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Structure–activity studies of new melanocortin peptides containing an aromatic amino acid at the N-terminal position

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Abstract

Cyclic melanotropin peptides, designed with an aromatic amino acid substitution at the N-terminal position of the MT-II-type scaffold, were prepared by solid-phase peptide synthesis and evaluated for their ability to bind to and activate human melanocortin-1, -3, -4, and -5 receptors. The structure–activity studies of these MT-II analogues have identified a selective antagonist at the hMC4R (H-Phe-c[Asp-Pro-D-Nal(2')-Arg-Trp-Gly-Lys]-NH₂, pA₂ = 8.7), a selective partial agonist at the hMC4R (H-D-Nal(2')-c[Asp-Pro-D-Phe-Arg-Trp-Gly-Lys]-NH₂, IC₅₀ = 11 nM, EC₅₀ = 56 nM), and a selective partial agonist at the hMC3R (H-D-Phe-c[Asp-Pro-D-Phe-Arg-Trp-Lys]-NH₂, IC₅₀ = 3.7 nM, EC₅₀ = 4.9 nM). Aromatic amino acid substitution at the N-terminus in conjunction with the expansion of the 23-membered cyclic lactam MT-II scaffold to a 26-membered scaffold by addition of a Gly residue in position 10 leads to melanotropin peptides with enhanced receptor selectivity.

Keywords

Melanotropins; N-Terminal aromatic amino acid substitution; Cyclic peptide lactams; Structure–activity; Receptor selectivity; Melanotropin receptors

1. Introduction

The melanotropin peptides are a group of neural and hormonal peptides which includes α -, β -, and γ -melanocyte stimulating hormone (MSH) and adrenocorticotrophic hormone (ACTH). They are referred to collectively as the melanotropin (or the synonymical melanocortin) peptides because they are derived from the same gene and pro-hormone (pro-opiomelanocortin) [23,42] and share an identical core amino acid sequence, His-Phe-Arg-Trp, recognized to be important for receptor binding and activation [10,27].

There are five known melanocortin receptor (MCR) subtypes, all of which belong to the family of seven transmembrane G-protein coupled receptors (GPCRs), and whose activation is linked to the generation of intracellular cAMP [3,7,8,12,14–16,22,31,34,38]. Importantly, the interaction of the melanocortin peptides with their receptors can be modified by two endogenous protein antagonists, agouti, and agouti-related protein, which are the products of two distinct genes [33,35]. Tissue-specific processing of the pro-opiomelanocortin precursor coupled to tissue-specific expression of MCR subtypes and antagonists has the potential to provide a wide spectrum of physiological responses from these neuropeptides. Indeed, as referenced below, over the past several decades, the melanocortin peptides have been implicated in an increasingly diverse number of physiological processes.

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The melanotropin peptides are traditionally known for their roles in pigmentation via the melanocyte α -MSH receptor or MC1R, and adrenocortical steroidogenesis via the classical adrenocortical ACTH receptor or MC2R [23,42]. More recently, the application of pharmacological studies and molecular biology techniques has identified α -MSH as an important hypothalamic satiety signal [11,28]. The MC3R is expressed in both central and peripheral tissues and although the physiological role for this MCR has not yet been identified, it is the MCR with the greatest affinity for γ -MSH and it appears to mediate some aspects of the central control of blood pressure [32]. The MC4R is expressed in virtually all brain regions and the distribution of this MCR in the CNS is both different and wider than that of the MC3R. Furthermore, the MC4R has been implicated in feeding behaviors and weight homeostasis [9,11,43]. Targeted deletion of the MC5R gene has implicated that this MCR subtype is involved in the coordination of exocrine gland function [6]. In addition, although not assigned to a single MCR, melanocortins have also been implicated in adipocyte lipolysis, thermal control, immunomodulation, sexual function, and cognition, among other functions [9,43].

In order to gain a thorough understanding of their physiological relevance in normal and disease states, as well as for the development of specific drugs for treatment of diseases with which they are associated, there is an urgent need for the development of MCR selective ligands that can be used for in vitro and in vivo studies. In this paper, we report on the design and synthesis of new melanotropin peptides characterized to have 23- and 26-membered lactam rings and containing an aromatic amino acid, such as Phe or Nal(2') in position 4 (as referred to the sequence of α -MSH, Ac-Ser¹-Tyr²-Ser³-Met⁴-Glu⁵-His⁶-Phe⁷-Arg⁸-Trp⁹-Gly¹⁰-Lys¹¹-Pro¹²-Val¹³-NH₂), in their respective isomers L and D. Here, we report the synthesis and biological activity at human MC3, MC4, and MC5 receptors of several analogues modified in position 4 (Table 1) with aromatic residues, which resulted in potent and selective ligands for these human melanocortin receptors.

2. Results and discussion

The melanotropin analogues listed in Table 1, were prepared by solid-phase peptide synthesis as reported in Section 3. These compounds were prepared to explore modification of position 4 in conjunction with various substitutions at the positions 6, 9, and 10, in order to improve the selectivity of the cyclic α -MSH analogues MT-II [1,2] and SHU-9119 [26] which were previously discovered in our laboratories, and are highly potent but non-selective agonist and antagonist analogues, respectively. They were further evaluated for their binding affinities to the human melanocortin receptors 3–5 in competitive binding assays using the radiolabeled ligand [¹²⁵I]-NDP- α -MSH and for their agonist or antagonist (when significant) potency in cAMP assays employing the HEK293 cells expressing these receptors.

In the past few years, several analogues of MT-II and SHU-9119, which are high affinity agonists and antagonists at the hMC3 and the hMC4 receptors were reported in literature, in which most of the modifications were made in positions 6–9 [19–21,24,25,40]. These modifications have suggested that amino acid residues at the 6 (His), 7 (Phe), 8 (Arg), and 9 (Trp) positions are important for molecular recognition and activation of the melanocortin receptors. In particular, positions 6 and 7 of cyclic and of linear derivatives of α -MSH appeared to be important for high affinity and selectivity at hMC3 and hMC4 receptors [1,17,26,36, 39,41].

Regarding position 4 and the role of the residue, Nle external to the lactam ring, we still have very little information concerning its influence on potency and selectivity at the hMC3 and hMC4 receptors. Therefore, in this study, we were interested in exploring the significance of the position 4 in 23- and 26-membered cyclic lactam analogues of MT-II and SHU-9119. In particular, we explored position 4 of the peptides with a 23-membered ring by replacing the

Nle residue with D-Phe , while position 4 of peptides with a 26-membered ring (Gly at position 10) were explored by Phe, D-Phe , Nal(2'), and $\text{D-Nal}(2')$. Thus, analogue **1** (PG-137) which possesses a Phe residue at position 4, was found in the binding assay to be 11-, 49-, and 9-fold less potent at the hMC3, hMC4, and hMC5 receptors, respectively, than MT-II. In the adenylate cyclase assay, PG-137 was 46-, 167-, and 12-fold less potent at the hMC3R, hMC4, and hMC5 receptor than MT-II (Table 1). The influence of the Phe⁴ substitution on the three-dimensional (3D) structure of this 26-membered cyclic lactam scaffold was then investigated by comparing the global minima (obtained by simulations using Kolossváry's large scale low mode conformational search method (LLMOD) [30] and the OPLS-AA force field) of PG-137 (Table 2) and our earlier published compound PG-933, which has Nle residue in the position 4 (Ac-Nle-c[Asp-Pro-D-Phe-Arg-Trp-Gly-Lys]-NH₂) [17]. As it is evident from Fig. 1, the backbone conformations of these peptides between the residues **8** and **11** are quite similar, whereas a considerable deviation in the backbone conformation was observed at the N-terminus, which stems from the Nle⁴ (PG-933) to Phe⁴ (PG-137) substitution. Such N-terminal distortion results in a change in the conformational space of the D-Phe^7 side chain, as exemplified by the corresponding χ^1 angles (Table 3). The D-Phe^7 side chain of PG-137 apparently prefers the *trans*-conformation ($\chi^1 = +172^\circ$), whereas in PG-933 the *gauche*(+) conformation ($\chi^1 = +64^\circ$) is more favored, similar to MT-II ($\chi^1 = +66^\circ$). This observation may explain the decline in potency by one to two orders of magnitude for PG-137 when compared to MT-II.

In contrast compound **2** (PG-138), in which the 7 position was modified with a $\text{D-Nal}(2')$ and the 4 position with Phe, was found to be a potent and selective antagonist at the hMC4 receptor (Table 1). In fact, in our assays, the compound PG-138 resulted to have a IC₅₀ of 15.1 nM and a pA₂ value of 8.7. Furthermore, PG-138 was found to be a partial agonist/antagonist at the hMC3 receptor. These results highlight that position 4 can be an additional structure site to regulate the potency and selectivity of cyclic melanotropin peptides at melanocortin 3–5 receptors. In addition, an examination of the global minimum conformation of PG-138 uncovered a shift of the β -turn structure from the residues 6–7, as reported for MT-II [44], to residues 9–10 due to the introduction of the Gly at the position 10 (Fig. 2). At the same time, the overall peptide pharmacophore topography remains similar to that of MT-II, which is consistent with the nanomolar range binding affinity observed for this peptide. The shift of the β -turn position, however, may be attributed to the hMC4R selective antagonist properties of PG-138. Subsequently, we examined the effect of chirality of the Phe⁴ residue on selectivity and potency by replacing Phe⁴ with its isomer D-Phe^4 in peptides with a 26-membered ring. Binding and functional assay data of compounds **3** (PG-975) and **4** (PG-976) showed a consistent reduction in affinity and potency at hMC3, hMC4, and hMC5 receptors as compared to MT-II. In addition, compound **4** resulted in an antagonist at the hMC4 (IC₅₀ = 53 nM, EC₅₀ > 1000), an antagonist at the hMC3 (IC₅₀ = 21.9 nM, EC₅₀ > 1000), with slight partial agonist activity (7% at 10 μM), and an antagonist at the hMC5 receptor (IC₅₀ = 540 nM, EC₅₀ > 5000) but without appreciable selectivity. In view of these results, we decided to further investigate position 4 by placing Nal(2') and $\text{D-Nal}(2')$ residues in it. Compound **5** (PG-141), with a Nal(2') residue in position 4, resulted in a weak agonist at the hMC3 and hMC4 receptors (EC₅₀ = 300 and 2300 nM, respectively), with weak binding affinity at the hMC5R, though 100% efficacious. On the other hand, the compound **6** (PG-143), with a Nal(2') residue in position 4 and a $\text{D-Nal}(2')$ in position 7, showed a better binding profile at the hMC3, hMC4, and hMC5 receptors, but a consistent decrease in potency at all receptors (EC₅₀ = 1600, 3400, and 1600 nM, respectively) compared to compound **5**. Interestingly, the compound **7** (PG-977), with a $\text{D-Nal}(2')$ residue in position 4 and D-Phe in position 7, was found to be a highly selective partial agonist at the hMC4R (EC₅₀ = 56 nM), an antagonist at the hMC3R with weak partial agonist activity (EC₅₀ > 1 μM) and a weak antagonist at the hMC5R (IC₅₀ = 970 nM). In contrast, compound **8** (PG-978), with a $\text{D-Nal}(2')$ residue at both positions 4 and 7, was found to be a partial agonist at hMC3 receptor, and a potent antagonist at hMC4 and hMC5 receptors, but without marked selectivity. These results obtained on peptides with a 26-membered lactam

ring suggest that position 4 can play an important role in the activity of melanotropin peptides. In particular, the data indicate that positioning of the bulky hydrophobic residues is crucial for potency at melanocortin receptors, and also selectivity in some cases.

We then decided to explore the same modification in peptides with a 23-membered lactam rings. In these cases, we decided to replace Nle⁴ with D-Phe residue only. Thus, compound **9** (PG-951), with a D-Phe⁴ and D-Phe⁷ substitution, resulted in a potent and selective partial agonist analogue at the hMC3R (EC₅₀ = 4.9 nM), although it lacks agonist activity at the hMC4 and hMC5 receptors. We also have examined the global minimum of PG-951 and compared it to the NMR structure of MT-II (Fig. 3). Interestingly, we found a generally good fit within the pharmacophore region of these peptides, but a striking difference in the orientation of the Trp⁹ residue side chain was also observed. In our earlier work, we made a note of a possible connection between the Chi-space of the Trp residue and melanocortin receptor selectivity [5]. In this light, the difference in the side chain conformations of the Trp residue of MT-II (*gauche*-(−), $\chi^1 = -78^\circ$) and PG-951 (*trans*, $\chi^1 = -177^\circ$) may be responsible for the hMC3R selectivity observed for PG-951. Compound **10** (PG-952), with D-Phe⁴ and D-Nal(2')⁷ residues, was found to be a weak partial agonist at the hMC3R (EC₅₀ = 55 nM), and an antagonist at hMC4 and hMC5 receptors with a low selectivity (hMC4/hMC5 = 14). Finally, since previously, we demonstrated that inserting a D-Nal(2') residue in 9 position makes it possible to modulate the activity on hMC5R [25], we synthesized two analogues in which we replaced the position 4 with a D-Phe and in position 9, the Trp with a D-Nal(2'). Thus, the compound **11** (PG-953), H-D-Phe-c[Asp-Pro-D-Phe-Arg-D-Nal(2')-Lys]-NH₂, was found to be an agonist which is 130-fold less potent at the hMC3R and 6-fold less potent at hMC5R, but it resulted in a weak antagonist at the hMC4R (IC₅₀ = 140 nM, EC₅₀ > 1 μM). On the other hand, compound **12** (PG-954), H-D-Phe-c[Asp-Pro-D-Nal(2')-Arg-D-Nal(2')-Lys]-NH₂, was found to be a partial agonist at the hMC3R (IC₅₀ = 9.3 nM, EC₅₀ = 16 nM), a weak agonist at the hMC5R (IC₅₀ = 570 nM, EC₅₀ = 95 nM), and a weak partial agonist/antagonist at the hMC4R (IC₅₀ = 32 nM, EC₅₀ = 1400 nM). These results demonstrate that an aromatic residue in position 4 can change the biological profile of melanocortin peptides at the hMC3–5 receptors. The structure–activity relationships information provided by this set of synthetic melanocortin analogues supports the hypothesis that the incorporation of a phenylalanine or naphthylalanine residue in the position 4 could be a factor for selectivity and potency at central melanocortin receptors. The information that we currently have is not sufficient to speculate on the possible involvement in receptor interaction of the bulky hydrophobic residue in position 4. However, these structure–activity studies of synthetic melanocortin ligands at the human receptors have identified a selective antagonist (**2**) at the hMC4R (pA₂ = 8.7), a selective partial agonist at hMC4R (**7**) and a selective partial agonist at hMC3R, **9** (IC₅₀ = 3.68 nM, EC₅₀ = 4.87 nM).

In conclusion, the results demonstrated that the modifications in position 4 by hydrophobic residues, such as Phe and Nal(2') in conjunction with the expansion of the 23-membered cyclic lactam MT-II scaffold to the 26-membered scaffold (by addition of a Gly residue in the position 10) can be used to obtain melanotropin peptides with enhanced receptor selectivity.

2.1. Peptide synthesis

The peptides (Table 1) were synthesized by methods previously developed in our laboratory [18] using *N*^α-Fmoc chemistry with an appropriate orthogonal protection strategy. Coupling was carried out with standard in situ activating reagents used *N*^α-Fmoc SPPS, such as the uronium salts (HBTU) in the presence of a tertiary base (DIPEA), to generate HOBT esters. The cyclization of the peptides was performed on the solid support after removing the Allyl and Alloc protecting groups under neutral conditions with catalytic amounts of Pd(PPh₃)₄ in the presence of PhSiH₃ and argon [18], assuring an orthogonal deprotection of the side chain-protecting groups used during the synthesis. One concern was that Trp residues are extremely

susceptible to alkylation by cations produced during the cleavage process. Trialkylsilanes, such as Et₃SiH, have been shown to be effective, particularly for peptides containing Arg(Pbf) and Trp(Boc) [13,37]. In our syntheses, cleavage of the peptide from the resin with a Et₃SiH-based TFA cocktail was adopted since the Trp residue had a Boc-side chain-protecting group.

The crude peptide obtained following cleavage from the resin showed a single major peak by analytical RP-HPLC. Purification was accomplished by preparative RP-HPLC. The physicochemical properties and purity of the final peptides were assessed by FAB-MS, RP-HPLC, TLC in three solvent systems (Table 4) and amino acid analysis (not shown).

3. Experimental

3.1. Materials

Thin layer chromatography (TLC) was done on Merck silica gel 60 F₂₅₄ plates using the following solvent systems: (A) 1-butanol/acetic acid/pyridine/water (5:5:1:4); (B) ethyl acetate/pyridine/acetic acid/water (5:5:1:3); (C) upper phase of 1-butanol/acetic acid/water (4:1:1). The peptides were detected on the TLC plates using iodine vapor. Final peptide purification was achieved using a semipreparative RP-HPLC C₁₈-bonded silica column (Vydac 218TP1010, 1.0 cm × 25 cm). The peptides were eluted with a linear acetonitrile gradient (10–50%) over 30 min at a flow rate of 5.0 mL/min, with a constant concentration of TFA (0.1%, v/v). The linear gradient was generated with a Dynamax HPLC solvent delivery system (Rainin Instrument Co. Inc., Woburn, MA). The separations were monitored at 280 and 230 nm, and integrated with a Dynamax dual wavelength absorbance detector model UV-D. Fractions corresponding to the major peak were collected, pooled, and lyophilized. Amino acid analyses were performed at the University of Arizona Biotechnology Core Facility. The system used was an Applied Biosystems model 420A amino acid analyzer with automatic hydrolysis (vapor phase at 160 °C for 1 h 40 min using 6N HCl), a pre-column phenylthiocarbamyl-amino acid (PTC-AA) analysis. No corrections are made for amino acid decomposition. The Rink Amide resin, *N*^α-Fmoc amino acids and amino acid derivatives were purchased from Advanced Chemtech (Louisville, KY). All purchased amino acids were of the L configuration. Fluorenylmethoxycarbonyl (Fmoc) was used for *N*^α protection, and the reactive side chains of the amino acids were protected as follows: Lys, with allyloxycarbonyl (Alloc); Asp, with Allyl ester (Allyl); Arg, with Pbf; Trp, with *tert*-butyloxycarbonyl (Boc). All reagents and solvents were ACS grade or better and were used without further purification. The purity of the finished peptides were checked by TLC in three different solvents and analytical RP-HPLC at 280 and 230 nm in all cases and they were greater than 95% pure as determined by these methods. The structures of the pure peptides were confirmed by fast atom bombardment (FAB) and in some cases with electrospray ionization (ESI) mass spectrometry. The analytical data for each compound is presented in Table 4.

3.2. General method for peptide synthesis and purification

The peptides were synthesized on 0.5 g of Rink amide resin (0.7 mmol of NH₂/g of resin) using *N*^α-Fmoc chemistry and an orthogonal side chain-protection strategy using manual solid-phase synthesis methods. The first amino acid, *N*^α-Fmoc-Lys(*N*^α-Alloc)-OH, was linked on to the resin which was previously deprotected by a 25% piperidine solution in DMF for 30 min. The following amino acids were then added to the growing peptide chain by stepwise addition: *N*^α-Fmoc-Trp(*N*^β-Boc)-OH, *N*^α-Fmoc-Arg(*N*^G-Pbf)-OH, *N*^α-Fmoc-D-Nal(2')-OH, *N*^α-Fmoc-Pro-OH, *N*^α-Fmoc-Asp(β-Allyl)-OH, *N*^α-Fmoc-Nle-OH, and *N*^α-Fmoc-(L or D)-Phe-OH or *N*^α-Fmoc-(L- or D-)-Nal(2)-OH using standard solid-phase methods. Each coupling reaction was achieved using a three-fold excess of amino acid, of HBTU/HOBt in presence of DIEA. The *N*^α-Fmoc protecting group was removed by treating the protected peptide resin with 25% piperidine solution in DMF (1 × 50 mL, 5 min, 1 × 50 mL, 20 min). The peptide resin was

washed with DMF (3× 50 mL), DCM (3× 50 mL) and again with DMF. Upon complete formation of the protected linear peptide, the final *N*^α-Fmoc protecting group was removed in the usual manner with 25% piperidine and acetylated with 25% acetic anhydride in DCM for 20 min. At this step, the Alloc and Allyl protecting groups of lysine and aspartic acid, respectively, were removed as detailed below. All procedures were done in an argon atmosphere.

In a typical example, to a peptide resin (500 mg) washed with DCM (3× 2 min) in the presence of argon, was added a solution of PhSiH₃ (24 equiv.) in 2 mL of DCM and the resin was manually stirred for 2 min. Subsequently, a solution of Pd(PPh₃)₄ (0.25 equiv.) in 6 mL of DCM was added as argon was bubbled continuously through the resin. The reaction was mechanically stirred for 30 min under argon. Then, the peptide resin was washed with DCM (3× 50 mL, 2 min), DMF (3× 50 mL, 1 min) and again with DCM (4× 50 mL, 2 min) and the process was repeated. The removal of Allyl and Alloc groups under neutral conditions with catalytic amounts of Pd(PPh₃)₄ in the presence of PhSiH₃ and complete absence of oxygen [18] permitted orthogonal deprotection of the side chain-protecting groups used during the synthesis. Then the peptideresin was suspended in 20 mL of *N*-methyl-pyrrolidone (NMP), followed by cyclization of the peptide via the free carboxylic acid side chain of Asp and the free amino group side chain of Lys by addition of HBTU (6 equiv.), HOBt (6 equiv.), and DIEA (12 equiv.) for 2 h. This process was repeated until a negative Kaiser test resulted [29]. The acylation step was accomplished using Ac₂O (20 equiv.) and DIEA (1 equiv.) in DCM.

The peptide was cleaved from the resin and the other side chain-protecting groups removed using the following mixture: TFA/TES/H₂O (9:0.5:0.5) for 3 h. The resin was removed from solution by filtration and the crude peptide was recovered by precipitation with cold anhydrous ethyl ether giving a white powder that was purified by preparative HPLC on a C18-bonded silica gel column (Vydac 218TP1010, 1.0 cm × 25 cm) eluted with a linear gradient of acetonitrile in aqueous 0.1% of trifluoroacetic acid (v/v). The purification was monitored at 280 nm, and the fraction corresponding to the major peak were collected, combined, and lyophilized to give final compound as a pure (>98%) white powder (yield: 45–50% as cyclic peptide). Analytical HPLC, TLC, and amino acid analysis was used to characterize the peptides (Table 4).

3.3. Receptor binding assays [4]

Competition binding experiments were performed on whole cells. Transfected HEK293 cells with hMCRs were seeded on 96-well plates, 48 h before assay, 100,000 cells/well. For the assay, the medium was removed and cells were washed twice with a freshly prepared binding buffer containing 100% minimum essential medium with Earle's salt (MEM, GIBCO), 25 mM HEPES (pH 7.4), 0.2% bovine serum albumin, 1 mM 1,10-phenanthroline, 0.5 mg/L leupeptin, and 200 mg/L bacitracin. Cells were then incubated with different concentrations of unlabeled peptide and labeled [¹²⁵I]-[Nle⁴, D-Phe⁷]-α-MSH (Perkin-Elmer Life Science, 100,000 cpm/well, 0.1386 nM) for 40 min at 37 °C. The medium was subsequently removed and each well was washed twice with the assay buffer. The cells are lysed by the addition of 250 μL of 0.1N NaOH, and 250 μL of 1% Triton X-100. The lysed cells were transferred to 12 mm × 75 mm glass tubes and the radioactivity was measured by Wallac 1470 WIZARD Gamma Counter. Data were analyzed using Graphpad Prism 3.1 (Graphpad Software, San Diego, CA).

3.4. Adenylate cyclase assay [4]

HEK293 cells transfected with human melanocortin receptors were grown to confluence in MEM medium (GIBCO) containing 10% fetal bovine serum, 100 units/mL penicillin and streptomycin, and 1 mM sodium pyruvate. The cells were seeded on 96-well plates 48 h before assay (100,000 cells/well). For the assay, the medium was removed and cells were rinsed with

1 mL of MEM buffer (GIBCO) or with Earle's balanced salt solution (EBSS, GIBCO). An aliquot (0.4 mL) of the Earle's balanced salt solution was placed in each well along with isobutylmethylxanthine (IBMX; 5 μ L; 0.5 mM) for 1 min at 37 °C. Varying concentrations of melanotropins (0.1 mL) were added and the cells incubated for 3 min at 37 °C. The reaction was stopped by aspirating the buffer and adding ice-cold Tris-EDTA buffer to each well (0.15 mL). The 96-well plates were covered and placed on ice. After dislodging the cells with the help of trypsin, the cells were transferred to polypropylene micro-centrifuge tubes, capped, and placed in a boiling water bath for 15 min. The cell lysates were then centrifuged for 2 min (6500 rpm), and 50 μ L of the supernatant was aliquoted into a clean Eppendorf tube. cAMP content was measured by competitive binding assay according to the assay kit instructions (TRK 432, Amersham Corp.).

3.5. Data analysis

IC₅₀ and EC₅₀ values represent the mean of duplicate experiments performed in triplicate. IC₅₀ and EC₅₀ estimates and their associated standard errors were determined by fitting the data using a non-linear least squares analysis, with the help of Graphpad Prism 3.1 (Graphpad Software, San Diego, CA).

4. Computational procedures

Molecular modeling experiments employed MacroModel 8.1 equipped with Maestro 5.0 graphical interface installed on a Linux RedHat 9.0 system. Peptide structures were built into extended structures with standard bond lengths and angles, and they were minimized using the OPLS-AA force field and the Polak-Ribier conjugate gradient (PRCG). Optimizations were converged to a gradient RMSD less than 0.05 kJ/($\text{\AA}$ mol) or continued until a limit of 50,000 iterations was reached. Aqueous solution conditions were simulated using the continuum dielectric water solvent model (GB/SA) as implemented in MacroModel. Extended cut-off distances were defined at 8 $\text{\AA}$ for van der Waals, 20 $\text{\AA}$ for electrostatics and 4 $\text{\AA}$ for H-bonds.

Conformational profiles of the cyclic peptides were investigated by MacroModel's large scale low mode (LLMOD) procedure of Kolossváry [30] using the energy minimization parameters as described above. A total of 25,000 search steps were performed and the conformations with energy difference of 50 kJ/mol from the global minimum were saved. Interatomic dihedral angles were measured for each peptide analogue using the standard Maestro measurement tool, and they are described in Tables 2 and 3. The superimpositions of peptide structures were performed using the α -carbons of the core sequence His(Pro)-D-Phe-Arg-Trp unless otherwise specified.

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Fig. 1. Stereoview of the superimposed LLMOD-derived global minima of PG-137 [red: black (print version)] and PG-933 [blue: grey (print version)] (rmsd = 0.91 Å). “For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article”.

Fig. 2.

Stereoview of the superimposed NMR conformation of core sequence His-D-Phe-Arg-Trp of MT-II [red: black (print version)] [44] and the LLMOD-derived global minimum of the core sequence Pro-D-Nal(2')-Arg-Trp of PG-138 [blue: grey (print version)] (rmsd = 1.27 Å). “For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article”.

Fig. 3. Stereoview of the superimposed NMR structure of MT-II [red: black (print version)] [44] and the LLMOD-derived global minimum of PG-951 [blue: grey (print version)] (rmsd = 0.77 Å). The Lys residues and the side chains of Asp⁵ are omitted for clarity. “For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article”.

Table 1
Binding and intracellular cAMP accumulation of the α -melanotropin analogues at human melanocortin receptors

No.	Peptide code	Structure	hMC3R			hMC4R			hMC5R		
			IC ₅₀ (nM)	EC ₅₀ (nM)	% Activity at 10 μ M	IC ₅₀ (nM) ^d	EC ₅₀ (nM) ^b	% Activity at 10 μ M	IC ₅₀ (nM) ^d	EC ₅₀ (nM) ^b	% Activity at 10 μ M
1	PG-137	H-Phe-c[Asp-Pro-D-Phe-Arg-Tyr-Gly-Lys]-NH ₂	14 $\pm$ 0.50	86 $\pm$ 0.50	100	53 $\pm$ 4.6	480 $\pm$ 50	48	69 $\pm$.6	40 $\pm$ 5.6	100
2	PG-138	H-Phe-c[Asp-Pro-D-Nal(2')-Arg-Tyr-Gly-Lys]-NH ₂	8 $\pm$ 2	>5000	28	15 $\pm$ 1.5	>10000	0, pA ₂ = 8.7	7.8 $\pm$ 2	11 $\pm$ 1.1	100
3	PG-975	H-D-Phe-c[Asp-Pro-D-Phe-Arg-Tyr-Gly-Lys]-NH ₂	2.5 $\pm$ 0.40	56 $\pm$ 10	56	5.9 $\pm$ 1	381 $\pm$ 3 30	50	44 $\pm$ 2	7 $\pm$ 1.7	10
4	PG-976	H-D-Phe-c[Asp-Pro-D-Nal(2')-Arg-Tyr-Gly-Lys]-NH ₂	22 $\pm$ 7.8	>1000	7.3	53 $\pm$ 10	>1000	0	540	>5000	0
5	PG-141	H-Nal-c[Asp-Pro-D-Phe-Arg-Tyr-Gly-Lys]-NH ₂	210 $\pm$ 22	300 $\pm$ 33	85.0	1500 $\pm$ 110	2300 $\pm$ 240	61	1000	900 $\pm$ 100	100.0
6	PG-143	H-Nal-c[Asp-Pro-D-Nal(2')-Arg-Tyr-Gly-Lys]-NH ₂	105 $\pm$ 11	1600 $\pm$ 100	15.0	160 $\pm$ 17	3400 $\pm$ 500	19	110 $\pm$ 15	1600 $\pm$ 160	100.0
7	PG-977	H-D-Nal-c[Asp-Pro-D-Phe-Arg-Tyr-Gly-Lys]-NH ₂	9.4 $\pm$ 3.1	>1000	13	11 $\pm$ 0.30	56.0	50	970	>5000	0
8	PG-978	H-D-Nal(2')-c[Asp-Pro-D-Nal(2')-Arg-Tyr-Gly-Lys]-NH ₂	1.8 $\pm$ 0.79	>1000	85	5 $\pm$ 1	>10000	0	37	>10000	0
9	PG-951	H-D-Phe-c[Asp-Pro-D-Phe-Arg-Tyr-Gly-Lys]-NH ₂	3.7 $\pm$.05	4.87 $\pm$ 0.50	61	700 $\pm$ 100	>10000	0	1100 $\pm$ 180	>1000	0
10	PG-952	H-D-Phe-c[Asp-Pro-D-Nal(2')-Arg-Tyr-Gly-Lys]-NH ₂	73 $\pm$ 10	54.5 3 5	14	36 $\pm$ 5	>10000	0	490 $\pm$ 60	>10000	0
11	PG-953	H-D-Phe-c[Asp-Pro-D-Phe-Arg-D-Nal(2')-Lys]-NH ₂	160 $\pm$ 12	63.2 $\pm$ 10	61	140 $\pm$ 20	>10000	0	46 $\pm$ 10	74.2 $\pm$ 10	100
12	PG-954	H-D-Phe-c[Asp-Pro-D-Nal(2')-Arg-D-Nal(2')-Lys]-NH ₂	9.3 $\pm$ 1.3	16.3 $\pm$ 2	11	32 $\pm$ 3	1400	0	570 $\pm$ 30	95 $\pm$ 12	90

No.	Peptide code	Structure	hMC3R			hMC4R			hMC5R		
			IC ₅₀ (nM)	EC ₅₀ (nM)	% Activity at 10 μM	IC ₅₀ (nM) ^a	EC ₅₀ (nM) ^b	% Activity at 10 μM	IC ₅₀ (nM) ^a	EC ₅₀ (nM) ^b	% Activity at 10 μM
MTII		Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Lys]-NH ₂	1.25 ± 0.2	1.85 ± 0.2	100	1.1 ± 0.30	2.9 ± 0.52	100	7.5 ± 0.20	3.3 ± 0.70	100
SHU9119		Ac-Nle-c[Asp-His-D-Nal(2')-Arg-Trp-Lys]-NH ₂	2.3 ± 0.20	>10000	0	0.6 ± 0.10	>10000	0	0.9 ± 0.20	1.2 ± 0.10	97

^a IC₅₀ = concentration of peptide at 50% specific binding (N = 4-6). The peptides were tested in a range of concentrations (from 10⁻¹⁰ to 10⁻⁴ M).

^b EC₅₀ = concentration of peptide at 50% maximal cAMP generation (N = 4). The peptides were tested in a range of concentrations (from 10⁻¹⁰ to 10⁻⁴ M).

Table 2 Backbone torsion angles (°) for the low-energy conformations for MT-II, and the novel cyclic melanotropin peptides based on LLMOD/OPLS-AA calculations

Sequence	Code	Nle/Xaa ³		Asp ⁴		His/Pro ⁵		D-Phe/D-Nal ⁶		Arg ⁷		Trp/Xaa ⁸		Lys/Gly ⁹		Lys ¹⁰	
		φ	ψ	φ	ψ	φ	ψ	φ	ψ	φ	ψ	φ	ψ	φ	ψ	φ	ψ
Ac-Nle-c [Asp-His-D- Phe-Arg- Trp-Lys]- NH ₂ (MT-II)	MT-II (NMR) ^a	-111	131	-85	113	-108	109	84	0	-122	90	-77	108	-101	103	—	—
H-Phe-c [Asp-Pro-D- Phe-Arg- Trp-Gly- Lys]-NH ₂	PG-137	—	-172	-59	155	-70	148	123	-161	-80	172	-83	144	94	-61	63	57
H-Phe-c [Asp-Pro-D- Nal(2)-Arg- Trp-Gly- Lys]-NH ₂	PG-138	—	164	-78	152	-73	-63	56	-102	-144	-179	-63	-42	-127	82	-67	-57
H-D-Phe-c [Asp-Pro-D- Phe-Arg- Trp-Lys]- NH ₂	PG-951	—	-167	-84	161	-81	48	60	46	-92	178	-73	128	-92	-33	—	—
Ac-Nle-c [Asp-Pro-D- Phe-Arg- Trp-Gly- Lys]-NH ₂	PG-933	-87	178	-67	133	-71	151	93	-72	-146	150	-69	-54	-96	81	-66	-54

^aRef. [44].

Table 3

Side chain torsion angles ($^{\circ}$) for the low-energy conformations for MT-II, and the novel cyclic melanotropin peptides based on LLMOD/OPLS-AA calculations

Sequence	Code	His/Pro ⁵			D-Phe/D-Nal ⁶			Arg ⁷		Trp ⁸	
		χ^1	$\chi^{2,3,1}$	$\chi^{2,3,2}$	χ^1	$\chi^{2,3,1}$	$\chi^{2,3,2}$	χ^1	χ^2	χ^1	$\chi^{2,3,1}$
Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Lys]-NH ₂	MT-II (NMR)	-179	-102	79	66	-120	60	-63	170	-78	94
H-Phe-c[Asp-Pro-D-Phe-Arg-Trp-Gly-Lys]-NH ₂	PG-137	29	-31	—	172	-70	107	57	163	-177	91
H-Phe-c[Asp-Pro-D-Nal(2)-Arg-Trp-Gly-Lys]-NH ₂	PG-138	28	-29	—	66	-93	87	59	176	61	98
H-D-Phe-c[Asp-Pro-D-Phe-Arg-Trp-Lys]-NH ₂	PG-951	33	-35	—	55	-113	68	59	155	-177	84
Ac-Nle-c[Asp-Pro-D-Phe-Arg-Trp-Gly-Lys]-NH ₂	PG-933	28	-31	—	64	-98	82	-173	167	55	102

^aRef. [44].

Table 4

Physicochemical properties of the melanocortin peptides

No.	Sequence	Solvent A ^a	Solvent B ^a	Solvent C ^a	M.W.	MS	HPLC ^b
1	H-Phe-c[Asp-Pro-D-Phe-Arg-Trp-Gly-Lys]-NH ₂	0.81	0.05	0.44	1033.2	1033.4	3.50
2	H-Phe-c[Asp-Pro-D-Nal(2')-Arg-Trp-Gly-Lys]-NH ₂	0.82	0.06	0.46	1083.3	1083.8	3.89
3	H-D-Phe-c[Asp-Pro-D-Phe-Arg-Trp-Gly-Lys]-NH ₂	0.82	0.05	0.44	1033.2	1033.0	3.51
4	H-D-Phe-c[Asp-Pro-D-Nal(2')-Arg-Trp-Gly-Lys]-NH ₂	0.85	0.07	0.48	1083.3	1083.6	3.93
5	H-Nal-c[Asp-Pro-D-Phe-Arg-Trp-Gly-Lys]-NH ₂	0.83	0.06	0.46	1083.3	1083.8	3.41
6	H-Nal-c[Asp-Pro-D-Nal(2')-Arg-Trp-Gly-Lys]-NH ₂	0.88	0.07	0.49	1133.3	1133.9	3.92
7	H-D-Nal-c[Asp-Pro-D-Phe-Arg-Trp-Gly-Lys]-NH ₂	0.84	0.06	0.46	1083.3	1083.5	3.45
8	H-D-Nal(2')-c[Asp-Pro-D-Nal(2')-Arg-Trp-Gly-Lys]-NH ₂	0.89	0.08	0.50	1133.3	1133.5	3.98
9	H-D-Phe-c[Asp-Pro-D-Phe-Arg-Trp-Lys]-NH ₂	0.78	0.04	0.38	976.2	976.5	3.09
10	H-D-Phe-c[Asp-Pro-D-Nal(2')-Arg-Trp-Lys]-NH ₂	0.83	0.05	0.40	1026.2	1026.6	3.76
11	H-D-Phe-c[Asp-Pro-D-Phe-Arg-D-Nal(2')-Lys]-NH ₂	0.84	0.07	0.41	987.2	987.5	3.20
12	H-D-Phe-c[Asp-Pro-D-Nal(2')-Arg-D-Nal(2')-Lys]-NH ₂	0.89	0.10	0.45	1036.5	1037.5	3.77

^a Solvent systems: (A) 1-butanol/HOAc/pyridine/H₂O (5:5:1:4); (B) EtOAc/pyridine/H₂O (5:5:1:3); (C) 1-butanol/AcOH/H₂O (4:1:1).

^b Analytical HPLC performed on a C18 column (Vydac 218TP104) using a gradient of acetonitrile in 0.1% aqueous TFA at 1 mL/min. The following gradient was used: 10–90% acetonitrile in 40 min.