The orphan G protein-coupled receptor GPR139 is activated by the peptides: adrenocorticotropic hormone (ACTH), α- and β-melanocyte stimulating hormone (α-MSH, and β-MSH), and the conserved core motif HFRW

Anne Cathrine Nørh1, Mohamed A. Shehata1, Alexander S. Hauser1, Vignir Isberg1, Jacek Mokrosinski2, I. Sadaf Farooqi2, Daniel Sejer Pedersen1, David E. Gloriam1*# and Hans Bräuner-Osborne1*#

1 Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Universitetsparken 2, 2100 Copenhagen, Denmark. 2 University of Cambridge Metabolic Research Laboratories, Wellcome Trust-MRC, Institute of Metabolic Science, Addenbrooke’s Hospital, Cambridge CB2 0QQ, United Kingdom.

*Corresponding authors. E-mail address: david.goriam@sund.ku.dk (D.E.G. computational chemistry), hbo@sund.ku.dk (H.B.-O. pharmacology)
#These authors contributed equally (shared last authors)
Abbreviations

Ac, N-terminal acetylation
ACTH, adrenocorticotropic hormone
CHO-GPR139, CHO-k1 cell line stably expressing the human GPR139 receptor
CHO-M1, CHO-k1 cell line stably expressing the human muscarinic acetylcholine receptor M₁
cmp1a, compound 1a (2-(3,5-dimethoxybenzoyl)-N-(naphthalen-1-yl)hydrazine-1-carboxamide) a GPR139 agonist
EC₅₀, the concentration giving 50% of maximum response
GPCR, G protein-coupled receptor
GPR139, G protein-coupled receptor 139
HPLC, high performance liquid chromatography
LC-MS, liquid chromatography-mass spectrometry
MALDI-TOF MS, matrix-assisted laser ionization-time-of-flight mass spectrometry
MC3R, the melanocortin 3 receptor
MC4R, the melanocortin 4 receptor
MCH, melanin-concentrating hormone
NH₂, C-terminal amidation
NPC%, net peptide content
PC1, prohormone convertase 1 enzyme
PC2, prohormone convertase 2 enzyme
Phe, phenylalanine
POMC, pro-opiomelanocortin
S%, similarity %
TRH, thyrotropin releasing hormone
Trp, tryptophan
α-MSH, α-melanocyte stimulating hormone
α-MSH₁₋₁₀, peptide with the sequence SYSMEHFRWG
α-MSH₁₋₉, peptide with the sequence SYSMEHFRW
α-MSH₄₋₁₀, peptide with the sequence MEHFRWG
α-MSH₄₋₁₃, peptide with the sequence MEHFRWGKPV
β-MSH, β-melanocyte stimulating hormone
β-MSH₅₋₂₂, peptide with the sequence DEGPYRMEHFRWGFPKPD
γ₁-MSH, γ₁-melanocyte stimulating hormone
Abstract
GPR139 is an orphan G protein-coupled receptor that is expressed primarily in the brain. Not much is known regarding the function of GPR139. Recently we have shown that GPR139 is activated by the amino acids L-tryptophan and L-phenylalanine (EC\textsubscript{50} values of 220 μM and 320 μM, respectively), as well as di-peptides comprised of aromatic amino acids. This led us to hypothesize that GPR139 may be activated by peptides. Sequence alignment of the binding cavities of all class A GPCRs, revealed that the binding pocket of the melanocortin 4 receptor is similar to that of GPR139. Based on the chemogenomics principle "similar targets bind similar ligands", we tested three known endogenous melanocortin 4 receptor agonists; adrenocorticotropic hormone (ACTH) and α- and β-melanocyte stimulating hormone (α-MSH and β-MSH) on CHO-k1 cells stably expressing the human GPR139 in a Fluo-4 Ca\textsuperscript{2+}-assay. All three peptides, as well as their conserved core motif HFRW, were found to activate GPR139 in the low μM range. Moreover, we found that peptides consisting of nine or ten N-terminal residues of α-MSH activate GPR139 in the submicromolar range. α-MSH\textsubscript{1-9} was found to correspond to the product of a predicted cleavage site in the pre-pro-protein pro-opiomelanocortin (POMC). Our results demonstrate that GPR139 is a peptide receptor, activated by ACTH, α-MSH, β-MSH, the conserved core motif HFRW as well as a potential endogenous peptide α-MSH\textsubscript{1-9}. Further studies are needed to determine the functional relevance of GPR139 mediated signaling by these peptides.

Keywords
Orphan GPCR; GPR139; pro-opiomelanocortin (POMC); adrenocorticotropic hormone (ACTH); melanocyte stimulating hormone (MSH); peptide receptor.
G protein-coupled receptors (GPCRs) constitute the largest family of cell surface receptors, encoded by more than 800 genes in humans (Fredriksson et al., 2003). Given the importance of GPCRs in many aspects of physiology, they are among the most pursued targets for drug development and they account for approximately 19% of the established drug-targeted portion of the genome (Rask-Andersen et al., 2011). However there is still a huge untapped potential for drug discovery within the GPCR field owing to the fact that these drugs only act on less than a quarter of the 400 non-olfactory GPCRs. Furthermore, 121 non-olfactory GPCRs are still classified as orphan GPCRs, meaning that their endogenous ligands have not yet been identified (Southan et al., 2016).

The class A GPCR GPR139 is an orphan receptor meaning that its endogenous agonist is unknown. The expression pattern of GPR139 has primarily been studied on the mRNA level and showed expression mainly in the central nervous system (Liu et al., 2015; Matsuo et al., 2005; Süsens et al., 2006; Wagner et al., 2016). Attempts to perform autoradiography on brain slices from mice with radioligands for GPR139 have failed (Kuhne et al., 2016; Liu et al., 2015). While several antibodies against GPR139 are commercially available, only one antibody against the N-terminal of GPR139 have been described in the literature, which locate GPR139 expression to the medial habenula and lateral septal nucleus (Liu et al., 2015). Furthermore, GPR139 mRNA has been detected in high levels in the hypothalamus, in the pituitary and in the habenula of the brain (Liu et al., 2015; Matsuo et al., 2005; Wagner et al., 2016). These findings have led to the hypothesis that GPR139 could be involved in the regulation of food consumption and/or energy expenditure (Wagner et al., 2016). Moreover, another study has shown that activation of GPR139 with a surrogate agonist (JNJ-63533054) leads to decreased spontaneous locomotion activity in rats (Liu et al., 2015).

We have previously shown that both D- and L-isomers of the aromatic amino acids tryptophan (Trp) and phenylalanine (Phe) activate GPR139 via Gαq-coupling (Isberg et al., 2014). Recently, this finding was confirmed by Liu et al., that reported plasma levels of L-Trp and L-Phe in rats in the dynamic range of GPR139 activity (30-300 μM) (Liu et al., 2015). However, the potencies of L-Trp and L-Phe on GPR139 are modest (EC50 values of 220 μM and 320 μM, respectively), and interestingly, GPR139 is also activated by di-peptides comprised of aromatic amino acids (Isberg et al., 2014). This contrasts with other amino acids receptors, such as GPRC6A, which require an intact
α-amino acid moiety for agonist activity (Christiansen et al., 2006). This has led us to hypothesize that GPR139 is not simply an aromatic amino acid sensing receptor, but could also be activated by peptides.

In the present study, we have searched for peptides that could activate GPR139. Based on the chemogenomics principle that "similar targets bind similar ligands", we aligned the binding cavity residues of GPR139 and all other class A GPCRs to identify known peptide-binding GPCRs with similar binding cavities. In this manner the melanocortin 4 receptor (MC4R) was identified as a peptide-binding GPCR with a similar binding cavity to that observed for GPR139. Peptide ligands acting on MC4R were tested for activity on GPR139 in a Fluo-4 calcium assay. It was found that the adrenocorticotropic hormone (ACTH), α-melanocyte stimulating hormone (α-MSH) and β-melanocyte stimulating hormone (β-MSH) all activate GPR139.

1. Methodology and Experimental Procedures

1.1 Chemogenomic inference of peptide receptor ligands

The GPCRdb similarity search tool was used to identify class A GPCRs with a similar binding pocket using the structure-based GPCRdb numbering scheme (Isberg et al., 2015). A first search focused on a set of 15 generic peptide receptor-ligand interacting residue positions annotated from all eight crystallized peptide GPCRs available at the time (Isberg et al., 2016). A second search involved all class A GPCR 44 accessible binding pocket residues (Gloriam et al., 2009). The endogenous ligands of the ten most similar peptide receptors were selected for testing on GPR139. We also included the suggested ligand; β-alanine, for the orphan MAS-related receptor (MRGRD_HUMAN) (Shinohara et al., 2004).

1.2 Commercially available endogenous peptides and amino acids

Ten peptide receptor ligands were purchased and tested. These were: Adrenocorticotropic hormone (1-39) (human) (Tocris, Oxford, UK, #3492, batch#3A) (ACTH); α-melanocyte stimulating hormone (Tocris, #2584, batch#3A and Bachem, H-1075, lot#1055067) (α-MSH); β-melanocyte stimulating hormone (Sigma-Aldrich, Brøndby, Denmark #M-6513, lot#095K14351) (β-MSH); and Melanotan II (Tocris, #2566, batch#4C and Bachem, H-3902, lot#1056060), all of which are melanocortin 4 receptor (MC4R) peptide agonists; γ1-melanocyte stimulating hormone (Tocris, #3424, batch#1A) (γ1-MSH), which share a common motif with ACTH, α-MSH and β-MSH;
thyrotropin releasing hormone (Sigma-Aldrich, #P1319, lot#BCBM8636V) (TRH), a thyrotropin releasing hormone receptor peptide agonist; [Arg⁸]-vasopressin (Tocris, #2935, batch#5A/162260), an arginine vasopressin receptor 1a and 1b peptide agonist; oxytocin (Tocris, #1910, batch#14A), a weak peptide agonist on the vasopressin receptor 1a; 26RFa (Tocris, #4402, batch#1A), a pyroglutamylated RFamide receptor peptide agonist; melanin-concentrating hormone (Tocris, #3806, batch#2E) (MCH), a melanin-concentration hormone receptor 2 peptide agonist; and the non-peptide β-alanine (Sigma-Aldrich, #146064), the suggested ligand for the orphan MAS-related receptor.

1.3 Quality control of commercially acquired ACTH, α-MSH, β-MSH and γ1-MSH

Analytic HPLC was carried out on a Dionex Ultimate 3000 system using an analytical Gemini-NX 3μm C18 column (4.6×250 mm). Flow rate 1 mL/min and UV detection at 200, 210, 225, 254 and 280 nm. Gradient: 0-30 min 0-100% B in A, 30-35 min 100% B, 30-35 min 100% Solvent A. Solvent A: 0.1% TFA in H₂O (v/v), Solvent B: 0.1% TFA, 10% H₂O in MeCN (v/v/v). Data analysis was carried out with Chromeleon Version 6.80 SP4 Software. LC-MS was carried out on an Agilent 1200 series system using an Xbridge 3.5 μm C18 column (4.6×100 mm). Flow rate 1 mL/min and UV detection at 215, 254 and 280 nm and mass detection m/z 100-3000. Gradient: 0-30 min: 5-95% B in A. Solvent A: 0.1 % formic acid, 5 % MeCN in H₂O (v/v/v), Solvent B: 0.1 % formic acid, 5 % H₂O in MeCN (v/v/v). Data analysis was carried out with Bruker Daltonics DataAnalysis Version 3.3 software. MALDI-TOF MS was carried out on a Bruker Microflex system. Matrix: ACCA (α-cyano-4-hydroxy-cinnamic acid, Sigma-Aldrich #C8982) in MeCN/H₂O/TFA (500:475:25, v/v/v). Data analysis was carried out with Bruker FlexAnalysis software version 3.4 (build 57).

1.4 Searching for novel endogenous peptides with similarity to ACTH, α-MSH and β-MSH

The GPR139 active peptides (ACTH, α-MSH and β-MSH) were used as queries to screen the entire SWISS-PROT database using TBLASTN, and BLASTP (Altschul et al., 1990; Bateman et al., 2015; Camacho et al., 2009). The aim was to potentially identify similar peptides with unknown function and/or unknown target that could activate GPR139.

1.5 Searching for alternative cleavage sites in the pre-pro-protein POMC
Peptide sequence information for all class A peptide GPCR ligands was retrieved from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016) and their location on their respective precursors (pre-pro-proteins) were mapped. The dataset was analyzed for flanking amino acids on the N- as well as C-terminal peptide ends. The identified cleavage motif was then applied on the pre-pro-protein POMC to search for alternative cleavage sites that could reveal possible unknown peptides as well as shorter versions of already known peptides.

1.6 Synthesized peptides

α-MSH_{4-13} (MEHFRWGKPV), α-MSH_{1-10} (SYSMEHFRWG), α-MSH_{1-9} (SYSMEHFRW), α-MSH_{4-10} (MEHFRW), conserved motif (HFRW), and β-MSH_{5-22} (DEGPYRMEHFRWGFPKP) were synthesized by GenScript, NJ, USA with and without capping; N-terminal acetylation (Ac) and C-terminal amidation (NH₂), indicated with the tag “cap-“. Additionally, α-MSH_{P-Ser} (SY[pSer]SMEHFRWKPV-NH₂) and ACTH_{1-16,P-Ser} (SY[pSer]SMEHFRWKGPKVGGK) were synthesized by GenScript, NJ, USA. GenScript assessed the purity of the custom-synthesized peptides by HPLC and MS. Furthermore, GenScript performed a solubility test for all peptides and determined the net peptide content (NPC%) using a Vario MICRO Organic Elemental Analyzer.

1.7 Ca^{2+}-Fluo-4 assay

All peptides/ligands were tested for agonistic and antagonistic effect on a CHO-k1 cell line stably expressing the human GPR139 (CHO-GPR139) kindly provided by H. Lundbeck A/S, Valby, Denmark, and also on a CHO-k1 cell line stably expressing the human muscarinic acetylcholine receptor M₁ (CHO-M1) (The cDNA Resource Center, Bloomsburg University, PA, USA, #CEM100TN00) to assess specificity. The peptides/ligands that did not show agonist activity on GPR139 were also tested as antagonists against EC_{80} (800 nM) of compound 1a (cmp1a) (Shi et al., 2011) (see structure in Fig. SI1), also kindly provided by H. Lundbeck A/S. The CHO-GPR139 cell line was grown in Dulbecco’s modified Eagle’s medium (DMEM) F12-Kaighn’s (Gibco, 21127, distributed by Invitrogen, Nærum, Denmark) supplemented with 10% dialyzed fetal bovine serum (Gibco, 26400, United States origin), 1% GlutaMAX™-I (100X) (Gibco, 35050), and 100 units/mL penicillin and 100 μg/mL streptomycin (Gibco, 15140) and 1.0 mg/mL genetin (Invitrogen, 1181103). CHO-M1 was grown in Ham’s F12 (Gibco, 21765) supplemented with 10% fetal bovine serum (Gibco, 10270, South America origin) + 100 units/mL penicillin and 100 μg/mL streptomycin and 0.25 mg/mL
geneticin. Cmp1a (H. Lundbeck, A/S) and carbamoylcholine chloride (Sigma-Aldrich, C4382) was used as positive controls in the two cell lines, respectively.

30,000 cells/well (either CHO-GPR139 or CHO-M1) were plated in black 96-well plates with flat clear bottoms (Corning, Falcon, ref 353219, distributed by VWR, Søborg, Denmark) and incubated overnight. The Fluo-4 dye loading solution (Invitrogen, F36206) was prepared according to the manufacturer’s instructions by dissolving it in HEPES buffer (HBBS (Invitrogen, ref 14175129) supplemented with 10mM HEPES, 1 mM MgCl$_2$, 1 mM CaCl$_2$, pH = 7.4) supplemented with 2.5 mM probenecid (HEPES-probenecid buffer). 50 μL dye loading solution was added to each well. Cells were incubated with the Fluo-4 dye for 60 min at 37 °C, then washed with 100 μL HEPES buffer.

**Agonist testing.** 100 μL HEPES-probenecid buffer was then added to each well and incubated for 10 minutes at 37 °C before measurement. All peptides/ligands were diluted according to the manufacturer’s or GenScript’s recommendation and in low-binding micronic tubes (Micronic, MP32022, distributed by In Vitro A/S, Fredensborg, Denmark). For testing agonist activity, the peptides were transferred to low-binding ligand plates (Greiner bio-one, PS-microplate, non-binding, U-bottom, ref# 650901, distributed by In Vitro A/S) before 33 μL of the test peptides (4× concentrated) were added automatically after baseline measurements.

**Antagonist testing.** 100 μL of the peptides/ligands dissolved in HEPES-probenecid buffer (in micronic tubes as described above) was added to each well and incubated for 10 minutes at 37 °C before measurement. The antagonist activity was tested against EC$_{80}$ of cmp1a (800 nM) added automatically as 33 μL (4×) after baseline measurements.

Changes in intracellular calcium levels was recorded on a FlexStation 3 (Molecular Devices, serial no. FV05958) at 37 °C with an excitation filter of 485 nm and an emission filter of 520 nm.

**1.8 cAMP accumulation assay**

α-MSH-derived peptides (α-MSH, α-MSH$_{1-9}$, cap-α-MSH$_{1-9}$ and Melanotan II) were functionally characterized in vitro in terms of their activity on Gα$_s$-coupled melanocortin receptors, MC3R and MC4R transiently expressed in COS7 cells using cAMP accumulation assay (HitHunter® cAMP assay, DiscoverX, Fremont, CA, USA, 90-0075SM). The COS-7 cell line was grown in Dulbecco’s modified Eagle’s medium (DMEM) with 1000 mg/l glucose (Sigma-Aldrich, D6046) supplemented with 10% fetal
bovine serum (Gibco, 10270, South America origin, lot#42G4850K), 1% GlutaMAX™
(100X) (Gibco, 35050), and 100 units/mL penicillin and 100 μg/mL streptomycin
(Sigma-Aldrich, P0781). 20,000 cells/well were plated in white opaque 96-well plates
(Perkin Elmer, CulturPlate-96, 6005680) and transfected the day after seeding using
Lipofectamine 2000™ reagent (Invitrogen, 11668) in serum-free Opti-MEM I medium
(Gibco, 31985) with 30 ng DNA of respective receptor or mock plasmid construct in
pcDNA3.1(+) according to the manufacturer’s protocol.
Assays were performed one day after transfection. Cells were washed with PBS and
incubated in 30 μL PBS supplemented with 1 mM 3-isobutyl-1-methylxanthine (IBMX,
Cayman Chemical, 13347) for 30 min prior to stimulation with agonist. All peptides/ligands were diluted according to the manufacturer’s or GenScript’s
recommendation and dilutions were prepared in low-binding micronic tubes (Micronic,
MP32022). Serial dilutions of agonist were added to PBS with IBMX (1.5 μL, 20x
dilution) and cells were stimulated for further 30 minutes at 37 °C. Intracellular cAMP
detection was carried out directly after the ligand stimulation. 10 μL anti-cAMP antibody
followed by 40 μL chemiluminescent substrate/lysis buffer/enzyme donor-cAMP
complex mix prepared according to manufacturer’s protocol were added and plates
were incubated shaking for 1 hour at ambient temperature. Finally, 40 μL enzyme
acceptor was dispensed and chemiluminescent signal was quantified after 4-5 hours in
TopCount Microplate Scintillation Counter (Packard, serial no. 409252) for 5 sec/well
with 1 min counting delay before each plate.

1.9 Data analysis
The peptide concentration was calculated using the net peptide content (NPC%) and
peptide purity. All pharmacological data were analyzed using GraphPad Prism (version
6.0h for Mac OS X Yosemite, GraphPad Software, La Jolla California, USA,
www.graphpad.com). Data presented is from at least three independent experiments in
triplicates. The responses in Fig. 2 were normalized to the responses of buffer (0%) and
E_{max} (8 μM) of cmp1a (100%). The responses in Fig. 4A is normalized to the
responses of buffer (0%) and E_{max} of α-MSH (100%). Fig. 4B were normalized to the
background signalling measured for mock-transfected cells (0%) and E_{max} of α-MSH
(100%). All graphs in Fig. 2+4 are shown as mean ± SEM. Fitting of concentration-
response curves was performed by non-linear regression.

\[ R = R_{\text{min}} + \frac{R_{\text{max}} - R_{\text{min}}}{1 + 10^{(\log EC_{50} - X) \cdot \sigma_x}} \]
Where X is the logarithm of the concentration, R is the response, EC$_{50}$ is the concentration giving 50% of maximum response and n$_H$ is the Hill coefficient, which describes the slope. In Fig. 2 for concentration response curves that reached a plateau (ACTH, α-MSH, β-MSH, α-MSH$_{1-9}$, α-MSH$_{1-10}$ and HWFR) $R_{\text{min}}$ was fixed to 0 and $R_{\text{max}}$ was fixed to the response of the maximum concentration tested. For the concentration response curves that did not reach a plateau (cap-α-MSH$_{1-9}$, cap-α-MSH$_{1-10}$, cap-HWFR, α-MSH$_{4-10}$ and cap-α-MSH$_{4-10}$) the relative $E_{\text{max}}$ ± SEM compared to $E_{\text{max}}$ of cmp1a is given for the highest peptide concentration tested. In Fig. 4A, α-MSH and α-MSH$_{1-9}$ was fitted where $R_{\text{min}}$ was fixed to 0 and $R_{\text{max}}$ was fixed to the response of the maximum concentration tested. In Fig 4B only melanotan II and α-MSH were fitted.

2. Results
2.1 Inferred endogenous peptide ligands
The binding cavity of GPR139 aligned to class A receptors revealed many orphan receptors similar to GPR139 (Fig. 1), among which GPR142 was the most similar with 80% similarity. Six peptide-binding GPCRs possess a similar ligand-binding pocket to GPR139, including MC4R with a sequence similarity of 49%. On this basis, we purchased and tested the ten known endogenous or synthetic peptide ligands on the human GPR139, which are; ACTH, α-MSH, β-MSH, γ1-MSH, Melanotan II, TRH, [Arg$^8$]-vasopressin, oxytocin, 26RFa, and MCH. No agonist or antagonist activity on GPR139 was detected for γ1-MSH, Melanotan II, TRH, [Arg$^8$]-vasopressin, oxytocin, 26RFa, and MCH (data not shown). However, ACTH, α-MSH, β-MSH all showed agonist activity on GPR139.

2.2 Activity of endogenously POMC-derived peptides
ACTH, α-MSH, β-MSH and γ1-MSH all originate from the pre-protein POMC. The concentration-response curves of ACTH, α-MSH and β-MSH on CHO-GPR139 cells showed that ACTH (EC$_{50}$ = 2.06 μM), α-MSH (EC$_{50}$ = 2.21 μM) and β-MSH (EC$_{50}$ = 6.3 μM) all have low micromolar potencies on GPR139, and are as efficacious as the reference surrogate agonist cmp1a (Fig. 2A and Table 1). ACTH, α-MSH and β-MSH had no detectable impurities (incl. L-Phe and L-Trp), as determined by rigorous HPLC, LC-MS and MALDI-TOF MS analysis (Fig. SI2). Furthermore, when we tested for specificity, none of these peptides showed any activity on the muscarinic acetylcholine M$_1$ (M1) receptor (data not shown).
2.3 Activity of peptides with the HFRW motif

Next, we examined which part of the peptides were important for GPR139 activation. The active peptides (ACTH, α-MSH and β-MSH) share the amino acid sequence motif MEHFRW (α-MSH4-10), and all POMC-derived peptides (includes also γ1-MSH) the sub-motif HFRW (Table 1). When peptides representing both motifs were tested on GPR139 only the HFRW peptide was found to be active (EC50 = 4 µM) (Fig. 2B). Since the longer sequence motif MEHFRWG (α-MSH4-10) itself was inactive, we set out to determine whether the C- or N-terminus of α-MSH is important for activating GPR139. Therefore, α-MSH was truncated in both ends for the amino acids flanking the MEHFRWG motif. This gave two 10-mer analogues of α-MSH with 3 residue truncations at the C- or N-terminus; α-MSH1-10 and α-MSH4-13, respectively, which were tested on GPR139 (Table 1). Interestingly, only the C-terminally truncated analogue α-MSH1-10 was active (EC50 = 318 nM) (Fig. 2C).

Subsequently, we looked for other peptides sharing the HFRW motif. Our BLASTP and TBLASTN searches for novel uncharacterized peptides with similarity to the queries did not result in any similar or homologues peptides. However, our search for peptides containing the conserved core motif HFRW, revealed 6 new proteins, two of which (PKD1_HUMAN and RM27_HUMAN) are known and predicted to be secreted, respectively. These proteins are well characterized but are not known to play any role in GPR139 signaling; besides, nothing is known about their proteolytic processing suggesting that the HFRW motif originates exclusively from POMC. We also looked for potential cleavage sites along the POMC and one additional truncated analogue of α-MSH (α-MSH1-9) that was active on GPR139 was identified (EC50 = 596 nM) (Fig. 2C and 3).

Endogenously ACTH and β-MSH have free N- and C-termini, unlike α-MSH that is acetylated at the N-terminus and amidated at the C-terminus. In addition to the naturally occurring peptides, capped and uncapped analogues were also custom-synthesized and tested on GPR139 (Table 1). All capped peptides were less potent than the non-capped versions. The exact reason for the observed difference in potency remains unclear at this time and future studies are needed to understand the underlying reasons for the reduced activity.
In order to compare the selectivity and rank-order potency of α-MSH, α-MSH_{1-9} and cap-α-MSH_{1-9} on GPR139 compared to the MC4R, we tested the peptides in a cAMP assay on COS7 cells transiently transfected with MC4R. α-MSH had a potency of 430 nM on MC4R and α-MSH_{1-9} did not show any effect on MC4R (Fig. 4B). Cap-α-MSH_{1-9} only induced a 50% response of α-MSH when tested in the highest concentration, which is comparable to the response on GPR139. Melanotan II was also tested on MC4R (EC_{50} = 11 nM) as control as it is a known synthetic agonist of the receptors, but melanotan II had no effect on GPR139 (Fig. 4). As an additional control experiment α-MSH, α-MSH_{1-9}, cap-α-MSH_{1-9} and melanotan II were tested on the MC3R in the cAMP assay and peptides showed the same rank-order potency of the agonists as on the MC4R (Fig. SI3).

During the preparation of this manuscript the Dr. Jesper V. Olsen group performed a large-scale mass spectrometry-based peptidomics of the rat hypothalamus and identified thousand of neuropeptides and their post-translational modifications including two novel neuropeptides cleaved from POMC; α-MSH_{P-Ser} and ACTH_{1-16,P-Ser} (Table 1), both of which are phosphorylated (Secher et al., 2016). However, neither of the two peptides showed any agonist or antagonist activity on GPR139 (data not shown).

All custom synthesized peptides were of >95% purity (UV detection at 220 nm) with no significant impurities observed when analyzed by HPLC and MS at GenScript (Fig. SI4+SI5) and the net peptide content (NPC%) was determined for all custom synthesized peptides at GenScript (Table SI1).

3. Discussion
We found that the binding cavity of GPR139 mainly resembles that of peptide receptors, including MC4R (Fig. 1). GPR139 has been shown to signal through Gα_s (Hu et al., 2009), Gα_i (Süsens et al., 2006) and Gα_q (Dvorak et al., 2015; Isberg et al., 2014; Liu et al., 2015; Matsuo et al., 2005; Shehata et al., 2016; Shi et al., 2011; Wang et al., 2015), however in CHO-k1 cells GPR139 only signals via the Gα_q-coupled signaling pathway (Isberg et al., 2014; Shehata et al., 2016). The main signaling pathway of MC4R is through Gα_s (reviewed in (Tao, 2010)). Accordingly, the Ca^{2+}-Fluo-4 assay and the cAMP were used to measure the activity of GPR139 and MC4R, respectively. Three physiological peptide ligands of MC4R, POMC-derived peptides ACTH, α-MSH
and β-MSH, were found to also activate GPR139 with potencies in the single micromolar range (Fig. 2 and Table 1). α-MSH had high nanomolar potency on MC4R (Fig. 4B) whereas γ1-MSH, which is also a POMC-derived peptide, did not show any activity on GPR139 (Table 1). Likewise, γ1-MSH did not have activity on the MC4R (Pritchard et al., 2004). We have shown that the rank-order potency of peptides at GPR139 are \( \alpha \)-MSH \( > \) α-MSH \( > \) cap-α-MSH\( _{1-9} \) \( >> \) melanotan II and at MC4R melanotan II \( > \) α-MSH \( > \) cap-α-MSH\( _{1-9} \) \( >> \) α-MSH\( _{1-9} \). Together this data concludes that GPR139 and the MC\(_4\)R have an overlapping but also divergent agonist profiles.

MC4R is a class A GPCR highly involved in mammalian energy homeostasis and body weight as Mc4r\(^{-/-}\) knockout mice show overt obesity (Huszar et al., 1997). Critically, heterozygous loss of function mutations in MC4R represent the commonest genetic form of human obesity (Farooqi et al., 2003, 2000; Vaisse et al., 2000). Studies in mice and humans have shown that, in addition to effects on food intake and body weight, melanocortin signalling is involved in the modulation of insulin secretion, linear growth and blood pressure by modulating sympathetic tone (Ellacott and Cone, 2006). Our data suggest that GPR139 may potentially also be important in mediating some of the effects attributed to melanocortin peptides.

Matsuo et al. and Liu et al. show that GPR139 mRNA is detected in high levels in the hypothalamus and in the pituitary (Liu et al., 2015; Matsuo et al., 2005) and Süsen et al. show that it is expressed in the CA1 area of the hippocampus (Süsens et al., 2006). Interestingly, POMC is expressed in the pituitary and in the hypothalamus and ACTH, α-MSH and β-MSH are secreted in those areas of the brain (Bergeron et al., 2000; Kato et al., 2004). ACTH and the MSH-peptides originate from the pre-pro-protein POMC (Fenger and Johnsen, 1988). POMC can be processed by the prohormone convertase 1 enzyme (PC1) and the prohormone convertase 2 enzyme (PC2) leading to a range of peptide hormones (Benjannet et al., 1991; Thomas et al., 1991). POMC and PC1 are expressed in corticotroph cells of the anterior pituitary, and POMC is processed by PC1 to ACTH in these cells (Kato et al., 2004). In the hypothalamus PC2 is additionally expressed (together with PC1 and POMC), which can process ACTH further to give α-MSH and POMC to β-MSH and γ1-melanocyte stimulating hormone (γ1-MSH) (Bergeron et al., 2000). It is notable that GPR139 is expressed in the same areas of the brain as POMC-derived hormones.
The MC4R is also expressed in the CNS particularly in the hypothalamus and in the CA1 and CA2 area of the hippocampus (Gantz et al., 1993; Kishi et al., 2003; Mountjoy et al., 1994). It is noteworthy that the GPR139 and MC4R receptors are expressed in broadly similar areas of the brain and that they share some of the same ligands, although with different potency and rank-order.

Interestingly, our data show that ACTH, α-MSH and β-MSH are a 100-fold less potent on GPR139 than on the MC4R. However, they are 20-120 fold more potent on the GPR139 receptor than \( L-\text{Trp} \) (EC\(_{50} = 220\mu\text{M}\)) and \( L-\text{Phe} \) (EC\(_{50} = 320\mu\text{M}\)) (Isberg et al., 2014) (Fig. 2A and Table 1). Moreover, the conserved HFRW motif alone (shared between ACTH, α-MSH, β-MSH and γ1-MSH) is able to activate GPR139 in the low micromolar range. This suggests that GPR139 is a peptide-binding receptor, since ACTH, α-MSH and β-MSH all are more potent on GPR139 than \( L-\text{Trp} \) and \( L-\text{Phe} \).

Concentrations of α-MSH and β-MSH in human cerebrospinal fluid are in the high picomolar range (Berrettini et al., 1985; Shuster et al., 1977), but can locally be present in higher concentration. α-MSH and β-MSH have nanomolar potencies at the MC4R (Fig. 4B) (Pritchard et al., 2004). Thus, it is reasonable to propose that α-MSH and β-MSH most likely do not activate GPR139 \textit{in vivo}, due to their lower potency at GPR139. However, it is well known that agonist potency at GPCRs is greatly affected by receptor expression levels and cellular context (e.g. G protein levels) (Bräuner-Osborne et al., 1996) and it is thus plausible that the peptides identified in the present study could display higher GPR139 receptor potency \textit{in vivo}. It is also possible that alternative peptides with an HFRW-like motif are the endogenous agonists or that another post-translational modified POMC-derived peptide, than tested here, displays higher GPR139 potency or physiological level and is the ‘true’ endogenous agonist.

Through computational analysis we found a new putative cleavage site on the POMC leading to the peptide α-MSH\(_{1,9}\). Although neither PC1 or PC2 can cleave at a single K residue, other peptides such as neuropeptide SF/FF (NPFF), growth hormone-release hormone (GHRH), corticotrophin-releasing hormone (CRH), urocortin 1 (UCN1), neuromedin S-33 (NMS) and RFamide-related peptide-1 (NPVF) are cleaved at a GK or GR motif. α-MSH\(_{1,9}\) incorporates the HFRW motif and displays a GPR139 potency in the submicromolar range, furthermore α-MSH\(_{1,9}\) does not activate MC4R or MC3R. However, the peptide was not found in the mass spectrometry proteomics compendium.
of peptideAtlas (Farrah et al., 2011); in the mass spectrometry peptidomics of the rat hypothalamus (Secher et al., 2016); or in the small open reading frames predicted peptides of similar genes (sORFs.org) (Olexiouk et al., 2015). Peptides that are secreted in low quantities are difficult to detect in proteomics experiments (Farrah et al., 2013) and this could provide an explanation to its apparent absence in the databases (vide supra).

Overall, the results reported herein strongly suggest that GPR139 is a peptide receptor and not simply a receptor for aromatic amino acids. If α-MSH$_{1-9}$ is present endogenously it could potentially be the endogenous ligand for GPR139, especially because α-MSH$_{1-9}$ is selective to GPR139 when compared to similar receptors (MC4R and MC3R). However, the α-MSH$_{1-9}$ peptide has to be identified physiologically in the brain in relevant concentrations to substantiate this hypothesis.

4. Conclusions
This is the first study to report that endogenously expressed peptides can activate the orphan receptor GPR139. ACTH, α-MSH, β-MSH, C-terminally truncated α-MSH (α-MSH$_{1-9}$ and α-MSH$_{1-10}$), and the conserved core motif HFRW can all activate GPR139 in the sub- to low micromolar range. These results indicate that the previously reported aromatic amino acids are not the most potent endogenous agonists of GPR139. GPR139 is therefore more likely to be a peptide receptor.

Conflict of interest
The authors declare no conflict of interest

Acknowledgement
We thank Dr. Kirsten Bayer Andersen for performing preliminary experiments for this study. This work was supported by the Lundbeck Foundation (separate grants to H.B.-O., D.S.P and D.E.G. [R169-2013-16327]), the A. P. Møller Foundation for the Advancement of Science (H.B.-O.), the European Research Council (DE-ORPHAN 639125; DEG), the Carlsberg Foundation (D.S.P.) and The Danish Council for Independent Research (DFF – 1331-00180) and the Wellcome Trust, NIHR Biomedical Research Centre, MRC, Bernard Wolfe Endowment and ERC (I.S.F.)
Author contributions
D.E.G. and H.B.-O. designed the study and supervised the computational and pharmacological work, respectively. V.I., M.A.S. and A.S.H. performed the computational part, A.C.N. performed the pharmacological characterization on GPR139, J.M. and I.S.F. performed the pharmacological characterization on the melanocortin receptors, and D.S.P did the chemical analysis. A.C.N. drafted the manuscript, with input from M.A.S. and A.S.H., which was commented on and approved by all authors.

References
Dvorak, C.A., Coate, H., Nepomuceno, D., Wennerholm, M., Kuei, C., Lord, B., Woody,


Huszar, D., Lynch, C.A., Fairchild-Huntress, V., Dunmore, J.H., Fang, Q., Berkemeier,


Mountjoy, K.G., Mortrud, M.T., Low, M.J., Simerly, R.B., Cone, R.D., 1994. Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control


**Figure 1.** GPR139 and peptide receptors have similar transmembrane ligand binding cavities. Top: A 2D snake-like diagram sequence representation of GPR139. Residues located in the proposed binding cavity are highlighted in red. Bottom: Alignment of the residues located in the proposed binding cavity of GPR139 to the corresponding residues in all other class A GPCRs. Circles in front of receptor name; Green: peptide, and Grey: orphan. Grey/Orange: GPER a target with a proposed ligand in several publications but not yet confirmed by IUPHAR (Prossnitz and Filardo, 2016). Abbreviations: I% = percent sequence identity, S% = percent sequence similarity, S = similarity score. The alignment was made in GPCRdb (Isberg et al., 2016). Residue numbering is according to GPCRdb numbering system (Isberg et al., 2015).

**Figure 2.** Concentration response curves of the active peptides on GPR139. GPR139 activates the G<sub>q</sub> signaling pathway leading to an increase in intracellular Ca<sup>2+</sup> in CHO-cells stably expressing the human GPR139, which was measured in a Fluo-4 Ca<sup>2+</sup>-assay. Compound 1a (cmp1a) is a surrogate GPR139 agonist used as a control. The Ca<sup>2+</sup> response induced by buffer and by 8 μM cmp1a (E<sub>max</sub> of cmp1a) are represented by dashed and dotted lines, respectively. Data are mean ± SEM of three experiments performed in triplicates and normalized to buffer and E<sub>max</sub> of cmp1a. A) Ca<sup>2+</sup>-response of the endogenously expressed peptides; ATCH, α-MSH and β-MSH on GPR139, B) Ca<sup>2+</sup>-response of the two conserved motifs; HFRW and α-MSH<sub>4-10</sub> (capped and un-capped), C) Ca<sup>2+</sup>-response of the two N-terminal ends of α-MSH; α-MSH<sub>1-9</sub> and α-MSH<sub>1-10</sub> (capped and un-capped).

**Figure 3.** A potential new cleavage site in the precursor POMC and physiological peptide. The blue arrows and asterisks indicate a potential new cleavage site on the precursor POMC represented by the motif GK right after the conserved motif HFRW on α-MSH<sub>1-9</sub>. Red asterisks and arrows indicate known cleavage motifs on POMC leading to α-MSH. The red box indicates α-MSH and the blue box indicates α-MSH<sub>1-9</sub>, which is a putative new physiological peptide of POMC.

**Figure 4.** α-MSH activates both GPR139 and MC4R, whereas α-MSH<sub>1-9</sub> only activates GPR139 and melanotan II only activates MC4R. Concentration-response curves of α-MSH, α-MSH<sub>1-9</sub>, cap-α-MSH<sub>1-9</sub> and melanotan II on A) GPR139 in a Ca<sup>2+</sup>-Fluo-4 assay. The Ca<sup>2+</sup> response induced by buffer is represented by a dashed line. The Ca<sup>2+</sup> responses are normalized to E<sub>max</sub> of α-MSH, B) MC4R in a cAMP assay. The
cAMP response induced in mock transfected cells is represented by a dashed line. The cAMP responses are normalized to $E_{\text{max}}$ of α-MSH.

**Table 1. Potencies, efficacies and peptide sequence alignment of the POMC derived peptides and analogues.** The mean $pE_{50} \pm \text{SEM}$, mean $E_{50}$ and the relative $E_{\text{max}} \pm \text{SEM}$ (compared to the surrogate GPR139 agonist cmp1a) of 3 experiments performed in triplicates and normalized to buffer (0%) and $E_{\text{max}}$ of cmp1a (100%) are shown to the left. To the right is shown a peptide sequence alignment of all POMC derived peptides and analogues. Abbreviations: Ac: N-terminal acetylation, NH$_2$: C-terminal amidation, cap-: the peptides are N-terminal acetylated and C-terminal amidated, SEM: standard error of mean, ND: not determined, NA: not active. ATCH, α-MSH, β-MSH, the conserved motif HFRW activate Ca$^{2+}$-responses in CHO-cells expressing the GPR139 in the low micromolar range, and the N-terminal of α-MSH (both α-MSH$_{1-10}$ and α-MSH$_{1-9}$) activate Ca$^{2+}$-responses in the submicromolar range.
--- GKRSYSMEHFRWGKPVVGK---

α-MSH

Potential peptide (α-MSH<sub>1-9</sub>)

Known cleavage motifs

Potential cleavage motif

Cleavage sites
<table>
<thead>
<tr>
<th>Peptide</th>
<th>mean pEC$_{50} \pm$ SEM</th>
<th>mean EC$_{50}$ (µM)</th>
<th>relative E$_{\text{max}} \pm$ SEM (%)</th>
<th>Peptide sequence alignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-MSH$_{1-10}$</td>
<td>6.47 ± 0.05</td>
<td>0.318</td>
<td>68 ± 4</td>
<td>-</td>
</tr>
<tr>
<td>α-MSH$_{1-9}$</td>
<td>6.23 ± 0.05</td>
<td>0.596</td>
<td>81 ± 4</td>
<td>-</td>
</tr>
<tr>
<td>ACTH</td>
<td>5.69 ± 0.05</td>
<td>2.06</td>
<td>90 ± 4</td>
<td>-</td>
</tr>
<tr>
<td>α-MSH</td>
<td>5.66 ± 0.04</td>
<td>2.21</td>
<td>89 ± 10</td>
<td>-</td>
</tr>
<tr>
<td>HFRW</td>
<td>5.4 ± 0.05</td>
<td>4.00</td>
<td>64 ± 6</td>
<td>-</td>
</tr>
<tr>
<td>β-MSH</td>
<td>5.20 ± 0.05</td>
<td>6.30</td>
<td>101 ± 11</td>
<td>-</td>
</tr>
<tr>
<td>cap-α-MSH$_{1-10}$</td>
<td>ND</td>
<td>ND</td>
<td>53 ± 9 (at 61 µM)</td>
<td>-</td>
</tr>
<tr>
<td>cap-α-MSH$_{1-9}$</td>
<td>ND</td>
<td>ND</td>
<td>31 ± 4 (at 14 µM)</td>
<td>-</td>
</tr>
<tr>
<td>cap-HFRW</td>
<td>ND</td>
<td>ND</td>
<td>23 ± 6 (at 122 µM)</td>
<td>-</td>
</tr>
<tr>
<td>cap-α-MSH$_{1-10}$</td>
<td>ND</td>
<td>ND</td>
<td>21 ± 8 (at 16 µM)</td>
<td>-</td>
</tr>
<tr>
<td>α-MSH$_{4-10}$</td>
<td>ND</td>
<td>ND</td>
<td>13 ± 2 (at 101 µM)</td>
<td>-</td>
</tr>
<tr>
<td>α-MSH$_{4-13}$</td>
<td>NA</td>
<td>NA</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>cap-α-MSH$_{1-13}$</td>
<td>NA</td>
<td>NA</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>α-MSH$_{P-Ser}$</td>
<td>NA</td>
<td>NA</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ACTH$_{1-16,P-Ser}$</td>
<td>NA</td>
<td>NA</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>β-MSH$_{5-22}$</td>
<td>NA</td>
<td>NA</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>cap-β-MSH$_{5-22}$</td>
<td>NA</td>
<td>NA</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>γ1-MSH</td>
<td>NA</td>
<td>NA</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Highlights

- Using GPCRdb we found that the binding cavity of GPR139 is 49% similar to MC4R
- ACTH, α-MSH and β-MSH activate GPR139 in the low μM-range
- We predicted a novel possible cleavage site in POMC leading to the peptide α-MSH_{1-9}
- α-MSH_{1-9} activates GPR139 in the high nM range
Supplementary Information

Fig. 1. Structure of cmp1a (Shi et al., 2011).

Fig. 2. Analytical data for ACTH, α-MSH and β-MSH (HPLC, LC-MS and MALDI-TOF).

A) ACTH (Adrenocorticotropic hormone)
Peptide: H-SYSHFRGWGPVKRRPVVKYPNGAEDEAEAFPLEF-OH

HPLC data for ACTH (UV absorbance at 280 nm)
LCMS spectrum for ACTH (Deconvoluted mass spectrum showing the 3+ to 8+ ions)

MALDI-TOF MS spectrum for ACTH (Mass spectrum showing the 1+ and 2+ ions)
B) α-MSH (α-Melanocyte-stimulating hormone)
Peptide: Ac-SYSMEHFRWGKPV-NH₂

HPLC chromatogram for α-MSH (UV absorbance at 280 nm)
By LCMS the minor peak at 11.2 min was identified as oxidized α-MSH (oxidation of the methionine residue to the corresponding sulfoxide, M+16).

LCMS spectrum for α-MSH (Deconvoluted mass spectrum showing the 1+ to 3+ ions)

<table>
<thead>
<tr>
<th>Charge</th>
<th>Monoisotopic mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>MH1+</td>
<td>1664.8</td>
</tr>
<tr>
<td>MH2+</td>
<td>832.9</td>
</tr>
<tr>
<td>MH3+</td>
<td>555.6</td>
</tr>
</tbody>
</table>
MALDI-TOF MS data for α-MSH (Mass spectrum showing the 1+ ion)
c) β-MSH (β-Melanocyte-stimulating hormone)
Peptide: H-AEKKDEGPYRMETHFRWGSPPKDOH

HPLC data for β-MSH (UV absorbance at 280 nm)

LCMS spectrum for β-MSH (Deconvoluted mass spectrum showing the 2+ to 6+ ions)

<table>
<thead>
<tr>
<th>Charge</th>
<th>Monoisotopic mass</th>
<th>Charge</th>
<th>Monoisotopic mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>MH1+</td>
<td>2660.3</td>
<td>MH4+</td>
<td>665.8</td>
</tr>
<tr>
<td>MH2+</td>
<td>1330.6</td>
<td>MH5+</td>
<td>532.9</td>
</tr>
<tr>
<td>MH3+</td>
<td>887.4</td>
<td>MH6+</td>
<td>444.2</td>
</tr>
</tbody>
</table>
MALDI-TOF MS spectrum for β-MSH (Mass spectrum showing the 1+ ion)
D) Phenylalanine (Phe) and Tryptophan (Trp)

HPLC chromatogram for Phe (UV absorbance at 254 nm)

HPLC chromatogram for Trp (UV absorbance at 254 nm)
Fig. SI3. α-MSH, cap-α-MSH\textsubscript{1-9} and melanotan II activate a cAMP response in cells transiently transfected with MC3R, but α-MSH\textsubscript{1-9} do not have any effect. cAMP response in the HitHunter® cAMP assay are all normalized to E\textsubscript{max} of α-MSH on MC3R.
Fig. SI4. HPLC at 220 nm for all custom-synthesized peptides (GenScript)
Fig. SI5. MS for all costum-synthesized peptides (GenScript)
Table SI1. The molecular weight, the net peptide content (NPC%) and HPLC Purity % for all costum-synthesized peptides made by GenScript. The molecular weight is calculated and in compliance with the MS in SI.Fig.3. The NPC% is determined using a Vario MICRO Organic Elemental Analyzer. The HPLC Purity % summarized from SI. Fig.2. Abbreviations: MW: molecular weight, NPC%: percent net peptide content.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>MW</th>
<th>NPC %</th>
<th>HPLC Purity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-MSH&lt;sub&gt;1-10&lt;/sub&gt;</td>
<td>1299.4</td>
<td>75.35</td>
<td>98.1</td>
</tr>
<tr>
<td>α-MSH&lt;sub&gt;1-9&lt;/sub&gt;</td>
<td>1242.4</td>
<td>75.46</td>
<td>98.4</td>
</tr>
<tr>
<td>HFRW</td>
<td>962.1</td>
<td>63.19</td>
<td>98.9</td>
</tr>
<tr>
<td>α-MSH&lt;sub&gt;4-10&lt;/sub&gt;</td>
<td>644.7</td>
<td>ND</td>
<td>97.2</td>
</tr>
<tr>
<td>α-MSH&lt;sub&gt;4-13&lt;/sub&gt;</td>
<td>1286.5</td>
<td>ND</td>
<td>98.6</td>
</tr>
<tr>
<td>β-MSH&lt;sub&gt;5-22&lt;/sub&gt;</td>
<td>2204.4</td>
<td>ND</td>
<td>95.5</td>
</tr>
<tr>
<td>cap-α-MSH&lt;sub&gt;1-10&lt;/sub&gt;</td>
<td>1340.5</td>
<td>76.35</td>
<td>98.4</td>
</tr>
<tr>
<td>cap-α-MSH&lt;sub&gt;1-9&lt;/sub&gt;</td>
<td>1283.4</td>
<td>79.59</td>
<td>98.7</td>
</tr>
<tr>
<td>cap-HFRW</td>
<td>1003.1</td>
<td>68.98</td>
<td>96.7</td>
</tr>
<tr>
<td>cap-α-MSH&lt;sub&gt;4-10&lt;/sub&gt;</td>
<td>685.8</td>
<td>63.85</td>
<td>99.1</td>
</tr>
<tr>
<td>cap-α-MSH&lt;sub&gt;4-13&lt;/sub&gt;</td>
<td>1327.6</td>
<td>62.43</td>
<td>98.5</td>
</tr>
<tr>
<td>cap-β-MSH&lt;sub&gt;5-22&lt;/sub&gt;</td>
<td>2245.4</td>
<td>70.85</td>
<td>96.4</td>
</tr>
<tr>
<td>ACTH&lt;sub&gt;1-16,P-Ser&lt;/sub&gt;</td>
<td>2017.2</td>
<td>66.22</td>
<td>98.1</td>
</tr>
<tr>
<td>α-MSH&lt;sub&gt;P-Ser&lt;/sub&gt;</td>
<td>1702.8</td>
<td>81.92</td>
<td>97.6</td>
</tr>
</tbody>
</table>