Synthesis and Pharmacology of α/β^3-Peptides based on the Melanocortin Agonist Ac-His-DPhe-Arg-Trp-NH_2 Sequence

Anamika Singh,[§,¶] Srinivasa R. Tala,[§] Viktor Flores,[¶] Katie Freeman,[§] and Carrie Haskell-Luevano[*,§,¶]

[§]Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, MN 55455, USA; [¶]Department of Pharmacodynamics, College of Pharmacy, University of Florida, Gainesville, FL 32610, USA.

Table of Contents:

Table 1: Analytical data of the peptides synthesized in this study.[a]

Peptide	Sequence	HPLC k' system 1	HPLC k' system 2	m/z Calcd.	m/z (M+1) Found
1	Ac-His-DPhe-Arg-Trp-NH_2	4.0	5.9	685.3	686.6
2	Ac-His-Phe-Arg-Trp-NH_2	4.6	6.8	685.3	686.6
3	Ac-DPhe-Arg-Trp-NH_2	3.5	6.4	548.3	549.2
4	Ac-Phe-Arg-Trp-NH_2	3.9	7.5	548.3	549.2
5	Ac-His-β^3hPhe-Arg-Trp-NH_2	4.3	6.6	699.4	700.6
6	Ac-His-β^3hDPhe-Arg-Trp-NH_2	3.6	6.4	699.4	700.4
7	Ac-His-DPhe-β^3hArg-Trp-NH_2	3.9	5.9	699.4	700.3
8	Ac-His-DPhe-Arg-β^3hTrp-NH_2	4.1	6.3	699.4	700.4

[a]The HPLC k' value equals [(peptide retention time – solvent retention time)/ solvent retention time]. Two different solvent systems were used. Solvent system 1 is 10% acetonitrile (MeCN) in 0.1% trifluoroacetic acid/H_2O with a gradient to 90% acetonitrile over 35 min. Solvent system 2 is 10% methanol (MeOH) in 0.1% trifluoroacetic acid/ H_2O with a gradient to 90% methanol over 35 min. An analytical Vydac C18 column (Vydac 218TP104) with a flow rate of 1.5 mL/min was used for analytical characterization. The peptide purity was determined by RP-HPLC in both solvent systems at a wavelength of 214 nm. All peptides were found to be >95% pure.

Experimental:

Chemistry. Metarials: Fmoc-β^3hPhe-OH, Fmoc-β^3hDPhe-OH, Fmoc-β^3hArg(Pmc)-OH), and Fmoc-β^3hTrp(Boc)-OH were purchased from Chem Impex International (Wood Dale, IL). The amino acids Fmoc-His(Trt)-OH, Fmoc-Phe-OH, Fmoc-DPhe-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Trp(Boc)-OH, 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-methylbenzhydrylamine resin (Rink Amide MBHA), and the coupling reagent 1-[bis(dimethylamino)methylene]-1H-benzotriazolium hexafluorophosphate 3-oxide (HBTU) were purchased from Peptides International (Louisville, KY). *N*,*N*-diisopropylethylamine (DIEA), triisopropylsilane (TIS), 1,2-ethanedithiol (EDT), *N*,*N*-dimethylformamide (DMF), trifluoroacetic acid, piperidine, and pyridine were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile (ACN), anhydrous ethyl ether, dichloromethane (DCM), and methanol (MeOH) were purchased from Fisher (Fair Lawn, NJ). All reagents were ACS grade or better and used without further purification.

General Peptide Synthesis Procedure: The peptides were synthesized on rink-amide-MBHA resin (0.37 meq/g substitution). Each peptide was synthesized individually using a 25 mL reaction vessel fitted with a coarse frit (CEM Corporation). Approximately 270 mg (0.1 mmol) of resin was added to the reaction vessel and swelled in DCM for two hours. The resin was washed with DMF and subsequently deprotected with the addition of a deprotecting reagent (20% piperidine in DMF) to the reaction vessel, which was stirred for 2 minutes at room temperature. The reaction vessel was then drained using vacuum and additional deprotecting reagent was added. The vessel was placed in the microwave and irradiated (75 °C, 30 W, 4 min). Following a positive Kaiser test, indicating the presence of a free amine, the first amino acid residue was coupled. The general protocol for coupling began with the addition of a 3-fold excess of the N-Fmoc-protected amino acid (5-fold for Arg) and a 3-fold excess of HBTU (5-fold for Arg) in DMF. A 5-fold excess of DIEA (7-fold for Arg) was added, and irradiated in the microwave synthesizer at 75 °C, 30 W (for His residues 50 °C, 30 W) for 5 min (10 min for Arg). Following a negative Kaiser test, the cycle was repeated until the final amino acid was coupled. The coupling and deprotection conditions were same as above for the β-amino acids.

After final deprotection and positive Kaiser test, the peptide was acetylated with the addition of acetic anhydride and pyridine (3:1 v/v mixture), and mixed for 30 min at room temperature. Following a negative Kaiser test, the peptide was washed in with DCM and dried in a desiccator. Side chain deprotection and peptide cleavage from the resin was performed by mixing the dried peptide-resin with a cleavage cocktail (91% TFA, 3% EDT, 3% TIS, and 3% water) for a minimum of 2 h. The peptides were precipitated using cold (4 °C) anhydrous diethyl ether. The precipitated crude peptide was washed with cold (4 °C) diethylether at least three times.

The crude peptide (~40 mg) was purified using a Shimadzu RP-HPLC system with a photodiode array detector and a semipreparative RP-HPLC C18 bonded column (Vydac 218TP1010, 1 cm x 25 cm) and then lyophilized. Analysis of the purified peptides by analytical RP-HPLC (Vydac 218TP104, 4.6 mm x 25 cm) indicated their purity was 95% or greater and electron spray ionization mass spectrometry (ESI-MS) indicated they had the correct molecular mass. The ESI-TOF spectra were recorded on a Bruker Bio TOF II at University of Minnesota and ABI 3200 Q Trap system at University of Florida

Biological Assay:

Pharmacological characterization of the synthesized peptides at the mouse melanocortin receptors mMC1R, mMC3-5Rs was achieved using the cAMP AlphaScreen™ (Perkin Elmer) assay following the manufacturer's instructions.

cAMP AlphaScreen Assay: AlphaScreen™ cAMP Assay kit (Perkin Elmer, Cat #6760625M) was purchased from Perkin Elmer. Peptide ligands were dissolved in DMSO at a stock concentration of 10^{-2} M and stored at -20 °C until assayed. HEK-293 cells were stably transfected with the mouse melanocortin receptors using a pCDNA3 expression vector using a calcium phosphate method and G418 selection as previously reported by our laboratory.[1] These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% newborn calf serum (NCS). Cells were 70-90% confluent on the day of the assay. Stimulation buffer (Hank's Balanced Salt Solution [HBSS 10X [-] sodium bicarbonate] and [-] phenol red, Gibco®], 0.5 mM isobutylmethylxanthine [IBMX], 5 mM HEPES buffer solution [1M, Gibco®], 0.1% bovine serum albumin [BSA] in Milli-Q water, pH=7.4) and the lysis buffer (5mM HEPES buffer solution [1M, Gibco®], 0.1% BSA, 0.3% Tween-20 [aqueous solution 10% w/v] in Milli-Q water, pH=7.4) were prepared on the day of the assay. The cells were dislodged with Versene (Gibco®) at 37°C followed by centrifugation at room temperature and 800 RPMs for 5 minutes (Sorvall Super T21 high speed centrifuge, swinging bucket rotor). The media was aspirated and cells were suspended in Dulbecco's phosphate buffered saline solution (DPBS 1X [-] calcium chloride and [-] magnesium chloride, Gibco®). 10 µL of cell suspension was mixed with 10 µL Trypan blue dye solution (BioRad) and 10 µL of this solution was counted manually by hemocytometer. The cells were re-suspended in stimulation buffer at 10 x 10^6 cells/mL. Anti-cAMP acceptor beads (0.2 unit/µL, AlphaScreen™) were added to the cells and ~10,000 cells were added to each well of 384-well plate (Optiplate™). The cells were stimulated with peptide (from 10^{-4} to 10^{-10} M; 10^{-5} to 10^{-11} M for α-MSH) or forskolin (10^{-4} M) in stimulation buffer and incubated in the dark at room temperature for 2 hours.

The lysis buffer containing 10 unit/µL Streptavidin donor bead (AlphaScreen™) and 3.3 unit/µL biotinylated cAMP (AlphaScreen™) was prepared in the dark at least an hour

prior to the addition. Following the 2 h incubation period lysis buffer containing 10 unit/μL donor bead and 3.3 unit/μL biotinylated cAMP was added in a subdued light environment to each well. The plate was incubated for 2 h in the dark at room temperature and read on the Enspire™ Alpha plate reader using a pre-normalized assay protocol (set by the manufacturer). The assays were performed using duplicate data points and repeated in at least three independent experiments. The potencies and their associated standard errors of mean, were determined by fitting the data to a nonlinear regression method using the PRISM program (v4.0, GraphPad Inc.).

1. Haslach, E. M.; Huang, H.; Dirain, M.; Debevec, G.; Geer, P.; Santos, R. G.; Giulianotti, M. A.; Pinilla, C.; Appel, J. R.; Doering, S. R.; Walters, M. A.; Houghten, R. A.; Haskell-Luevano, C. Identification of Tetrapeptides from a Mixture Based Positional Scanning Library that can Restore nM Full Agonist Function of the L106P, I69T, I102S, A219V, C271Y, and C271R Human Melanocortin-4 Polymorphic Receptors (hMC4Rs). *J. Med. Chem.* **2014**, *57*, 4615-4628.