STRUCTURE-ACTIVITY RELATIONSHIPS OF AN AGOUTI-RELATED PROTEIN-DERIVED DECAPEPTIDE AT THE MURINE MELANOCORTIN RECEPTORS

By

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This document is dedicated to my husband, Joseph, not only for being a loving, supportive and dedicated partner, but for being mom and dad to our kids and to me when I needed it. Any success I have is because of you.

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Abstract of Thesis Presented to the Graduate School
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STRUCTURE-ACTIVITY RELATIONSHIP OF AN AGOUTI-RELATED PROTEIN-DERIVED DECAPEPTIDE AT THE MURINE MELANOCORTIN RECEPTORS

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Obesity is a complex, multi-factorial chronic disease involving several contributing factors. The melanocortin neurohormone messenger system is thought to play a role in modulating the obesity phenotype. In particular, Agouti-related protein (AGRP), an endogenous antagonist of melanocortin receptors, interacts with the human brain melanocortin receptors hMC4R and hMC3R. When AGRP is over-expressed centrally in transgenic mice, the result is an obese phenotype. Thus, AGRP expression in the hypothalamus is thought to be involved in the regulation of energy homeostasis at the brain melanocortin receptors. We hypothesized that the AGRP antagonist activity is determined by the structure-function relationship between specific amino acids in AGRP and its receptor.

The decapeptide, Tyr-[Cys-Arg-Phe-Phe-Asn-Ala-Phe-Cys]-Tyr, which is derived from the AGRP C-terminal domain, has been reported to possess μM antagonistic properties at the hMC4R. We synthesized this decapeptide using a manual solid-phase

peptide synthetic strategy. In addition, we pharmacologically characterized this decapeptide at the murine melanocortin receptors, mMC1R, mMC3R, mMC4R and mMC5R. In particular, we used the competitive displacement of I^{125}-MTII, a radio-labeled melanocortin agonist, to determine binding and the β-galactosidase bioassay to determine receptor activity.

We found the decapeptide, Tyr-[Cys-Arg-Phe-Phe-Asn-Ala-Phe-Cys]-Tyr, to be a weak antagonist at the mMC4R ($pA_2 = 6.79$) and to possess agonist activity (2.89 ± 2.26 μM) at the skin mMC1R. Further, the decapeptide did not have appreciable activity at the mMC3R or mMC5R. The agonist activity at the mMC1R skin receptor was unexpected and contrasts with a previous report that AGRP has no activity at the MC1R. Our study provides experimental evidence that the C-terminal portion of AGRP contains a specific recognition sequence for melanocortin receptor activity. Furthermore, we conclude that the conformational shape which the AGRP protein attains is a significant determinant of AGRP activity at the melanocortin receptors.

CHAPTER 1
BACKGROUND AND SIGNIFICANCE

Obesity is a highly prevalent chronic disease in humans. Based on data from the National Health and Nutrition Examination Survey (NHANES) from 1999-2002, 30 percent of adults 20 years of age and older (greater than 60 million people), had a body mass index (BMI) of 30 or greater. This is significantly increased from 23 percent in 1994.[1] The high prevalence of obesity gives rise to increasing numbers of patients with obesity-related complications such as musculoskeletal disorders, cardiovascular disease, decreased respiratory function and diabetes mellitus. These sequelae have a significant negative impact on patients' quality of life as well as on health care cost. Furthermore, because of these serious complications, obesity is now considered the second leading cause of preventable death in the U.S. [1, 2] Current research on controlling obesity in humans involves focus on the factors involved in obesity development.

Several factors, including genetic make-up, physiology, environment, behavior and individual psychology all modulate obesity risk and severity. In particular, previous studies identify the function of neuroendocrine messenger systems such as the melanocortin system as a genetic and physiological factor which plays an important role in human weight homeostasis in both animal models and humans[3-6]. Specifically, the melanocortin messengers interact with several other central and peripheral neurohormone messengers to affect weight homeostasis by modifying feeding behavior and thermogenesis. By characterizing the key structural elements of the components of the melanocortin system, researchers hope to uncover a powerful therapeutic target for drug

therapy in an effort to control obesity in humans. The following is a brief overview of the melanocortin system and how it is purported to fit into the complex system of central weight regulation.

Focus on the Melanocortin System Components

The melanocortin system consists of melanotrophic peptides, endogenous peptide inhibitors and receptors. In particular, the melanotrophic peptide hormones are derived from the proopiomelanocortin (POMC) gene transcript. POMC is the source of multiple hormones including adrenocorticotropin (ACTH), ß-lipotropin, met-enkephalin and β-endorphin[7]. The POMC product that is produced at a given time depends on the hormone system that activates POMC transcription. When POMC is activated and transcribed as part of melanocortin activation, it gives rise to α-, γ- and β- melanocyte stimulating hormone (Table 1-1). Most research has focused on α-melanocyte stimulating hormone

Table 1-1 Melanocyte-stimulating hormone peptide structures

Chemical	Sequences
α-MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2
β-MSH	NH_2-Asp-Glu-Gly-Pro-Tyr-Lys-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH
γ-MSH	NH_2-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH

(α-MSH), a 13-amino acid peptide (Figure 1-1) which is the prototype endogenous agonist of the melanocortin system receptors. It is produced both centrally and peripherally and has been found to stimulate the five melanocortin receptor subtypes.

The melanocortin receptors are G-protein coupled receptors (GPCRs). They have the classic GPCR secondary structure consisting of seven lipophilic α-helical membrane-spanning regions. The N-terminus is exposed on the extracellular portion of the membrane while the C-terminus remains intracellular. In this construct, the melanocortin peptides putatively interact with the helical pocket that is formed by the surrounding

transmembrane regions of the receptor. Interaction of the ligand and receptor induces a conformational change in the target receptor resulting in activation of the receptor.

Activated melanocortin receptors interact with a trimeric G-protein. In the case of the melanocortin receptors, this interaction stimulates G_s which activates adenylate cylase (AC). AC triggers an intracellular cascade of signaling in which adenosine triphosphate (ATP) is converted to cyclic adenosine monophosphate (cAMP). Cyclic AMP then acts as a second messenger and modulates subsequent physiological activity[7]. Specifically, melanocortin receptor activation causes an increase in cAMP-mediated phosphokinase A (PKA) activity which activates the cAMP response element binding protein (CREB). Then, CREB interacts with cAMP response element (CRE) on deoxyribonucleic acid (DNA) to activate cell-specific transcription of mRNA resulting in a physiological response.

Five subtypes of melanocortin receptors in several species have been identified and are numbered in the order in which they were originally cloned. Further, murine receptor isolates are homologous to human receptors[8]. The melanocortin 1 receptor (MC1R) is located peripherally in both humans and mice and is found in melanocytes, melanoma cells and macrophages. MC1R is known to affect pigmentation and are reputed to be involved in the inflammatory response[9], however all of the mediators and specific effects of MC1R involvement are as yet unknown. The melanocortin 2 receptor (MC2R) is responsive only to ACTH and is located in all three layers of adrenal cortex as well as on adipocytes. These receptors are involved in adrenal steroidigenesis and also have adrenocorticotrophic effects[10]. The melanocortin 3 receptor (MC3R) is expressed in various areas, particularly in the brain, including the arcuate nucleus and the anterior

ventral periventricular nucleus. In addition, the MC3R is expressed in the heart, placenta, pancreas and gut. MC3R, like the melanocortin 4 receptor (MC4R), discussed below, plays a role in weight homeostasis[11]. The melanocortin 5 receptor (MC5R) is expressed ubiquitously in muscle, liver, spleen, lung, brain, adipocytes, and a variety of other tissues. Deletion of MC5R in animal experiments results in exocrine gland dysfunction, however more research regarding its specific function is needed[12].

The MC4R is primarily expressed in the CNS, specifically in cortex, hippocampus, thalamus and hypothalamus [13]. In terms of function, MC4R is involved in feeding behavior and weight homeostasis in both animal and human models. MC4R knockout mice develop obesity, hyperphagia, hyperinsulinemia and hyperglycemia, indicating that receptor activation is important in inducing satiety and preventing obesity[14].

The melanocortin system is currently the only GPCR system that is known to involve both endogenous receptor agonists *and* antagonists. In particular, the *agouti* gene locus (*ASIP*) encodes Agouti Signaling Protein (ASP), a known antagonist of the melanocortin receptors in animal models[15]. This protein is expressed in the periphery and is responsible for the phenotype of the lethal A_y mouse, in which ASP is ectopically expressed. Phenotypically, lethal A_y mice are obese with a yellow coat color[16] as a result of this ectopic ASP expression. The yellow coat color is a result of a shift in production of eumelanin to pheomelanin[17] that occurs as a result of ASP interaction with MC1R. Also, ASP antagonizes central melanocortin receptors in lethal A_y mice, leading to the obesity trait. Although this antagonism occurs centrally, ASP is not known to be expressed endogenously in the central nervous system. [18] Researchers have used this

result to look for a protein analogous to ASP that is produced centrally and may contribute to the obese phenotype in mice and humans.

Homology studies with ASP in several species lead to the discovery of Agouti-Related Protein, or AGRP, which is a centrally-expressed analog to ASP in both murine and human models[19]. ASP and AGRP are 131-amino-acid proteins in mouse and 132-amino-acid proteins in humans. Further, both have cysteine-rich C-termini with secondary structure that is maintained through disulfide bridges[19-21]. The sequence homology between ASP and AGRP is 40% percent in the C-terminus[19] (Figure 1). AGRP is known to bind and antagonize both MC3R and MC4R, while ASP is active at both MC1R and MC4R[15, 22, 23]. Previous research has identified the triplet of amino acids

```
Human ASP      MDVTRLLLATLLVFLCFFTANSHLPPEEKLRDDRSLRSNSSVNLLDVPSV 50
Human AGRP     -----MLTAAVLSCALLLALPATRGAQMGLAPMEGIRRPDQALLPELPGL 45

               SIVALNKKSKQIGRKAAEKKRSSKKEASMKKVVRPRTPLSAP-CVATRNS 99
               GLRAPLKKTT--AEQAEEDLLQEAQALAEVLDLQDREPRSSRRCVRLHES 93

               CKPPAPACCDPCASCQCRFFRSACSCRVLSLNC------ 132
               CLGQQVPCCDPCATCYCRFFNAFCYCRKLGTAMNPCSRT 132
```

Figure 1-1 Alignment of Human ASP and Human AGRP in ClustalW[24]. The C-terminus is in bold-face and the portion used to derive the decapeptide is indicated in italics. The common Arg-Phe-Phe (RFF) motif is shaded.

at positions hAGRP (111-114), Arg-Phe-Phe, as necessary for MC4R antagonism.[25] Further, structural studies indicate that these residues form an active loop region in the peptide which interacts with the melanocortin receptors[20]. Short cyclic fragments of the C-terminus of ASP and AGRP that contain the Arg-Phe-Phe domain were shown by Tota et al to possess potent antagonist activity at MC4R but 10-fold lower activity and binding than that of the full C-terminus[25]. In addition, no activity was seen for these fragments at MC3R.

The purpose of the current study is to identify binding interactions between synthetic melanocortin receptor antagonists and the melanocortin receptors in order to logically design molecules to modulate the activity of melanocortin receptors and also to provide information about the structure of these receptors and antagonists *in vivo*. Specifically, this study looks at the interaction of a synthetic cyclic decapeptide derived from AGRP with all five murine melanocortin receptors. A brief overview of the synthetic strategy used to synthesize the test compound follows. The specific synthetic scheme is given in the Methods section of Chapter 2.

Solid Phase Peptide Synthesis: "FMOC" Chemistry

Solid-phase peptide synthesis was conceived by R.B. Merrifield in 1959[26]. The strategy involves the covalent attachment of a nascent peptide chain to an insoluble polymeric support resin. The resin is maintained in a filter system that allows the peptide to be anchored and easily separated from reaction reagents. This procedure allows the use of excess soluble reagents which can be filtered and washed from the reaction vessel once amino-acid coupling reactions have terminated. The use of excess reagents allows the reactions to be driven to high yields (>90%). In addition, the catalysis of these reactions minimizes the formation of side products. By using a series of deprotection and coupling steps, the reasearcher can select specific reactive moieties on the peptide to participate in chemical reactions while others remain protected. Since this rationally designed system of synthesis is generous in allowing high concentrations of reagents and the synthesized molecule is easily separated from reagents, the system lends itself well to automation[27].

Synthetic methods of solid phase peptide synthesis are differentiated based on the protection scheme used. Protection schemes require the use of both N-α- and side chain

protection moieties so that the exposed reactive portions of the nascent peptide can be manipulated separate from each other. During peptide synthesis, temporary N-α-protecting groups are removed with each amino-acid coupling step to reveal the portion of the amino acid which will be coupled to the next residue. Permanent side chain protecting groups are used to protect vulnerable side chain groups during coupling reactions and are removed altogether after synthesis has been completed.

The "FMOC" synthetic strategy was used to synthesize the test compounds in this study. This strategy is based on an orthogonal system of protecting groups developed by Carpino and Han[28]. Orthogonal schemes are based on using two or more classes of protecting groups that can be removed by differing chemical mechanisms. FMOC chemistry utilizes molecules constructed using a base labile "temporary" Nα-9-fluorenylmethyloxycarbonyl (FMOC) group. The permanent protecting groups are primarily based on tertiary-butanol esters and ethers which provide bulky, base-stable protection to otherwise labile amino acid side chains. During synthesis, the temporary FMOC group is removed sequentially after each amino acid is added in order to form the growing peptide chain while the permanent protecting groups remain intact until the end of synthesis[29].

The FMOC synthetic scheme described above is used in this study to produce synthetic peptides for use in research on the melanocortin system. In particular, this study is focused on Agouti-related protein (AGRP), a component of the melanocortin system that is of particular interest as an obesity target. In particular, previous research establishes that AGRP participates in normal signaling for human feeding behavior and energy homeostasis[30]. Therefore, structural alterations in AGRP may affect this normal

signaling and thereby contribute to the development of obesity. To structurally characterize the activity of AGRP at the melanocortin receptors, biochemists have isolated the cyclic decapeptide Tyr-[Cys-Arg-Phe-Phe-Asn-Ala-Phe-Cys]-Tyr from the AGRP C-terminus[25]. According to Tota et al., this small portion of the AGRP molecule binds to and antagonizes the human MC4R. In order to verify this finding which establishes the cyclic decapeptide as the active portion of AGRP and further analyze its interaction with melanocortins receptors that were previously unstudied, we synthesized the decapeptide using manual solid phase peptide synthesis using the FMOC strategy described by Stewart et al. [31]. We then pharmacologically characterized the peptide at the 5 murine melanocortin receptors. We used the murine model of melanocortin receptor action to test our hypothesis that the cyclic decapeptide Tyr-[Cys-Arg-Phe-Phe-Asn-Ala-Phe-Cys]-Tyr is the minimally active fragment of the AGRP molecule and will thus have antagonist properties at the mMC3R and mMC4R. Further, the decapeptide will not have significant activity, similar to its parent AGRP, at other murine melanocortin receptors.

CHAPTER 2
MATERIALS AND METHODS

The synthesis of the cyclic decapeptide Tyr-[Cys-Arg-Phe-Phe-Asn-Ala-Phe-Cys]-Tyr was accomplished by manual solid phase peptide synthesis using the FMOC synthetic strategy and then tested *in vitro* for binding and activity at the murine melanocortin receptors.

Synthesis of the Tyr-[Cys-Arg-Phe-Phe-Asn-Ala-Phe-Cys]-Tyr Cyclic Decapeptide

Solid Phase Peptide Synthesis Preparation

Reaction reagents and their structures are listed in the Appendix and corresponding reaction quantities are shown in Table 2-1. All peptide reagents and resin were obtained from Peptides International (Louisville, KY, USA). All reagents and solvents were American Chemical Society (ACS) grade or higher.

Table 2-1 Amino acids used and quantities

Amino Acid	Protection Scheme	Equivalents	Molar quantity (mmol)	Molecular Weight	Amount (g)
Cys	FMOC/Trt	3.13	0.76	585.73	0.45
Phe	FMOC	3.13	0.76	387.44	0.29
Ala	FMOC·H_2O	3.13	0.76	329.36	0.25
Asn	FMOC/Trt	3.13	0.76	596.69	0.45
Arg	FMOC/Pbf	3.13	0.76	648.78	0.49
Tyr	FMOC/tBu	3.13	0.76	459.55	0.35

The manual FMOC synthesis was conducted in a reaction vessel consisting of a glass-fritted filter and a valve separating two ports. First, the glass reaction vessel was acid-washed with chromerge and concentrated H_2SO_4. The vessel was then silanized using 10% dichloromethylsilane (DCMS) in dry toluene and mixed using helium gas for

15 to 30 minutes. After mixing, the reaction vessel was rinsed with toluene, then treated with dry methanol for 15 minutes, and then dried with acetone.

Upon assembly of the reaction apparatus, one port of the reaction vessel was attached to nitrogen gas and the other to a vacuum line for waste removal. The reaction vessel was then mounted to a ring stand inside a vertical laminar flow hood. A waste flask was secured beneath the reaction vessel (Figure 2-1). The gas and vacuum lines were primed.

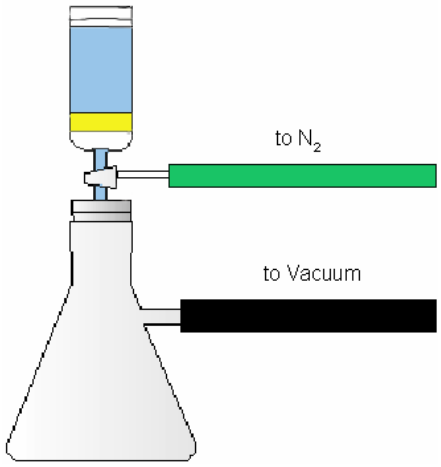


Figure 2-1 This glass fritted filter glass container contains a stem with a bidirectional valve. The filter flask is attached to a ring stand and placed above and connected to a waste flask. The bidirectional valve is connected to a nitrogen gas line and the waste flask is attached to a vacuum source.

FMOC-Wang resin[32] beads (Appendix) were added to the pre-weighed reaction vessel and weighed. Since the dry polystyrene resin beads have an average diameter of 50 µm, the beads were then soaked in dimethylformamide (DMF) for two hours to promote swelling. After this process, the beads obtained 2.5 to 6.2 times their dry volume and formed a well-solvated gel. This maximally increases the bead surface area and exposes the reagent-accessible chemical attachment points.

Manual Solid Phase Peptide Synthesis

The synthesis procedure is outlined in Table 2-2**.** After copious rinsing of the solvated resin with DMF, the beads were rinsed with 20% piperidine for 2 minutes, drained, and then mixed again for 18 minutes in 20% piperidine to remove the FMOC protecting group (Figure 2-2). The resin was then rinsed with DMF and the Kaiser ninhydrin test [33] (described below) was conducted to observe the extent of deprotection.

Table 2-2 Protocol for manual FMOC chemistry synthetic strategy

Cycle	Function	Time
1	DMF wash	5 X 1min
2	20% Piperidine in DMF rinse	2 min
3	20% Piperidine in DMF deprotection	18 min
4	DMF wash	4 X 1 min
5	Ninhydrin Test	3 min
6a-d	Add : FMOC-AA dissolved in DMF (3 eq.) BOP (3 equiv.) HOBt (3 equiv.) DIEA (5.1 equiv.)	
7	Coupling	
8	Ninhydrin Test	3 min
9	DMF wash	3 X 1

For the ninhydrin test, a small sample of the resin was removed by capillary pipette and placed into a small glass vial. Two drops of each of the Kaiser ninhydrin test solutions A, B and C (Appendix) were added to the vial. The vial was then heated to approximately 100°C for 3 minutes. A positive test indicating complete deprotection of the free peptide chain amino group was given by a homogeneous deep blue color. If a negative test was obtained, the piperidine deproctection step was repeated until the ninhydrin test was positive.

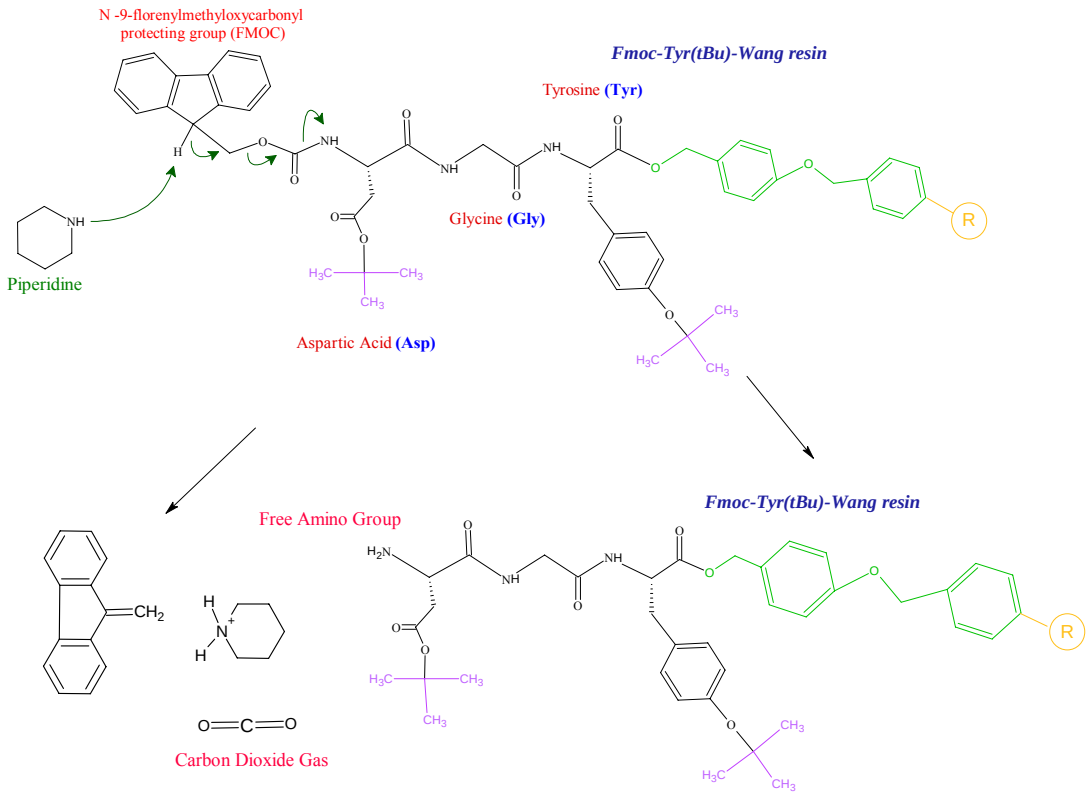


Figure 2-2 An example of the deprotection scheme using FMOC chemistry. R = resin bead.

After confirmation of successful deprotection, the next amino acid in the decapeptide sequence was added to the reaction vessel in the amount given in Table 2.1. In addition, benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) in 3-fold excess (Appendix) was added to accelerate the formation of the -Obt ester form of the FMOC amino acids (Figure 2-3). Also, 1-hydroxybenzotriazole (HOBt) (Appendix) in 3-fold excess was used for three reasons: (1) to accelerate the cabodiimide-mediated couplings, (2) to suppress racemization and (3) to inhibit dehydration of the carboxamide side chains of Asn and Gln to the corresponding nitriles. Finally, N,N-diisopropylethylamine (DIEA) in 3.1 fold excess was used to stabilize side products of the amino acid coupling reaction (Figure 2.3). Completion of the amino acid

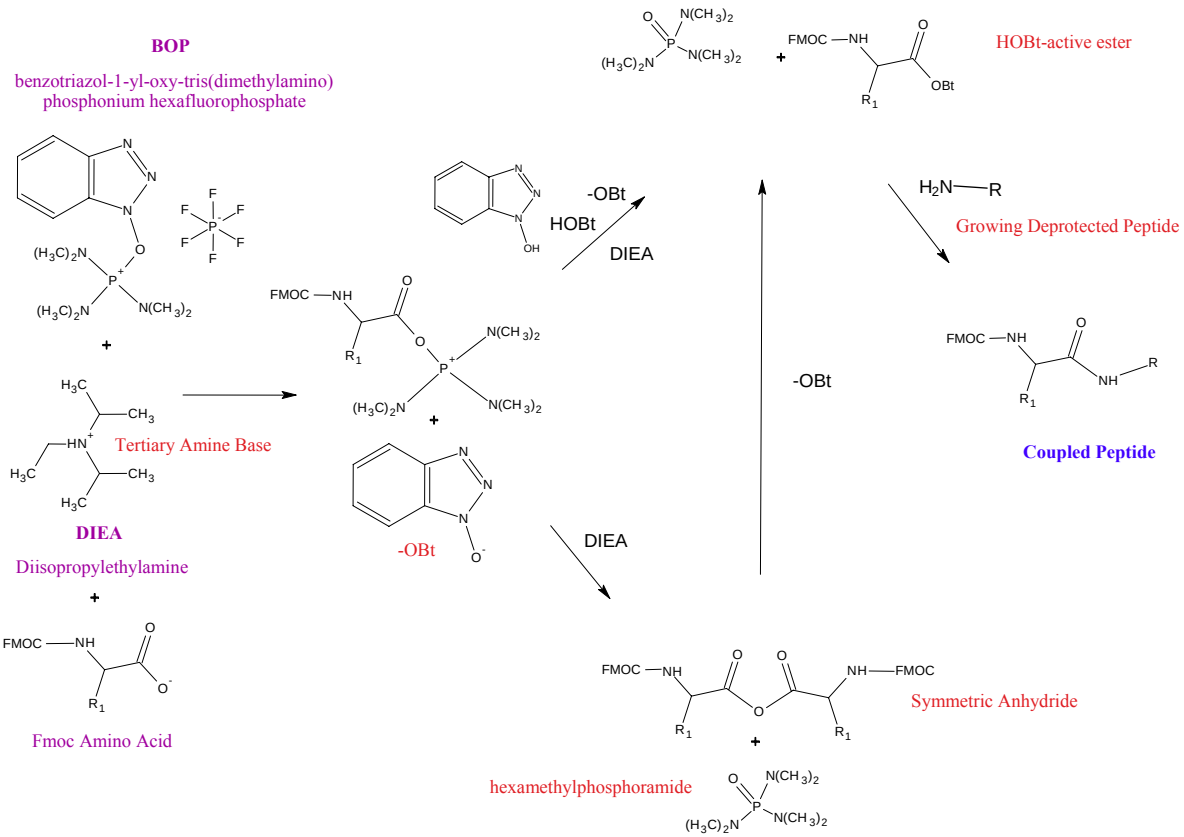


Figure 2-3 Activation of the coupling reaction by BOP.

addition and FMOC deprotection was monitored using the ninhydrin test. After repeating the procedure nine times to complete the linear peptide Tyr-Cys-Arg-Phe-Phe-Asn-Ala-Phe-Cys-Tyr, the reaction vessel containing the resin and newly formed peptides was dried under vacuum overnight. The weight of the peptides and resin combination was determined after drying was complete.

Removal of Orthogonal Protecting Groups and Cleavage of the Peptide from the Resin

The orthogonal protecting groups on the peptide amino acids were cleaved from the peptide in the same step as the peptide cleavage from the resin beads. This was accomplished using a trifluoroacetic acid (TFA), 1,2 ethanedithiol, p-cresol, and water cleavage cocktail in a 15:3:1:1 ratio (Figure 2-4).

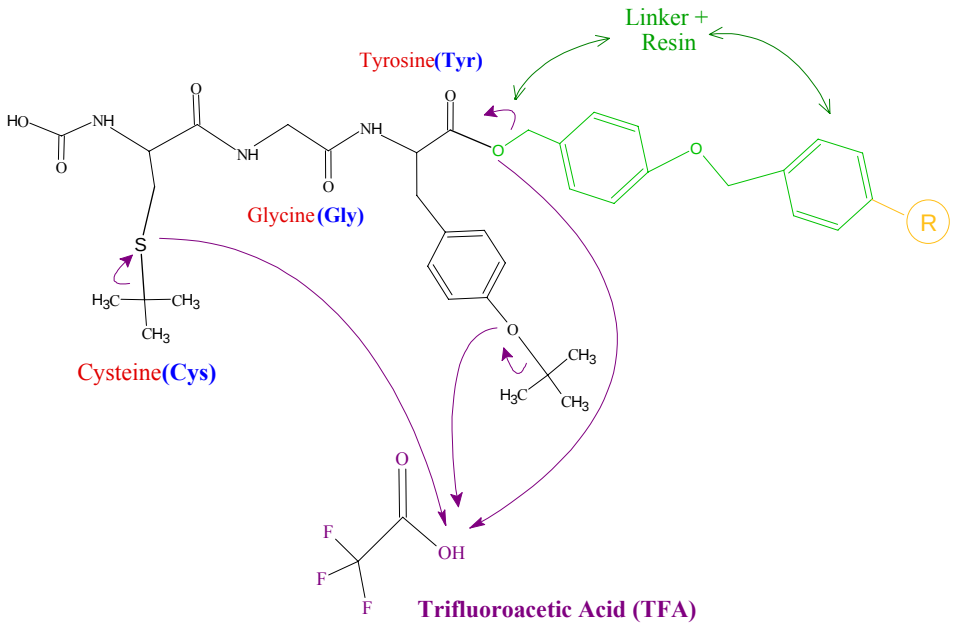


Figure 2-4 Cleavage of the peptide chain from the resin bead by TFA.

Five milliliters of the cleavage cocktail were added to a medium round bottom flask with the dried resin and allowed to mix for 2 hours at 0°C and then 30 to 45 minutes at room temperature. The decapeptide was then separated from the resin by filtration and the resulting solution was concentrated. After concentration, the peptide was extracted using water and ethyl ether and the extract was concentrated until a white flocculent appeared. The resulting crude peptide was then lyophilized.

Analysis of the Linear Peptide

The success of the manual linear peptide synthesis was confirmed using high performance liquid chromatography (HPLC) purification with a Shimadzu® chromatography system with a photodiode array. The structure was confirmed with gas chromatography/ mass spectroscopy (GC/MS).

Cyclization of the Linear Peptide

Upon confirmation of the weight of the linear peptide by analytical methods, a disulfide bridge was formed[34] between the two cysteine residues on the linear decapeptide Tyr-Cys-Arg-Phe-Phe-Asn-Ala-Phe-Cys-Tyr. Half of the crude peptide was dissolved in 1.5 L 0.1% degassed acetic acid in water. The pH was adjusted from 3.3 to 8.5 with concentrated ammonium hydroxide and the solution was then mixed overnight. After mixing, the pH of the solution was lowered to 4.0 with glacial acetic acid. A non-measured amount of HCl-amber-lite ion exchange resin was added and this combination was mixed until the yellow solution turned clear (~1 hour). The exchange resin was then filtered into a large round bottom flask and concentrated. The resulting compound, Tyr-[Cys-Arg-Phe-Phe-Asn-Ala-Phe-Cys]-Tyr was lyophilized.

Analysis of the Cyclized Peptide

The final cyclized product was analyzed by MALDI-TOF mass spectroscopy at the University of Florida Protein Core Facility. Final peptide purification was achieved using a semi-preparative RP-HPLC C_{18}- bonded silica column (Vydac® 218TP1010, 1.0 X 25 cm). The structure was assessed by analytical RP-HPLC and 2D ^{1}H NMR. (University of Florida protein core facility).

Biological Assays

Cell Culture and Transfection

HEK-293 cells (human embryo kidney cells transformed with human adenovirus type 5) were maintained in Dulbecco's modified Eagle's medium (DMEM), 10% penicillin-streptomycin and 10% fetal calf-serum. The cells were seeded 1 day prior to transfection at 1-2 x 10^6 cells/100mm. Murine melanocortin receptor cDNA (coding deoxyribonucleic acid) (20 μg) cloned into in the pcDNA3 expression vector in a

previous experiment was transfected into the seeded HEK-293 cells using the calcium phosphate method[35]. G418 sulfate aminoglycoside ($C_{20}H_{40}N_4O_{10}.2H_2SO_4$) selection[36] was used to isolate stable receptor populations (1 g/mL).

Receptor Binding Assays

HEK-293 cells that were transfected and were stably expressing the various melanocortin murine receptors (mMC1R, mMC3R, mMC4R and mMC5R) were maintained in Dulbecco's modified Eagle's medium (DMEM), 10% penicillin-streptomycin and 10% fetal calf-serum. One day prior to conducting the binding studies, transfected cells were plated into Primera 24 well plates (Falcon) at a density of 0.1-0.3 x 106 cells perwell. Concentrations of 10^{-6} to 10^{-12} M MTII (Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Lys]-NH_2), a potent melanocortin receptor agonist[37], and 10^{-4} to 10^{-10} M Tyr-[Cys-Arg-Phe-Phe-Asn-Ala-Phe-Cys]-Tyr were used to competitively displace MTII that was radio-labeled with I^{125} (100000 cpm/well; NEN Life Sciences). A 450 µL solution of the concentration of decapeptide being tested was added to each well. Next, 50 µL of I^{125} MTII was added to each well and incubated at 37°C for 1 hour. The medium was then rinsed and washed with assay buffer (DMEM, 0.1 mg/ml and Bovine Serum Albumin (BSA)). The cells were lysed with 0.5 mL 0.1 M NaOH and 0.5 mL 1% Triton X-100 for 10 minutes, and then transferred to 16 X 150 mm glass tubes.

Quantification of the Receptor Binding Assays

The binding of I^{125}-radiolabeled MTII was measured using a γ-counter. Dose-response curves and IC_{50} values for 10^{-6} to 10^{-12} M MTII and 10^{-4} to 10^{-10} M Tyr-[Cys-Arg-Phe-Phe-Asn-Ala-Phe-Cys]-Tyr were generated and analyzed by nonlinear least-squares analysis. The IC_{50} values represent the mean ($\pm$ standard deviation) of duplicate wells generated in at least two independent experiments.

β-galactosidase Bioassay

HEK-293 cells stably expressing melanocortin receptors (mMC1R, mMC3R, mMC4R, mMC5R) were transfected with 4 μg of the CRE/β-galactosidase reporter gene[38] using the same transfection method described above. Primera 96-well plates were treated with 5000-15000 post-transfection cells and incubated overnight. Forty-eight hours post-transfection, cells were stimulated with MTII (with 10^{-6} to 10^{-12} M), Tyr-[Cys-Arg-Phe-Phe-Asn-Ala-Phe-Cys]-Tyr (10^{-4} to 10^{-10} M) and forskolin (10^{-4} M) in assay medium (DMEM containing 0.1mg/mL BSA and 0.1 mM isobutylmethylxanthine) for 6 hours. The assay medium was aspirated and 50 μL of lysis buffer (250 mM TrisHCl, pH 8.0, and 0.1% Triton X-100) was added to each well. The plates were then stored at -80°C overnight. The next day, the plates containing the cells lysates were thawed and10 μL aliquots were taken from each well and transferred to another 96-well plate. The 10 μL samples were set aside for relative protein determination. Phosphate-buffered saline (PBS) with 0.5% BSA (40 μL) was then added to each well of the original plates of cell lysate. Further, 150 μL of substrate buffer (60 mM sodium phosphate, 1 mM $MgCl_2$, 10 mM KCl, 5 mM β-mercaptoethanol, 200 mg/mL 2-Nitrophenyl-β-D-galactopyranoside (ONPG)) was added to each well and the plates were incubated at 37°C.

Quantification of β-galactosidase Bioassay

The sample absorbance OD_{405} was measured using a 96-well plate reader. The relative protein value was determined by adding 200 μL 1:5 dilution of G250 protein dye (Bio-Rad):water to the 10 μL cell lysate aliquots taken previously. In addition, the OD_{595} was measured. Furthermore, the transfection efficiency of the CRE-β-galactosidase reporter assay was determined using 10^{-4} M forskolin treatments of 6 wells of each plate as controls. Data points were normalized both to the relative protein content and non-.

Analysis of Bioassay Data

Data analysis was conducted and IC_{50} and EC_{50} values were determined by using nonlinear regression analysis with the PRISM program (v2.0, GraphPad Inc.). In addition, pA_2 values quantifying antagonism were generated using the Schild analysis method.[39] The EC_{50} and pA_2 values represent the mean (+standard deviation) of triplicate wells examined in at least two independent experiments.

CHAPTER 3
RESULTS

The synthesis of the melanocortin decapeptide, Tyr-[Cys-Arg-Phe-Phe-Asn-Ala-Phe-Cys]-Tyr, was conducted with a yield of 0.5g of product. We confirmed that each of the coupling steps in the synthesis was completed using the ninhydrin test as previously described. The purified peptide was > 99% pure and the sequence was determined by methods described previously to be the sequence of the desired decapeptide with the correct molecular weight.

Both the binding activity and function of the decapeptide at the murine melanocortin receptors was determined.

Receptor Binding

The hAGRP decapeptide bound extensively to the mMC4R (IC_{50} = 275 ± 62 nM). Additionally, the decapeptide possessed μM binding IC_{50} values at mMC3R (IC_{50} = 11.7 ± 3.9 μM), mMC5R (IC_{50} = 38.7 ± 23.5 μM), and mMC1R (IC_{50} = 3.09 ± 2.19 μM).

Receptor Antagonist Activity

The hAGRP decapeptide was an antagonist of mMC4R (pA2 = 6.8 ± 0.4) (Figure 3-1). In addition, it has only slight antagonist activity at the mMC3R which was not significant enough to quantify, and lacked antagonist activity at mMC5R at concentrations up to 100 μM.

Receptor Agonist Activity

The hAGRP decapeptide was found to have agonist activity at mMC1R (EC_{50} = 2.89 ± 2.26 μM) (Figure 3-2).. No agonist activity was detected for the decapeptide at any of the other melanocortin receptors (mMC3R, mMC4R, mMC5R).

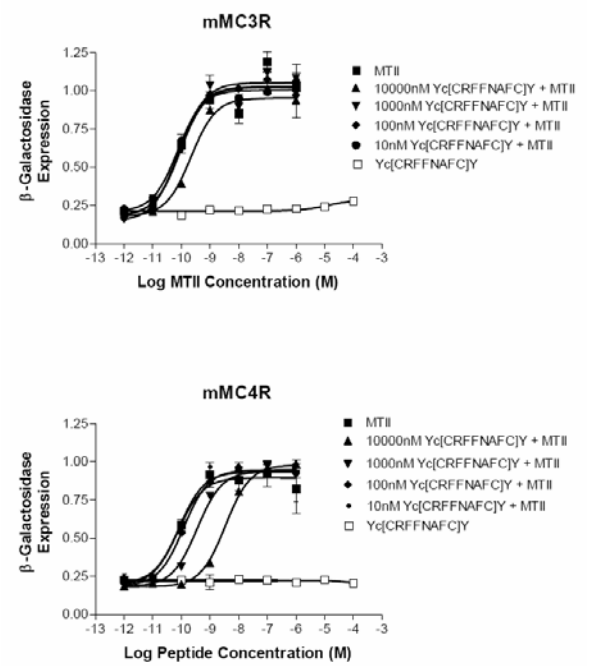


Figure 3-1 β-galactosidase expression is given for varying concentrations of decapeptide with the varying concentrations of MTII**.**

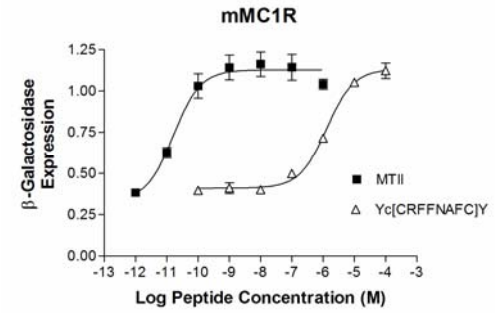


Figure 3-2 β-galactosidase expression is given for varying concentrations of decapeptide peptide concentration. For comparison, the β-galactosidase expression for MTII is given.

CHAPTER 4
DISCUSSION AND CONCLUSION

Discussion

Our most significant finding in this study is that the AGRP decapeptide Tyr-[Cys-Arg-Phe-Phe-Asn-Ala-Phe-Cys]-Tyr has both unexpected agonist activity at the mMC1R while also being an antagonist of the mMC4R. The antagonist activity at mMC4R that we observed is consistent with our hypothesis and previous research[30], however, the agonist activity at mMC1R has not been described in previous studies. Furthermore, our findings imply that the conformational space that AGRP occupies *in vivo* is related to the activity of AGRP at the melanocortin receptors.

The finding that the AGRP decapeptide has activity at the mMC1R is in contrast with previous research which has shown that the parent AGRP protein has no activity at the skin mMC1R[22]. These previous studies are supported by the finding that transgenic mice ectopically expressing AGRP have the brown (wild-type) coat phenotype. This previous result indicates that AGRP does not shift the production of eumelanin to pheomelanin in these transgenic mice and, thus, AGRP has no activity at skin mMC1R. In our study, we also expected to find that the AGRP decapeptide that we studied would have no agonist activity at the mMC1R. However we observed that the AGRP decapeptide has micromolar agonist activity at the mMC1R and sufficient binding at this receptor (3.09 ± 2.19 μM) to explain this activity as a direct receptor effect.

Since the AGRP decapeptide amino acid sequence is derived from the sequence of its parent protein, AGRP, the contrasting activity between the decapeptide and its parent

peptide at the mMC1R cannot be explained by the decapeptide amino acid sequence alone. Specifically, the decapeptide possesses the identical amino acid sequence as the active portion of the human and murine AGRP C-terminus (108-177)[25]. Although the decapeptide has some distinct chemical moieties in analogous positions of the human and murine Agouti Signal Protein (Figure 4.1), the decapeptide retains the Arg-Phe-Phe motif in a conserved position between AGRP and ASP. This Arg-Phe-Phe sequence is thought to be essential for the antagonist activity of the melanocortin antagonists[40]. Moreover, in contrast to AGRP, when mASP is ectopically expressed in transgenic mice, mice with the agouti coat color are produced. The resulting agouti coat color indicates that mASP is an antagonist at the skin mMC1R[41]. Thus, although the AGRP decapeptide sequence is derived from a consensus sequence of the AGRP and ASP C-terminus, the AGRP decapeptide itself has different pharmacological activity than both of its parent proteins.

Previous research has indicated that the conserved Arg-Phe-Phe (RFF) motif found in both the ASP and AGRP active sites may be important for receptor recognition of these endogenous antagonists. In support of this idea, studies have shown that mutation of these Arg-Phe-Phe residues in AGRP results in considerably less efficacious antagonism at the melanocortin receptors.[4] Furthermore, homology exists between the Arg-Phe-Phe motif found in the melanocortin endogenous antagonists and the conserved His-Phe-Arg-Trp residues in the endogenous melanocortin ligands. Specifically, the Arg-Phe-Phe motif may be mimicking the His-Phe-Arg-Trp molecular interactions and allowing the melanocortin receptors to recognize AGRP and ASP. Moreover, previous studies have found that the tetrapeptide Ac- His-Phe-Arg-Trp -NH_2 is the minimal fragment of melanocortin agonists required to produce a physiological response (μM) in

the classic frog skin bioassay[42]. Therefore, our study provides the first experimental data to support the hypothesis that the conserved antagonist Arg-Phe-Phe residues may be mimicking the agonist His-Phe-Arg-Trp interactions with melanocortin receptors, specifically at the skin mMC1R.

Our finding that the decapeptide Tyr-[Cys-Arg-Phe-Phe-Asn-Ala-Phe-Cys]-Tyr has agonist activity at MC1R is consistent with a model in which the conformational shape that AGRP adopts determines its activity at its receptor. Since the decapeptide was synthesized to mimic a single portion of the AGRP C-terminus, our study supports the hypothesis that the amino acids adjacent to the decapeptide residues on the parent AGRP peptide influence the conformational shape of the entire peptide. Thus, the spatial relationships between the antagonist residues and the residues on the receptor are important for determining the activity of AGRP at the melanocortin receptors.

Some experimental characteristics of our study resulted in some differences in our findings and those from previous research in human melanocortin receptors and may limit the comparison between our results and those previous. In particular, the binding affinity that we found for the hAGRP at the mMC3R is 10-fold less potent than the binding affinity previously reported for hMC3R. In addition, the binding affinity we report for mMC4R is 4-fold less potent than the binding affinity reported for hMC4R. We attribute these differences to two main experimental differences between our study and previous studies: the species of receptor used and the differences in the radio-labeled compound used. In our research, we used murine melanocortin receptors with the intention of further reproducing our results *in vivo* in murine models, whereas previous researchers have used human melanocortin receptors. Species differences have been

previously noted with melanocortin receptors[42] and therefore further studies should be conducted with human subjects to confirm our results. In addition, structural differences distinguish the linear compound I^{125}-NDP-MSH used by Tota et al. for binding activity assays and the cyclic compound I^{125}MTII that we used for these assays. However, despite the differences in the radio-labeled compound used, we tested both I^{125}-NDP-MSH and I^{125}MTII at mMC1R, mMC3R, mMC4R, and mMC5R and found them to have similar IC_{50} values at each receptor. Therefore, our binding assay results using I^{125}MTII are comparable to previous research with either compound.

Conclusion

Researchers have proposed that the Arg-Phe-Phe sequence located in the C-terminus of agouti signal protein and AGRP is intimately involved in molecular recognition and antagonist activity of these molecules and the melanocortin receptors. The Arg-Phe-Phe sequence conserved in both of these compounds is structurally related to the conserved sequence His-Phe-Arg-Trp found in the melanocortin-stimulating hormone compounds. Furthermore, the Arg-Phe-Phe sequence may be mimicking the interactions of the sequence His-Phe-Arg-Trp with the melanocortin receptors. The results of this study provide evidence that supports this hypothesis.

APPENDIX
CHEMICAL STRUCTURES

Table A-1 Chemical structures for FMOC amino acids

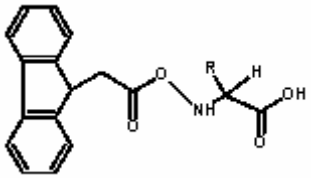
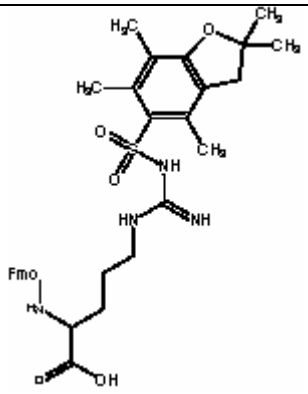
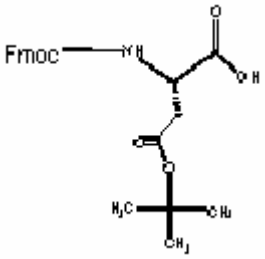
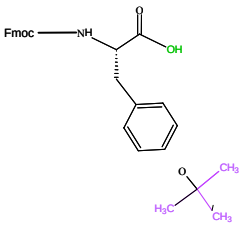
Reagent	Structure
9-fluorenyl-methoxy-carboxyl with (FMOC) Amino Acid	
Fmoc-Arg(Pbf)	
Fmoc-Asp(tBu)	
Fmoc-Tyr (tBu)	

Table A-1 Continued

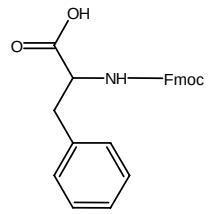
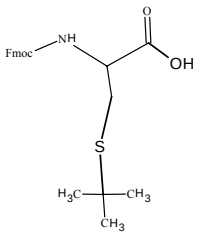
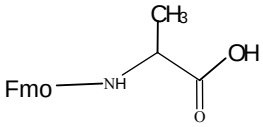
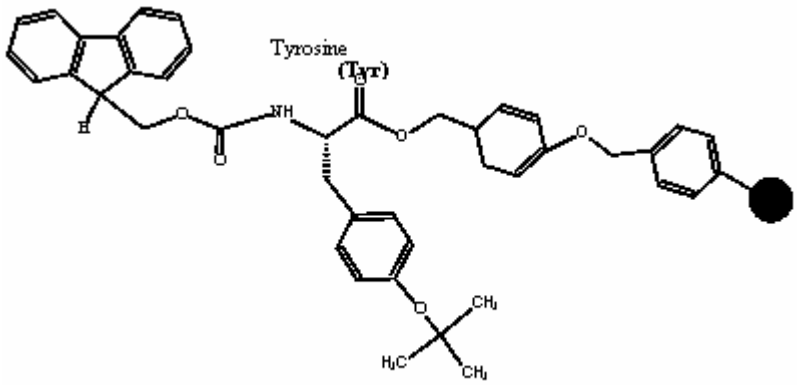
Reagent	Structure
Fmoc-Phe	
Fmoc-Cys (Trt)	
Fmoc-Ala	
Fmoc-Tyr(tBu)-Wang resin	

Table A-2 Chemical structures for FMOC reagents

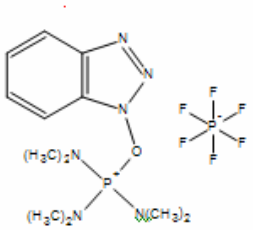
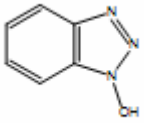
Reagent	Structure
benzotriazol-1-yl-oxy-tris(dimethylamino phosphonium hexafluorophosphate (BOP)	
1-hydroxybenzotriazole (HObt)	

Table A-3 Various reagents for Solid Phase Synthesis

Benzotriazol-1-yl-oxy-tris(dimethylamino) phosphonium hexafluorophosphate (BOP):	3-fold excess, 0.34g/amino acid
N,N-diisopropylethylamine (DIEA)	5.1-fold excess, 218 µL 10%: 50mL DIEA q.s. to 500mL with DMF
1-hydroxybenzotriazolemonohydrate (HOBt) (1M)	(light sensitive): 27.2g HOBt q.s. to 200mL with DMF (FW= 135.13) 760 uL (1 mmol)
N,N-dimethylformamide (DMF)	(freshly distilled on sieves before use)
20% Piperidine in DMF (2:1)	
Kaiser Solution A	0.01M potassium cyanate (KCN) (33mg KCN q.s to 50mL H_2O) added to 98mL Pyridine (bubbled with helium)
Kaiser Solution B	2.5g Ninhydrin q.s. to 50mL with n-butanol
Kaiser Solution C	80g phenol in 20mL n-butanol (leave in warm place overnight)

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BIOGRAPHICAL SKETCH

Anzeela Mulaiya Schentrup was born in Mississauga, Ontario, Canada, to parents of Indian descent. She came to Florida at the age of 9 and grew up near Ft. Lauderdale. She first attended the University of Florida in 1994 and pursued a Bachelor of Arts degree in the history of science, in which she received highest honors for her thesis on pharmacy in Germany at the onset of the Industrial Revolution. Through exposure to the field of pharmacy sciences in the course of writing this thesis, Anzeela decided to pursue a scientific career leading to a graduate degree in medicinal chemistry. After two years, her scientific training stimulated her interest in a career in clinical medicine, so Anzeela left medicinal chemistry to pursue a Doctor of Pharmacy degree, which she received in 2004. During this time, she was encouraged by her former mentor, Carrie Haskell-Luevano, PhD, to pursue the Master of Science in medicinal chemistry for the work she had already completed. In addition, after the birth of her second child, and due to the faith of her current mentor, Julie Johnson, PharmD, Anzeela decided to return to graduate research in pursuit of the Doctor of Philosophy degree in pharmacogenomics. While in this degree program, Anzeela completed the Master of Science degree in medicinal chemistry and continues to pursue her doctorate. Although her educational background is diverse, all of the disciplines in which she has studied and developed a level of expertise have contributed to her current understanding of pharmacy practice and ability to perform translational research. Currently, Anzeela lives in Gainesville, Florida, with her husband, Joseph Côté, and two children, Ena Marie and Maximilian Côté.