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Serendipitous discovery of a new class of agonists for the melanocortin 1 and 4 receptors and a new class of cyclophanes

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ABSTRACT

A new class of melanocortin 4 receptor (MC4r) agonists was discovered from an unexpected sidereaction in which formaldehyde caused cyclization. These cyclophanes were found to be sub micromolar agonists of the MC1 and MC4 and were less potent on the MC3 and MC5 receptor. They were shown to compete with the peptidic antagonist SHU9119 for binding to the MC4 receptor. In an acute feeding study in Sprague Dawley rats, food intake was reduced more than 50% versus vehicle after 3 h at a dose of 1 mg/kg.

The melanocortin receptors constitute a group of five receptors (MC1R-MC5R) belonging to the family A of seven transmembrane G-protein coupled receptors.[1,2] The endogenous ligands are derived from proopiomelanocortin (POMC) and are small peptides.[3,4] In addition, two endogenously expressed antagonists, agouti and agouti gene related peptide (AgRP), are belonging to the system.[5] The MC3 and MC4 receptors have been linked to sexual behavior and energy homeostasis.[6–8] Mutations in the MC4 receptor are coupled to obesity in humans,[9] and in rodents subchronic treatment with the synthetic peptide ligand MT-II (**1**) resulted in weight loss.[10] α-MSH (**2**), which is one of the endogenous ligands for the MC1, MC3, MC4 and MC5 receptors, is a tridecapeptide. Structure–activity analysis by Hruby et al. revealed the pharmacophores important for the interaction between α-MSH and the melanocortin receptors.[11–14] Scheme 1 depicts MT-II and α-MSH with the pharmacophores emphasized.

Despite the urgent and growing need for a pharmacological treatment of obesity very few effective treatment options are currently available.[15,16] With the aim to satisfy this unmet need, a research program focused on the discovery and development of orally available MC4R agonists was initiated.

In essence the overall approach was to synthesize small molecule libraries based on in silico design and subsequently screen the libraries for MC4R agonistic activity. Thus, using the modeling package SYBYL[17] a model of MT-II was overlayed with new scaffold candidates containing the pharmacophores. Scaffolds which were predicted to spatially present the pharmacophores in a manner that allowed for strong receptor interactions were identified. Small focused libraries were synthesized using solid phase technology and after cleavage from the solid support the crude compounds were without further purification screened for MC4R agonistic activity using a cell based assay. MC4R was stably transfected into baby hamster kidney (BHK) cells with a reporter system consisting of a cyclic-AMP responsive element (CRE) coupled to a luciferase reporter gene.[18] Responses were compared to the effect of MT-II and expressed relative (in %) to the maximum activity of MT-II (E_{max}). MT-II was considered to be a full agonist at the MC4R. Compounds identified by this method were further characterized in binding and cAMP assays for other relevant MC receptors.

MC4R agonistic activity was detected in a library based on a scaffold carrying four pharmacophores and synthesized using simple acylation and reductive alkylation chemistry as outlined in Scheme 2.

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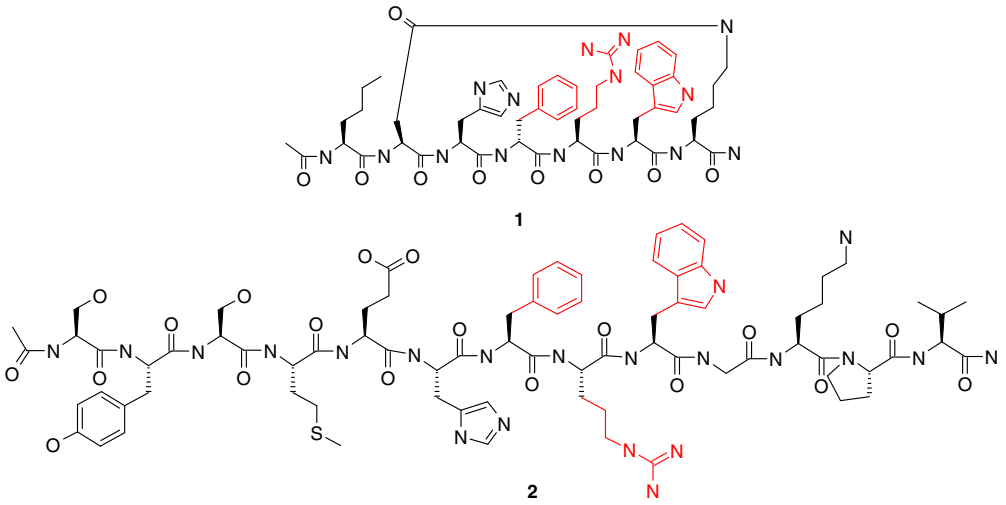
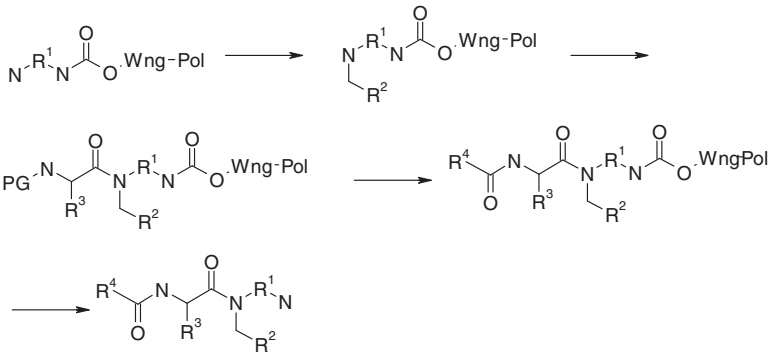


Scheme 1. MT-II (**1**) and α-MSH (**2**). The important pharmacophores are shown in red.



Scheme 2. Outline of the synthesis.

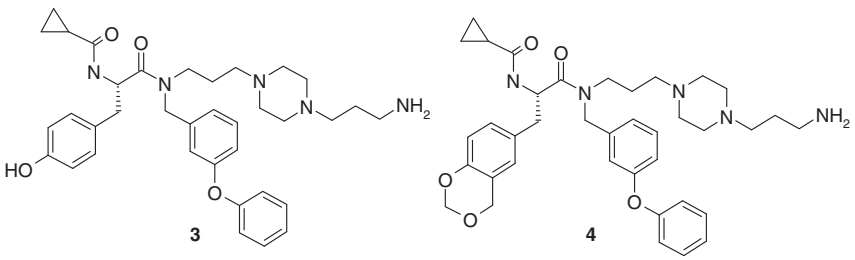
The library consisted of 384 random compounds out of 7776 possible ($6 \times 9 \times 8 \times 18$), which covers 5% of the chemical space represented by these building blocks and was synthesized in our Parallel Synthesis Lab using a ACT384 robot. According to the library design, the active compound was expected to have the structure **3** shown in Scheme 3.

The crude material exhibiting MC4R agonistic activity was subjected to LC–MS analysis. Surprisingly, the LC–MS data demonstrated that the major component had a molecular mass of 655 Da corresponding to a net mass increase of 42 Da compared to the mass of the expected compound (**3**) (613 Da). Also present in the mixture was a minor component exhibiting a surplus mass of 54 Da compared to that of structure **3**. Thus the screening hit was of unknown structure formed by an unknown side reaction.

By manual synthesis using the same chemistry as applied in the automated library synthesis compound **3** was indeed the major product formed as evidenced by LC–MS analysis (613 Da). Compound **3** was devoid of any activity in the MC4R agonist assay.

The active crude material from the library synthesis was subjected to HPLC-fractionation and a fraction accounting for the MC4R agonistic activity was collected. The NMR spectrum of the active fraction indicated the presence of several components and could not be analyzed in depth. However, the NMR data served to rule out simple interpretations such as isopropylation or acetylation for the mass difference of 42 Da.

An important hint to the nature of the +42 Da components and the underlying chemistry was the fact that all library samples carrying a tyrosine in the R^3 position contained components with increased molecular masses of 42 Da. One possible explanation for the presence of the +42 Da products is a reaction between formaldehyde of an yet unknown source and a tyrosine residue to form 4*H*-benzo[1,3]dioxin compounds. Thus by reaction with



Scheme 3. Intended structure **3** and 4*H*-benzo[1,3]dioxin **4**.

formaldehyde compound **3** could form compound **4** (Scheme 3) with a molecular mass of 655 Da.

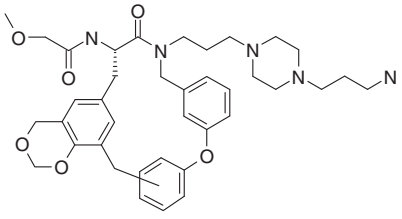
To challenge this hypothesis, compound **3** was synthesized using the manual procedure described with the only exception that paraformaldehyde was deliberately added during the TFA cleavage step. This experiment provided a product exhibiting MC4R agonist activity and the major product was indeed the desired 4*H*-benzo[1,3]dioxin **4** corresponding to the +42 Da product of compound **3**. Interestingly, in this sample produced by the deliberate addition of formaldehyde to the cleavage step, the +42 Da compound **4** was accompanied by a minor +54 Da component which was also observed in the active library sample. This indicated that the +54 Da component was also formaldehyde derived.

Using the chemistry including formaldehyde, a small library (24 compounds) of 4*H*-benzo[1,3]dioxin compounds was synthesized and screened for MC4R agonistic activity without purification after cleavage from the solid support. The data implied that compounds where R^4 was a methoxyacetyl group were more potent than the cyclopropyl analogues. Thus, the next step was to synthesize compound **5** (the methoxyacetyl analogue of **4**) under more controlled conditions. The synthesis of **5** is outlined in Scheme 4.

Surprisingly, the purified compound **5** also tested negative in the MC4R agonist assay. Now the additional +54 Da minor byproducts observed both in the original active library sample and in the product obtained when including formaldehyde in the manual synthesis of **3** came under scrutiny. Using a chiral analytical HPLC column (Chiralpak AD 250 × 4.6 mm, heptane/ethanol + 0.1% DEA 1:4) it was possible to separate the +54 Da component from the main compound **5**. The +54 Da component was found to account for the MC4R agonistic activity and structure **6** was assigned to this compound (Scheme 5).

Both MS and NMR analysis provided compelling evidence supporting the proposed structure **6** for the +54 Da component. Comparison of LC–MS/MS data for the +54 Da component with those of **5** demonstrated that the fragment originating from cleavage of the amide bond between the R^3–R^4 moiety and the R^1–R^2 fragment, are observed in the spectra of **5** but are absent in those recorded for the +54 Da component. This is in accordance with the presence of an additional stable bond tethering the two fragments as illustrated in **6**. NMR data were in accordance with the proposed overall structure **6** and demonstrated the presence of both the *para*- and the *ortho*-isomer in a 2:1 ratio. Finally, the structure (**6**) was unambiguously proven by synthesis of the *para*-isomer (**6a**) using a ring closure reaction under acidic conditions (Scheme 6).

Structure **6a** was simplified to *S*-5[6]-Methoxy-9-(3-(4-(3-aminopropyl) piperazin-1-yl)propyl)-7-(2-methoxyacetylamino)-1(1,3),3(1,4),5(1,3)-tribenzena-2-oxa-9-aza-cyclodecaphane (**7**),[19,20] synthesized using similar conditions. The NMR spectra of **6a** and **7** both exhibited duplication of signals due to the presence of amide bond rotamers and line broadening attributable to ring flipping of the piperazine ring. Additionally, individual resonances were observed for each of the four protons in the *p*-substituted ring indicative of hindered ring rotation, a phenomenon also observed in similar systems, for example, acerogenin.[21] While the signal duplication due to hindered rotation around the amide bond disappeared upon heating to 120 °C, degeneration of the signals



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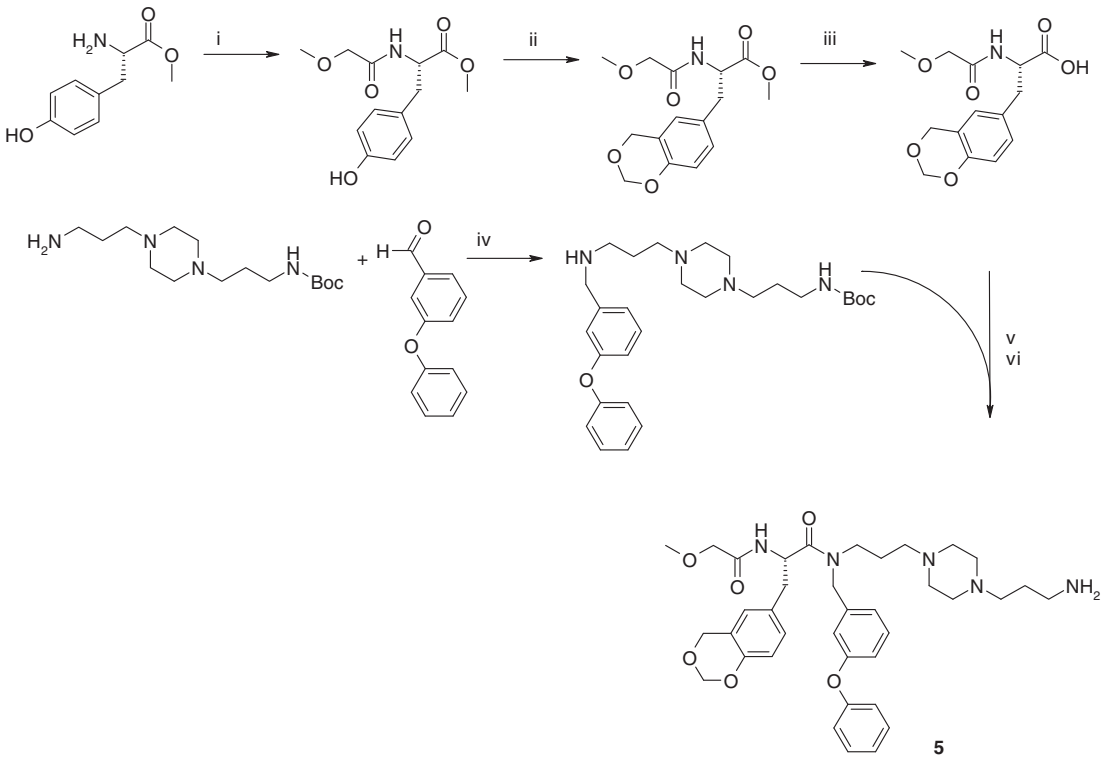
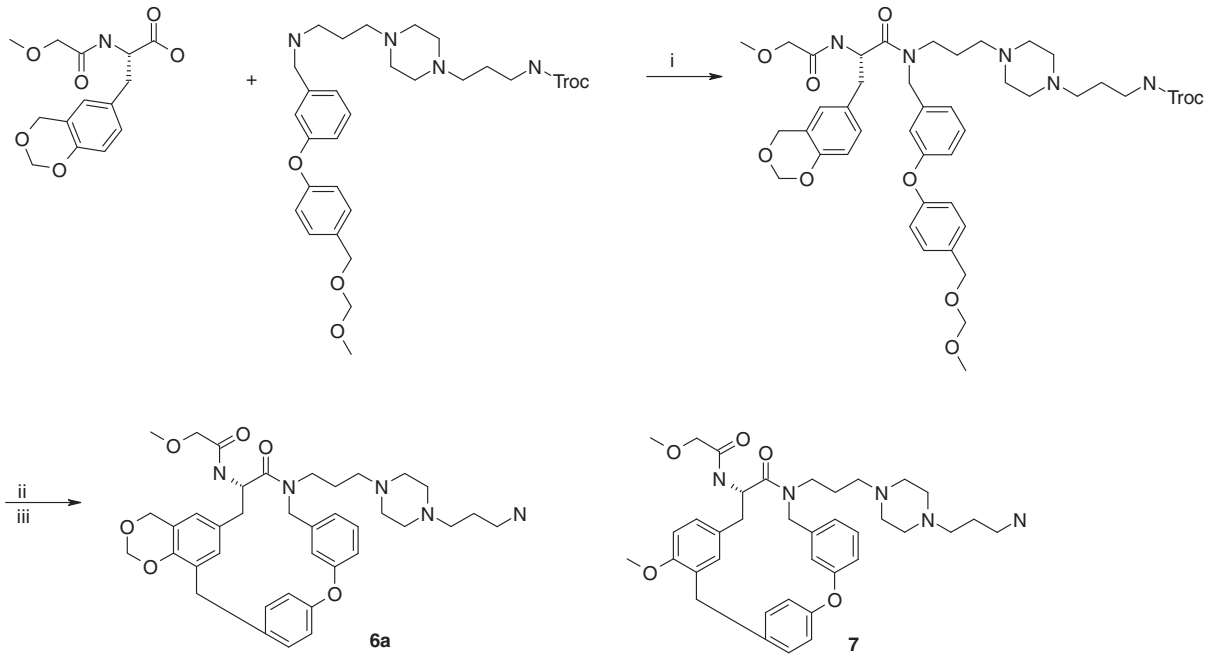
Scheme 5. Proposed structure **6**.



5

Scheme 4. Synthesis of compound **5**. Reagents and conditions: (i) 1 equiv HOBt, 1 equiv TBTU, 1.5 equiv DIPEA; (ii) TFA/DCM 1:1, 2 equiv paraformaldehyde, 1 h; (iii) LiOH, H_2O. (iv) ethanol, Raney-Ni, H_2; (v) 1.2 equiv 2,4,6-tripropyl-1,3,5,2,4,6-trioxatriphosphorinane-2,4,6-trioxide (PPAA), 5.9 equiv *N*-methyl morpholin, DCM; (vi) TFA.



Scheme 6. Synthesis of macrocycle **6a** and structure of macrocycle **7**. Reagents and conditions: (i) 1.5 equiv collidine, 1.5 equiv HATU, DCM; (ii) TFA/DCM 1:1 90 min; (iii) Zn, HOAc.

Table 1
Binding data for compounds **6a** and **7** (mean ± SEM, *n*)

	hMC1 K_i (nM)	hMC3 K_i (nM)	hMC4 K_i (nM)	hMC5 K_i (nM)
6a	328 ± 16, 3	4530 ± 1846, 3	403 ± 171, 3	1532 ± 364, 3
7	116 ± 12, 3	1182 ± 360, 3	392 ± 69, 3	388 ± 46, 3

corresponding to protons in the *p*-substituted aromatic ring was not observed.

The macrocycle **7** was further tested in vitro in binding and cAMP assays and for effect on food intake in vivo. Tables 1 and 2 show binding and functional data for compounds **6a** and **7** for the relevant melanocortin receptors. Both compounds are agonists for the MC1 and MC4 receptors and do not show any detectable activation of the MC3 and MC5 receptors (Table 1 and 2). Using the established peptidic MC4 receptor antagonist SHU9119[22] it is possible to show competitive antagonism, indicating a similar binding site for the peptide SHU9119 and the macrocycle **6a** (Fig. 1).

Compound **7** was tested in an acute feeding model using male Sprague Dawley rats and was able to reduce food intake with a potency comparable to or better than Sibutramine (Fig. 2) in the first 3 h after ip injection.

Attempts to obtain a structure–activity relationship (SAR) were not successful. Changes in the aromatic ring on the amino acid (R^3) lead to structural isomers which were usually hard to separate, while replacement of the amine (R^1) with shorter and simpler amines lead to inactive compounds, thus indicating the importance of a having certain distance between the cyclophane and the terminal amine.

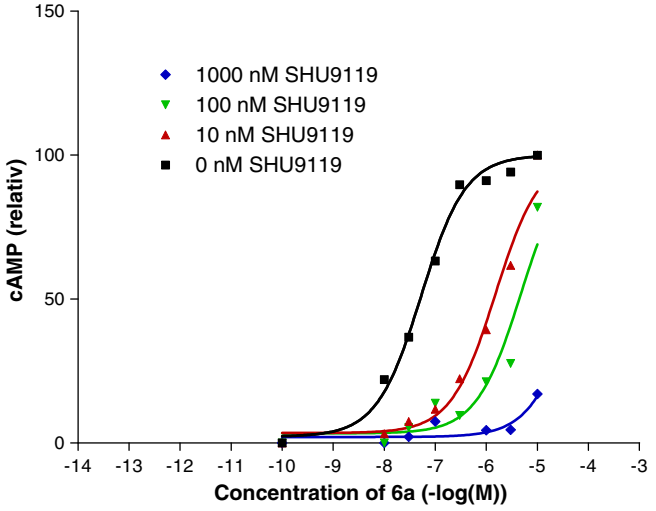


Figure 1. Inhibition of the agonist **6a** with 10, 100 or 1000 nM of the antagonist SHU9119. The maximal stimulation with compound **6a** is set to 100% and the other result is calculated as relative values to maximal stimulation with compound **6a**.

The source of the formaldehyde is most likely the rack in which the samples were stored after cleavage and evaporation of the trifluoroacetic acid. The racks were composed of Bakelite, a phenol/formaldehyde polymer. In conclusion, a novel class of cyclophanes was discovered as potent and selective MC4R agonists. The active components were formed by an unintended side reaction involving formaldehyde. Despite an in vitro activity of around a half a

Table 2
Functional data for compounds **6a** and **7** (mean ± SEM, *n*)

	hMC1 EC_{50} (nM)	hMC1 E_{max} (%)	hMC3 EC_{50} (nM)	hMC4 EC_{50} (nM)	hMC4 E_{max} (%)	hMC5 EC_{50} (nM)
6a	12 ± 1, 3	85 ± 4	>10,000, 3	697 ± 138, 13	98 ± 5, 13	>10,000, 3
7	16 ± 3, 3	83 ± 1	>10,000, 3	466 ± 56, 16	61 ± 5, 16	>10,000, 3

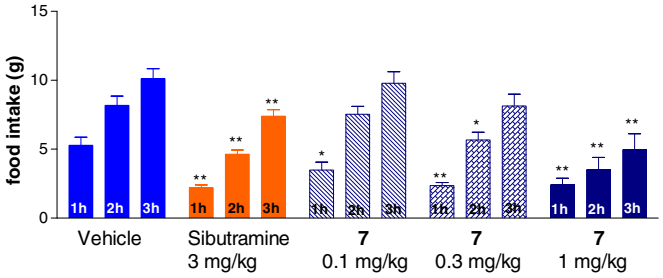


Figure 2. In vivo effect on food intake in scheduled fed male Sprague Dawley rats, which have been trained to consume a 24 h food ration during 5 h. Macrocycle **7** (0.1, 0.3 and 1 mg/kg) was administered ip to 8 rats just before food was presented. Sibutramine[16] (3 mg/kg) was used as positive control. Food intake was followed for 3 h and compared to a vehicle treated group ($^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001). A mixture of 20% Tween 20, 20% propylene glycol and 50 mM phosphate buffer pH 6.0 was used as vehicle.

micromolar, the compounds displayed good in vivo potency. We suspect this may be due to a high penetration rate of the blood brain barrier. In conclusion, a novel class of cyclophanes was discovered as potent and selective MC4R agonists.

Acknowledgments

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.01.011. These data include MOL files and InChiKeys of the most important compounds described in this article.

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