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A Survey of Stapling Methods to Increase the Affinity, Activity and Stability of Ghrelin Analogues

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Abstract

The growth hormone secretagogue receptor (GHSR) is a G protein-coupled receptor which regulates various important physiological and pathophysiological processes in the body. Ghrelin is the primary high affinity endogenous ligand for GHSR and has limited secondary structure in solution, which makes it proteolytically unstable. This inherent instability in ghrelin can be overcome by incorporating helix-inducing staples that stabilize its structure and improve affinity and activity. We present an analysis of different stapling methods at positions 12 and 16 of ghrelin(1-20) analogues with the goal of increasing proteolytic stability and to retain or improve affinity and activity towards GHSR. Ghrelin(1-20) analogues were modified with a wide range of chemical staples, including a lactam staple, triazole staple, hydrocarbon staple, Glaser staple, and xylene-thioether staple. Once synthesized, the analogue affinity and α-helicity were measured using competitive binding assays and circular dichroism spectroscopy, respectively. Generally, an increase in alpha-helicity using a flexible staple linker led to improved affinity towards GHSR. Ghrelin(1-20) analogues with a lactam, triazole, and hydrocarbon staple resulted in helical analogues with stronger affinity towards GHSR than unstapled ghrelin(1-20), a compound that lacks helical character. Compounds were also investigated for their agonist activity through β-arrestin 1 & 2 recruitment BRET assays and for their metabolic stability through serum stability assays.

Keywords

Ghrelin receptor, GHSR-1a, G-protein coupled receptors, ghrelin receptor agonists, cancer cachexia, molecular imaging, peptide staples, helix-inducing staples, solid-phase peptide synthesis.

Summary for Lay Audience

The ghrelin receptor is a receptor in our bodies that is responsible for our ability to feel hunger. This receptor is activated by a hormone called ghrelin, which is a peptide molecule made up of 28 amino acids. When the ghrelin receptor is activated by ghrelin, it produces appetite, releases growth hormone, and controls our metabolism. Due to its effects on stimulating appetite, ghrelin has been studied as a potential therapeutic for the treatment of diseases like cancer cachexia, which is the debilitating complication of cancer that causes wasting of the body. Furthermore, the ghrelin receptor has been identified as a potential biomarker for ovarian and prostate cancers, as this receptor is expressed differently in those cancers than in normal tissue. As a result, ghrelin's peptide structure has been used as a template for the design of therapeutics and molecular imaging agents. However, ghrelin is very unstable to the enzymes in our bodies that are effective at breaking down peptides (proteases). Proteases are efficient at breaking down peptides when they have no secondary structure, however studies have demonstrated that they are not able to break down peptides if they are in an alpha-helical confirmation. Thus, peptide chemists have developed methods of inducing alpha-helicity into a peptide structure to improve peptides' biological properties. One of these methods is using chemical staples to lock the peptide into the desired confirmation. The work in this thesis demonstrates introducing 5 different chemical staples into the structure of truncated ghrelin(1-20) compounds and seeing the effects the different staples have on producing helical compounds and their affinity towards the ghrelin receptor. The ability of these compounds to activate the receptor will also be discussed, as well as their stability in human serum.

Co-Authorship Statement

Chapter 1 was written by Juan Esteban and edited by Dr. Leonard G. Luyt.

Chapter 2 is a manuscript in preparation and was written by Juan Esteban and edited by Dr. Leonard G. Luyt. All chemical synthesis, purification, characterization, cell work, bioassays and analysis were performed by Juan Esteban. Materials and lab space for chemical synthesis and chemical purification and characterization were provided by Dr. Leonard G. Luyt. Materials and lab space for cell work and bioassays were provided by Dr. Rithwik Ramachandran.

Chapter 3 was written by Juan Esteban and edited by Dr. Leonard G. Luyt.

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List of Abbreviations

Alloc	Allyloxycarbonyl
Aib	Aminoisobutyric acid
CuAAc	Copper(I)-catalyzed alkyne-azide cycloaddition
CD	Circular Dichroism
Dpr	2,3-Diaminopropionic acid
DCE	1,2-Dichloroethane
DCM	Dichloromethane
DIPEA	N,N-Diisopropylethylamine
DMF	N,N-Dimethylformamide
DMSO	Dimethyl sulfoxide
EDT	Ethane-1,2-dithiol
Fmoc	Fluorenylmethyloxycarbonyl
GHSR	Growth hormone secretagogue receptor
GOAT	ghrelin O-acyl transferase
GPCR	G-protein coupled receptor
HATU	1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate
HCTU	O-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HEK293	Human embryonic kidney 293 cells
HPLC	High-performance liquid chromatography
IC_{50}	Half maximal inhibitory concentration
EC_{50}	Half maximal effective concentration
E_{Max}	Maximum effect
MS	Mass spectrometry
Mtt	4-Methyltrityl
NMR	Nuclear magnetic resonance
PET	Positron emission tomography
Pip	2-phenylisopropyl ester
RCM	Ring closing metathesis

SPECT	Single-photon emission computed tomography
SPPS	Solid-phase peptide synthesis
TBME	Tert-butyl methyl ether
TFA	Trifluoroacetic acid
TIPS	Triisopropylsilane

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Chapter 1

1.0 Introduction

1.1 GHSR-1a and Ghrelin

G-protein coupled receptors (GPCR) have been a prevalent target for pharmaceutical companies due to their wide array of functions in the human body. These transmembrane receptors act as one of the primary messengers in cells for receiving intercellular, nutritional, and environmental information which activate a cellular response. GPCRs are estimated to be targeted by about 35% of all approved drugs in the US market and their global market is expected to reach $3.7 billion by 2027.[1,2] A GPCR that has garnered attention in recent years is the growth hormone secretagogue receptor (GHSR) or ghrelin receptor. This 7-transmembrane GPCR exists as two isoforms, GHSR-1a and GHSR-1b, with only the 1a isoform being functionally active.[3] One of the ghrelin receptor's endogenous ligands is the 28 amino acid peptide, ghrelin. The activation of the ghrelin receptor by ghrelin controls a number of physiological functions such as energy homeostasis, growth hormone secretion and regulation of appetite.[4,5] Due to these physiological properties of GHSR-bound ghrelin, it has been theorized as a potential target for the treatment of cancer cachexia, a cancer complication that affects half of all cancer patients.[6] Additionally, in the last decade, a number of studies have demonstrated that GHSR differential expression is present in prostate and ovarian cancers, making it a suitable target for the molecular imaging of these diseases.[7,8] In more recent years, GHSR has also been shown to be differentially expressed in the myocardium of dilated cardiomyopathy patients and to be upregulated in end-stage heart failure.[9,10] As a result, it is postulated that the ghrelin-GHSR axis has the potential to be a biomarker for heart disease.[10] The imaging of this molecular target may aid in the understanding of fundamental biological processes as well as the molecular basis of these diseases.

Human ghrelin was first discovered and identified by Kojima and coworkers in 1999.[11] This peptide hormone originates from a 117 polypeptide that is post-

translationally cleaved to the 94 amino acid proghrelin. Prohormone convertase (1/3) then processes this peptide into the 28 amino acid des-octanoyl ghrelin.[12] It is at this point that ghrelin's unique n-octanoyl acid chain is added to the serine at position 3, through a post-translational modification by ghrelin O-acyl transferase (GOAT) to produce the bioactive form of ghrelin (Figure 1).[4] A study by Bednarek et al. discovered that this n-octanoyl chain was crucial for receptor binding, as des-octanoyl ghrelin showed poor activity at even micromolar concentrations (IC_{50} >10 000 nM).[13] In the same study, it was also determined that replacing the ester linkage with an amide linkage by substituting the serine residue with diaminopropionic acid (Dpr) resulted in no change in activity. This substitution produces a much more stable bond from hydrolysis in comparison to the ester bond present in natural human ghrelin. Furthermore, an alanine-scan study determined the importance of the N-terminal positive charge and Phe^4, as well as confirming the importance of the octanoylated Ser^3. It also revealed that excluding the aforementioned positions, most other positions could potentially be substituted to optimize affinity towards GHSR.[14]

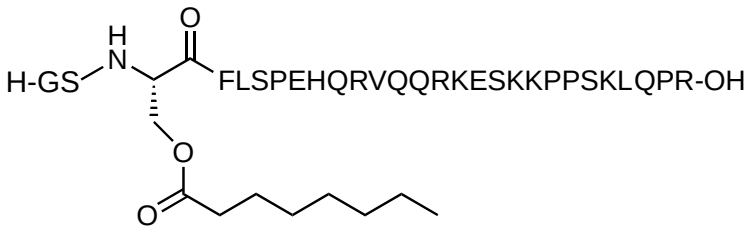

Figure 1. Peptide sequence of human ghrelin(1-28).

1.2 Peptide Synthesis

To synthesize ghrelin analogues and peptides in general, the technique commonly used due to its efficiency and ease is automated fluorenylmethyloxycarbonyl (Fmoc) based solid-phase peptide synthesis. This peptide synthesis technique involves continuous cycles of Fmoc deprotection and coupling of amino acids to the growing peptide chain from the C-terminus to the N terminus, with the C-terminus being conjugated to a solid support (Figure 2). The peptide synthesis cycle begins with the loading of an Fmoc protected amino acid onto the solid support using standard coupling conditions. Then, the fmoc is

deprotected using 20% piperidine, exposing the free amine that will take part in the subsequent coupling reaction. The coupling reaction is then performed using an aminium coupling agent to create the active ester that is attacked by the free amine. The base DIPEA is used here to deprotonate the tertiary amine of the amide moiety. This deprotection/coupling cycle is then repeated until the desired peptide sequence is synthesized and any required post-synthesis modifications are made. Once complete, the peptide can then be simultaneously deprotected and cleaved off the solid support using a cleavage cocktail containing a high concentration of trifluoroacetic acid (TFA). This method can minimize reaction times, as it can shift the equilibrium of coupling reactions to the products side using excess coupling reagents. It can also facilitate the removal of side products and excess reagents through solvent washes that leave the resin-anchored peptide product intact.

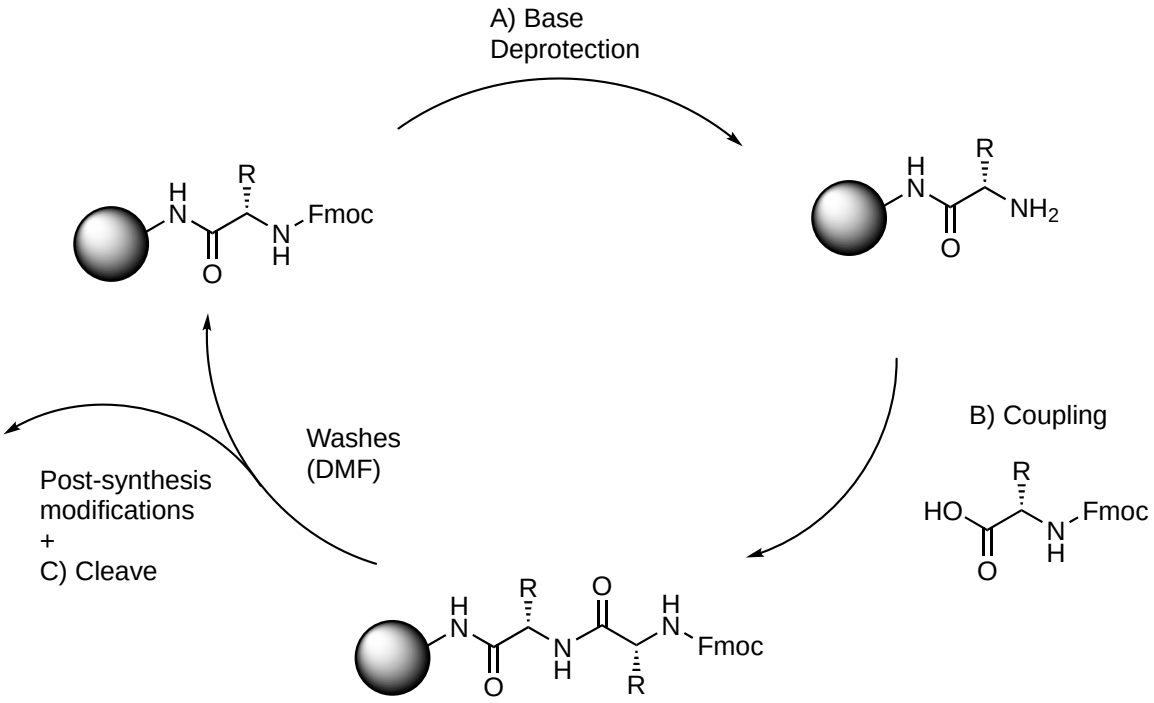

Figure 2. General procedure for SPPS. Reagents and Conditions: A) 20% Piperidine in DMF, B) HCTU, fmoc protected amino acid, DIPEA, DMF, C) 95% TFA, 2.5% H_2O, 2.5% TIPS

1.3 Molecular Imaging

The field of molecular imaging attempts to visualize, characterize and quantify cellular and molecular processes by using imaging probes. Often the biologic processes that are being imaged have relevance to specific disease states, where an effective imaging probe may lead to a better understanding of the disease and improve patient outcomes. The more commonly used molecular imaging modalities include fluorescent probes for optical imaging, radioactive isotopes for either positron emission tomography (PET) imaging, and single photon-emission computed tomography (SPECT) imaging. Fluorescence imaging involves the visualisation of molecular processes through the use of fluorophores. Fluorophores are light-absorbing compounds that emit a specific wavelength when excited with electromagnetic radiation.[15] When conjugated to high affinity ligands, these fluorophores can provide imaging of important biological targets with high spatial resolution and sensitivity. This makes fluorescence imaging a key modality in *ex vivo* applications and in pre-clinal testing of pharmaceuticals. However, a limitation of this modality is that due to the inability of fluorescent light to pass through layers of tissue, it is not commonly used *in vivo.* PET imaging uses compounds labelled with radioactive isotopes to visualize a variety of physiological processes and most commonly for the imaging of cancers. This imaging technique is based on measuring two high energy gamma rays that are produced in opposite directions from the annihilation of a positron (produced from the decay of the radioisotope) and an electron.[16] The radioisotopes used in PET imaging include ^{11}C, ^{13}N, ^{15}O, ^{18}F, ^{64}Cu, ^{68}Ga, ^{76}Br and ^{94m}Tc. Each of these radioisotopes has a different half-life and require unique chemistry for their incorporation into ligands of interest.[17] PET imaging offers high sensitivity and has the unique characteristic of being quantifiable, as the amount of radiotracer administered is proportional to the signal emitted.[16] Similar to PET, SPECT imaging also uses high energy photons to visualize organs and biological processes. However, only one gamma photon is produced and is measured using a gamma camera. The radioisotopes used in SPECT include ^{99m}Tc, ^{123}I, and ^{133}Xe.[18] While SPECT offers lower sensitivity than PET, it's radioisotopes are more readily available and have longer decay times, making SPECT imaging more feasible. Like

PET, the SPECT imaging probe's concentration is also quantifiable.[19] SPECT has applications in tumor imaging, infection imaging and can provide information about cardiac or brain function.

1.3.1 Ghrelin Analogues as Imaging Agents

Due to the ghrelin receptor's potential as a biomarker in a wide range of diseases and biological processes, there have been a variety of imaging agents developed in the last decade to target this receptor.[20] Ghrelin's natural high affinity towards the receptor has made it an ideal template for the design of potential imaging agents targeting the ghrelin receptor. For their design, it is common for ghrelin imaging agents to be truncated to the first 8-20 amino acids, as several studies have demonstrated that these truncations still allow for high affinity towards the ghrelin receptor while also making the potential imaging agent more cost efficient.[13,14,21] The first example of a ghrelin analogue being studied as a potential imaging agent was reported by Rosita et al.[22] Here, the addition of PET and SPECT moieties on the terminal end of an aliphatic chain at position 3 on truncated ghrelin(1-14) analogues were investigated (Figure 3). It was concluded that the addition of a fluorine atom (surrogate for ^{18}F) for PET imaging and a rhenium metal complex (used as a surrogate for ^{99m}Tc) for SPECT imaging at the terminal aliphatic chain were able to retain high affinity for the ghrelin receptor with IC_{50} values of 28 nM and 35 nM, respectively.

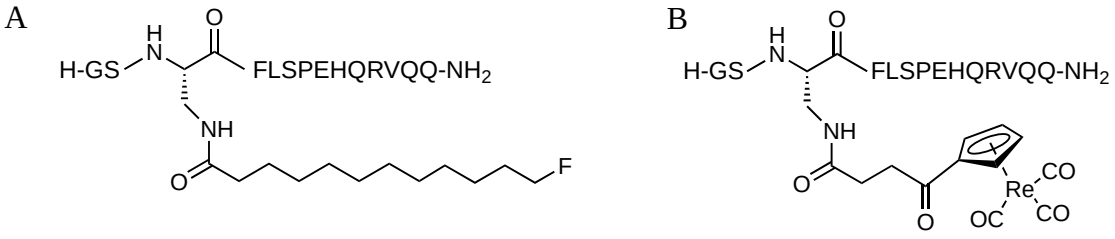

Figure 3. Lead compounds by Rosita et al. for PET (A) and SPECT (B) radiolabelling.[22]

Following the success of conjugating PET and SPECT moieties into the aliphatic chains of ghrelin analogues, a shift was made to incorporate fluorescent dyes for optical imaging. While the short-length, high affinity peptides previously mentioned were efficient for the appending of PET and SPECT moieties, their short nature made them difficult to conjugate with bulky dye molecules without affecting the peptide's affinity to their target. The incorporation of fluorescein isothiocyanate onto the C-terminal lysine residue of a ghrelin(1-19) peptide (Figure 4.A) resulted in a fluorescent probe targeting the ghrelin receptor with an IC_{50} value of 9.5 nM. This dye-labeled probe was also the first to provide visualization of the ghrelin receptor without an antibody.[23] The success of this probe at visualizing the ghrelin receptor in tissue prompted a study to investigate the efficacy of this probe as a tool to detect GHSR in prostate cancer tissue vs normal tissue.[8] Using the same principal of appending an imaging moiety off the C-terminal lysine residue, a study demonstrated that the incorporation of a ^{68}Ga radionuclide at this position resulted in a ghrelin(1-19) analogue with similar affinity (5.9 nM) to full length human ghrelin (Figure 4.B).[24] However, *in vivo* studies showed significant accumulation of this probe in the kidneys which demonstrated a need to optimize the pharmacokinetic properties of truncated ghrelin analogues.

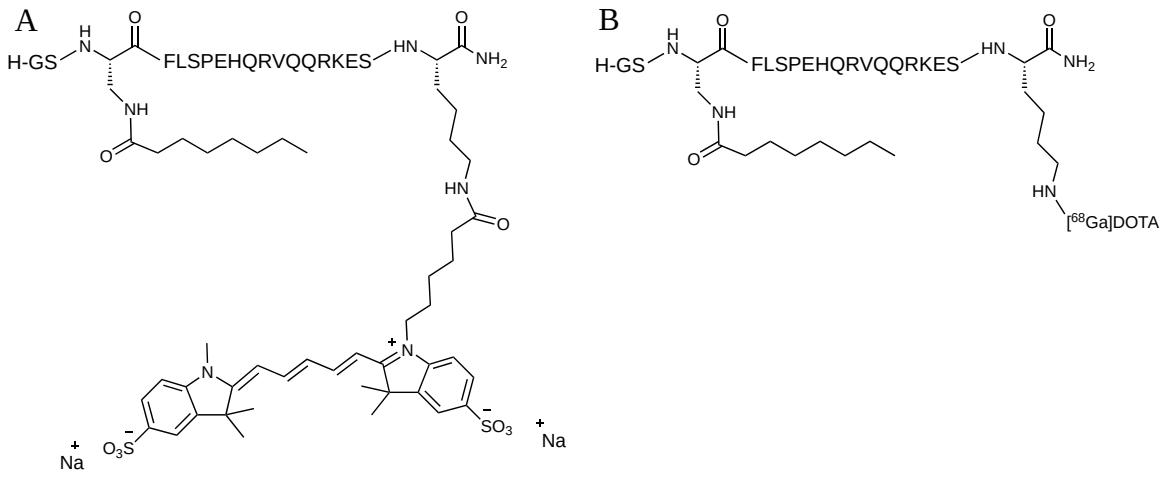

Figure 4. Lead compound by McGirr et al. (A) and lead compound by Charron et al. (B).[23,24]

In more recent years, a structure-activity study was conducted on ghrelin(1-8) analogues that resulted in the highest affinity ghrelin analogue reported to date with an IC_{50} value of 0.11 nM. The study revealed how modifications at residues 1, 3, 4, and 8 were crucial for the large increase in affinity towards the ghrelin receptor.[21] The subsequent lead compound was then appended with a 6-fluoro-2-pentafluorophenyl naphthoate prosthetic group to incorporate fluorine-18 for PET imaging (Figure 5). This lead compound was also assessed for stability in human serum which resulted in 60% intact peptide after 24 hours incubation.

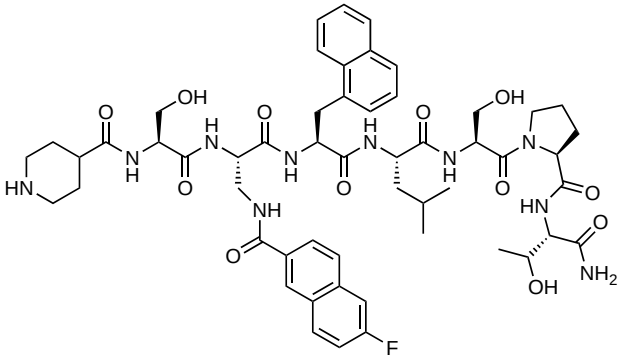

Figure 5. Lead compound from SAR study on ghrelin(1-8) analogues.[21]

1.4 Cancer Cachexia

Cancer cachexia is a wasting disease that is a frequent complication for cancer patients. Its symptoms are characterized as a reduction in lean body mass, reduced strength in limbs and biochemical abnormalities (anemia or inflammation). While most often associated with cancer, cachexia can also result as a complication from other illnesses such as Alzheimer's disease, chronic heart failure, chronic kidney disease and infectious diseases, amongst others.[6] Cancer Cachexia is reported to affect 50% of all cancer patients and 60-80% of advanced cancer patients. Its mortality rate is also described to be as high as 80% for cancer per year, while also being associated with poor-symptom status and poor quality of life for patients living with the complication. Treatment approaches for this

disease range from anabolic steroids, anti-catabolic therapies and nutritional interventions.[25] However, to date there has not been an effective treatment developed for this illness.

1.4.1 Ghrelin as a Therapeutic Agent for the Treatment of Cancer Cachexia

As previously mentioned, the GHSR is a GPCR that regulates various important physiological processes in the body including appetite, growth hormone secretion and adipocyte metabolism. More specifically, ghrelin, which is the gastric-derived endogenous ligand of the ghrelin receptor, regulates energy homeostasis by transmitting peripheral nutritional information to the brain thereby exhibiting orexigenic effects.[26] Activation of the central nervous system GHSRs by ghrelin has been demonstrated to control food anticipation and food-based learning in rodents.[27] Studies have also shown that administration of ghrelin *in vivo* increases AMP-activated protein kinase activity in the hypothalamus, which is linked to hypothalamic appetite regulation.[28]

Interestingly, levels of acylated ghrelin have been found to be 50% higher in cancer patients with cachexia when compared to those without cachexia.[29] This suggests that cachexia's anorexic effects overwhelm ghrelin's orexigenic properties and thus supraphysiologic doses must be used to yield positive results.[30] In 2004, human trials targeting increased appetite in patients with melanoma, breast and colon cancers with a 450 pmol/kg ghrelin IV infusion for 90 min resulted in a 31% increase in food intake when compared to IV infusion of saline.[31] Similar results were seen in another human trial in 2008, where patients with a wide range of cancers were administered with two 2.4 nmol/kg IV infusions of ghrelin. This treatment regimen saw an increase of 56% food intake for one day for those administered with ghrelin in comparison to placebo.[32] As a result, it is theorized that ghrelin's peptide structure may serve as an effective structural template for the design of therapeutic agents for the treatment of this wasting disease.

1.5 Peptide Stability and Ghrelin's Helical Character

While natural peptides serve as efficient blueprints for targeting their endogenous receptors, linear peptides are well noted for their lack of stability towards proteolytic degradation. This results from proteases in the body being efficient at hydrolyzing the amide backbones of linear or extended peptide confirmations. However, studies of protease structures have found that most proteases have active sites that are too small to permit an α-helix to bind, indicating that peptides with more α-helical character are less prone to enzymatic degradation.[33] In addition, interactions between peptides and their receptor proteins reveal that the peptide's secondary structure plays a vital role in ligand recognition, suggesting that an increase in α-helical character could improve affinity and activation towards their receptors.[34]

Full length ghrelin is comprised of 28 amino acids with a post-translational octanoyl modification at Ser^3. An NMR study revealed that part of ghrelin's structure contains an α-helical confirmation in the presence of the ghrelin receptor.[35] The two most stable conformations which present different proline rotamers, indicate the presence of what the author's propose as a rare left-handed α-helical region between Glu^8 and Lys^{20}, which are not common in nature. However, a molecular dynamics study which looked at simulations in water and in a dimyristoyl phosphatidylcholine-lipid bilayer/water system determined that a short right-handed α-helix is present from residues Pro^7 to Glu^{13}.[36] Similarly, a cryo-electron microscopy structure of the GHSR-ghrelin bound complex revealed a right-handed α-helical region present in the peptide structure between Pro^7 and Gln^{14} (Figure 6).[37] In this study, the authors importantly noted that extensive interactions are present between the extracellular regions of the ghrelin receptor and the helical region of ghrelin, which further stabilizes the binding of the ligand to the receptor. These studies demonstrate that regions of the ghrelin peptide structure, approximately in the middle of the peptide sequence, contain an α-helix both in a water/lipid bilayer system and when in the presence of the ghrelin receptor.

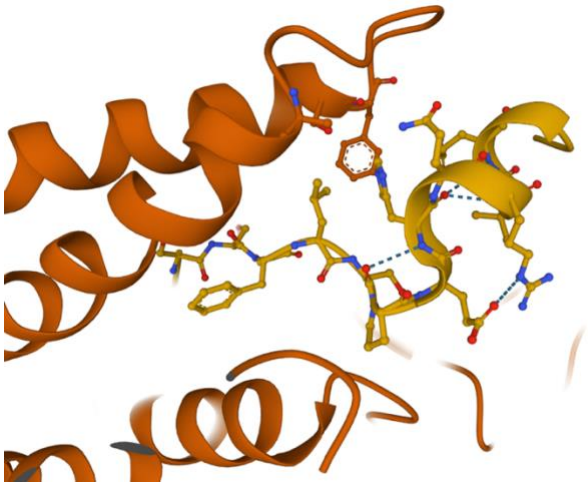

Figure 6. Cryo-electron microscopy structure of ghrelin bound to the ghrelin receptor showing ghrelin's helical character.[37]

1.6 Helix-Inducing Chemical Staples

A portion of ghrelin's structure has been proven to be helical when bound to the ghrelin receptor or in similar water/lipid environments. As a result, its linear or disordered character when not bound to the ghrelin receptor suggests it is quickly degraded by proteases in the body and thus lacks in vivo stability. A possible solution to increasing the *in vivo* stability of ghrelin analogues is to introduce helix-inducing chemical staples into their peptide structure. An α-helix is composed of 3.6 residues per complete turn which leaves the *i,i+4, i+7, i+11* side chains on the same face of the helix. To stabilize an α-helix, covalent bonds are formed between the side chains of amino acid residues that are located at these positions (Figure 7).

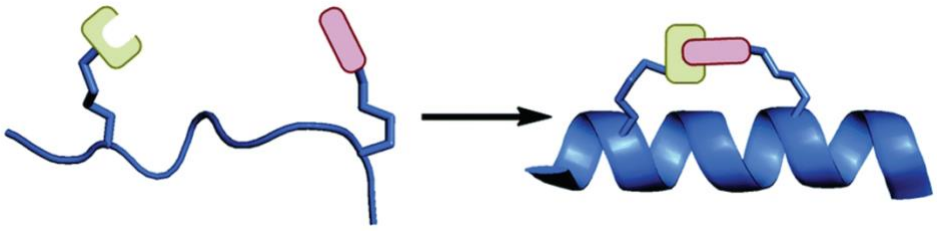

Figure 7. Formation of covalent linkage at *i,i+4, i+7, i+11* positions results in induction of helicity.[38]

The goal of these helix inducing staples is to constrain the peptide into its bioactive helical confirmation and thereby overcome the energetic penalty related to folding.[38] Inducing this secondary confirmation on peptides has been shown to increase target affinity, cell penetration and protection against proteolytic degradation. One of the first examples of the uses of a helix inducing staple was by Felix and coworkers where they effectively stabilized an α-helix by a lactamisation of an aspartic acid and a lysine residue with *i,i+4* spacing.[39] Here, the authors used CD and NMR to describe the α-helical nature of the stapled growth hormone releasing factor which also retained bioactivity upon lactimization. Perhaps the most well-known staple and where the term "peptide staple" was coined came from the publication by Verdine and coworkers in 2000.[40] In their impactful paper, the group investigated the optimal length and stereochemistry of α, α-disubstituted unnatural amino acids which they tethered together in what became known as the hydrocarbon staple. This staple proved to be highly efficient in inducing helicity and increasing metabolic stability in their peptide target. [40] In the following decade, a wide range of helix inducing staples would be designed and tested towards a variety of targets. Amongst them was triazole staple which was first described by Chorev, D'Ursi and coworkers where they cyclized propargyl glycine and lysine azide residues together using the prevalent copper(I)-catalyzed alkyne-azide cycloaddition reaction.[41] Using this "click" chemistry to from a macrocycle, they were able to successfully induce helicity in their model parathyroid hormone-related peptide. These new developments in the field of peptidomimetics inspired the Luyt group to investigate the effects of introducing a helix inducing staple in ghrelin with the goal of improving receptor affinity and stability through cyclization. This culminated in a study where a "staple scan" was able to identify the locations of an *i,i+4* staple that generated the highest affinity towards the ghrelin receptor for ghrelin(1-20).[42] By cyclizing a glutamic acid and a lysine reside to create a lactam macrocycle, positions 12 and 16 of ghrelin(1-20) were identified as the location for an *i,i+4* staple which yielded the highest affinity to the ghrelin receptor with an IC_{50} value of 7.9 nM (Figure 8).

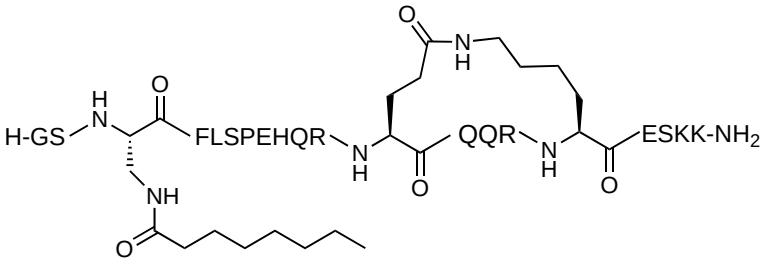

Figure 8. Structure of Lalonde et al.'s *i,i+4* lactam stapled ghrelin(1-20) analogue.[42]

The ability of a peptide staple to induce helicity is contingent on factors such as linker length, the spacing between residues, and the amino acid residues present between the staple. However, there have been many examples in the literature of comparative studies that aim to determine the efficiency of chemical staples at inducing helicity in a peptide structure. One of these studies concluded that the lactam staple, the hydrocarbon staple, the triazole staple and the bis-thioether staple achieved the greatest helicity for an *i,i+4* staple on a range of peptides when measured by circular dichroism (CD) spectroscopy.[38] The study noted that the hydrocarbon, triazole and lactam staples have the optimal flexibility and atom length to induce helicity in a peptide structure. Furthermore, the hydrocarbon staple has the advantage of increased helix propensity due to the disubstituted nature of the amino acid building block (Figure 9). The most common disubstituted amino acid, α-aminoisobutyric acid (Aib) has been shown to strongly favour helical structures.[43] Therefore, it is believed that combining the helix propensity from disubstituted amino acids and the helix stabilizing effect of peptide staples would yield highly helical peptide structures.

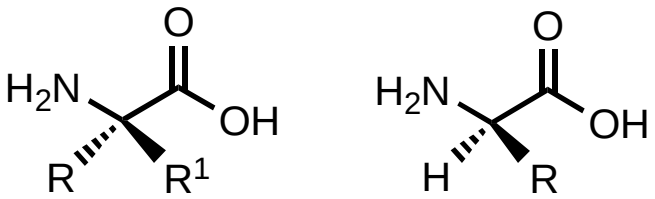

Figure 9. A disubstituted amino acid (left) and a standard amino acid (right), where R^1 is not H.

More recently, a novel helix inducing stapling method efficiently yielded highly helical stapled BCL-9 peptides through what is referred to as a Glaser staple. By utilizing a Glaser-Hay coupling reaction of propargyl serine residues to form the peptide macrocycle, this stapling method resulted in a 56% increase in peptide helicity in comparison to their linear counterparts.[44] This large and rigid peptide staple has the ability to induce helicity in a *i,i+4* and an *i,i+7* spacing.

1.7 Project Goals

As previously described, positions 12 and 16 of ghrelin(1-20) analogues have been identified as the ideal positions for the introduction of a chemical staple to increase affinity towards the ghrelin receptor. As a result, an investigation of different stapling methods at these positions of ghrelin(1-20) analogues is presented with the goals of increasing affinity and activity towards the ghrelin receptor and increasing stability from enzymatic degradation. In total, five helix-inducing chemical staples will be studied, their corresponding ghrelin(1-20) analogues will be synthesized and their affinities for the ghrelin receptor will be measured through inhibition radioligand displacement assays in HEK293 cell transiently transfected with GHSR. Their ability to activate the ghrelin receptor will also be measured using beta-arrestin recruitment BRET assays. The five chemical staples are: lactam (Lys-Asp) staple, triazole staple, bisthioether staple, hydrocarbon staple, and Glaser staple (Figure 10). These specific staples have been chosen due to their known ability to induce helicity in a peptide structure.

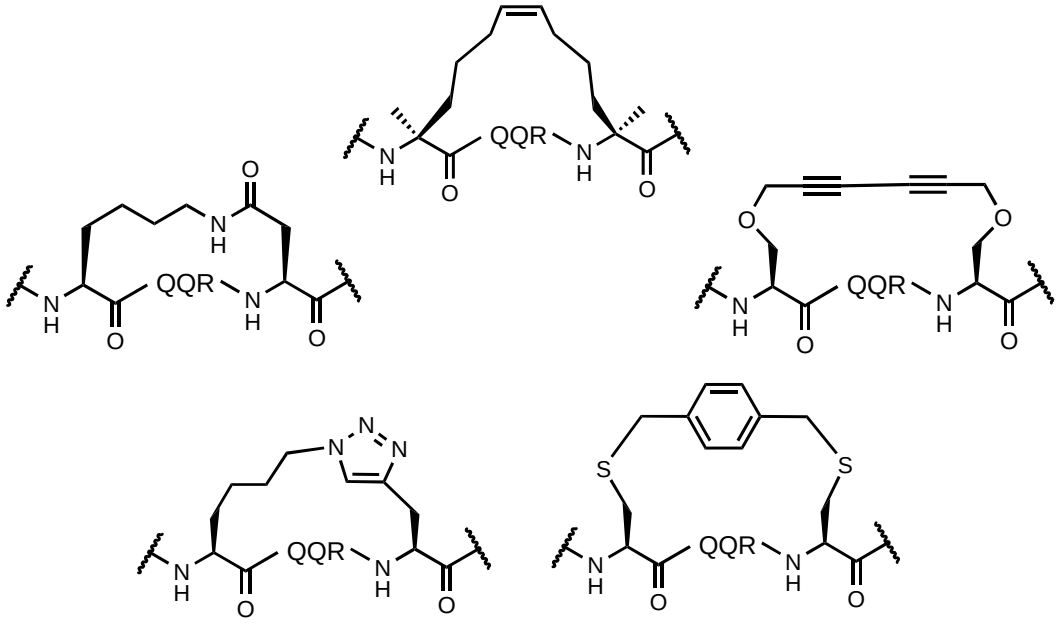

Figure 10. The five staples investigated (clockwise from the top): hydrocarbon staple, Glaser staple, xylene-thioether staple, triazole staple, and lactam staple (Lys-Asp).

Once the receptor affinity of the outlined peptides has been evaluated and their ability to activate the ghrelin receptor (potency and efficacy) has been measured, their stability from enzymatic degradation will be determined through serum stability assays. The goal of this is to test the hypothesis that increasing helicity through the use of chemical staples in ghrelin analogues, can provide increased stability from enzymatic degradation. Stapled ghrelin(1-20) analogues that are determined to have high affinity and activity towards the ghrelin receptor and robust stability from enzymatic degradation, will then be evaluated as imaging agents with the incorporation of fluorescent dyes or as potential therapeutic agents for cancer cachexia by evaluating feeding behaviour in mice.

Chapter 2

2.0 Integrated Research Article

2.1 Introduction

Chemical staples have become recognized as an effective molecular tool for peptide chemist to modulate the pharmacodynamic and pharmacokinetic properties of peptides. More specifically, introducing helix-inducing chemical staples into a peptide structure has become a popular synthetic method for increasing affinity towards the target protein while also potentially increasing peptide stability to enzymatic degradation and improving cell permeability. By effectively locking the peptide into their desired biological confirmation, staples can enhance a peptide's ability to form stronger interactions with their native receptor, thereby increasing their affinity towards their target. Additionally, studies have demonstrated that the binding sites of many proteases found in humans are not large enough to permit an α-helical confirmation to bind, thus suggesting that they may increase the serum stability of peptides.[33] However, with the wide range of chemical staples that have been developed in the last decades, the question arises as to which staples are consistent at inducing helicity when incorporated into a peptide structure.

Strategies for inducing α-helicity in peptides have been around for decades, including metal ion clips,[45] unnatural amino acids,[46] and helix-nucleating templates.[47] Staples have been notably effective at stabilizing the secondary structure of a peptide by reducing the entropic penalty associated with folding. The term 'peptide staple' was coined by Verdine and coworkers in their seminal paper published in 2000.[40] In their work, they developed a hydrocarbon cross link using α,α-disubstituted amino acid residues bearing olefin tethers. This was the first example of a peptide where the use of a hydrocarbon tether was effective at enhancing its helicity and metabolic stability. In years to come, the hydrocarbon staple would be regarded as one of the most effective staples at increasing α-helicity in range of peptide sequences and biological applications. Other peptide staples that are known for their ability to induce α-helicity are the lactam staple, [48] triazole

staple,[49] xylene-thioether staple,[50] and the recently developed Glaser staple.[44] Comparative studies have demonstrated that the lactam and triazole staples are as efficient as the hydrocarbon staple at increasing helical character in a peptide structure.[38] The xylene-thioether staple is known for its efficient two-component cross-link which allows for bifunctionality of the linker unit, while the Glaser staple allows for increased rigidity in the linker structure.

Stapled peptides have been developed for a wide range of cellular targets such as protein-protein interactions,[51] G-protein coupled receptors (GPCRs),[52] and enzyme inhibitors for antiviral therapeutics.[53] One GPCR that has garnered attention in recent years is the growth hormone secretagogue receptor (GHSR) 1a or as it most known, the ghrelin receptor. The ghrelin receptor gets its name from its 28 amino acid endogenous peptide ligand, ghrelin. This peptide hormone, discovered by Kojima and co-workers in 1999, possesses an octanoyl chain that is post-translationally coupled on to the serine at the third position by ghrelin *O*-acyl transferase.[11] This unique modification has been determined to be crucial for receptor recognition, as des-acyl ghrelin has no affinity (IC_{50} > 10,000 nM) towards the ghrelin receptor.[13] When ghrelin binds to its native receptor, it has been reported to exhibit orexigenic effects and has since been labelled as the "hunger hormone". Due to this biological role that ghrelin plays, studies have probed its potential as a therapeutic agent for the treatment of cancer cachexia, the wasting illness that affects roughly half of all cancer patients.[29,32] Also, this receptor has been shown to be differentially expressed in ovarian[7] and prostate[8] cancer, and in the hearts of dilated cardiomyopathy patients and patients with end-stage heart failure.[9,10] Thus, it has become a target of interest in the field of molecular imaging.

Like many natural peptides, ghrelin's linear structure makes it susceptible to enzymatic degradation by proteases. As a result, many ghrelin analogues that were developed as imaging agents have been noted as having poor in vitro stability in human serum.[54] However, studies have demonstrated that when ghrelin binds to its receptor, part of its peptide structure folds into an α-helical confirmation approximately from Pro^7 to Gln^{14}.[35-37] This suggest that a section of ghrelin's sequence has helical character in certain environments and could be stabilized using a helix-inducing chemical staple. In prior work,

a lactam staple (Glu-Lys) was introduced into the peptide structure of ghrelin(1-20) analogues in what was referred to as a "staple scan" to discover the optimal location that yields a cyclic peptide with the highest affinity towards the ghrelin receptor. In the resulting publication, Lalonde and coworkers described how an i,i+4 staple between the 12 and 16 position of ghrelin(1-20) resulted in the highest affinity towards the ghrelin receptor with a reported IC_{50} of 7.9 nM.[42] The resulting compound was then appended with a Cy5 fluorescent dye and were successful at imaging the ghrelin receptor in selected ovarian cancer cell lines using confocal microscopy.

Since the optimal position for an *i,i+4* lactam (Glu-Lys) staple in ghrelin(1-20) analogues yielded the highest affinity towards the ghrelin receptor was determined to be positions 12 and 16. The question arose whether different stapling techniques at this same location would yield compounds with greater affinity towards the ghrelin receptor, and if these techniques would have varied influence on the metabolic stability of ghrelin analogues. As a result, we present an investigation of five different helix inducing staples at positions 12 and 16 in truncated ghrelin(1-20) analogues. The five staples investigated will be the triazole, lactam (Lys-Asp), hydrocarbon, xylene-thioether, and Glaser staples. This selection of stapling techniques will provide a range of conformations as it includes varying degrees of flexibility in the linker region. Once synthesized these compounds will be evaluated for their affinity towards the ghrelin receptor, for their ability to activate the ghrelin receptor, and for their metabolic stability in human serum. Selected compounds with desirable pharmacological properties have the potential to be evaluated as molecular imaging agents or therapeutic agents targeting cancer cachexia.

2.2 Results and Discussion

To determine whether different stapling techniques would increase the bioactivity and stability of ghrelin(1-20), five ghrelin analogues bearing different helix-inducing staples were synthesized. For their synthesis, the natural ghrelin sequence had substitutions at positions 12 and 16 with residues bearing the functional groups required for the diverse stapling techniques with an *i,i+4* spacing. Ghrelin's natural affinity towards the ghrelin

receptor is largely due to its octanoyl chain present at Ser^3. However, the instability of the ester bond requires that the Ser^3 be substituted with the amine analogue, diaminopropionic acid (Dpr), in order to have the more stable amide bond connecting the octanoyl chain to the peptide. Due to the different cyclization methods used in this report, two different orthogonal protecting groups had to be used to protect the reactive amine of the Dpr^3 residue to prevent any unwanted side reactions from occurring. For most peptides, an Alloc protecting group was used, which deprotects easily and effectively with a palladium catalyst and phenyl silane. For any suspected cyclizations that posed a problem with the Alloc protecting group, the protecting group Mtt, which deprotects with dilute acid (1% TFA), was used. Following the successful stapling and coupling of the octanoyl chain, peptides are cleaved from their solid support, purified by preparative HPLC and characterized by ESI+ mass spectrometry.

2.2.1 Triazole Stapled Ghrelin (1-20) Analogue

Triazole tethers have been documented to stabilize secondary peptide structures and produce helicity that is comparable to that of the lactam staples.[41,49] Furthermore, the amino acid residues used for the formation of the triazole moiety are readily accessible and the optimal conditions of the copper assisted "click chemistry" reaction are established.[38] As a result, the first stapled ghrelin analogue synthesized was the triazole stapled ghrelin(1-20) analogue (Figure 11). To synthesize the 20 amino-acid long peptide, Fmoc-based automated SPPS was used with modifications of the truncated ghrelin amino acid sequence at positions 3, 12 and 16. Position 3 used an Alloc protecting group on the Dpr residue for the coupling of the octanoyl chain as described in **2.2**. Positions 12 and 16 were occupied with a lysine azide residue and a propargyl glycine residue, respectively. The side chains of these two residues were then used in the copper-assisted azide-alkyne cycloaddition reaction to create the macrocycle. This cycloaddition reaction was carried with copper (I) bromide, sodium ascorbate, 2,6-lutidine and DIPEA. The reaction was stirred overnight, and the formation of the macrocycle was confirmed by micro cleaving a small sample of the resin-attached peptide product and analyzing it using HPLC/MS. Once the

macrocyclization was confirmed to have been successful, the deprotection of the Alloc protecting group of Dpr^3 and the subsequent coupling of the octanoyl chain was carried out.

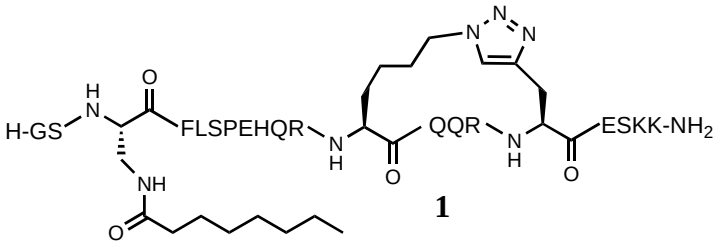

Figure 11. Structure of triazole stapled ghrelin(1-20) analogue.

2.2.2 Lactam (Lys-Asp) Stapled Ghrelin (1-20) Analogue

Previous work demonstrated the efficiency of a lactam (Glu-Lys) staple at increasing the helicity and affinity towards GHSR-1a of ghrelin(1-20) analogues.[42] The lactam staple offers an increased helicity in peptides by using readily available natural amino acids to form a lactam macrocycle through amide bond formation. While the lactam (Glu-Lys) staple was able to provide a significant increase in helicity, studies have suggested that a lactam (Lys-Asp) staple also provides an increase of helicity in peptides, and may perhaps stabilize the secondary structure of certain peptides due to the smaller stapling unit.[48,55] Thus, a lactam (Lys-Asp) stapled ghrelin(1-20) analogue (Figure 12) was synthesized as part of this project to further investigate the differences that the amide position of the lactam staple and ring size have on helicity and binding affinity towards the ghrelin receptor.

The first attempt at synthesizing this compound involved using orthogonal protecting groups, with the Lys^{12} residue containing an Alloc group and the Asp^{16} containing an allyl ester group. However, analysis of a small resin sample post-SPPS with an LCMS revealed that a considerable amount of aspartimide formation had occurred during the automated synthesis. Aspartimide formation is a sequence dependent cyclization that occurs to aspartic acid residues in the presence of base. More specifically to Fmoc-

based SPPS, it occurs due to the repetitive piperidine treatments required for Fmoc deprotection.[56] The result of aspartimide formation is a series of side reactions that can ultimately lead to up to four different products: the desired compound and three side products that may have very similar retention times by HPLC. Therefore, a new strategy had to be designed for the synthesis of this stapled analogue while considering ways to limit this unwanted cyclization.

The second attempt at the synthesis involved using alternative orthogonal protecting groups. The orthogonal phenylisopropyl ester (Pip) protecting group, which can be removed under mild acidic conditions, can provide sufficient steric bulk to prevent unwanted side reactions such as the removal of the amide hydrogen by piperidine seen in aspartimide formation.[48] Thus, the Asp^{16} was protected with the Pip group and the Lys^{12} residue was protected with an Mtt group, another acid labile group. Since both protecting groups are acid labile, the Dpr^{3} residue contained the orthogonal Alloc group as this protecting group is inert to acidic conditions. After the SPPS of the peptide, a small sample confirmed through LCMS that aspartimide formation was successfully kept to a minimum. Next, the lactamisation reaction using the coupling agent HATU and the base DIPEA was able to yield the desired resin-bound peptide macrocycle.

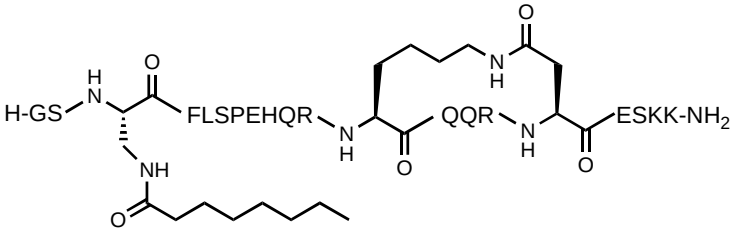

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Figure 12. Structure of lactam (Lys-Asp) stapled ghrelin(1-20) analogue.

2.2.3 Hydrocarbon Stapled Ghrelin (1-20) Analogue

The last stapling technique that was investigated was the hydrocarbon staple. This stapling technique is formed from a ring closing metathesis (RCM) reaction between two non-natural amino acids bearing olefin tethers. Once the olefin tethers undergo the ring

closing metathesis and the peptide macrocycle is formed, it provides a stabilization of the secondary structure of the peptide and has been observed to significantly increase protease resistance.[57,58] To attempt the synthesis of the hydrocarbon stapled ghrelin(1-20) analogue (Figure 13), Fmoc-based SPPS was carried out using the disubstituted unnatural amino acid residue (4'-pentenyl) alanine at positions 12 and 16. Once again, Dpr^3 was protected with the orthogonal Mtt group. Following SPPS however, it was discovered that a noticeable amount of incomplete coupling of the (4'-pentenyl) alanine residue had occurred. It was theorized that this was caused due to the increased steric bulk present near the amine caused by the disubstituted character of the amino acid. As a result, a manual coupling of the unnatural amino acid was completed with longer reaction times as well as the more efficient coupling agent, HATU. Following the successful synthesis with complete coupling of the unnatural amino acid, the deprotection of Mtt and coupling of octanoyl acid chain was carried out. The ring closing metathesis reaction was then attempted using 20 mol% of Grubbs 2nd generation catalyst in DCE in an open-air system. An open-air system was used to allow for the ethylene by product of the RCM reaction to escape, thereby shifting the equilibrium of the reaction to the products.

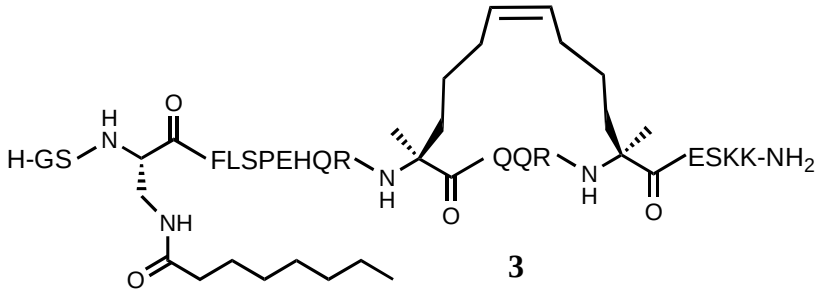

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Figure 13. Structure of proposed hydrocarbon stapled ghrelin(1-20) analogue.

2.2.4 Glaser Stapled Ghrelin (1-20) Analogue

A more recent development in helix-inducing stapling techniques is the Glaser staple. This rigid staple is formed from a Glaser-Hay coupling reaction involving two 1,3-diyne motifs. When incorporated into a peptide structure in both *i,i+4* and *i,i+7* residue

spacing, a significant increase in helicity was observed, likely due to the added rigidity that is produced from the cyclization reaction.[43,59] This stapling technique also offers increased solubility which is a characteristic not shared by all the other peptide techniques.[38] To synthesize this stapled peptide analogue, Dpr^3 was protected with Mtt so that unwanted side reactions could not occur during the cyclization reaction. Following the deprotection of the Mtt group and coupling of the octanoyl chain, the cyclization reaction which is formed from a Glaser-Hay coupling reaction was attempted. This reaction involves the formation of a macrocycle between two propargyl serine residues, and was achieved by adding copper chloride, a 4,4'-bis(hydroxymethyl-2,2'-bipyridine ligand and DIPEA to a vessel containing the resin-anchored peptide and allowing it to react for three days. The resulting stapled peptide product was the Glaser stapled ghrelin(1-20) analogue (Figure 14)

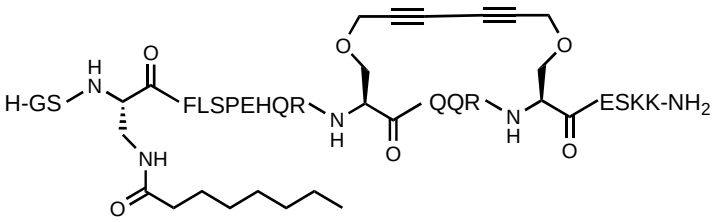

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Figure 14. Structure of Glaser stapled ghrelin(1-20) analogue.

2.2.5 Xylene-thioether Stapled Ghrelin (1-20) Analogue

The inclusion of semi-rigid cross-linkers, through the formation of thioethers, has been a popular technique for stabilizing alpha-helical secondary structures. This is an example of a two-component stapling technique which is formed from the bisalkylation of sulfide moieties most commonly in an *i,i+4* residue spacing. This stapling technique has the advantage of allowing for more flexibility in design, as the cross-linking reactions are selective and compatible with unprotected peptides.[50] While many cross-linkers can be used to react with Cys residues, a recent study showed that more rigid systems like bis(bromomethyl)benzene can provide increased helicity due to their ability for low-strain intramolecular cyclizations.[60] While studies report that the different constitutional isomers

of α,α′-dibromo-xylene (meta, ortho and para) are all able to provide significant increases in helicity, the α,α′-dibromo-p-xylene isomer was chosen for evaluation due to availability.[50] Thus, a synthesis of a bis-thioether stapled ghrelin(1-20) analogue (Figure 15) using the α,α′-dibromo-p-xylene linker was planned. The synthesis of ghrelin analogue first involved SPPS with two trityl protected cysteine residues at positions 12 and 16. Since the trityl protecting group is acid labile, the Dpr^3 was orthogonally protected with an Alloc protecting group. Following the deprotection of the Alloc group and the coupling of the octanoyl chain, the peptide was deprotected and cleaved from the solid support using 92.5% TFA, 2.5% H_2O, 2.5% ethane-1,2-dithiol (EDT), and 2.5% TIPS. EDT was included in the cleavage cock to prevent oxidation of the free thiols that are present after deprotection. For the cross-linker coupling reaction, the deprotected peptide product was then mixed in a NH_4HCO_3 buffer solution with tris(2-carboxyethyl)phosphine and α, α′-dibromo-p-xylene.

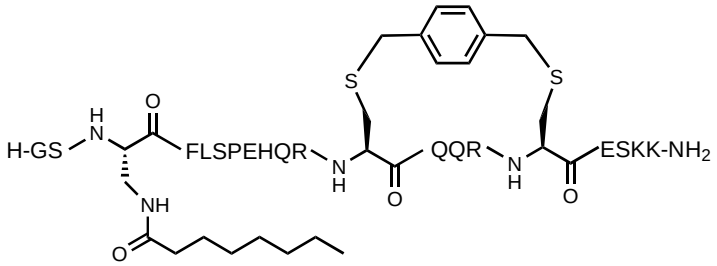

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Figure 15. Structure of xylene-thioether stapled ghrelin(1-20) analogue.

2.3 Circular Dichroism

The helical character of the stapled ghrelin(1-20) analogues was determined by obtaining the respective circular dichroism (CD) spectra of each peptide. CD measurements were performed at a concentration of 0.1mM in sodium phosphate buffer. The distinctive spectra for an α-helical peptide contains a large positive band at 192 nm and two negative bands at 208 nm and 222 nm. It is common practice to report the mean residue ellipticity (MRE) of the negative band at 222 nm to compare the relative helicity of peptides, as seen in Table 1. Perhaps a more useful method for comparison, however, is to report the percent

helicity of the peptide. Using the MRE value at 222 nm, a percent helicity for an individual peptide can be determined using a formula that considers the temperature, the number of peptide units, and the theoretical MRE values for a fully helical peptide and a random coil at 222 nm.[55] The calculated value approximates what fraction of the peptide is in a helical confirmation. The results from the CD experiments showed that highest increase in helicity in comparison to the linear ghrelin (1-20) analogue were the triazole, lactam and hydrocarbon stapled analogues (**1-3)** (Figure 16). These staples were able to produce peptides that were more helical than the unstapled ghrelin(1-20) analogue, which shows 0% helical character in water, and full-length human ghrelin. However, it should be noted that human ghrelin's CD was measured in water, and reports indicate that this peptide becomes more helical once it has interacted with the ghrelin receptor or in a lipid bilayer/water system.[36] The Glaser and bisthioether staples (**4** and **5**) were also able to increase helicity in comparison to the unstapled ghrelin(1-20) analogue but were significantly less helical than **1-3.** This could indicate that a more rigid peptide staple is not compatible with the peptide sequence of ghrelin (1-20) for increasing helicity in contrast to more flexible staples.

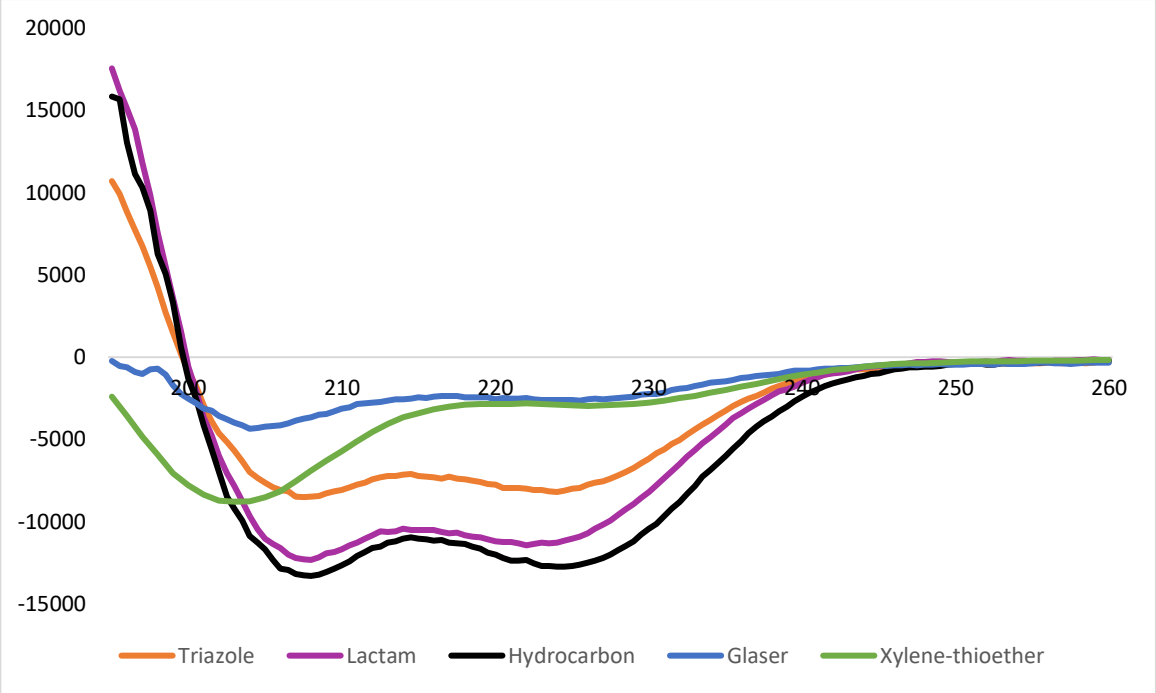

Figure 16. Circular Dichroism spectra for the hydrocarbon, triazole and lactam stapled ghrelin(1-20) analogues.

Consistent with the literature, the results from the circular dichroism experiments indicate that the lactam, triazole and hydrocarbon staple are the most efficient at inducing α-helicity in a peptide structure.[38] Interestingly, the lactam (Lys-Asp) staple saw a modest increase in percent helicity (35%) when compared to the previously reported lactam (Glu-Lys) staple which had a calculated percent helicity value of 27%.[42] This difference in helicity could be explained by the compatibility of ghrelin(1-20)'s sequence with the shorter stapling unit of the lactam (Lys-Asp) staple. Not surprisingly, the hydrocarbon stapled ghrelin analogues saw the greatest induction of α-helicity in ghrelin(1-20), as was predicted from the literature. It is postulated that part of the reason this staple is so effective at inducing helicity is due in part to the disubstituted nature of its unnatural amino acid residue from which the linker unit is formed. In fact, peptides have been reported to have achieved α-helical induction even when the unnatural amino acids have not been cyclized.[61] A logical progression of this work would be to investigate if using disubstituted

amino acid with the other staples investigated would yield similar percent helicity values as the hydrocarbon staple. Probing for the effect that this unique feature of the unnatural amino acid building blocks has on helicity would give insight for the design of more helical peptide compounds.

Compound	[θ]222 (water)	% Helicity
Human Ghrelin	-2 884	12%
Ghrelin(1-20)	2 460	0%
1	-7 996	26%
2	-11 436	35%
3	-12 332	39%
4	-2 497	10%
5	-2 840	12%

Table 1. Results from circular dichroism spectroscopy experiments ([θ]222 values) and calculated percent helicity of synthesized stapled ghrelin(1-20) analogues, human ghrelin, and ghrelin(1-20).

2.4 GHSR-1a Binding Assays

To evaluate the binding affinity of the synthesized stapled ghrelin(1-20) analogues towards the ghrelin receptor, radioligand inhibition binding assays using [^{125}I]-ghrelin on HEK-293 cells transiently transfected with the ghrelin receptor were performed. These cells were specifically chosen for their rapid proliferation and their compatability with X-tremeGENE 9 DNA transfection. The averaged results of these assays, measured by calculating the concentration of the different stapled ghrelin analogues at 50% radioligand inhibition (IC_{50}) can be seen in Table 2 along with their respective peptide sequences**.** In order to compare the efficiency of the different staples at increasing the affinity of ghrelin(1-20) analogues towards the ghrelin receptor, values obtained through a binding assay for both human ghrelin (7.57 nM) and ghrelin(1-20) (2.28 nM) were also included in the table. The difference between these two values indicates that truncation of the native

human ghrelin sequence to the first 20 amino acids leads to an increase binding affinity towards the receptor, which is consistent with the data found in the work by Bednarek and coworkers.[13] In their paper, they used radioligand binding assays with a potent ghrelin receptor binder [35S]MK-0677 to compare the binding affinity of full-length human ghrelin (IC_{50} = 0.25 nM) and truncated ghrelin(1-23) (IC_{50} = 0.16) and noticed an almost two-fold difference between the two. The results from the binding assays demonstrate that the triazole, lactam (Lys-Asp) and hydrocarbon staples (**1-3**) were able to increase the binding affinity of ghrelin(1-20) analogues. The three staples saw a varied increase in binding affinity to the ghrelin receptor in comparison to truncated ghrelin(1-20), with the triazole having almost a twofold increase (0.92 nM) (Figure 17). In contrast, the bulkier and more rigid stapling units, the Glaser and xylene-thioether staples (**4** and **5**), saw a decrease in binding affinity in comparison to ghrelin(1-20).

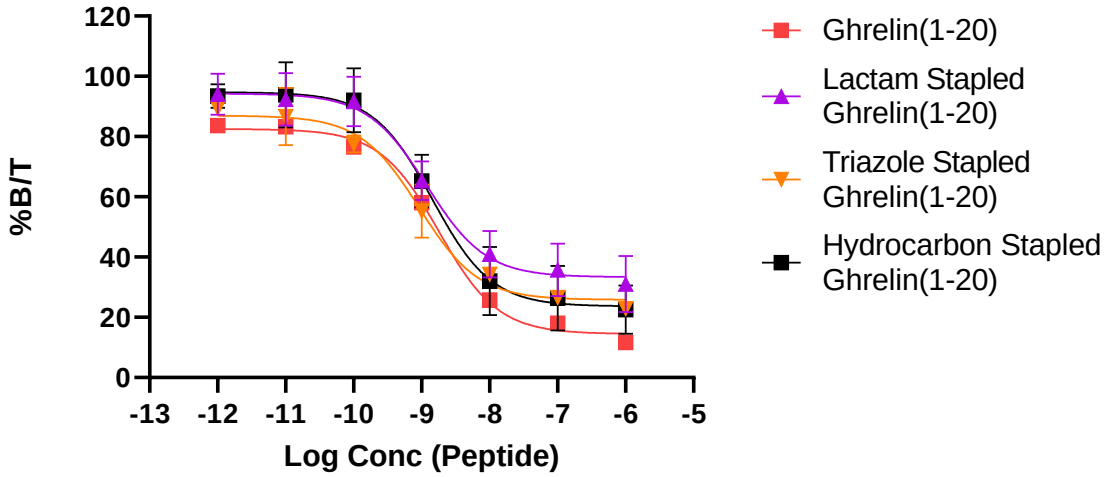

Figure 17. Radioligand binding assay IC_{50} curves for lactam stapled, triazole stapled and hydrocarbon stapled ghrelin(1-20) analogues.

Clear differences are present in the degrees of freedom of the staple linker region, for peptides that saw an increase in binding affinity towards the ghrelin receptor (triazole, lactam, and hydrocarbon) in comparison to those that saw a decrease (Glaser, xylene-

thioether). As a result, it is hypothesized that the rigidity of the stapling unit may influence the confirmation of the peptide when bound to the ghrelin receptor, which may affect the interactions that take place in the binding pocket. Furthermore, a cryo-EM study published by Qin and coworkers described how the ECL2 of the ghrelin receptor sits directly over the ligand-binding pocket, thereby suggesting that interactions with the c-terminal region of a ghrelin analogue could occur.[37] This indicates that the different stapling moieties may interact with these regions and could partly explain differences found in binding affinities for stapled analogues with similar degrees of freedom. Comparing the CD results to the binding affinity data towards the ghrelin receptor, there does not appear to be a clear correlation between increased helicity and increased binding affinity towards the ghrelin receptor. While the most helical peptides (**1-3)** were able to significantly increase binding affinity towards the receptor, the other two staples (**4** and **5**) that also saw an increase in helicity were weaker binders to the ghrelin receptor in comparison to the linear ghrelin (1-20).

Compound	**Sequence**	**IC_{50} (nM)**
Human Ghrelin	H-GSS(octanoyl)FLSPEHQRVQQRKESKKPPAKLQPR-OH	7.57 ± 1.51
Ghrelin (1-20)	H-GS-Dpr(octanoyl)-FLSPEHQRVQQRKESKK-OH	2.28 ± 0.48
1	H-GS-Dpr(octanoyl)-FLSPEHQR[XQQRY]ESKK-OH	0.92 ± 0.31
2	H-GS-Dpr(octanoyl)-FLSPEHQR[KQQRD]ESKK-OH	1.19 ± 0.40
3	H-GS-Dpr(octanoyl)-FLSPEHQR[OQQRO]ESKK-OH	1.44 ± 0.16
4	H-GS-Dpr(octanoyl)-FLSPEHQR[ZQQRZ]ESKK-OH	4.03 ± 0.65
5	H-GS-Dpr(octanoyl)-FLSPEHQR[CQQRC]ESKK-OH	4.93 ± 1.08

Table 2. Sequence and radioligand binding assays results (IC_{50} values) of synthesized stapled ghrelin(1-20) analogues, human ghrelin, and ghrelin(1-20), n = 2-3. X and Y correspond to lysine azide and propargyl glycine residues, respectively. O corresponds to 2-(4-pentenyl)alanine and Z corresponds to propargyl serine residues. See Figures 2.1-2.5 for structure details.

2.5 ß-Arrestin Recruitment BRET Assays

As previously described, ghrelin has been proposed as a potential molecular template for the design of therapeutics targeting cancer cachexia due to ghrelin's orexigenic properties when bound to its receptor. The ghrelin receptor is expressed in the hippocampus and hypothalamic arcuate nucleus where it is said to play a role in regulating feeding behavior.[62] In GPCRs, ligands activate the receptor by binding to the active site and causing a conformational change in the receptor that activates G-protein dissociation from the receptor. For the ghrelin receptor specifically, it exerts intracellular effects through G-protein activation primarily via $G_{q/11}$ and $G_{i/o}$.[63] Once these G-proteins have dissociated and activated their respective pathways, ß-arrestins normally bind to the intracellular c-terminus of the receptor and internalize the receptor through endocytosis. ß-arrestins were first described as being part of this desensitization pathway for the ghrelin receptor but recently they have been shown to act as molecular modulators for G-protein dependent signalling. More specifically, ß-arrestins have been linked to the adipogenic functions of the ghrelin/GHSR system.[64] As a result, to measure the ability of the stapled ghrelin analogues to activate the ghrelin receptor, ß-arrestin recruitment BRET assays were performed with transfected HEK293 cells using a GHSR-1a construct and either ß-arrestin 1 or 2 . Here, the concentration required to exhibit a 50% activation response (EC_{50}) can be obtained, which is referred to as the potency of the compound. As well, the maximum efficacy (E_{max}) exhibited by each stapled ghrelin analogue can also be obtained to compare which compounds activate the receptor with a greater signal response.

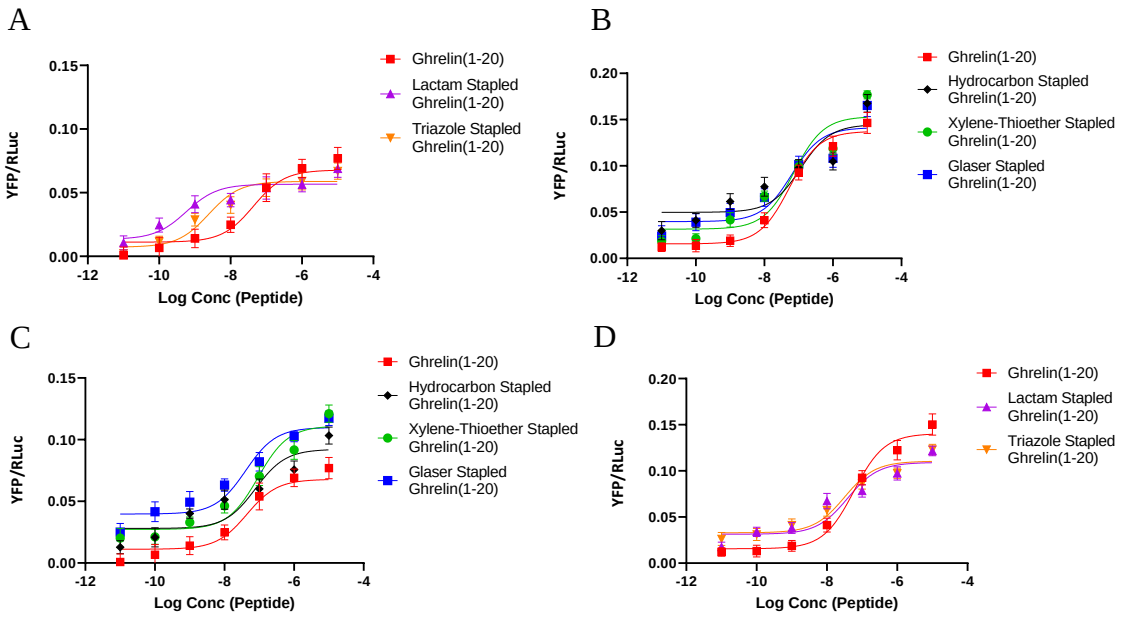

Figure 18. ß-arrestin recruitment BRET assay curves for the outlined compounds. (A) and (B) are for ß-arrestin 1, (C) and (D) are for ß-arrestin 2.

The results from the BRET assays can be found in Table 2.3. Notably, these assays revealed that the triazole and lactam (Lys-Asp) stapled analogues (**1** and **2**) activated the receptor with greater potency than unstapled ghrelin(1-20) with EC_{50} values for ß-arrestin 1 of 2.4 nM and 0.6 nM, and values for ß-arrestin 2 of 32.0 nM and 38.7 nM, respectively (Figure 18. A and C). However, both analogues did so with E_{max} values that were lower than unstapled ghrelin(1-20). In contrast, the hydrocarbon, xylene-thioether and Glaser stapled analogues (**3-5**) activated the receptor with greater efficacy than unstapled ghrelin(1-20) with E_{max} values for ß-arrestin 1 of 0.922, 0.110, and 0.111 and values for ß-arrestin 2 of 0.145, 0.141 and 0.153, respectively (Figure 1.8. B and D). These compounds activated the receptor with slightly lower potency than the unstapled ghrelin analogue.

Compound	ß-arr 1 E_{max}	ß-arr 1 EC_{50}	ß-arr 2 E_{max}	ß-arr 2 EC_{50}
Ghrelin (1-20)	0.068 ± 0.021	44.1 ± 13.6 nM	0.137 ± 0.013	54.0 ± 5.12 nM
1	0.059 ± 0.007	2.43 ± 0.29 nM	0.111 ± 0.011	32.0 ± 3.17 nM
2	0.057 ± 0.008	0.57 ± 0.08 nM	0.109 ± 0.010	38.7 ± 3.55 nM
3	0.092 ± 0.019	77.2 ± 15.9 nM	0.145 ± 0.054	114 ± 42.5 nM
4	0.110 ± 0.013	40.5 ± 4.78 nM	0.141 ± 0.031	62.8 ± 13.8 nM
5	0.111 ± 0.013	91.7 ± 10.7 nM	0.153 ± 0.015	71.6 ± 7.02 nM

Table 3. Results from ß-Arrestin Recruitment BRET Assays, n = 3-8. See Figures 2.1-2.5 for structure details.

Differences between the bioactivity of the stapled ghrelin analogues could be explained by variations in the flexibility of the staple units. The stapled compounds with the more flexible staples (**1** and **2**) appeared to activate the ghrelin receptor as potent partial agonists for both ß-arrestin 1 & 2 recruitment. Partial agonists are ligands that are unable to induce maximal activation of a receptor population, suggesting that the more flexible staples allow for confirmations of ghrelin(1-20) that only stabilize the active conformation of the receptor partially but do so with great affinity. This is consistent with the data from the radioligand binding assays performed, where compounds **1** and **2** binded to the ghrelin receptor with higher affinity than unstapled ghrelin(1-20). Conversely, the more rigid peptide staples (**4** and **5**) acted as super agonist of the ghrelin receptor. This suggest that increased rigidity of the peptide staple unit allows for interactions of ghrelin(1-20) that effectively stabilize the active conformation of the ghrelin receptor. Though an outlier with this trend does exist, where the hydrocarbon stapled ghrelin analogue (**3**) which has a flexible stapling unit like compounds **1** and **2**, exhibited super agonist activity at a slightly reduced potency when compared to ghrelin(1-20). While it is not known why this stapled compound demonstrates this unique bioactivity, it is postulated that its disubstituted amino acid residues may play a role in the ligand confirmations that lead to the stabilization of the active conformation of the ghrelin receptor.

2.6 Serum Stability Assays

Like many natural peptides, ghrelin suffers from having poor *in vivo* stability due to the efficiency of proteases at hydrolysing amide bonds.[65] To test our hypothesis that increasing the α-helical character of ghrelin analogues would increase their metabolic stability from protease degradation, serum stability assays were conducted. For these assays, the most helical compound (**3**) and unstapled ghrelin(1-20) were evaluated to see if there would be a significant difference in metabolic stability. Peptides were incubated in a solution of 25% human serum in PBS and aliquots would be periodically removed for quantification by HPLC/MS.

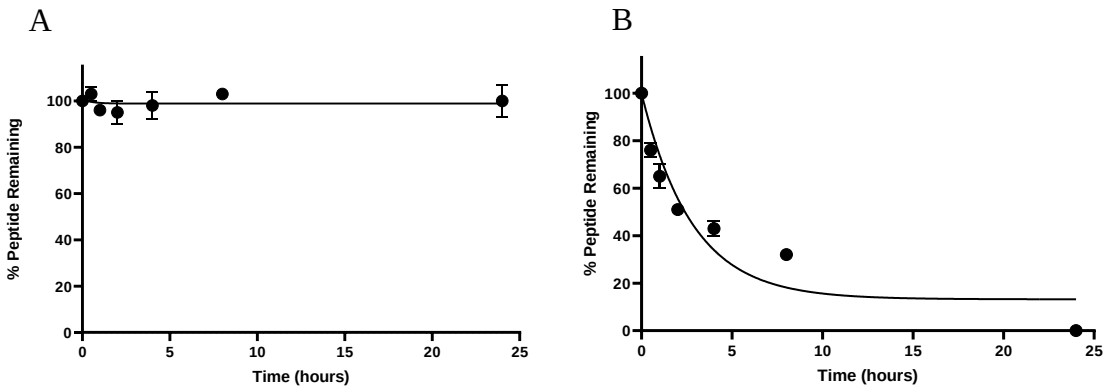

Figure 19. Human serum stability assay results for (A) **3** and (B) ghrelin(1-20).

It was determined that compound **3** was stable from proteolysis by serum proteins for over 24 hours. This is in stark contrast with the unstapled ghrelin(1-20) compound which had a calculated $t_{1/2} < 5$ hours. Therefore, it can be stated that inducing significant helicity into the peptide structure using chemical staples does yield increased metabolic stability from human serum proteins. As mentioned previously, studies have demonstrated that the active sites of human proteases do not permit an α-helical confirmation to bind, which is consistent with the results seen from these assays.[34] The proteolytic resistance exhibited by the hydrocarbon staple has also been well documented in the literature. In the

work by Bird and coworkers, they demonstrated how the degree of α-helix stabilization and the number of inserted staples correlated with increased proteolytic resistance.[66]

2.7 Conclusion

The five ghrelin(1-20) analogues showcased in this work all contained distinct helix-inducing staples, providing cyclic peptides that have varying flexibility, lipophilicity, and potential for functionality. The stapled peptides were evaluated for their affinity towards and their ability to activate the ghrelin receptor. The results from the bioassays suggested that the flexibility of the stapling unit influenced both affinity and activity towards the ghrelin receptor. The more flexible peptide staples (**1**-**3**) were determined to have the greatest increase in binding affinity towards the ghrelin receptor in comparison to unstapled ghrelin(1-20), whereas the more rigid staples (**4** and **5**) saw a decrease. Interestingly, some of the flexible linkers (**1** and **2)** activated the ghrelin receptor as potent partial agonists when compared to their unstapled counterpart. This contrasts with the more rigid linkers (**4** and **5**) which acted as super agonists for the ghrelin receptor. An outlier, in the activity assays was also discovered where compound **3**, which has a flexible linker unit, also acted as a super agonist for the ghrelin receptor. The stapled peptides were also evaluated for their helical content and for their stability in human serum. It was discovered that the introduction of all peptide staples into the ghrelin(1-20) structure resulted in an increase in α-helicity compared to their linear counterpart albeit to different extends. A trend was seen where the more flexible linker units (**1-3**) saw the greatest increase in percent helicity. Moreover, compound **5** was found to yield the greatest increase in helicity out of all the stapling techniques investigated with a 39% helical character. Compound **5** was then further evaluated in serum stability assays and was determined to be metabolically stable for over 24 hours. The work presented demonstrates that the incorporation of a peptide staple into ghrelin(1-20) analogues could be used as a template for the design of imaging agents targeting diseases such as ovarian and prostate cancer. The retained agonist properties of the stapled peptides suggests promise for their use as therapeutic agents for diseases such as cancer cachexia.

Chapter 3

3.0 Outlook

The ghrelin receptor has become a target of interest for the pharmaceutical industry since its discovery by Howard and coworkers in 1996 due to its wide range of biological functions.[67] As such, a variety of agonist, antagonists and inverse agonists have been developed to target the ghrelin receptor for diseases such as anorexia, obesity, cachexia, and diabetes.[68-70] The ghrelin receptor's differential expression in cancers and other diseases have also made it a promising target for molecular imaging. While peptidomimetics and small molecules have been developed to target the ghrelin receptor, its high affinity endogenous ligand ghrelin can serve as an effective template for the design of molecular imaging agents and therapeutics. As highlighted in this report, the linear nature of ghrelin analogues makes them susceptible to hydrolysis by proteases and such new strategies are needed to stabilize ghrelin's structure from metabolic degradation. Ghrelin's partial helical character when bound to the ghrelin receptor[37] and the previous work showcasing that staples at selective locations in the ghrelin structure can yield high affinity compounds has set the foundation for this project.[42] Thus, we presented a survey of helix-inducing staples to investigate which cyclization techniques would yield the highest affinity and activity towards the ghrelin receptor, while also increasing the peptides stability from proteolytic degradation.

The staples showcased in this report were selectively picked for their ability to induce α-helicity in a peptide structure with an *i,i+4* spacing. Most of these staples are formed from unnatural amino acid residues, which adds a level of complexity and cost when the objective is to translate these compounds into therapeutics. As a result, some efforts in peptide chemistry have been dedicated to produce efficient helix-inducing staples from natural amino acids and inexpensive linkers. One example of this was shown by Fairlie and coworkers where they appended D-glucuronic acid to a lysine and a serine residue in an *i,i+4* spacing.[71] This stapling technique proved to be successful at inducing helicity for pentapeptides at of varying sequences, with some compounds reaching over

50% helical character. Other efforts in stapling techniques have focused on producing highly functional staples that show significant induction of helicity in peptide structures. One example of this was demonstrated through an oxime staple that was derived from acetone-linked peptides.[72] The method is comprised of bis-alkylating homocysteine residues at either *i,i+4* and *i,i+7* spacing to yield a highly functional peptide staple which effectively induced helicity in their model peptide. This technique allowed for the functionalization of the oxime moiety to which the authors appended a variety of molecular structures such as PEG linkers, fluorescent dyes, cell penetrating peptides and targeting moieties. More recently, a unique stapling technique showcased that a fluorescent dye could be used as the linker unit for a peptide staple in a wide range of spacings (*i,i+2*-*i,i+7*).[73] Using dibromobimane as a linker and a variety of cysteine analogues, the authors were able to successfully cyclize their model peptides using thiol-alkylation chemistry. The resulting stapled peptides saw a significant increase in helical character and were effectively used to image NIH/3T3 cells using confocal microscopy. Overall, it can be said that the field of peptide stapling is veering to more cost effective cyclizations using natural amino acids, and for stapling techniques with highly functional linkers.

One of the applications where the effective targeting of the ghrelin receptor is still required is in the development of imaging probes for prostate cancer. Prostate cancer is categorized as a leading malignancy in men with roughly 270,000 new cases and 34,500 deaths in 2022. Statistical analysis of prostate cancer incidence over the last decade reveals that progress in diagnosis has stalled due to a decline in routine screening for men aged 75 and older and an increase distant stage diagnosis (advanced cancers).[74] While the use of prostate specific antigen (PSA) test has been attributed to a halving of death rates in the last quarter century, the development of new imaging probes targeting this cancer can aid in the earlier detection of this malignancy.[75] The ghrelin receptor has also been shown to be differentially expressed in prostate cancer tissue.[8] While a fluorescein appended ghrelin analogues was developed and effectively imaged differential binding *ex vivo*, no ghrelin-based probes have been developed that have entered the clinic. Also for imaging, high affinity peptidomimetic PET agents have been developed to target prostate cancer.[76] However, these potent ghrelin receptor agonists require *in vivo* evaluation to assess their

potential as clinical imaging agents. As such, there remains a need for radiopharmaceuticals targeting the ghrelin receptor for prostate cancer imaging.

Another potential application for the targeting of the ghrelin receptor is for the treatment of cancer cachexia. This metabolic disorder, which is a complication of cancer causes weight and muscle loss and affects roughly half of all cancer patients.[6] It is reported that cancer cachexia is the cause of 30% of cancer deaths and has no known therapeutic to date.[6] The ghrelin receptor has become a target of interest for the treatment of this disorder due to the appetite stimulating effects that occur when the receptor is activated. Consequently, studies have used ghrelin as a treatment for cancer cachexia to study its orexigenic effects.[30,32] While results from studies using intravenous ghrelin have shown increased appetite, muscle tissue, and reduction of adipocyte tissue loss, it is unclear if human ghrelin can act as a suitable therapeutic for this disease.[77] Moreover, due to ghrelin's pharmacological profile, potential side-effects and short half-life, well-monitored parenteral administration is required, which puts into to question its efficacy in the clinic.[78] Efforts to try to target the ghrelin receptor for the treatment of cancer cachexia have also included quinolone derivatives.[79] These potential therapeutics showed super agonist character in calcium influx assays in hypothalamic neurons, suggesting their potential to activate appetite stimulating pathways in the brain. However, these potent compounds have only been reported in *in vitro* studies and require animal testing to evaluate their appetite stimulating potential. Therefore, a robust therapeutic for the treatment of cancer cachexia is still in need for development.

Peptides and small molecule therapeutics, like the ones described above, have varying pharmacological profiles. Traditionally, small molecules have been known for their small size (>500 Da) and offer high metabolic stability and oral bioavailability. However, small molecules are also known to produce significant side effects due to their poor target selectivity. Peptides are seen as the middle ground between small molecules and the larger biologics (>5000 Da). Endogenous peptides, like ghrelin, offer high target selectivity and potency but have poor metabolic stability and oral bioavailability, requiring intravenous administration of the therapeutics. Due to the unique pharmacology of such peptides, efforts at modifying their structure and improving their poor pharmacokinetic properties

have been of interest to peptide chemist. One of the most popular methods of improving these properties are by introducing peptide staples into the peptide structure. Cyclizing peptides has been shown to improve metabolic stability, oral bioavailability and at times affinity towards the target receptor.[80] A successful example of this is the hydrocarbon stapled peptide ATSP-7041, a potent inhibitor of MDM2 and MDMX for p53-dependent cancer therapy.[81] In their work, the author's noted that the stapled peptide demonstrated robust cell penetrating properties and was resistant to proteolytic degradation. This stapled compound also showed nanomolar affinity towards its target and potent inhibition of a protein-protein interaction had been previously targeted by small molecules, unsuccessfully. As such, stapled peptides are a promising form of therapeutics for molecular targets that where once thought as undruggable. Moreover, modifying peptides through the incorporation of staples has become a significant molecular tool to optimize the pharmacological properties of peptides in the design of robust therapeutics.

In this thesis, five ghrelin(1-20) analogues bearing different helix inducing staples were successfully synthesized, characterized, and evaluated using bioassays. Unique and distinct cyclization methodologies were employed to synthesize these compounds including lactam bond formation, copper assisted alkyne-azide cycloaddition, ring closing metathesis of olefins, two component thioether linker formation, and Glaser-Hay cross coupling. Some trends were seen linking the flexibility of the linker unit and helical character of the stapled ghrelin analogues as well as their respective bioactivity. Firstly, the ghrelin analogues bearing the more flexible staple units (lactam, triazole and hydrocarbon staples) saw the greatest increase in percent helicity. Similar trends were seen in the results from the bioassays which also suggested that the flexibility of the peptide stapled had an influence on both the affinity and activity towards the ghrelin receptor. In terms of binding affinity towards the ghrelin receptor, the more flexible staples had the greatest increase in binding affinity towards the ghrelin receptor whereas the more rigid staples (xylene-thioether and Glaser staples) saw a decrease when compared to the unstapled ghrelin analogue. Similarly, the flexibility of the stapling unit appeared to have some influence on how the ghrelin receptor was activated. This was seen as the triazole and lactam staples acted as potent partial agonists for the ghrelin receptor, which contrasts with the Glaser and

xylene-thioether staples that acted as super agonists. However, the hydrocarbon staple which has increased flexibility in its linker unit, also acted as a super agonist. The hydrocarbon staple was subjected to serum stability assays to probe its metabolic stability due its increased helical character. In these studies, it was determined to be stable from proteolysis for over 24 hours, confirming the hypothesis that increasing α-helical character significantly improves metabolic stability.

Future work in this project involves more comprehensive probing of the pharmacology of the stapled analogues, possibly through Ca^{2+} mobilization assays. These assays consist of measuring intracellular calcium with a fluorescent-dye to quantify receptor activation and are effective at determining the potency of ghrelin receptor agonists and antagonists.[82] These functional assays would provide further insight and validation to the receptor activating properties of the stapled ghrelin analogues. Other functional assays that would provide similar insight are inositol trisphosphate turnover and cAMP accumulation assays. The outlined compounds that are then determined to have the desired combination of high affinity and activity towards the ghrelin receptor and high stability from enzymatic degradation, may then be subjected to animal feeding studies to investigate their potential as therapeutics for the treatment of cancer cachexia. Likewise, the high affinity and highly protease-resistant compounds may also be appended to molecular imaging moieties (radionuclide or fluorescent compounds) for their evaluation as imaging agents. These probes have the potential to be used as imaging agents targeting ovarian and prostate cancer, as well as aid in studies to identify if the ghrelin-GHSR axis may serve as a biomarker for cardiomyopathy patients.

4.0 Experimental

4.1 Peptide synthesis and Characterization

Peptides were synthesized using standard fluorenylmethyloxycarbonyl (Fmoc) based solid-phase peptide synthesis (SPPS) conditions on a Biotage SyroWave automated peptide synthesizer. Syntheses were done on a 0.1 mmol scale using Fmoc-protected Rink amide MBHA resin, which was allowed to swell before each synthesis with 2 mL of dichloromethane for 10 minutes. Fmoc deprotection was completed by adding 20% piperidine in dimethylformamide (DMF) for 12 minutes with 30 second vortexing, washing 3 times with DMF, and repeating the 20 % piperidine in DMF for another 12 minutes with 30 second vortexing. Coupling of the desired amino acid was accomplished by adding the fmoc-protected amino acid (4 eq) in DMF, HCTU (4 eq) in DMF and DIPEA in N-methyl pyrrolidinone for 1 hour in vortex. This amino acid coupling and deprotection cycle was continued until the desired sequence of the peptide was completed. In the case of (**5**), the coupling reaction for the disubstituted pentenyl alanine residues was performed twice with the coupling agent HATU due to incomplete coupling seen with standard coupling conditions (see 3.2.5). After each synthesis, and in between modifications of the peptides, small samples of the resin-bound peptides were obtained and "micro-cleaved" by adding to the small sample with a 300 µL cleavage cocktail of 95% trifluoroacetic acid, 2.5% triisopropylsilane, and 2.5% water for 1 hour in vortex. The resulting liquid would then be precipitated with 1 mL of cold tert-butyl methyl ether (TBME), centrifuged for 10 minutes at 3000 rpm, and the mother liquor decanted. The resulting product was characterized using analytical reverse-phase high-performance liquid chromatography tandem mass spectrometry (HPLC/MS) over gradients of acetonitrile (0.1% TFA) and water (0.1% TFA). The same procedure was used when fully cleaving the peptides from the resin with 5 mL cleavage cocktails and 20 mL of TBME for precipitating the peptides. Fully cleaved peptides were then purified using a preparative reverse-phase HPLC/MS instrument.

4.1.1 Deprotection and octanoyl coupling of Dpr^3

The Dpr^3 residue was orthogonally protected in each synthesis with either an allyloxycarbonyl (Alloc) group or 4-methyltrityl (Mtt) group. For the selective deprotection of the Alloc group, 24 eq of phenylsilane resin-bound peptide containing vessel and allowed to vortex for 5 minutes. Then, 0.1 eq of tetrakis(triphenylphosphine) palladium (0) was added to the mixture and vortexed for a further 10 min to yield the deprotected free amine after thorough washes. Selective deprotection of the Mtt was achieved by adding a mixture of 1% TFA, 4% TIPS, and 95% DCM to the peptide vessel and vortexing for 2 minutes. This was repeated 10 times with thorough washes of DCM in between. Once the free amine was present after the deprotection of the respective group, the coupling of the octanoyl chain was immediately performed. This was achieved by mixing 3 eq of octanoic acid and 3 eq of HATU coupling reagent in 2 mL of DMF and adding this mixture to the peptide vessel. After 30 seconds of vortexing, 6 eq of DIPEA was added to the vessel and allowed to stir for 2 hours.

4.1.2 Triazole Stapled Ghrelin (1-20) Analogue Cyclization **(1)**

The cyclization of the triazole stapled ghrelin analogue involved a copper-assisted azide-alkyne cycloaddition reaction. This was performed on the resin-bound peptide by adding to the peptide vessel: 5.5 mL of anhydrous DMSO, 1 eq of copper (I) bromide, 1 eq of sodium ascorbate in 750 uL of water, 10 eq of 2,6-lutidine and 10 eq of DIPEA. The reaction was the allowed to stir overnight. The resin-bound peptide thoroughly washed with isopropanol and DMSO. After further modifications as described in section 2.1.1, cleavage of the peptide bound resin and purification using preparative HPLC/MS compound **1** was successfully synthesized. MS (ESI+) $[M+H]^{3+}$ m/z calculated: 834.7788, $[M+H]^{3+}$ m/z observed: 834.8093.

4.1.3 Lactam (Lys-Asp) Stapled Ghrelin (1-20) Analogue Cyclization **(2)**

The lactam cyclization reaction involved the formation of an amide bond between the lysine and aspartic acid residues. This was achieved by adding a solution of 3 eq of HATU in 2 mL of DMF to the peptide vessel. After vortexing for 30 seconds, 6 eq of DIPEA were

added to the mixture and the resulting solution was allowed to vortex for two hours. Cleavage and purification of the peptide resulted in compound **2**. MS (ESI+) $[M+H]^{3+}$ m/z calculated: 826.7750, $[M+H]^{3+}$ m/z observed: 827.1233.

4.1.4 Hydrocarbon Stapled Ghrelin (1-20) Analogue Cyclization **(3)**

The ring closing metathesis reaction was used to cyclize the hydrocarbon stapled ghrelin analogue. This reaction was completed using 20% mol of Grubb's 2nd generation catalyst in DCE. Almost complete cyclization was achieved when the reaction was repeated twice using fresh reagents. Following successful identification of the cyclic product, the resulting compound was then purified using preparative HPLC/MS to yield compound **3**. MS (ESI+) $[M+H]^{3+}$ m/z calculated: 835.1273, $[M+H]^{3+}$ m/z observed: 835.4740.

4.1.5 Glaser Stapled Ghrelin (1-20) Analogue Cyclization **(4)**

The cyclization of the Glaser stapled ghrelin analogue involved a Glaser-Hay coupling reaction. This was achieved by first making a solution using 10 mL of anhydrous DMSO and 10 eq of copper (I) chloride and 15 equivalents of biphenyl-2,3-diol (BPY-diol) ligand. The mixture was then added to the peptide vessel and vortexed for 30 seconds. Immediately after, 20 eq of DIPEA were added to the peptide vessel and the resulting solution was allowed to vortex for 3 days. Cleavage and purification of the compound was able to successfully yield compound **4**. MS (ESI+) $[M+H]^{3+}$ m/z calculated: 834.4312, $[M+H]^{3+}$ m/z observed: 834.7562.

4.1.6 Bis-thioether Stapled Ghrelin (1-20) Analogue Cyclization **(5)**

The two-component cyclization reaction for the bis-thioether stapled ghrelin analogue involved thioether formation by coupling the xylene crosslinker to the free sulfides on the peptide. This was performed by adding the cleaved peptide product to a mixture of ACN:H_2O solution containing 100mM of NH_4CO_3 buffer. To this mixture 1.5 eq of TCEP was added and allowed to stir for 1 hour. Then, 3 eq of the α, α′-dibromo-p-xylene linker and allowed to stir for 2 hours. The reaction was the quenched using 5% HCl and purified

using preparative HPLC/MS to effectively yield compound **5**. MS (ESI+) $[M+H]^{3+}$ m/z calculated: 854.4263, $[M+H]^{3+}$ m/z observed: 854.7375.

4.2 Circular Dichroism and Percent Helicity

Circular Dichroism (CD) spectra was obtained using a Jasco J-810 spectropolarimeter and recorded in the range of 185–260 nm. Peptide solutions were prepared with sodium phosphate buffer at a pH of 7.2 and a concentration of 0.1 mM. Measurements were performed using a cuvette with a path length of 1 mm and a scanning speed of 10-50 nm/min. Five individual scans were averaged by the instrument to obtain the CD spectra. All spectra were collected at a temperature of 20ºC. The percent helicity for each peptide was calculate using equation 2.1. In the equation, $[\theta]_{222}$ corresponds to the mean residue ellipticity of the peptide at 222 nm and $[\theta]_0$ corresponds to the mean residue ellipticity of a random coil conformation. $[\theta]_{max}$ corresponds to the maximum theoretical mean residue ellipticity for a helix of n residues and is described in equation 2.2, where x is an empirical constant equal to 3.

$$f\ helix = \frac{[\theta]_{222} - [\theta]_0}{[\theta]_{max} - [\theta]_0}$$

Equation 3.1 Percent helicity equation

$$[\theta]_{max} = [\theta]_{\infty}\frac{(n-x)}{n}$$

Equation 3.2 Maximum theoretical mean residue ellipticity equation

4.3 Radioligand-Displacement Binding Assay

4.3.1 GHSR-1a Transfection of HEK293 Cells

HEK293 cells were maintained in T175 flask containing RPMI medium supplemented with 10% fetal bovine serum and were passaged 2-3 times per week. On the day of transfection,

a fully confluent T175 flask of cells was seeded into four 10 cm^3 plates. A solution was made for each plate containing 480 µL of opti-MEM and 15 µL of X-tremeGENE 9 DNA Transfection Reagent in a glass vial and set aside for 15 min to allow for complex to form. Then, 5 µg of DNA of GHSR-1a-eYFP construct was added to each vial and allowed to sit for 20 minutes. The resulting 500 µL DNA complex solution was then added dropwise to each 10 cm^3 plate and allowed to incubate for 24 hours. The following day the cells were harvested and allowed to freeze gradually to a temperature of -150ºC and stored until needed.

4.3.2 Binding Assay

The binding affinity towards GHSR-1a of the ghrelin(1-20) analogues were determined through radioligand competitive binding assays. Assays were performed using the human [His[^{125}I]]-ghrelin radioligand and GHSR-1a transfected HEK293 cells. A suspension of 50 000 cells assay tube were incubated with the outlined ghrelin(1-20) analogues at concentrations of 10^{-5} to 10^{-11} M and 15 pM of human [His[^{125}I]]-ghrelin radioligand in binding buffer. The binding buffer consisted of 25 mM HEPES, 5 mM magnesium chloride, 1 mM calcium chloride, 2.5 mM EDTA, and 0.4% BSA, pH 7.4). Cell suspension was incubated for 20 min at 37.5ºC with stirring at 550 rpm. Immediately following incubation, the resulting cell suspension was centrifuged to produce a cell pellet which was then washed with a tris-HCl solution. The assay tubes were then place in a γ counter where the unbound radioligand was measured.

4.4 Beta-arrestin BRET Assays

4.4.1 GHSR-1a Transfection of HEK293 Cells

HEK293 cells were maintained in T175 flask containing RPMI medium supplemented with 10% fetal bovine serum and were passaged 2-3 times per week. On the day of the transfection, two vials with 300 µL of opti-MEM and 9 µL of X-tremeGENE 9 DNA Transfection Reagent were set aside for 20 min to allow for a complex to form. Then, 7.9 µg of DNA of GHSR-1a-eYFP construct and 300 µg of Beta-Arrestin 1 or 2 tagged with

RLuc was added to each vial and allowed to sit for 40 minutes. 2mL were then taken from a fully confluent HEK293 T175 flask, diluted to 12 mL and split evenly in a 6 well plate. The resulting DNA complex solution was then added dropwise to each well and allowed to incubate for 24 hours. The following day the cells were harvested and transferred onto a 96 well plate with each well containing 100 µL of the transfected HEK293 cell solution. 24 hours after transferring to the 96 well plate, the cells were ready for the BRET assay.

4.4.2 BRET Assay

The ability of the stapled ghrelin analogues to activate the GHSR was determined using beta-arrestin BRET assays. Once the 96 well plate with the transfected HEK293 cells (see above) has been plated for 24 hours, a drug plate containing a serial dilution of the outlined stapled analogues with a 10^{-4} to 10^{-10} concentration range was prepared using Hank's balanced salt solution (HBSS). The cell media from the 96 well plate was then discarded and to each well 90 µL of HBSS and 10 µL of drug was added for a final concentration range of 10^{-5} to 10^{-11}. The plate was then allowed to sit at room temperature for 20 minutes. At the 15-minute mark, 10 µL of a 50 µM H-coelenterazine solution is added to each well for a final concentration of 5 µM. The plate is then allowed to sit for the remaining 5 minutes and then placed in a Mithras LB940 plate reader to measure the corresponding BRET signal.

4.5 Serum Stability Assays

The stability of the stapled ghrelin analogues in human serum was assessed by incubating each peptide (1 mM) at 37 °C in a 25% serum PBS solution at pH 7.4. Sample aliquots were collected after 10 minutes of incubation in serum to allow for equilibrium between the peptide and serum. 15 µL aliquots of the peptide solution were removed in triplicate at 0, 30 min, 1h, 2h , 4h , 8h , and 24 h. 60 µL of 4% NH_4OH(aq) was used to quench the reaction and residual salts and proteins were removed using a Waters Oasis HLB microextraction plate. The cartridge was activated using 200 µL of MeOH followed by 200 µL of water. The sample solution was loaded onto the cartridge and the column was washed

with 200 μL of 5% MeOH (aq). The peptide was then eluted using 30 μL (x2) of 2% formic acid in MeOH. The resulting 60 μL aliquot of the eluted peptide was then analyzed by HPLC-MS. The selected ion chromatogram corresponding to the m/z value of the peptide was obtained and the resulting MS peak was integrated to quantify the amount of intact peptide remaining at each time-point.

5.0 References

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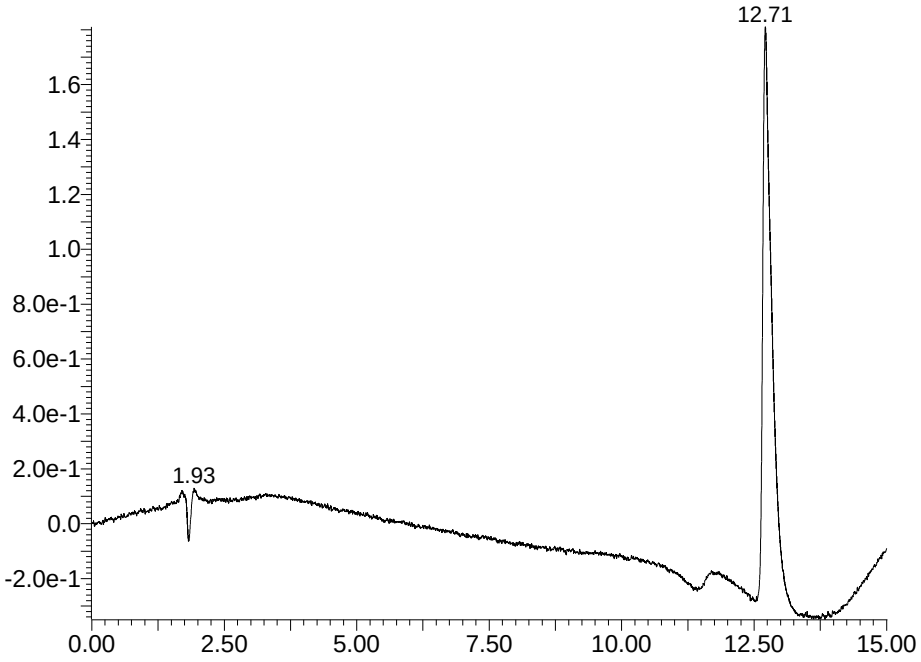
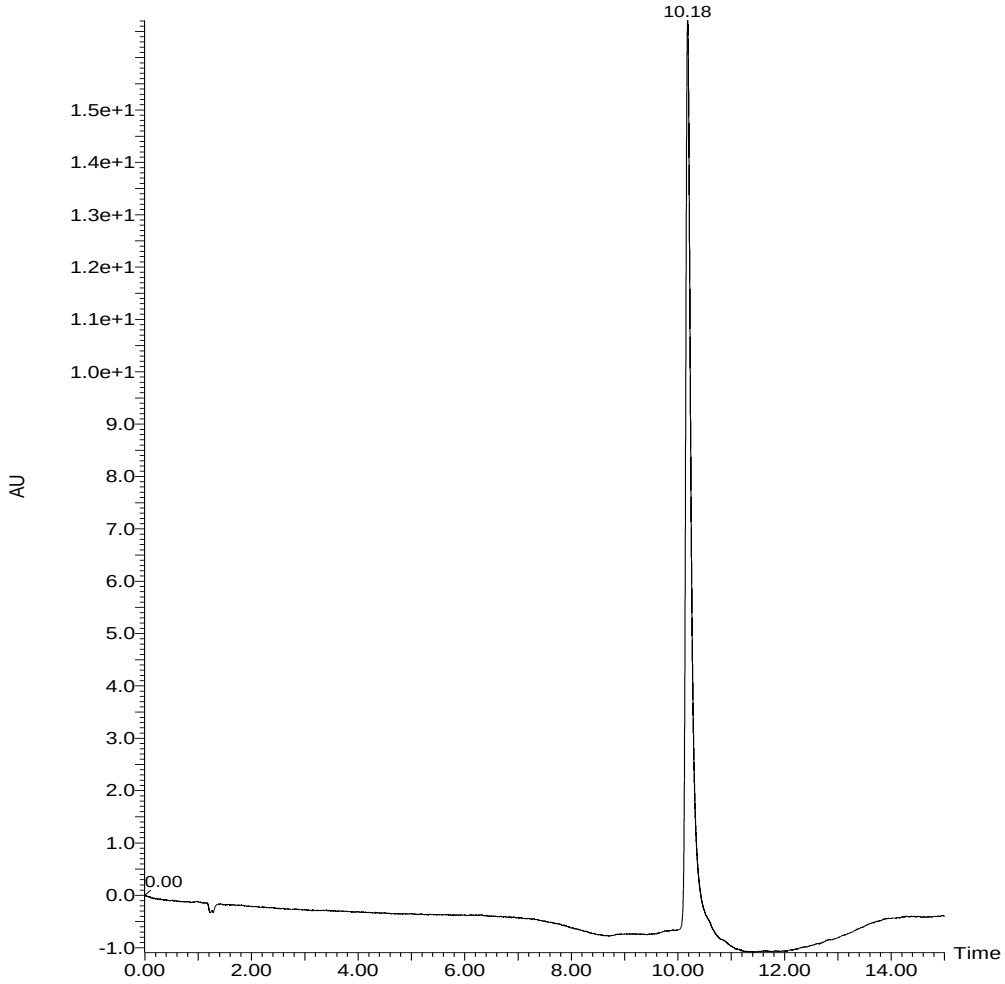
6.0 Appendices

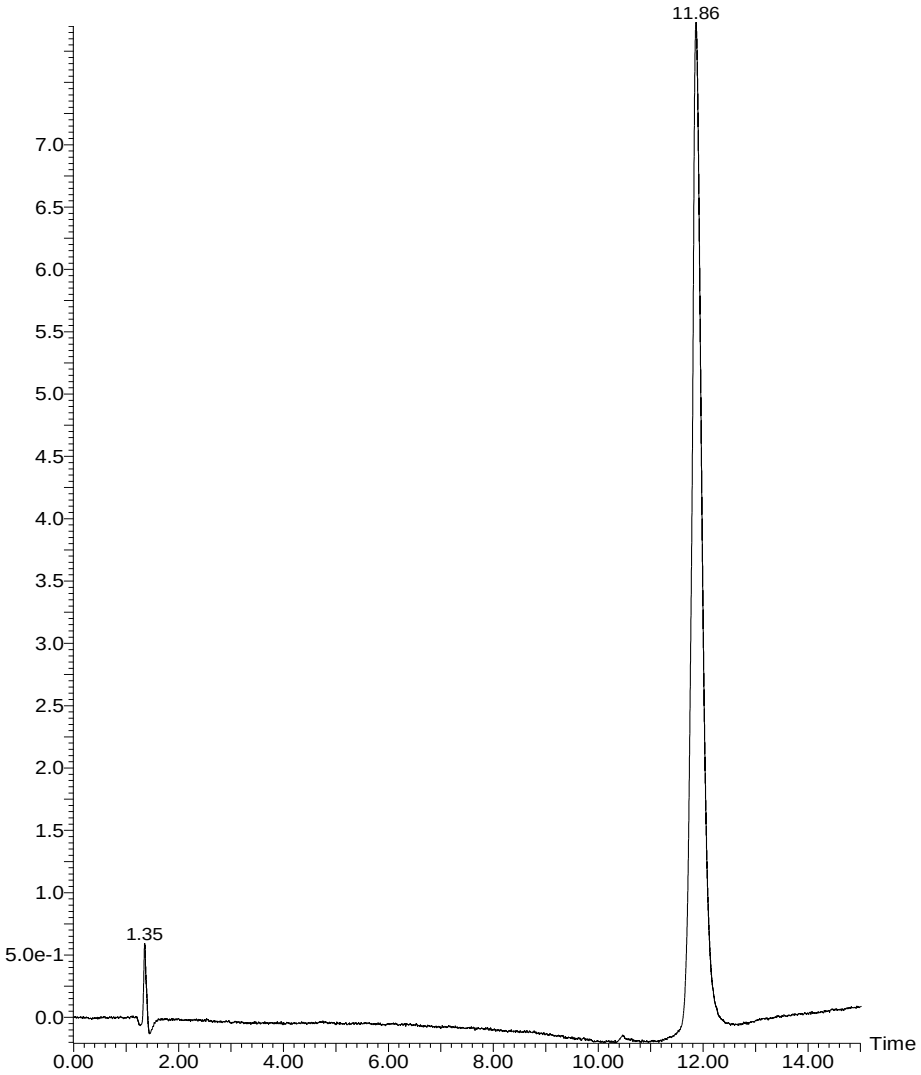
6.1 Appendix A: Peptide Chromatograms



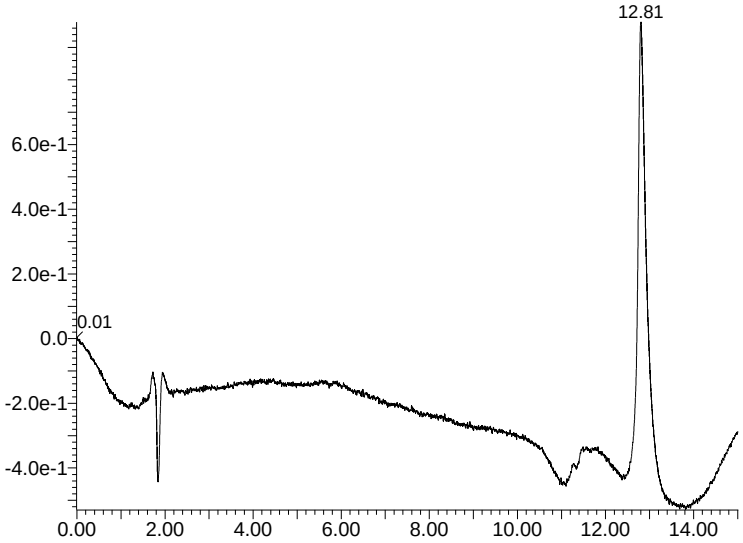
Analytical UV chromatogram from HPLC of purified compound **1** (20-80% Acetonitrile/Water)



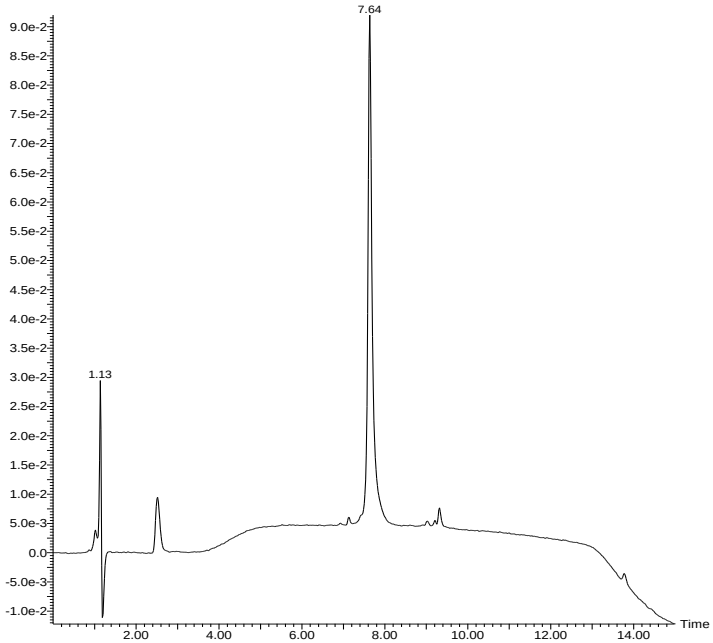
Analytical UV chromatogram from HPLC of purified compound **2** (20-80% Acetonitrile/Water)



Analytical UV chromatogram from HPLC of purified compound **3** (20-50% Acetonitrile/Water)

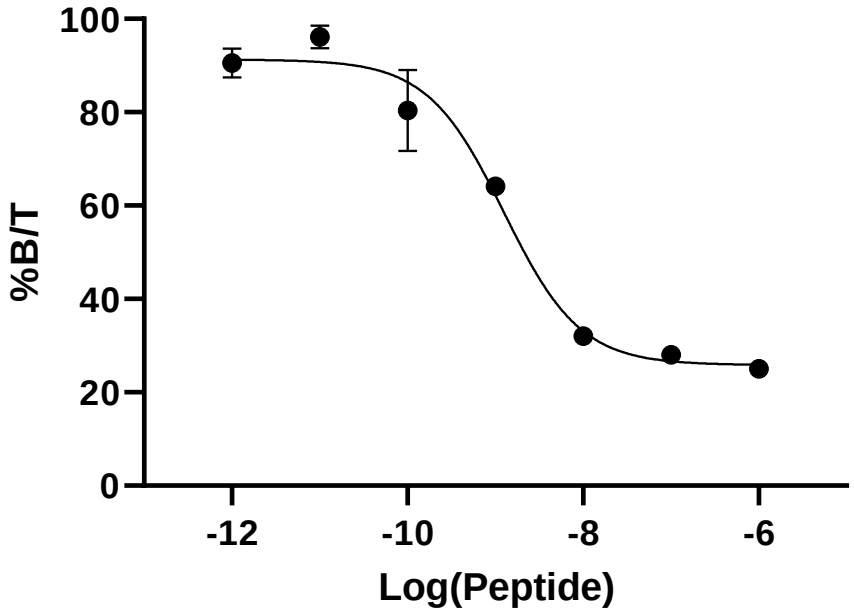


Analytical UV chromatogram from HPLC of purified compound **4** (20-80% Acetonitrile/Water)

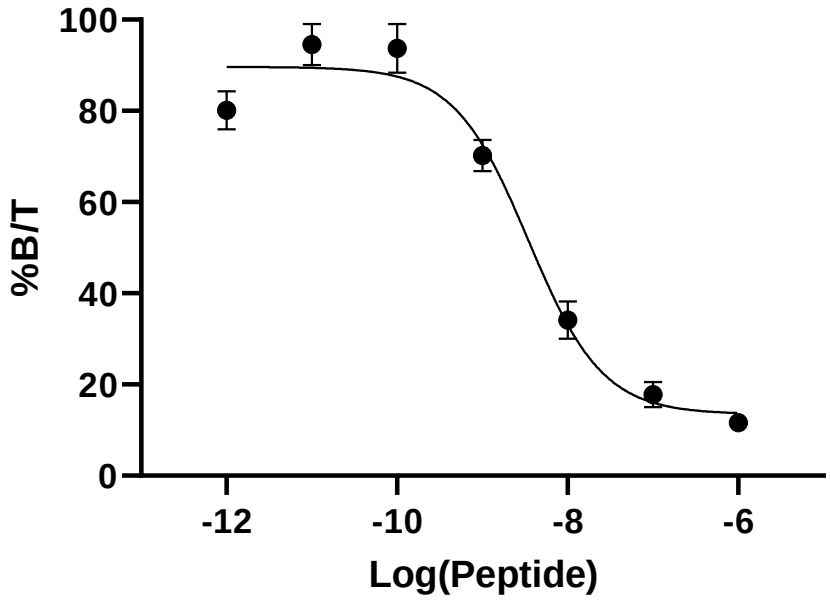


Analytical UV chromatogram from HPLC of purified compound **5** (10-70% Acetonitrile/Water.

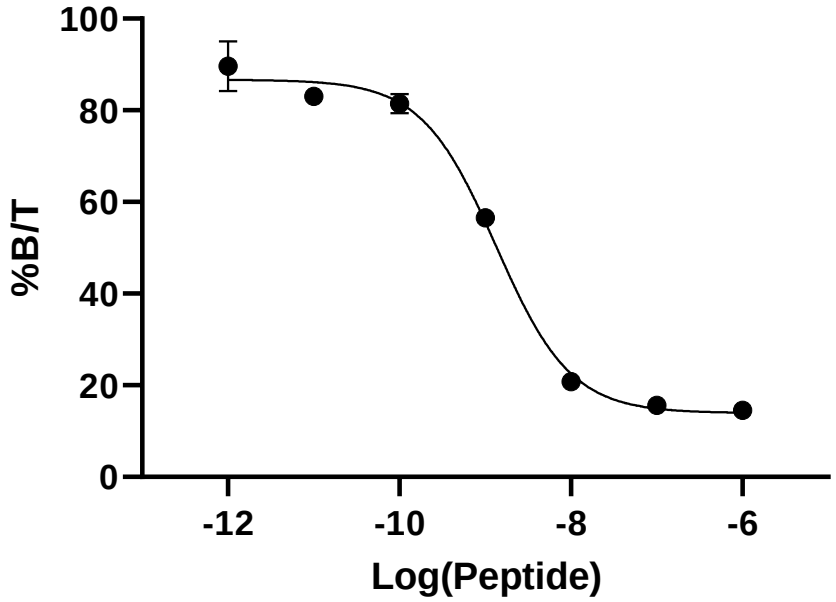
6.2 Appendix B: Displacement curves



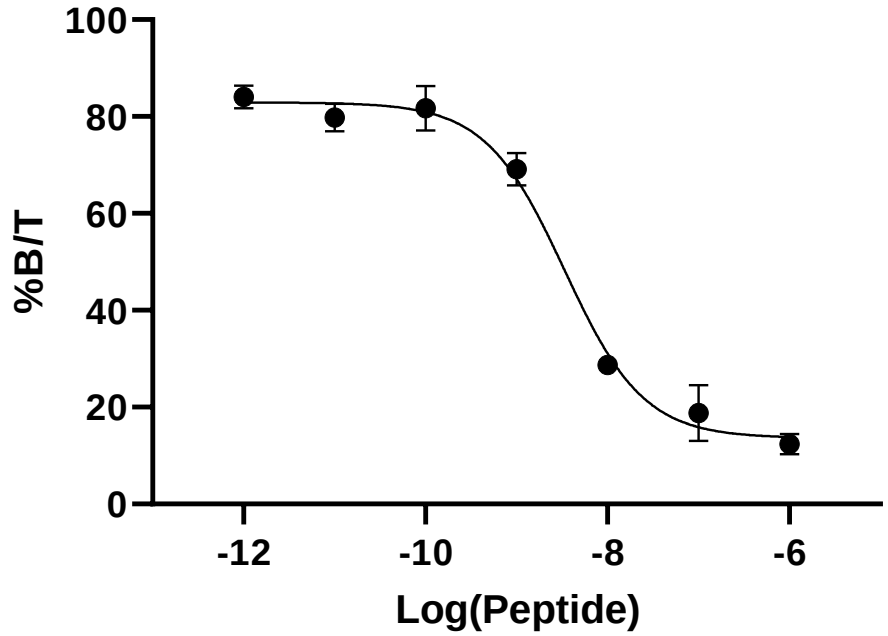
IC_{50} curve for compound **1**.



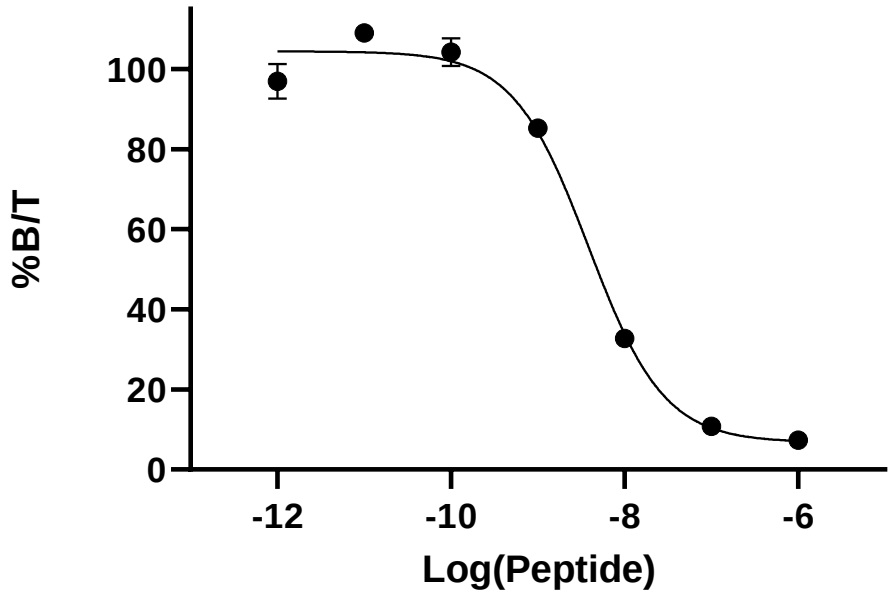
IC_{50} curve for compound **2**.



IC_{50} curve for compound **3**.

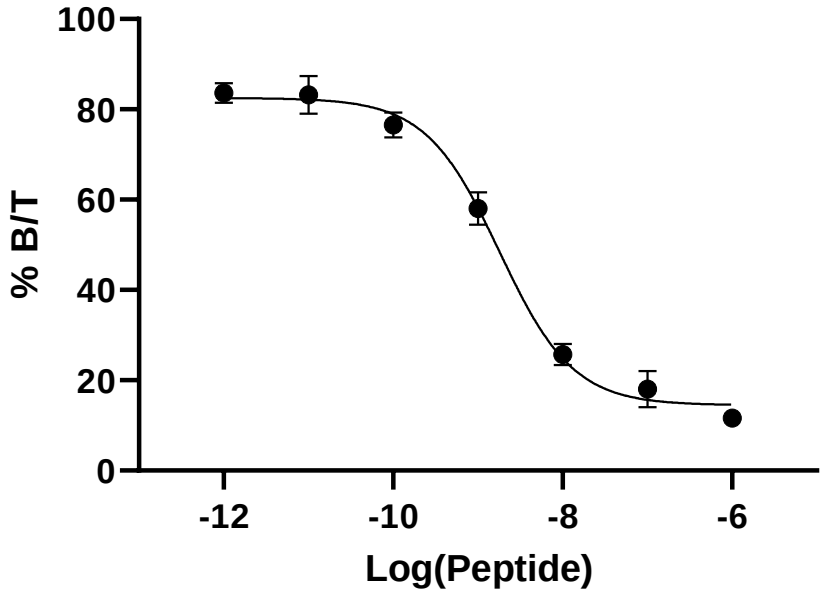


IC_{50} curve for compound **4**.

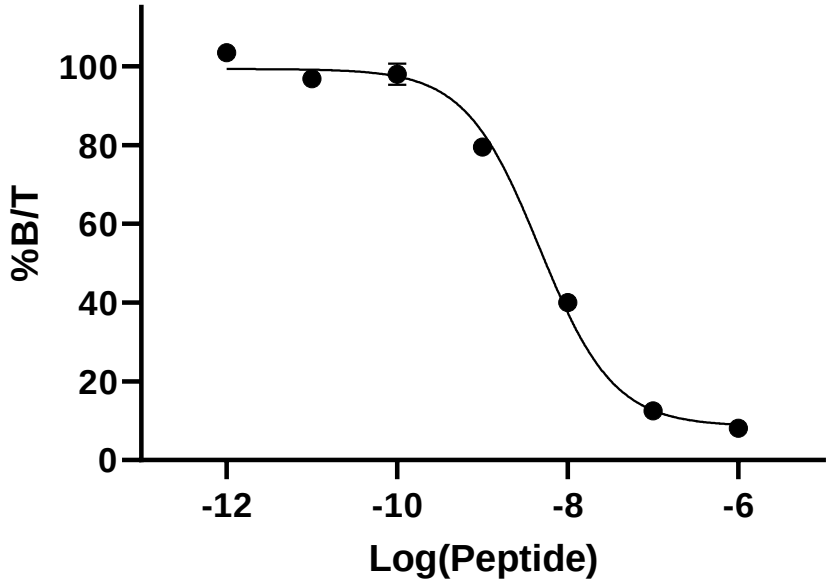


IC_{50} curve for compound **5**.

xxx

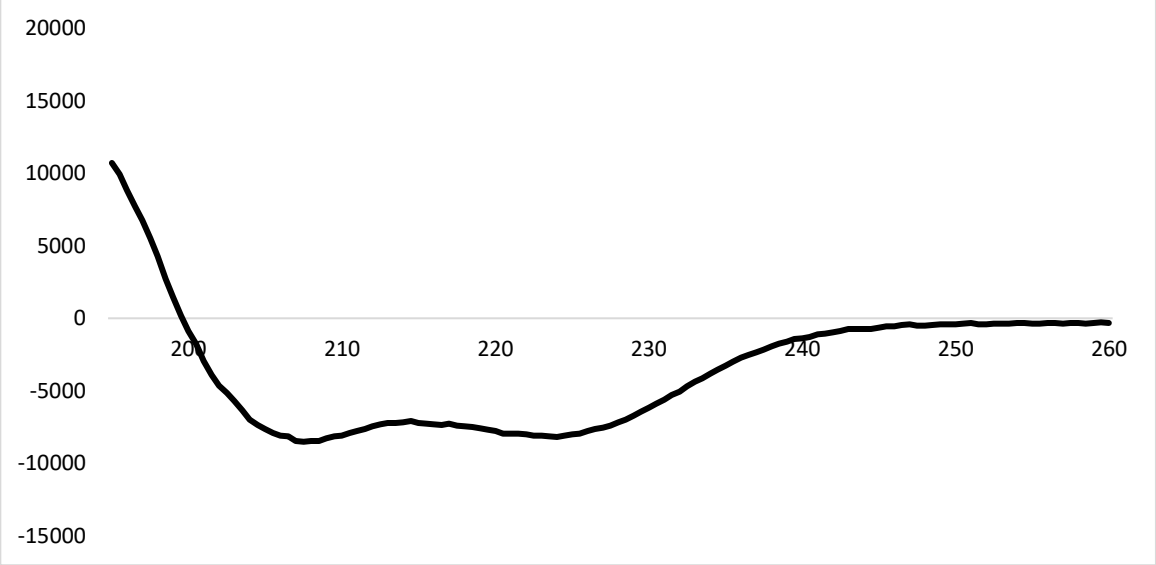


IC_{50} curve for ghrelin(1-20)

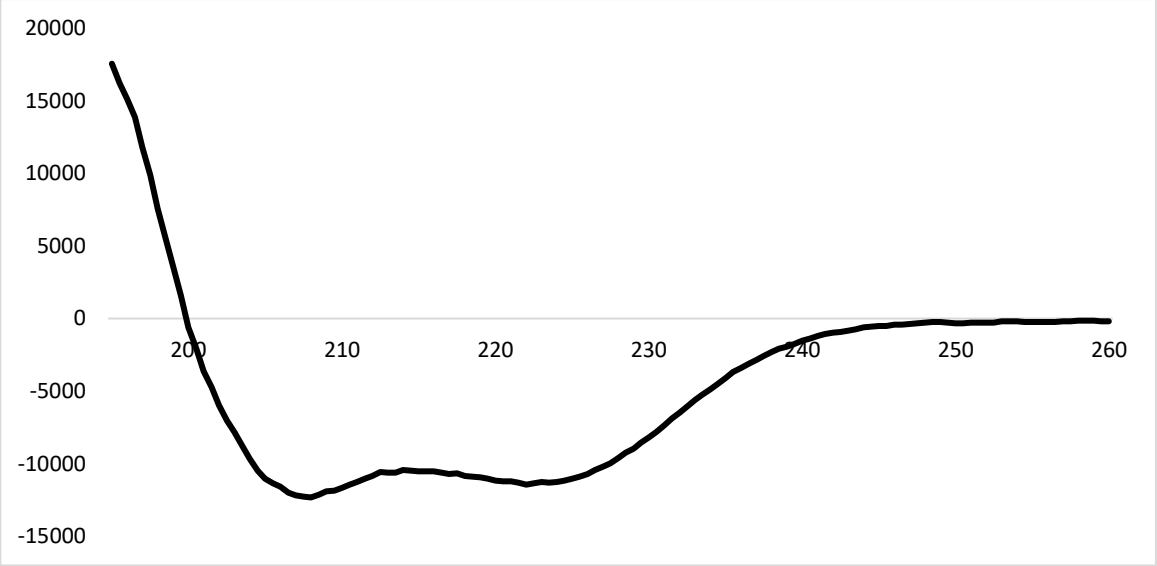


IC_{50} curve for Human ghrelin.

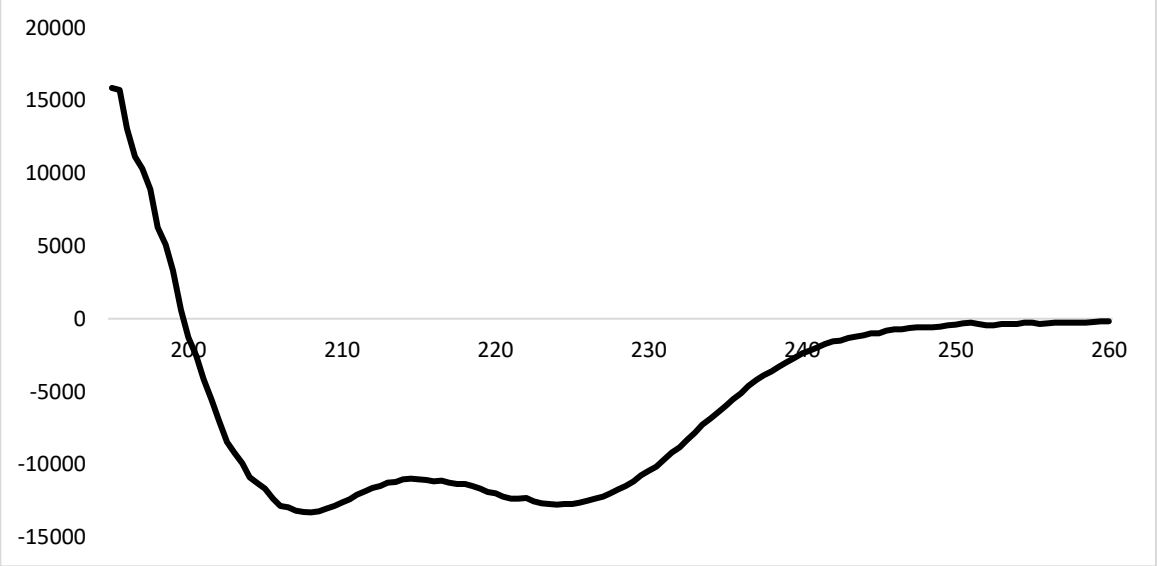
6.4 Appendix C: CD spectra



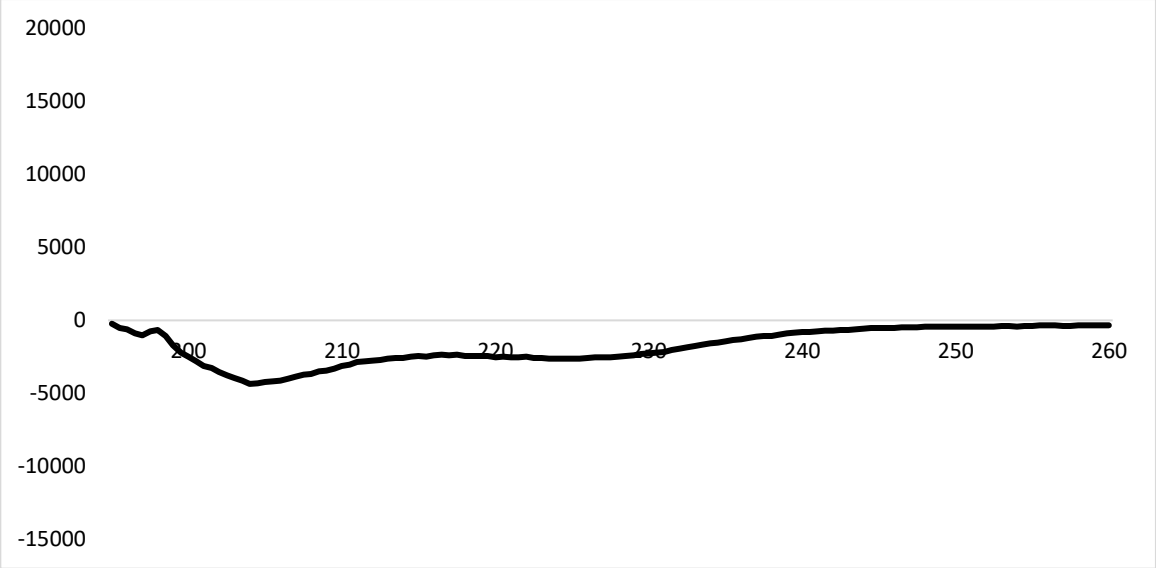
CD spectra for compound **1**



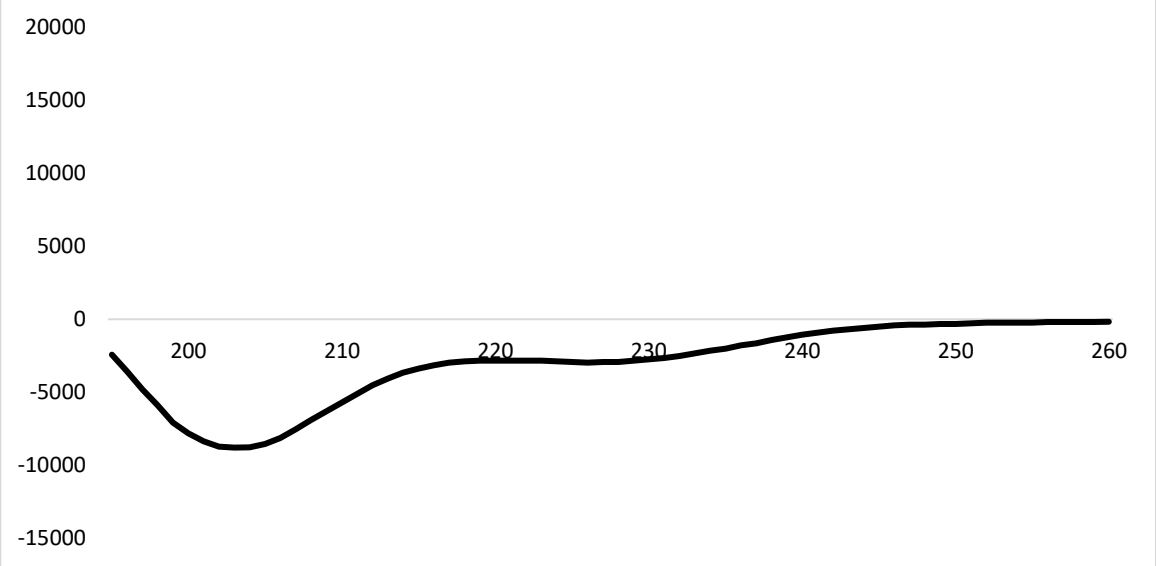
CD spectra for compound **2**



CD spectra for compound **3**



CD spectra for compound **4**



CD spectra for compound **5**

7.0 Curriculum Vitae

1. NAME: Juan Esteban **Date:** 23/08/2022

2. EDUCATION (include titles and supervisor names for research projects carried out as part of degrees):

Degree	University	Department	Year
BSc.	University of Western Ontario	Chemistry	2020

3. TA and OTHER RESEARCH or TEACHING RELATED EMPLOYMENT HISTORY:

Date	Position	Course	Supervisor	Department and Institution
Fall 2021	TA	2213A	Workentin	Chemistry, Western
Winter 2021	TA	3393B	Luyt	Chemistry, Western
Fall 2020	TA	2273A	Wisner	Chemistry, Western

4. HONORS AND AWARDS:

J. Peter Guthrie Conference Travel Award (2022)
APS Travel Award (2022)
Dean's honour list, University of Western Ontario (2020)
Outstanding Oral Presentation Award, 48th Southern Ontario Undergraduate Student Chemistry Conference (2020)

5. SCHOLARSHIPS RECEIVED: (BSc and graduate work only)

Scholarship type and Organization	Years awarded	Amount awarded
QEII-GSST, University of Western Ontario	**2021**	**$15,000**

6. COURSES TAKEN: List all Graduate Level courses taken

Date	Course Number and Title	Instructor	Grade
Jan 2021	9518 Molecular Imaging	Foster	093
Oct 2020	9713 Advanced Medicinal Chemistry	Luyt	091
Sep 2020	9507 Advanced Chemical Communications	Gillies	086

7. PRESENTATIONS: (underline presenter, identify format (oral or poster) of each presentation)

27th American Peptide Symposium (Oral presentation)	2022
18th Annual Oncology Research Day (Poster presentation)	2022
1st Canadian Peptide and Protein Community Virtual Symposium (Poster presentation)	2021
48th Southern Ontario Undergraduate Student Chemistry Conference (Oral presentation)	2020