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Synthesis of a novel potent cyclic peptide MC4-ligand by ring-closing metathesis

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Abstract—The synthesis of a novel potent cyclic peptide MC4-ligand by ring-closing metathesis (RCM) is described. Based on the Ac-Nle-Gly-Lys-D-Phe-Arg-Trp-Gly-NH_2–MC4 ligand, Ac-Nle-Alg-Lys-D-Phe-Arg-Trp-Alg-NH_2 was designed and synthesized followed by cyclization using RCM. Both compounds are high affinity and selective MC4-R-agonists. The cyclic RCM-peptide was more potent in a rat-grooming assay.

1. Introduction

The MC4-receptor (MC4-R) is a member of the melanocortin receptor family, of which five subtypes (MC1-R - MC5-R) have been cloned.[1] The physiological functions regulated by the different melanocortins vary greatly,[1] from coat pigmentation to exocrine gland function. The melanocortin receptors are activated by the endogenous ligands α-, β-, and γ-melanocyte stimulating hormones (MSH) and adrenocorticotropin (ACTH).

The MC4-R is of particular interest because of its likely involvement in the regulation of body weight in mammals,[2–12] which makes it an attractive target for drug design. The sequences of several relevant MC4-R ligands are shown in Table 1. Synthetic MC4-R ligands are generally derived from native α-MSH, with each modification bringing increased activity, such as in NDP-MSH and in MT-II,[14,15] a shortened cyclic NDP-MSH(4–10)[13] derivative, or increased selectivity for the MC4-R (JK1, JK64[16]). Two modifications to α-MSH are commonly incorporated into synthetic melanocortin peptide ligands to increase stability and potency. Firstly, methionine is replaced by norleucine, which increases the activity and stability of the peptides.[22] Secondly, the chirality of the phenylalanine residue is often inverted to give D-phenylalanine, which results in a dramatic increase in activity. One other method commonly used to increase the activity or selectivity of peptide ligands is to reduce their conformational freedom through cyclization. Relevant examples in this respect are the MT-II ligand,[23] which is a cyclic peptide derived from $NDP\text{-}MSH^{4-10}$ and VJH-085, which is a derivative of MT-II (Fig. 1).[24]

In general, selectivity between the melanocortin receptors has been difficult to attain, since the selectivity is determined by both the primary structure and the conformation of the core tetrapeptide, with the latter probably being influenced by the surrounding sequence.[46] However, replacement of His-6 by Lys-6 increases selectivity for the MC4-R, as was the case in the JK1 peptide.[16b] To date, this is the only melanocortin ligand that has a lysine residue in place of a histidine.[16] The

Abbreviations: ESI-MS, electrospray ionization mass spectrometry; RCM, ring-closing metathesis; SPPS, solid phase peptide synthesis; SPE, solid phase extraction; Boc, *tert*-butyloxycarbonyl; DCM, dichloromethane; DiPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; EDT, 1,2-ethanedithiol; MTBE, methyl *tert*-butyl ether; Nle, norleucine; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; TFA, trifluoroacetic acid; TIS, triisopropylsilane.

Keywords: MC4-agonists; Cyclic peptide; Metathesis; Solid phase synthesis; Melanocortin.

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Table 1. Common melanocortin receptor ligands

Peptide	Sequence													
		1	2	3	4	5	6	7	8	9	10	11	12	13	
α-MSH	Ac-	Ser	Tyr	Ser	Met	Glu	**His**	**Phe**	**Arg**	**Trp**	Gly	Lys	Pro	Val-	NH_2
NDP-MSH	Ac-	Ser	Tyr	Ser	Nle	Glu	**His**	**DPhe**	**Arg**	**Trp**	Gly	Lys	Pro	Val-	NH_2
MT-II				Ac-	Nle-c	[Asp	**His**	**DPhe**	**Arg**	**Trp**	Lys]-	NH_2			
JK1				Ac-	Nle	Gly	**Lys**	**DPhe**	**Arg**	**Trp**	Gly-	NH_2			
JK64				Ac-	Nle	Gly	**Arg**	**DPhe**	**Arg**	**Trp**	Gly-	NH_2			

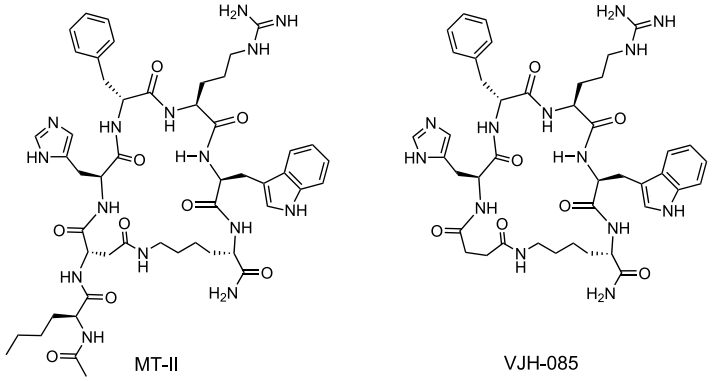


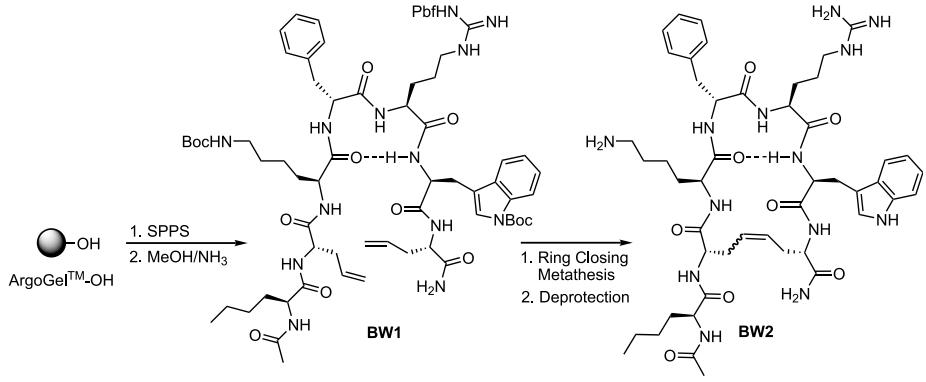
Figure 1.

His-6 residue is part of the tetrapeptide Ac-His[6]-Phe[7]-Arg[8]-Trp[9]-NH_2, which is the minimal peptide fragment that elicits a melanotropic response.[17] The His residue is probably not necessary for binding at the MC4-R, as illustrated by the presence of activity in several synthetic tripeptide fragments such as Ac-DPhe-7-Arg-8-DTrp-9-NH_2,[18] but seems to have potency-enhancing effect.[19–21]

As is shown in Figure 1, the VJH-085 ligand is a truncated derivative of MT-II, which is missing the norleucine residue. This has a positive effect on the MC4-R selectivity,[24] but it was unclear whether this is caused by removal of the norleucine residue, or a change in ring structure resulting from the removal of one amino acid residue. Additional small changes to the MT-II macrocycle might therefore give a further improvement in selectivity.

To this end, JK1[16] and MT-II were taken as starting points in the synthesis of new selective peptide ligands for the MC4-R. The replacement of the His-6 residue in NDP-MSH(4-10) by Lys-6, which originally resulted in JK1, had a pronounced effect on MC4-R selectivity. A similar effect was expected to occur in cyclic peptide ligands, and thus an MT-II derivative was designed in which the histidine residue was replaced by a lysine residue.

As an attractive alternative to lactamization, ring-closing metathesis (RCM) was used, which would enable replacement of the lactam bridge of MT-II by an aliphatic bridge, constructed from two allylglycine residues. For this purpose, it was decided to synthesize the linear peptide **BW1** by solid phase synthesis, followed by cyclization to **BW2** using RCM, shown in Scheme 1. The macrocyclic ring of **BW2** is shortened by three methylene groups as compared to MT-II, and incorporates all the functionalities present in JK1. This was expected to lead to an increase in selectivity compared to MT-II, while maintaining a similar activity.



Scheme 1. Solid phase peptide synthesis (SPPS) and cleavage of **BW1** from the resin by aminolysis, followed by RCM to **BW2**.

So far most work on RCM has involved the synthesis of relatively small and unfunctionalized macrocycles. Still, the number of peptide cyclizations using RCM have been previously carried out[25–37] is fairly limited. In addition, the cyclization efficiencies, vary greatly and are hard to predict, because preorganization of the linear peptides probably plays an important role.[25]

2. Results: synthesis

Peptide **BW1** was synthesized on ArgoGel™-OH resin using an automated peptide synthesizer on a 0.25 mmol scale. Initial attempts to perform the ring-closing metathesis prior to cleavage of the peptide from the resin were unsuccessful, which is in agreement with earlier observations in our group.[29] However, other groups have been more successful with respect to this.[25,28] Therefore, the peptide was cleaved into solution by aminolysis of the ester linkage using a saturated solution of ammonia in methanol, thus preserving the side chain protecting groups. The resulting peptide was obtained in high yield and good purity.

Unfortunately, the fully protected peptide was only soluble in DMF, and sparingly soluble in all solvents commonly used for RCM. Because of the extremely poor solubility of protected **BW1** in organic solvents, simple TLC analysis of the crude linear peptides and the cyclization reactions was hardly possible. The compounds showed excessive tailing, preventing any comparison of linear and cyclized peptides on TLC. Therefore, analysis had to be performed after cyclization and deprotection of the peptides.

Two common catalysts for RCM are available (Fig. 2). The first widely applied RCM catalyst is **1**, the original Grubbs' catalyst. Second-generation Grubbs' catalyst **2** is more stable and more reactive. Since it was not clear at the outset, which catalyst would be superior in the cyclization of large peptide systems, the use of both catalysts was investigated. Because the protected peptide only dissolved in DMF and similar solvents, cyclization solutions were prepared by addition of a stock solution of the linear peptide in DMF to the desired solvents for cyclization. In most cases, the subsequent precipitation of the peptide was slow enough to allow RCM to proceed in the meantime. In each case, cyclization was performed at a concentration of 1.6 mM and using 20 mol % catalyst, for 2 h. In Table 2 the conditions and results are listed.

After RCM, all peptides were deprotected using TFA in the presence of scavengers, precipitated from MTBE/hexane, and lyophilized followed by determination of the extent of cyclization by ESI-MS.

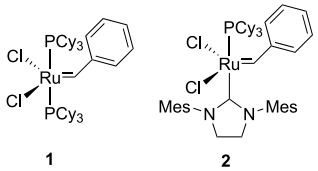


Figure 2. First- (**1**) and second-generation (**2**) Grubbs' catalyst.

Table 2. Attempted cyclization conditions

No.	Solvent	Catalyst	Temperature (°C)	Result[a]
1	DCM	**2**	40	Linear
2	1,2-Dichloroethane	**2**	50	Linear
3	Toluene	**2**	70	Linear
4	1,1,2-Trichloroethane	**2**	70	Linear
5	1,1,2-Trichloroethane	**2**	100	Linear
6	Toluene	**1**	70	Linear
7	1,1,2-Trichloroethane	**1**	100	Linear
8	1,1,2-Trichloroethane	**1**	100, 1 h; 40, 2 h.[b]	Linear/**cyclic**

[a] As determined by ESI-MS.
[b] Second portion of 20 mol % catalyst added after 1 h.

Only *one* combination of solvent, catalyst, and temperature (entry 8 in Table 2) turned out to be effective in performing ring-closing metathesis, using the first-generation Grubbs' catalyst. In all cases, cyclization using the second-generation Grubbs' catalyst **2** was unsuccessful, despite its supposedly greater activity and stability over **1**. At reaction temperatures below approximately 70 °C the protected peptide usually precipitated quickly. In order to prevent precipitation of the starting material, higher temperatures were used. This in turn resulted in decomposition of both catalysts, as was observed from the rapid brown coloration of the reaction mixture and the eventual failure of the cyclization reaction. Therefore, cyclization was performed at a starting temperature of 100 °C, and after 1 h an additional 20 mol % catalyst **1** was added and the reaction mixture slowly allowed to cool to 40 °C. Using this procedure, most of the peptide stayed in solution and a relatively high yield of the cyclized product was obtained as a mixture of cis and trans isomers in 63% yield after deprotection and purification. Unfortunately, we were unable the separate the isomers by preparative HPLC.

3. Results: biological activity

3.1. Binding

BW1 and **BW2** were tested for binding to receptors transiently expressed in HEK293 cells, in a competition assay with iodinated NDP-MSH. K_i values were calculated using the formula $K_i = (IC_{50}\ (\text{compound})/IC_{50}\ (\text{NDP-MSH})) * K_d$ (^{125}I-NDP-MSH). Table 3 shows the K_i values determined for ligands **BW1** and **BW2**, together with the corresponding values for MT-II,[38] JK1,[16] and JK64.[16]

The K_i values obtained for **BW1** and **BW2** were very similar. The affinities of the peptides listed in Table 3 for the MC4-R did not vary greatly, and with the exception of JK64 were within experimental error. The benefit to selectivity gained by the His-6 to Lys-6 replacement, which was present in the **BW** peptides and JK1 was clearly observed, as JK64 and MT-II both possessed

Table 3. K_i values for the melanocortin receptors of **BW1** and **BW2**

Ligand	hMC3-R	hMC4-R	hMC5-R
BW1	119 nM ± 46	1.9 nM ± 1.0	IC_{50} >300 nM[a]
BW2	126 nM ± 46	2.3 nM ± 1.0	IC_{50} >300 nM[a]
MT-II	4.8 nM ± 2.1	1.7 nM ± 0.8	—
JK1	213 nM ± 38	2.4 nM ± 0.3	268 nM ± 26
JK64	12 nM ± 1	0.53 nM ± 0.1	26 nM ± 1

[a] The ligands were tested to a maximum concentration of 1 μM. At this concentration no complete inhibition of the labeled ligand was observed.

reduced selectivity. Although the difference in selectivity between linear **BW1** and cyclic peptide **BW2** was within experimental error, **BW2** seemed to show a small improvement in selectivity for the MC4-R over the MC5-R compared to JK1. In addition, a small reduction in MC4-R versus MC5-R selectivity as compared to JK1 was detected.

3.2. Activation

Activation of MC receptors by ligands was determined in a reporter gene assay using the CRE-*LacZ* construct described by Chen et al.[39] This method detects melanocortin receptor activation through elevation of intracellular cAMP levels. This is detected by a β-galactosidase (*LacZ*) gene, cloned downstream of promoter with five copies of the cyclic AMP response element (CRE). Activation of CRE-binding protein by increased intracellular cAMP levels thus causes *LacZ* gene expression, which can be measured using a simple colorimetric assay, and is a measure for melanocortin receptor activation. Table 4 shows the EC_{50} values determined for **BW1** and **BW2**, compared to those found for MT-II, JK1,[16] and JK64.[16]

Both **BW1** and **BW2** were full agonists of the MC3-5 receptors, and very similar in potency and selectivity, which is in agreement with the results from the binding experiments described above. Both have reduced potency compared to MT-II and JK64 peptides, but are slightly more potent than JK1. The overall potency of **BW1** and **BW2** at all receptors is in between that of MT-II and JK1. This is an indication that the high activity of the MT-II ligand, was at least partially conferred to the **BW** peptides by cyclization.

3.3. Effects on grooming behavior

In the rat, melanocortin-induced grooming behavior is most probably mediated by the MC4 receptor, since MC4 selective agonists induce excessive grooming behavior, whereas MC3 selective agonists do not.[38] Furthermore, SHU9119, an antagonist for the MC4 receptor, but an agonist for the MC1 and MC5 receptors, antagonises grooming behavior.[38] Therefore we tested **BW1** and **BW2** for activity in a rat-grooming assay, as a measure for in vivo MC4 receptor activity. Administration of MCR-agonists has previously been shown to induce grooming behavior, mediated through the MC4-R.[38] This allows comparison of the in vivo activities of both peptides. The previously reported JK64 peptide (Table 1), was the most potent compound in a previously performed grooming assay,[16] and was included for comparison purposes.

Table 4. EC_{50} values of the **BW** peptides at the MC3–5-R

Ligand	MC3-R	MC4-R	MC5-R
BW1	1.5 nM ± 0.4	0.21 nM ± 0.01	24 nM ± 8
BW2	2.0 nM ± 0.6	0.34 nM ± 0.02	44 nM ± 21
MT-II	0.8 nM ± 0.2	0.01 nM ± 0.004	—
JK1	9 nM ± 2.5	1 nM ± 0.6	30 nM ± 6
JK64	0.5 nM ± 0.07	0.052 nM ± 0.02	14 nM ± 6

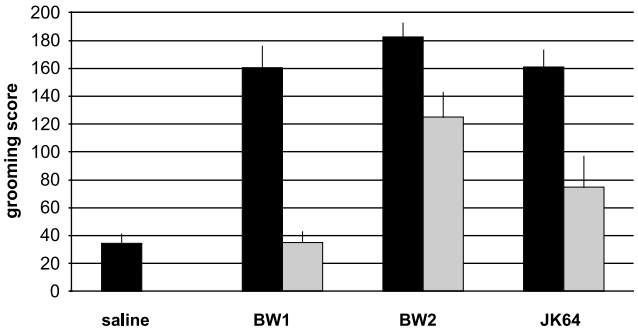


Figure 3. Grooming scores after i.c.v. injection of 3 μg in 3 μL (black bars) or 0.3 μg in 3 μL (gray bars).

As can be seen in Figure 3, intracerebroventricular (i.c.v.) injections of all three of the peptides induce grooming behavior. Cyclic peptide **BW2** was clearly the most potent, followed by JK64 and then linear peptide **BW1**. This order of potency was unexpected, since both **BW1** and **BW2** had shown similar activity and potency in the in vitro assays. Earlier data on JK64 showed that this peptide had approximately a sevenfold increase in activity over the **BW** peptides, and was thus expected to be the most potent compound in the assay.

4. Discussion and conclusions

Linear peptide **BW1** and RCM-cyclic peptide **BW2** are novel peptides with high affinity and selectivity for the MC4-R. However, the difference in binding affinity and selectivity between linear peptide **BW1** and its cyclic derivative **BW2** were small. EC_{50} values were lower than IC_{50} values. This is most probably caused by the high sensitivity of the reporter gene assay that was used to determine EC_{50} values.[39] It has been shown before that for MC receptors EC_{50} values are lower than IC_{50} values, when using this assay.[38] Although cyclization was expected to lead to an increased activity of cyclic peptide **BW2** because of reduction of conformational freedom, this was not observed. A possible cause for the similar affinity and the ensuing activity of linear **BW1** and cyclic peptide **BW2** might be enthalphy–entropy compensation, which may also have been the case in a previously studied cyclic phosphopeptide.[40] Nevertheless, upon testing of peptides **BW1–2** in an activation assay, an

improvement in activity over the native JK1 peptide was observed, although the resulting potency still did not match that of the MT-II peptide (Table 3). A second measure of potency was the effect on rat-grooming behavior. In this assay, RCM-cyclized **BW2** was the most potent compound, which was somewhat unexpected in view of the affinity and activity data. The discrepancy between the in vitro binding and activation potencies, which were similar for **BW1** and **BW2**, and the in vivo assay (rat-grooming behavior), in which **BW2** was more potent, suggest that the pharmacokinetics of **BW1** and **BW2** differ. A plausible explanation for this observation is that due to cyclization, the proteolytic stability of **BW2** is higher than that of **BW1**.

The results of the activation assay indicate that the **BW1** and **BW2** peptides both behave as hybrids of JK1 and MT-II, as had been expected to occur in **BW2**. While the replacement of His-6 by Lys-6 is responsible for an increase in binding selectivity, the presence of merely allylglycine residues in **BW1** and **BW2** is apparently responsible for the greater activity of these peptides in the activation assay as compared to JK1. Earlier comparisons of MT-II and its uncyclized analogue had demonstrated a large difference in activity and selectivity,[23] and a similar effect was expected going from linear peptide **BW1** to RCM-cyclized peptide **BW2**. However, since these peptides were equipotent, this may indicate that the increase in potency upon cyclization to MT-11 might not be entirely due to cyclization. Finally, the in vivo potency was shown to be affected by cyclization of the peptides, suggesting that in these measurements the stability of the peptides also may play an important role.

In conclusion, we have developed novel peptide ligands, which display high activity in vivo. These were designed as hybrids of two previously described peptides, and displayed the favorable properties of both. This resulted in peptide **BW2**, which is a novel cyclic ligand with high potency and increased selectivity for the MC4-R as compared to the earlier developed JK1 peptide.

5. Experimental

5.1. General

Unless stated otherwise, chemicals were obtained from commercial sources and used without further purification. ArgoGel™-OH resin was purchased from Argonaut (Hengoed, United Kingdom). Fmoc-amino acids were purchased from Alexis corporation (Läufelingen, Switzerland). Solvents were purchased from Biosolve (Valkenswaard, The Netherlands) and were stored on molecular sieves (4 Å). DiPEA, pyridine, and collidine were distilled from ninhydrin and KOH prior to use. Solid phase synthesis was carried out on an ABI 433A automated peptide synthesizer, using the FastMoc 0.25 protocol.[41] ESI-MS experiments were performed on a Shimadzu LCMS QP8000 apparatus. Analytical HPLC was performed on a Shimadzu Class-VP automated HPLC with UV detector system operating at 214 and 254 nm using (**A**) Phenomenex® Jupiter™ C18 (300 Å, 5 μm, 250 × 4.6 mm) analytical reversed-phase column or (**B**) Phenomenex® Jupiter™ C4, (300 Å, 5 μm, 250 × 4.6 mm) analytical reversed-phase column. Eluents system **1**: A = 0.1% TFA in water/acetonitrile 95/5 and B = 0.1% TFA in acetonitrile/water 95/5 or **2**: A = 0.1% TFA in water/acetonitrile 95/5 and B = 0.1% TFA in acetonitrile/water 60/40. Elution was effected using a gradient starting at 100% A for 2 min, rising to 100% B over a period of 48 min at a flow rate of 1 mL/min. SPE was performed on Isolute® C8 SPE columns using a similar gradient. TLC R_f values were determined on Merck pre-coated silica gel 60 F-254 plates; spots were visualized by UV-light (254 nm), ninhydrin, or Cl_2-TDM.[42] Column chromatography was performed using ICN silica gel 60 Å, 32–63 μm.

5.2. Peptide synthesis

ArgoGel™-OH resin (1 g, 0.48 mmol) was weighed into a round-bottom flask, and Fmoc-L-Allylglycine-OH (0.684 g, 1.92 mmol) was added. After overnight drying of the resin in vacuo over P_2O_5, DMF (10 mL) was added, and the resin allowed to swell for 10 min. To the swollen resin was added 2,6-dichlorobenzoylchloride (0.28 mL, 1.9 mmol, 4 equiv) and pyridine (0.31 mL, 3.8 mmol, 8 equiv), and the resin was shaken overnight. Subsequently, the resin was filtered off, washed with 4 × 10 mL DMF, 4 × 10 mL DCM, and 3 × 5 mL Et_2O, and dried in vacuo. The loading was determined by treating a resin sample with 20% piperidine in DMF and determining the UV absorbance at 301 nm, and was found to be 0.357 mmol g^{-1}.

The loaded resin (0.7 g, 0.25 mmol) was subsequently placed into the peptide synthesizer, and the **BW1** peptide synthesized using the ABI FastMoc 0.25 protocol.[41] After completion of the peptide synthesis, the resin was placed into a round-bottom flask and dried in vacuo. The resin was treated overnight with 25 mL of a saturated solution of ammonia in methanol, yielding a cloudy suspension. The resin was filtered off, and washed with 6 × 10 mL DMF and 3 × 10 mL DCM. The organic solvents were combined and removed in vacuo, yielding 0.413 g (0.19 mmol) of a clear gel. ESI-MS: mass calcd for $C_{73}H_{106}N_{13}O_{15}S$ 1436.77 $[M+H]^+$. Found 1436.85. HPLC R_t (**A1**) = 43.54 min.

5.3. Cyclization

A stock solution of protected peptide **BW1** (0.413 g) in 5 mL DMF was prepared under gentle heating to 40 °C. Cyclization took place in a two-necked round-bottom flask, heated using an oil bath, under a continuous flow of dry N_2. Prior to cyclization, the solvents (20 mL, table) were heated to the desired temperature under a continuous N_2 flow for 20 min. Subsequently, 0.5 mL of the stock solution of the protected peptide was added (0.032 mmol, final concentration 1.6 mM), dry N_2 passed through for an additional 5 min, and catalyst **I** or **II** added (20 mol %, 3 mg). Cyclization was carried out for 2 h, after which the solvent was removed in vacuo. Cyclization entry 8 was performed for 1 h at

100 °C, an additional 20 mol % catalyst added, and cyclized for a further 2 h at 40 °C.

5.4. Deprotection and analysis

The eight cyclization samples were deprotected by treatment with 2 mL of a cocktail containing TFA/TIS/EDT/H_2O (85/2/4.5/8.5 v/v) for 2 h. Subsequently, the peptides were precipitated by addition of 50 mL MTBE/hexane (1/1), centrifuged, and the pellet washed with 2×30 mL MTBE/hexane (1/1). The resulting dark brown pellets were dissolved in 5 mL water/acetonitrile (2/1) and applied to a solid phase extraction (SPE) column. After washing with 2×10 mL water, the peptides were eluted with 20 mL water/acetonitrile (1/1). The organic solvents were removed in vacuo, and the remaining water layers lyophilized and analyzed using ESI-MS.

Only cyclization attempt (Table 2, entry 8) contained a significant amount of cyclic product as judged by mass spectrometry. This was purified by preparative HPLC, yielding 2.9 mg **BW2** (0.02 mmol, 63%) after lyophilization as a mixture of cis and trans isomers, which could be detected as a double peak on analytical HPLC, and 0.5 mg **BW1**. Additional **BW1** was obtained by pooling the product obtained in cyclizations 4 and 6, followed by purification with preparative HPLC, yielding a total of 6.6 mg (0.067 mmol, 108%) **BW1**. The latter yield is presumably due to a weighing error. **BW1**. ESI-MS: mass calcd for $C_{50}H_{74}N_{13}O_8$ 984.58 $[M+H]^+$. Found 984.65 $[M+H]^+$, 493.25 $[M+2H]^{2+}$. HPLC R_t (**A2**) = 31.90 min; **BW2** ESI-MS: mass calcd for $C_{48}H_{70}N_{13}O_8$ 956.55 $[M+H]^+$. Found 956.65 $[M+H]^+$, 479.25 $[M+2H]^{2+}$. HPLC R_t (**B2**) = 25.86 and 27.07 min (ratio *E*/*Z* or *Z*/*E*: 3/2).

5.5. Binding assay

For binding experiments, receptors were transiently expressed in HEK293 cells. IC_{50} and K_i values were determined with iodinated [Nle4-D-Phe7]-α-MSH (NDP-MSH) as tracer. NDP-MSH was iodinated using bovine lacto-peroxidase (Calbiochem, La Jolla, CA) and ^{125}I-Na (ICN, Aurora, OH) according to Oosterom et al.[43] and subsequently high pressure liquid chromatography-purified on a C_{18} column (μBondapak 3.9 mm × 300 mm, Waters). 293 HEK cells growing in 24-well plates were washed with tris-buffered saline (TBS) supplemented with 2.5 mM calcium chloride and incubated for 30 min at room temperature with peptides and tracer diluted in Ham's F10 medium (Gibco) supplemented with 2.5 mM calcium chloride, 0.25% bovine serum albumin (BSA) (ICN, Aurora, OH), and 200 KIU/mL aprotinin (Sigma, Steinheim, Germany). After two washes with ice-cold TBS buffer (+2.5 mM calcium chloride) to remove non-bound tracer, the cells were lysed in 1 M sodium hydroxide and samples were counted in a γ-counter. In each binding experiment, IC_{50} values of non-iodinated NDP-MSH were determined to allow calculation of K_i values using the formula: $K_i = (IC_{50}\ (\text{compound})/IC_{50}\ (\text{NDP-MSH})) \times K_d$ (^{125}I-NDP-MSH). The K_d values of ^{125}I-NDP-MSH for the receptors were calculated from saturation isotherms of ^{125}I-NDP-MSH (two independent experiments per receptor).

5.6. Activation assay

Activation of receptors was determined using *LacZ* as a reporter gene.[39] HEK293 cells growing in 10-cm dishes were cotransfected with 100–200 ng receptor and 7 μg of CRE*LacZ* construct.[16,39] After transfection, the cells were plated into 96-well plates (BectonDickinson). Two days after plating, the cells were incubated with peptides at the appropriate concentrations in serum-free medium (DMEM containing 0.2% BSA (ICN, Aurora, OH) glutamine (Gibco) and NEAA (Gibco)). After 5–6 h of incubation, the assay medium was aspirated and 40 μL of lysis buffer (PBS containing 0.1% Triton X-100 (Boehringer, Mannheim, Germany)) was added. The plates were stored at −20 °C and after thawing 80 μL of substrate mix (0.1 M phosphate buffer, pH 7.4 containing 1.6 g/L *o*-nitrophenyl-β-D-galactopyranoside (ONPG, Molecular Probes, Leiden, The Netherlands), 67.5 mM β-mercaptoethanol (Merck, Darmstadt, Germany), and 1.5 mM magnesium chloride) was added. Absorbance at 405 nm was determined in a Victor2 microplate reader (Perkin–Elmer, Brussels, Belgium).

5.7. Grooming assay

For grooming assays, rats received an intracerebroventricular (i.c.v.) cannula as described by Brakkee et al.[44] and were allowed to recover for 3 days. Before the experiment, the rats were kept for at least 1 h in the experimenting room to reduce novelty-induced grooming. Grooming assays were performed as described in Gispen et al.[45] Briefly, observation started 10 min after i.c.v. injection of compounds diluted in saline (3 μL fluid). Grooming behavior was scored at timepoints with intervals of 15 s for a period of 60 min. The grooming score per rat is expressed as the number of timepoints that the rat showed grooming behavior.

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