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Discovery of 1-amino-4-phenylcyclohexane-1-carboxylic acid and its influence on agonist selectivity between human melanocortin-4 and -1 receptors in linear pentapeptides

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Abstract—Linear pentapeptides (Penta-*cis*-Apc-DPhe-Arg-Trp-Gly-NH_2) containing 1-amino-4-phenylcyclohexane-1-carboxylic acid (*cis*-Apc) and substituted Apc are potent hMC4R agonists and they are inactive or weakly active in hMC1R, hMC3R, and hMC5R agonist assays. This study, together with our earlier report on 5-BrAtc, demonstrated the importance of replacing His^6 with phenyl-containing rigid templates in achieving good hMC4R agonist potency and selectivity against hMC1R in linear pentapeptides.

In the last decade, five human melanocortin receptor subtypes (hMC1R–hMC5R) have been cloned and characterized.[1] These melanocortin receptors are seven-transmembrane G-protein coupled receptors (GPCRs) which mediate a wide range of physiological functions including pigmentation regulation (MC1R), glucocorticoid production (MC2R), food intake and energy expenditure (MC3R and MC4R) as well as exocrine gland function (MC5R).[1] MC4R, in particular, has become a current drug target for the treatment of obesity due to its involvement in feeding behavior.[2,3] It was recently suggested that MC4R also plays a role in sexual function.[4] Our laboratories are interested in the design of potent and selective human melanocortin-4 receptor (hMC4R) agonists for the treatment of obesity.[5]

We previously reported that using linear pentapeptide Bu-His^6-$DPhe^7$-Arg^8-Trp^9-Gly^{10}-NH_2 (**1**, α-MSH numbering) as the template, replacement of His with racemic 2-aminotetraline-2-carboxylic acid (Atc) gave two separable diastereomers, one of which (**2**) showed modest hMC4R potency (EC_{50} = 290 nM) and selectivity against hMC1R (about 15-fold).[6] N-cap modification of peptide **2** from *n*-butanoyl (Bu-) to *n*-pentanoyl (Penta-) gave peptide **3** which showed slight improvement in agonist potency at both hMC1R and hMC4R, compared with peptide **2**.[6] To improve hMC4R potency and selectivity of Penta-Atc-DPhe-Arg-Trp-Gly-NH_2 (**3**), one strategy involves systematic substitution of the Atc phenyl ring which resulted in 5-BrAtc.[6] An alternative approach involves the use of phenyl-containing rigid templates[7] which led to the discovery of 1-amino-4-phenylcyclohexane-1-carboxylic acid (Apc) (Fig. 1). As described in this report, linear pentapeptides containing *cis*-Apc and substituted Apc showed excellent hMC4R agonist potency and are inactive or weakly active in hMC1R, hMC3R, and hMC5R agonist assays.

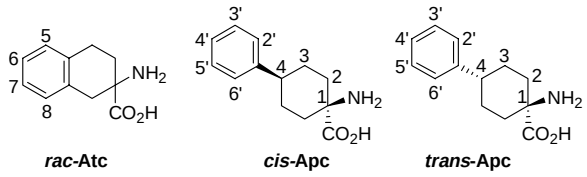

Figure 1. 2-Aminotetraline-2-carboxylic acid (Atc) and 1-amino-4-phenylcyclohexane-1-carboxylic acid (Apc).

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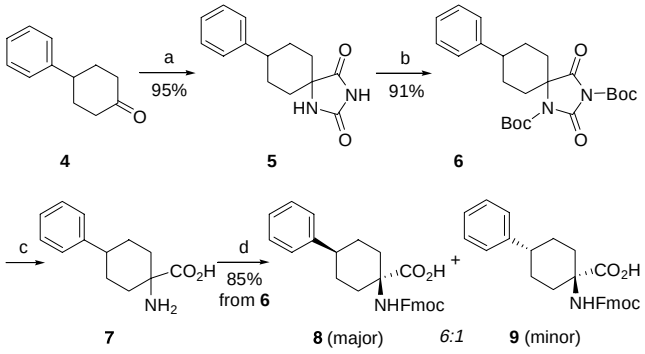
Synthesis and stereochemical determination of Fmoc-protected 1-amino-4-phenylcyclohexane-1-carboxylic acid. Commercially available 4-phenyl cyclohexanone (**4**) was converted via Bucherer–Bergs reaction[8] into hydantoin **5** as a mixture of two isomers (~6:1 by NMR analysis). Direct hydrolysis of hydantoin **5** into Apc amino acid **7** involved drastic reaction conditions (prolonged reflux with concentrated aqueous NaOH solution) and lengthy work-up procedures. An improved protocol was adopted in which the hydantoin N–H's of **5** were first protected with *tert*-butoxylcarbonyl (Boc) groups[9] (giving compound **6**) which allowed the subsequent hydrolysis of the hydantoin group to proceed under mild conditions (1 N NaOH, room temperature, overnight). Without work-up, the pH of the above basic reaction mixture containing Apc amino acid **7** was adjusted to ~11 and Fmoc-protection gave a readily separable mixture of *cis*-Apc **8** and its minor *trans*-isomer **9** (Scheme 1).

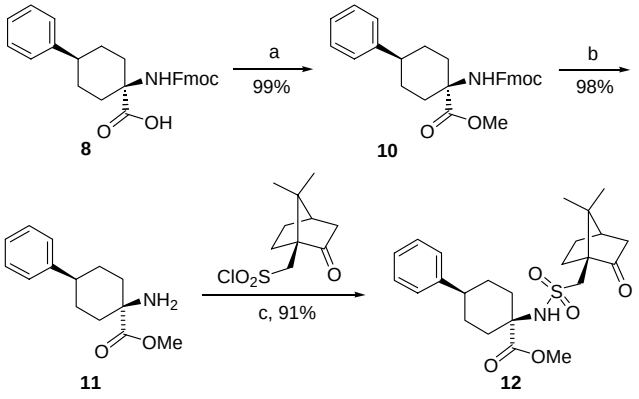
The *cis*-configuration between the phenyl and amino groups of the major isomer **8** was unambiguously established by X-ray crystallographic study of the corresponding camphorsulfonamide **12**[10] prepared via a three-step sequence (Scheme 2).

Synthesis of Fmoc-protected substituted Apc. Addition of various aryl lithium reagents (generated from the corresponding aryl bromides/iodides **14** and *n*-BuLi) to 1,4-cyclohexanedione mono-ethylene ketal (**13**) gave alcohol **15**. Dehydration of the tertiary alcohol of **15**, hydrogenation of the resulting olefin, followed by hydrolysis of the cyclic ketal gave substituted phenyl cyclohexanone **17**. Using a four-step sequence previously used in the synthesis of *cis*-Apc **8**, ketone **17** was converted into the corresponding Fmoc-protected substituted Apc **20**. As in the case of the unsubstituted Apc isomers **8** and **9**, the major *cis*-isomer **20** was readily separated from its minor *trans*-isomer by column chromatography (Scheme 3).

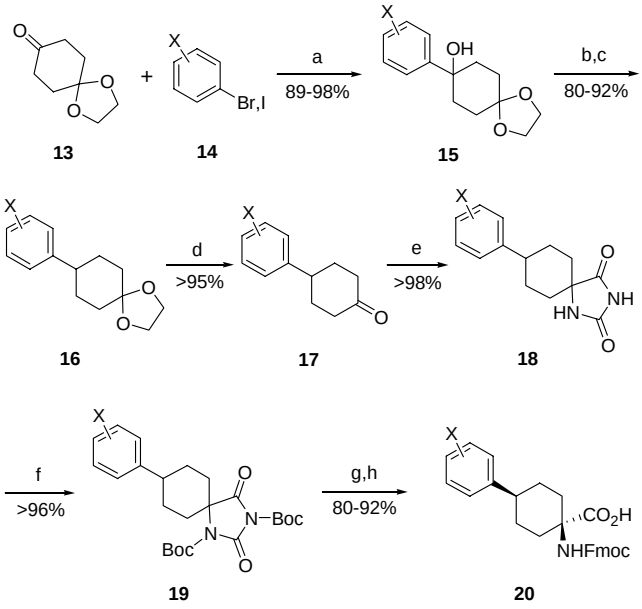
All new peptides[11] were synthesized on solid phase from Fmoc-protected Apc (or Fmoc-protected substituted Apc) and other suitably protected amino acids using



Scheme 1. Reagents and conditions: (a) KCN, $(NH_4)_2CO_3$, EtOH/H_2O, 90 °C, overnight; (b) $(Boc)_2O$, Et_3N, DMAP, THF, rt, overnight; (c) 1 N NaOH, DME, H_2O, rt, overnight; (d) Fmoc-Cl, pH 11, dioxane/H_2O, rt.



Scheme 2. Reagents and conditions: (a) CH_2N_2, Et_2O, 0 °C, 30 min; (b) piperidine, DMF, rt, 20 min; (c) $(iPr)_2EtN$, DMAP, CH_2Cl_2, rt, 18 h.



Scheme 3. Reagents and conditions: (a) *n*-BuLi, −78 °C, THF, 2 h; (b) cat. TsOH, reflux, benzene, 3–4 h; (c) H_2, 5 wt% Pd/C, EtOAc, 3 h; (d) acetone, TsOH, 60 °C, 4–6 h; (e) KCN, $(NH_4)_2CO_3$, EtOH/H_2O, overnight; (f) $(Boc)_2O$, Et_3N, DMAP, rt, THF, overnight; (g) 1 N NaOH, DME/H_2O, rt, overnight; (h) Fmoc-Cl, pH 11, dioxane/H_2O, rt, overnight.

standard Fmoc methodology. The crude peptides were purified to homogeneity using reversed-phase HPLC and characterized by fast atom bombardment mass spectroscopy.

Agonist assays were performed using HEK293 cells transfected with hMC1R–hMC5R as reported in detail elsewhere.[11,12] The EC_{50} values reported in Tables 1 and 2 are the average of at least two separate experiments. Binding assays were performed using radiolabeled NDP-MSH as reported in detail elsewhere.[12] The IC_{50} values reported in Table 3 are the average of at least two separate experiments.

Table 1. Agonist activity of His[6] modified pentapeptides at the human melanocortin receptors

Peptide	Amino acid sequence	EC_{50}[a] (nM)	
		hMC4R	hMC1R
1	Bu-His-(D)Phe-Arg-Trp-Gly-NH_2	20	10
2	Bu-Atc-(D)Phe-Arg-Trp-Gly-NH_2 (2nd isomer)[b]	290	4400
3	Penta-Atc-(D)Phe-Arg-Trp-Gly-NH_2 (2nd isomer)[b]	45	830
21	Penta-*cis*-Apc-(D)Phe-Arg-Trp-Gly-NH_2	2	25% at 50 μM[c]
22	Penta-*trans*-Apc-(D)Phe-Arg-Trp-Gly-NH_2	88	1300
23	Penta-*cis*-4′-MeApc-(D)Phe-Arg-Trp-Gly-NH_2	3	55% at 50 μM[c]
24	Penta-*cis*-4′-ClApc-(D)Phe-Arg-Trp-Gly-NH_2	4	45% at 50 μM[c]
25	Penta-*cis*-4′-MeOApc-(D)Phe-Arg-Trp-Gly-NH_2	9	0% at 50 μM[c]
26	Penta-*cis*-4′-EtOApc-(D)Phe-Arg-Trp-Gly-NH_2	13	0% at 50 μM[c]
27	Penta-*cis*-4′-*i*PrOApc-(D)Phe-Arg-Trp-Gly-NH_2	6	60% at 50 μM[c]
28	Penta-*cis*-4′-HOApc-(D)Phe-Arg-Trp-Gly-NH_2	5	1300
29	Penta-*cis*-3′-MeOApc-(D)Phe-Arg-Trp-Gly-NH_2	2	690
30	Penta-*cis*-3′,5′-*dii*PrApc-(D)Phe-Arg-Trp-Gly-NH_2	60% at 50 μM[c]	210
31	Penta-*cis*-Apc-(D)Phe-Arg-Trp-NH_2	130	1300
32	*trans*-Pcc-(D)Phe-Arg-Trp-Gly-NH_2	550	60% at 50 μM[c]

Bu stands for $CH_3CH_2CH_2C(=O)$ and Penta stands for $CH_3CH_2CH_2CH_2C(=O)$.

[a] Concentration of peptide at 50% maximum cAMP accumulation or the % of cAMP accumulation (relative to NDP-MSH) observed at the highest peptide concentration tested.

[b] 1st isomer and 2nd isomer refer to the order in which the two diastereomers eluted under our HPLC conditions.[13]

[c] Not tested for antagonist activities.

Table 2. Agonist activity of pentapeptides containing various His surrogates at the human melanocortin receptors

Peptide	EC_{50}[a] (nM)			
	hMC1R	hMC3R	hMC4R	hMC5R
1	10	0% at 50 μM[b]	20	0% at 50 μM[b]
21	25% at 50 μM[b]	57% at 50 μM[b]	2	36% at 50 μM[b]
26	0% at 50 μM[b]	28% at 50 μM[b]	13	46% at 50 μM[b]

[a] Concentration of peptide at 50% maximum cAMP accumulation or the % of cAMP accumulation (relative to NDP-MSH) observed at the highest peptide concentration tested.

[b] Not tested for antagonist activities.

Table 3. Binding affinity of pentapeptides containing various His surrogates at the human melanocortin receptors

Peptide	IC_{50}[a] (nM)			
	hMC1R	hMC3R	hMC4R	hMC5R
1	580	4000	150	13000
21	7100	Not determined	13	Not determined
26	4700	Not determined	43	Not determined

[a] Concentration of peptide at 50% radiolabeled NDP-MSH displacement.

As shown in Table 1, the lead pentapeptide Bu-His-DPhe-Arg-Trp-Gly-NH_2 (**1**) is a potent hMC4R agonist (EC_{50} = 20 nM) but is not selective against hMC1R (EC_{50} = 10 nM). Using Atc as a His replacement, modestly hMC4R potent and selective peptides **2** and **3** were obtained.[6] Apc, in which the phenyl group is attached to C-4 of the cyclohexane ring in an exocyclic fashion, is structurally quite different from Atc (Fig. 1). We were delighted to find that peptide **21** (made from *cis*-Apc **8**) is over 22-fold (the standard error in our assays is about 2-fold) more potent as a hMC4R agonist compared with peptide **3** and at the same time possessed minimal agonist activity at hMC1R (25% activation at 50 μM). In contrast, peptide **22** (made from *trans*-Apc **9**) is about 40-fold less potent as a hMC4R agonist and is significantly less hMC4R selective, compared with peptide **21**. The different agonist profiles of peptides **21** and **22** clearly showed that the relative orientation of the phenyl group in Apc has a significant impact on the hMC4R agonist potency and selectivity of the resulting peptide. Due to the highly flexible nature of linear peptides, we were unable to determine the solution structures of peptides **1** and **21** using NMR spectroscopy. On the other hand, incorporation of *cis*-Apc into conformationally constrained (MT-II-like) cyclic peptide templates gave highly rigid solution structures which led to a predictive pharmacophore model of hMC4R.[14]

In an effort to optimize *cis*-Apc, a series of substituents (methyl-, chloro-, methoxy-, ethoxy-, isopropoxy-, hydroxy-, and isopropyl-) were introduced into *cis*-Apc (general structure **20**) and the agonist activities of the resulting peptides (peptides **23**–**30**) are shown in Table 1. Peptides **23**–**28**, bearing 4′-substituents with different electronic and steric properties, all showed hMC4R EC_{50} values within a 4-fold range (3–13 nM). Since peptide **27**, containing bulky 4′-isopropoxy group, retains excellent hMC4R agonist activity, it should be interesting to use Apc with bulkier 4′-substitution (e.g., *t*-butoxy-, phenoxy- or phenyl) to establish the size limit of the hMC4R His binding pocket.

Peptide **29**, which contains 3′-MeOApc, possessed the same hMC4R potency (EC_{50} = 2 nM) as peptide **21**. Peptide **30**, with isopropyl groups at both 3′ and 5′ positions of Apc, is weakly active as a hMC4R agonist, perhaps due to an unfavorable steric interaction with the hMC4R binding pocket. Peptide **30** was not tested for its hMC4R binding and antagonist activity. Interestingly, both peptides **29** and **30** with 3′-substitution showed higher hMC1R agonist activities compared with peptide **21**. More diverse substitutions need to be explored at the

2′- and 3′-positions of *cis*-Apc to fully establish the structure–activity relationship in this region.

In an attempt to reduce the molecular weight of peptide **21**, truncated peptides such as **31** and **32** were prepared. Tetrapeptide **31**, with the Gly residue removed, is significantly less potent and selective as a hMC4R agonist, compared to pentapeptide **21**. Peptide **32**, containing *trans*-4-phenylcyclohexane-1-carboxylic acid (*trans*-Pcc) (Fig. 2), which could be considered as Apc with its N-cap truncated, is only moderately potent (EC_{50} = 550 nM) as a hMC4R agonist. The reduced hMC4R agonist activities of peptides **31** and **32** clearly demonstrate the important roles played by the Gly residue and N-cap of peptide **21**.

The most hMC4R selective peptides described above were tested in hMC3R and hMC5R agonist assays and the results are shown in Table 2. The lead pentapeptide **1** (Bu-His-DPhe-Arg-Trp-Gly-NH_2) was inactive in both hMC3R and hMC5R agonist assays. The low agonist activities in hMC3R and hMC5R are maintained when His of peptide **1** was replaced by *cis*-Apc (peptide **21**) and *cis*-4′-EtOApc (peptide **26**). The above peptides were also, in certain cases, tested in hMC1R–hMC5R binding assays (Table 3). The binding affinities of the peptides **1**, **21**, and **26** toward hMC4R, track with their agonist activities in the order of **21** > **26** > **1**. Peptides **21** and **26** bind to hMC1R with micromolar affinity, but showed no significant agonist activity.

Hruby et al. proposed that 'the position 6 of the synthetic melanocortin ligands is important for enhancing potency and selectivity at MC3 and MC4 melanocortin receptors'.[15,16] Hruby's study was carried out using a cyclic peptide antagonist as the template (SHU9119) and the resulting compounds are all hMC4R antagonists with no hMC1R biochemical data.[15,16] In a study by Haskell-Luevano et al., 17 histidine surrogates were introduced into a Ac-His^6-DPhe^7-Arg^8-Trp^9-NH_2 tetrapeptide template[17] and the tetrapeptide containing amino-2-naphthylcarboxylic acid (Anc) showed good mouse MC4R agonist potency (EC_{50} = 21 nM) and good selectivity against mouse MC1R (EC_{50} = 7900 nM). The biological activities of these tetrapeptides at human melanocortin receptors were not reported.[17] Our work described herein showed that linear pentapeptides (Penta-*cis*-Apc-DPhe-Arg-Trp-Gly-NH_2) containing *cis*-Apc and substituted Apc are potent hMC4R agonists and they are inactive or weakly active in hMC1R, hMC3R, and hMC5R agonist assays. This report on Apc and substituted Apc, together with our earlier report on 5-BrAtc and related Atc,[6] demonstrated the importance of replacing His^6 with phenyl-containing rigid templates in achieving good hMC4R agonist potency and selectivity against hMC1R in linear pentapeptides.[18] Further modification of Apc amino acid, incorporation of Apc and related analogs into cyclic peptides, and in vivo studies using Apc-containing linear/cyclic peptides would be reported in due course.

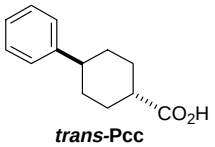


Figure 2. Structure of *trans*-4-phenylcyclohexane-1-carboxylic acid (*trans*-Pcc).

Acknowledgments

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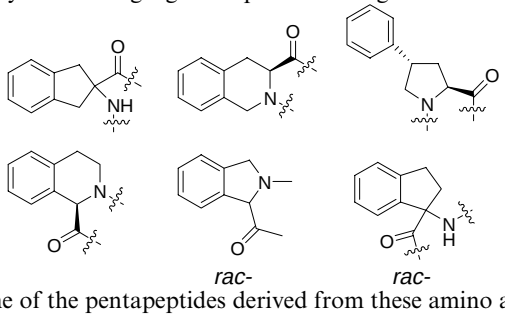

None of the pentapeptides derived from these amino acids is as potent or hMC4R selective as peptide **3** (data not shown).
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