chain.

In conclusion, the 21-membered cyclic peptide **2**, *c*(His-D-Phe-Arg-Trp-Ahp), was the 4.7 times more potent agonist at hMC-4R than α-MSH but the 3.8 times less potent agonist than *c*(CO-CH_2-CH_2-CO-His-D-Phe-Arg-Trp-Dab)-NH_2. Furthermore, compound **2** with a straight chain linker of one ω-amino acid exhibited low hMC-4R selectivity over hMC-1R compared with *c*(CO-CH_2-CH_2-CO-His-D-Phe-Arg-Trp-Dab)-NH_2. This finding would provide us with useful information concerning ring size and chemical structure of the linker for the further development of potent agonists with hMC-4R selectivity.

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15. The protected linear peptides were prepared on 2-chlorotritylchloride resin using standard solid-phase procedures based on Fmoc-chemistry. The side-chain protecting groups of amino acids were Boc (Trp); Pbf (Arg); and Trt (His). The NH_2-peptidyl-resin was treated with DCM/trifluoroethanol (TFE) mixture (7:3, v/v) twice to produce the side-chain protected linear peptide. The head-to-tail cyclization of the peptide was performed using PyBOP/HOBt (3 equiv, 1:1) and diisopropylethylamine (DIEPA, 6 equiv) in DMF for 2 h to yield the protected cyclic peptide. The protected cyclic peptides were then treated by TFA/H_2O/triisopropylsilane (TIS) (95:2.5:2.5 by volume).
16. The MS spectra of compounds were obtained by ESI (ABI, QSTAR pulsar-i). The analytical reverse-phase C-18 HPLC was performed using Nakarai COSMOSIL AR-II column (4.6×250 mm). Compound **1**: HRMS (*m*/*z*) $[M+1]^+$ = 768.4315 (calcd 768.4309), t_R = 27.1 min, compound **2**: HRMS (*m*/*z*) $[M+1]^+$ = 754.4166 (calcd 754.4152), t_R = 24.0 min, compound **3**: HRMS (*m*/*z*) $[M+1]^+$ = 740.3978 (calcd 740.3996), t_R = 22.8 min, compound **4**: HRMS (*m*/*z*) $[M+1]^+$ = 726.3863(calcd 726.3839), t_R = 20.0 min, compound **5**: HRMS (*m*/*z*) $[M+1]^+$ = 712.3671 (calcd 712.3683), t_R = 22.3 min, compound **6**: HRMS (*m*/*z*) $[M+1]^+$ = 698.3503 (calcd 698.3526), t_R = 19.2 min, compound **7**: HRMS (*m*/*z*) $[M+1]^+$ = 768.4327 (calcd 768.4327), t_R = 28.4 min.
17. CD spectra were recorded at room temperature in a JASCO J-725 spectropolarimeter using quartz cuvettes with a 0.02 cm path length.
18. One- and two-dimensional NMR experiments were performed at 400 MHz in a JEOL LA-400 spectrometer at 25 °C. The NMR samples were prepared by dissolving 1 mg of the compound in DMSO-d_6 (0.5 ml). COSY and NOESY were collected by the methods of States et al. (*J. Magn. Reson*. **1982**, *48*, 286). Resonance assignments were determined by COSY spectrum using PFG technique.
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