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# Characterization of aliphatic, cyclic, and aromatic N-terminally "capped" His-D-Phe-Arg-Trp-$NH_2$ tetrapeptides at the melanocortin receptors

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**Abstract**

The melanocortin system is implicated in multiple physiological pathways including pigmentation, inflammation, erectile function, feeding behavior, energy homeostasis, weight homeostasis, and exocrine gland function, just to list a few. The endogenous agonists for the melanocortin receptors include the gene transcripts derived from the proopiomelanocortin gene and include the core tetrapeptide His-Phe-Arg-Trp sequence postulated to be important for melanocortin receptor selectivity and stimulation. Posttranslational processing of the proopiomelanocortin derived agonists results in the N-terminal acetylation and C-terminal amidation of α-melanocyte stimulation hormone (α-MSH). In this study we generated 25 N-terminally "capped" tetrapeptides containing the core sequence X-His-D-Phe-Arg-Trp-$NH_2$ and pharmacologically characterized them at the mouse melanocortin $MC_1$ receptor, melanocortin $MC_3$ receptor, melanocortin $MC_4$ receptor, and melanocortin $MC_5$ receptor. The N-terminal "capping" groups consisted of linear, cyclic, or aromatic moieties and all resulted in full agonist activity at the melanocortin receptors examined in this study. Increasing aliphatic chain length increased potency of the tetrapeptide derivatives, with the addition of octanoyl capping group resulting in 70- to 110-fold increased tetrapeptide potency at the melanocortin $MC_1$ receptor ($EC_{50}$ = 0.4 nM), melanocortin $MC_3$ receptor ($EC_{50}$ = 4.0 nM), and melanocortin $MC_4$ receptor ($EC_{50}$ = 0.4 nM) while only enhancing potency at the melanocortin $MC_5$ receptor ($EC_{50}$ = 0.8 nM) by 8-fold, compared to the tetrapeptide His-D-Phe-Arg-Trp-$NH_2$. This octanoyl derivative surprisingly resulted in a 14-fold greater potency than α-MSH ($EC_{50}$ = 5.4 nM) at the mouse melanocortin $MC_4$ receptor implicated in feeding behavior and obesity. The 3,3,3-triphenylpropionyl derivative resulted in greater than 14 μM agonist potencies at the melanocortin $MC_1$ receptor, melanocortin $MC_3$ receptor, and melanocortin $MC_4$ receptor and possessed a 140 nM agonist $EC_{50}$ value at the melanocortin $MC_5$ receptor. This 3,3,3-triphenylpropionyl-His-D-Phe-Arg-Trp-$NH_2$ peptide is a 100-fold selective agonist for the melanocortin $MC_5$ receptor, versus the other melanocortin receptors studied herein, and is the first melanocortin $MC_5$ receptor selective tetrapeptide derivative reported to date with nanomolar potency.


*Keywords:* Obesity; G-protein-coupled receptor; Melanocortin receptor; Melanotropin

## 1. Introduction

The melanocortin receptors belong to the superfamily of seven transmembrane spanning G-protein-coupled receptors and stimulate the cAMP signal transduction pathway (Cone et al., 1996; Eberle, 1988). The endogenous agonist ligands for these melanocortin receptors are derived by posttranslational modification of the proopiomelanocortin gene transcript, which upon differential processing, results in the generation of the α-, β-, and γ-melanocyte-stimulating hormones (MSH) and adrenocorticotropin hormone (ACTH). The melanocortin receptor subtypes are activated by all of the endogenous melanocortin peptides, with the exception of the melanocortin $MC_2$ receptor which is only stimulated by adrenocorticotropin. All these melanocortin peptide agonists contain a core His-Phe-Arg-Trp tetrapeptide sequence that has been attributed to the ligand selectivity and stimulation of the melanocortin receptors (Castrucci et al., 1989; Haskell-Luevano et al., 1996a; Hruby et al., 1987). The melanocortin receptor family also has two endogenous antagonists, agouti (Lu et al., 1994) and the agouti-related protein (AGRP) (Ollmann et al., 1997; Shutter et al., 1997), which are the only known

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naturally occurring antagonists of G-protein-coupled receptors discovered to date. The centrally located melanocortin $MC_3$ and $MC_4$ receptors have been identified in knockout mice to be involved in feeding behavior, obesity, metabolism, and energy homeostasis (Butler et al., 2000; Chen et al., 2000; Huszar et al., 1997). In addition to the involvement of the melanocortin $MC_4$ receptor in feeding behavior and obesity, it has been implicated to participate in grooming behavior, modulation of algesia, and erectile function and sexual behavior (Adan and Gispen, 2000; Adan et al., 1999; De Wied, 1999; MacNeil et al., 2002; Martin et al., 2002; Van der Ploeg et al., 2002; Vrinten et al., 2001).The most well-studied melanocortin receptor ligands are for the skin melanocortin $MC_1$ receptor which are involved in pigmentation and animal coat coloration (Eberle, 1988; Hruby et al., 1993; Lerner and McGuire, 1961; Mountjoy et al., 1992). Additionally, the melanocortin $MC_5$ receptor has been identified as playing a role in exocrine gland function (Chen et al., 1997; Van der Kraan et al., 1998).

The melanocortin receptor agonist α-MSH is a 13-amino-acid linear peptide that is posttranslationally processed to include the N-terminal acetyl and C-terminal amide moieties (Eberle, 1988; Hadley, 1989; Lerner and McGuire, 1961). Due to the endogenous posttranslational processing of the melanocortin agonist ligands, melanocortin peptide structure–activity studies have generally included the N-terminal acetyl and C-terminal amide on peptide derivatives, which is also proposed to increase enzymatic stability by peptidases (Castrucci et al., 1984; Eberle, 1988). Previous reports modifying melanocortin peptides by the addition of fatty acid conjugates (Al-Obeidi et al., 1990; Hadley et al., 1991), biotin (Chaturvedi et al., 1984), and chlorotriazinylaminofluorescein (Chaturvedi et al., 1985) at the N-terminus resulted in enhanced and decreased potencies, depending upon the modification, in the classical pigmentation frog or lizard skin assays, tyrosinase assays, or melanoma cell assays. At the cloned melanocortin receptors, few studies have been reported examining the involvement of the N-terminal groups in receptor potency. The compounds RO27-3225 (2-{2-[2-Butyrylamino-3-(1*H*-imidazol-4-yl)-propionylamino]-3-phenyl-propionylamino}-5-guanidino-pentanoic acid [1-(carbamoylmethyl-methyl-carbamoyl)-2-(1*H*-indol-3-yl)-ethyl]-amide, $N^{\alpha}$-butyryl-His-D-Phe-Arg-Trp-$N^{\alpha}$-methyl-Gly-$NH_2$) and RO27-4680 (2-{2-[2-Butyrylamino-3-(1*H*-imidazol-4-yl)-propionylamino]-3-naphthalen-2-yl-propionylamino}-5-guanidino-pentanoic acid [1-(carbamoylmethyl-methyl-carbamoyl)-2-(1*H*-indol-3-yl)-ethyl]-amide, $N^{\alpha}$-butyryl-His-DNal(2′)-Arg-Trp-$N^{\alpha}$-methyl-Gly-$NH_2$), which are novel modified peptide derivatives containing the N-terminal butyryl moiety, resulted in potent in vivo biological activity (Benoit et al., 2000). The tetrapeptide His-D-Phe-Arg-Trp-$NH_2$ was reported at the human melanocortin $MC_4$ receptor to possess a 8 nM agonist $EC_{50}$ value (Yang et al., 2000) which is similar to the 10 nM agonist $EC_{50}$ value of the tetrapeptide Ac-His-D-Phe-Arg-Trp-$NH_2$ reported at the mouse $MC_4$ receptor (Haskell-Luevano et al., 2001b). The study reported herein was performed to determine if various linear, cyclic, or aromatic acyl modifications at the N-terminus of the tetrapeptide His-Phe-Arg-Trp-$NH_2$ would result in increased agonist potency and/or enhanced melanocortin receptor selectivity.

## 2. Materials and methods

### 2.1. Chemicals

The $N^{\alpha}$-9-fluorenylmethyloxycarbonyl (Fmoc) amino acids Fmoc-Trp-(Boc), Fmoc-Arg-(Pbf), Fmoc-D-Phe, and Fmoc-His-(Trt) were purchased from Peptides International (Louisville, KY). The peptides were assembled on Rink-amide-4-methylbenzhydrylamine (MBHA) resin (0.44 meq/g substitution), purchased from Peptides International. The coupling reagents 2-(1-*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt) were purchased from Peptides International. Glacial acetic acid, dichloromethane, methanol, acetonitrile, and anhydrous ethyl ether were purchased from Fisher Scientific (Fair Lawn, NJ, USA). *N*,*N*-dimethylformamide was purchased from Burdick and Jackson (McGaw Park, IL, USA). Trifluoroacetic acid, 1,3-diisopropylcarbodiimide, pyridine, piperidine and acetic anhydride were purchased from Sigma (St. Louis, MO, USA). *N*,*N*-diisopropylethylamine and triisopropylsilane were purchased from Aldrich (Milwaukee, WI, USA). The carboxylic acid capping groups 2-biphenylcarboxylic acid, 4-butoxybenzoic acid, 3,3-diphenylpropionic acid, *n*-trityl-glycine, 3,3,3-triphenylpropionic acid, *p*-tolylacetic acid, 2,4,6-trimethylbenzoic acid, cyclohexanecarboxylic acid, 2-naphthoic acid, 4-isopropylbenzoic acid, 9-anthracenecarboxylic acid, phenylacetic acid, benzoic acid, diphenylacetic acid, 3-cyclopentylpropionic acid, *tert*-butylacetic acid, butyric acid, octanoic acid, 2-ethylhexanoic acid, hexanoic acid, and 4-methylvaleric acid were purchased from Aldrich. Pivalic acid and 4-bromobenzoic acid were purchased from Fluka (Milwaukee, WI, USA). All reagents and chemicals were ACS grade or better and were used without further purification.

### 2.2. Peptide synthesis

Peptide synthesis was performed using standard Fmoc methodology (Carpino and Han, 1970, 1972) manually or on an automated synthesizer (Advanced ChemTech 440MOS, Louisville, KY, USA). Approximately 2.5 g of Rink-amide-MBHA resin (1.1 mmol) was added to a manual reaction vessel (Peptides International). The resin was allowed to swell for 2 h in dichloromethane followed by $N^{\alpha}$ deprotection of the Fmoc group using 20% piperidine in *N*,*N*-dimethylformamide for 2 min followed by a 20-min 20% piperidine treatment. A positive Kaiser test

(Kaiser et al., 1970) resulted, indicating free amine groups on the resin. The growing peptide chain was added to the amide resin using the general amino acid cycle as follows: the addition of 3-fold excess amino acid starting from the C-terminus, 3-fold excess 1-hydroxybenzotriazole, and 3.1-fold excess of 1,3-diisopropylcarbodiimide in *N*,*N*-dimethylformamide. The coupling reaction is mixed by bubbling with nitrogen gas for 2 h, followed by emptying of the reaction vessel under vacuum. The resin-$N^{\alpha}$-protected peptide is washed with *N*,*N*-dimethylformamide (5 × 1 min) to remove excess reagents followed by $N^{\alpha}$-Fmoc deprotection with 20% piperidine in *N*,*N*-dimethylformamide as described above. The reaction vessel is washed with *N*,*N*-dimethylformamide to remove the piperidine, and the next coupling cycle is performed as described above. Upon complete synthesis of the side chain protected $N^{\alpha}$-Fmoc-His-D-Phe-Arg-Trp–resin conjugate, the peptide–resin was washed with dichloromethane (4 × 1 min) and dried in vacuo. The dry peptide–resin was split into equal aliquots for the subsequent N-terminal addition of the various "capping" groups (Figs. 1–3). The peptide–resin was solvated in *N*,*N*-dimethylformamide for 1 h, the $N^{\alpha}$-Fmoc

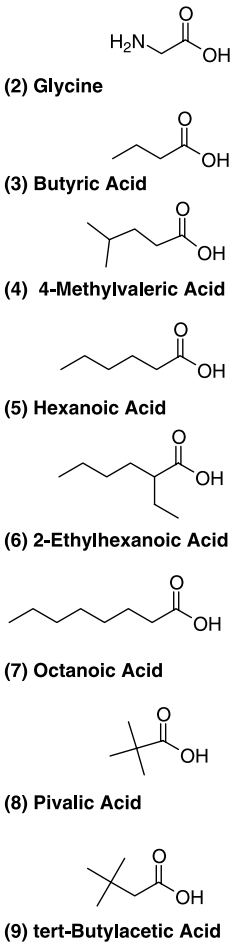


Fig. 1. Structures, names, and compound number of the aliphatic N-terminal capping groups of the tetrapeptide His-D-Phe-Arg-Trp-$NH_2$ used to prepare analogues 2 through 9.

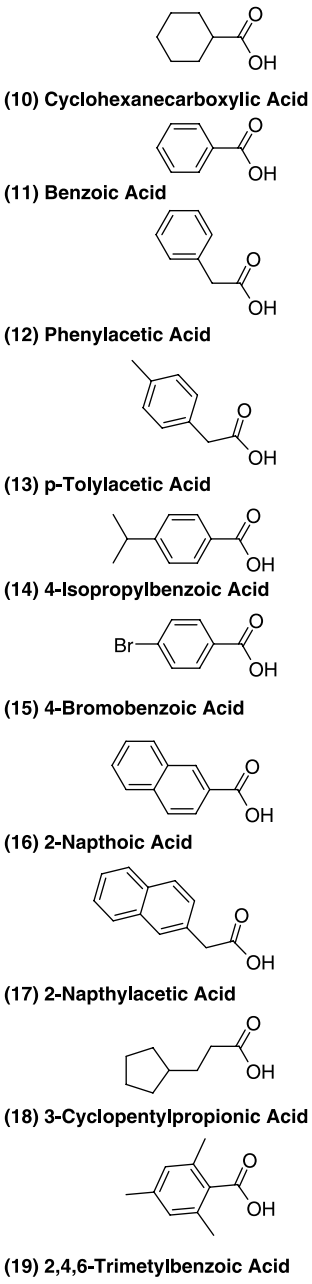


Fig. 2. Structures, names, and compound number of the cycloalkyl and aromatic N-terminal capping groups of the tetrapeptide His-D-Phe-Arg-Trp-$NH_2$ used to prepare analogues 10 through 19.

group was removed by 20% piperidine in *N*,*N*-dimethylformamide, and the "capping" group (6-fold excess) was coupled using 6-fold excess of 1,3-diisopropylcarbodiimide and 6-fold excess of 1-hydroxybenzotriazole. Deprotection of the amino acid side chains and cleavage of the "capped" peptide from the resin was performed using an automated synthesizer (Advanced ChemTech 440MOS). The reactions were performed using a 40-well Teflon reaction block with a course Teflon frit. The side chain deprotection and peptide–resin cleavage were achieved by incubation with 3 ml of cleavage cocktail (95% trifluoroacetic acid, 2.5%

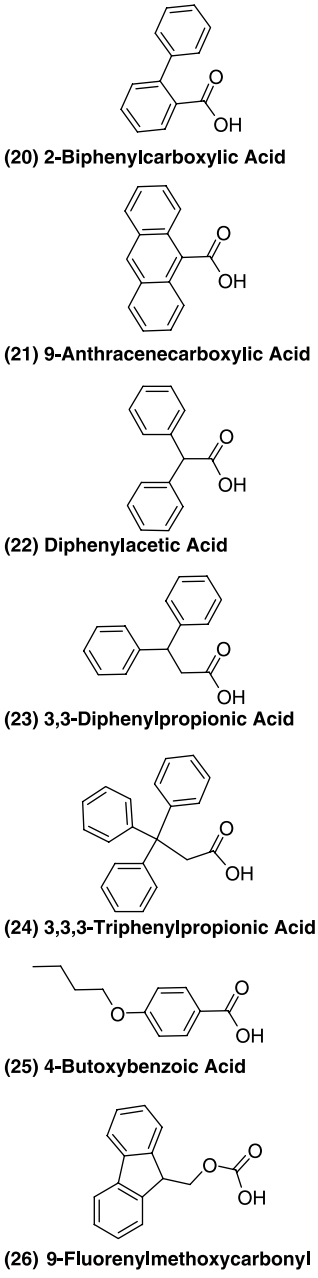


Fig. 3. Structures, names, and compound number of the bulky aromatic N-terminal capping groups of the tetrapeptide His-D-Phe-Arg-Trp-$NH_2$ used to prepare analogues 20 through 26.

water, 2.5% triisopropylsilane) for 3 h at 500 rpm. The cleavage product was transferred from the reaction block into a cleavage block containing 7 ml collection vials by positive nitrogen gas pressure. The resin was washed with 1.5 ml of cleavage cocktail for 5 min at 500 rpm and transferred to the previous cleavage solution. The peptides were transferred to preweighed 50 ml conical tubes and precipitated with cold (4 °C) anhydrous ethyl ether (up to 50 ml). The flocculent peptide was pelleted by centrifugation (Sorval Super T21 high-speed centrifuge using the swinging bucket rotor) at 4000 rpm for 5 min, the ether was decanted off, and the peptide was washed one time with cold anhydrous ethyl ether, pelleted, and the ether was decanted off. The crude peptide was dried in vacuo for 48 h. A 10- to 15-mg sample of crude peptide was purified by reversed-phase high performance liquid chromatography (RP-HPLC) using a Shimadzu chromatography system with a photodiode array detector and a semipreparative RP-HPLC $C_{18}$ bonded silica column (Vydac 218TP1010, 1.0 × 25 cm) and lyophilized. The purity of the peptides was assessed by analytical RP-HPLC in two diverse solvent systems (Table 1), one-dimensional $^1H$ nuclear magnetic resonance spectroscopy and had the correct molecular mass (University of Florida protein core facility, Table 1).

### 2.3. Cell culture and transfection

Briefly, human embryonic kidney (HEK)-293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum and seeded 1 day prior to transfection at $1-2 \times 10^6$ cell/100-mm dish. Melanocortin receptor DNA in the $pCDNA_3$ expression vector (20 μg) was transfected using the calcium phosphate method. Stable receptor populations were generated using G418 selection (1 mg/ml) for subsequent bioassay analysis.

### 2.4. Functional bioassay

HEK-293 cells stably expressing the melanocortin receptors were transfected with 4 μg cAMP response element (CRE)/β-galactosidase reporter gene as previously described (Chen et al., 1995; Haskell-Luevano et al., 2001b). Briefly, 5000–15,000 post transfection cells were plated into 96-well Primera plates (Falcon) and incubated overnight. Forty-eight hours post-transfection, the cells were stimulated with 100 μl peptide ($10^{-4}$–$10^{-12}$ M) or forskolin ($10^{-4}$ M) control in assay medium (DMEM containing 0.1 mg/ml bovine serum albumin (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) was added. The plates were stored at −80 °C overnight. The plates containing the 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. To the cell lysate plates, 40 μl phosphate-buffered saline with 0.5% BSA was added to each well. Subsequently, 150 μl substrate buffer (60 mM sodium phosphate, 1 mM $MgCl_2$, 10 mM KCl, 5 mM β-mercaptoethanol, 200 mg *o*-nitrophenyl-β-D-galactopyranoside (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 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 non-receptor-dependent forskolin stimulation.

Table 1
Analytical data for the peptides synthesized in this study

| Peptide | Structure/capping group | HPLC $k'$ (System 1) | HPLC $k'$ (System 2) | Purity | Mass spectral analysis ($M+1$) |
|---|---|---|---|---|---|
| | Ac-His-D-Phe-Arg-Trp-$NH_2$ | 4.0 | 6.4 | >99 | 687.2 |
| 1 | $NH_2$-His-D-Phe-Arg-Trp-$NH_2$ | 3.7 | 3.7 | >99 | 644.2 |
| 2 | Glycine | 3.6 | 6.1 | >99 | 701.2 |
| 3 | Butyryl | 4.5 | 7.5 | >99 | 714.2 |
| 4 | 4-Methylvaleryl | 5.4 | 8.9 | >99 | 742.2 |
| 5 | Hexanoyl | 5.1 | 9.1 | >99 | 741.9 |
| 6 | 2-Ethylhexanoyl | 6.1 | 6.3 | >99 | 771.1 |
| 7 | Octanoyl | 6.6 | 6.7 | >96 | 771.0 |
| 8 | Pivalyl | 4.9 | 7.3 | >99 | 728.4 |
| 9 | *tert*-Butylacetyl | 5.2 | 8.8 | >99 | 742.9 |
| 10 | Cyclohexanecarboxyl | 5.5 | 5.5 | >99 | 755.1 |
| 11 | Benzoyl | 5.1 | 5.1 | >99 | 748.8 |
| 12 | Phenylacetyl | 5.2 | 8.3 | >99 | 761.9 |
| 13 | *p*-Tolylacetyl | 5.3 | 9.0 | >96 | 777.1 |
| 14 | 4-Isopropylbenzoyl | 6.0 | 10.0 | >99 | 790.1 |
| 15 | 4-Bromobenzoyl | 5.8 | 9.3 | >99 | 827.1 |
| 16 | 2-Napthoyl | 5.9 | 9.5 | >96 | 798.9 |
| 17 | 2-Naphthylacetyl | 6.3 | 6.2 | >98 | 812.4 |
| 18 | 3-Cyclopentylpropionyl | 6.0 | 9.0 | >99 | 769.2 |
| 19 | 2,4,6-Trimethylbenzoyl | 5.3 | 9.4 | >99 | 790.3 |
| 20 | 2-Biphenylcarboxyl | 6.0 | 9.7 | >99 | 825.1 |
| 21 | 9-Anthracenecarboxyl | 6.2 | 9.9 | >96 | 849.1 |
| 22 | Diphenylacetyl | 6.5 | 10.3 | >99 | 839.1 |
| 23 | 3,3-Diphenylpropionyl | 6.8 | 6.8 | >99 | 852.2 |
| 24 | 3,3,3-Triphenylpropionyl | 7.8 | 11.2 | >99 | 928.0 |
| 25 | 4-Butoxybenzoyl | 6.8 | 10.6 | >99 | 820.7 |
| 26 | 9-Fluorenylmethoxycarbonyl | 7.1 | 10.4 | >99 | 866.3 |

HPLC $k'$=[(peptide retention time − solvent retention time)/solvent retention time] in solvent system 1 (10% acetonitrile in 0.1% trifluroacetic acid/water and a gradient to 90% acetonitrile over 40 min) or solvent system 2 (10% methanol in 0.1% trifluroacetic acid/water and a gradient to 90% methanol over 40 min). An analytical Vydac $C_{18}$ column (Vydac 218TP104) was used with a flow rate of 1.5 ml/min. The percentage peptide purity is determined by HPLC at a wavelength of 214$\lambda$.

### 2.5. Data analysis

$EC_{50}$ values represent the mean of duplicate experiments performed in quadruplet or more independent experiments. $EC_{50}$ estimates and their associated standard errors were determined by fitting the data to a nonlinear least-squares analysis using the PRISM program (v3.0, GraphPad). The results are not corrected for peptide content, although all the peptides examined in this study were determined to have approximately equal peptide content as determined by using Beers Law.

## 3. Results

The tetrapeptide **1**, His-D-Phe-Arg-Trp-$NH_2$, has been modified at the N-terminus by acylation with carboxylic acid "capping" groups which terminates peptide synthesis. These "capping" groups are summarized in Figs. 1–3 and consist of linear, cyclic, and aromatic hydrophobic moieties. Table 2 summarizes the agonist $EC_{50}$ values characterized at the mouse melanocortin $MC_1$ receptor, melanocortin $MC_3$ receptor, melanocortin $MC_4$ receptor, and melanocortin $MC_5$ receptor.

The tetrapeptide Ac-His-D-Phe-Arg-Trp-$NH_2$ is the lead peptide for this study and has been previously reported at the mouse melanocortin receptors (Haskell-Luevano et al., 1996a, 2001b), and possesses 23, 422, 40, and 5 nM agonist activity at the melanocortin $MC_1$ receptor, melanocortin $MC_3$ receptor, melanocortin $MC_4$ receptor, and melanocortin $MC_5$ receptor, reported herein. Tetrapeptide (**1**), His-D-Phe-Arg-Trp-$NH_2$, possesses a free amine at the N-terminus, and is within experimental error of the N-terminally acetylated tetrapeptide. Fig. 4 summarizes the pharmacology of the peptides with linear aliphatic modifications of the N-terminus of tetrapeptide **1** at the melanocortin receptors examined in this study. Addition of all linear acyl groups selected in this study to the N-terminus of the tetrapeptide resulted in increased potency or equipotency at each of the melanocortin receptors tested. The addition of the octanoyl group to the tetrapeptide **7** resulted in the most significant increase in potency of the compounds prepared in this study, with ca. 100-fold increased potency at the melanocortin $MC_1$ and melanocortin $MC_3$ receptor, a 70-fold increased potency at the melanocortin $MC_4$ receptor, but only a 8-fold increased melanocortin $MC_5$ receptor potency, compared with the "uncapped" tetrapeptide **1**.

Table 2
Functional activity of the N-terminally modified tetrapeptide His-D-Phe-Arg-Trp-$NH_2$ at the mouse melanocortin receptors

| Peptide | Structure/capping group | mMC1R | | mMC3R | | mMC4R | | mMC5R | |
|---|---|---|---|---|---|---|---|---|---|
| | | $EC_{50}$ (nM) | Fold difference | $EC_{50}$ (nM) | Fold difference | $EC_{50}$ (nM) | Fold difference | $EC_{50}$ (nM) | Fold difference |
| α-MSH | Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-$NH_2$ | 0.55 ± 0.09 | | 0.79 ± 0.14 | | 5.37 ± 0.62 | | 0.44 ± 0.09 | |
| NDP-MSH | Ac-Ser-Tyr-Ser-Nle-Glu-His-D-Phe-Arg-Trp-Gly-Lys-Pro-Val-$NH_2$ | 0.038 ± 0.012 | | 0.098 ± 0.013 | | 0.21 ± 0.03 | | 0.071 ± 0.012 | |
| MTII | Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Lys]-$NH_2$ | 0.020 ± 0.003 | | 0.16 ± 0.03 | | 0.087 ± 0.008 | | 0.16 ± 0.03 | |
| | Ac-His-D-Phe-Arg-Trp-$NH_2$ | 23.4 ± 6.44 | | 422 ± 150 | | 40.7 ± 9.65 | | 5.60 ± 1.87 | |
| 1 | $NH_2$-His-D-Phe-Arg-Trp-$NH_2$ | 39.8 ± 10.0 | 1 | 421 ± 132 | 1 | 27.5 ± 3.71 | 1 | 6.23 ± 1.92 | 1 |
| 2 | Glycine | 22.2 ± 5.4 | | 111 ± 41 | − 4 | 2.16 ± 0.54 | − 13 | 1.35 ± 0.67 | − 5 |
| 3 | Butyryl | 11.3 ± 3.73 | − 4 | 179 ± 45 | | 3.36 ± 0.29 | − 8 | 2.94 ± 1.34 | |
| 4 | 4-Methylvaleryl | 2.91 ± 0.82 | − 14 | 42.1 ± 9.5 | − 10 | 2.78 ± 0.35 | − 10 | 0.74 ± 0.015 | − 8 |
| 5 | Hexanoyl | 0.87 ± 0.19 | − 45 | 35.5 ± 6.5 | − 12 | 1.63 ± 0.55 | − 17 | 0.44 ± 0.07 | − 14 |
| 6 | 2-Ethylhexanoyl | 2.65 ± 0.59 | − 15 | 16.2 ± 5.17 | − 26 | 2.05 ± 0.13 | − 13 | 0.25 ± 0.077 | − 25 |
| 7 | Octanoyl | 0.36 ± 0.24 | − 111 | 4.01 ± 0.74 | − 105 | 0.38 ± 0.063 | − 72 | 0.79 ± 0.25 | − 8 |
| 8 | Pivalyl | 22.6 ± 3.10 | | 89 ± 16 | − 5 | 12.7 ± 5.35 | | 0.77 ± 0.11 | − 8 |
| 9 | *tert*-Butylacetyl | 32.7 ± 11.0 | | 648 ± 50 | | 9.56 ± 2.08 | | 4.15 ± 0.63 | |
| 10 | Cyclohexanecarboxyl | 70.7 ± 54.2 | | 154 ± 30 | | 4.85 ± 0.85 | − 6 | 0.78 ± 0.26 | − 8 |
| 11 | Benzoyl | 14.4 ± 2.06 | | 253 ± 72 | | 3.35 ± 0.57 | − 8 | 0.96 ± 0.41 | − 6 |
| 12 | Phenylacetyl | 5.00 ± 0.31 | − 8 | 47.0 ± 18.7 | − 9 | 2.21 ± 0.65 | − 12 | 0.71 ± 0.12 | − 9 |
| 13 | *p*-Tolyacetyl | 5.36 ± 0.69 | − 7 | 41.8 ± 12 | − 10 | 0.86 ± 0.14 | − 32 | 0.31 ± 0.041 | − 20 |
| 14 | 4-Isopropylbenzoyl | 14.7 ± 5.20 | | 151 ± 32 | | 6.53 ± 0.82 | − 4 | 3.08 ± 0.57 | |
| 15 | 4-Bromobenzoyl | 22.5 ± 10.4 | | 270 ± 74 | | 5.28 ± 1.06 | − 5 | 1.89 ± 0.55 | |
| 16 | 2-Napthoyl | 19.0 ± 4.4 | | 89.0 ± 10 | − 5 | 4.94 ± 0.38 | − 6 | 2.33 ± 0.47 | |
| 17 | 2-Naphthylacetyl | 4.10 ± 1.26 | − 10 | 16.8 ± 4.62 | − 25 | 0.90 ± 0.23 | − 30 | 0.49 ± 0.23 | − 13 |
| 18 | 3-Cyclopentylpropionyl | 1.33 ± 0.64 | − 30 | 51 ± 16.5 | − 8 | 21.9 ± 6.67 | | 0.88 ± 0.27 | − 7 |
| 19 | 2,4,6-Trimethylbenzoyl | 24.0 ± 5.36 | | 78.1 ± 16.3 | − 5 | 6.43 ± 0.51 | − 4 | 1.06 ± 0.41 | − 6 |
| 20 | 2-Biphenylcarboxyl | 78.5 ± 41 | | 925 ± 246 | | 8.49 ± 2.23 | | 6.78 ± 1.10 | |
| 21 | 9-Anthracenecarboxyl | 31.1 ± 9.7 | | 535 ± 96 | | 28.7 ± 4.18 | | 43.4 ± 19.0 | 7 |
| 22 | Diphenylacetyl | 6.22 ± 1.51 | − 6 | 31.0 ± 5.44 | − 14 | 5.50 ± 0.90 | − 5 | 0.97 ± 0.40 | − 6 |
| 23 | 3,3-Diphenylpropionyl | 44.0 ± 7.67 | | 529 ± 220 | | 44.0 ± 12.7 | | 4.18 ± 0.65 | |
| 24 | 3,3,3-Triphenylpropionyl | 14,400 ± 3,100 | 362 | 15,400 ± 6,800 | 37 | 14,300 ± 2,300 | 520 | 140 ± 99 | 22 |
| 25 | 4-Butoxybenzoyl | 9.96 ± 1.87 | − 4 | 142 ± 19 | | 5.75 ± 0.69 | − 5 | 2.47 ± 0.66 | |
| 26 | 9-Fluorenylmethoxycarbonyl | 12.1 ± 4.46 | | 103 ± 34 | − 4 | 98.1 ± 24.9 | 4 | 4.38 ± 1.60 | |

The indicated errors represent the standard error of the mean determined from at least four independent experiments. The fold differences listed are considered significant beyond the inherent 3-fold experimental error. A negative fold difference value indicates the resulting capping group increased the potency of the His-D-Phe-Arg-Trp-$NH_2$ tetrapeptide.

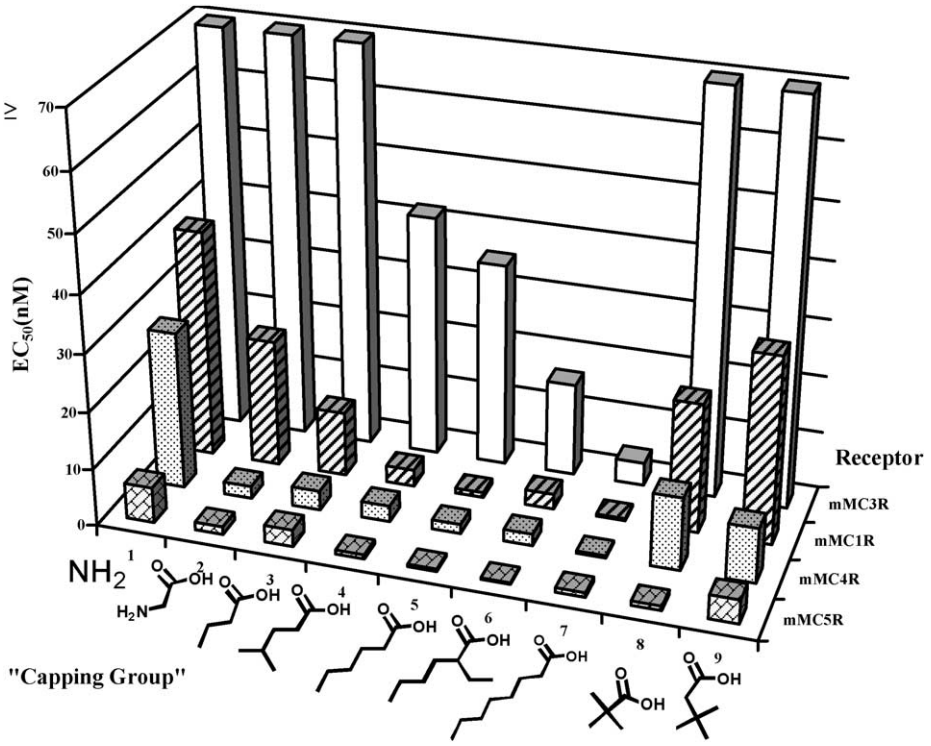


Fig. 4. Graphical representation summarizing the effect on melanocortin receptor (*Y*-axis) agonist $EC_{50}$ values (*Z*-axis) of the indicated N-terminal aliphatic capping group moieties on the X-His-D-Phe-Arg-Trp-$NH_2$ tetrapeptides.

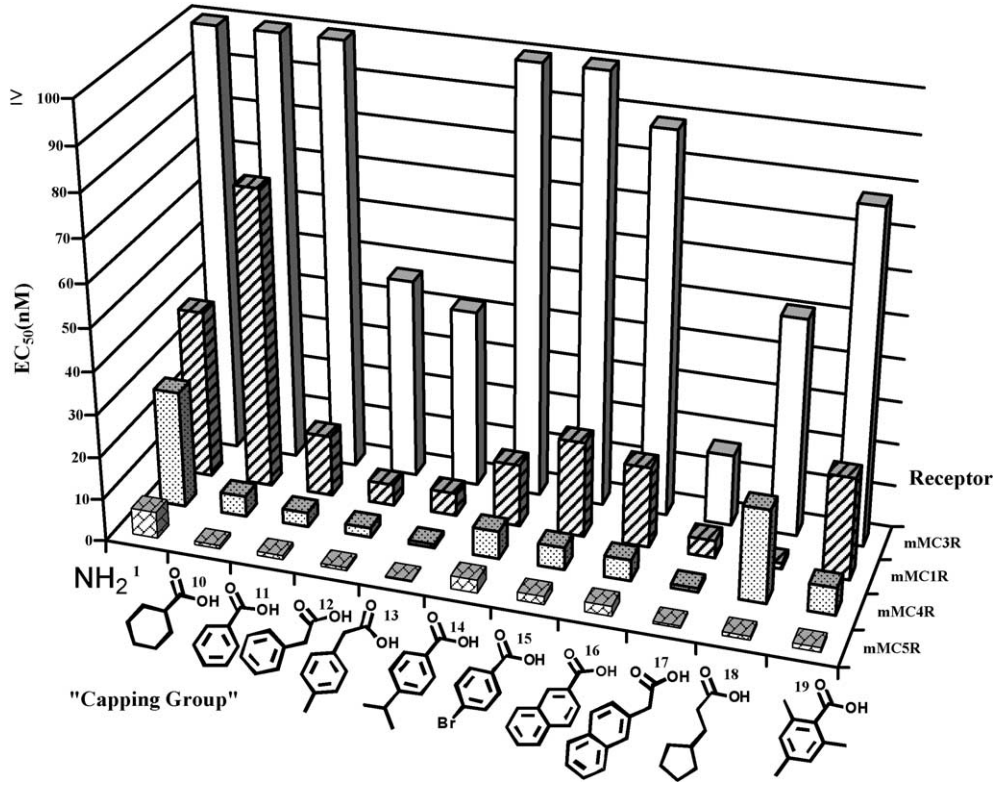


Fig. 5. Graphical representation summarizing the effect on melanocortin receptor (*Y*-axis) agonist $EC_{50}$ values (*Z*-axis) of the indicated N-terminal cyclic and aromatic capping group moieties on the X-His-D-Phe-Arg-Trp-$NH_2$ tetrapeptides.

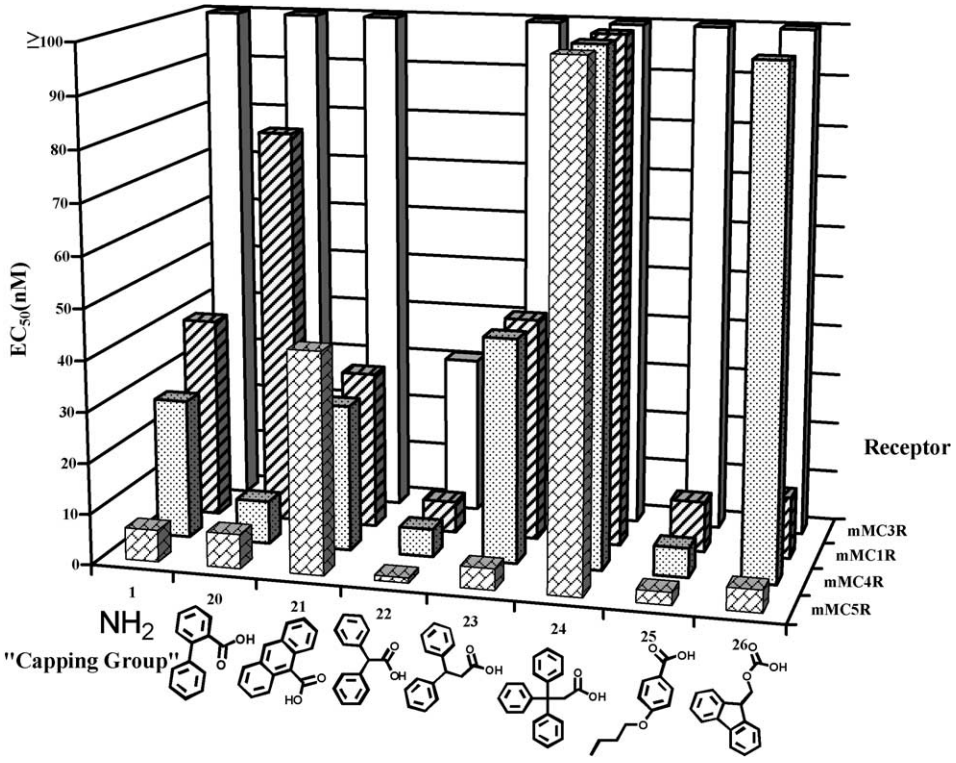


Fig. 6. Graphical representation summarizing the effect on melanocortin receptor (*Y*-axis) agonist $EC_{50}$ values (*Z*-axis) of the indicated N-terminal multi-aryl capping group moieties on the X-His-D-Phe-Arg-Trp-$NH_2$ tetrapeptides.

Fig. 5 summarizes the pharmacology of the cycloalkyl and aryl N-terminal modifications of the His-D-Phe-Arg-Trp-$NH_2$ tetrapeptide at the melanocortin receptors. Surprisingly, all tetrapeptides in this study with monocyclic modifications had an increase in potency, or retained equipotency, as compared with the control. The most notable enhancement to peptide potency resulted from the addition of either *p*-tolylacetyl or 2-naphthylacetyl to the N-terminus of the tetramer. The peptide that contains the *p*-tolylacetyl group (**13**) resulted in 32-fold increased melanocortin $MC_4$ receptor potency and 20-fold melanocortin $MC_5$ receptor potency to subnanomolar $EC_{50}$ values, and 7- to 10-fold increased melanocortin $MC_1$ and $MC_3$ receptor potency, compared with **1**. Incorporation of the 2-naphthylacetyl moiety in compound **17** resulted in a 10-fold increase in melanocortin $MC_1$ and MC receptor potency, and was notably 25- to 30-fold more potent at the melanocortin $MC_3$ and $MC_4$ receptors, as compared with **1**.

Fig. 6 summarizes the pharmacology of peptides modified at the N-terminus with acyl groups that contain two or more aromatic moieties. Interestingly, the peptides that contain either the bulky 2-biphenylcarboxyl group (**20**) or the 3,3-diphenylpropionyl group (**23**) retained equipotency compared with free amine containing tetrapeptide **1** at the melanocortin receptors. Addition of the 9-anthracenecarboxyl moiety in compound **21** resulted in 7-fold decreased melanocortin $MC_5$ receptor potency, but was equipotent at the melanocortin $MC_1$, $MC_3$, and $MC_4$ receptors, compared with **1**. Compound **24** contains a 3,3,3-triphenylpropionyl group and resulted in $EC_{50}$ values greater than 14,000 nM at the melanocortin $MC_1$, $MC_3$, and $MC_4$ receptors, but possessed a 140 nM melanocortin $MC_5$ receptor agonist potency.

## 4. Discussion

The central melanocortin receptors, $MC_3$ and $MC_4$, have been implicated in participating in feeding behavior, obesity, weight and energy homeostasis (Butler and Cone, 2001; Chen et al., 2000; Haskell-Luevano et al., 2001b; Huszar et al., 1997). Therefore, these melanocortin receptor isoforms have become drug target candidates in industry, as well as academia, for the treatment of obesity-related diseases such as heart disease, type II diabetes mellitus, stroke, hypertension, and morbidity. This study was undertaken to explore the topographical space (Hruby et al., 1997) available at the N-terminus of the melanocortin agonist tetrapeptide His-D-Phe-Arg-Trp-$NH_2$ to enhance ligand potency and melanocortin receptor subtype selectivity.

Figs. 1 and 4 illustrate the aliphatic capping groups used in this study and their effects on agonist pharmacology of the His-D-Phe-Arg-Trp-$NH_2$ tetrapeptide at the melanocortin $MC_1$, $MC_3$, $MC_4$, and $MC_5$ receptors. Increasing the alkyl chain length from $n=3$ (**3**) to $n=5$ (**5**) or $n=7$ (**7**) resulted in an increased potency trend (5- and 45-fold, respectively)

at the melanocortin $MC_3$ receptor (Fig. 4), suggesting that increasing the chain length beyond that of octanoyl may further result in increased potencies. This increased potency may be attributed to increased ligand–receptor interactions where the aliphatic capping group may be resembling the Nle side chain of the parent molecule NDP-MSH peptide (Benoit et al., 2000), or alternatively the increased aliphatic length may additionally increase lipid–peptide interactions which may increase the availability of the ligand to the melanocortin receptor in the lipid bilayer, which may therefore increase ligand potency (Sargent and Schwyzer, 1986). Previously, four fatty acids (palmitoyl, myristoyl, decanoyl, and hexanoyl) were added to the N-terminal of the MTII (Al-Obeidi et al., 1989a,b) agonist template and found to be 10–100 times more potent than the native hormone α-MSH in the mouse S91 melanoma tyrosinase assay (Al-Obeidi et al., 1992). Interestingly, using the lizard skin pigmentation assay, the hexanoyl- and decanoyl-modified peptides were equipotent to α-MSH, whereas the longer myristoyl and palmitoyl derivatives were 100 times less potent than α-MSH. Peptide **5**, containing the hexanoyl moiety on the tetrapeptide template, resulted in a 1.6 nM melanocortin $MC_4$ receptor agonist which is equipotent with α-MSH (within the inherent 3-fold experimental error), consistent with the observations of the hexanoyl conjugated to the MTII peptide template in the mouse S91 melanoma tyrosinase assay.

Modified peptides RO27-3225 and RO27-4680, based upon the His-Phe-Arg-Trp-$N^{\alpha}$-methyl-Gly-$NH_2$ and His-DNal(2′)-Arg-Trp-$N^{\alpha}$-methyl-Gly-$NH_2$ templates, respectively, contain a butyryl N-terminal functional moiety identical to the capping group of analogue **3** presented herein (Benoit et al., 2000). RO27-3225 was reported as a 1 nM melanocortin $MC_4$ receptor agonist that is selective for the melanocortin $MC_4$ receptor versus the $MC_3$ receptor. Peptide **3** herein is a 3 nM melanocortin $MC_4$ receptor agonist that is 53-fold selective for the melanocortin $MC_4$ receptor versus the melanocortin $MC_3$ receptor (Table 2), which is consistent with the observations of Benoit et al. Interestingly, the *tert*-butylacetyl group in analogue **9** was the only aliphatic N-terminal modification in this study that did not enhance ligand potency, compared to the uncapped peptide **1**, at any of the melanocortin receptors examined herein.

(Figs. 2–3 and 5–6) illustrate the aromatic and cycloalkyl capping groups used in this study and their effect on agonist pharmacology of the His-D-Phe-Arg-Trp-$NH_2$ tetrapeptide at the melanocortin $MC_1$ receptor, melanocortin $MC_3$ receptor, melanocortin $MC_4$ receptor, and melanocortin $MC_5$ receptor. Unexpectantly, all but one of the cycloalkyl and aryl capping groups used in this study resulted in equipotency or increased potency at the melanocortin receptors, compared with the uncapped peptide **1**. At the melanocortin $MC_3$ receptor, the most potent cyclic capping group was the 2-naphthylacetyl group (**17**) that resulted in a 25-fold increased potency compared with **1**, and is 20-fold less potent than α-MSH. At the melanocortin $MC_4$ receptor, addition of the *p*-tolylacetyl group (**13**) and 2-naphthylacetyl group (**17**) increased potency by 30-fold compared to the uncapped peptide **1** (ca. 0.9 nM agonist $EC_{50}$ values, 6-fold more potent than α-MSH). At the melanocortin $MC_5$ receptor, the peptide that contained the *p*-tolylacetyl group (**13**) resulted in 20-fold enhanced potency, compared with **1**, and the 2-naphthylacetyl (**17**) analogue was 13-fold more potent than **1**. Both **13** and **17** resulted in agonist potency equal to α-MSH at the melanocortin $MC_5$ receptor. Based upon homology molecular modeling and receptor mutagenesis studies of the melanocortin $MC_1$ and $MC_4$ receptors, similar putative ligand binding pockets consisting of a hydrophobic–aromatic "network" of Phe receptor residues interacting with the ligand Phe and Trp amino acids have been postulated (Haskell-Luevano, 1996b, 2000, 2001a; Lu et al., 1997, 1998; Yang et al., 1997, 2000). The increase in potency of the peptides presented herein may be a result of interactions of the N-terminal aromatic moieties of the peptides with the hydrophobic–aromatic "network" of melanocortin receptor residues. These data suggest that these types of modifications may be used to slightly enhance ligand potency at the melanocortin receptors.

The 3,3,3-triphenylpropionyl N-terminal capping group derivative **24** resulted in greater than 14 μM agonist potencies at the melanocortin $MC_1$, melanocortin $MC_3$, and $MC_4$ receptors but possessed an agonist $EC_{50}$ value of 140 nM at the melanocortin $MC_5$ receptor (Table 2). Thus, the capping group of **24** resulted in a ligand that is 100-fold selective for the melanocortin $MC_5$ receptor versus the melanocortin $MC_1$, $MC_3$, and $MC_4$ receptors. This is the most selective melanocortin $MC_5$ receptor tetrapeptide agonist reported to date.

The central melanocortin $MC_3$ and $MC_4$ receptors have both been implicated in playing an important role in weight and energy homeostasis using several nonselective ligands and knockout mice (Butler et al., 2000; Chen et al., 2000; Cowley et al., 2001; Fan et al., 1997; Huszar et al., 1997). Recently, there have been reports on the in vivo biological activities of selective ligands for the melanocortin $MC_4$ receptor that have further implicated this receptor as playing an important role in weight and energy homeostasis (Benoit et al., 2000; Kask et al., 1999; Vergoni and Bertolini, 2000; Vergoni et al., 1998, 2000). However, there still remains a high demand for additional ligands selective for the central melanocortin $MC_3$ and $MC_4$ receptors, as well as the remaining melanocortin receptors. In the N-terminally modified tetrapeptide His-D-Phe-Arg-Trp-$NH_2$ derivatives studied herein, the most melanocortin $MC_4$ versus $MC_3$ receptor selective capping group is 2-biphenylcarboxylic acid (**20**), which resulted in 109-fold selectivity (Table 2). Other peptide derivatives that are selective for the melanocortin $MC_4$ versus the $MC_3$ receptor contain benzoyl (**11**, 76-fold), *tert*-butylacetyl (**9**, 68-fold), glycine (**2**, 50-fold), butyryl (**3**, 50-fold), *p*-tolylacetyl (**13**, 50-fold), and 4-bromobenzoyl (**15**, 50-fold). None of these single modifications resulted in the melanocortin $MC_4$ versus $MC_3$

receptor selectivity observed for the cyclic c[Asp-(racemic)Atc-D-Phe-Arg-Trp-Lys]-$NH_2$ peptide, possessing 65 nM agonist activity at the human melanocortin $MC_4$ receptor while possessing only slight agonist activity at the human melanocortin $MC_3$ receptor (Danho et al., 2001). Tetrapeptides possessing unique melanocortin $MC_4$ versus $MC_3$ receptor pharmacology include Ac-Anc-D-Phe-Arg-Trp-$NH_2$ (a 21 nM melanocortin $MC_4$ receptor agonist and a 2.5 μM melanocortin $MC_3$ receptor antagonist, >4700-fold $MC_4$ versus melanocortin $MC_3$ selective agonist) (Holder et al., 2002a) and Ac-His-D-Phe(pI)-Arg-Trp-$NH_2$ (a 25 nM melanocortin $MC_4$ receptor agonist and a 56 nM melanocortin $MC_3$ receptor antagonist) (Holder et al., 2002b). However, these data would suggest that enhanced melanocortin $MC_4$ versus $MC_3$ receptor selectivity may be obtained by the addition of the 2-biphenylcarboxyl or benzoyl N-terminal "capping" groups, and that these moieties may be considered in the design of non-peptide melanocortin $MC_4$ receptor selective molecules. This speculation remains to be verified experimentally, however, since it is well recognized in the literature and structure–activity studies that additive effects proposed by the combination of individually identified ligand potency enhancers in combination may not enhance ligand potency more than any single modification alone.

This study reports modifications of the N-terminus of the melanocortin tetrapeptide His-D-Phe-Arg-Trp-$NH_2$ agonist with various linear, cyclic, or aromatic acyl moieties and their pharmacology at the mouse melanocortin receptors. This study identified N-terminal functional groups attached to the melanocortin tetrapeptide His-D-Phe-Arg-Trp-$NH_2$ that resulted in increased agonist potency and melanocortin receptor selectivity. The most notable results include the identification of the 3,3,3-triphenylpropionyl-His-D-Phe-Arg-Trp-$NH_2$ peptide which is a high nM selective $MC_5$ receptor agonist (100-fold selective versus the melanocortin $MC_1$, $MC_3$, and $MC_4$ receptors). The octanoyl N-terminally modified tetrapeptide resulted in the most significant enhancement of potency, possessing nM or sub-nM agonist $EC_{50}$ values at the melanocortin $MC_1$, $MC_3$, $MC_4$, and $MC_5$ receptors. Finally, the 2-biphenylcarboxyl N-terminally modified tetrapeptide resulted in 100-fold melanocortin $MC_4$ versus $MC_3$ receptor selectivity.

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