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Identification and in Vivo and in Vitro Characterization of Long Acting and Melanocortin 4 Receptor (MC4-R) Selective α-Melanocyte-Stimulating Hormone (α-MSH) Analogues

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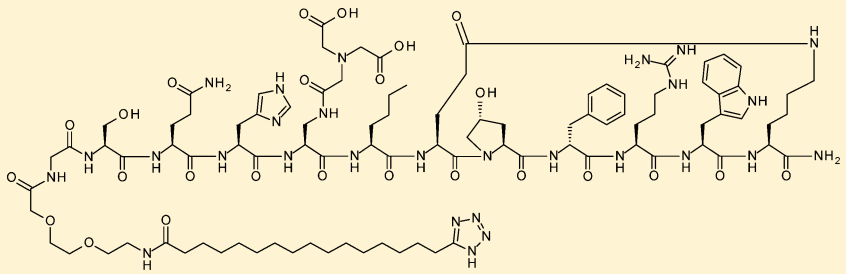
Supporting Information



ABSTRACT: We report in vitro and in vivo data of new α-melanocyte-stimulating hormone (α-MSH) analogues which are N-terminal modified with a long chain fatty acid derivative. While keeping the pharmacophoric motif (D-Phe-Arg-Trp) fixed, we tried to improve selectivity and physicochemical parameters like solubility and stability of these analogues by replacing amino acids further away from the motif. Receptor specific changes in binding affinity to the melanocortin receptors were observed between the acetyl derivatives and the fatty acid analogues. Furthermore, amino acids at the N-terminal of α-MSH (Ser-Tyr-Ser) not considered to be part of the pharmacophore were found to have an influence on the MC4/MC1 receptor selectivity. While the acetyl analogues have an in vivo effect for around 7 h, the long chain fatty acid analogues have an effect up to 48 h in an acute feeding study in male Sprague−Dawley rats after a single subcutaneous administration.

INTRODUCTION

The melanocortin system is one of the most important players in the central regulation of energy balance. The melanocortin system is comprised of peptide hormones and neuropeptides derived from the common hormone precursor pro-opiomelanocortin (POMC)[1,2] and five G-protein coupled receptors (melanocortin receptor 1−5, MC1−5R).[3−9] The melanocortin peptides and receptors mediate a number of different physiological functions, for example regulation of skin pigmentation,[10] sexual function,[11] feeding behavior[12] and energy homeostasis,[13] steriodogenesis,[14] and inflammation.[15,16] In the rodent brain, α-MSH (**1**)[17,18] is one of the main products of the POMC precursor, and in rodents peptide **1** is believed to mediate the central effects of melanocortins on energy regulation. In humans, both peptide **1** and β-MSH[19−21] are expressed and both are believed to be involved in the regulation of body weight. This effect is mainly mediated through the MC4 receptor (MC4-R), which is expressed in several regions of the brain including the hypothalamus and the brain stem.[22] The MC4-R has been shown to be the key melanocortin receptor involved in the regulation of body weight and is recognized as a promising target to therapeutically approach obesity. It has been shown in rodents that activation of the MC4-R suppresses appetite and increases the metabolic rate, leading to a significant weight loss. In vivo studies in rodents and data from MC4-R deficient humans underscore the importance of the MC4-R in body weight regulation; both mice and humans deficient in MC4-R are obese.[23−25] Both homozygous and heterozygous mice become

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Table 1. Binding (MC1-R, MC3-R, MC4-R, MC5-R) and Functional Data (MC4-R) for the Prepared Peptides[a]

name[b]	hMC1 K_i/nM	hMC3 K_i/nM	hMC4 K_i/nM	hMC5 K_i/nM	hMC4 cAMP[c] EC_{50}/nM
Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2 (α-MSH) (**1**)	1.5 ± 0.4	46 ± 5	26 ± 9	150 ± 30	3.4 ± 1.1
Ac-Ser-Tyr-Ser-Nle-Glu-His-D-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2 (NDP-α-MSH) (**2**)	0.046 ± 0.007	0.78 ± 0.16	0.30 ± 0.09	0.48 ± 0.11	0.14 ± 0.03
Ac-Nle-c[Asp-HisD-Phe-Arg-Trp-Lys]-NH_2 (MT-II) (**3**)	6.4 ± 2.4	53 ± 5	0.25 ± 0.07	17 ± 5	0.20 ± 0.03
Ac-Gly-Ser-Gln-His-Ser-Nle-c[Glu-Hyp-D-Phe-Arg-Trp-Lys]-NH_2(**4**)	200 ± 40	45 ± 11	4.6 ± 2.1	5.1 ± 1.6	2.0 ± 0.7
hexadecanoyl-Gly-Ser-Gln-His-Ser-Nle-c[Glu-Hyp-D-Phe-Arg-Trp-Lys]-NH_2(**5**)	8.0 ± 2.9	2.5 ± 0.6	0.12 ± 0.02	1.6 ± 0.15	0.17 ± 0.04
Ac-Gly-Ser-Gln-homoArg-Ser-Nle-c[Glu-Hyp-D-Phe-Arg-Trp-Lys]-NH_2 (**6**)	73 ± 17	22 ± 6	1.0 ± 0.1	4.3 ± 0.9	0.47 ± 0.12
hexadecanoyl-Gly-Ser-Gln-homoArg-Ser-Nle-c[Glu-Hyp-D-Phe-Arg-Trp-Lys]-NH_2 (**7**)	3.2 ± 1.5	2.0 ± 0.5	0.37 ± 0.17	0.47 ± 0.08	0.04 ± 0.01
4-(hexadecanoylsulfamoyl)butanoyl-Gly-Ser-Gln-His-Ser-Nle-c[Glu-Hyp-D-Phe-Arg-Trp-Lys]-NH_2 (**8**)	230 ± 110	8.2 ± 2.1	0.11 ± 0.05	1.2 ± 0.2	0.15 ± 0.03
(16-(tetrazol-5-yl)hexadecanoyl)-Oeg-Gly-Ser-Gln-His-SerNle-c[Glu-Hyp-D-Phe-Arg-Trp-Lys]-NH_2 (**9**)	1200 ± 340	67 ± 18	0.2 ± 0	15 ± 5.5	170 ± 60
hexadecanoyl-Gly-Gly-Gly-Gly-Gly-Nle-c[Glu-Hyp-D-Phe-Arg-Trp-Lys]-NH_2 (**10**)	130 ± 30	8.0 ± 2.7	0.33 ± 0.09	3.0 ± 0.9	1.0 ± 0.4
(16-(tetrazol-5-yl)hexadecanoyl)-Oeg-Gly-Ser-Gln-His-Dap-Nle-c[Glu-Hyp-D-Phe-Arg-Trp-Lys]-NH_2 (**11**)	400 ± 110	42 ± 10	0.17 ± 0.03	10 ± 3	62 ± 29
(16-(tetrazol-5-yl)hexadecanoyl)-Oeg-Gly-Ser-Gln-His-ßDap-Nle-c[Glu-Hyp-D-Phe-Arg-Trp-Lys]-NH_2 (**12**)	700 ± 250	121 ± 12	0.17 ± 0.03	40 ± 5	85 ± 20
(16-(tetrazol-5-yl)hexadecanoyl)-Oeg-Gly-Ser-Gln-His-Arg-Nle-c[Glu-Hyp-D-Phe-Arg-Trp-Lys]-NH_2 (**13**)	44 ± 13	27 ± 7	0.08 ± 0.02	3.0 ± 1.7	4.7 ± 2.0
(16-(tetrazol-5-yl)hexadecanoyl)-Oeg-Gly-Ser-Gln-His-Dap(iPr)-Nle-c[Glu-Hyp-D-Phe-Arg-Trp-Lys]-NH_2 (**14**)	270 ± 72	13 ± 2	0.26 ± 0.04	3.4 ± 0.7	15 ± 3
(16-(tetrazol-5-yl)hexadecanoyl)-Oeg-Gly-Ser-Gln-His-Dap(Me2)-Nle-c[Glu-Hyp-D-Phe-Arg-Trp-Lys]-NH_2 (**15**)	440 ± 100	11 ± 3	0.13 ± 0.03	2.9 ± 0.9	130 ± 36
(16-(tetrazol-5-yl)hexadecanoyl)-Oeg-Gly-Glu-Glu-Glu-Glu-Nle-c[Glu-Hyp-D-Phe-Arg-Trp-Lys]-Glu-NH_2 (**16**)	>10000	1600 ± 260	250 ± 98	710 ± 180	nd[d]
(16-(tetrazol-5-yl)hexadecanoyl)-Oeg-Gly-Glu-Glu-Arg-Glu-Nle-c[Glu-Hyp-D-Phe-Arg-Trp-Lys]-Glu-NH_2 (**17**)	>10000	1100 ± 280	35 ± 12	270 ± 150	270 ± 78
(16-(tetrazol-5-yl)hexadecanoyl)-Oeg-Gly-Glu-Glu-His-Glu-Nle-c[Glu-Hyp-D-Phe-Arg-Trp-Lys]-NH_2 (**18**)	8100 ± 1900	450 ± 81	7.8 ± 1.5	110 ± 21	nd
(16-(tetrazol-5-yl)hexadecanoyl)-Oeg-Gly-Ser-Gln-His-Dap{[bis(carboxymethyl)amino]acetyl}-Nle-c[Glu-Hyp-D-Phe-Arg-Trp-Lys]-NH_2 (**19**)	2700 ± 620	71 ± 12	0.58 ± 0.08	13 ± 4	88 ± 21

[a]Numbers given represent averages of replicates ($n \geq 3$) ± SEM. All binding assays were performed in ovalbumin to minimize interference with albumin binding, however this was not possible for the functional cAMP assay, which was performed in 0.1% human serum albumin (HSA). [b]Oeg is the amino acid 8-amino-3,6-dioxaoctanoic acid (H_2N-$(CH_2CH_2O)_2$-CH_2COOH). [c]All analogues except **17** and **19** were full agonists as compared to the E_{max} of α-MSH. The E_{max} of **17** and **19** were found to be 77% and 85% respectively. [d]Not determined.

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obese although the obesity of the homozygous mice is more severe than that of the heterozygous mice, which demonstrates a gene dosage effect for receptor deletion.[23] Data from studies of MC4-R knockout mice treated with the nonselective melanocortin agonist, melanotan II (MT-II, **3**),[26] supports that the MC4 receptor is important in the mediation of melanocortins effect on energy homeostasis as no effect on food intake, energy expenditure, and body weight was observed in MC4-R knockout mice treated with peptide **3**.[27−29] However, recent work from Haskell-Luevano and co-workers in both MC3-R and MC4-R knockout mice shows a partial effect on food intake in both MC3-R and MC4-R knockout mice after icv injecting of compound **3**, demonstrating that both the MC3-R and MC4-R are involved in the anorectic action of **3**,[30] in contrast Kumar et al., found that the presence of MC4-R was necessary for the effects on body weight of a novel nonselective melanocortin receptor agonist (BIM-22511) whereas the effect on hyperinsulinemia was independent of the presence of the MC4-R.[31] Furthermore, the MC4-R is involved in both sides of the energy balance equation: appetite and energy expenditure.[32,33] Several pharmaceutical companies have been developing oral available small molecules targeting the MC4-R for the treatment of obesity, however, with limited success,[34] and although the involvement of the MC4-R in body weight regulation was demonstrated 15 years ago, no drug development program has progressed past phase I, showing that obtaining orally available selective small molecule compounds targeting the MC4-R is a difficult task.[35] Thus a long acting MC4-R selective peptide agonist for subcutaneous administration could be an attractive alternative to a small molecule for the treatment of obesity.

Early structure−activity relationship (SAR) work by Hruby et al.[36] revealed the important amino acids in peptide **1** for the activation of the melanocortin receptors. The sequence Phe-Arg-Trp appears to be essential, while the amino acids in 4 (Met) and 6 (His) position are less important. Replacement of the other amino acids with an alanine (Ala-scan) does not have an impact on the binding affinity. Inversion of the stereochemistry of the phenylalanine in position 7 and substituting the methionine with a norleucine increases the binding affinity by at least ahundred fold as well as rendering the peptides, Ac-Nle^4, D-Phe^7-α-MSH-NH$_2$, or NDP-α-MSH (**2**),[37] more stable toward proteolysis or oxidation. A cyclic truncated version of compound **1** is peptide **3**, which contains a lactam bridge from position 5 (Glu→Asp) to position 10 (Gly→Lys): Ac-Nle^4, c[Asp^5, D-Phe^7, Lys^{10}]-α-MSH [4−10]-NH$_2$. The binding affinities of these three reference peptides toward MC1-R, MC3-R, MC4-R, and MC5-R and functional activity toward the MC4-R are shown in Table 1.

For the development of an α-MSH analogue for the treatment of obesity, a more selective receptor profile than **3** is desired. Previous SAR work has implicated the histidine in position 6 as being involved in receptor selectivity of the MC4-R versus the MC1/3-R.[38−40] The desired target profile of the development candidate was decided to comprise the following parameters: a pharmacokinetic profile for a once daily subcutaneous administration and a selectivity for the MC4-R against the MC1-R greater than 1000-fold. The selectivity against the MC1-R is prioritized because it appears to be involved in the regulation of the immune systems[41] as well as pigmentation.[42,43] Some selectivity versus MC3-R and MC5-R was desired to have a specific drug but not considered as important as the selectivity versus the MC1-R. Besides the effects on energy homeostasis,[29] the MC3-R has been implicated in control of inflammation.[44] The MC5-R has been discussed to be involved in lipolysis,[45] and furthermore it has been implicated in the function of the sebaceous gland.[46]

RESULTS AND DISCUSSION

Generally, for binding to the MC1-R, MC3-R, MC4-R, and MC5-R, the obtained K_i values of the three reference compounds, **1**, **2**, and **3** (Table 1) correspond to the data found in the literature,[47−50] however some differences exists which are likely to be due to differences in binding assay protocols, e.g., binding to whole cells or membranes and some publications giving IC_{50} values instead of K_i. Also, differences in the literature are reported like the values for peptide **2** on the MC5-R that were reported to be 0.86 nM by Haskell-Luevano[47] et al. and 5.05 by Schiöth[48] et al. Also, the values for compound **1** binding to the MC4-R varies greatly in the literature from a IC_{50} of 38 nM[47] to a K_i of 900 nM in a paper from Schiöth et al.[51] These large differences may reflect different conditions used in the binding assay. For the purpose of increasing the selectivity and comparing to the reference peptides, it is important to compare the values obtained from the same assays, both for the reference peptides and the new analogues. It was not possible to establish a robust assay with the exact same binding conditions across the four binding assays, but for the purpose of comparing the changes to reference compounds within each receptor assay and for the comparison of the changes in the relative selectivity between the receptors, the present assays were considered acceptable, although it should be kept in mind that recombinant expression systems are artificial systems and do not completely reflect the conditions in the body. With regard to the functional MC4-R assay the EC_{50} of peptides **1**, **2**, and **3** are within the range of published values.[47,50,52−54] Concerning the relation between K_i and EC_{50}, the EC_{50} for **3** and **2** correspond to the K_i values, whereas for peptide **1**, the EC_{50} is lower than the K_i, which may suggest the presence of spare receptors as this would left-shift[55] the functional curves. However, if this were the case, the EC_{50} values for compounds **2** and **3** should also be lower than the corresponding K_i. In the functional assay used here, we are also able to detect partial agonists,[56,57] which also speaks against a high level of spare receptors.

The half-life in vivo in humans of the natural peptide hormone **1** is around 21 min,[58] while PT-141,[59] a shorter and cyclic analogue of ligand **1** and a close analogue to peptide **3**, has a half-life of 2 h. To achieve a half-life appropriate for a once-daily injection, we decided to attach a fatty acid or fatty acid analogue to a peptide with a suitable selectivity profile. As a selective starting peptide, we used analogue **4**, with the histidine in position 6 substituted with a hydroxy proline in the pharmacophore quartet. In Table 1, the binding data of the analogues to MC1-R, MC3-R, MC4-R, and MC5-R are shown. An acylated analogue of **4** is peptide **5**, possessing a hexadecanoyl moiety at the *N*-terminal. To our surprise, the binding affinity of the acylated peptide **5** increased approximately 30-fold for the MC4-R but less for the MC3-R (approximately 20-fold) and not for the MC5-R. For the MC1-R, a similar increase in binding affinity as for the MC4-R of approximately 25-fold was observed, causing the acylated peptide **5** to have a different selectivity profile than the original peptide **4** (Table 1). In addition, a similar effect was observed for the peptides **6** and **7** but with differences between the receptors in the fold increase in binding affinity. This change in

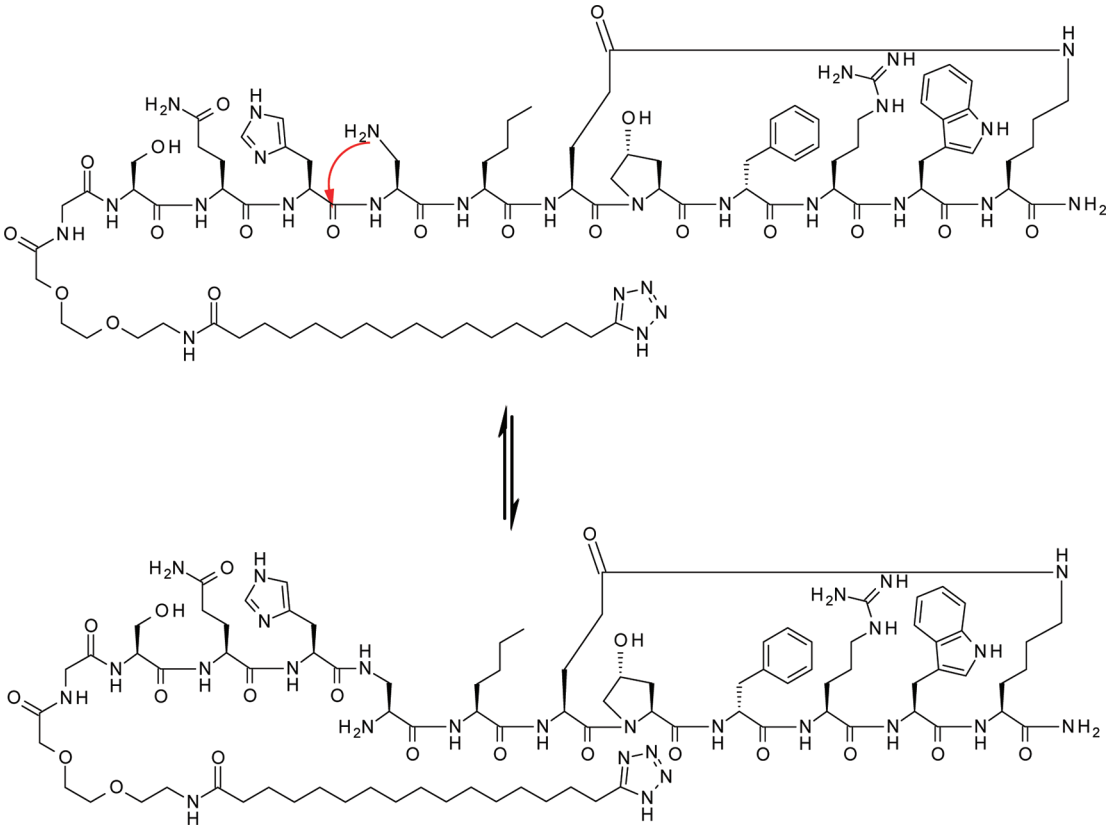

Figure 1. Transpeptidation of peptides **11** and **12**.

the selectivity profile indicates a specific interaction of the fatty acid chain with the receptor and not an unspecific interaction with the membrane.

Adding negative charged groups close to the fatty acid as in peptides **8** (the *N*-acyl-sulfamoyl moiety has a pK_a around 2−5)[60] or **9** decreases binding to the MC1-R significantly but not the binding to the MC4-R.

To probe for the receptor interaction of the five amino acids between the norleucine and the fatty acid, an all Gly analogue was prepared (**10**). Again, only the affinity for the MC1-R decreased, supporting the theory about a specific interaction of this part of the peptide with the MC1-R, whereas the other receptors are less sensitive to the amino acid residues in this area of the molecule.

When comparing the K_i values of the acylated analogues with the EC_{50} values, a shift in potency was observed with the potency of the acylated analogues in the cAMP assay being lower than expected from the binding K_i value (see Table 1). This is most probably due to interference from albumin binding in the cAMP assay, which was performed in the presence of 0.1% human serum albumin (HSA), whereas the binding assay was performed in ovalbumin to avoid interference from albumin. It was not possible to eliminate the presence of albumin in the functional assay, and the data from the cAMP assay is more used to demonstrate agonism on the MC4-R than to discuss selectivity. With compounds **1**, **2**, and **3**, no such right shift was observed when the EC_{50} values are compared to the K_i values, reflecting the low albumin interaction with these compounds.

In the course of our search for a lead compound, we had to add a new optimization parameter: solubility. Preparing sterile filtered solutions with peptide concentrations around 1−3 mg/mL was a challenge, most likely due to aggregation. Only when we introduced an additional charge next to the norleucine position the solubility of the peptides increased at a pH below 5.5.

Peptide **11** was the first compound in a series of potential candidates having a diaminopropionic acid (Dap) as isosteric replacement for the Ser in **9**. However, one interesting feature was discovered upon closer investigation of the stability of the molecule. Under neutral and basic conditions, peptide **11** undergoes transpeptidation to **12** (Figure 1). The exact ratio is pH dependent; a more basic pH shifts the equilibrium toward **12**. The reaction is much slower under acidic conditions and the equilibrium is shifted toward **11**. In agreement with the findings by Blodgett and Loudon,[61] the equilibration could be catalyzed with anions like acetate and phosphate (data not shown).

Keeping the positive charge in this position, we prepared a few analogues **13**, **14**, and **15**, which could not isomerize but had a similar profile on the receptors. Most of these peptides were active in an acute feeding model (Table 2).

The acute effect on food intake of all analogues except peptides **6** and **16** was tested at the onset of dark in ad libitum fed male lean Sprague−Dawley rats where food intake was followed for 48 h, measuring food intake at 7, 24, and 48 h. Analogues that were able to reduce food intake past the 7 h time point were defined as long acting compounds compared to the reference compound **3** that reduced food intake for up to 7 h but were not different from vehicle when food intake for time periods 7−24 h or 24−48 h was measured.

In general, the peptides have an effect in the acute feeding study for 24 h up to 48 h, except for the two *N*-terminal acetylated peptides **3** and **4**.

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Table 2. Results of Feeding Experiments after a Single Injection of 3 mg/kg Peptide or Vehicle in Male Sprague–Dawley Rats[a].

	% of vehicle food intake		
entry	0–7 h	7–24 h	24–48 h
3	23***[b]	102	101
	58***	122*[d]	100
	49***	118	100
	56***	91	103
4	55***	99	103
5	73	61***	81***
	63***	58***	96
7	97	57**[c]	82*
	71**	92	103
8	40***	36***	58***
9	79	56***	92
10	110	76*	94
11	79	45***	73***
	70	68***	85**
	49***	55***	82***
12	82	54***	81***
13	46***	51***	83***
14	60**	40***	73***
	50***	53***	72***
15	62*	60***	82***
17	74**	90	98
18	75	79	88*
19	53***	39***	66***

[a]The data are shown as % food intake compared to the vehicle food intake in the same time periods. A vehicle group of 7–8 animals was included in each separate experiment and each treatment group was from 7–8 animals. For absolute food intake and SEM of each experiment see Supporting Information. Multiple results for the same entry indicate multiple experiments under the same conditions. [b]***$p < 0.001$. [c]**$p < 0.01$. [d]*$p < 0.05$ as compared to the respective vehicle group.

After the introduction of the extra charge *N*-terminal to the norleucine, the solubility of the peptides was largely improved at pH below 5.5. The earlier less soluble peptides had a tendency toward being associated with injection site reactions, but also the more positive charged peptides containing extra arginines were prone to injection site reactions, most likely due to the release of subcutaneous histamine as seen with several positively charged peptides such as NPY,[62] substance P,[63,64] and VIP.[65−67] To have access to peptides soluble around pH 7, we introduced negative charges into the peptide as in compounds **16**, **17**, **18**, and **19**. Peptide **16** dropped around 400-fold in affinity to the MC4-R, and also dropped in affinity to the other MC-R, indicating that too high a negative charge in this area influences binding to all MC-R, but by reducing the total negative charge by replacing a glutamate with an arginine (peptide **17**) or histidine (peptide **18**), we could rescue the affinity toward the MC4-R. Unfortunately, these two peptides were less active in vivo (Table 2).

Peptide **19**, derived from **11** by adding a nitrilotriacetic acid moiety to the sidechain of the Dap amino acid, had the desired solubility profile (Figure 2) as well as good affinity toward the MC4-R, an attractive selective profile (Table 1) and an in vivo effect suitable for at least an once daily treatment (Table 2). A few key peptides (**11**, **12**, **19**) have also been tested in a rat functional MC4-R assay and found to be full agonist (data not shown).

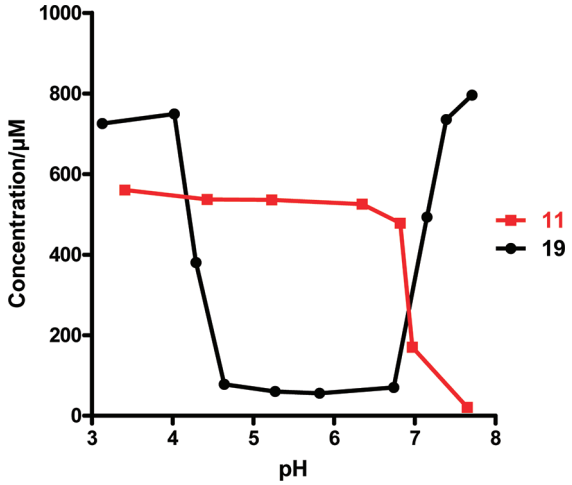

Figure 2. Solubility curves of peptides **11** and **19**.

CONCLUSION

In this publication, we present 16 MC4-R peptide agonists. They are composed of a macrocyclic lactam containing the pharmacophores and a norleucine attached at the N-terminal. This part we kept fixed for this study. From previous SAR work (data unpublished) inspired by work of Cai et al.,[68] we considered the substitution of the histidine in position 6 with an hydroxy proline a good starting point to achieve selectivity over the MC1-R. Whether the selectivity was gained via removal of the imidazole or by a change in the conformation of the macrocycle backbone is unclear. A peptide chain of five amino acids followed by a fatty acid derivative was attached to the α-amine of the norleucine. The five amino acids were introduced as a linker between the pharmacophore and the fatty acid to keep the high affinity binding to the MC4-R. By modulating the properties of these five amino acids, we could change physicochemical parameters without affecting binding to the MC4-R, except in the case **16**, which contains too many negative charges. The affinities toward the MC3- and MC5-R are only affected to a lesser degree, in contrast to the affinity for the MC1-R, which seems to be very sensitive to this part of the molecule. This demonstrated that this part of the molecule is also important for receptor interaction and may be viewed as an additional pharmacophore for adjusting the affinity and selectivity at the MC-R. Furthermore, our data supports the idea of a hydrophobic site in the MC1/3/4-R in contrast to the MC5-R. Todorovic[69] has published data of fatty acid derivatives where the fatty acid was attached to the His-D-Phe-Arg-Trp tetra peptide without observing similar differential effects on the MC receptors, arguing for an unspecific membrane interaction, however the fatty acid was in this case attached directly to the pharmacophoric tetrapeptide and may therefore influence the effect on binding differently than our peptides which have a spacer between the pharmacophoric unit and the fatty acid.

The profile regarding selectivity, solubility and stability of peptide **19** fulfill the criteria set at the time for progressing the compound into further development.

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EXPERIMENTAL SECTION

Peptide Synthesis. Peptides were synthesized using standard Fmoc chemistry[70] using 4 equiv of diisopropyl carbidiimde (DIC) and 4 equiv of hydroxybenzotriazole (HOBt) in *N*-methylpyrrolidone (NMP) on Rink amide polystyrene resin (~0.55 mmol/g). The *N*-terminal Fmoc protecting group was removed after treatment with 20% piperidine for 20 min. The amino acids involved in the cyclization were protected on their side chain by 2-phenylisopropyl on Glu or 4-methyltrityl (Mtt) on Lys. Cyclizations were done after the synthesis of the linear peptide on resin (**4**–**18**) or after the coupling of the second amino acid (Glu) involved in the cyclization (**19**) using 4 equiv of benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), 4 equiv of HOBt, and 8 equiv of ethyldiisopropylamine in NMP. Removal of the protecting groups prior to cyclization was accomplished by repeated treatment (10×) with 1–2% trifluoroacetic acid (TFA) and 3–4% triethylsilane or triisopropylsilane (TIPS) in dichloromethane. The fatty acids or fatty acid derivatives were coupled using amino acid coupling conditions.

The side chain modification of peptide **19** was introduced by using Fmoc-Dap(Mtt)-OH for the backbone assembly, deprotection of the amino group using the condition described above, and coupling of *N*,*N*-bis[(*tert*-butoxycarbonyl)methyl]glycine[71] to the side chain amine using standard peptide coupling conditions.

Peptide were cleaved from resin with 93% TFA, 2% TIPS, 2.5% water, and 2,5 dithiotreitol (Dtt) for 2 h and isolated by precipitation with ether.

The crude peptides were purified on a reverse phase preparative HPLC using a C18 column (Waters XBridge PrepC18, 5 μm, 50 mm × 250 mm), with an acetonitrile/water 0.1% TFA gradient from 35–55% over 40 min. Fractions containing the pure peptide were collected and lyophilized. Typical yields for isolated peptides were between 10 and 20%. The peptides were stored in this form at 4 °C. All peptides were analyzed by LC-MS (Agilent 6230 TOF LC-MS), purity was ≥95% (peptide content), and freeze-dried powder usually contained also water and trifluoroacetate. Peptide content was determined using a HPLC with a chemiluminescent nitrogen detector (CLND)[72] from Antek Instruments (Houston, TX, USA).

Analytical data for

11: calcd mass 1918.07 Da, found m/2 = 960.05 Da, m/3 = 640.38 Da.

12: calcd mass 1918.07 Da, found m/2 = 960.06 Da, m/3 = 640.39 Da.

19: calcd mass 2091.10 Da, found m/2 = 1046.59 Da, m/3 = 698.06.

Binding Assays. The human MC1, 3, and 5 receptors were cloned by PCR and subcloned into the pcDNA3 expression vector. The MC4-R was bought from Euroscreen in pcDNA3. Cells stably expressing the human MC-R were generated by transfecting the expression vector into BHK570 cells (ATTC) and using 1 mg/mL G418 to select for stable clones. The stable cell lines were cultured in DMEM with glutamax, 10% FCS, 1% pen/strep, and 1 mg/mL G418 at 37 °C and 5% CO_2.

The compounds were characterized in vitro using ^{125}I-NDP-α-MSH binding to membranes from recombinant BHK570 cells expressing the relevant human melanocortin receptor using a filtration system to separate bound from unbound radioligand (K_d: MC1-R 45 ± 12 pM (n = 3), MC3-R 1.46 ± 0.34 nM (n = 3), MC4-R 0.34 ± 0.13 nM (n = 5), and MC5-R 0.96 ± 0.23 nM (n = 4)). In each assay, the amount of membranes were adjusted to avoid depletion in the assay, i.e., total binding of the radioligand less than 10% in each assay. ^{125}I-NDP-α-MSH was bought from either Perkin-Elmer (NEX352) and before 2007 from Amersham biosciences (IM316). The binding assays were performed in the presence of ovalbumin to avoid interaction of the analogues with fatty acids with 0.1% albumin. All binding assays were performed in duplicates and at least three times for each compound on membranes prepared in the following way: The cells were rinsed with phosphate buffered saline (PBS) and incubated with Versene for approximately 5 min before harvesting. The cells were flushed with PBS, and the cell-suspension was centrifuged at 4 °C for 10 min at 27000*g*. The pellet was resuspended in 20 mL of ice-cold buffer (20 mM Tris pH 7.2 + 5 mM EDTA + 1 complete protease inhibitor tablet/50 mL (Boehringer Mannheim 1 873 580)) and homogenized with glass-Teflon homogenizer, 10 times and low speed. The cell-suspension was centrifuged at 4 °C at 4100*g* for 20 min. Pellets were resuspended in the relevant ice-cold assay buffer (see description under each assay), and the membranes were diluted to a protein concentration of 1 mg/mL in relevant assay buffer, aliquoted, and kept at −80 °C until use. Protein content was determined using the Bio-Rad protein assay (cat. no. 500−0006). For the MC4-R, the cell pellet was resuspended in 20 mM HEPES pH 7.1, 5 mM $MgCl_2$ and 1 mg/mL bacitracin, and the final protein concentration was 15−17 mg/mL. Test compounds were dissolved in dimethylsulfoxide (DMSO) to a concentration of 4 mM, and the compounds were tested in the range of concentrations from 10^{-11} to 10^{-5} M, except for NDP-α-MSH in the MC1-R assay, where the range was $10^{-12}$to 10^{-6} M due to the high affinity to the MC1 receptor. Saturation analysis was used for K_d determinations. For the MC1-R, 10 concentrations of ^{125}I-NDP-α-MSH in the range of 0.1−100 pM were used, for the MC3-R and the MC5-R, 12 concentrations of ^{125}I-NDP-α-MSH in the range of 12.5 pM to 5 nM were used, and for the MC4-R, 11 concentrations of ^{125}I-NDP-α-MSH in the range of 2.6−800 pM were used. Nonspecific binding was determined in the presence of 10 μM ^{125}I-NDP-α-MSH.

Melanocortin Receptor 1 (MC1-R) Binding Assay. The assay was performed in duplicates in a total volume of 250 μL; 25 μL of ^{125}I-NDP-α-MSH (100 pM in final concentration), 25 μL of test compound/control, and 200 μL of cell membrane (35 μg/mL). Radioligand, membranes, and test compounds were diluted in buffer (25 mM HEPES pH 7.4, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM EDTA, 0.1% ovalbumin, 0.005% Tween-20, 5% hydroxypropyl-β-cyclodextrin (97%, Acros organics, code 297561000)). The samples were incubated at 30 °C for 90 min in the Greiner microtiter plates, and incubation was separated with GF/B filters that were prewetted for 60 min in 0.5% polyethyleneimine, and washed 2−3 times with NaCl (0.9%) before separation of bound from unbound radioligand by filtration in a Packard Filtermate harvester. After filtration, the filters were washed with ice-cold 0.9% NaCl 10 times. The filters were dried at 50 °C for 30 min, sealed, and 30 μL of Microscint 0 (Packard, cat no. 6013616) were added to each well and the plates were counted in a Topcounter 1 min/well.

Melanocortin Receptors 3 and 5 (MC3-R and MC5-R) Binding Assay. The assay was performed in duplicates in a volume of 100 μL and the ingredients were mixed in the following order: 25 μL of test-compound, 25 μL of ^{125}I-NDP-α-MSH (250 pM in final concentration), and 50 μL of membranes (30 μg/well) and incubated for 1 h in Costar round-bottom wells microtiter plate (catalogue no. 3365). Radioligand, membranes, and test compounds were diluted in buffer (25 mM HEPES pH 7.4, 1 mM $CaCl_2$, 5 mM $MgSO_4$, 0.1% ovalbumin (Sigma A-5503), 0.005% Tween-20, 5% hydroxypropyl-β-cyclodextrin (97% Acros organics, code 297561000)). The assay mixture was incubated for 1 h at 20−25 °C. The rest of the procedure was similar to the procedure described above for MC1-R binding.

MC4-R Binding Assay. The membrane suspension was diluted in binding buffer to give maximal 10% specific binding, approximately 50−100-fold dilution. The assay was performed in duplicates in a 96-well filter plate, Millipore MADVN 6550 in a volume of 200 μL. Then 50 μL of cell membrane suspension, 50 μL of ^{125}I-NDP-α-MSH (79 pM in final concentration), 50 μL of test compound, and 50 μL of binding buffer were mixed and incubated for 2 h at 25 °C. Radioligand, membranes, and test compounds were diluted in binding buffer; 25 mM HEPES pH 7.0, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM EGTA, 0.02% bacitracin (Sigma B-0125), 0.005% Tween-20, and 0.1% ovalbumin (Sigma A-5503). The incubation was terminated by placing the 96-well filter plates on a Millipore vacuum manifold, and the supernatant was sucked through the filters. The wells were washed twice with 100 μL of ice-cold 0.9% NaCl, and the plates were then air-dried and punched into counting vials. The radioactivity retained on the filters was counted using a Cobra II auto γ counter.

MC4-R cAMP Assay. The assay was performed in duplicate in BHK570 cells expressing the MC4-R, which were stimulated with increasing concentrations of potential MC4-R agonists in a range of 10^{-11} to 10^{-5} M, and the degree of stimulation of cAMP was measured using the Flash Plate cAMP assay kit (NEN Life Science Products cat no SMP004) using the supplied materials and buffers. The assay was performed in duplicates and each compound was tested at least three times. The cAMP assay was performed on whole cells in the presence of human serum albumin. Cells at approximately 80−90% confluence were washed 3 times with PBS, lifted from the plates with Versene, and diluted in PBS and then centrifuged 2 min at 365*g* and the supernatant removed. The cells were washed twice with stimulation buffer from the kit, and 75000 cells/well were seeded in the plates in a volume of 50 μL. The test compounds and reference compound were dissolved in DMSO to a concentration of 4 mM and diluted in PBS with 0.1% human serum albumin and 0.005% Tween-20 and added to the plates in a volume of 50 μL. MT-II or compound **5**, which is a full agonist when compared with MT-II, was used as internal standard in the cAMP test and for calculations of Emax. MT-II and α-MSH had similar E_{max} when they were tested in the same assay. The amount of released cAMP was measured according to the Flash Plate cAMP assay protocol, except that the cAMP standards were diluted in PBS with 0.1% human serum albumin and 0.005% Tween-20 and not in stimulation buffer as described in the protocol from the kit.

Acute Food Intake. Male Sprague−Dawley (SD) rats from Taconic Europe, Denmark, are used for the experiments. The rats weighted 200−250 g at the start of experiment. The rats arrived 14 days before start of experiment to allow acclimatization to experimental settings. During this period, the animals were handled two times. After arrival rats were housed individually for one week in a reversed light/dark phase (meaning that lights are off during day time and on during night time) for two weeks. Because rats are normally active and eat their major part of their daily food intake during the dark period, rats were dosed in the morning right before lights are turned off. At this time, the rats will be hungry even though they have free access to food before and throughout the test period. This setup results in the lowest variation in data and highest test sensitivity. The experiment was conducted in the rats' home cages, and rats had free access to food and water throughout the acclimatization period and the experiment period. Each analogue was tested in a group of 7−8 rats, and a vehicle group of 7−8 rats was included in each set of testing. Rats were dosed once according to body weight with 3.0 mg/kg, 1.0 mL/kg administered subcutaneously (sc). Peptides were dissolved in 0.9% NaCl/5% hydroxypropyl-β-cyclodextrin (HPβCD). After dosing, the rats were returned to their home cages, where they had access to food and water. Individually consumed food was manually weighed each hour up to 7 h and after 24 and 48 h. At the end of the experimental session, the animals were euthanized. Outliers were excluded after applying the Grubbs statistical evaluation test for outliers. Data was reported as accumulated food intake as functions of time. Comparisons of accumulated food intake for specified time points were made between vehicle group and test groups by two-way ANOVA with Bonferroni post-test. The animals were observed for abnormal activity and behavior, but because nothing abnormal was observed, no specific behavioral tests were performed.

Calculations. IC_{50} values were calculated by nonlinear regression analysis of binding curves (6 data points minimum) using the program GraphPad Prism, GraphPad software, USA. K_i values were calculated according to the Cheng−Prusoff equation ($K_i = IC_{50}/[1 - L/K_d]$, where L is the concentration of the radioligand and K_d is the dissociation constant of NDP-α-MSH calculated from saturation binding experiments and IC_{50} values = the concentration of compound when 50% of the radioligand is displaced). K_d values were calculated using the one site binding model (hyperbola) in Prism. Agonist values are represented as EC_{50} (= the concentration with 50% stimulation) calculated by nonlinear regression analysis of binding curves (6 points minimum) using the windows program GraphPad Prism, GraphPad software, USA.

■ ASSOCIATED CONTENT

Supporting Information

Tables with analytical data and absolute food intake data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): All authors are or were employed by Novo Nordisk and are minor share holders.

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■ ABBREVIATIONS USED

ANOVA, analysis of variance; CLND, chemiluminescent nitrogen detector; DMSO, dimethylsulfoxide; HPβCD, hydroxypropyl-β-cyclodextrin; HPLC, high performance liquid chromatography; HSA, human serum albumin; MC, melanocortin; MSH, melanocyte stimulating hormone; NMP, *N*-methylpyrrolidone; Oeg, 8-amino-3,6-dioxaoctanoic acid; PBS, phosphate buffered saline; POMC, pro-opiomelanocortin; SAR, structure−activity relationship

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