Supporting Information

Azepinone-Containing Tetrapeptide Analogues of Melanotropin Lead to Selective *h*MC4R Agonists and *h*MC5R Antagonist

Olivier Van der Poorten,[†,‖] Krisztina Fehér,[‡,‖] Koen Buysse,[†] Debby Feytens,[†] Ioanna Zoi,[§] Steven D. Schwartz,[§] José C. Martins,[‡] Dirk Tourwé,[†] Minying Cai,[§] Victor J. Hruby,[§] Steven Ballet[†,*]

† Department of Organic Chemistry, Vrije Universiteit Brussel, Pleinlaan 2, B-1050, Brussels, Belgium

‡ Department of Organic Chemistry, University of Ghent, Krijgslaan 281 S4, 9000 Ghent, Belgium

§ Department of Chemistry and Biochemistry, University of Arizona, Tucson, AZ, 85721, USA

1. General Information

High Performance Liquid Chromatography (HPLC) analysis of the tetrapeptides **1-5** samples was conducted on a Waters 717 plus autosampler equipped with a Waters 1525 Binary HPLC pump and a Waters 2487 Dual Absorbance Wavelength detector over a Grace Vydac C18 column (25 cm x 4.6 mm x 5 µm). The solvent system consists of 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B). Samples were prepared by dissolving a compound in a 1:1 mixture of acetonitrile and water. The samples were eluted through the column using a gradient from 3% B to 100% B in 20 minutes at a flow rate of 1 mL/min.

MS spectra were recorded on a VG Quattro II mass spectrometer using electrospray ionization (positive or negative ion mode). Data analysis was done with Masslynx 2.2 software. Alternatively MS spectra were recorded on a Micromass QTOF-micro system. Data analysis was done with Masslynx 4.1 software.

Concerning Thin Layer Chromatography (TLC) analysis, a small spot of solution was applied to a glass plate or plastic sheet coated with silica gel $60F_{254}$ (Merck). After the run, the spot was visualized under UV light or by spraying with permanganate solution for non-UV active compounds. The permanganate spray is a solution of $KMnO_4$ (3 g), K_2CO_3 (20 g) and NaOH (0.025 g) in water (300 mL).

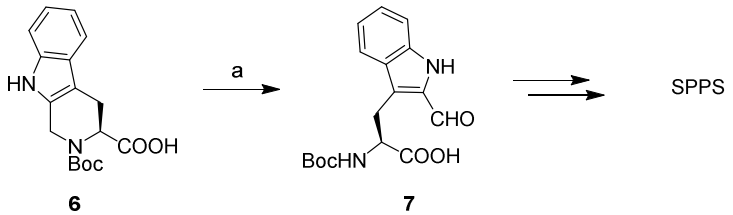
Flash chromatography was performed using Davisil silicagel 60 (0.040-0.063 nm; Grace Davison) as the stationary phase.

Purification of compounds was conducted on a Gilson HPLC system equipped with Gilson 322 pumps and a UV/Vis-156 detector (215 nm) over a Supelco Discovery® BIO Wide Pore C_{18} column (25 cm x 2.21 cm x 5 µm). A flow rate of 20 mL/min was applied. Per injection of 1.8 mL, a maximum of 20 mg of crude product was injected. The collected fractions were lyophilised to obtain the purified compound with a final purity of > 99%.

2. Synthesis of Melanocortin Tetrapeptides 1-5

*2.1 Aia Peptides **1-3***

The synthesis of the Aia containing tetrapeptides **1-3** was performed according to a previously described solid phase methodology (**Scheme 1**).[1,2] Peptide synthesis was performed applying standard Boc methodology and using MBHA resin as the solid phase carrier. DIC and HOBt in DMF/CH_2Cl_2 (1:1, v/v) were used for standard coupling reactions. Final cleavage of the peptide from the resin was done by treatment with HF_{liq} for 1 h at 0 °C. Anisol (1 mL) and HF_{liq} (10 mL) were used for 1 g peptide resin.

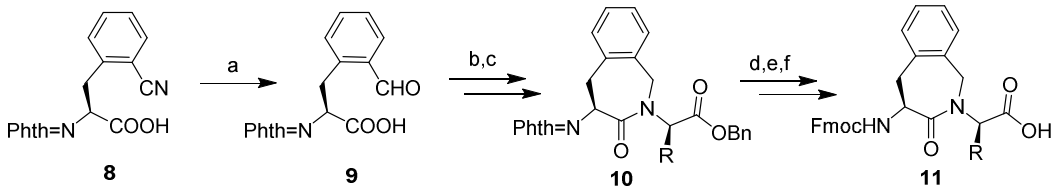


Scheme 1. a) SeO_2 (1.4 equiv), dioxane, reflux.

1. Pulka, K.; Feytens, D.; Van den Eynde, I.; De Wachter, R.; Kosson, P.; Misicka, A.; Lipkowski, A.; Chung, N. N.; Schiller, P. W.; Tourwé, D. Synthesis of 4-Amino-3-Oxo-Tetrahydroazepino[3,4-*b*]indoles: New Conformationally Constrained Trp Analogs. *Tetrahedron* **2007,** *63*, 1459-1466.
2. Feytens, D.; De Vlaeminck, M.; Tourwé, D. A Novel Solid Phase Approach to Aia-Containing Peptides. *J. Pept. Sci.* **2009,** *15*, 16-22.

*2.2 Aba Peptide **4***

Aba analogue **4** was prepared using Fmoc-based SPPS with Fmoc-Aba-*D*-Phe **11** as one of the building blocks (**Scheme 2**).[3]

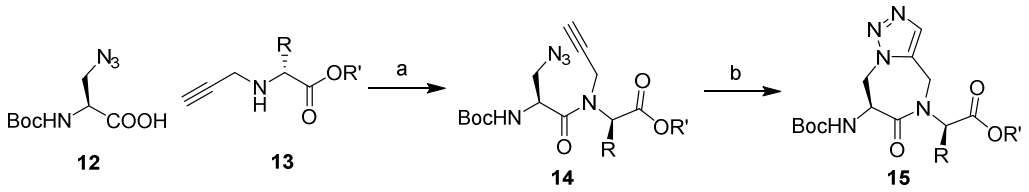


Scheme 2. a) Raney-Ni, H_2 (50 psi), H_2O/AcOH/pyridine (1:1:1, v/v), 50 °C ; b) amino acid benzyl ester HCl, $NaCNBH_3$ (2.5 equiv), $MgSO_4$ anhydrous (4.5 equiv), CH_2Cl_2, *N*-Methylmorpholine pH 6, rt ; c) EDC.HCl (1.5 equiv), pyridine (2.5 equiv), H_2O/acetonitrile (1:1, v/v), rt ; d) H_2 (50 psi), 10% Pd/C, dioxane/H_2O (3:2, v/v) ; e) NH_2NH_2 (6.0 equiv), EtOH, reflux ; f) Fmoc-OSu (1.1 equiv), Na_2CO_3 (1.1 equiv), H_2O/acetone (1:1, v/v), rt.

3. Van Rompaey, K.; Van den Eynde, I.; De Kimpe, N.; Tourwé, D. A Versatile Synthesis of 2-Substituted 4-Amino-1,2,4,5-Tetrahydro-2-Benzazepine-3-Ones. *Tetrahedron* **2003,** *59*, 4421-4432.

*2.3 Ata Peptide **5***

Tetrapeptide **5** contains the Ata scaffold, which was incorporated using standard Boc-based SPPS. The Ata building block **15** was synthesized according to the more recent solution phase protocol reported by Buysse *et al.* (**Scheme 3**).[4]



Scheme 3. a) EDC.HCl (1.5 equiv), HOBt or HOAt (2.5 equiv), DMF, rt ; b) DMF, reflux.

4. Buysse, K.; Farard, J.; Nikolaou, A.; Vanderheyden, P.; Vauquelin, G.; Pedersen, D. S.; Tourwé, D.; Ballet, S. Amino Triazolo Diazepines (Ata) as Constrained Histidine Mimics. *Org. Lett.* **2011,** *13*, 6468-6471.

3. Characterization

Table 1. Characterization of Melanocortin Analogues **1**-**5**.

Peptide	Sequence	t_R (min)	R_f (EBAW[a])	MS $(M+H)^+$	Yield (%)
1	Ac-Aia-*D*-Phe-Arg-Trp-NH_2	13.05	0.52	747.23	19
2	Ac-Aia-*p*F-*D*-Phe-Arg-Trp-NH_2	13.94	0.52	765.36	33
3	Ac-Aia-*p*Br-*D*-Phe-Arg-Trp-NH_2	14.85	0.55	825.09	/
4	Ac-Aba-*D*-Phe-Arg-Trp-NH_2	13.35	0.49	708.12	18
5	Ac-Ata-*D*-Phe-Arg-Trp-NH_2	10.43	0.45	699.27	7

[a] EBAW = ethyl acetate/butanol/acetic acid/water (1:1:1:1, v/v)

4. Binding Assays and Functional Assay

4.1 Competition Binding Experiments

Competition binding experiments were carried out using whole HEK293 cells stably expressing human MC1, MC3, MC4, and MC5 receptors. HEK293 cells transfected with *h*MCRs[5-7] 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 (Perkin-Elmer 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-phenanthroline, 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 x 75 mm borosilicate glass tubes, and the radioactivity was measured by a Wallac 1470 WIZARD Gamma Counter.

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4.2 Adenylate Cyclase Assay

HEK 293 cells transfected with human melanocortin receptors[5-7] were grown to confluence in MEM (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 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 concentrations 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 2300g. 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 MultiScreenTM-FB 96-well plates (Millipore, Billerica, MA). The total [^{3}H]cAMP was measured by a Wallac MicroBeta TriLux 1450 LSC and Luminescence Counter (Perkin-Elmer Life Science, Boston, MA). The cAMP accumulation data for each peptide analogue were determined with the help of a cAMP standard curve generated by the same method as described above.

4.3 IC_{50} and EC_{50}

IC_{50} and EC_{50} values represent the mean of four experiments performed. IC_{50} and EC_{50} estimates were determined by fitting the data using a nonlinear least squares analysis, with the help of GraphPad Prism 4 (GraphPad Software, San Diego, CA).

5. NMR Spectroscopy and Computational Procedures

The NMR samples contained ± 3 mg peptides dissolved in 600µl DMSO-d_6. NMR experiments were performed at 298 K on Bruker Avance II NMR spectrometer operating at 700.13 MHz and 500.13 MHz equipped with a 5 mm inverse TXI-Z probe. Proton and carbon chemical shift scales were calibrated to the DMSO-d_6 solvent signal at 2.500 ppm for ^{1}H and 39.95ppm for ^{13}C. The ^{1}H resonances were assigned using 2D COSY,[8] 2D TOCSY[9] and 2D NOESY[10] experiments in combination with ^{1}H-^{13}C as well as ^{1}H-^{15}N HSQC heteronuclear correlations[11]. The raw datasets typically consisted of 1–2Kx512 complex data points, for processing the spectra were zero filled to produce a 2Kx2K data matrix, multiplied with a squared cosine bell function followed up with automatic base line correction in both dimensions.

All assignments were carried out using the CARA program[12]. The distance constraints were obtained from an off-resonance ROESY spectrum[13] with 300 ms mixing time. The ROE intensities were evaluated from the volume of the cross-peaks and calibrated using the distances between protons of the Trp aromatic protons. ROE cross peaks were assigned manually. The simulation engine CNS 1.21[14] employed simulated annealing algorithm in torsion angle space with a subsequent refinement in explicit DMSO[15].

The CNS force field used for peptides and proteins with explicit hydrogens (protein-allhdg.top, protein-allhdg.param, protein.link) were supplemented with topologies and parameters developed for the explicit Aba, Aia, *D*-Phe and *p*F-*D*-Phe residues as well as for the new type of peptide bond connecting Aba to *D*-Phe and Aia to *p*F-*D*-Phe residues.

For the final structure calculations 65 ROEs for tetrapeptide **2** and 45 ROEs for tetrapeptide **4** were used, respectively. Furthermore 3 homonuclear $^3J_{HN,HA}$ couplings (typically 7 – 9 Hz) were employed for restraining main chain conformations in both cases. An ensemble of 10 structures was generated using acceptance test

selecting structures with no bond length, valence angle, NOE violations and having lowest total internal energies. The accepted structures are clustered on the basis of pairwise RMSD matrix calculated in Pymol[16].

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16. The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC.

6. Molecular Modeling and Ligand Docking

For *h*MC4R the active state model proposed by Mosberg *et al.*[17] was used (*h*MC4R models, http://mosberglab.phar.umich.edu/resources/). The model for *h*MC5R was generated based on 3eml and 2ydo used templates, which are antagonist- and agonist-bound A2A adenosine receptors respectively, by homology modeling using MODELLER 9.11[18] using the Chimera[19] interface. This produced a reasonable *h*MC5R model with unblocked entry to the binding site. Dockings have been carried out with Autodock Vina.[20] The receptor binding poses were analyzed using LigPlot+.[21] Visualization and analysis of the molecules were done in PyMOL.[16]

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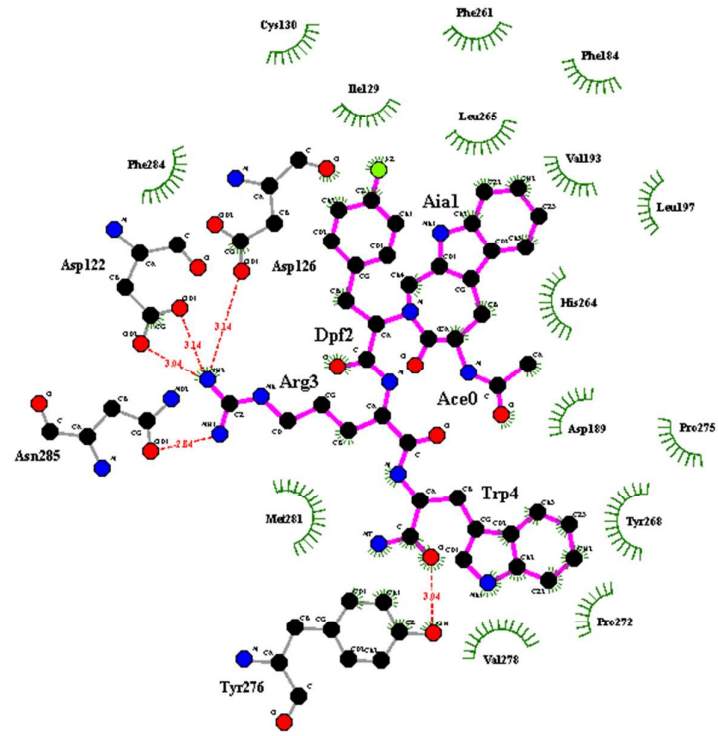
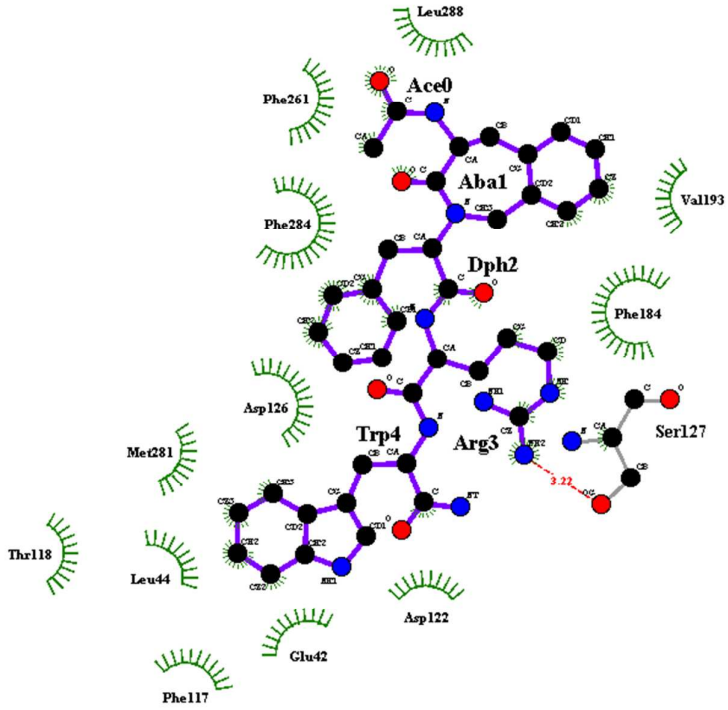
7. 2D Ligand Interaction Maps

Figure 1. 2D ligand-receptor interaction maps in *h*MC4R for **A)** Aia-containing peptide **2**, **B)** Aba-containing peptide **4**, and for **C)** [Nle^4,*D*-Phe^7]-α-MSH. Hydrophobic contacts are colored in green and polar contacts are indicated by a red dashed line (Residue code: Ace = Gly; Aba = aminobenzazepinone; Aia = aminoindoloazepinone; Dph = *D*-Phe; Dpf = *p*F-*D*-Phe)

A)



B)



C)

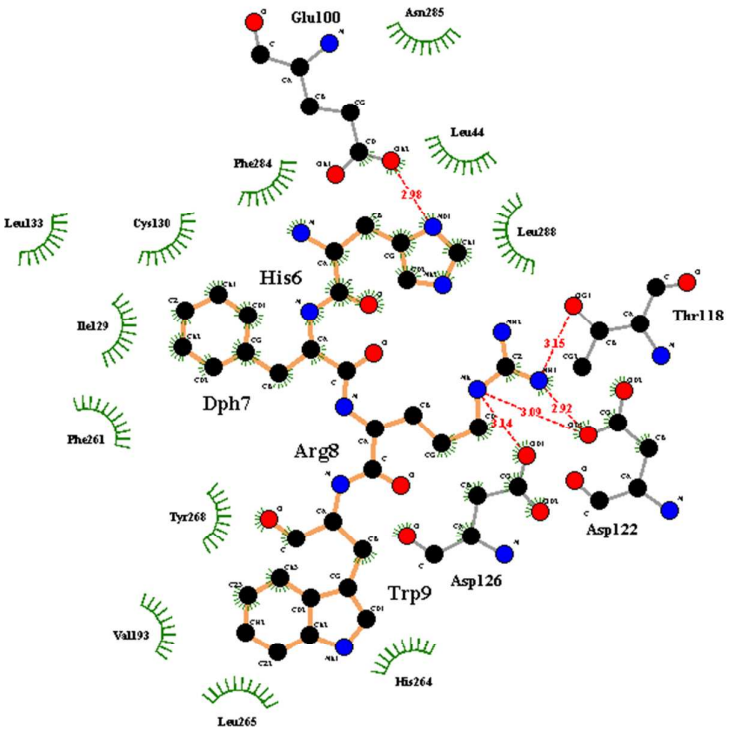
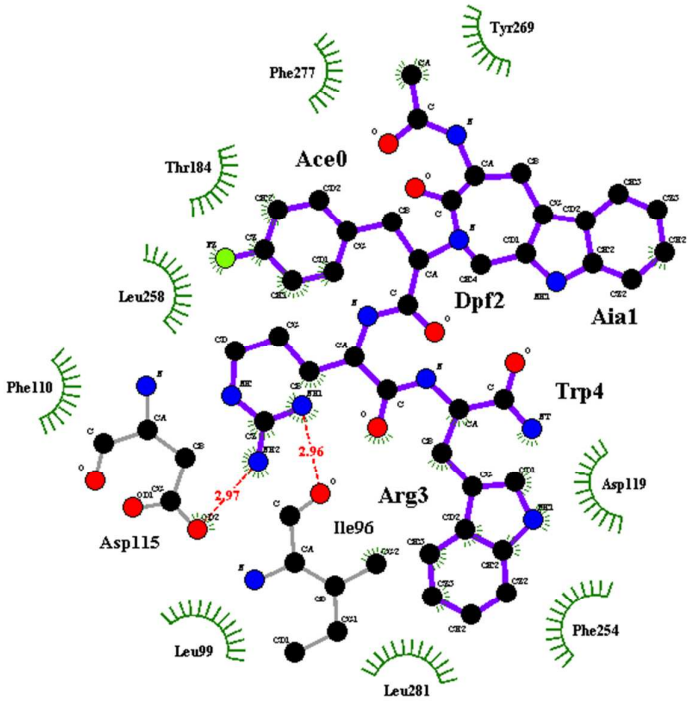

Glu100
Asn285
Leu44
Phe284
2.98
Leu133
Cys130
Leu288
His6
Ile129
Thr118
3.15
Dph7
3.09
2.92
3.14
Phe261
Arg8
Tyr268
Asp122
Asp126
Trp9
Val193
His264
Leu265

Figure 2. 2D ligand-receptor interaction maps in *h*MC5R for **A)** Aia-containing peptide **2**, **B)** Aba-containing peptide **4**. Hydrophobic contacts are colored in green and polar contacts are indicated by a red dashed line (Residue code: Ace = Gly; Aba = aminobenzazepinone; Aia = aminoindoloazepinone; Dph = *D*-Phe; Dpf = *p*F-*D*-Phe)

A)



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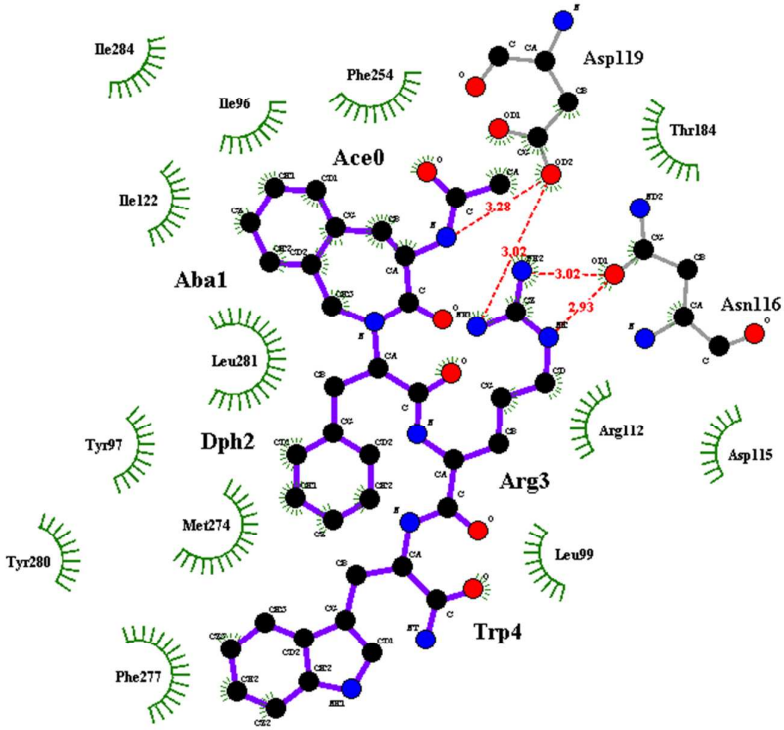

8. Comparison of Binding Pose Interactions

Table 2. Comparison of the *h*MC4R and *h*MC5R ligand-receptor interactions. Hydrophobic contacts are listed as well as polar interactions, the latter are underlined and in *italics*, conserved residues involved in the orthosteric binding are shown in **bold**.

NDP-α-MSH	*h*MC4R	Aia peptide 2	*h*MC4R	*h*MC5R	Aba peptide 4	*h*MC4R	*h*MC5R
		Ace^{0}	Asp189 Tyr268	Tyr269 Phe277	Ace^{0}	**Phe261** Leu288	***Asp119*** Thr184 **Phe254**
His^{6}	Leu44 Asn285 Phe284 ***Glu100***	Aia^{1}	Phe184 Val193 Leu197 **Phe261** **His264** Leu265	Leu99	Aba^{1}	Val193 Phe184	Ile96 Ile122 **Phe254** Ile281 Ile284
Dph^{7}	Ile129 Cys130 Leu133 **Phe261**	Dpf^{2}	Ile129 Cys130 **Asp126** **Phe261** Phe284 **His264**	Thr184 Leu258 **Phe254**	Dph^{2}	**Asp126** Phe284 Leu288	Tyr97 Met274 Phe277 Ile281 Tyr280
Arg^{8}	*Thr118* ***Asp122*** ***Asp126***	Arg^{3}	***Asp122*** ***Asp126*** *Asn285*	Ile96 Ile96 Leu99 Phe110 **Asp115** Leu281	Arg^{3}	**Asp126** Phe184 *Ser127*	Arg112 **Asp115** *Asn116* ***Asp119*** Thr184
Trp^{9}	Val193 **Trp258** **His264** Leu265 Tyr268	Trp^{4}	Tyr268 Pro272 Pro275 Val278	**Asp119** **Phe254** Leu281	Trp^{4}	Glu42 Leu44 Phe117 Thr118 Met281	Met274 Phe277
		NH_2	Tyr276	Leu281	NH_2	Asp129	Leu99
hydrophobic	12		18	14		15	18
H bond	4		3	2		1	3