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# SYNTHESIS AND BIOLOGICAL EVALUATION OF α-MSH PEPTIDE -PEPTOID HYBRIDS

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## Abstract

Peptoids or N-substituted glycines are a class of peptidomimetics where the side chain in an amino acid is attached to nitrogen instead of α-carbon. Peptoids mimic the critical structural features of peptides but are shown to overcome some of their problems such as poor absorption and rapid degradation by proteolytic enzymes. Therefore, we anticipate that these molecules would be more stable in vivo and might possess better pharmacokinetic properties. Our aim was to synthesize stable and effective peptoid α-MSH ligands for human melanocortin receptors (MCR). The melanocortin receptors are found to be involved in a number of physiological functions including pigmentation, memory and learning, antipyretic and anti-inflammatory effects, immunomodulation, regulation of food intake and energy homeostasis.

We were interested in making peptoid analogs of Ac-His-D-Phe-Arg-Trp-$NH_2$, which has been shown to be the minimum sequence required for agonist activity for human melanocortin receptors, MC1R and MC4R. A set of tetrapeptides was synthesized, where the amino acids histidine, phenylalanine and arginine in the oligomer Ac-His-D-Phe-Arg-Trp-$NH_2$, were replaced by their respective peptoid analog one at a time. The tetrapeptide, Ac-NHis-D-Phe-Arg-Trp-$NH_2$ (**28**) was found to have agonist activity at mouse MC4R ($EC_{50}$ = 1.71μM) and MC5R ($EC_{50}$ = 0.75 μM). While tetrapeptide, Ac-His-D-Phe-NArg-Trp-$NH_2$ (**30**) had agonist activity at mMC1R ($EC_{50}$ = 1.47 μM).

Another set of tetrapeptides was synthesized, where phenylalanine in the oligomer Ac-His-D-Phe-Arg-Trp-$NH_2$, was replaced with various aromatic peptoids. Although, this modification did not prove fruitful for agonist activity at all mouse melanocortin receptors, it does introduce specificity for mMC1R. Increasing the stearic bulk and lipophilicity of the phenylalanine side chain imparts specificity to mMC1R. PJ2078A (**32**) and PJ2079C (**33**) showed modest agonist activity at mMC1R ($EC_{50}$ = 7.66 μM & $EC_{50}$ = 12.2 μM respectively) but poor agonist activity at other mouse melanocortin receptors.

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## List of Abbreviations

| | |
|---|---|
| Ac | Acetyl |
| Ala | Alanine |
| Arg | Arginine |
| Asn | Asparagine |
| Asp | Aspartic Acid |
| Boc | *tert*-Butyloxycarbonyl |
| $Boc_2O$ | di-*tert*-Butyloxy dicarbonate |
| Boc-ON | 2-(*tert*-Butoxycarbonyloxyimino)-2-phenylacetonitrile |
| Bom | Benzyloxymethyl |
| Cbz | Benzyloxycarbonyl |
| CIP | 2-Cholro-1,3-dimethylimidazolidium hexaphosphate |
| Cys | Cysteine |
| DCHA | Dicyclohexylamine |
| DCM | Dichloromethane |
| DIC | N, N-Diisopropylcarbodiimide |
| DIEA | Diisopropylethylamine |
| DMF | Dimethylformamide |
| DMSO | Dimethylsulfoxide |
| EDT | Ethanedithiol |
| Fmoc | 9-Fluorenylmethoxycarbonyl |

| | |
|---|---|
| Glu | Glutamic Acid |
| Gly | Glycine |
| HF | Hydrogen fluoride |
| His | Histidine |
| HOAT | 1-Hydroxy-7-aza-benzotriazole |
| HOBT | N-Hydroxybenzotriazole |
| i-PrOH | Isopropyl alcohol |
| Leu | Leucine |
| Lys | Lysine |
| *p*-MBHA | *p*-Methyl-benzhydrylamine |
| Me | Methyl |
| MeOH | Methanol |
| Met | Methionine |
| Nle | Norleucine |
| OPfp | Pentafluorophenyl ester |
| Pbf | 2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl |
| Phe | Phenylalanine |
| Pmc | 2,2,5,7,8-Pentamethylchroman-6-sulfonyl |
| Pro | Proline |
| PyBOP | Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate |
| Ser | Serine |
| SAR | Structure Activity Relationship |

| | |
|---|---|
| SPPS | Solid Phase Peptide Synthesis |
| TEA | Triethylamine |
| TFA | Trifluoroacetic Acid |
| TIS | Triisopropylsilane |
| TMS | Trimethylsilane |
| Tos | Tosyl (*p*-Toluenesulfonyl) |
| Trp | Tryptophan |
| Trt | Trityl (Triphenylmethyl) |
| Tyr | Tyrosine |
| Val | Valine |
| Z | Benzyloxycarbonyl |

## Introduction

The melanocortins are a family of peptides derived from differential cleavage of proopiomelanocortin (POMC) peptide in the pituitary gland. This family of peptides includes corticotropin (ACTH), melanotropins namely α-MSH (α-melanocyte stimulating hormone), β-MSH, γ-MSH, β-endorphin and β-LPH (lipotropic hormone). α-MSH has been known for a long time to be involved in the regulation of integumental pigmentation in vertebrates. ACTH (adrenocorticotropin hormone) has been known to regulate steroid production [1,2]. More recently the melanocortin peptides have been shown to have a variety of other central and peripheral effects including improvement of memory [3], sexual behavior [4], obesity [5], cardiovascular regulation [6], anti-pyretic, analgesia, anti-inflammatory [7] and immunomodulatory effects [8].

Five human melanocortin receptors [9, 10, 11, 12, 13] (MCR) have been identified to date. They are G-protein coupled seven transmembrane receptors (GPCR) with 40-60 % sequence homology between subtypes and are coupled to a cAMP signal transduction pathway [14]. α-MSH is the most potent endogenous agonist for all melanocortin receptors except melanocortin receptor MC2R. These receptors are distributed among various tissue regions in the body including the skin, adrenal glands, hypothalamus, brain etc. The physiological role of each of the melanocortin receptor subtypes is starting to become evident but not completely understood [15].

Melanocortin receptor MC1R or MSH-R, one of the earliest known receptors in the family, is expressed in both normal and malignant melanocytes and keratinocytes in the skin epidermis [16]. α-MSH and ACTH (α-MSH = ACTH > β-MSH > γ-MSH) are both equipotent agonists of human MC1R [17]. Stimulation of these receptors has been shown to induce melanin synthesis and cell proliferation [18]. They are thought to be involved in regulation of skin pigmentation. Agonists of MC1R are being investigated for cosmetic tanning and hypopigmentary disorders (including wound healing) [19]. Apart from the skin, MC1R is also found to be expressed in cells involved in inflammatory responses such as macrophages, monocytes and neutrophils. Melanocortic peptides are known to have anti-pyretic and anti-inflammatory effects [20], but the possible involvement of MC1R in these effects is not clearly established.

Melanocortin receptor MC2R or ACTH-R is expressed primarily in the adrenal cortex, which is also the site for corticosteroidogenesis. As the name suggests, ACTH is the most potent endogenous agonist for this receptor. ACTH stimulates corticosteroidogenesis, which is regulated by MC2R [21]. MC2R is also expressed in the adrenal medulla, however its function there is as yet unknown [21]. Apart from the adrenal glands, MC2R is also found to be expressed in cultured human melanocytes [22]. ACTH is known to stimulate proliferation and melanogenesis of human melanocytes [17] and also to induce DNA synthesis and cell proliferation of keratinocytes [23].

Melanocortin receptor MC3R is expressed in brain, hypothalamus, heart, placenta and gut. α-MSH, ACTH and γ-MSH are all equipotent agonists of MC3R. The physiological role of this receptor is poorly understood, although recent studies in rats indicate it may play some role in regulation of energy homeostasis and obesity [24]. The presence of MC3R in the nucleus accumbens in the brain where opiate receptors are also present, suggests a possible role in opiate addiction and other psychiatric diseases [25].

Melanocortin receptor MC4R is expressed primarily in the brain regions of cortex, thalamus, brain stem and hypothalamus. It is expressed extensively in hypothalamic sites including the paraventricular (PVN) nuclei, which plays an important role in feeding behavior. α-MSH, β-MSH and ACTH are all equipotent endogenous agonists for this receptor. The physiological role of MC4R is better understood and has generated a considerable amount of interest in the area of eating disorders and obesity research [26]. MC4R has been shown to be involved in regulation of energy balance and body weight [27]. It is thought to regulate food intake and onset of obesity through the leptin signaling pathway [28]. Agonists of MC4R have been shown to decrease food intake and reduce body weight [29] and are being investigated for treatment of obesity. MC4R may also be involved in controlling erection and a study has shown melanotropic peptides initiate erection in psycogenic erectile dysfunctional men [30].

Melanocortin receptor MC5R is expressed in a host of human tissues including brain, adrenal glands, skeletal muscle, fat cells, kidney, liver, pituitary, testis and uterus. α-MSH is the most potent agonist amongst all melanocortin peptides for this receptor. The physiological role of MC5R is very poorly understood. However, a study has shown involvement of this receptor in the control of lipid and pheromone production in exocrine glands of mice [31], suggesting a similar role in lipid metabolism in humans.

The identification of five subtypes of melanocortin receptors and observation of various physiological effects of melanocortic peptides, have led to the possibility of developing new drugs for skin tanning, obesity treatment, inflammation etc. However, due to the lack of adequate understanding of some of the melanocortin receptors, especially MC3R and MC5R, it is necessary to develop subtype specific ligands. Subtype specific ligands are powerful tools for elucidating the pharmacology of receptors and could be possible drug candidates. α-MSH, being a potent endogenous agonist for most melanocortin receptors except MC2R, would be a model for developing both potent agonists and antagonists for MCRs.

α-MSH is a tridecapeptide with its N-terminal acetylated and C-terminal amidated and extensive structure activity studies have been carried out on this molecule. Alanine substitution studies [32, 33] have shown that the core sequence $His^6$-$Phe^7$-$Arg^8$-$Trp^9$, common to all melanocortic peptides (Chart 1), is crucial for

both activity and binding. Although $His^6$ is observed to be less important for the binding of α-MSH to rat melanocortin receptors, $Arg^8$ and $Trp^9$ are most crucial for binding. The N-terminal residues $Ser^1$-$Tyr^2$-$Ser^3$ were found not to be critical for activity of rat MC1R, while the C-terminal residues $Lys^{11}$-$Pro^{12}$-$Val^{13}$ were important for both binding and activity. Both N- and C-terminal residues were necessary for binding at rat MC3R. $Glu^5$ and $Gly^{10}$ were observed not to be important for the binding, at least for rat MC1R and MC3R.

**Chart 1. Amino acid sequence of melanocortins and NDP-MSH**

**α-MSH**

Ac-Ser-Tyr-Ser-Met-Glu-**His-Phe-Arg-Trp**-Gly-Lys-Pro-Val-$NH_2$ (**1**)

**β-MSH**

Asp-Glu-Gly-Pro-Tyr-Lys-Met-Glu-**His-Phe-Arg-Trp**-Gly-Ser-Pro-Pro-Lys-Asp (**2**)

**γ-MSH**

Tyr-Val-Met-Gly-**His-Phe-Arg-Trp**-Asp-Arg-Phe-Gly (**3**)

**ACTH**

Ser-Tyr-Ser-Met-Glu-**His-Phe-Arg-Trp**-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe (**4**)

**NDP-MSH**

Ac-Ser-Tyr-Ser-Nle-Glu-His-D-Phe-Arg-Trp-Gly-Lys-Pro-Val-$NH_2$ (**5**)

One of the first, potent analogs of α-MSH to be synthesized was NDP-MSH (**5**) (Chart 1). It was observed that heat-alkali treatment of α-MSH resulted in prolonged biological activity. Quantitative gas-chromatographic analysis of the

heat-alkali treated α-MSH revealed partial racemization at $Met^4$ and $Phe^7$ positions. Replacing phenylalanine with D-phenylalanine and methionine with norleucine in α-MSH resulted in $Nle^4$-D-$Phe^7$-α-MSH or NDP-MSH [34]. Methionine was replaced with its bioisostere norleucine because it has sulfur, which is known to oxidize easily, resulting in a loss of potency of α-MSH. Incorporating D-phenylalanine makes the molecule more resistant to enzymatic degradation, thereby making NDP-MSH more potent and longer acting than α-MSH, but non-selective between MCR subtypes. One study [35] has shown that the N-terminal sequence ($Ser^1$-$Tyr^2$-$Ser^3$) of NDP-MSH to be unimportant for binding at human MC1R and MC4R but necessary for MC3R and MC5R, while the C-terminal sequence ($Gly^{10}$-$Lys^{11}$-$Pro^{12}$-$Val^{13}$) is important for binding at all four human MCR subtypes.

α-MSH and NDP-MSH are both linear, flexible molecules. Studies have been carried out to identify the biologically relevant conformation of the core bioactive sequence (His-Phe-Arg-Trp). Knowledge of the biologically active conformation is important for designing selective and potent analogs. Cyclization between amino acid side chains in peptides brings constraint and rigidity to the molecule that might mimic the biologically active conformation. It was proposed that the potency and long-acting effect of NDP-MSH was due to a β-turn conformation of the molecule [34]. This led to the design and synthesis of cyclic α-MSH analogs that stabilized the β-turn (Chart 2).

**Chart 2. MTII and Important Cyclic α-MSH Analogs**

Ac-Ser-Tyr-Ser-c[Cys-Glu-His-Phe-Arg-Trp-Cys]-Lys-Pro-Val-$NH_2$ (**6**)

Ac- c[Cys-Glu-His-D-Phe-Arg-Trp-Cys]-Lys-Pro-Val-$NH_2$ (**7**)

Ac-Ser-Tyr-Ser-c[Cys-Glu-His-D-Phe-Arg-Trp-Cys]-Lys-Pro-Val-$NH_2$ (**8**)

Ac- -c[Cys-Glu-His-D-Phe-Arg-Trp-Cys]-$NH_2$ (**9**)

Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Lys]-$NH_2$ (**MTII**) (**10**)

Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Ala-Lys]-$NH_2$ (**11**)

Studies revealed that cyclization of α-MSH between positions four and ten stabilized the β-turn and also resulted in good melanotropic activity, which led to the synthesis of the analog Ac-c[$Cys^4$, $Cys^{10}$]- α-MSH (**6**) [36]. By replacing $Met^4$ and $Gly^{10}$ with cysteine (pseudoisostere), the cyclization was brought by forming a disulfide bridge. This analog was 1000-10000 times more potent than α-MSH in lizard and frog skin assays respectively. Further structure activity studies showed removal of the N-terminal tripeptide sequence ($Ser^1$-$Tyr^2$-$Ser^3$) does not have any significant effect on activity. Also the D-$Phe^7$ substituted analogs were more potent than their L-$Phe^7$ counterparts in the frog and lizard bioassay [37]. Some potent compounds identified from this series are Ac-c[$Cys^4$, D-$Phe^7$, $Cys^{10}$]- α-MSH(4-13)-$NH_2$ (**7**) and Ac-c[$Cys^4$, D-$Phe^7$, $Cys^{10}$]- α-MSH-$NH_2$ (**8**). A more recent study [38] on human MCRs has shown similar results. Ac-c[$Cys^4$, $Cys^{10}$]- α-MSH (**6**) has less affinity than Ac-c[$Cys^4$, D-$Phe^7$, $Cys^{10}$]- α-MSH (**8**)in all subtypes. The N-terminal sequence seems to be not very important for most subtypes, since Ac-c[$Cys^4$, D-$Phe^7$, $Cys^{10}$]- α-MSH(4-13)-$NH_2$ had similar affinity

as Ac-c[Cys$^4$, D-Phe$^7$, Cys$^{10}$]- α-MSH, except perhaps for MC5R. The preference order for compounds **6**, **7** and **8** was MC1R > MC4R > MC3R > MC5R. Another compound in the study, Ac-c[Cys$^4$, D-Phe$^7$, Cys$^{10}$]- α-MSH(4-10)-$NH_2$ (**9**) had a unique preference order of MC4R > MC1R > MC3R > MC5R. This suggests that the C-terminal sequence (Lys$^{11}$-Pro$^{12}$-Val$^{13}$) is not as critical for affinity for MC4R as it is for MC1R, which allows incorporation of some degree of selectivity for MC4R.

Besides the disulfide cyclics were the lactams, where cyclization was achieved by forming a lactam bridge between the acidic fifth residue and basic tenth residue. Some analogs synthesized [39] in this class were 23-membered rings of the general formula, Ac-Nle$^4$-c[Yaa$^5$-D-Phe$^7$, Lys$^{10}$]- α-MSH(4-10)-$NH_2$, where Yaa is Asp or Glu. MTII or Ac-Nle$^4$-c[Asp$^5$-D-Phe$^7$, Lys$^{10}$]- α-MSH(4-10)-$NH_2$ (**10**), was found to be a super potent, prolonged acting but non-selective agonist. Several structure activity studies have been carried out on MTII.

One study [40] was that the 26-membered lactam, Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Ala-Lys]-$NH_2$ (**11**) formed by inserting alanine between tryptophan and lysine had similar, but non-selective, activity as MTII (**10**) in all human MCRs. This suggests that the 23-membered ring may not be optimal for human MCRs, while the 23-membered ring was found to be optimal for lizard skin bioassay (lizard skin bioassay usually has given parallel relationship to mammalian assay). Further modification was carried out on analog **11** by inverting chiralities of Asp,

Phe and Trp respectively, in order to affect the conformation and bring some selectivity between human MCR subtypes. Inverting the chiralities of Asp and D-Phe resulted in a drastic drop in binding. Although no analogs more potent than MTII or **11** were seen, a pattern in the structure activity between the human MCRs was observed. It seems that human MC1R and MC4R share a similar structure activity profile and so does MC3R and MC4R. This suggests that certain changes in conformation can bring selectivity for certain MCRs.

Further structure activity studies on MTII (Ac-Nle$^4$-c[Asp$^5$-His$^6$-D-Phe$^7$-Arg$^8$-Trp$^9$- Lys$^{10}$]-$NH_2$) involved replacing each amino acid with proline, one at a time [41]. Replacing with a conformationally constrained amino acid such as proline may stabilize a conformation that could be biologically relevant. Substituting D-Phe, Arg or Trp in MTII with proline resulted in a drastic loss in activity and binding. The analog with proline in place of histidine had similar activity as MTII but with no selectivity. However, the MTII analog with Nle substituted by proline, was selective for human MC4R with agonist activity comparable to MTII. This led to extensive structure activity studies on the N-terminus of MTII (Ac-Nle$^4$) [41]. The Ac-Nle$^4$ portion, which is outside the lactam ring, was substituted with hydrophobic, hydrophilic and proline-like residues. Some conclusions drawn from this study are that the Ac-Nle segment is not critical for binding or activity at MC4R but very important for full agonist activity of MC5R. This allows introduction of selectivity into the molecule. Flexible, hydrophobic groups at Ac-Nle$^4$ position are necessary for agonist activity while bulky hydrophobic and

hydrophilic groups decrease agonist activity at MC5R. Analogs without the terminal acetyl group were as potent as MTII but non-selective.

Introduction of bulky aromatic amino acids in place of D-Phe in MTII was another way of introducing specificity between human MCRs [42]. It was observed that Ac-Nle$^4$-c[Asp$^5$-His$^6$-D-Nal(2)$^7$-Arg$^8$-Trp$^9$- Lys$^{10}$]-$NH_2$ (**12**)or SHU9119 was a potent antagonist of human MC4R, less potent antagonist of MC3R and a full agonist of MC1R and MC5R. SHU9119, an analog of MTII, has a D-2'-naphthylalanine (Nal) in place of D-Phe. The D-*p*-iodophenylalanine analog, Ac-Nle$^4$-c[Asp$^5$-His$^6$-D-Phe(pI)$^7$-Arg$^8$-Trp$^9$- Lys$^{10}$]-$NH_2$ (**13**) was also a potent antagonist for human MC4R, partial agonist for MC3R and a potent agonist of MC1R. Interestingly, the D-*p*-chloro and D-*p*-fluorophenylalanine analogs had no antagonist activity at any MCRs.

**Chart 3. SHU9119 and other analogs**

Ac-Nle-c[Asp-His-D-Nal(2')-Arg-Trp-Lys]-$NH_2$ (**SHU9119**) (**12**)

Ac-c[Cys-Glu-His-D-Nal(2')-Arg-Trp-Cys]-$NH_2$ (**HS9510**)(**14**)

Ac-c[Cys-Glu-His-D-Nal(2')-Arg-Trp-Gly-Cys]-$NH_2$ (**HS964**)(**15**)

Ac-c[Cys-Arg-His-D-Nal(2')-Arg-Trp-Gly-Cys]-$NH_2$ (**HS007**)(**16**)

Ac-c[Cys-Nle-Glu-His-D-Nal(2')-Arg-Trp-Gly-Cys]-$NH_2$ (**HS010**)(**17**)

Ac-c[Cys-Nle-Arg-His-D-Nal(2')-Arg-Trp-Gly-Cys]-$NH_2$ (**HS024**)(**18**)

A similar study [38] was also done in the cyclic disulfide series and similar selectivity for human MC4R was seen for the antagonist Ac-c[$Cys^4$-D-Nal(2')$^7$-$Cys^{10}$]-$NH_2$ (**14**) or HS9510. However its affinity was less compared to SHU9119. The 26-membered Ac-c[$Cys^4$, D-Nal(2')$^7$, $Cys^{11}$]-α-MSH(4-11) (**15**) or HS964 had a higher degree of selectivity for MC4R when compared to both HS9510 and SHU9119 [43]. HS964 also had a unique order of selectivity of MC4R > MC5R > MC3R > MC1R. It was one of the first analogs with some human MC5R affinity. Interestingly, substituting the acidic $Glu^5$ with a basic arginine in HS964, HS007 (**16**) (Chart 3), resulted in higher affinity for MC3R and similar affinity for MC4R. The 29-membered ring Ac-c-[$Cys^3$, D-Nal(2')$^7$, $Cys^{11}$]-α-MSH(3-11) (**17**) or HS010 was found to be selective for MC3R, while HS024, or Ac-c-[$Cys^3$, $Arg^5$, D-Nal(2')$^7$, $Cys^{11}$]-α-MSH(3-11) (**18**) (Chart 3) with $Arg^5$ in place of $Glu^5$, is a potent and selective antagonist of MC4R [44].

A slight change in SHU9119 was achieved by coupling $Asp^5$ to $Lys^{10}$ through its β-carboxyl group to give βSHU9119 (**19**) [45]. This analog was more selective for human MC4R when compared to SHU9119. Further modifications on SHU9119 around Ac-$Nle^4$ or $His^6$ and/or $Asp^5$ were also carried out. MBP10 or c-[succinyl$^6$-D-Nal(2')$^7$-$Arg^8$-$Trp^9$-$Lys^{10}$]-NH2 (**20**), the 20-membered lactam without Ac-$Nle^4$, $His^6$ and $Asp^5$ replaced with succinic acid (des-amino-aspartic acid) was identified to be one of the most potent and highly selective antagonists of human MC4R. It was also concluded that the D-Nal(2')$^7$-$Arg^8$-$Trp^9$ sequence

was essential for high affinity and selectivity for MC4R and the $His^6$-D-Nal(2')$^7$-$Arg^8$-$Trp^9$ sequence was necessary for high affinity for MC3R and MC5R.

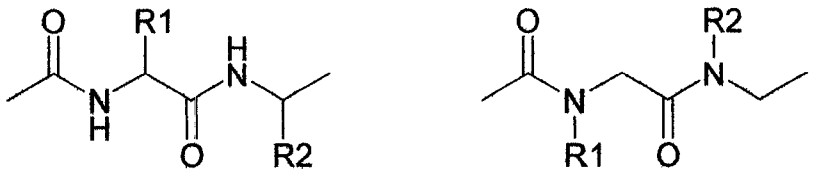
Such short tripeptide and tetrapeptide sequences could be a starting point for developing small molecule agonists and antagonists for melanocortin receptors. Although there are several known peptide drugs, peptides with their large molecular weight, susceptibity to enzymatic degradation, and poor oral bioavailability, are not ideal drug candidates. Peptidomimetics are compounds that can mimic the critical structural features of the peptides but may not have their disadvantages. Therefore it would be useful to identify short sequences of α-MSH that may serve as prototypes for developing peptidomimetic agonists and antagonists for the melanocortin receptors.

One of the earlier truncation studies involving a systematic analysis of fragments of NDP-MSH and their stereochemical modifications had identified several tripeptide agonists in the frog skin bioassay [46]. The tripeptide, Ac-D-Phe-Arg-D-Trp-$NH_2$ (**21**) was identified to be the most potent agonist in this study. A similar study around the core sequence (His-D-Phe-Arg-Trp) of NDP-MSH identified tripeptide and tetrapeptide agonists for human MCRs [47]. The tetrapeptide, Ac-His-D-Phe-Arg-Trp-$NH_2$ (**22**) was the most potent agonist for both human MC1R and MC4R in this study while Ac-His-Phe-Arg-D-Trp-$NH_2$ (**23**) had agonist activity at human MC1R. The tripeptides, Ac-D-Phe-Arg-Trp-$NH_2$

(**24**) and Ac-D-Phe-Arg-D-Trp-$NH_2$ (**21**) had some agonist activity at human MC4R.

Extensive structure-activity relationship studies on the tetrapeptide Ac-His-D-Phe-Arg-Trp-$NH_2$ (**22**) have been carried out at the mouse melanocortin receptors (mMCRs). It has been shown that the His residue plays an important role for selectivity at mMC4R [48]. Replacing His with other amino acids is well tolerated by mMC4R when compared to other mMCR's. The tetrapeptide, Ac-Anc-D-Phe-Arg-Trp-$NH_2$ (**25**) (Anc = amino-2-naphthylcarboxylic acid) was found to be the most potent agonist ($EC_{50}$ = 21 nM) of mMC4R, with > 4700-fold agonist selectivity over mMC3R. This molecule also has good selectivity for mMC1R but not for mMC5R. Similar studies [49] with replacements of the Phe residue led to identification of a tetrapeptide, Ac-His-D-Nal(2') -Arg-Trp-$NH_2$ (**26**) (D-Nal(2') = 3-(2-naphthyl)-D-alanine), a mMC4R antagonist that is only 425 fold less potent than SHU9119 (**12**). Tetrapeptide **26** could be a prototype molecule for designing mMC4R antagonists. But [50] replacements of the Trp residue indicated that this residue is necessary for potency at mMCRs, especially at mMC3R. Substitution of Trp with Nal(2') or D-Nal(2') did not alter potency significantly at mMC1R, mMC4R and mMC5R, when compared to parent tetrapeptide, Ac-His-D-Phe-Arg-Trp-$NH_2$ (**22**). This suggests that Nal(2') or D-Nal(2') could be a good replacement for the chemically reactive indole side chain of the Trp residue, which is susceptible to oxidation.

**Figure 1. (a) Peptide, (b) Peptoid**




**(a)** **(b)**

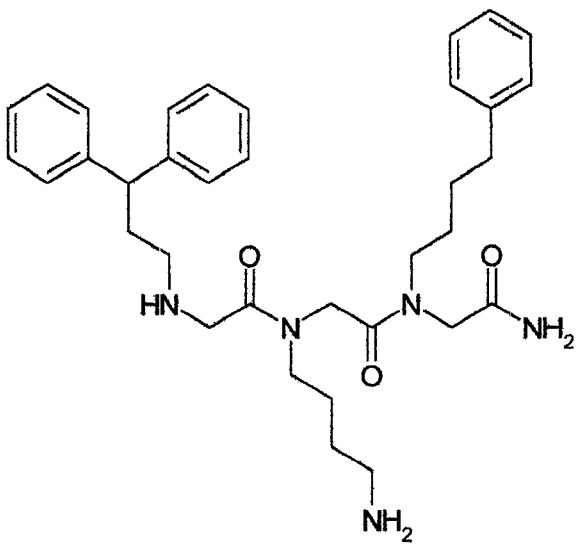
The tetrapeptide, Ac-His-D-Phe-Arg-Trp-$NH_2$ (**22**) was also chosen as a prototype in this study to develop peptoid agonists for melanocortin receptors. Peptoids [51], a class of peptidomimetics, are N-substituted glycines, where the side chain of an amino acid is attached to the α-amino nitrogen instead of the α-carbon (Figure 1). Peptoids mimic the spatial arrangement of the peptide side chain and therefore may retain its biological activity. They are achiral, an attractive feature for a drug molecule, as it avoids the challenges involved in synthesizing chiral drugs. Single isomers are preferred as drugs rather than racemic mixtures. These molecules have been shown to be less susceptible to enzymatic degradation [51], possibly due to the increase in stearic bulk around the amide bond. Peptoids, being tertiary amides, lack hydrogen-bonding capability that possibly increases their hydrophobicity and makes them better absorbed than peptides [52]. Peptoid ligands for MC1R and bombesin receptors have been successfully identified by combinatorial approaches. A tripeptoid (**27**) (Figure 2) was identified as a potent ligand for MCR in mouse melanoma cell assay [53].

In this study, the aim was to synthesize and observe the biological effect of two sets of peptoid analogs of Ac-His-D-Phe-Arg-Trp-$NH_2$ (**22**). One set is

where each amino acid in the sequence, Ac-His-D-Phe-Arg-Trp-$NH_2$ (**22**), is substituted sequentially with its peptoid analog. A study on peptoid analogs of

**Figure 2. Tripeptoid (27)**

**(27)**




tetrapeptide, Ac-His-D-Phe-Arg-Trp-$NH_2$ (**22**) has however been reported after this study was initiated [54]. Another tetrapeptide set of Ac-His-D-Phe-Arg-Trp-$NH_2$ (**22**) has been proposed, where the phenylalanine residue is replaced with different aromatic peptoids. Introduction of peptoids would perhaps alter the conformation of the tetrapeptide (**22**) and varying the phenylalanine sidechain could possibly affect the biological activity and selectivity for the various MCRs. Also, by introducing peptoids into the sequence, the molecules may have better pharmacokinetic properties (better absorption, stability against enzymatic degradation) attributed to peptoids. If these molecules are found to be active, they could be novel ligands for melanocortin receptors.

## Specific Aims

1. To study the effect of replacing each amino acid of the tetrapeptide Ac-His-D-Phe-Arg-Trp-$NH_2$ with its peptoid analog, the following tetrapeptides were planned to be synthesized.

   a) Ac-NHis-D-Phe-Arg-Trp-$NH_2$ (**28**)

   b) Ac-His-NPhe-Arg-Trp-$NH_2$ (**29**)

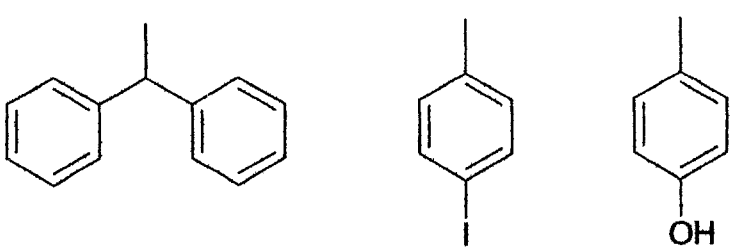
   c) Ac-His-D-Phe-NArg-Trp-$NH_2$ (**30**)

   d) Ac-His-D-Phe-Arg-NTrp-$NH_2$ (**31**)

NHis, NPhe, NArg and NTrp are the peptoid analogs of His, Phe, Arg and Trp, respectively.

2. To study the effects of varying the bulk and lipophilicity of the aromatic D-Phe residue on activity and selectivity at MCRs. By increasing the bulk and lipophilicity, a range of activity from agonist to antagonist is seen at MCRs [42]. A series of tetrapeptides of general formula Ac-His-Nxx-Arg-Trp-$NH_2$ (**32**, **33**, **34**), where Nxx are N-aryl substituted glycines, were planned to be studied. Aryl groups that are more lipophilic and bulkier (diphenylethyl), more lipophilic (4-iodobenzyl ) and less lipophilic (tyramine) than a phenyl group were chosen.

**(32)** **(33)** **(34)**



## Materials And Methods

$^1$H NMR spectra were recorded with a Bruker AC 300 spectrometer in $d_6$-DMSO (TMS as internal standard); the values of chemical shifts are expressed in ppm and coupling constants (J) in Hz. MALDI-TOF mass spectra were performed on a Micromass, model TOF Spec SE, made in Beverly, MA. The samples were prepared with 50 % water, 50 % $CH_3CN$ and 0.1 % TFA using α-cyano-4-hydroxycinnamic acid (purchased from Sigma, St.Loius, MO) as the matrix. Electrospray mass spectra were performed on a Waters corporation model Q-TOF-2, made in Milford, MA. The samples were prepared with 50% water, 50% $CH_3CN$ and 0.1 % TFA. Thin-layer chromatography (TLC) was done on Merck silica gel 60 $F_{254}$ plates using either or all of the following solvent systems: (A) 1-butanol / acetic acid / pyridine / water (5:5:1:4); (B) ethyl acetate / pyridine / acetic acid / water (5:5:1:3); (C) 1-butanol / acetic acid / water (4:1:1). The compounds were detected on the TLC plates using iodine vapor and UV absorption. Final peptide purification of **28**, **29** and **30** was done on a semipreparative RP-HPLC $C_{18}$ silica column (Vydac, 1.0 x 25 cm). The peptides were eluted with a linear acetonitrile gradient (10-100 %) over 60 min at a flow rate of 4 mL/min with a constant concentration of TFA (0.1 % v/v). The separation was monitored at 280 nm. Purity of the peptides was determined by analytical RP-HPLC $C_{18}$ silica column (Vydac, 4.6 x 220 mm), using a linear gradient of acetonitrile (0-100 %) over 60 min at a flow rate of 1 mL/min, monitored at 214 nm. Final peptide purification of **32**, **33** and **34** was performed

on a Waters Corporation HPLC, made in Milford, MA using a semipreparative RP-HPLC $C_4$ silica column (Dynamac, 2.14 x 25 cm). The peptides were eluted with a linear acetonitrile gradient (5-100 %) over 40 min at a flow rate of 10 mL/min with a constant concentration of TFA (0.1 % v/v). The separation was monitored at 254 nm. Purity of the peptides was determined by analytical RP-HPLC $C_4$ silica column (Microsorb - MV, 4.6 x 250 mm), using a linear gradient of acetonitrile (5-100 %) over 20 min at a flow rate of 1 mL/min, monitored at 214 nm.

The reagents $(Boc)_2O$, Boc-ON and 9-fluorenylmethyl chloroformate, were purchased from Fisher Biotech (Fairlawn, NJ). Indole-3-carboxaldehyde, bis-benzyloxythiourea, glyoxlylic acid, N-benzyl glycine, imidazole-4-carboxaldehyde, diisopropylcarbodiimide, triisopropylsilane, sodium cyanoborohydride and sodium borohydride were purchased from Aldrich (Milwaukee, WI). 1,3-Diaminopropane, 4-iodobenzylamine and bromoacetic acid were purchased from Lancaster Synthesis (Windham, NH). Tyramine and 2,2-diphenylethylamine were purchased from ACROS (New Jersey, USA). CIP was purchased from Fluka (Steinheim, Switzerland). HOAT was purchased from PerSeptive Systems (Framingham, MA). The pMBHA resin (1.03 meq / g) and Rink amide resin (0.7 mmol / g) were purchased from Peptides International (Louisville, KY). The Rink amide resin (0.61 mmol / g) was purchased from Novabiochem (La Jolla, CA). The amino acids $N^{\alpha}$-Boc-D-Phe and $N^{\alpha}$-Boc-Trp(CHO) were also purchased from

Peptides International (Louisville, KY). $N^{\alpha}$-Boc-Arg($N^{\gamma}$-Tos) was purchased from Fisher Biotech (Fairlawn, NJ). $N^{\alpha}$-Boc-His($N^{im}$-Bom) was purchased from Peninsula Lab Inc (Torrance, CA). $N^{\alpha}$-Fmoc-Arg($N^{\gamma}$-pmc)OPfp and $N^{\alpha}$-Fmoc-His($N^{im}$-Trt)OPfp were purchased from Bachem Bioscience (Torrance, CA). $N^{\alpha}$-Fmoc-D-Phe, $N^{\alpha}$-Fmoc-Trp($N^{in}$-Boc)-OH, $N^{\alpha}$-Fmoc-Arg($N^{\gamma}$-Pbf)-OH, $N^{\alpha}$-Fmoc-His($N^{im}$-Trt)-OH and PyBOP were purchased from Advanced Chemtech (Loiusville, KY). All solvents were ACS grade and used without further purification. DMF and 1,4-dioxane were dried using 4A° Molecular sieves.

## Results And Discussion

### Chemistry

The tetrapeptides **28**, **29** and **30** were synthesized successfully by solid phase peptide chemistry. The peptoid monomers of arginine (NArg), tryptophan (NTrp) and phenylalanine (NPhe) were synthesized with their α-amine group and side chain functionalities orthogonally protected, while attempts to synthesize the peptoid analog of histidine (NHis) were not successful. The peptoid analogs of arginine (NArg) and phenyalanine (NPhe) were incorporated into the tetramer Ac-His-D-Phe-Arg-Trp-$NH_2$. The tetramer Ac-NHis-D-Phe-Arg-Trp-$NH_2$ (**28**) was synthesized by alkylating the terminal amino group of the resin-bound tetrapeptide H-Gly-D-Phe-Arg-Trp-$NH_2$ (**35**). However, the tryptophan peptoid (NTrp) could not be incorporated successfully.

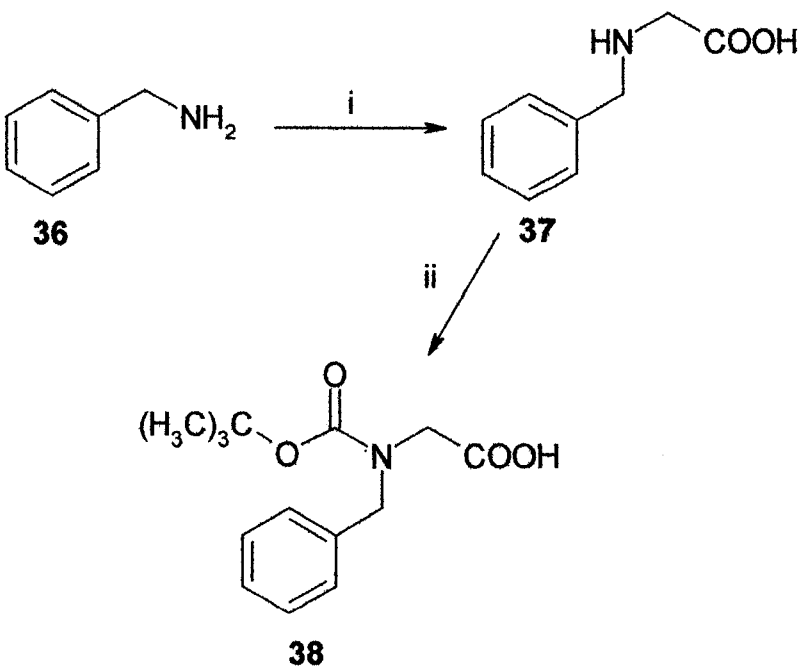
**Table 1. Analytical properties of α-MSH peptoid analogs**

| Compound | TLC(Rf)[a] | | | MS | $(M + H^+)^b$ | HPLC |
|---|---|---|---|---|---|---|
| | A | B | C | Observed | Calculated | rt (min)[c] |
| **28**(PJ2042) | 0.72 | 0.57 | 0.26 | 685.73 | 686.79 | 22 |
| **29**(PJ2038) | 0.79 | 0.71 | 0.37 | 686.35 | 686.79 | 20 |
| **30**(PJ2036) | 0.81 | 0.78 | 0.45 | 686.35 | 686.79 | 27 |

*[a]Solvent systems: (A) 1-butanol / acetic acid / pyridine / water (5:5:1:4); (B) ethyl acetate / pyridine / acetic acid / water (5:5:1:3); (C) 1-butanol /acetic acid / water (4:1:1). [b]MALDI-TOF mass spectroscopy. [c]Analytical RP-HPLC performed on $C_{18}$ silica column (Vydac, 4.6 x 220 mm), using a linear gradient of acetonitrile (0-100 %) over 60 min at a flow rate of 1 mL/min.*

The phenylalanine analog, N-*tert*-butyloxycarbonyl N-benzylglycine (Boc-NPhe) (**38**) was synthesized as shown in Scheme 1. N-benzylglycine (**37**) was commercially available and was also synthesized by alkylation of benzylamine with chloroacetic acid. The secondary amine was protected with a *tert*-butyloxycarbonyl (Boc) group. The tetrapeptide, Ac-His-NPhe-Arg-Trp-$NH_2$ (**29**) was synthesized successfully by solid phase peptide synthesis using the $N^{\alpha}$-Boc protecting group strategy. The coupling of amino acids was done using PyBOP as the coupling agent. The coupling reactions were monitored by the Kaiser test [55]. Final cleavage of the tetrapeptide **29** from the resin and removal of all protecting groups were carried out with HF and the final purification was done by RP-HPLC.

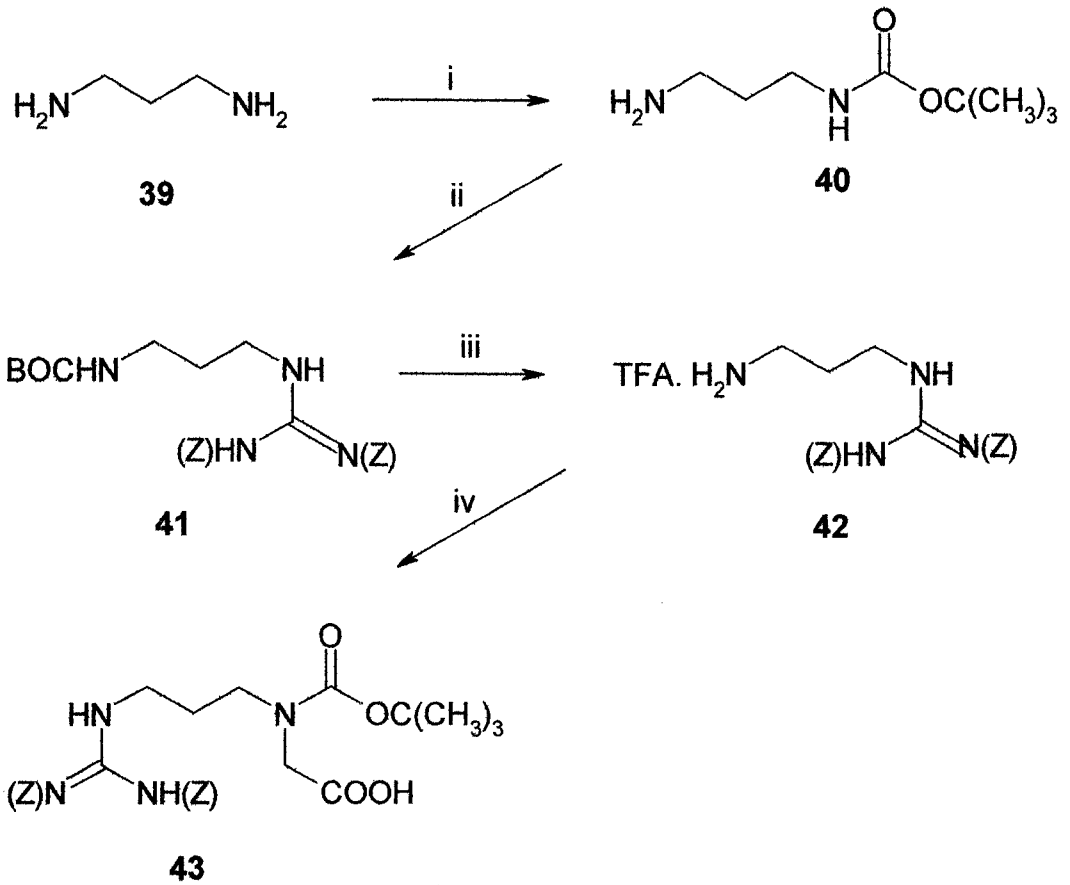
**Scheme 1. Synthesis of Boc-NPhe (38)**




*Reagents: (i) chloroacetic acid, MeOH; (ii) Boc-ON, TEA, 1, 4 dioxane / water.*

The peptoid analog of arginine (NArg) was synthesized as shown in Scheme 2. The starting material, 1,3 diaminopropane (**39**) was first mono-protected with a Boc group. It was observed that slow addition of the $Boc_2O$ was crucial for obtaining a good yield of the mono-protected compound (**40**) otherwise more of the bis-protected compound was obtained. The Cbz-protected guanidine group was incorporated in a single step by using 1,3-bis(benzyloxycarbonyl)-2-methyl-2-thiopseudourea. Originally the guanidine group was first synthesized and then protected with a tosyl group. However in anticipation of difficulties in removing the tosyl group during the peptide synthesis, the Cbz protecting group was used, which also shortened the synthetic scheme. The next step involved removal of the Boc group with TFA. It was important to remove all residual TFA or else it could cleave the Cbz groups as well. This was followed by reductive amination with glyoxylic acid and subsequent protection of the secondary amine with the Boc group. The reductive amination and Boc- protection were carried out in one pot in order to avoid aqueous work up of the secondary amine. The peptoid (**43**) was recystallized as a DCHA salt. The tetrapeptide Ac-His-D-Phe-NArg-Trp-$NH_2$ (**30**) was synthesized successfully by solid phase peptide synthesis using the Boc protecting group strategy. The coupling conditions, final cleavage and purification were the same as conditions used for **29**. It was also observed that coupling between α-amino acids and secondary amine of peptoids, in general was slower than coupling between α-amino acids. Secondary amines of peptoids being relatively stearically hindered might be a cause for the slow reactivity.

**Scheme 2. Synthesis of Boc-NArg(Cbz) (43)**

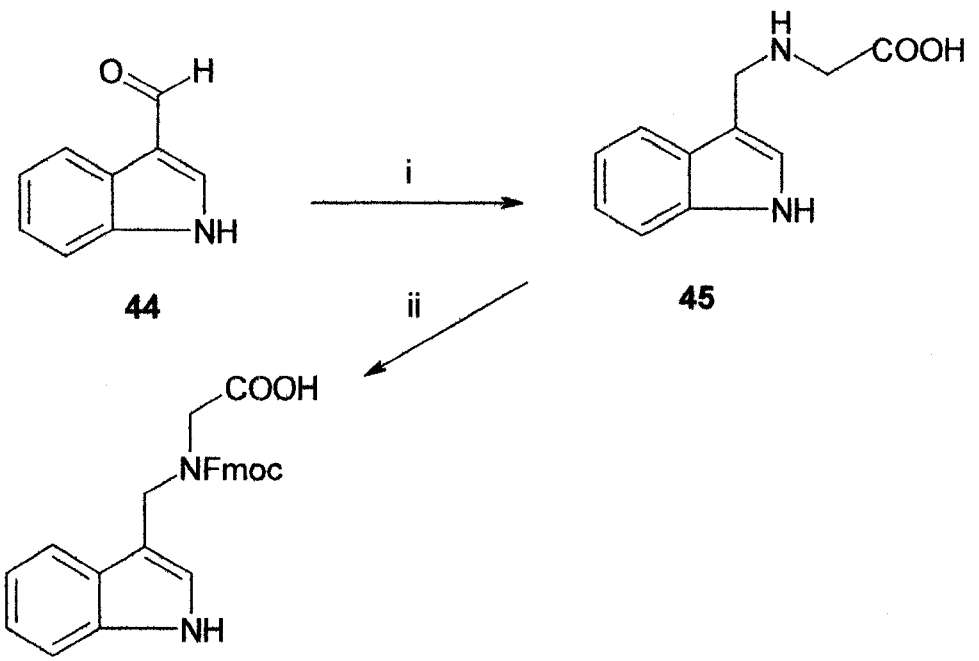



*Reagents: (i) $Boc_2O$, NaOH, 1,4 dioxane / water; (ii)Bis-N,N-benzyloxy thiourea, TEA, DMF; (iii) TFA; (iv) (a) glyoxylic acid, $NaBH_3CN$, MeOH, (b) $Boc_2O$, TEA, 1,4 dioxane / water.*

The peptoid analog of tryptophan was synthesized starting from indole-3-carboxaldehyde undergoing reductive amination with glycine, followed by Boc protection of the secondary amine, while the indole nitrogen was left unprotected. Unfortunately during the solid phase peptide synthesis, the indole system was oxidized during the repeated TFA deprotection procedure. Therefore, the

secondary amine was protected with a fluorenylmethoxycarbonyl (Fmoc) group as shown in Scheme 3, since the Fmoc synthesis procedure involved base deprotection and the indole group was therefore less susceptible to oxidation. However the tryptophan peptoid could not be incorporated successfully in the peptide. The product after the final purification did not contain the peptoid residue. It may have been due to the difficulty in coupling the stearically bulky peptoid to the resin. Perhaps coupling agents other than PyBOP might have been successful. We could have also replaced the indole side chain with a

**Scheme 3. Synthesis of Fmoc-NTrp (46)**



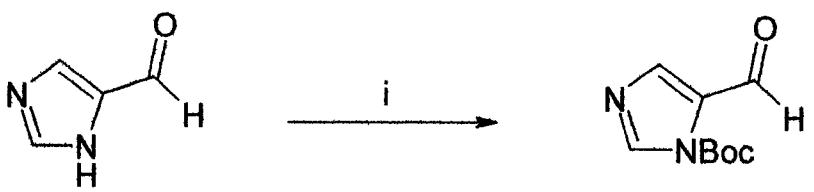

**46**

*Reagents: (i) glycine, TEA, $NaBH_4$, MeOH; (ii) Fmoc-Cl, $Na_2CO_3$, 1,4 dioxane / water.*

naphthyl side chain. Substituting indole with a naphthyl group in that position has been shown not to alter the biological activity significantly [50]. This alteration could have also avoided some of the chemical instability experienced with the indole moiety.

The peptoid analog of histidine (NHis) was also attempted unsuccessfully using several strategies. Starting from conversion of 4-(hydroxymethy) imidazole to a halide (Cl, Br) and subsequent displacement of the halide with glycine to form the secondary amine. However, this was not the best method to synthesize a secondary amine. The peptoid was therefore constructed by reductive amination of $N^{im}$-Boc- imidazole-4-carboxaldehyde (**48**) with the tetrapeptide H-Gly-D-Phe-Arg-Trp-$NH_2$ (**35**). $N^{im}$-Boc- imidazole-4-carboxaldehyde (**48**) was synthesized as shown in Scheme 4. The imidazole ring had to be protected in order to prevent reaction at the imidazole nitrogen during the final capping of the peptide with an acetyl group.

**Scheme 4. Synthesis of $N^{im}$-Boc- imidazole-4-carboxaldehyde (48)**

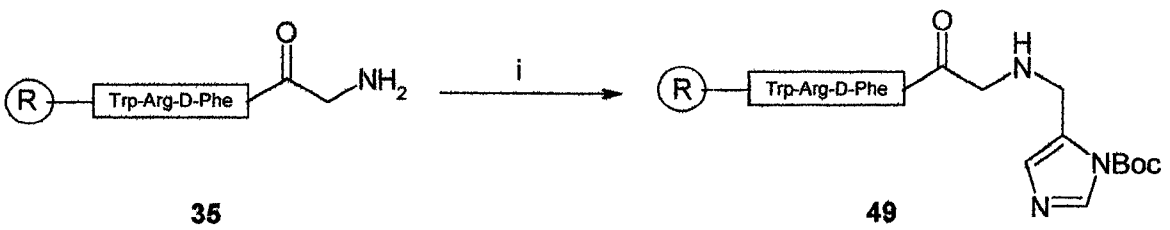



**47** **48**

*Reagents: (i) Boc-ON, TEA, 1,4 dioxane / water.*

The tetrapeptide (**35**) was synthesized by solid phase peptide chemistry used in synthesis of **29** and **30**. The reductive amination was carried out on the terminal glycine as shown in Scheme 5 and subsequently the secondary amine was capped with an acetyl group. The tetrapeptide, Ac-NHis-D-Phe-Arg-Trp-$NH_2$ (**28**) was cleaved from resin with HF and purified by RP-HPLC. Maybe this reductive amination of glycine directly on the resin might have been a successful method for synthesizing peptoids, at least in case of the NTrp (using indole-3-carboxaldehyde) and NPhe (using benzaldehyde).

**Scheme 5. Synthesis of NHis on H-Gly-D-Phe-Arg-Trp-$NH_2$ (35)**



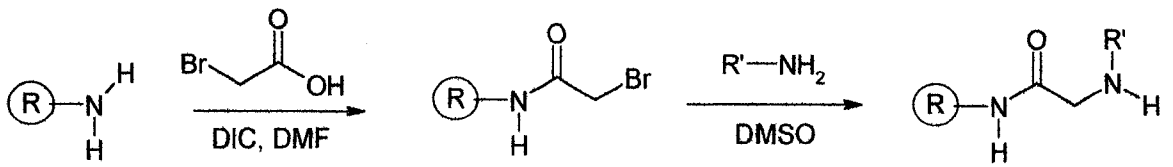

**35** **49**

*Reagents: (i) $N^{im}$-Boc- imidazole-4-carboxaldehyde (**48**),$NaBH_3CN$, DMF.*

Due to the challenges encountered in synthesizing the peptoid analogs using the above methods and the need to synthesize substantial amounts of peptoids for solid-phase synthesis, the peptoids in **32**, **33** and **34** were prepared by 'submonomer' solid-phase synthesis [56] (Scheme 6.). Here, the first step is the acylation of a resin-bound secondary amine with a haloacetic acid (like chloro, bromo or iodoacetic acid) using coupling agents such as DIC. The second step is to introduce the side chain by nucleophilic displacement of the

halogen with an excess of commercially available primary amine. By using two readily available submonomers, the disadvantage of having to prepare large quantities of protected peptoid monomers is overcome.

**Scheme 6. Solid-phase synthesis of peptoid from two submonomers**




Step 1 Step 2

**Table 2. Analytical properties of α-MSH aromatic peptoid analogs**

| Compound | Aromatic Side Chain | MS $(M + H^+)^a$ Observed | Calculated | HPLC rt (min)[b] |
|---|---|---|---|---|
| **32**(PJ2078A) | 2,2-Diphenylethyl | 776.40 | 776.92 | 35 |
| **33**(PJ2079C) | 4-Iodophenyl | 812.22 | 812.89 | 33 |
| **34**(PJ2080A) | Tyramine | 716.35 | 716.82 | 32 |

*[a]Electrospray mass spectroscopy. [b]Analytical RP-HPLC performed on $C_{18}$ silica column (Vydac, 4.6 x 220 mm), using a linear gradient of acetonitrile (0-100 %) over 60 min at a flow rate of 1 mL/min.*

The tetrapeptides, **32**, **33** and **34** were prepared on solid support using $N^{\alpha}$-Fmoc protecting group strategy. Amino acids, tryptophan and arginine were first coupled to Rink amide resin using PyBOP as the coupling agent. Followed by coupling of bromoacetic acid, which was found to be optimal for the acylation

step [56], to the arginine residue using DIC until a negative Kaiser test was observed. The resin was then split into three portions and the bromine was displaced with respective amines (2,2-diphenylethylamine, 4-iodobenzylamine and tyramine) in DMSO. This displacement step was repeated until a positive Kaiser test was observed. The final amino acid, histidine was then coupled to the secondary amine using 2-chloro-1,3-dimethyl imidazolidinium hexaphosphate (CIP) and HOAT, which was found to be particularly effective for difficult couplings [57]. The final deprotection and cleavage from resin were performed using TFA with scavengers (see experimental). The peptides were isolated using RP-HPLC.

**Biological Evaluation**

The peptoid-peptide hybrids were all evaluated for their activity at mouse melanocortin receptors using the β-galactosidase reporter gene bioassay (Table 3 & 4). In general, tetrapeptides **28**, **29** and **30** (Table 3) were found to be at least $10^5$ times less active than MT-II (**10**). However MT-II, one of the most potent agonist for melanocortin receptors, is structurally quite different from these tetrapeptides. Whereas, these tetrapeptides are just simple modifications of the core sequence, His-Phe-Arg-Trp. However, even the simple modification of the core sequence resulted in dramatic loss in activity. The tetrapeptide, Ac-His-D-Phe-Arg-Trp-$NH_2$ (**22**) had nanomolar agonist activity in β-galactosidase reporter gene bioassay at mMC1R ($EC_{50}$=25.6 nM), mMC3R ($EC_{50}$=195 nM), mMC4R ($EC_{50}$=10.2 nM) and mMC5R ($EC_{50}$=34.6 nM) [58]. Amongst the peptoid-peptide

hybrids, PJ2042 (**28**) was found to be the most active agonist at both mMC4R ($EC_{50}$=1.71 μM) and mMC5R ($EC_{50}$=0.75 μM). While, PJ2036 (**30**) was found to be the most active agonist at mMC1R ($EC_{50}$=1.47 μM). The results concurred with those of the same compounds determined by Haskell-Leuvano *et al* at the University of Florida, Gainesville [54].

The substitution of amino acid with its peptoid analog is best tolerated at the N-terminal position of the tetrapeptide, Ac-His-D-Phe-Arg-Trp-$NH_2$. The tetrapeptide, PJ2042 with the histidine residue replaced by its peptoid analog, showed relatively good agonist activity at all three mouse receptors (MC1R, MC4R and MC5R) but lacked specificity. It was 30-40 times more active than PJ2038 (**29**) and PJ2036 at mMC4R and 8-10 times more active at mMC5R.

**Table 3. Biological evaluation of α-MSH peptoid analogs at mouse melanocortin receptors.**

| Compound | $EC_{50}$ (μM)[a] | | |
|---|---|---|---|
| | mMC1R | mMC4R | mMC5R |
| MT-II (**10**)(nM) | 0.029 ± 0.017 | 0.053 ± 0.027 | 70 ± 121.21 |
| PJ2042(**28**) | 3.18 ±1.26 | 1.71 ± 1.24 | 0.75 ± 0.51 |
| PJ2038(**29**) | 25.6 ± 8.55 | 34.46 ± 11.41 | 9.03 ± 3.59 |
| PJ2036(**30**) | 1.47 ± 0.6 | 43.66 ± 19.32 | 10.53 ±3.67 |

[a] *$EC_{50}$ values determined by β-Galactosidase bioassay. $EC_{50}$ values represent the mean of experiments performed in triplicate.*

The tetrapeptide, PJ2038 with phenylalanine peptoid is most active at mMC5R ($EC_{50}$ = 9.03 μM). The compound is 3-4 times less active at mMC1R

($EC_{50}$ = 25.6 μM) and MC4R ($EC_{50}$ = 34.46 μM) and so has slight specificity for mMC5R. Replacing an amino acid with a peptoid can alter the amide backbone and orientation of residue side chains. Modifications to orientation of side chain of residues could be challenging, in order to achieve good activity. We know that stereochemical modification of residues significantly alters activity and receptor subtype specificity [46, 47].

The tetrapeptide, PJ2036 with arginine peptoid, was found to be the most active agonist at mMC1R. It is 2-fold and 25-fold better than PJ2042 ($EC_{50}$ = 3.18 μM) and PJ2038 ($EC_{50}$ = 25.6 μM), respectively. PJ2036 also showed good specificity between the three MCR's. Its activity at mMC1R was 10- and 43-times better than at mMC5R ($EC_{50}$ = 10.53 μM) and mMC4R ($EC_{50}$ = 43.66 μM), respectively. Unlike PJ2038, alteration to the amide backbone of PJ2036 due to introduction of arginine peptoid is found to be useful for activity and selectivity.

The second set of tetrapeptides (**32**, **33**, **34**), where the phenylalanine side chain of Ac-His-D-Phe-Arg-Trp-$NH_2$ is replaced with various aromatic groups, resulted in mMC1R selective agonists (Table 4). It appears that increase in stearic bulk and lipophilicity of phenylalanine side chain results in loss of agonist activity at all mMCR's except mMC1R. PJ2078A (**32**) and PJ2079C (**33**) showed modest, yet selective activity at mMC1R and poor agonist activity at the other MCR's. However, it would be useful to test the binding affinity of these tetrapeptides at the mMCR's. Since these compounds could still bind to the other

mMCR's and be antagonists, that information will not be revealed by β-galactosidase reporter gene bioassay because it is an assay for agonist activity.

**Table 4. Biological evaluation of α-MSH aromatic peptoid analogs at mouse melanocortin receptors.**

| Compound | $EC_{50}$ (μM)[a] | | | |
|---|---|---|---|---|
| | mMC1R | mMC3R | mMC4R | mMC5R |
| α-MSH (**1**)(nM) | 3.62 ± 0.16 | 2.70 ± 0.14 | 8.44 ± 0.13 | 5.60 ± 0.12 |
| PJ2078A (**32**) | 7.66 ± 0.13 | SA | SA | SA |
| PJ2079C (**33**) | 12.2 ± 0.2 | SA | SA | SA |
| PJ2080A (**34**) | SA | SA | SA | SA |

[a] *$EC_{50}$ values determined by β-Galactosidase bioassay. SA or slight agonist denotes that some stimulatory response was observed but not enough to determine an $EC_{50}$ value.*

The tetrapeptide, PJ2078A has a diphenylethyl side chain that is stearically bulkier than a phenyl side chain. As mentioned earlier, increasing stearic bulk of the phenylalanine side chain results in antagonists at MC3R and MC4R but remains agonists at MC1R and MC5R [42]. It would be interesting to test the binding affinity of PJ2078A at mMCR's and see if it has any significant antagonist activity at mMC3R and mMC4R. The tetrapeptide, PJ2079C with a more lipophilic *p*-iodobenzyl side chain, also exhibits similar selective agonist activity at mMC1R and no significant agonist activity at other mMCR's. In the same above mentioned study [42], Ac-Nle$^4$-c[Asp$^5$-His$^6$-D-Phe(pI)$^7$-Arg$^8$-Trp$^9$-Lys$^{10}$]-$NH_2$ (**13**), where phenylalanine side chain is replaced by *p*-

iodophenylalanine, was found to be an antagonist for human MC4R, partial agonist for MC3R and an agonist of MC1R. Therefore, PJ2079C could also exhibit similar behavior at other MCR's. The tetrapeptide, PJ2080A (**34**) with a tyramine side chain showed no significant agonist activity at any of the four mMCR's. The tyramine side chain is less bulky and lipophilic than the diphenylethyl and *p*-iodobenzyl side chains. Unlike the other two tetrapeptides, presence of a polar hydroxyl group on the residue might have decreased the hydrophobic interactions that are considered to be important for binding at these receptors [59] and resulted in subsequent loss of activity. However, this theory still remains to be verified.

## Summary

The tetrapeptides **28**, **29** and **30**, with peptoids NHis, NPhe and NArg replacing amino acids in the sequence Ac-His-D-Phe-Arg-Trp-$NH_2$, were synthesized. But attempts to synthesize the tetrapeptide **31**, with the tryptophan analog were unsuccessful. The peptoid monomers of phenylalanine, arginine and tryptophan, with the side chain and α-nitrogen protected, were synthesized successfully. An easy synthetic scheme for synthesizing protected NTrp and NArg has also been worked out, but this strategy to synthesize peptoids is not very convenient or versatile, since large amounts of peptoid are needed for solid-phase chemistry. Therefore, the peptoids in tetrapeptides **32**, **33** and **34** were synthesized by the 'submonomer' method. The tetrapeptide, Ac-His-D-Phe-Arg-NTrp-$NH_2$ (**31**) could have also been synthesized by the 'submonomer' method, as shown successfully by Haskell-Leuvano *et al* in their synthesis of the same compound [54].

The tetrapeptides **28**, **29** and **30**, are direct analogs of Ac-His-D-Phe-Arg-Trp-$NH_2$, which has been identified as the sequence critical for human MC1R and MC4R agonistic activity. Introduction of peptoids to the prototype Ac-His-D-Phe-Arg-Trp-$NH_2$ has led to varying biological activity in mouse melanocortin receptors. Modification of the histidine residue (**28**) has been most suitable for achieving relatively good activity at mouse MC1R, MC4R and MC5R. While modification to the phenyalanine residue (**29**) has not been very effective. But,

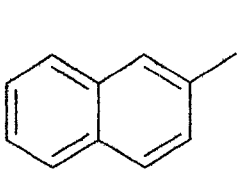
modification to the arginine residue (**30**) led to identification of a molecule with relatively good agonist activity and selectivity at mouse MC1R.

The tetrapeptides **32**, **33** and **34**, where the phenylalanine side chain of **29** is replaced with various aromatic groups, led to the identification of compounds with selective agonist activity for mMC1R. Although, the compounds with diphenyethyl (**32**) and *p*-iodobenzyl (**33**) side chains had only modest activity at mouse MC1R, they had no significant agonist activity at other MCR's. Increasing stearic bulk and lipophilicity of phenylalanine side chain has led to compounds that are selective agonists for mMC1R. Perhaps, modifications with bulkier and more lipophillic aromatic side chains, such as the naphthyl (Nal(2')) and biphenyl side chains, might produce more potent and selective mMC1R agonists (Figure 3). The naphthyl and biphenyl moiety are present in many potent ligands of melanocortin receptors [42, 43, 45, 49]. Interestingly, PJ2080A (**34**) with the tyramine side chain did not show significant agonist activity at any of the mMCR's. It is likely that the presence of a polar functionality (hydroxyl group) might have diminished the binding capacity of this molecule producing the subsequent loss in activity.

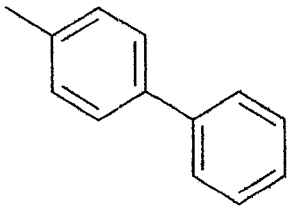
Based on the observation that Ac-NHis-D-Phe-Arg-Trp-$NH_2$ (**28**) has been the most active yet non-selective agonist at the various mMCR's, it might be fruitful to examine the effects of modifications to the histidine side chain such as the one carried out on Ac-His-NPhe-Arg-Trp-$NH_2$ (**29**). In the past, modifications

**Figure 3. Suggested analogs of Ac-His-NPhe-Arg-Trp-$NH_2$ (29).**

Ac-His-Nxx-Arg-Trp-$NH_2$, where Nxx are peptoids with aromatic side chains as shown below:



**(50)**



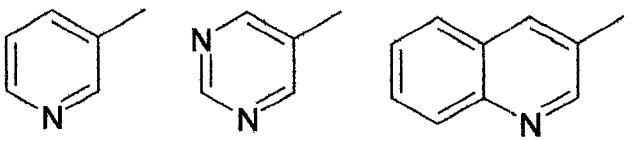
**(51)**

to histidine residue by substituting other heterocycles (pyridine, indole, thiophene) and aromatic rings (phenyl, naphthyl) have been explored [48]. Some (pyridine, thiophene) have been successful in introducing selectivity and good agonist activity to the molecules. Perhaps, it might be worthwhile to replace the basic imidazole side chain of the histidine residue with different nitrogen heterocycles, including pyridine (Figure 4). Among the chosen analogs are a bulky heterocycle (quinoline) and isosteres of imidazole (triazole, isoxazole, thiazole) and pyrimidine. Introduction of heterocyclic rings of varied size and basicity in that position might provide valuable SAR information at the melanocortin receptors.

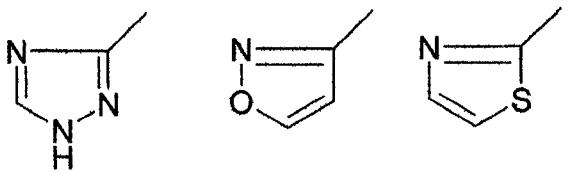
It would be interesting to study these α-MSH peptide-peptoid hybrids for their activity at human melanocortin receptors. If these molecules are found active, they could be novel ligands for human melanocortin receptors. In recent

**Figure 4. Suggested analogs of Ac-NHis-D-Phe-Arg-Trp-$NH_2$ (28).**

Ac-Nxx-D-Phe-Arg-Trp-$NH_2$, where Nxx are peptoids with heterocyclic side chains as shown below:



**(52)** **(53)** **(54)**



**(55)** **(56)** **(57)**

years, melanocortin receptor ligands especially for MC4R, are being actively studied for treatment of obesity and eating disorders. Agonists for MC1R are being investigated for cosmetic tanning and treatment of hypopigmentary disorders, while antagonists for MC1R could be potentially used for skin whitening. These peptoid-peptide hybrids could serve as a lead for developing peptidomimetic ligands with better activity and selectivity. By introducing peptoids in the sequence, these molecules may have better stability towards enzymatic degradation and better absorption, thereby possessing good pharmacokinetic behavior.

## Experimental

### Chemistry

**N- *tert*-butyloxycarbonyl N-benzylglycine (38)**. N-benzylglycine (6 mmol, 1 g) was taken up into dioxane (5 mL) and water (5 mL). To it, triethylamine (9 mmol, 1.3 mL) and 2-(tert-butoxycarboxyloxyimino)-2-phenylacetonitrile (6.7 mmol, 1.6 g) were added and the mixture stirred at room temperature for 36 h. Dioxane was removed under reduced pressure and the mixture poured into water (50 mL). The mixture was extracted with ethyl acetate (3 X 25 mL). The aqueous layer was acidified to pH 2 and extracted again with ethyl acetate (3 X 25 mL). The organic layers were combined, washed with water (2 X 50 mL), dried over magnesium sulfate ($MgSO_4$) and concentrated under reduced pressure to give the product as a yellow oil, which solidifies on standing. The product (1.5 g, 93.8 %) was washed in ethyl acetate-hexane. m.p.102-103$^0$C. $^1$H NMR ($d_6$-DMSO): 1.34 (s, 9H), 3.36 (s, 2H), 4.39 (s, 2H), 7.24-7.33 (m, 5H).

**N - *tert*-butyloxycarbonyl aminopropane (40)**. 1,3 diaminopropane (1.4 mL, 1.5 mmol) was taken up into 10 mL of a dioxane -water (1 : 1) mixture and stirred in an ice-water bath. To this mixture, *tert*-butyloxy dicarbonate (0.55 g, 2.5 mmol) in dioxane (2 mL) was added slowly over two hours with rapid stirring. It was stirred for an additional hour at 0°C, then continued overnight at room temperature. The dioxane was removed under reduced pressure and the residue poured into water (25 mL). Insoluble particles were removed by filtration. The

filtrate was extracted with dichloromethane (3 X 25 mL) and the organic layers was washed with saturated NaCl solution, dried over magnesium sulfate and concentrated under reduced pressure to give the product as a pale yellow oil (0.32 g, 72.7 %).$^1$H NMR ($d_6$-DMSO): 1.39 (s, 9H), 1.40-1.46 (m, 2H), 2.47-2.52 (m, 2H), 2.91-2.97 (m, 2H), 6.78(s, 1H).

**1-[1,3 bis(benzyloxycarbonyl)]guanidino-3-*tert* (butyloxycarbonyl) – diaminopropane (41).** N - *tert*-butyloxycarbonyl aminopropane (**40**) (0.8 g, 4.3 mmol) was taken up into DMF (5 mL) and TEA (0.8 mL, 9.6 mmol). To it, 1,3 bis(benzyloxycarbonyl)-2-methyl-2-thiopseudourea (1.53 g, 4.3 mmol) was added and stirred at room temperature for 15 h. 30 mL of ethyl acetate was added to the reaction mixture and washed with 10 % citric acid (3 X 30 mL). The organic layer was dried over $MgSO_4$ and evaporated to give the product as a yellow oil (1.5 g, 68.2 %). $^1$H NMR ($d_6$-DMSO): 1.37 (s, 9H), 1.58-1.60 (m, 2H), 2.28 (s, 1H) 2.88-2.94 (m, 2H), 3.30-3.37 (t, $J$ = 7 Hz, 2H), 5.01 (s, 1H), 5.16 (s, 4H), 5.21 (s, 1H), 7.33-7.38 (m, 10H).

**1-[1,3 bis(benzyloxycarbonyl)]guanidino-3- aminopropane (42).** 1-[1,3 bis(benzyloxycarbonyl)]guanidino-3-*tert* (butyloxycarbonyl)–diaminopropane (**41**) (1.5 g, 3.1 mmol) was stirred in 5 mL of TFA in an ice bath for 30 min. TFA was removed under reduced pressure to give the product as a yellow film (1.5 g, 99.9 %). This was used immediately in the next step without further purification.

**N-tert(butyloxycarbonyl)-N-(3-[1,3 bis(benzyloxycarbonyl) guanidino]propyl)-glycine (42).** 1-[1,3 bis(benzyloxycarbonyl)]guanidino-3-aminopropane (**41**) (1.5 g, 3.1 mmol) was dissolved in 5 mL of methanol and neutralized with 1N KOH solution. Glyoxylic acid (0.3 g, 3.1 mmol) was added and the mixture stirred in an ice bath for an hour. Sodium cyanoborohydride (0.2 g, 3.1 mmol) was then added slowly to the mixture. After stirring in an ice bath for 3 h, the reaction mixture was allowed to warm to room temperature. After an additional 3 h, the solvent was removed under reduced pressure. The residue was dissolved in water (5 mL) and dioxane (5 mL). To it, TEA (0.7 mL, 4.7 mmol) and Boc-ON (0.84 g, 3.4 mmol) were added and stirred at room temperature for 48 h. Dioxane was removed under reduced pressure and the residue poured into water (30 mL). The aqueous layer was first extracted with ethyl acetate (3 X 30 mL), acidified to pH 2 with 1N $H_2SO_4$ and extracted with ethyl acetate (3 X 30 mL). The combined organic layers were washed with water and sat. NaCl, dried over $MgSO_4$ and concentrated to give the product as a yellow oil (1.1 g, 65 %). This oil was dissolved in ethyl acetate and dicyclohexylamine (2 mmol) was added giving a precipitate. The precipitate was collected and recrystallized from ethyl acetate-hexane to give a crystalline salt melting at 205-209 °C. $^1$H NMR ($d_6$-DMSO): 1.15-1.19 (m, 2H), 1.51 (s, 9H), 3.05-3.09 (m, 2H), 3.12-3.16 (t, $J$ = 4 Hz, 2H), 4.99-5.03 (d, $J$ = 12 Hz, 2H), 5.17 (s, 2H), 7.35-7.39 (m, 10H), 7.88 (s, 1H), 8.31 (s, 1H)

**Fmoc-N-methylindolyl-glycine (46).** Indole-3-carboxaldehyde (**44**) (1 g, 6.9

mmol) and TEA (2 mL, 13.8 mmol) were added to glycine (0.5 g, 6.9 mmol) dissolved in methanol (10 mL) and stirred at room temperature for 2 h. $NaBH_4$ (0.54 g, 13.8 mmol) was added slowly to the reaction mixture while stirring in an ice/water bath. The reaction was maintained at 0 °C for 3 h and then at room temperature for 15 h. The solvent was evaporated under reduced pressure and the crude residue was taken up into 18 mL of 10 % aqueous $Na_2CO_3$ solution and 20 mL of dioxane and stirred at 0 °C. 9-Fluorenyl-methyl-chloroformate (0.86 g, 3.4 mmol) was then added. The reaction was maintained at 0 °C for 4 h followed by room temperature for 8 h. The reaction mixture was poured into 50 mL of water and extracted with ethyl acetate (2 X 50 mL). The aqueous layer was acidified to pH 2 with 1N $H_2SO_4$ and cooled overnight. The resulting precipitate was filtered off to give the product (76 %, 2.2g) melting at 164-166 °C. $^1H$ NMR ($d_6$-DMSO): 3.86 (d, $J$ = 4 Hz, 2H), 4.24 (s, 2H), 4.30 (d, $J$ = 4 Hz, 2H), 4.42(t, $J$ = 4 Hz, 1H), 7.31-7.45 (m, 7H), 7.61-7.73 (m, 3H), 7.90 (d, $J$ = 4 Hz, 2H), 10.95 (d, $J$ = 4Hz, 1H).

**$N^{im}$–(*tert*-butyloxycarbonyl) imidazole-4-carboxaldehyde (48).** Imidazole-4-carboxaldehyde (**47**) (0.5 g, 5.2 mmol) was dissolved in 10 mL of dioxane -water (1:1) mixture. To it, TEA (1.1 mL, 7.8 mmol) and di-*tert*-butylpyrocarbonate (1.3 g, 5.7 mmol) were added and the mixture was stirred in an ice/water bath for an hour and at room temperature for 2 h. The solvent was removed under reduced pressure and the residue poured into water (30 mL) and extracted thrice with ethyl acetate (3 X 30 mL). The organic layer was washed with sat. NaCl, dried

over $MgSO_4$ and concentrated to give an oil (1 g, 99 %).

**General procedure for synthesis and cleavage of peptides 29 and 30.**

*p*-MBHA resin (1.03 meq/g, 1mmol) was neutralized with 10 % DIEA in DCM for 10 min. $N^{\alpha}$-Boc-Trp($N^{in}$-For) (3 eq) was coupled to the resin in DMF using PyBOP (3 eq) and DIEA (9 eq) for 2 h. The coupling was monitored by the Kaiser test [55]. The $N^{\alpha}$-Boc protecting group was removed by washing with 45 % TFA, 10 % anisole in DCM for 20 min, followed by washing the resin thrice with DCM. The other amino acids, $N^{\alpha}$-Boc-Arg($N^{Y}$-Tos), $N^{\alpha}$-Boc-Phe and $N^{\alpha}$-Boc-His($N^{im}$-Bom) or the peptoid analogs, $N^{\alpha}$-Boc-NArg($N^{Y}$-cbz) and $N^{\alpha}$-Boc-NPhe were coupled sequentially using the same coupling conditions. The reactions were carried out till they gave a negative Kaiser test. After removal of $N^{\alpha}$-Boc protecting group of $N^{\alpha}$-Boc-His($N^{im}$-Bom), N-terminal acetylation was carried out by acetic anhydride ( 3 eq) and DIEA ( 9 eq) in DMF for an hour. The resin was washed thrice with DMF, DCM, MeOH and dried in vacuo. The resin (0.2 g) was suspended in 20 % piperidine (20 mL) in DMF and shaken at room temperature for an hour to remove $N^{in}$-Formyl protecting group. The resin was then washed with DMF, i-PrOH, DCM and dried under vacuum. The peptide was cleaved off the resin using anhydrous HF (25 mL / g of resin), anisole (2 mL / g of resin) and thiocresol (0.5 mL / g of resin). The mixture was stirred at 0 °C for an hour. HF and scavengers were removed under reduced pressure. The resin was then washed with diethyl ether (4 X 10 mL) and extracted with 30 % acetic acid (3 X 15 mL). The extract was lyophilized and purified by preparative RP-HPLC.

**Ac-NHis-D-Phe-Arg-Trp-$NH_2$ (28).** *p*-MBHA resin (1.03 meq/g, 1mmol) was neutralized with 10 % DIEA in DCM for 10 min. $N^{\alpha}$-Boc-Trp($N^{in}$-For) (3 eq) was coupled to the resin in DMF using PyBOP (3 eq) and DIEA (9 eq) for 2 h. The coupling was monitored by the Kaiser test. The $N^{\alpha}$-Boc protecting group was removed by washing with 45 % TFA, 10 % anisole in DCM for 20 min followed by three washes with DCM. The other amino acids, $N^{\alpha}$-Boc-Arg($N^{\gamma}$-Tos), $N^{\alpha}$-Boc-Phe and $N^{\alpha}$-Boc-Gly were coupled sequentially using the same coupling conditions. The reactions were carried out till they gave a negative Kaiser test. After removal of $N^{\alpha}$-Boc protecting group off $N^{\alpha}$-Boc-Gly, $N^{im}$-Boc-imidazole-4-carboxaldehyde (3 eq) in DMF was added and shaken for 30 min. Reductive amination was carried out using sodium cyanoborohydride (3 eq) for 90 min. The resin was then washed thrice with DMF, DCM and MEOH. N-terminal acetylation was carried out by acetic anhydride ( 3 eq) and DIEA ( 9 eq) in DMF for an hour. The resin was washed thrice with DMF, DCM, MeOH and dried under vacuum. The resin (0.2 g) was suspended in 20 % piperidine (20 mL) in DMF and shaken at room temperature for an hour to remove the $N^{in}$-Formyl protecting group. It was then washed with DMF, i-PrOH, DCM and dried under vacuum. The peptide was cleaved off the resin using anhydrous HF (25 mL / g of resin), anisole (2 mL / g of resin) and thiocresol (0.5 mL / g of resin). The mixture was stirred at 0 °C for 90 min. HF and scavengers were removed under under reduced pressure. The resin was washed with diethyl ether (4 X 10 mL) and extracted with 30 % acetic acid (3 X 15 mL). The extract was lyophilized and purified by preparative

RP-HPLC.

**General procedure for synthesis and cleavage of peptides 32, 33 and 34.**

Rink amide resin (0.61 mmol/g, 0.58 mmol) was deprotected with 20 % piperidine in DMF for 30 min. $N^{\alpha}$-Fmoc-Trp($N^{in}$-Boc) (3 eq) was coupled to the resin in DMF using PyBOP (3 eq) and DIEA (9 eq) for 2 h. The coupling was monitored by the Kaiser test [55]. The $N^{\alpha}$-Fmoc protecting group was removed by washing with 20 % piperidine in DMF for 30 min, followed by washing the resin with DMF, *i*-PrOH and DCM. The amino acid, $N^{\alpha}$-Fmoc-Arg($N^{\gamma}$-Pbf) was coupled to the tryptophan residue using the same coupling conditions. The reactions were carried out till they gave a negative Kaiser test. After removal of the $N^{\alpha}$-Fmoc protecting group, 0.5 M solution of bromoacetic acid (0.56 g, 4.06 mmol) in DMF, followed by a 2M solution of DIC (0.73 mL, 4.64 mmol) in DMF were added to the resin. The reactions were carried out till they gave a negative Kaiser test. The resin was then dried and split into three portions. To each portion, 20 equivalents of the amine (2,2-diphenylethylamine, 4-iodobenzylamine or tyramine) were added as 1-2M solutions in DMSO and shaken for 8 h. The resin was then washed thoroughly with DMF, MeOH and DCM. A mixture of $N^{\alpha}$-Fmoc-His($N^{im}$-Pbf)(3 eq), HOAT (1.5 eq), CIP (3 eq) and DIEA (12 eq) in DMF was then added to the resin and shaken till a negative Kaiser test result was achieved. After the removal of $N^{\alpha}$-Fmoc protecting group, N-terminal acetylation was carried out by acetic anhydride (3 eq) and DIEA (9 eq) in DMF for an hour. The resin was washed with DMF, DCM, MeOH and dried in vacuo. The resin was suspended in 7 mL

solution of TFA : water : phenol : TIS (88:5:5:2) and shaken at room temperature for 40 min. The resin was then filtered and washed with MeOH (4 X 10 mL). The extract was concentrated in vacuo, lyophilized and purified by preparative RP-HPLC.

**Biology** (Conducted at the University of Florida, College of Pharmacy, in the laboratory of Prof. Carrie Haskell-Leuvano)

**Cell Culture and Transfection**

Briefly, HEK-293 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum and seeded one day prior to transfection at 1 to 2 x $10^6$ cell/100 mm dish. Melanocortin receptor cDNA subcloned into the $pCDNA_3$ expression vector (Invitrogen) was transfected (20 µg) using calcium phosphate method. Stable receptor populations were generated using G418 selection 1(1 g/mL) for subsequent bioassay [60].

**β-Galactosidase Bioassay**

Cells stably expressing wild-type receptors were transfected with 4 µg of CRE/ β-Galactosidase receptor gene as previously described [60]. Briefly, 5000 to 15000 post-transfection cells were placed into 96-well Primeria plates (Falcon) and incubated overnight. At 48 h post-transfection, cells were stimulated with compound, or forskolin ($10^{-4}$ M), in assay medium (DMEM containing 0.1 mg/mL BSA and 0.1 mM isobutylmethylxanthine) for 6 h. The assay media was

aspirated, and 50 µL of lysis buffer (250 mM Tris-HCl pH = 8.0 and 0.1% Triton X-100) were added. The plates were stored at –80 °C overnight. The plates containing cell lysates were thawed the following day. Aliquots of 10 µL were taken from each well and transferred to another 96-well plate for relative protein determination. Phosphate-buffered saline (40 µL) with 0.5% BSA was added to each well. Subsequently, 150 µL of substrate buffer (60 mM sodium phosphate, 1 mM $MgCl_2$, 10 mM KCl, 5 mM β-mercaptoethanol, 200 mg of ONPG) was added to each well, and the plates were incubated at 37 °C. The sample absorbance, $OD_{405}$, was measured using a 96-well plate reader (Molecular Devices). The relative protein was determined by adding 200 µL of 1:5 dilution Bio Rad G250 protein dye:water to the 10 µL cell lysate sample taken previously and the $OD_{595}$ was measured on a 96-well plate reader (Molecular Devices). Data points were normalized both to the relative protein content and nonreceptor dependent forskolin stimulation. $EC_{50}$ values represent the mean duplicate experiments performed in triplicate. $EC_{50}$ values were determined by fitting the data to a nonlinear regression analysis using PRISM program (v3.0. Graphpad Inc.).

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