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Solid-phase peptide head-to-side chain cyclodimerization: Discovery of C_2-symmetric cyclic lactam hybrid α-melanocyte-stimulating hormone (MSH)/agouti-signaling protein (ASIP) analogues with potent activities at the human melanocortin receptors☆

Alexander V. Mayorov[1], Minying Cai, Erin S. Palmer, Zhihua Liu, James P. Cain, Josef Vagner, Dev Trivedi, Victor J. Hruby*

Department of Chemistry and Biochemistry, University of Arizona, 1306 E. University Blvd., Tucson, AZ 85721, USA

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ABSTRACT

A novel hybrid melanocortin pharmacophore was designed based on the pharmacophores of the agouti-signaling protein (ASIP), an endogenous melanocortin antagonist, and α-melanocyte-stimulating hormone (α-MSH), an endogenous melanocortin agonist. The designed hybrid ASIP/MSH pharmacophore was explored in monomeric cyclic, and cyclodimeric templates. The monomeric cyclic disulfide series yielded peptides with hMC3R-selective non-competitive binding affinities. The direct on-resin peptide lactam cyclodimerization yielded nanomolar range (25–120 nM) hMC1R-selective full and partial agonists in the cyclodimeric lactam series which demonstrates an improvement over the previous attempts at hybridization of MSH and agouti protein sequences. The secondary structure-oriented pharmacophore hybridization strategy will prove useful in development of unique allosteric and orthosteric melanocortin receptor modulators. This report also illustrates the utility of peptide cyclodimerization for the development of novel GPCR peptide ligands.

1. Introduction

Peptide cyclization is a well-established approach to improving peptide biological activity [38,53,59], which stems from reduced conformational freedom of the parent peptide and thus a better defined secondary structure required for efficient receptor–ligand interaction, *i.e.*, "bioactive conformation" [39,54]. It has been previously noted that the success of a peptide cyclization depends strongly on the probability of juxtaposition of the reactive groups of the linear peptide precursor, and is usually encumbered by side reactions, most notably, oligomerizations and cyclooligomerizations [20,57]. These side reactions become prevalent in syntheses of small strained cyclic peptides, such as *N*-unsubstituted tri- and tetrapeptides, typically incurring formation of cyclic dimers and trimers (Scheme 1) [57].

Although several peptide natural products, such as gramicidin S [27] and marine cyclodepsipeptide IB-01212 [19], are known to possess C_2-symmetry, there are few examples of peptide cyclodimerizations pertinent to development of peptides with enhanced biological activities, which include syntheses of cyclic biphalin [70] and morphiceptin [96] analogues. Thus, practical aspects of this area of peptide chemistry and its applications to development of novel GPCR ligands remain relatively unexplored. Recent reports on preparative peptide cyclodimerizations including syntheses of dimeric disulfides [76,80,91], depsipeptides [19], lactams [98], and peptide cyclodimerizations involving azide-alkyne cycloaddition "click chemistry" [14,62,77] prompted us to disclose our results on preparation of structurally unique C_2-

Abbreviations: All, allyl; AgRP, Agouti-related protein; ASIP, agouti-signaling protein; Boc, *tert*-butyloxycarbonyl; Fmoc, fluorenylmethoxycarbonyl; CH_3CN, acetonitrile; Cl-HOBt, 1-hydroxy-6-chlorobenzotriazole; DCM, dichloromethane; DIPEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DIC, diisopropyl carbodiimide; HOBt, *N*-hydroxybenzotriazole; *h*MCR, human melanocortin receptor; *m*MCR, mouse melanocortin receptor; MSH, melanocyte-stimulating hormone; Nal(2′), 2′-naphthylalanine; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; TFA, trifluoroacetic acid; SPPS, solid-phase peptide synthesis; RP-HPLC, reverse-phase high performance liquid chromatography; hMC3R, human melanocortin-3 receptor; α-MSH, α-melanocyte-stimulating hormone Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2; NDP-α-MSH, Ac-Ser-Tyr-Ser-Nle-Glu-His-*D*-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2; *h*ASIP (116–123), human agouti-signaling protein c [Cys-Arg-Phe-Phe-Arg-Ser-Ala-Cys]; MT-II, Ac-Nle-c [Asp-His-*D*-Phe-Arg-Trp-Lys]-NH_2.

☆ Abbreviations used for amino acids and designation of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in J. Biol. Chem. 1972, 247, 977–983.

* Corresponding author. Tel.: +1 520 621 6332; fax: +1 520 621 8407.
E-mail address: hruby@u.arizona.edu (V.J. Hruby).
[1] Current address: Department of Chemistry, The Scripps Research Institute, La Jolla, CA 92037, USA.

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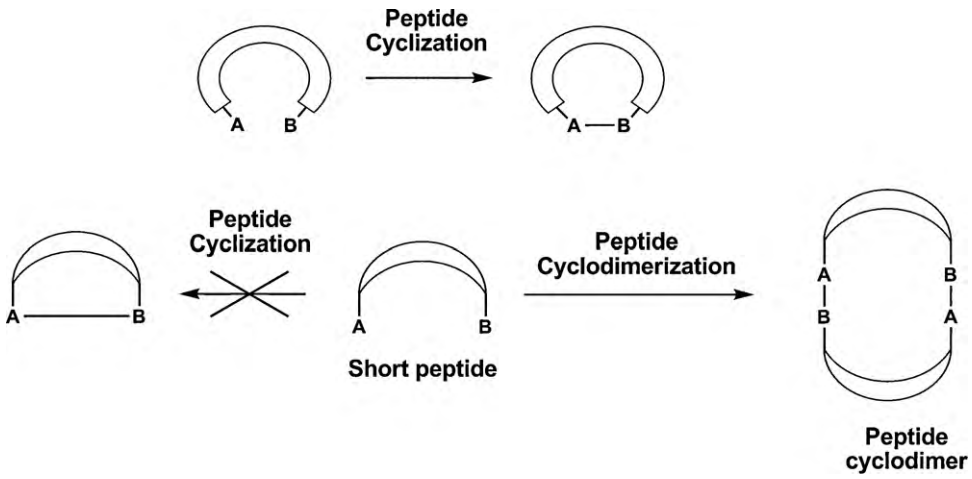


Scheme 1. Peptide cyclization and cyclodimerization.

symmetrical hybrid analogues of α-MSH/agouti-signaling protein *via* solid-phase head-to-side chain lactam cyclodimerization, and their biological evaluation at the human melanocortin receptors.

The melanocortin system [16,17,25] remains a challenging target for rational peptide and peptidomimetic design since the 3D-topographical requirements for the specific melanocortin receptor subtype recognition have not been fully elucidated [28,33,100]. At the same time, the numerous multifaceted physiological functions of the five known subtypes of human melanocortin receptors (hMC1-5R), including skin pigmentation [15,32], control of the immune system [15,32], erectile function [25,55,99,100], blood pressure and heart rate [60,74], control of feeding behavior and energy homeostasis [6,16,22,23,94,95,100,102], modulation of aggressive/defensive behavior [71,72], and mediation of pain [50,69,97], continue to provide a strong stimulus for development of potent and selective melanocortin agonists and antagonists. Several general approaches to development of such compounds have been described in literature [9,24,40,41], and include: (a) *D*-amino acid scan/unnatural amino acid substitutions in linear α-, β- and γ-MSH-derived sequences [29,83]; (b) hybridizations of the native MSH sequences with each other and with sequences of other bioactive peptides [4,8,34]; (c) implementation of various global and local conformational constraints *via* peptide cyclizations and employment of constrained amino acids [1,5,7,30,31,65,66,82]; and (d) manipulation of steric factors that influence receptor–ligand interactions [3,64,65]; as well as (e) construction of small molecules based on β-turn peptidomimetics and "privileged structure" scaffolds [10,73,79,85,89]. Until recently, this work had primarily been focusing on the hMC4R due to its direct involvement in the regulation of feeding behavior and energy homeostasis [6,16,22,23,94,95,100,102], as well as sexual behavior [25,55,93,99,100]. The hMC3 receptor has been acknowledged to play a complementary role in weight control [6,21,102], and current reports suggest that hMC3R is an inhibitory autoreceptor on POMC neurons [16] based on the observed stimulation of food intake by peripheral administration of an MC3R-selective agonist [63], and MC3R agonist-induced inhibition of spontaneous action of POMC neurons [18], although the full scope of physiological functions of this receptor is still poorly understood. Development of selective ligands for the hMC1 and hMC5 receptors is also receiving some attention lately due to the roles of these receptors in regulation of skin pigmentation [15,32] and control of the immune system [15,32] (hMC1R), and in regulating exocrine gland function [13], and coordinating central and peripheral signals for aggression (hMC5R) [71,72].

Agouti-signaling protein (ASIP) [61] and agouti-related protein (AgRP) [75] (Fig. 1) were originally described as endogenous antagonists for the melanocortin-1 and melanocortin-3/-4 receptors, respectively, although recent reports ascribe inverse agonist activity to both proteins [11,36]. There is some evidence that AgRP mediates orexigenic signaling of ghrelin [12], which makes it an important regulator of feeding behavior [44]. Initial structure–function relationship studies have attributed the melanocortin receptor antagonist activities to the Arg-Phe-Phe tripeptide pharmacophore, which is a part of the central loop within the inhibitor cystine knot (ICK) motif in both agouti proteins [68], as displacement of these three key amino acids with alanine within the AgRP/ASIP pharmacophore sequence results

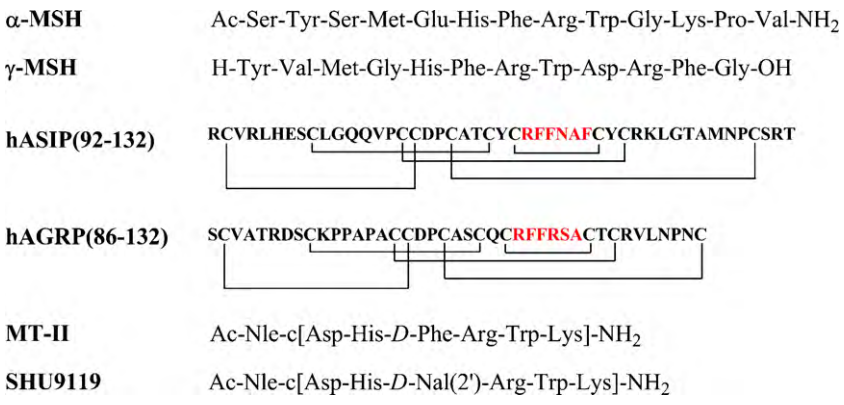


Fig. 1. Sequences of some endogenous and synthetic melanotropin peptides.

in a loss of activity [101]. Truncation of both ASIP and AgRP sequences results in substantial loss of both binding affinities and antagonist/inverse agonist potencies [47,90], and Ac-mini-AGRP(87-120, C105A)-NH_2 has been reported to be the minimal AgRP sequence equipotent to the full-length AgRP [45], indicating possible significance of *N*- and *C*-terminal sequences of these proteins in receptor–ligand interactions. Replacement of the Arg-Phe-Phe tripeptide sequence with the His-*D*-Phe-Arg-Trp MSH tetrapeptide pharmacophore, quite predictably, led to conversion of AgRP/ASIP-derived melanocortin antagonists to relatively potent agonists [46,101], since the His-*D*-Phe-Arg-Trp tetrapeptide sequence is known to induce melanocortin agonist activity in a wide variety of linear and cyclic peptide templates [9,24,37,43]. In other instances, the agouti Arg-Phe-Phe tripeptide sequence was embedded into the linear and cyclic α-MSH templates to yield nanomolar range mMC1R agonists, although the agonist potencies of these peptides were reported to be >300-fold below that of the super-agonist MT-II as determined by the *m*MCR CRE/β-galactosidase assay [48].

The structural uniqueness of the Agouti protein pharmacophores presents intriguing opportunities for exploration of novel melanocortin templates beyond the tetrapeptide pharmacophore His-Phe-Arg-Trp of the endogenous agonists α-, β- and γ-MSH to expedite development of highly potent and selective melanocortin ligands.

2. Materials and methods

2.1. Materials

N^{α}-Fmoc-amino acids, peptide coupling reagents and Rink amide AM resin were obtained from Novabiochem (San Diego, CA), except N^{α}-Fmoc-Glu(OAll)-OH, which was purchased from NeoMPS (San Diego, CA). The following side chain protecting groups were used: Trp(N^{in}-Boc), Arg(N^{ε}-Pbf), Cys(S-Trt). ACS grade organic solvents were purchased from VWR Scientific (West Chester, PA), and other reagents were obtained from Sigma–Aldrich (St. Louis, MO) and used as commercially available. The polypropylene reaction vessels (syringes with frits) were purchased from Torviq (Niles, MI). The purity of the peptides was checked by analytical reverse-phase HPLC using a Vydac C_{18} 218TP104 column (Western Analytical Products, Murrieta, CA) monitored at 230 and 254 nm, and was determined to be >97% for all peptides reported herein. Additionally, the purity of the peptides was evaluated by thin-layer chromatography (TLC), which was performed using three different solvent systems. Analytical thin-layer chromatography (TLC) was carried out on 0.25 mm glass-backed silica gel 60 F_{254} plates (EM Science 5715, VWR Scientific). The TLC chromatograms were visualized by UV light, and by dipping in potassium permanganate solution followed by heating (hot plate).

2.2. Peptide synthesis

All peptides in this study were synthesized manually by the N^{α}-Fmoc solid-phase methodology [64,65], using Bromophenol Blue pH indicator to monitor the extent of coupling reactions as described by Krchnak et al. [58] Rink amide AM resin (4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin, 0.5 g, 0.637 mmol/g) was placed into a 50 mL polypropylene syringe with the frit on the bottom and swollen in DMF (20 mL) for 1 h. The Fmoc protecting group on the Rink linker was removed by 25% piperidine in DMF (1× 5 min and 1× 15 min). The resin was washed with DMF (4× 15 mL), then washed with 0.02 M HOBt solution in DMF, stained with 0.05 mM solution of Bromophenol Blue in 0.02 M HOBt/DMF solution, and washed with 0.02 M HOBt/DMF solution (4× 15 mL). The first N^{α}-Fmoc amino acid was coupled using pre-activated ester (3 equiv. of N^{α}-Fmoc amino acid, 3 equiv. of HOBt, and 3 equiv. of DIC) in DMF. The coupling mixture was transferred into the syringe with the resin and shaken for 90 min, at which point the blue color of the resin changed to yellow, indicating complete coupling. The resin was washed with DMF (3× 15 mL), and thrice with DCM (3× 15 mL), and the unreacted amino groups were capped using acetic anhydride (2 mL) and pyridine (2 mL) in DCM (15 mL) for 30 min, and the resin was once again washed with DMF (6× 15 mL). The peptide sequences were completed by consecutively coupling the appropriate amino acids using the procedure described above.

2.3. Disulfide cyclizations

Upon completion of the peptide sequences, the resin was washed with DMF (5× 15 mL), DCM (3× 15 mL), methanol (5× 15 mL), and diethyl ether (5× 15 mL), and dried under reduced pressure (16 h). The peptides were cleaved off the solid support with 95% (v/v) TFA, 2.5% water, and 2.5% triisopropylsilane (5 mL, 3 h), and the crude peptides were precipitated out by the addition of a chilled 3:1 mixture of diethyl ether and petroleum ether (50 mL) to give white precipitates. The resulting peptide suspensions were centrifuged for 10 min at 6,500 rpm, and the liquid was decanted. The crude peptides were washed with diethyl ether (4× 50 mL), and after the final centrifugation, the peptides were dried under vacuum (2 h). The resulting white residues were dissolved in 2 M acetic acid, the insoluble impurities were removed by passing the solutions through Gelman Laboratory Acrodisc 13 mm syringe filters with 0.45 μM PTFE membranes (Pall Corporation, East Hills, NY), and the clear filtrates were lyophilized. The resulting crude linear peptides were cyclized by air oxidation as follows: the obtained crude peptides (~200–250 mg) were dissolved in glacial acetic acid (0.35 mL), diluted with water (450 mL) and acetonitrile (130 mL) to a peptide concentration of about 0.5 mM, and to the resulting solution aqueous ammonia was added to adjust the pH to about 7.0, as determined with pH paper. The resulting peptide solution in a 0.01 M ammonium acetate buffer was stirred for 36–72 h, until the cyclization was complete, as determined by HPLC and MS analysis. The peptide solution was then acidified by addition of glacial acetic acid (1 mL), acetonitrile was removed under reduced pressure, and the residual solution was lyophilized. The obtained white powders (200–250 mg) were re-dissolved in glacial acetic acid (2 mL), the resulting solutions were diluted with water (8 mL) to a peptide concentration of about 20 mg/mL, and passed through a Sephadex G-15 column (520 mm × 30 mm) using 1 M aqueous acetic acid as the eluent. Fractions containing the target peptides, as determined by TLC, were combined and lyophilized. Final purification was accomplished by preparative RP-HPLC on a C_{18}-bonded silica column (Vydac 218TP152022, 250 mm × 22 mm, 15–20 μm, 300 Å) using a Shimadzu SCL-10A HPLC system. The peptides were eluted with a stepwise gradient of 10% (0–5 min), 20–22% (7–17 min), 34–35% (20–30 min), and 38–40% (32–45 min) acetonitrile in 0.1% aqueous TFA solution with 10 mL/min flow rate. The purified peptides were isolated in 20–25% overall yield.

2.4. Solid-phase lactam cyclodimerizations (method A)

The orthogonal allyl ester protection for the side chain of Glu was removed with 0.1 equiv. $Pd(PPh_3)_4$/20 equiv. $PhSiH_3$ in DCM (2× 30 min) prior to the peptide cyclization [88]. The deprotected resin-bound peptide was washed with DCM (6× 5 mL), and DMF (3× 5 mL). The peptide cyclodimerizations were found to proceed in facile manner under the standard cyclization conditions described previously [64], with 6 equiv. DIC, 6 equiv. Cl-HOBt in THF (72 h) [81], and were monitored by Kaiser ninhydrin test [49].

Upon completion of cyclization the resin was treated with 5% solution of sodium diethyldithiocarbamate trihydrate in DMF (20 min) to remove any remaining traces of the Pd catalyst [64,65], then washed with DMF (5× 15 mL), DCM (3× 15 mL), methanol (5× 15 mL), and diethyl ether (5× 15 mL), and dried under reduced pressure (16 h). The cyclized peptides were cleaved off the solid support with 95% (v/v) TFA, 2.5% water, and 2.5% triisopropylsilane (5 mL, 3 h), and the crude peptides were precipitated out by the addition of a chilled 3:1 mixture of diethyl ether and petroleum ether (50 mL) to give white precipitates. The resulting peptide suspensions were centrifuged for 10 min at 6500 rpm, and the liquid was decanted. The crude peptides were washed with diethyl ether (4× 50 mL), and after the final centrifugation, the peptides were dried under vacuum (2 h). The resulting white residues were dissolved in 2 M acetic acid, and the insoluble impurities were removed by passing the solutions through Gelman Laboratory Acrodisc 13 mm syringe filters with 0.45 μM PTFE membranes (Pall Corporation, East Hills, NY), and the clear filtrates were lyophilized. Purification was accomplished by preparative RP-HPLC on a C_{18}-bonded silica column (Vydac 218TP152022, 250 mm × 22 mm, 15–20 μm, 300 Å) using a Shimadzu SCL-10A HPLC system. The peptides were eluted with a stepwise gradient of 10% (0–5 min), 20–22% (7–17 min), 34–35% (20–30 min), and 38–40% (32–45 min) acetonitrile in 0.1% aqueous TFA solution with 10 mL/min flow rate. The purified peptides were isolated in 20–25% overall yield. The molecular compositions of the purified peptides were confirmed by ^{1}H NMR in DMSO-d_6, and the dimeric structures were assigned by high resolution electrospray ionization (ESI) mass-spectrometry using an IonSpec Fourier transform mass spectrometer with a HiRes ESI source.

2.5. Targeted synthesis of cyclodimeric analogue 12 (method B)

The Fmoc-Arg(Pbf)-*D*-Phe-Glu(OAllyl)-Trp(Boc)-NH_2 tetrapeptide sequence was constructed on highly acid-labile Sieber amide resin (0.46 g, 0.64 mmol/g) [86]. The Glu side chain allylic ester protection was removed as previously described [64,88]. The cleavage of the resulting partially protected Fmoc-Arg(Pbf)-*D*-Phe-Glu(OH)-Trp(Boc)-NH_2 carboxylic acid peptide off the solid support was achieved by treating the resin with 2% TFA/1% triisopropylsilane (TIPS) in dichloromethane (5× 2 min), while collecting the TFA/DCM solution into a 300 mL round-bottom flask, containing 50 mL pyridine and 100 mL methanol. After the cleavage process was complete, the resin was washed thrice with methanol and the washes were added to the same pyridine/methanol mixture. The resulting solution was concentrated under reduced pressure and the syrupy residue was triturated with deionized water to remove pyridinium-TFA salts. The resulting white precipitate was centrifuged for 10 min at 6500 rpm, and the liquid was decanted. The crude peptide was washed with water (4× 50 mL), and after the final centrifugation, the precipitate was dried under vacuum (12 h) to yield 172 mg of crude partially protected peptide (48%). In parallel, the same sequence was prepared on Rink amide AM resin (~0.3 mmol scale), and its *N*-terminal Fmoc protection was removed with 25% piperidine/DMF. The crude partially protected tetrapeptide Fmoc-Arg(Pbf)-*D*-Phe-Glu(OH)-Trp(Boc)-NH_2 was coupled onto the exposed *N*-terminal amino group with DIC/HOAt over 4 h, followed by washing the resin with DMF (4×) and DCM (4×), and capping of the remaining amino groups with acetic anhydride/pyridine/DCM (1:2:20, v/v/v) to preclude cyclodimerization of the residual monomeric peptide during the cyclization of the dimer. The *N*-terminal Fmoc and Glu side chain allyl ester protecting groups were removed and macrocyclization was induced with DIC/Cl-HOBt in THF as described above. TFA cleavage/deprotection and purification followed the procedures described above for the method A. The peptide **12** samples prepared by methods A and B were determined to be identical according to HPLC, HR-MS and ^{1}H NMR analyses.

2.6. Receptor binding assay

Competition binding experiments were carried out using whole HEK293 cells stably expressing human MC1, MC3, MC4, and MC5 receptors as described before [64–66]. HEK293 cells transfected with hMCRs [7,26,35] were seeded on 96-well Plates 48 h before assay (50,000 cells/well). For the assay, the cell culture medium was aspirated and the cells were washed once with a freshly prepared MEM buffer containing 100% minimum essential medium with Earle's salt (MEM, GIBCO), and 25 mM sodium bicarbonate. Next, the cells were incubated for 40 min at 37 °C with different concentrations of unlabeled peptide and labeled [^{125}I]-[Nle^4,*D*-Phe^7]-α-MSH (PerkinElmer Life Science, 20,000 cpm/well, 33.06 pM) diluted in a 125 μL of freshly prepared binding buffer containing 100% MEM, 25 mM HEPES (pH 7.4), 0.2% bovine serum albumin, 1 mM 1,10-phenanthrolone, 0.5 mg/L leupeptin, 200 mg/L bacitracin. The assay medium was subsequently removed, the cells were washed once with basic medium, and then lysed by the addition of 100 μL of 0.1 M NaOH and 100 μL of 1% Triton X-100. The lysed cells were transferred to 12 mm × 75 mm borosilicate glass tubes, and the radioactivity was measured by a Wallac 1470 WIZARD Gamma Counter.

2.7. Adenylate cyclase assay

HEK 293 cells transfected with human melanocortin receptors [7] 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 (50,000 cells/well). For the assay, the cell culture medium was removed and the cells were rinsed with 100 μL of MEM buffer (GIBCO). An aliquot (100 μL) of the Earle's balanced salt solution with 5 nM isobutylmethylxanthine (IBMX) was placed in each well along for 1 min at 37 °C. Next, aliquots (25 μL) of melanotropin peptides of varying concentration were added, and the cells were incubated for 3 min at 37 °C. The reaction was stopped by aspirating the assay buffer and adding 60 μL ice-cold Tris/EDTA buffer to each well, then placing the plates in a boiling water bath for 7 min. The cell lysates were then centrifuged for 10 min at 2300 × *g*. A 50 μL aliquot of the supernatant was transferred to another 96-well plate and placed with 50 μL [^{3}H] cAMP and 100 μL protein kinase A (PKA) buffer in an ice bath for 2–3 h. The PKA buffer consisted of Tris/EDTA buffer with 60 μg/mL PKA and 0.1% bovine serum albumin by weight. The incubation mixture was filtered through 1.0 μm glass fiber filters in MultiScreen^{TM}-FB 96-well plates (Millipore, Billerica, MA). The total [^{3}H] cAMP was measured by a Wallac MicroBeta TriLux 1450 LSC and Luminescence Counter (PerkinElmer Life Science, Boston, MA). The cAMP accumulation data for each peptide analogue was determined with the help of a cAMP standard curve generated by the same method as described above. IC_{50} and EC_{50} values represent the mean of two experiments performed in triplicate. IC_{50} and EC_{50} estimates and their associated standard errors were determined by fitting the data using a non-linear least squares analysis, as implemented in GraphPad Prism 4 (GraphPad Software, San Diego, CA). The maximal cAMP produced at 10 μM concentration of each ligand was compared to the amount of cAMP produced at 10 μM concentration of the standard agonist MT-II, and is expressed in per cent (as % max effect) in Table 2. The antagonist properties of the lead compounds were evaluated by their ability to competitively displace the MT-II agonist in a dose-dependent manner, at up to 10 μM. The pA_2 values were obtained using the Schild analysis method [84].

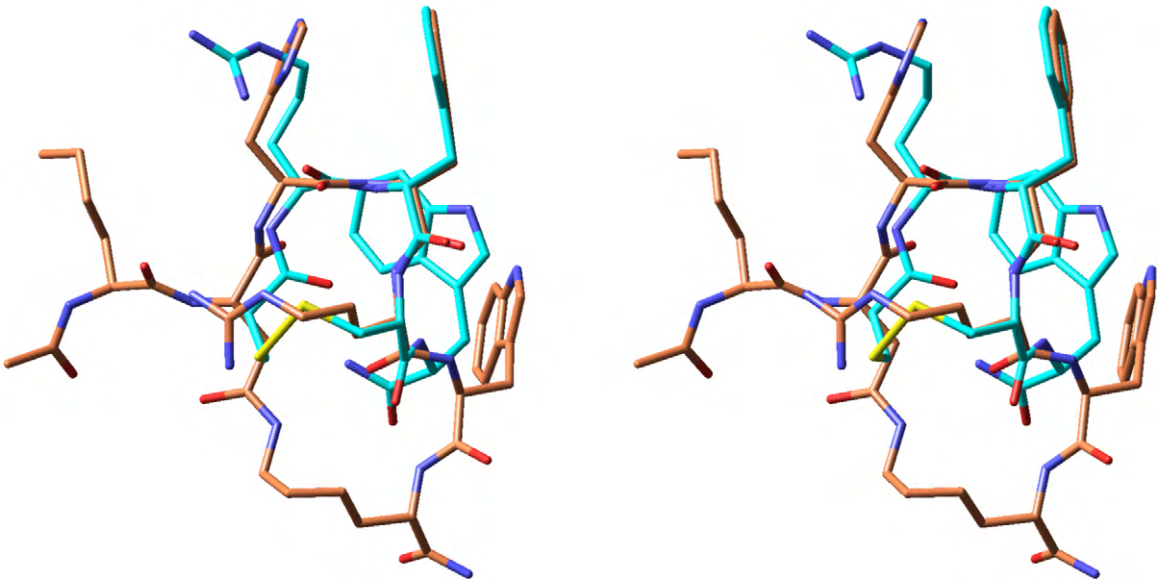


Fig. 2. Stereo view of the superimposed global minimum of analogue **6** (blue) obtained by MCLM (Monte Carlo/Low Frequency Mode)/OPLS_2005 (GB/SA) simulation with the NMR structure of non-selective super-agonist MT-II (gold) (rmsd = 0.12 Å, non-hydrogen backbone atoms of the Xaa-*D*-Phe-Yaa-Trp pharmacophores only). Hydrogens 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.)

2.8. Computational procedures

Molecular modeling experiments employed MacroModel version 9.1 equipped with Maestro 7.5 graphical interface (Schrödinger, LLC, New York, NY, 2005) installed on a Linux Red Hat 9.0 system, and were performed as previously described [3,64,65]. Peptide structures were built into extended structures with standard bond lengths and angles, and they were minimized using the OPLS_2005 force field [51] and the Polak–Ribier conjugate gradient (PRCG). Optimizations were converged to a gradient RMSD less that 0.05 kJ/Å 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) [87]. Extended cut-off distances were defined at 8 Å for Van der Waals, 20 Å for electrostatics and 4 Å for H-bonds.

Conformational profiles of the cyclic peptides were investigated by the hybrid Monte Carlo/Low Frequency Mode (MCMM/LMCS) [56] procedure as implemented in Macromodel using the energy minimization parameters as described above. MCMM torsional variations and Low Mode parameters were set up automatically within Maestro graphical user interface. A total of 20,000 search steps were performed and the conformations with energy difference of 50 kJ/mol from the global minimum were saved. The superimpositions of peptide structures were performed using the α-carbons of the core sequences Arg-Phe-Xaa-Trp and His-*D*-Phe-Arg-Trp.

3. Results

3.1. Design of the MSH/ASIP hybrid pharmacophore

The design of the MSH/ASIP hybrid pharmacophore capitalized on the reported 3D NMR structures of ASIP [67] and the cyclic α-MSH analogue MT-II [104]. Both structures feature a β-turn-like motif within the pharmacophore region, which spans over the first two residues in the His-D-Phe-Arg-Trp MSH pharmacophore, and the over the last two residues of the Arg-Phe-Phe-Arg ASIP pharmacophore. The chosen approach to the hybridization of these two seemingly distinct tetrapeptide sequences involved replacement of His^6 and Arg^8 residues within the MSH pharmacophore with Arg and Xaa, respectively, where the Xaa was to be an amino acid with functionality suitable for macrocyclization of the hybrid peptide analogue, *i.e.*, a "scaffolding" residue. Such hybridization would maintain the order of amino acid functionality characteristic for the agouti proteins (Arg-aromatic AA-aromatic AA), while preserving the secondary structure and the spacing between the aromatic amino acids observed in MSH analogues. The global constraint was applied *via* macrocyclization using a cyclic disulfide template similar to the one reported previously by our laboratories to furnish a highly hMC4R-selective allosteric antagonist series [103], and a lactam cyclodimerization. This design approach was expected to result in the series of hybrid MSH/ASIP analogues that would retain some biological properties of both parent compounds, leading to novel SAR trends and new potent and highly selective melanotropin peptides.

3.2. Molecular modeling

The hybrid Monte Carlo/Low Frequency Mode (MCMM/LMCS) [56] simulations employed the OPLS_2005 force field [51] and GB/SA implicit aqueous solvation model [87], as implemented in Macromodel v9.1 modeling software package, and predicted the expected β-turn-like secondary structure centered over the Arg-Phe residues in the cyclic disulfide series, similar to the 3D structures of the previously reported cyclic disulfide N^{α}-guanidinylbutyl-Cys^8 analogues of α-MSH, obtained by simulated annealing-MD/OPLS-AA experiments [103]. Comparison of the structures of the hybrid ASIP/MSH peptides and the cyclic α-MSH analogue MT-II revealed that the binding space of Arg^8 (MT-II) is blocked in the new peptides by the disulfide bridge (Fig. 2). While steric hindrance of Arg^8 binding space may be beneficial for development of highly selective melanocortin peptides, as demonstrated by our earlier reports [64,65], we recognized that it could also impair the receptor–ligand interactions and lead to a decline in binding affinity and agonist potency.

The computational experiments predicted the cyclodimeric peptides to possess C_2-symmetrical secondary structures, stabilized by two β-turns, also centered at the Arg-Phe residues (Fig. 3). The presence of the β-turn feature in the 3D structures of the designed peptides, requisite for the melanocortin receptor recog-

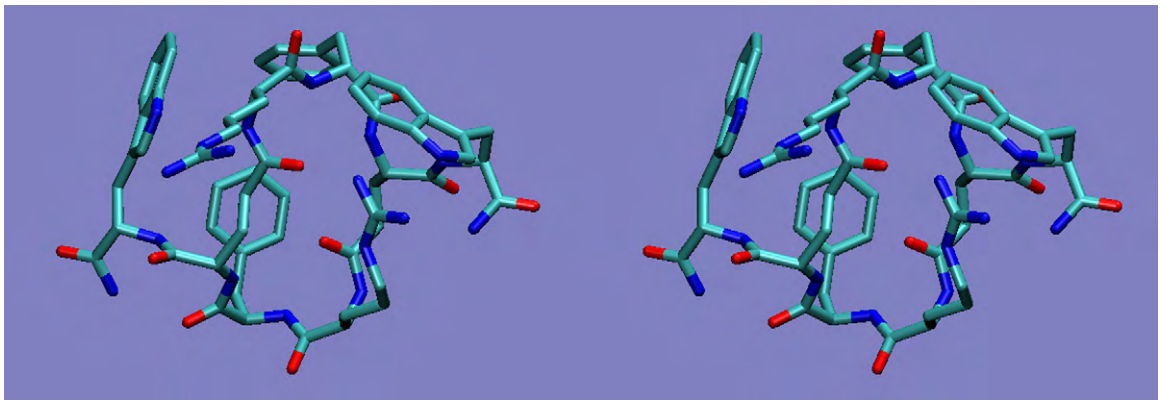
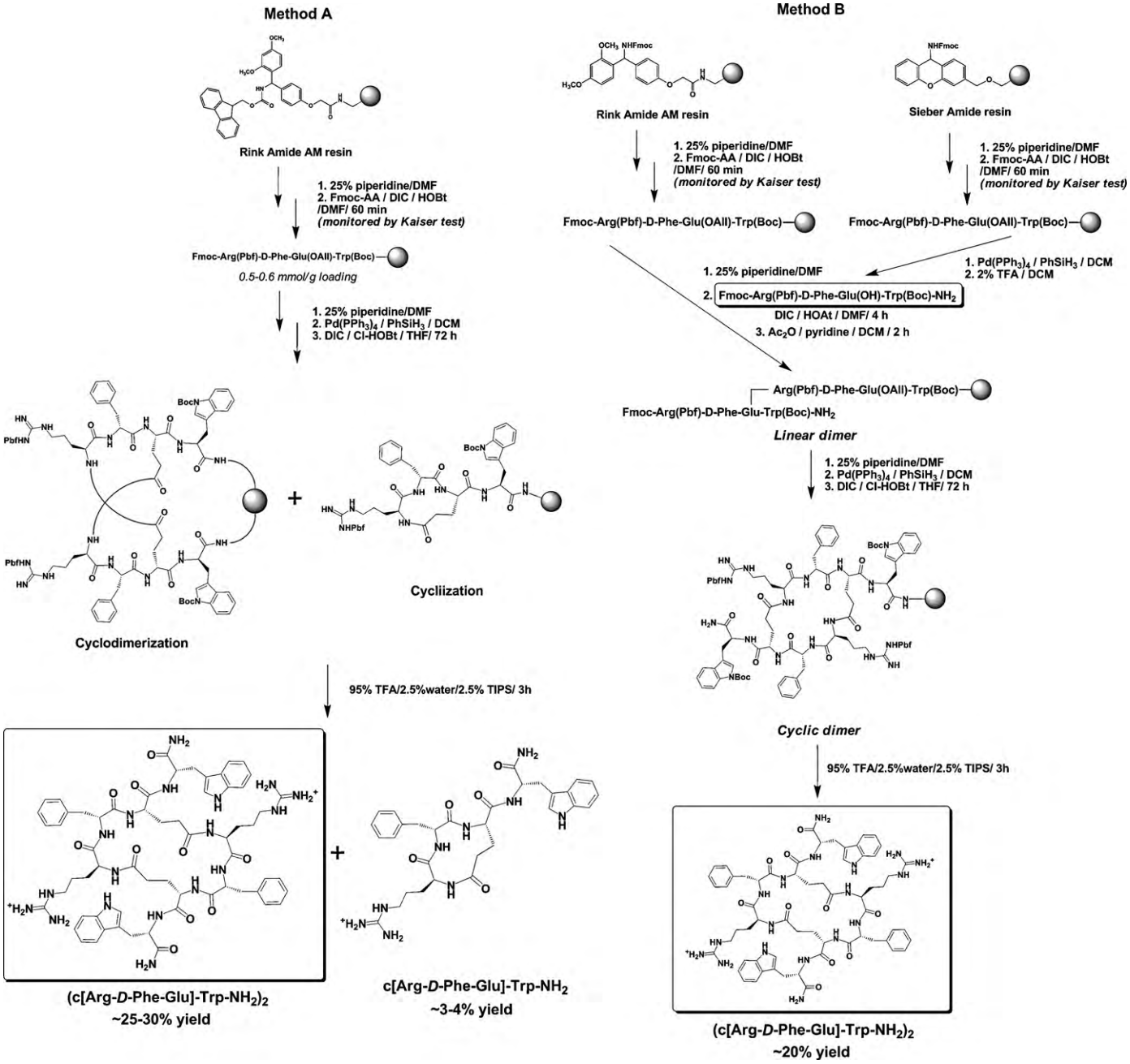


Fig. 3. Stereo view of the global minimum of analogue **12**, obtained by MCMM/LMCS (Monte Carlo multiple minima-low frequency mode)-OPLS_2005 (GB/SA) simulations. Hydrogens are omitted for clarity.



Scheme 2. Solid-phase synthesis of cyclodimeric α-MSH/ASIP hybrid peptides.

Table 1
Sequences and the physicochemical properties of the cyclic homodimeric α-MSH/AgRP analogues.

No.	Sequence	*m/z* (M+H$^+$)		*m/z* (M+2H$^+$)		HPLC retention time, min[a]		TLC R_f[b]	
		Calcd.	Obsd. (ESI)	Calcd.	Obsd. (ESI)	1	2	1	2
1	Ac-c[Cys-Arg-Phe-Cys]-Trp-NH_2	753.2965	753.2934	–	–	15.667	24.958	0.64	0.38
2	Ac-c[Cys-Arg-*D*-Phe-Cys]-Trp-NH_2	753.2965	753.2931	–	–	14.742	23.517	0.64	0.41
3	Ac-c[Cys-Arg-*D*-Phe-Cys]-*D*-Trp-NH_2	753.2965	753.2939	–	–	14.567	23.650	0.63	0.39
4	Ac-c[Cys-Arg-Phe-Cys]-*D*-Trp-NH_2	753.2965	753.2946	–	–	15.725	25.675	0.61	0.43
5	c[S(CH_2)$_2$CO-Arg-Phe-Cys]-Trp-NH_2	696.2750	696.2716	–	–	16.525	25.583	0.64	0.47
6	c[S(CH_2)$_2$CO-Arg-*D*-Phe-Cys]-Trp-NH_2	696.2750	696.2733	–	–	15.283	23.792	0.65	0.45
7	c[S(CH_2)$_2$CO-Arg-*D*-Phe-Cys]-*D*-Trp-NH_2	696.2750	696.2723	–	–	15.050	23.358	0.64	0.43
8	c[S(CH_2)$_2$CO-Arg-Phe-Cys]-*D*-Trp-NH_2	696.2750	696.2733	–	–	16.350	24.967	0.64	0.41
9	c[Arg-*D*-Phe-Glu]-Trp-NH_2	618.3152	618.3161	–	–	14.700	24.892	0.54	0.35
10	c[Arg-*D*-Phe-Glu]-*D*-Trp-NH_2	618.3152	618.3159	–	–	14.825	23.533	0.53	0.34
11	(c[Arg-Phe-Glu]-Trp-NH_2)$_2$	1235.6227	1235.6351	618.3153	618.3221	17.200	28.083	0.57	0.34
12	(c[Arg-*D*-Phe-Glu]-Trp-NH_2)$_2$	1235.6227	1235.6264	618.3153	618.3180	17.325	26.642	0.62	0.34
13	(c[Arg-*D*-Phe-Glu]-*D*-Trp-NH_2)$_2$	1235.6227	1235.6173	618.3153	618.3127	17.292	26.858	0.61	0.33
14	(c[*D*-Arg-Phe-Glu]-Trp-NH_2)$_2$	1235.6227	1235.6046	618.3153	618.3181	17.283	27.450	0.56	0.33
15	(c[Arg-Nal(2′)-Glu]-Trp-NH_2)$_2$	1335.6534	1335.6915	668.3306	668.3377	19.650	31.400	0.61	0.34
16	(c[Arg-*D*-Nal(2′)-Glu]-Trp-NH_2)$_2$	1335.6534	1335.6729	668.3306	668.3323	19.742	31.442	0.66	0.41
17	(c[*D*-Phe-Arg-Glu]-Trp-NH_2)$_2$	1235.6227	1235.6147	618.3153	618.3183	16.958	27.875	0.59	0.33
18	(c[*D*-Nal(2′)-Arg-Glu]-Trp-NH_2)$_2$	1335.6534	1335.6855	668.3306	668.3360	20.042	32.000	0.66	0.40

[a] HPLC column: Vydac 218TP104, 250 mm × 4.6 mm, 10 μm, 300 Å; HPLC solvent system 1: solvent A, 0.1% TFA in water; solvent B, 0.09% TFA in acetonitrile; gradient: 10–90% B in A over 40 min, flow rate 1.0 mL/min; HPLC solvent system 2: solvent A, 0.1% TFA in water; solvent B, 0.09% TFA in methanol; gradient: 10–90% B in A over 40 min, flow rate 1.0 mL/min.
[b] TLC system 1: *n*-butanol/acetic acid/water/pyridine (4:1:2:1); TLC system 2: *n*-butanol/acetic acid/water (4:1:1).

nition, binding and receptor activation [92,104], encouraged us to synthesize several analogues using both monomeric cyclic disulfide and cyclodimeric lactam templates, and explore the dihedral space of these new peptides *via D*-amino acid substitutions of the Arg, Phe, and Trp residues.

3.3. Chemistry

The peptides of the cyclic disulfide series were synthesized by standard Fmoc/*t*Bu chemistry on Rink amide AM resin. TFA deprotection/cleavage off the solid support provided the crude linear peptides, which were cyclized by air oxidation in 0.01 M ammonium acetate buffer at pH 7.5. The peptide lactam cyclodimerizations were achieved by construction of the linear tetrapeptide sequences on polystyrene-based Rink amide AM resin at 0.5–0.6 mmol/g loading, consecutive removal of the *N*-terminal Fmoc and Glu(OAll) protecting groups, and exposure of the deprotected resin-bound peptide to DIC/6-Cl-HOBt in THF over 72 h (Scheme 2). This procedure resulted in a predictably low yield (<5%) of the monomeric cyclic tetrapeptide products due to the high strain within their 11-membered lactam ring [57], and led predominantly to the corresponding 22-membered cyclodimers, which were isolated in 25-30% yields after TFA cleavage off the solid support and HPLC purification (Scheme 2, method A).

The cyclodimeric structure was assigned to these peptides on the basis of HR-MS and ^{1}H NMR data (Table 1, Fig. 4), and unequivocally confirmed through the targeted synthesis of analogue **12** (Scheme 2, method B). The partially protected Fmoc-Arg(Pbf)-*D*-Phe-Glu(OH)-Trp(Boc)-NH_2 tetrapeptide was obtained by constructing the sequence on highly acid-labile Sieber amide resin (9-Fmoc-amino-xanthen-3-yloxy-Merrifield resin) [86], Pd^0-mediated deprotection of the Glu side chain [88], and cleavage of the resulting carboxylic acid peptide off the solid support with 2% TFA/1% TIPS in dichloromethane. In parallel, the same sequence was prepared on Rink amide AM resin, its N^{α}-terminal Fmoc protection was removed, and the crude partially protected tetrapeptide Fmoc-Arg(Pbf)-*D*-Phe-Glu(OH)-Trp(Boc)-NH_2 was coupled onto the exposed N^{α}-terminal amino group with DIC/HOAt over 4 h, followed by capping of the remaining amino groups with acetic anhydride to preclude cyclodimerization of the residual monomeric peptide during the cyclization of the dimer. The *N*-terminal Fmoc and Glu side chain allyl ester protecting groups were removed and macrocyclization was induced with DIC/Cl-HOBt. TFA cleavage/deprotection followed by HPLC purification gave the cyclodimeric analogue **12**, which was chromatographically and spectroscopically undistinguishable from the peptide obtained through a direct on-resin cyclodimerization thus unambiguously establishing its intrinsically anti-parallel cyclodimeric structure (Fig. 4).

3.4. Competition binding and cAMP assays

Biological evaluation of the ASIP/α-MSH hybrid peptides was performed using human melanocortin receptors (hMC1R, hMC3-5R) expressed in whole HEK293 cells, as summarized in Table 2. The monomeric cyclic disulfide analogues **1**–**4** showed no binding affinity or receptor activation at the hMC1R, hMC4-5R. At the same time, these peptides exhibited weak binding affinity to the hMC3R within the 0.42–5.00 μM range, and competitively displacing only 35-59% of ^{125}I-labeled NDP-α-MSH, similar to the previous precedents [10,103]. In addition, analogue **2** exhibited a weak partial agonist activity at the hMC3R (EC_{50} = 2.4 μM, 28% maximal cAMP). Replacement of the chiral *N*-acetyl-Cys linker arm in the cyclic disulfide template with an achiral β-mercaptopropionic acid (Mpa) linker arm (analogues **5-8**) resulted in a complete loss of both binding affinity and agonist activity at all four receptor subtypes.

The small quantities of the monomeric cyclic lactam analogues **9** and **10**, isolated as by-products of direct on-resin cyclodimerizations leading to the peptides **12** and **13**, were examined in binding and cAMP assays, and were found to possess neither binding affinity nor agonist activity at all four human melanocortin receptor subtypes.

The all *L*-diastereomer **11** showed faint non-competitive binding affinity at the hMC5R (IC_{50} = 2.6 μM) and a weak partial agonist activity at the hMC1R (EC_{50} = 2.5 μM, 25% maximal cAMP). The *D*-Phe^7 analogue **12** displayed an improved competitive binding at the hMC1R and the hMC4R (IC_{50} = 1.9 μM and 1.4 μM, respectively), while binding at the hMC3R and the hMC5R remained apparently non-competitive (IC_{50} = 2.9 μM, 45 and 62% binding efficiency, respectively). At the same time, the cAMP assay revealed a nanomo-

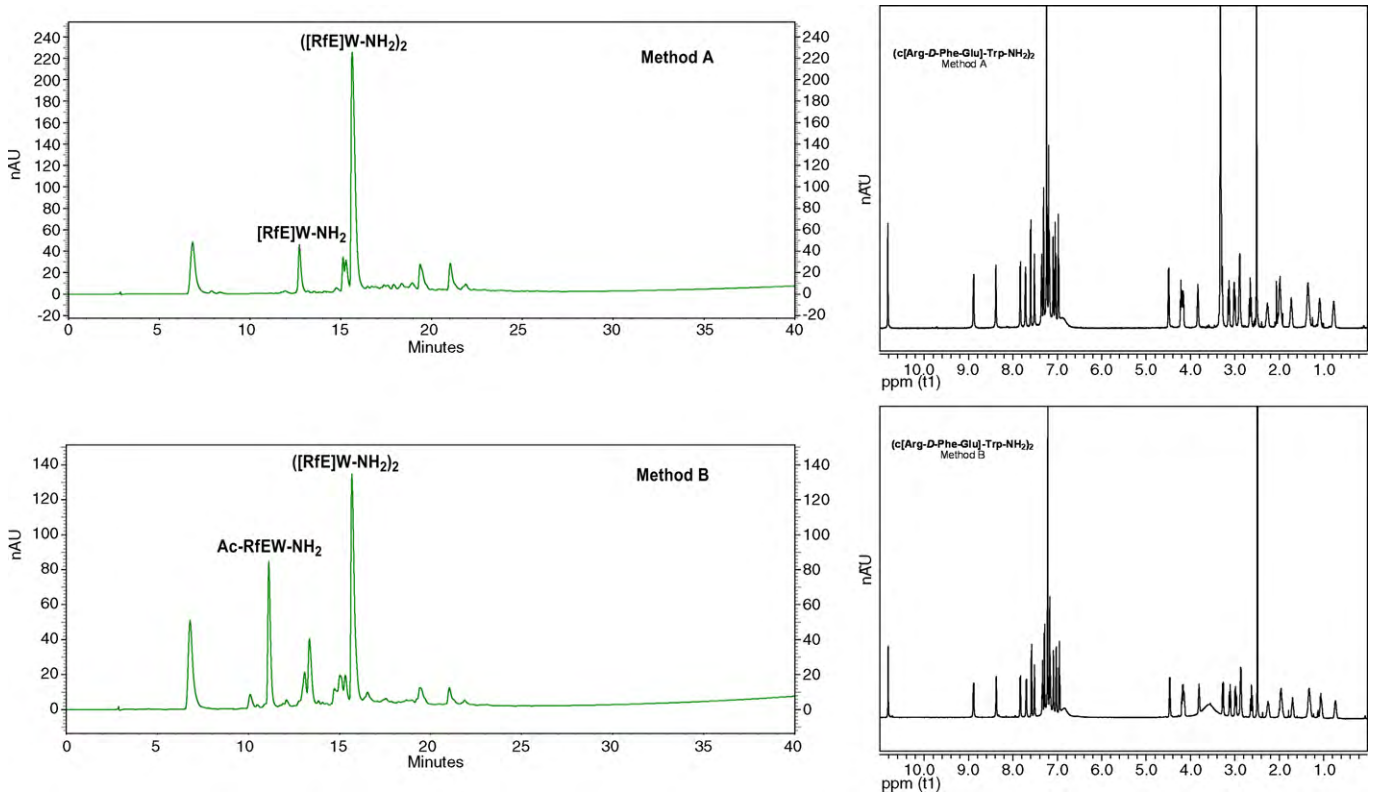


Fig. 4. HPLC chromatograms of the crude cyclodimeric peptide **12** synthesized by methods A and B. ^{1}H NMR spectra of the purified cyclodimeric peptide **12** synthesized by methods A and B.

lar range full agonist activity at the hMC1R (EC_{50} = 120 nM) and partial agonist activity at the hMC4R (EC_{50} = 22 nM, 46% cAMP stimulation), and weak partial agonist activities at the other receptor subtypes (hMC3R: EC_{50} = 1.7 μM, 70% cAMP stimulation; hMC5R: EC_{50} = 1.6 μM, 59% cAMP stimulation). A similar pharmacological profile was observed for the *D*-Phe^{7}, *D*-Trp^{9} analogue **13**, which exhibited weak micromolar range binding at four receptor subtypes, weak partial agonist activity at the hMC3R (EC_{50} = 4.2 μM, 60% cAMP stimulation) and the hMC5R (EC_{50} = 2.4 μM, 72% cAMP stimulation), and nanomolar range partial agonist activity at the hMC1R (EC_{50} = 240 nM, 75% cAMP stimulation) and the hMC4R (EC_{50} = 26 nM, 40% cAMP stimulation).

D-Arg^{6} substitution (analogue **14**) resulted in slightly improved non-competitive binding affinities at the receptor subtypes, compared to the *L*-diastereomeric peptide **11**. The weak hMC1R agonist activity observed in analogue **11** also substantially increased in analogue **14** to yield a potent (EC_{50} = 25 nM, 43% cAMP stimulation) and highly selective (>400-fold) hMC1R partial agonist.

Phe^{7} → Nal(2′)^{7} substitutions (analogues **15** and **16**) resulted in improved competitive binding affinities, compared to the Phe^{7}/*D*-Phe^{7} peptide analogues **11** and **12**, with the *D*-Nal(2′)^{7} peptide **16** showing IC_{50} values in the high nanomolar range of 360–800 nM. Expectedly, the hMC1R partial agonist activity was retained in these two peptides (analogue **15**: EC_{50} = 470 nM, 34% cAMP stimulation; analogue **16**: EC_{50} = 25 nM, 55% cAMP stimulation), although selectivity was lost, with partial agonist activity detected at the hMC3R and the hMC5R.

Finally, the effects of cyclodimerization were examined on the MSH pharmacophore *D*-Phe-Arg-Trp through inserting of a Glu linker arm between Arg^{8} and Trp^{9} and subjecting the resulting *D*-Phe^{7}-Arg^{8}-Glu^{9}-Trp^{10} sequence to cyclodimerization. The obtained 22-membered cyclodimeric peptide **17** showed good partial non-competitive binding at the hMC1R (IC_{50} = 120 nM, 22% binding efficiency), the hMC3R (IC_{50} = 80 nM, 23% binding efficiency) and the hMC5R (IC_{50} = 450 nM, 45% of ^{125}I-NDP-α-MSH displaced), and retained a weak partial agonist activity at the hMC1R (EC_{50} = 2.7 μM, 42% cAMP stimulation). The *D*-Nal(2′)^{7} analogue **18** showed diminished, albeit competitive, micromolar range receptor binding, and slightly improved nanomolar range partial agonist activity at the hMC1R (EC_{50} = 200 nM, 42% cAMP stimulation).

4. Discussion

The outcome of the biological evaluation of analogues **1–18** was analyzed from the perspective of the performance of the hybrid MSH/ASIP pharmacophore in monomeric cyclic disulfide and lactam templates, as well as the impact of its cyclodimerization on the biological profiles of the resulting cyclodimeric peptides. The weak non-competitive binding affinities and micromolar range partial agonist activities determined for the monomeric cyclic disulfide analogues **1-8** suggest that the designed ASIP/MSH hybrid pharmacophore in this template may not be sufficient to induce a potent binding affinity and agonist activity at the human melanocortin receptors-1, -4, and -5, while possessing a potential for development of non-competitive (possibly, allosteric) molecular probes for the hMC3R. Also, the observed strong influence of the linker arm structure on the peptide binding affinity is completely congruent with our previous reports [52,65,66].

Furthermore, the monomeric cyclic lactam analogues **9** and **10** exhibited no binding affinity or agonist activity at the hMC1,3-5R, and one possible explanation for this result was found by examination of ^{1}H NMR spectra of these peptides. The complex ^{1}H NMR spectra of the monomeric cyclic lactams (Fig. 5) indicates a presence of three possible *cis*/*trans* ω-rotamers, in dynamic equilibrium with one another, as could be expected from a dihedrally

Table 2
Binding affinities and cAMP activities of cyclic and cyclodimeric α-MSH/ASIP hybrid analogues at hMCRs.[a]

No.	Structure	hMC1R				hMC3R				hMC4R				hMC5R			
		IC_{50} (nM)	% BE	EC_{50} (nM)	Act%	IC_{50} (nM)	% BE	EC_{50} (nM)	Act%	IC_{50} (nM)	% BE	EC_{50} (nM)	Act%	IC_{50} (nM)	% BE	EC_{50} (nM)	Act%
1	Ac-c[Cys-Arg-Phe-Cys]-Trp-NH_2	NB	0	NA	0	1200 ± 200	42	NA	0	NB	0	NA	0	NB	0	NA	0
2	Ac-c[Cys-Arg-*D*-Phe-Cys]-Trp-NH_2	NB	0	NA	0	420 ± 30	45	2400 ± 300	28	NB	0	NA	0	NB	0	NA	0
3	Ac-c[Cys-Arg-*D*-Phe-Cys]-*D*-Trp-NH_2	NB	0	NA	0	>5000	35	NA	0	NB	0	NA	0	NB	0	NA	0
4	Ac-c[Cys-Arg-Phe-Cys]-*D*-Trp-NH_2	NB	0	NA	0	1500 ± 130	59	NA	0	NB	0	NA	0	NB	0	NA	0
5	c[$S(CH_2)_2$CO-Arg-Phe-Cys]-Trp-NH_2	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0
6	c[$S(CH_2)_2$CO-Arg-*D*-Phe-Cys]-Trp-NH_2	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0
7	c[$S(CH_2)_2$CO-Arg-*D*-Phe-Cys]-*D*-Trp-NH_2	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0
8	c[$S(CH_2)_2$CO-Arg-Phe-Cys]-*D*-Trp-NH_2	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0
9	c[Arg-*D*-Phe-Glu]-Trp-NH_2	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0
10	c[Arg-*D*-Phe-Glu]-*D*-Trp-NH_2	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0
11	(c[Arg-Phe-Glu]-Trp-NH_2)$_2$	>10,000	59	2500 ± 200	25	>10,000	37	NA	0	>5000	25	NA	0	2600 ± 500	65	NA	0
12	(c[Arg-*D*-Phe-Glu]-Trp-NH_2)$_2$	1900 ± 710	100	120 ± 10	100	2900 ± 400	45	1700 ± 300	70	1400 ± 480	91	22 ± 8	46	2900 ± 400	62	1600 ± 500	59
13	(c[Arg-*D*-Phe-Glu]-*D*-Trp-NH_2)$_2$	1300 ± 330	100	240 ± 42	75	>5000	93	4200 ± 500	60	2800 ± 300	100	26 ± 12	40	500 ± 80	99	2400 ± 300	72
14	(c[*D*-Arg-Phe-Glu]-Trp-NH_2)$_2$	1700 ± 200	60	25 ± 2	43	>5000	30	NA	0	2700 ± 100	75	NA	0	110 ± 10	47	NA	0
15	(c[Arg-Nal(2′)-Glu]-Trp-NH_2)$_2$	2700 ± 600	94	470 ± 20	34	4700 ± 500	85	1300 ± 210	28	3800 ± 400	100	NA	0	2300 ± 400	95	500 ± 60	11
16	(c[Arg-*D*-Nal(2′)-Glu]-Trp-NH_2)$_2$	460 ± 34	96	25 ± 2	55	580 ± 87	86	820 ± 80	20	360 ± 82	100	NA	0	800 ± 80	100	65 ± 21	16
17	(c[*D*-Phe-Arg-Glu]-Trp-NH_2)$_2$	120 ± 20	22	2700 ± 300	42	80 ± 36	23	NA	0	>10,000	40	NA	0	450 ± 50	45	NA	0
18	(c[D-Nal(2′)-Arg-Glu]-Trp-NH_2)$_2$	>5000	78	200 ± 30	42	3000 ± 360	78	NA	0	1900 ± 230	95	NA	0	>5000	88	NA	0
MT-II	Ac-Nle-c[Asp-His-*D*-Phe-Arg-Trp-Lys]-NH_2	2.3 ± 0.9	100	1.02 ± 0.4	100	8.0 ± 1.3	100	5.1 ± 0.3	100	2.3 ± 0.85	100	2.1 ± 0.6	100	4.2 ± 1.3	100	5.7 ± 2.2	100

[a] IC_{50} = concentration of peptide at 50% specific binding ($N = 4$). NB = 0% of ^{125}I-NDP-α-MSH displacement observed at 10 μM. % BE = maximal % of ^{125}I-NDP-α-MSH displacement observed at 10 μM. EC_{50} = Effective concentration of peptide that was able to generate 50% maximal intracellular cAMP accumulation ($N = 4$). Act% = % of cAMP produced at 10 μM ligand concentration, in relation to MT-II. NA = 0% cAMP accumulation observed at 10 μM. The peptides were tested at a range of concentration from 10^{-10} to 10^{-5} M.

Fig. 5. The convoluted ^{1}H NMR spectra of the monomeric cyclic lactam **9** indicates presence of three possible *cis*/*trans* ω-rotamers.

strained 11-membered lactam ring [57]. The lack of a well-defined secondary structure in the monomeric cyclic lactam peptides may impair receptor–ligand interaction, and, by the same token, the less strained larger macrocycles may have more stable secondary structures, as evident from the ^{1}H NMR spectra of the cyclodimeric peptides (such as analogue **12**, Fig. 4), which would lead to augmented receptor binding affinity and agonist potency. This hypothesis was put to the test in screening of the biological properties of the cyclodimeric peptides **11–18**. Indeed, the cyclodimeric analogues exhibited a marked improvement in their binding affinities as well as displayed nanomolar range full and partial agonist activities at the hMC1R and the hMC4R. Thus, the designed ASIP/MSH hybrid pharmacophore in the cyclodimeric template clearly favors the hMC1R and the hMC4R subtypes, which may be due to the tight secondary structure of the cyclodimeric template (Fig. 3), unencumbered by steric or hydrophobic influence of the neighboring amino acids, which has been previously shown to favor the hMC3R and the hMC5R, and produce agonists and antagonists selective for these receptor subtypes [3,7,64,65].

D-substitution in the position 6 (analogue **14**), normally associated with complete loss of all binding affinity and potency in MSH-derived templates [37], in the ASIP/MSH hybrid template led to somewhat improved non-competitive binding affinities at the receptor subtypes, compared to the *L*-diastereomeric peptide **11**. This finding suggests that the secondary structure requirements for non-competitive (allosteric) agonists are not limited to a particular type of β-turn, as is the case for orthosteric agonists derived from MSH peptides [104].

$Phe^7 \rightarrow Nal(2')^7$ substitutions in MSH peptides are generally known to lead to enhanced binding affinity and conversion of hMC3R/hMC4R agonists into antagonists, while retaining hMC1R/hMC5R agonist activities [42]. Similar substitutions in the ASIP/MSH hybrid pharmacophore-based cyclodimeric peptides (analogues **15** and **16**) indeed resulted in improved competitive binding affinities, compared to the Phe^7/*D*-Phe^7 peptide analogues **11** and **12**, with the *D*-$Nal(2')^7$ peptide **16** showing IC_{50} values in the high nanomolar range of 360-800 nM, while retaining nanomolar range hMC1R partial agonist activity. This SAR trend is in a good agreement with our earlier reports, which linked the augmented binding affinity with increased hydrophobicity of the $Nal(2')^7$/*D*-$Nal(2')^7$ peptides [64,65].

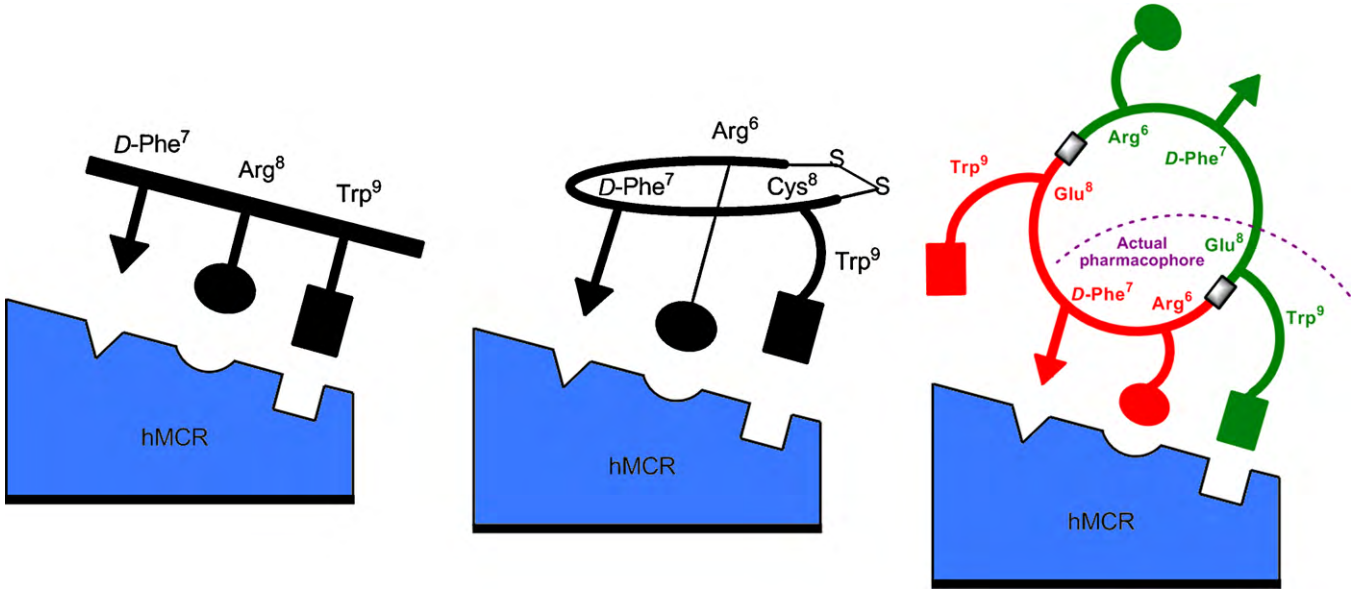


Fig. 6. Schematic representations of putative receptor–ligand interactions of α-MSH-derived peptides, monomeric ASIP/MSH hybrid peptide **6**, and a cyclodimeric peptide **12**, showing one possible mode of receptor binding.

The MSH pharmacophore-bearing peptides **17** and **18** showed reasonable (high nanomolar range) non-competitive binding affinities at the hMC1,3,5R but unremarkable (micromolar range) partial agonist activities at the hMC1R. This result indicates that the cyclodimeric template described in this report was perhaps unable to maintain the optimal topography for the MSH pharmacophore. A more in-depth *D*-amino acid scan of the template will allow to determine the full scope of its capabilities.

In summary, the design of a novel ASIP/MSH hybrid pharmacophore has yielded peptides with hMC3R-selective non-competitive binding affinities in the monomeric cyclic disulfide series. The reported direct on-resin peptide cyclodimerization yielded nanomolar range (25–120 nM) hMC1R-selective full and partial agonists in the cyclodimeric lactam series, which represents a 25-120-fold lower agonist potency than that of the super-agonist MT-II but demonstrates an improvement over the previous attempts at hybridization of MSH and agouti protein sequences [46]. Among the possible reasons for the acquired agonist activity are the well-established factors, such as increased charge [64] and increased hydrophobicity [64,65], or thermodynamic aspects, such as the rotational entropy factor [2,78]. In addition, a possibility that receptor recognition occurs at an overlap region of the dimeric pharmacophore structures (Fig. 6) cannot be discounted.

5. Conclusions

The direct on-resin peptide lactam cyclodimerization described in this report was found to be quite reliable and reproducible, depending little on the amino acid configuration. Molecular modeling and ^{1}H NMR experiments revealed high dihedral strain in the monomeric cyclic lactam structures, which is likely responsible for the observed predominant cyclodimerization pathway under the conventional solid-phase lactam cyclization conditions. The resulting C_2-symmetrical cyclodimeric peptides bearing the ASIP/MSH hybrid pharmacophore were determined to possess nanomolar range partial and full agonist activities at the hMC1R. These findings are consistent with the previous literature reports on agouti-signaling protein/MSH hybrid cyclic peptides, and indicate high potential of such hybrid structures as well as the peptide cyclodimerization for development of potent and selective melanotropin peptides.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.peptides.2010.06.026.

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