DESIGN AND ENGINEERING OF NEW GLUCAGON-LIKE-PEPTIDE-1 ANALOGUES

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ABSTRACT

Diabetes mellitus (DM) is a chronic disease that manifests itself with impaired control of blood glucose levels and affects hundreds of millions of individuals worldwide. Among various types of DM, Type II diabetes is the most prevalent. In addition to conventional therapies for diabetes, increasing knowledge of the pathology of the disease has led to new leads for drug targets. Recent studies have revealed that glucagon like peptide (GLP-1), an incretin hormone, has a significant effect in lowering blood glucose levels without serious side effects. A naturally occurring analog of GLP-1, Exenedin-4 (Byetta®), is an approved compound for diabetes. It stimulates insulin secretion in a glucose-dependent manner, and prevents and reverses the destruction of pancreatic β-cells.

We have synthesized new analogs of GLP-1 using two different strategies. First, we synthesized a lipidated GLP-1 analog with a flexible linker that can anchor itself in cell membranes. Restriction of GLP-1 to the membrane could increase the effective concentration of the peptide around the GLP-1 receptor (GLP-1R); resulting in a more efficient binding and activation of downstream signals. Our results showed that lipidated construct had the same efficacy as the GLP-1, however it exhibited less potency. Nevertheless this construct includes a number of sites for optimization, which may allow for enhanced activity.

We have also investigated longer lasting analogues of GLP-1 by installing unnatural amino acids at the sites that are susceptible to proteolysis. Using β-amino acid substitutions at the hydrolytic site, we intended to overcome protease degradation caused by dipeptidyl peptidase-IV (DPP-IV) enzyme. In addition to using well-known side chains of glutamic acid (Glu) and leucine (Leu), we also introduced a fluorinated amino acid with a hexafluoroleucine side chain. We describe a novel and efficient synthesis of β-hexafluoroleucine along with its use in other systems. Our results indicate that βGLP-1 analogues had similar efficacies as GLP-1, but showed diminished activities. Protease stability assays for β-Glu and β-Leu substituted analogs have revealed that they were more stable than GLP-1, which holds promise for overall insulinotropic effects of these molecules *in vivo*.

Our strategies for designing longer lasting analogues of GLP-1 have been successful to create ligands with similar efficacy for GLP-1R, although stimulation of signal transduction has not been sufficiently achieved. Due to their efficient binding to the cognate receptor, GLP-1 analogues described in this study may still yet exhibit improved glucose lowering properties *in vivo*. Moreover, using these constructs as models, further modifications can be made to engineer superior GLP-1 analogues.

To the memory of

Hamide (Amida) Pamuk

(1922-2000)

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CHAPTER 1 INTRODUCTION: THERAPEUTIC PEPTIDES

1.1 Peptides & Proteins in Drug Discovery Research

Discovery and development of new drugs is a tedious process that may take decades and cost billions of dollars to pharmaceutical companies. In addition to this, the failure rate of development of the new drug candidates is more than 90%. [1] Almost 40% of the drug candidates are eliminated at Phase I clinical trials due to their toxicity and approximately 60% of the remaining fail to pass Phase II clinical trials due to low efficacy or low bioavailability. Only half of the candidates can make it through Phase III, yet, even the molecules that complete all three trials are still subject to rejection by the Food and Drug Administration (FDA) prior to commercialization. Producing a safe and efficient prescription drug that goes through preclinical and clinical trials costs US$ 1,300 million on average.[2] Therefore drug targets are carefully chosen.

For a long time, peptides and proteins have not attracted much attention in drug research due to their low oral bioavailability and rapid degradation.[3] Even then, some peptide/protein drugs have been in common use due to their unique interactions that cannot be replaced by any other molecule.[4] Insulin, for instance, has been the standard therapy for diabetes since its purification from animal resources in 1920s. After its recombinant preparation in the 1980s, it is used either alone or in combination with newer pharmaceuticals, but cannot be substituted by any other diabetes drug despite the fact that it requires daily injections that are a burden for patients. Even though convenient routes of administration is not possible for some of those peptide/protein drugs, their complex mechanism of action cannot be matched by any orally available, small-

molecule pharmaceuticals. As our understanding of peptide-protein interactions increased, and techniques for production and purification of peptides have improved, more and more peptide candidates are picked for further development in pharmaceutical industry. New formulations and advances in administration routes have also been helpful to increase the number of peptide and protein drugs in the clinic.

1.2 Peptides & Proteins as Therapuetics:

The most frequently used peptide/protein drugs can be classified in three main groups.[5] Group I includes exogenously administered protein drugs in case of a deficiency. Insulin for Type I diabetes, growth hormones for treatment of growth factor deficiency in children, Protein C supplements for prevention of venal thrombosis or blood clotting factors in case of haemophilia are few examples. Group II consists of monoclonal-antibody-based drugs for autoimmune diseases such as allergy, arthritis and even for certain cancers. This group actively binds and manipulates a molecule or an organism that is the cause of malfunction or disease. Monoclonal antibodies that target and neutralize the effects of tumor necrosis factor (TNF) have been successful for treatment of rheumatoid arthritis and inflammatory bowel syndrome. Rituximab and cetuximab are important antibody treatments in lymphoma and colorectal cancer respectively. While the former binds to an over-expressed transmembrane protein on cancer cells and facilitates their destruction by the immune system, the latter targets epidermal growth factor of affected cells to impede their growth and proliferation. Group III includes vaccines as a preventative way to overcome infectious diseases. Hepatitis

B vaccine significantly protects those who are administered with a non-infectious protein of hepatitis B surface antigen. A recently approved vaccine against human papillomavirus (HPV) is shown to prevent not only genial warts, but also certain cervical cancers. Another example is the anti-Rhesus D antigen that is a long-used vaccine for desensitization of Rh-negative mothers towards Rh-positive fetus, in order to prevent immune reactions and miscarriages in subsequent pregnancies.[5]

Peptides and proteins are classified under the name biopharmaceuticals, however they show some differences in their therapeutic properties. Most of the above examples are chosen from proteins that are more than 50 amino acids and/or >5 kDa in molecular weight.

Currently, over 200 biopharmaceuticals that include peptide, protein and antibody-based drugs are in use in clinic.[4] Within these molecules, therapeutic peptides with <50 amino acids make up more than 60 brand-name drugs. Some of these are antidiabetic peptides such as amylin, exentide and liraglutide for treatment of Type I and/or Type II diabetes, angiotensin converting enzyme (ACE) inhibitors for treatment of hypertension, anti-HIV peptides against AIDS infection, calcitonins for treatment of postmenopausal osteoporosis and Paget's disease, central nervous system (CNS) agents to manage severe chronic pain, gonadotropin releasing hormone derivatives for treatment of advanced forms of prostate cancer and breast cancer, oxytocin for initiation of uterine contractions and prevention of postpartum haemorrhage, somatastatin analogues for treatment of acromegaly and carcinoid tumors and vasopressin analogues for diabetes insipidus. In addition, some peptides are used solely for diagnostic purposes such

as, secretins (for pancreatic dysfunction) and adrenocorticotropic hormone derivatives (for detection of adrenocortical insufficiency).[4]

1.3 Challenges and drawbacks of peptide drugs:

Among many reasons that peptides have been held back from being developed as drugs, low bioavailability is significant. Administration is generally intravenous injection in order to reach the target quickly without getting degraded along the way.[6] Due to numerous proteolytic enzymes in the digestive tract and harsh gastric acid, oral administration is not applicable for peptides. Blood plasma also contains proteases that are responsible for degradation of any immunogenic peptide, therefore shortening the half-life of peptide drugs. Clearance can also be very rapid (mins) via hepatic (liver) and renal (kidneys) routes. Hydrophilicity of a peptide chain can also make it difficult to pass through biological membranes and reach to target organs.[4] Therefore, even for an injectable peptide drug, chemical elaborations of the peptide structure are required to overcome these problems. Compared to protein and antibody drugs, peptides have more potential to be modified and mutated without compromising their activity.[7] Several modifications that are commonly used are amino acid sequence mutations, PEGylation, cyclisation and glycosylation.

In order to enhance the plasma half-life of peptide drugs, chemical alterations such as C-terminal amidation and N-terminal acetylations, and amino acid substitution at protease sensitive sites are often utilized. Most of the time, poor protease stability of α-peptides cannot be improved by using natural L-α-

amino acids. In order to maintain a longer plasma life, unnatural amino acids such as D-α-amino acids, side chain modified L-α-amino acids[8] and amino acids with unnatural backbone (β- or γ-amino acids) are used. [9,10,11] In our lab, fluorinated analogs of two anti microbial peptides, buforin and magainin were prepared by modifying proteolytic sites with L-Hexaflurorleucine.[12] The resulting peptides were shown to resist hydrolysis by a series of proteases and retain their biological activity. Other groups have also reported anti-microbial peptides made out of β-aminoacids as well as α/β-mixed amino acids.[13,14,9,15] These examples were all resistant to known proteases and also shown to have antibiotic properties against a panel of bacteria with minimal toxicity to red blood cells. [16,17,18]

Cyclization is another method for increasing proteolytic stability. It can be done via disulfide bond between two cysteine residues or head-to-tail cyclization via amide bond.[7,3,19]

Attachment of polyethylene glycol (PEG) increases plasma resistance of the peptides and improves the solubility of larger proteins without affecting their activity.[20] For example, PEGylation of Interferon-α has been shown to extent its serum life.[21] In another case, high molecular weight PEG attachment to trichosantin has yielded low immunogenicty in the new conjugate, due to transfer of non-immunogenic properties of the PEG to the new compound. [22]

Glycosylation is also shown to have dramatic effects on half-life and the immunogenicity of protein drugs.[3] The half-life of the native erythropoietin[23] has been improved by extra glycosylations on the protein.

1.4 Advantages of Peptide Drugs over Small Molecules and Other Biopharmaceuticals

Compared to higher molecular weight biopharmaceuticals (protein and antibody drugs), peptides have more potential to be modified and mutated without compromising their activity. Owing to their small size, penetration at the target site can be obtained when proper modifications are done. Manufacturing small peptides are more affordable than synthesis of larger proteins or production of monoclonal antibodies, and the shelf-life of peptides is generally longer.

Peptide drugs carry even superior advantages over small organic compounds.[5-6] First, they can target a larger interface on a protein/enzyme that leads to better specificity, efficacy and selectivity. Most peptide drugs are receptor agonists and are derived from natural ligands of the target receptor.[24] Because of the exclusive interactions of the peptide drugs with their target receptors/proteins, they do not display cross-reactivity, thus minimizing the side effect. In addition, they are less likely to elicit an immune response since they are derived from naturally occurring peptides of the body. Moreover, small amounts of the peptides suffice for activation of their receptors, which reduces the dosage of the drug.[25] Although proteolysis is a big problem for small peptides, their degradation by-products are just small peptide fragments or amino acids and are not toxic. [3]Clearance of these molecules is easily done without any damage or accumulation in organs.

1.5 Peptide Therapies in Diabetes Mellitus

Diabetes mellitus is a metabolic syndrome that is defined by raised plasma glucose levels, accompanied by complete or partial deficiency of insulin. Type II diabetes contributes to more than 90 % of the diabetic cases and it generally manifests itself at older ages or with related diseases (e.g. gastrointestinal tract disorders and obesity).[26] It is a widespread disease that is in continuous rise due to sedentary life styles, excessive food intake and/or genetic predisposal. The International Diabetes Foundation reported that DM would affect more than 300 million people around the world by the year 2025. This number may further increase with the contribution of other metabolic disorders that lead to diabetes. For instance, obese patients often develop Type II diabetes in later stages of their condition. The end result of the disease is often damage to important organs such as eyes, kidney and heart that is caused by the high levels of circulating glucose. Therefore, the main goal of treatment is maintenance of glycemic control with proper drugs in combination with controlled diet and regular exercise.

Recently, discovery of an incretin hormone that can efficiently regulate glucose homeostasis brought about a new therapeutic target in diabetes research. Glucagon-Like–Peptide-1 (GLP-1) is an incretin hormone that is secreted from small intestine in response to glucose levels in plasma. After binding to its cognate receptor (GLP-1R), GLP-1 stimulates release of insulin from pancreatic islets that starts the insulin-signaling cascade and ends up with storage of excess glucose as glycogen. Apart from its glucose lowering properties, GLP-1 also induces satiety after meals and slows down digestion by ceasing gastric emptying

which both prevent overeating. This aspect of the peptide makes it even a more potent target as a therapeutic due to possible uses in obesity treatment. GLP-1 itself is also well controlled in the body. First, it is strictly glucose-dependent and therefore secreted when there is an increase in glucose levels. Secretion proceeds in a dose-dependent manner. Second, immediately after GLP-1 reaches circulation, a ubiquitous protease dipeptidyl peptidase IV (DPP-IV) hydrolyses the N-terminus end, rendering it inactive. Although this second feedback mechanism plays an important role in glucose homeostasis in healthy individuals, it constitutes a challenge for using GLP-1 as a therapeutic compound for diabetic patients due to stability problems discussed in section 1.2.

It should be mentioned here that GLP-1 is not the only peptide drug option for diabetes. Amylin is another gastrointestinal peptide that has been identified as a potential therapeutic target. It is secreted after food intake from pancreatic cells, along with insulin. Advanced diabetic patients lack amylin due to loss of β-islets. Although mechanism of action is not well understood, it was shown to suppress glucagon secretion in a glucose-dependent manner and delay gastric emptying. Pramlintide is a synthetic amylin analog that has been approved by the FDA in 2005 for treatment of both Type I and Type II diabetes.

1.6 Modifications of GLP-1:

GLP-1 had been an excellent candidate for Type II dibetes theraphy except for its short life in plasma. Within 1-3 mins after its secretion, GLP-1 is completely degraded by DPP-IV. In order to overcome proteolysis, inhibitors of

DPP-IV have also been used and shown to be successful in extending the lifetime of GLP-1.[27] However, the substrate pool of this protease is large and includes incretins, chemokines, and neural system hormones.[28] It is not known if long-term consequences of DPP-IV inhibition would cause negative effects in other important pathways. Therefore, for GLP-1 to exert its full incretin effect, it should either be constantly secreted into circulation, or stabilized against DPP-IV hydrolysis. The former strategy has been tried with human subjects, where Type II dibetic patients have received intravenous GLP-1 during a 6-week period. At the end of the study, in addition to the reductions in fasting and postprandial plasma glucose levels, sensitivity to insulin and secretion of insulin had increased compared to the control group.[29] However continuous delivery of exogenous GLP-1 immobilizes the patient and is not a preferable method of treatment for a long-term. Rather, administration of the drug should be convenient enough for diabetic patients to perform on their own. This led to the search of more stable analogues of GLP-1 that can withstand proteolysis.

DPP-IV targets the amide bond between Ala8 and Glu9 on the GLP-1 peptide. (Figure 1) DPP-IV resistant analogues have been typically modified at this proteolytic site. GLP-1 peptides modified at Ala8, to D-Ala, Ser, Aib, Val or Abu display DPP-IV resistance and have conserved or improved biological activity. Additionally, a stable GLP-1 analogue, Exenatide, owes its protease stability to an Ala→Gly mutation on the penultimate N-terminal amino acid. It shows better stability in plasma (26 min. after intravenous delivery) and has the same effects as GLP-1.[30,31] Exenetide is derived from a naturally occurring

peptide isolated from the saliva of Gila Lizard (*Heloderma Suspectum*)[32] and is the first FDA-approved peptide drug for treatment of Type II diabetes. Due to its superior protease stability, it can be administered by two subcutaneous (S.C.) injections per day in 5 to 10μg quantities, and is sufficient for lowering plasma glucose efficiently. Exenetide, is a very potent mimic of GLP-1 and binds tightly to GLP-1R.[30,33]

Superior binding or potency may not be always achieved with mutated analogues. In some cases, protease stability may translate into a less active compound. For example replacements of Ala8, with L-Valine (Val8GLP-1), 2-amino butyric acid (Abu8GLP-1) [34] and L-Ser (Ser8GLP-1)[35] resulted in reduced potency compared to wild type GLP-1. This was correlated with reduced receptor affinities, which is usually expected for Ala8 modified analogues.[36] Nevertheless, in vivo experiments in mice had revealed that glucose-dependent insulin secretion was just as well regulated by these analogs due their superior stability towards DPP-IV (hrs vs. mins for GLP-1). Longer-lasting analogues can be strong candidates for enhancing insulinotropic activity even with seemingly reduced potencies.[37,35,38]

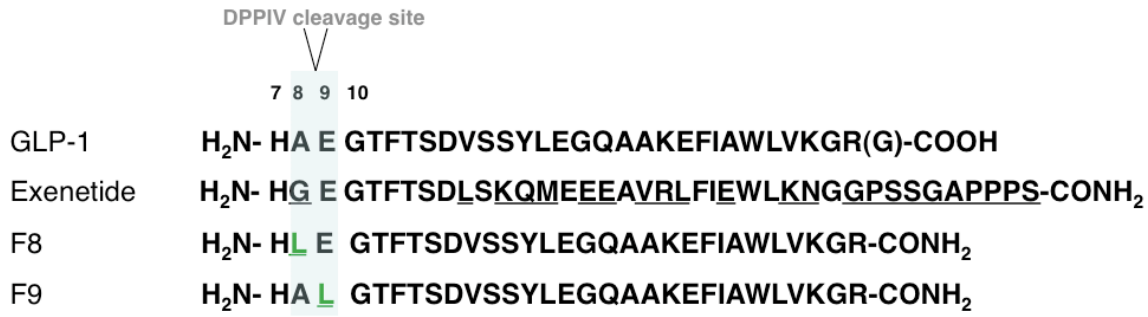

Figure 1. Primary sequences of GLP-1 Exenetide, and two fluorinated GLP-1 analogues are compared to wild type peptide. Mutated residues are underlined, fluorinated residues are shown in green. Arrow shows the site where dipeptidyl peptidase IV enzyme cleaves GLP-1.

Fluorinated amino acids have also been used as a way to resist hydrolysis at the proteolytic sites of GLP-1. As mentioned in section 1.2., of this chapter, introduction of fluorinated amino acids in several antimicrobial peptides has resulted in improved stability against various proteases without tempering their activity. Taking these successful examples into account, a series of GLP-1 mutants were prepared in our lab by substituting L-Hexafluoroleucine in place of Ala8, Glu9, Gly10, both Ala8 and Glu9 positions.[8] Among all, Ala8 and Glu9 substitutions (F8GLP-1 and F9GLP-1) yielded the most successful analogues in terms of displaying enhanced insulinotropic activities. F8GLP-1 was completely resistant to hydrolysis and F9GLP-1 displayed 1.5 fold better stability compared to GLP-1. The binding capacity of F8GLP-1 was diminished, whereas F9GLP-1 remained comparable to GLP-1, which suggests that the bulky fluorinated side chain was more tolerable at the 9th residue than it was at 8th. Overall, in vivo experiments revealed that both analogues had the same effect on blood glucose levels. They could efficiently stimulate insulin production to the same extent as GLP-1.

Encouraging results from these experiments, combined with the ones from β-peptides led us to work on protease stability of GLP-1 while protecting its incretin effect. Using different strategies, we have prepared DPP-IV resistant analogues. In the second chapter of this thesis, we will describe the synthesis of a lipidated GLP-1 analog that is proposed to anchor itself to the cell membrane and therefore enhance the downstream activities by more effectively binding to its receptor relative to GLP-1. A broad background for diabetes and its pathology,

insulin signaling, incretin hormones and alternative therapies for Type II diabetes is also given. In the following chapter (3rd), we will describe synthesis of β-aminoacid containing GLP-1 analogues. In this chapter we also present an efficient and enenatiopure synthesis of a fluorinated β-amino acid: L-β-Hexafluoroleucine. Chemistry of β-amino acids and uses of β-amino acid in synthesizing β-peptides for a variety of uses are also described.

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CHAPTER 2 THERAPEUTIC POTENTIAL OF GLP-1 IN TREATMENT OF TYPE II DIABETES

2.1 Type II Diabetes and Complications

2.1.1 Diabetes: A Growing Epidemic

Diabetes Mellitus is indicated by the body's impaired control of blood glucose levels and categorized in three main groups: Type I, Type II, and gestational. In all three cases, the main danger is hyperglycemia and subsequent damage to vital organs such as eyes, kidneys, and nerves.

In Type I diabetes, production of the hormone insulin is diminished as a result of β-cell loss in the pancreas. The causes could be genetic, autoimmune, or environmental, and there are few opportunities for prevention. β-cell loss may occur as a result of an autoimmune process, which starts with a chronic inflammation and results in β-cell destruction.[1] After β-cells have been lost, the only reliable treatment options are regular monitoring of glucose levels and injections of insulin to maintain homeostasis.

Type II diabetes occurs due to the body's resistance towards insulin and/or reduced ability of β-cells to produce enough insulin.[1] As the disease progresses, β-cell loss is also observed due to overexposure of the cells to high levels of glucose.[2] Type II is the most widespread form of diabetes. Currently, 2% of the world population and 10 % of US population has diabetes among which, the Type II form accounts for more than 90% of the cases.[3,4] Treatment options vary depending on age and the progression of the disease.

Gestational diabetes is a temporary condition during pregnancy where cells develop transient resistance towards insulin. In most cases of gestational

diabetes, the condition ceases after delivery. However, in some cases it may recur later in the life of the mother. Therefore, women with previously diagnosed gestational diabetes are in a high-risk group for Type II diabetes. [1]

Lifestyles that include a lack of exercise and excessive intake of calories increase predisposition to Type II diabetes. Until very recently, Type II diabetes was generally associated with older age, hence also named adult-onset diabetes. However recently, it is equally prevalent among young adults and especially in obese children.[4] While the disease is preventable up to some point with controlled diet and regular exercise,[5] it typically requires life-long efforts to regulate blood sugar with strict dietary control once it has been diagnosed.[3] Even then, complications arising from diabetes can cause life-threatening conditions and hospitalization. Therefore, an emerging epidemic of diabetes affects individuals in their normal daily life activities and reduces life expectancy.[6]

2.1.2 Insulin Signaling

Insulin, Mechanism of Action:

Insulin is a hormone released by pancreatic β-cells of the islet of Langerhans as a result of high levels of blood glucose. Insulin signaling triggers glucose uptake and promotes its storage in the form of glycogen and fat.[12] Its secretion also moderates the breakdown of glycogen and the release of glucose from the liver after meal ingestion; thus maintaining glucose homeostasis (Figure 1). Insulin works in an opposite to the hormone glucagon hormone, which release glucose from glycogen during starvation.[7]

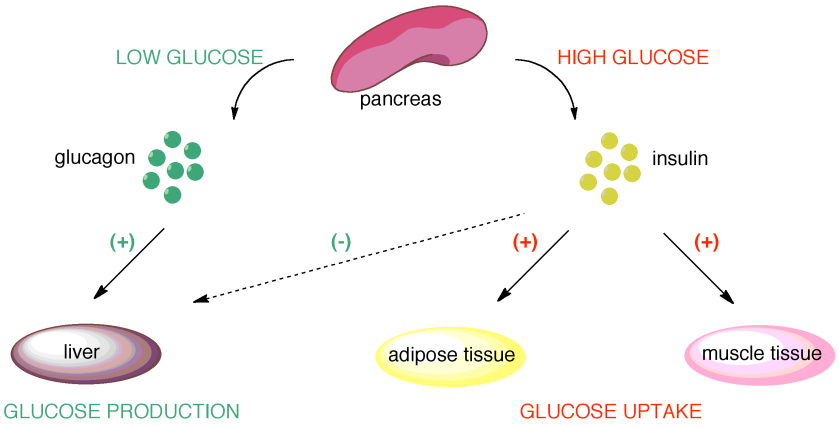

Figure 1. The role of pancreas in regulating glucose levels. At low glucose levels, glucagon upregulates glucose production from glycogen in liver, shown in green (+). At high glucose concentrations, insulin down- regulates glucose production, shown in green (-), and stimulates glucose uptake into tissues is shown in red (+).

After food intake, insulin from pancreatic islet cells is transported to the cell membrane and released. The three major sites for insulin action are skeletal muscle, adipose, and liver tissues.[8]

Although the insulin-signaling pathway is still the subject of ongoing research, many of the details have been elucidated. The insulin receptor consists of two extracellular α-subunits that are responsible for ligand binding and two intracellular β-subunits. Upon binding of insulin to its receptor's α-subunits, it activates tyrosine kinase activity within the β-subunit of the receptor.[9] Tyrosine residues on the β-subunit are autophosphorylated, providing a docking site for insulin receptor substrates (IRSs). Phosphorylation of the IRSs direct Src Homology 2 (SH2) containing proteins onto the activated β-subunits.[10] Type IA phosphatidylinositol 3-kinase (PI3K), among these proteins, is especially important for phosphorylation of phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P_2) to phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P_3). [11] Soon after insulin binding, concentrations of PtdIns(3,4,5)P_3 increases dramatically, and its binding partner, protein kinase B (PKB or Akt) is recruited from the cytosol to the plasma membrane. Interaction of PtdIns(3,4,5)P_3 with PKB at the plasma membrane recruits two other protein kinases (3-phosphoinositide-dependent protein kinase-1 (PDK1) and PDK2 that are responsible for phosphorylating PKB.[12] Activated PKB detaches from the cytosolic membrane and subsequently activates several metabolic processes. For one, it phosphorylates and deactivates glycogen synthase kinase-3 (GSK-3) that causes glycogen synthase to dephosphorylate and get activated for glycogen

synthesis. PKB also phosphorylates trancription factors of the Foxo-family that play a crucial role in insulin-dependent regulation of blood glucose. Finally, it recruits the glucose transporter, GLUT4, to stimulate glucose uptake into cells by withdrawing glucose from plasma.[13] (Figure 2)

An alternative PI3K-independent pathway is also suggested to play a role in glucose uptake via GLUT4 recruitment (Kahn&Pessin2002Diabetol). In this pathway, activated IR directly phosphorylates proto-oncogene Cbl, which in turn, activates GTP-binding proteins. (Figure 2) GTP-binding protein activation leads to insulin-induced GLUT transport and glucose assimilation via unknown effectors. [14]

In both of these signaling cascades, the increased population of GLUT4 on membranes increases uptake of glucose into insulin-responsive cells and decreases plasma glucose concentrations.

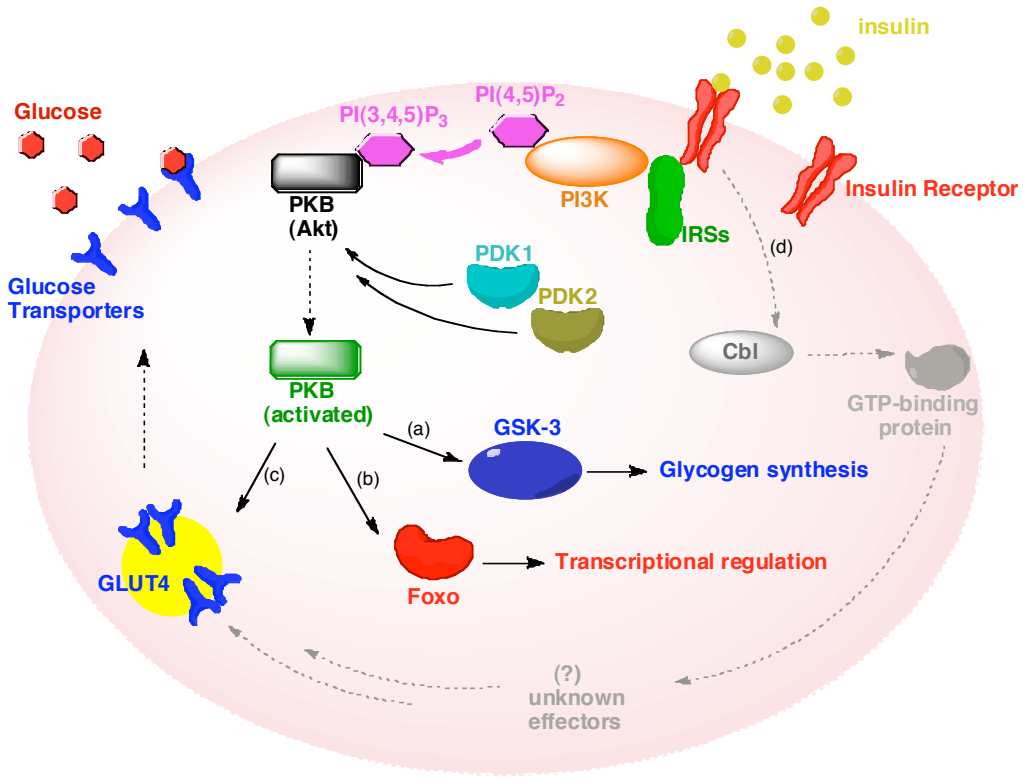

Figure 2. Insulin signaling pathway. Insulin binds to its receptor and activates a cascade of phosphorylation events, which leads to glucose transport and related regulatory mechanisms. a) Glycogen synthesis upregulated via glycogen synthase, b) Transcriptional regulation of insulin pathway is induced by Foxo family of proteins, c) Glucose is internalized via glucose transporter, GLUT4, d) Alternative PI3K-independent pathway is suggested to lower blood glucose through relocation of same transporter, GLUT4.

2.1.3 Insulin resistance and β-cell decline in Type II diabetes

Insulin Resistance:

In earlier stages of Type II diabetes, regardless of circulating insulin in the bloodstream, glucose levels tend to remain high. Glucose uptake is inefficient in fat and muscle tissues and the liver does not properly moderate glucose production from glycogen.[15] [16] These are the first signs of insulin resistance. As a healthy individual's fasting glucose level is 5.6 mmol/L or less, any level between this and 7.8 mmol/L is categorized as "prediabetic".[17] People with prediabetes have a higher risk of developing Type II diabetes, although it may take several years for the symptoms to manifest. Approximately 30% of individuals who are prediabetic do not develop diabetes at all.[18] When fasting plasma glucose is higher than 8 mmol/L, and the 2–hour glucose tolerance test shows more than 11 mmol/L glucose in bloodstream, the person is defined as diabetic.[4] At that point, insulin secretion is impaired; resistance towards insulin has already developed and progressed to a disease level.

Insulin resistance may be the combined outcome of more than one dysfunction in insulin signaling pathway. IRS-1 and IRS-2, PKBβ, forkhead transcription factor Foxo1a, and the irregularities in lipid metabolism all impact insulin resistance and development of Type II diabetes. Mutations of IRS-1 in human subjects result in insulin resistance. IRS-2 knockout-studies in mice revealed that the main insulin responsive tissues acquire resistance, followed by β-cell decline.[16] Downstream of IRSs is PI3K, and diminished or absent activity of PI3K inhibits insulin responses due to lack of PtdIns(3,4,5)P_3 in cells.[19] In

addition, overexpression of a constitutively active mutant of PI3K can recover the signaling events by stimulating GLUT4 relocation to the cell surface, establishing PI3K's key role in the signaling pathway.[8] Dysfunctions of PKB and transcription factor Foxo1a were found to cause insulin resistance independent of PI3K. Deficiency of a specific form of PKB, called the PKBβ, results in diabetes in mice. Mutations of PKBβ in humans are also associated with insulin resistant phenotype at the gene level.[16] Foxo1a plays an important role in transcriptional regulation of gluconeogenesis. Apart from possible upstream effects of PKB on Foxo1a, Foxo1a independently affects glucose homeostasis. Foxo1a partial-knockout mice display insulin desensitization and lower insulin levels.[16] Adipose tissue also plays an important role in glucose homeostasis by regulating free fatty acids (FFA) in plasma.[7,20] Excessive FFA in circulation is found to interfere with glucose uptake into cells via improper phosphorylation of IRSs, impairing insulin signaling and reduced translocation of GLUT4 to the cell membrane. Disruption of GLUT4 function was found to cause insulin resistance in muscle and liver tissues.[21]

Current research still has not pinpointed the causes of insulin resistance nor assigned exact roles to these molecules during the signaling process. Combined genetic and pharmacological efforts continue to provide reliable results for understanding progression of this complicated disease.

β-cell Decline:

Type II diabetes is characterized primarily by insulin resistance and β-cell dysfunction. Insulin sensitivity decreases with the progress of the disease, and β-cells produce larger amounts of insulin in order to compensate for the low response. Exhaustion eventually results in apoptosis of β-cells and the condition is further aggravated by the concomitant decrease of insulin production.[22] Apoptosis of β-cells results from increased endoplasmic reticulum (ER) activity, which is accelerated to produce larger amounts of insulin in response to metabolic overload.[2] High stress in ER often causes protein misfolding and self-destruction of cells.[23,24] The necessity to transport and store glucose rises, but the insulin signal and the responsiveness of critical cells are diminished. This vicious cycle leads to higher risks of hyperglycemia since the cells cannot consume circulating glucose in the blood. Most treatment options for Type II diabetes concentrate on preventing hyperglycemia rather than reconstituting β-cell function.[25] However, if β-cells could be restored to their normal condition, at least one face of the disease would be ameliorated and this could further aid in treatment of other symptoms.

Incretin therapy seems be the answer for this need, since recent analogues of Glucagon-Like Peptide-1 (GLP-1) have shown dramatic improvement in β-cell repair as well as sensitizing cells towards insulin in diabetic patients.[26,27] Understanding the nature of these incretins is necessary for developing better treatment options and finding how these molecules work.

2.2 GLP-1 as the Therapeutic Target

2.2.1 Incretin Hormones and Homeostasis

Incretins are gastrointestinal-tract hormones that stimulate insulin secretion after oral ingestion of food. This is called the "incretin effect" and it depends linearly on the amount of glucose consumed (i.e. the more glucose taken, the more insulin secreted).[28] Circulating glucose concentrations in blood needs to be kept low and balanced after a meal. Incretins do their job by increasing the ability of pancreatic β-cells to respond to glucose. Oral ingestion of food is the key point for this effect, since intravenous administration of glucose cannot induce the same level of insulin production.[29] Two crucial incretins are GLP-1 and Gastric Inhibitory Peptide (GIP).[30,31,32] GLP-1 and GIP are both secreted in the small intestine, but in varying concentrations and at slightly different locations. GIP is predominantly produced from the upper small intestine when easily absorbed nutrients and small loads of food are ingested. GLP-1 is secreted in the lower small intestine, after complex nutrients or a large amount of food are ingested.[28] A mixed meal causes both hormones to be produced, however blood concentrations differ: GIP concentrations can reach to a few hundred picomolar concentration, whereas GLP-1 concentrations do not exceed 50 picomolar.[33,34] Several studies have suggested that GIP can be stimulatory for GLP-1 secretion.[34] The link between GIP and GLP-1 hormones and their proximal secretion in the small intestine may be the purpose of having two incretin hormones in order to co-regulate the incretin effect. In addition to their insulin regulation effect, GLP-1 and GIP induce satiety after food ingestion and cease gastric motility.[32,35] These

aspects are important to prevent overeating and to maintain a reasonable body weight.

In Type II diabetes, the incretin effect is dramatically low or absent.[36] The insulinotropic effect of GIP is nearly lost, however the amount of the secreted hormone is the same.[37] On the other hand, the amount of GLP-1 production is lower in diabetic patients, whereas the potency of the peptide is reduced but not lost.[38,39] In addition to intrinsic diminished β-cell mass and subsequently poor insulin secretion, insulin production is further decreased by these gastrointestinal irregularities, and contributes to the disease state. Mechanisms causing GIP and GLP-1 to be ineffective still remain to be answered. It is known, however, that the loss of the incretin effect is not the primary reason for a diabetic condition.[36,40] Secretion and the activity of GLP-1 and GIP were found to be at normal levels in pre-diabetic patients or in women who are at high risk for developing Type II diabetes because of their previous gestational glucose intolerance. Therefore, hormonal abnormalities emerge secondary to the development of diabetes.[36] It is still not obvious at which stage of the disease the incretins become inactive.

GLP-1 treatment holds a clinical value in Type II diabetes since it is able to restore insulin secretion and sensitization by improving β-cell function. GIP analogues have not been developed for clinical trials because they are not found to be as effective as GLP-1 analogues. Future work regarding GIP analogues may include combined use of GLP-1 and GIP derivatives to mimic the natural incretin effect in the body.

2.2.2 Benefits of Incretin Therapy, β-cell Regeneration

β-cell destruction is the most challenging aspect of Type II diabetes to treat; and, unfortunately, most of the therapies, including insulin, do not reverse this dysfunction. GLP-1 therapies, on the other hand, have shown promising results in restoration of β-cell function and increasing insulin synthesis. In a clinical study by Zander et al., Type II diabetic patients were administered exogenous GLP-1 during a six-week period. Patients underwent multiple β-cell function tests before and after the trials. The insulinogenic index, which reflects the ratio between the circulating glucose and insulin levels, is a measure of β-cell sensitivity and was improved in GLP-1 receiving patients after six weeks.[26] In addition to the reductions in fasting and postprandial plasma glucose levels, sensitivity to insulin and secretion of insulin had increased compared to the control group.[26] Additionally, a steady weight loss was observed. An average of 1.9 kg loss in patients is considerable because conventional drugs for Type II diabetes, such as sulfonylureas, come with the problem of weight gain.[41,42] These results are not surprising since the incretin hormones inhibit gastric emptying and diminish appetite.[43] This is a two-fold advantage for patients with obesity, who often develop Type II diabetes at some stage in their life.[44] Existing treatments for diabetes put an extra burden on obese patients to control blood glucose levels and lose weight in order to have a positive therapeutic outcome. The option of using incretins in obesity treatment has also been suggested as a future therapy.[23,41]

2.2.3 GLP-1; Structure, Function and Biology

Physiology:

Glucagon-like-peptide-1 (GLP-1) is encoded by the same gene as glucagon, and post-translationally modified from the 180 residue proglucagon polypeptide into a 30 residue peptide.[45] The glucagon-encoding mRNA structure and glucagon gene structure were determined in 1983 and revealed two glucagon-like peptides embedded in proglucagon: GLP-1 and GLP-2.[46] GLP-1 is the effective peptide and many therapeutic analogues have been made, whereas GLP-2 has possible other roles such as intestinal regulation of nutrient absorption.[47,48]

GLP-1 is made up of residues 72-107 of the proglucagon peptide. Sequencing of naturally occurring GLP-1 revealed that an N-terminus extension is absent in its native and active form.[49] In the L-cells of the small intestine, GLP-1 is excised from its precursor into an active peptide by prohormone convertase 1/3 (PC1/3).[28] Active GLP-1 peptide has been designated as GLP-1 (7-37) amide or GLP-1 (7-36) amide since the C-terminus Gly extended form and the C-terminus amidated forms are the two active variants (Figure 3).[50] In humans, almost all GLP-1 secreted from the small intestine is C-terminus amidated.[51] Compared to the free carboxyl end, the amidated isoforms were found to be slightly more stable in plasma.

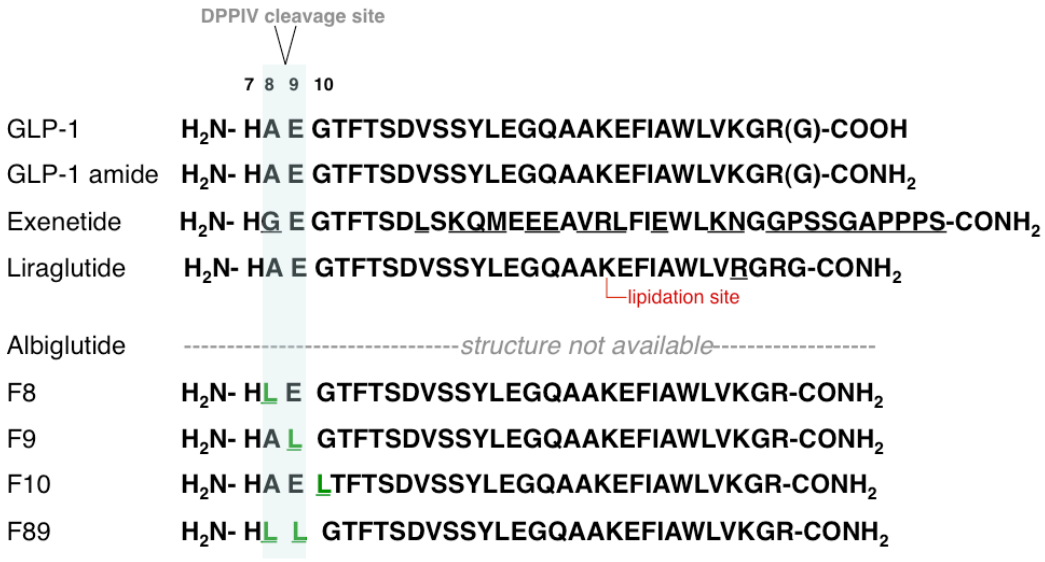

Figure 3. **Primary sequences of GLP-1 variants with and without C-terminal Gly-extensions and amidated or free carboxy forms are shown. Analouges of GLP-1, Exenetide, Liraglutide, Albiglutide and fluorinated peptides are compared to wild type peptide. Mutated residues are underlined, fluorinated residues are shown in green and lipidation site in Liraglutide is indicated in red. Arrow shows the site where dipeptidyl peptidase IV enzyme cleaves GLP-1.**

After its secretion from L-cells in the upper intestine, GLP-1 moves through the basal lamina and reaches the lamina propria.[46] Lamina propria is the connective tissue between endothelia of the intestine and the membrane containing capillaries. GLP-1 metabolism starts at this interface where the peptide travels through the capillaries. Endothelial-lining of the capillaries produce dipeptidyl peptidase IV (DPP-IV), a protease that is responsible for cleavage of GLP-1.[52] Despite the fact that GLP-1 degradation is very fast, incretin function of GLP-1 takes effect very quickly after meals. The quick response after food intake is thought to occur not only by increased circulation levels of GLP-1, but also by stimulation of the nervous system, when GLP-1 passes through intestinal membranes. GLP-1 may interact with sensory neurons in lamina propria, which stimulates the hypothalamus and signals the pancreas to secrete insulin.[53] Effects of peripheral secretion of GLP-1 on the nervous system may be important for inducing satiety and suppressing excessive eating.

GLP-1 is a short-lived species and cleared from circulation in 1 to 2 minutes.[54] Prior to its renal clearance, DPP-IV is the enzyme responsible for rapid inactivation of GLP-1. DPP-IV specifically cleaves two amino acids from the N-terminus, leaving GLP-1 (9-37) and GLP-1 (9-36) amide as inactive metabolites.[55] Inactivation by DPP-IV starts in the gut by immediate cleavage of 75% of the newly secreted GLP-1 and 50% of the remaining active molecules are further degraded in the liver.[56] Therefore, only 10-15 % of the secreted GLP-1 reaches circulation in its active and intact form. Inactive metabolites are cleared rapidly in kidneys within 4-5 minutes. Recent research has shown that DPP-IV

inhibitors could enhance the amounts of circulating active GLP-1 peptide[52] and some are in active use along with other medications.[57,58] Although studies so far have suggested that DPP-IV inhibitors may improve therapy, involvement of DPP-IV in other cell functions may still lead to unforeseen complications.[59,60,61] Instead, GLP-1 analogues that resist DPP-IV action may pose a lesser risk and provide an effective treatment of Type II diabetes.[62]

DPP-IV targets the amide bond between Ala8 and Glu9 on the GLP-1 peptide. (Figure 3) DPP-IV resistant analogues have been typically modified at this proteolytic site.[63] GLP-1 peptides modified at Ala8, to D-Ala, Ser, Aib, Val or Abu display DPP-IV resistance and have conserved or improved biological activity. Glu9 replacements resulted in varying resistance toward proteolysis as well as exhibiting varying degrees of biological activity.[64]

The sequence of GLP-1 is highly conserved among mammalian species. GLP-1 peptide adopts a random coil structure in pure water,[65] however structures determined by NMR spectroscopy in trifluoroethanol[66] or in micelles[67] showed that there is a significant helical content in these more hydrophobic environments. The overall structure of GLP-1 from the NMR studies is shown to consist of an N-terminal random coil (7-13), and two helical regions betwee ((13-20) and (24-36)), separated by an undefined region (21-23). The N-terminal region of GLP-1 is important for receptor affinity and a free amine at the terminus is required for activation. Therefore, N-terminal extensions of GLP-1 are found to be either inactive or exhibit reduced activity. Specifically, His7 on the N-terminus seems to be crucial for receptor signaling. Alanine scanning experiments have revealed

other conserved residues that are indispensable for receptor binding.[68] In addition to His7; substitutions of Gly10, Phe12, Thr13, Asp15, Phe28, and Ile29 have very limited affinity towards GLP-1R, and very low corresponding activities. Peptides, which include Ala substitutions of Gly10, Phe12, Thr13, Asp15 do not show difference in their structural analyses as assessed by CD experiments. However, Ala substitutions of Phe28 and Ile29 result in diminished helicity on the C-terminal end.[68] C-terminal residues are more likely to play a role in the secondary structure of the molecule that is required to fit in the binding cavity of the cognate receptor. Residues such as Phe28 and Ile29 exert selectivity by stabilizing a secondary structure rather than directly interacting with GLP-1R side chains. In contrast to the N-terminal part, C-terminal modifications are tolerable, as long as the amino acid replacements do not disturb α-helical content. For example Exenedin, which is a very potent mimic of GLP-1, includes a nine-amino acid extension on the C-terminus, yet is able to bind tightly to GLP-1R.

GLP-1 Receptor and Mechanism of Action

GLP-1 receptor (GLP-1R) is a class B, G-protein coupled receptor (GPCR) and is in the same family as GIP and glucagon receptors. It has seven transmembrane domains with an N-terminal extra-cellular, ligand-binding domain.[69] A recent crystal structure of GLP-1 in complex with the soluble extracellular domain of GLP-1R revealed that GLP-1 adopts a unique structure upon binding.[65] The region from Thr13 through Val33 forms a continuous α-helix with a kink at the Gly22 residue. (Figure 4) The hydrophobic face of GLP-1 consists of conserved residues which are important for binding: Ala24, Ala25,

Phe28, Ile29, Leu32, and Val33. As mentioned previously, Phe28 and Ile29 substitutions have been shown to have dramatic effects on ligand binding by alanine scanning experiments. Phe28 seems to be the key residue for hydrophobic interactions for binding and is located in the middle of the receptor-GLP-1 interface. Val 33 is the last residue on the well-defined α-helical region and also the last residue that is in contact with the receptor. The hydrophobic side chain of Val33 interacts with Tyr69 and Leu123 of the GLP-1R, while the backbone carbonyl group forms a hydrogen bond with the Arg121 side chain. The hydrophilic face of the bound GLP-1 helix is less influential on receptor affinity. Lys26 is the only residue of the hydrophilic face that interacts with the receptor via hydrogen bonding.[65]

GLP-1 binds mainly with its C-terminal residues to GLP-1R. N-terminal residues, from Thr13 to Glu21, do not make strong contacts with the receptor. Upon binding, the segment from Ala24 to Val33 is stabilized by a helical conformation at the receptor-ligand interface. It is suggested that conformational change at the C-terminus extends to the N-terminal residues that are random when unbound. A continuous helix forms as an allosteric consequence. Correct positioning of the N-terminal residues inside the GLP-1 extracellular domain then results in receptor activation.[65]

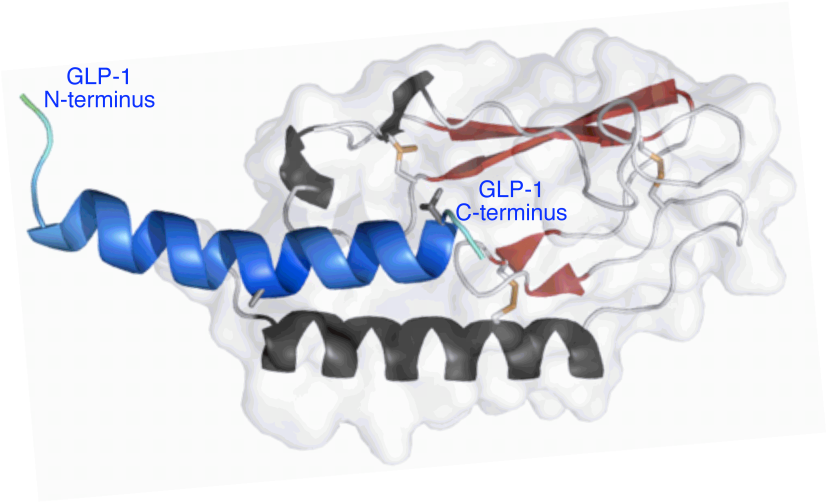

Figure 4. Crystal structure of GLP-1 peptide, bound to its receptor's extracellular domain, is shown in blue. (PDB ID: 3IOL) Extracellular domain's α-helices are shown in black and β-sheets are shown in red. (reproduced with permission[65])

Receptor Signaling:

GLP-1R is located in GI tract, brain, heart, kidney, and pancreatic tissues.[70,71] Binding of GLP-1 to its receptor activates adenylate cyclase through a G-protein, and increases cAMP levels in the cell. Protein kinase A and cAMP-regulated guanine nucleotide exchange factor (cAMP-GEFII or Epac2) are stimulated by high cAMP levels and subsequently change ion channel activity, raise the calcium concentrations within the cells and eventually affect insulin secreting granules to be transported from β-cells.[72] (Figure 5)

An elevated level of cAMP also has an effect on cell survival and proliferation of β-cells through activation of the cAMP response element binding protein (CREB) and the PI3K pathway.[73,60]

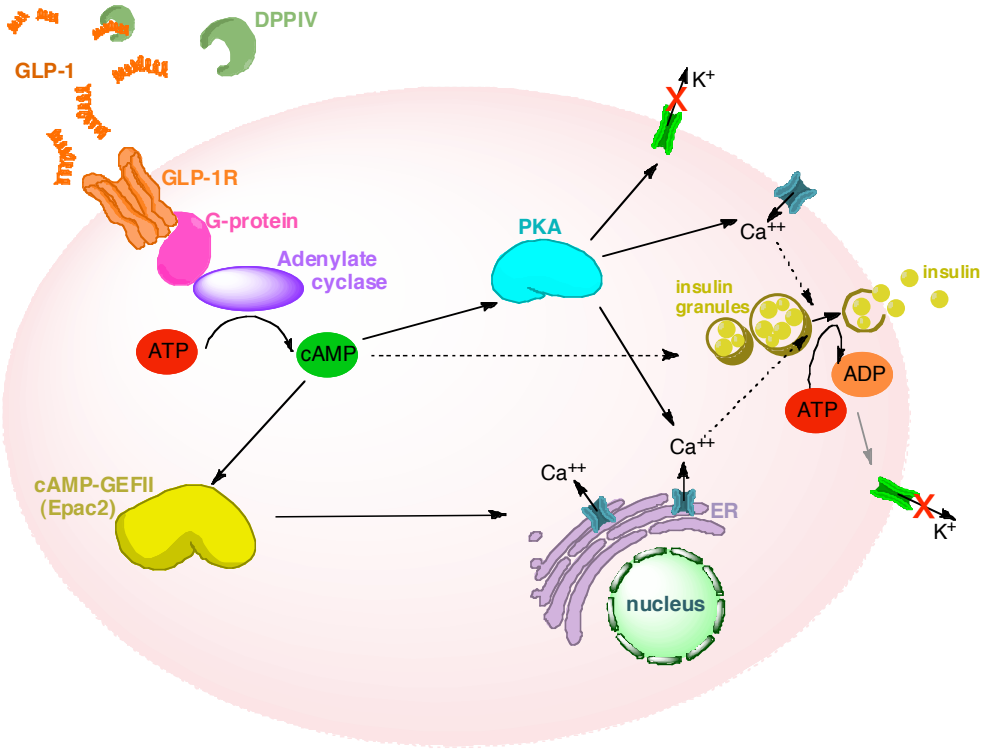

Figure 5. GLP-1 action in target cells. Upon binding to its receptor, GLP-1 stimulates the pathway to elevate cellular cAMP levels. As a result, two important proteins, protein kinase A (PKA) and cAMP-regulated guanine nucleotide exchange factor (cAMP-GEFII or Epac2) are activated and cause membrane depolarization via closing the ATP-sensitive K-channels. Change in electronic activity results in Ca^{++} mobilization from cellular Ca^{++} stores and also increases influx through Ca^{++} channels. High Ca^{++} concentrations in cytoplasm triggers exocytosis of insulin which is also affected by cAMP levels that accelerate granule mobilization in the cell. (reproduced with permission[30])

2.3 Current GLP-1 Based Therapies

2.3.1 GLP-1 Mimetics

Exenatide

A synthetic GLP-1 analogue, Exenatide, is a naturally occurring peptide isolated from the saliva of Gila Lizard (*Heloderma Suspectum*).[74] It shows better stability in plasma (26 min. after intravenous delivery) and has the same effects as GLP-1.[75] [76] Protease stability of the peptide is due to an Ala→Gly mutation on the penultimate N-terminal amino acid. (Figure 3) Exenatide is the first FDA approved peptide drug for treatment of Type II diabetes, and is available since 2005 under the commercial name Byetta (www.byetta.com). It is administered by two subcutaneous (S.C.) injections per day in 5 to 10μg quantities per injection. [77]

Liraglutide

Liraglutide is a lipidated analogue of GLP-1 with a further extended half-life: 12 hours after I.V. injection.[78] Owing to a lipid tail on Lys24, liraglutide can bind to albumin in plasma and remain in the blood stream in its active form for longer periods of time without proteolysis and clearance,[72] retaining an effect similar to continuous GLP-1 infusion to the plasma. Liraglutide is dosed once daily, S.C.[79], and has been recently approved by the FDA for treatment of Type II diabetes.

Albiglutide

A longer-lasting analogue, Albiglutide, has been developed as a once-weekly treatment.[72] Considering the advantages of albumin binding for

liraglutide, this construct was designed to covalently link albumin to two protease resistant GLP-1 peptides. The molecule is in phase III clinical trials.

2.3.2 DPP-IV Inhibitors:

DPP-IV digestion of both GLP-1 and GIP is a very rapid process in the plasma that has been the main impediment for incretin-based therapies so far. In order to overcome this problem and enhance the incretin effect, inhibitors of DPP-IV have been considered as potential drug candidates.[57] Initial studies showed that injection of DPP-IV inhibitors prevented degradation of both endogenous and exogenous GLP-1 in humans.[59] While lowering blood glucose levels effectively, inhibitors did not cause adverse effects such as hypoglycemia. Since the mechanism of action of these molecules is inhibition of the DPP-IV protease, hence enhancing the effects of GLP-1 and GIP, glucose levels are only dropped within normal range by natural incretin effect. Currently approved DPP-IV inhibitors including sitagliptin,[58,80] vildagliptin,[81] and saxagliptin[82] are all orally available. They are in use for Type II treatment, either alone or in combination with existing drugs.[83]

DPP-IV inhibitors seem to be well tolerated although there are still unknown areas where DPP-IV may act as an important enzyme or as a key-binding partner.[84,85] DPP-IV is a ubiquitous serine protease and the repertoire of substrates includes incretins, chemokines, and neural system hormones.[62] Processing of such species can be severely impaired when DPP-IV inhibitors are used continuously.[62,60] Beyond its proteolytic role, DPP-IV is also known as T-cell antigen CD26, and presents itself in a variety of tissues; the kidney, intestines,

and the vascular endothelium.[61] It interacts with membrane antigen CD45 and may have a role in T-cell activation and proliferation, however its contribution in immune function is not completely understood. There is evidence for several other members of the DPP family where inhibition results in reduced T-cell activity. Therefore, specificity and dosage of the inhibitors carry great importance, and the long-term consequences of DPP-IV inhibition need more in-depth analysis.[84]

2.3.3. Non-peptidic small molecule drugs for insulin signaling pathway:

Insulin resistance is the key pathological factor in Type II diabetes and can occur due to multiple defects in signal transduction.[7] Therefore, even before wide preparation and use of insulin, several molecular targets in the insulin signaling pathway are investigated for repairing or improving insulin mediated glucose control. (Figure 6)

Sulfonylureas:

Sulfonylureas are orally used insulin secretagogues. They stimulate insulin secretion by acting on ATP-sensitive potassium channels (K^+ATP) on pancreatic β-cells. Binding of a sulfonylurea analogue to a specific receptor causes K^+ATP channel to close and results in membrane depolarization. This process is followed by subsequent influx of calcium ions and release of insulin granules.[86] There are two side effects of these types of drugs. First, the risk of hypoglycemia is common for all sulfonylureas and is life threatening, however it can be controlled by strict control of dosage. Second, weight gain and increase in appetite is unavoidable for those who use sulfonylureas as the main treatment. Therefore it is

not the best choice for overweight or obese diabetic patients.[87] Glyburide is the most common sulfonylurea drug that has been used either alone, or most commonly with Metformin (Figure 6).

Meglitinides:

Meglitinides bind to ATP-dependent potassium channels on β-cells and increase insulin production in a similar manner to sulfonylureas.[88] The route by which the insulin is stimulated is same as sulfonylurea action, except binding sites of meglitinides are different -they bind to K^+ATP channels themselves. Two clinical agents are called Repaglinide and Netaglinide. The main risk for these molecules is hypoglycemia, as in sulfonylurea drugs, and requires careful dosing.

Thiazolidinedions :

Thiazolidinedions (TZDs) are insulin sensitizers that act mainly on adipocytes and muscle. They activate peroxisone proliferator-activated γ nuclear receptors (PPARγ) that are ligand activated transcription factors. PPARγ activation in adipocytes lowers triglyceride and free fatty acid levels.[89] Roughly 20 to 40% decrease in circulating free fatty acid levels is suggested to enhance insulin signaling peripherally and also affect muscle and liver cells.[90] Rosiglitazone and Pioglitazone are two TZD analogues that are in clinical use.[91] Both have desirable effects on control of high density lipoproteins due to their ability of fat redistribution.[92] Hypoglycemia is not a problem for TZDs when used as a single agent in a therapy, since insulin secretion is not affected. Edema and weight gain are implicated side effects for this group, and are associated with increased adipocyte mass.

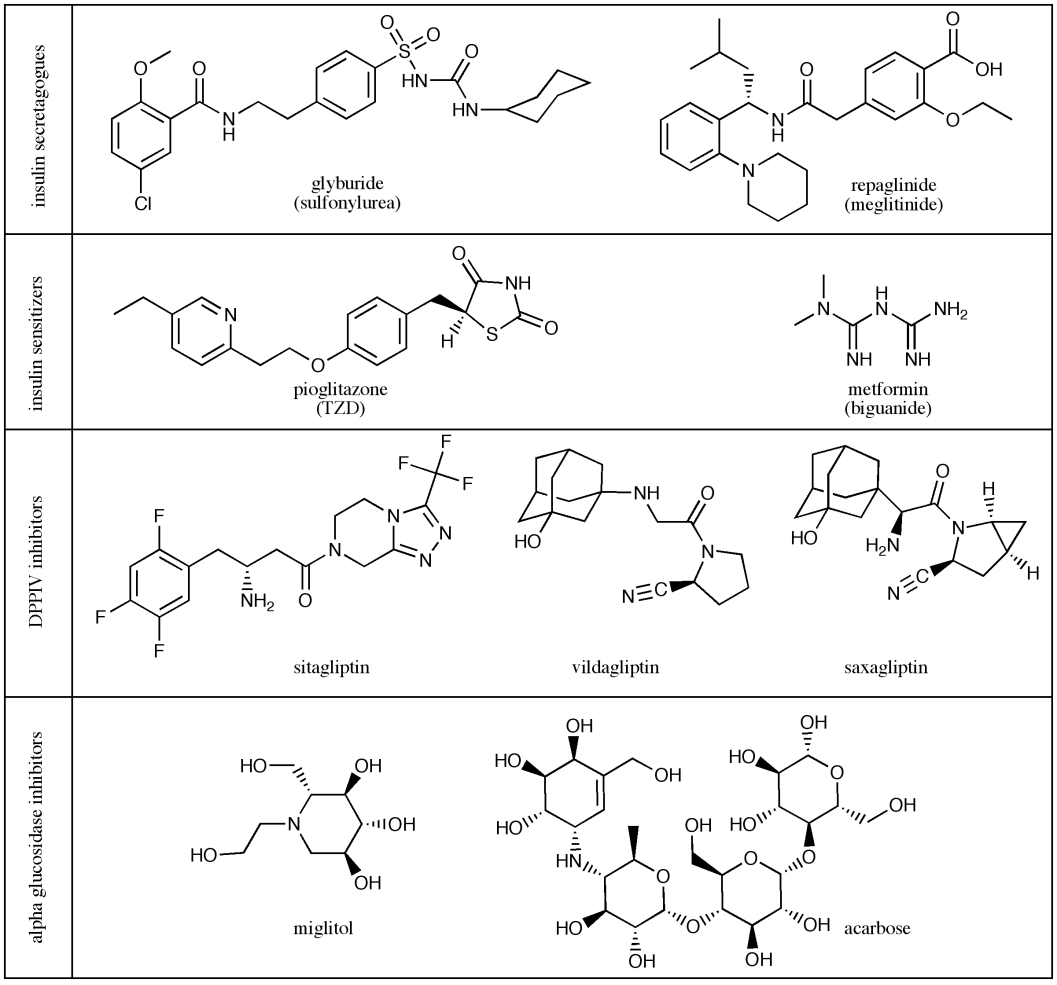

Figure 6. **Examples of non-peptidic, small molecule drugs for type II diabetes that are commonly used. Compounds are grouped by their mechanism of action.**

Biguanides:

Biguanides affect insulin sensitivity, specifically in muscle cells. Similar to TZDs, they do not portend risk of hypoglycemia, except in cases when they are used in combination with sulfonylureas and insulin. Metformin is the only drug that has remained in use for treatment of Type II diabetes from this group, however the mechanism of action is still not well understood. It is especially preferred for obese patients because it is the only small molecule drug that is not associated with weight gain and side effects are minimal. Lactic acidosis may occur due to over-dosing. Metformin is widely used especially as a counterpart in combination therapies of Type II diabetes, where a few types of drugs are used for glucose control.

2.3.4. Others

Apart from signal transduction targets and insulin itself, which have now many preparations and are also used in combination with many other drugs, there are various recent therapeutic molecules for Type II diabetes that are effective in other peripheral systems: Alpha-glucosidase inhibitors, acarbose and miglitol;[89] amylin analogue, pramlintide[93,94] are currently in use; and the cannabinoid receptor antagonist, rimonabant[95] had been in the clinic for a brief period. There is also ongoing research on small molecule activators of GLP-1R that can mimic the incretin effect.[96]

2.4 DESIGN OF LIPIDATED GLP-1 ANALOGUES

2.4.1 Lipidation of Therapeutic Molecules

About 2-4% of translated proteins are lipidated for their function and localization in the cell.[97] Lipidation provides interactions of target proteins with membranes and directs them to organelles or cell membranes. This process not only facilitates their passage through cell membranes but also helps them interact with membrane bound proteins or enzymes. Membrane proteins consist of a large variety of important molecules such as ion channels, small molecule transporters, and receptors. The majority of communications between cells, and signaling within the cells, depend upon these membrane proteins and the lipidated mediators.[98] Lipidation affords unique properties not easily achieved by other means.[99,100]

Newly developed pharmaceuticals often face problems when it comes to delivering the active drug to the desired targets in the body. Lipidation of drug molecules has been previously used in order to improve bioavailability and localization.[101] For peptide and protein drugs, lipidation is especially advantageous. Polypeptides gain amphiphilic character for membrane affinity, and increased lipophilicity often improves stability. One relevant example is Liraglutide, a potent GLP-1R agonist. It is a lipidated GLP-1 (7-37) amide with 97% homology to native GLP-1.[102] A-sixteen carbon fatty acid chain is attached to Lys26, where Lys34 is substituted with Arg to allow for specific acylation of the terminal lysine residue. Self-aggregation of the resulting molecule is

advantageous for slow release *in vivo*. Inclusion of a hexadecanoyl chain causes liraglutide to bind albumin and thereby slows renal clearance and degradation.

Protease resistance and slow clearance are proven benefits of lipidation in the case of liraglutide. As it will be further explained in section 2.4.3, our additional goal in this study for lipid attachment to GLP-1 is to provide membrane anchoring, thereby increasing the effective concentration of the peptide around its receptor. Binding of the ligand to its receptor would be improved by restricting the peptide to the cell membrane. Dissociation of the active analogue therefore would be prevented.[99] Lipids may also provide an advantage of delivery if the final construct can be stably transformed into liposomes.

2.4.2 PEG Conjugates of Therapeutic Molecules in Medicinal Chemistry

Polyethylene glycol (PEG) is a non-toxic polymer that has been widely used to modify the physical and chemical properties of biomolecules. Altered folding of the proteins, solubility of very hydrophobic biomolecules, stabilization of labile bonds, improved pharmocokinetics, and increased resistance to protease activity are some benefits that have been realized by the use of PEG conjugation.[103,104,105] Since initial development in 1970s, PEGylation has given rise to development of many successful human therapies.[106]

PEG itself can dissolve in both aqueous and organic solvents, thereby making bio-conjugation possible in various media. Because the properties of PEG typically transferres to the attached molecule, the resulting bioconjugate is expected to have improved pharmacokinetics. Even when short segments of PEG are used, the PEG linker may still have an impact on solubility. We utilized short

PEGs as linkers between lipid and peptide chains. In addition to its contribution to solubility, we also aimed to obtain a flexible lipid-peptide construct by using eight or twelve unit PEGs. They provide 20 and 35 Å spacing, respectively, between the C-terminus of the GLP-1 peptide and the lipid. We hypothesized that flexibility between the anchoring unit and the ligand would be important.

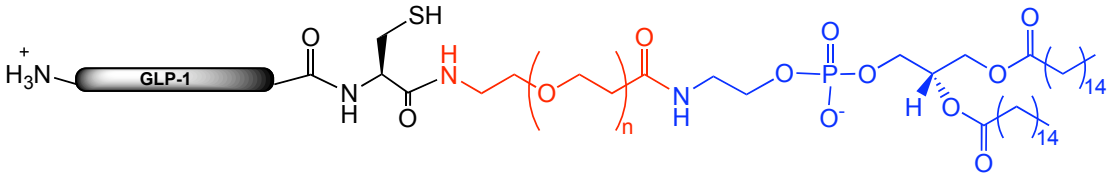

Figure 7. **GLP-1 analogue with a PEG linker to attach the dipalmitoyl lipid chain to the C-terminus of the molecule.**

2.4.3 Design and Characterization of Lipid-PEG Conjugates of GLP-1

In this study, we constructed a lipidated GLP-1 that includes a PEG linker between the C-terminus of the peptide and the hydrocarbon tail. (Figure 8)

In order to target cell membranes, we chose to use 1,2-dipalmitoyl-sn-glycerol-phosphoethanolamine (DPPE) as the lipid tag, which is naturally present in biological membranes. Phosphoethanolamine is a zwitterionic lipid component of most membranes and is a donor for palmitoylation *in vivo*.[98] A dipalmitoyl tail insures the anchoring of the attached cargo to the membrane and provides lateral motility. We used the free amine functionality of DPPE for covalently linking an activated ester of the PEG8 linker that will enhance solubility and provide flexibility. The resulting amphiphilic PEG-DPPE molecule was coupled with GLP-1 peptide via native chemical ligation.[107,108] In order to prevent any disturbance of binding between the construct and the receptor, we installed DPPE on C-terminus end of GLP-1, where the extensions are well tolerated.[65]

2.5 Structure-Activity Studies of Lipidated GLP-1 Analogues

2.5.1 Luciferase Coupled cAMP Accumulation in Cells:

Ligand induced signaling of hGLP-1R was measured in HEK293 cells using a cAMP responsive luciferase reporter gene assay.[109,110]

As with many members of GPCR family, functional response of GLP-1R is traditionally measured by cAMP accumulation in the cell in response to binding of ligands.[111] For complete pharmacological profile of new analogues, a combination of ligand binding and receptor activation assays are necessary to

acquire binding data and activity.[112] Binding experiments are performed by using radiolabelled agonists/antagonists and results are dependent on competition between the tested molecules and the displaced ligand. For assessment of the activity, cAMP accumulation is directly measured via radioimmunoassay.

Recently, reporter constructs have been designed and applied to a variety of the receptors that employ downstream cAMP stimulation[110,113,114], including GLP-1 receptor.[109] As a reporter construct, firefly luciferase gene was cloned downstream of a basal promoter that includes multiple repeats of cAMP response elements (CREs).[115] Expression of the luciferase gene is then responsive to cAMP production within the cell. In this transcription based GLP-1R assay, the outcome is amplified due to measurement of downstream signaling cascade of cAMP pathway and also the sensitivity of luciferase assay.[116] Therefore fewer number of cells can be used especially if response elements are increased. It is an advantage for high throughput screening of new ligands. In addition to increased sensitivity, the luminescence response of luciferase is linear to the amount of cAMP produced over broad range of concentrations, whereas, direct measurement of cAMP accumulation has a narrower linear range.[111] Most importantly, a luciferase coupled cAMP reporter assay could provide simultaneous efficacy and potency information for tested compounds, without tedious binding experiments.[117] Ligand activation curves provide half maximal effective concentrations (EC_{50}) and also maximal response of the ligands as well as concentration dependent response of compounds. When necessary, estimation of ligand-receptor binding constants can further be derived using an adaptation of the Gaddum

equation[116,118], although comparative experiments have already shown that similar results are deduced for receptor affinities whether estimated by reporter gene assay or individual ligand binding assay.[119] Therefore we used this quick and efficient technique for assaying GLP-1 derivatives in this study.

Our results include a series of GLP-1 analogues some of which have been synthesized as a part of the Chapter 3 and details are explained therein.

All GLP-1 analogues, including lipidated GLP-1 (shown as K1) had similar efficacies to GLP-1 however potencies were severely reduced compared to GLP-1. (Figure 9) Lipidated GLP-1 is lowest in activity with EC_{50} value of 2.3×10^{-8} M compared to wild type GLP-1 ($EC_{50}=3.3 \times 10^{-12}$ M). K2, K3 and K4 are point mutation analogues at the 9th position of GLP-1, where Glu is substituted with βHFL, βLeu, β-Glu respectively. They also displayed reduced activities, however better than the lipidated construct: EC_{50} measured for K2 was 8.2×10^{-8} M; for K3, 2.5×10^{-8} M; and for K4, 4.7×10^{-8} M.

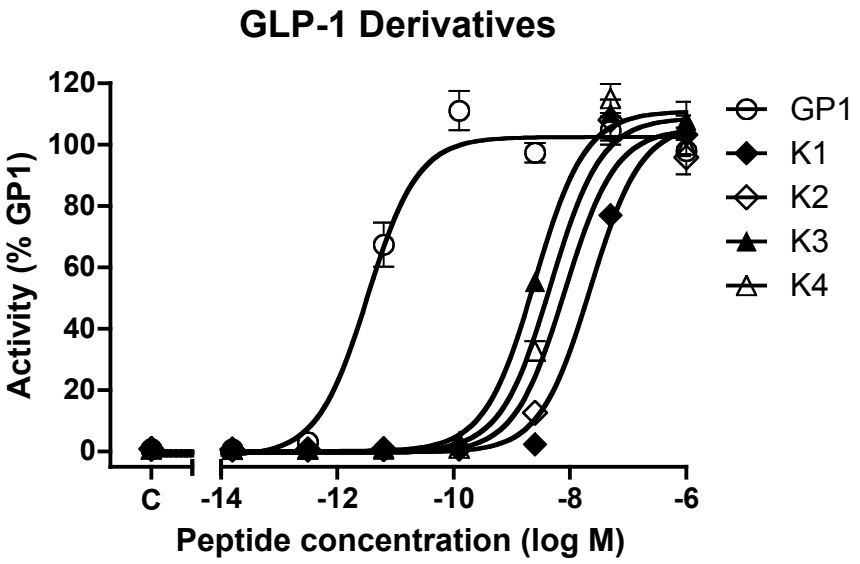

Figure 8. Lucifearse coupled cAMP production induced by wild type GLP-1 and synthetic analogues. K1 indicates lipidated GLP-1 analogue (EC_{50}=2.3x10^{-8}M). K2, K3, K4 are βHFL, βLeu, β-Glu substituted GLP-1 analogues at 9th position and have EC_{50} values of 8.2 x10^{-8}M, 2.5 x10^{-8}M, 4.7 x10^{-8} M, respectively.

Compound	Amino Acid Sequence
GLP-1	H_2N- HA E GTFTSDVSSYLEGQAAKEFIAWLVKGRA-$CONH_2$
K1 (GLP-1-lipid)	H_2N- HA E GTFTSDVSSYLEGQAAKEFIAWLVKGRA-PEG_8DPPE
K2 (βHFLGLP-1)	H_2N- HAβLGTFTSDVSSYLEGQAAKEFIAWLVKGRA- $CONH_2$
K3 (βLeuGLP-1)	H_2N- HAβLGTFTSDVSSYLEGQAAKEFIAWLVKGRA- $CONH_2$
K4 (βGluGLP-1)	H_2N- HAβEGTFTSDVSSYLEGQAAKEFIAWLVKGRA- $CONH_2$

Table 1. Amino acid sequences of GLP-1 analogues K1, K2, K3 and K4.

2.5.2 Results and Discussion:

2.5.2.1 Activity Profiles:

Peptidic GLP-1 mimics such as Exenetide and Liraglutide have so far been successful as GLP-1R agonists and have found use as the first incretin-based therapeutics in diabetes treatment. Identifying even more efficient, more protease resistant analogues is challenging but necessary for practicality in clinical use. We chose to attack this problem by creating a macromolecule that is able to anchor itself in the lipid bilayer and proximally interact with the receptor more efficiently. Ideally activation by the native GLP-1 peptide would be enhanced when the membrane-anchoring lipid is attached. Moreover, this conjugation may also help prevent degradation by DPP-IV via molecular crowding around GLP-1. Luciferase coupled cAMP formation assays have shown that our construct, K1, has the same affinity as the native GLP-1 to the receptor, whereas activation is 6800-fold less than GLP-1. There could be several reasons for this observed decrease in activity. The most possible scenario is that the lipid component of the molecule has failed to integrate into the cell membranes during incubation. Therefore construct may be interacting with its receptor in the same way as the anchor-free GLP-1 ligand. In this case, decrease in activity is attributable to conformational disturbance of the ligand due to the large C-terminal linker. Even though we aimed to cause minimal disturbance in conformation by attaching the anchor on the C-terminal part of the molecule where the mutations and attachments are most tolerated, the linker may be interfering with the positioning of the bound peptide and its structure within the GLP-1R active site. Whether the construct is membrane bound or not can be tested by synthesizing a chromogenic

analogue that can be visualized on the cell-surface. In our design, a free thiol moiety is available via cysteine residue on C-terminus for such modifications. Chances of dimerization of the cysteine residues are weak due to the fact that the binding to the receptor is comparable to wild type. In order to rule out the possibility of dimerization completely, the construct could be treated with Raney/Ni in order to reduce and inactivate the thiol group.

In a second scenario of interpreting the results, the designed construct may have been incorporated into the cell membrane as planned and is interacting with GLP-1R. Because binding is unaffected, but the activation of the receptor is diminished, the C-terminal attachment could be the responsible component mentioned previously. The PEG linker could prevent proper organization of the GLP-1 peptide for activation of GLP-1R. This hypothesis can be tested by synthesizing GLP-1 molecules with varying lengths of C-terminal PEG linkers and analyzing their binding and activation profile to investigate how well PEG linkers are tolerated on the C-terminus of the peptide.

We have also prepared a second set of GLP-1 analogues that consist of β-amino acid insertions at the proteolytic site of GLP-1. Background information about these analogues is given in Chapter 3. Alanine scaning experiments for replacement of glutamic acid at position 9 has been successful for obtaining more protease stable analogues of GLP-1.[120] Fluorinated side chain modifications have been shown to be tolerated and successful toward proteolysis. Previously in our lab, Ala8, Glu9, Gly10, both Ala8 and Glu9 positions were substituted with α-hexafluoroleucine residue to confer resistance. These fluorinated analogues are

called F8GLP-1, F9GLP-1, F10GLP-1, F89GLP-1 respectively. (Figure 3) F89GLP-1 and F8GLP-1 were completely resistant to hydrolysis, where F9GLP-1 and F10GLP-1 displayed 1.5 and 2.9 fold better stability. Binding capacity of the analogues was diminished except for F9GLP-1 (IC_{50} = 5.1 nM), which had a comparable affinity to the receptor as native GLP-1 (1.9 nM). F8GLP-1, F9GLP-1 and F10GLP-1 were found to be full agonists of GLP-1R, and F89GLP-1 was only a partial agonist. Notably, the F9 analogue was comparable to GLP-1 in its activity and with its improved stability, suggesting bulky fluorinated side chains are tolerated at this position in during binding.[121]

Backbone modifications on the other hand, are under-investigated. There is no example of β-amino acid containing GLP-1 analogue to our knowledge. The three analogues that we synthesized possess either β-Glu, βLeu or βHFL at the 9th position and are the first examples of β-amino acid modified GLP-1 peptides.

When α-amino acid substitutions are analyzed at position 9, wild type GLP-1 with Glu 9 is found to have a binding affinity of IC_{50} = 1.9 nM and Leu9GLP-1 and HFL9GLP-1 follow it with 2-fold and 2.5-fold less affinity, respectively. The fact that uncharged isopropyl and hexafluoroisopropyl groups at 9th position display similar binding affinity as wild type and activation of the receptor only a few fold less, suggests that large hydrophobic groups are tolerable at this position. It is also hypothesized that polar-hydrophobic fluorine atoms may mimic the glutamic acid side chain due to their high electron density and create a similar environment as a carboxylate group.[121] We expect a similar pattern of binding and activation of GLP-1R with β-amino acid inserted analouges, unless

backbone manipulation causes a large decrease in recognition and binding of these molecules.

Luciferase coupled cAMP accumulation experiments have shown that the efficacy of these peptides to be similar to native GLP-1, however the activation of receptor diminished greatly compared to the native peptide: 1400-fold less for β-Glu, 750-fold less for β-Leu, and 2500-fold less for βHFL-substituted analogue. Lower activation capacity of these peptides may be because of possible distruption of N-terminus structure within the receptor binding site. Due to extra methylene group in β-amino acids, the backbone is extended by one more carbon unit compared to the wild type, which, in turn may cause repositioning of the side chains around it. This may also disrupt the secondary structure that the peptide adopts when bound to the receptor. Without any crystal structural data it is not possible to know whether the peptide is only partially disturbed around the substituted β-amino acid, or the whole structure is affected. It is not very surprising that a backbone modification in this region decreases activity of the analogues because the N-terminus region includes crucial residues for activity and the indispensable N-terminus His is proximal to the modification site. The intriguing part of these results was that β-Leu containing analogue had superior activity than β-Glu containing peptide. In principle, the β-Glu substituted compound should be more like the wild type GLP-1 because the only difference at the mutated position is the backbone of the amino acid, not the side chain. The β-Leu substituted compound on the other hand has both backbone and side chain mutations with respect to native GLP-1. It is known from the previous studies that

α-Leu substitutions at the 9th position of GLP-1 does not lead to improved receptor activity compared to wild type (i.e. α-Glu containing peptide).[68] Thus, modification of side chain should not have any effect on the receptor activation.

2.5.2.2 Protease Stabilities:

Although neither the lipidated nor backbone modified GLP-1 analogues possess sufficiently good activation profiles compared to native GLP-1, protease stability assays show an advantageous aspect of these compounds. When these compounds are incubated with DPPIV, they maintained their stabilities longer than control GLP-1 peptide. Rate constants for degradation reactions could not be determined for βHFL and the lipidated analogue DP-1 due to restricted amount of peptide used in the digestion reactions. Rate constants for β-Leu, β-Glu and wild type GLP-1 were found to be 0.0054 (± 0.0026) min^{-1}, 0.0089 (± 0.0016) min^{-1} and 0.0520 (± 0.0044) min^{-1}, respectively. Better protease stability can be compensatory for the diminished receptor activity due to extended presence of the compounds around the receptor. Previous studies have reported such compounds. For example, replacement of Ala8 with valine or 2-amino butyric acid (Val^8GLP-1 and Abu^8GLP-1) resulted in 1.5 and 3.5-fold reduced potency, respectively, compared to wild type GLP1.[122] Receptor affinities were also reduced with respect to GLP-1. Loss in receptor binding is previously reported for Ala8 modified analogues[123] and was the reason for reduced cAMP production. However, for Val^8GLP-1 and Abu^8GLP-1, diminished receptor binding and cAMP response did not translate to lower insulinotropic activities. Both analogues were able to maintain glucose-dependent insulin secretion as well as, or even

better than GLP-1 *in vitro* and *in vivo* due to their superior stability towards DPP-IV (12 hours compared to 1-3 minutes). The Val^{8}GLP-1 analogue was later used for sub-chronic activation of GLP-1R in aging mice.[124] Minimum daily injections of Val^{8}GLP-1 (25 nmol/kg) for 12 days resulted in improved glucose-induced insulin secretion and insulin sensitivity, suggesting that extended use of stable GLP-1 analogues is possible to counteract symptoms of Type II diabetes.[125] Another example of this kind is the Ser^{8}GLP-1 analogue that displayed enhanced insulinotropic activity in animals due its improved plasma stability[126] even though its receptor affinity was reduced 2-fold and the potency was lowered 3-fold.[127] Fluorinated analogues of GLP-1, mentioned above, had shown similar profiles when tested in animals via oral glucose tolerance test.[121] Respective binding affinities of F8-GLP-1 and F9GLP-1 were diminished by 27.3 and 2.7-fold and their activities were lower by 73.8 and 2.1-fold compared to GLP-1. Stability experiments towards DPP-IV digestion revealed that F8-GLP-1 had completely resisted to hydrolysis, whereas F9-GLP-1 was only 1.5-fold better than GLP-1 in protease stability. Even then, insulinotropic activity of F9-GLP-1 was as good as F8-GLP-1; they both decreased blood glucose levels in live animals to same extent as GLP-1.[121] Thus stability of these analogues successfully compensated for lower binding and lower activation *in vivo*.

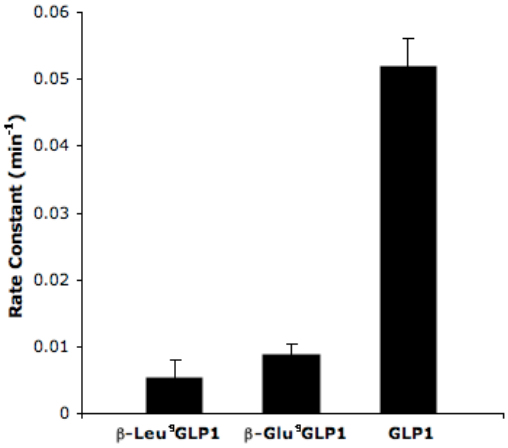

Figure 9. **Comparative rate constants of β-Leu9-GLP-1 and β-Glu9-GLP-1 with respective to GLP-1.**

Compound	% Efficacy	cAMP accumulation EC_{50}	ratio	Protease resistance k (min^{-1})	ratio
GLP-1	100.0	3.3×10^{-12}	1	0.052	1
K1	104.1	2.3×10^{-9}	0.0002	-	
K2	102.6	8.2×10^{-9}	0.0004	-	
K3	108.2	2.5×10^{-9}	0.0013	0.005	9.6
K4	106.1	4.7×10^{-9}	0.0007	0.009	5.8

Table 2. **Summary of luciferase coupled cAMP accumulation assays and protease stability experiments for K1 (lipidated GLP-1), K2 (βHFL-GLP-1), K3 (βLeu-GLP-1) and K4 (βGlu-GLP-1) compared to wild type GLP-1.**

Results of our experiments show that protease stability of β-Leu9-GLP-1 and β-Glu9-GLP-1 analogues are 9.6 and 5.8-fold higher than GLP-1, which may reflect on their insulinotropic activities if compounds were to be used for *in vivo* trials. Although potencies of these analogues are 750 and 1400-fold less than wild type GLP-1, superior protease stability may result in appreciable glucose control as seen in other examples before. To what extent this insulin-mediated response can be attained is unpredictable since there is not a direct correlation of how much the stability would overcome the diminished potencies of the compounds. It is challenging to draw a conclusion from the existing data since the experimental procedures are different in each study and so are analytical outcomes.

2.6 Conclusion

In this study we sought for longer lasting analogues of GLP-1 and synthesized i) GLP-1 with C-terminal membrane anchoring unit that can attach itself to the cell membrane and activate the receptor for extended period of time and ii) GLP-1 analogues with β-amino acid substitutions proximal to the N-terminus end. All analogues displayed similar efficacies compared to GLP-1, however their potencies were lower than GLP-1. The N-terminal modifications are known to abolish GLP-1 activity, however in our case, lower activities are not the result of diminished binding. All analogues were able to stimulate maximal response from GLP-1R and had similar affinities to GLP-1R compared to GLP-1. So if any structural perturbation was introduced by β-backbone, it did not affect binding of these peptides to the receptor, however it could cause incorrect positioning of the

important side chains once the peptide is bound. As a result, receptor signaling would be attenuated. Nevertheless, protease stability assays revealed that the β-amino acid substituted analogs are 5 to 10-fold more stable than GLP-1, which may compensate for reduced potency. Only further studies in animal models can reveal whether DPP-IV stability can result in a desired insulinotropic effect. If results are positive, then various β-amino acid substitutions can be tried at multiple sites on GLP-1. For a better proteolytic stability for instance, 8^{th} or both 8^{th} and 9^{th} positions can be modified.

For lipidated GLP-1, suggestions for improving the design have been given in section 2.4.2.1. It must also be mentioned though; recently Fortin et al. presented a similar case where a membrane anchoring GLP-1 ligand has been intrinsically expressed in human embryonic kidney 293 (HEK293) cell membranes.[128] The ligand is then shown to localize on the membrane of the cells and activate GLP-1 receptor. Such an endogenous ligand with similar properties holds promise that a synthetic construct can also attain activity when optimized, as suggested in 2.4.2.1.

2.7 Experimental:

2.7.1 Synthesis of Membrane Anchoring Units:

Synthesis of Boc-Cys(Trt)-PEG_n-COOH (n = 8 or12): Boc-Cys(Trt)-COOH (126 mg, 0.27 mmol) and HBTU (92.8 mg, 0.24 mmol) were dissolved in 0.5mL of dry DMF. DIEA (47 μL, 0.25 mmol) was added to the clear solution and gently mixed for 2 mins. In a separate vial, either H_2N-PEG_8-COOH (60 mg, 0.14 mmol), or H_2N-PEG_{12}-COOH (86 mg, 0.14 mmol) was solubilized in 0.5 mL dry DMF by addition of DIEA (47 μL, 0.25 mmol) in two equivalent excess of the H_2N-PEG_n-COOH and immediately transferred into activated Boc-Cys(Trt)-COOH solution. The reaction was monitored by TLC (9:1:0.1/CH_2Cl_2:MeOH:AcOH). After complete consumption of H_2N-PEG_n-COOH, the reaction mixture was concentrated under vacuum and redissolved in CH_2Cl_2. Silica gel column chromatography included two steps of purification: EtOAc followed by 0% to 5% MeOH gradient in CH_2Cl_2. Pure Boc-Cys(Trt)-PEG_8-COOH was obtained in 49% yield and pure Boc-Cys(Trt)-PEG_{12}-COOH was obtained in 41% yield. Boc-Cys(Trt)-PEG_8-COOH ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.4-7.18 (m, 15H), δ 6.58 (bs, 1H), δ 4.97 (d, 1H, *J* = 8.0 Hz), δ 3.89 (m, 1H), δ 3.76-3.71 (m, 2H), δ 3.62-3.54 (m, 28H, (PEG)), δ 3.48 (Ψt, 2H, *J* = 4.7 Hz), δ 3.38 (Ψt, 2H, *J* = 5.1 Hz), δ 2.68 (m, 1H), δ 2.59 (Ψt, 2H, *J* = 6.1 Hz), δ 2.48 (m, 1H), δ 1.39 (s, 9H).

Synthesis of Boc-Cys(Trt)-PEG$_n$-DPPE (n=8): Boc-Cys(Trt)-PEG$_8$-COOH (11 mg, 0.012 mmol) and HBTU (4.1 mg, 0.011 mmol) were dissolved in 0.5 mL of dry DMF, followed by DIEA addition (2.2μL, 0.12mmol). H_2N-DPPE (8.6 mg, 0.012 mmol) was suspended separately in 1 mL of $CHCl_3$ and DIEA (6.6 μL, 0.036 mmol) were added in three fold excess at 37 °C with vigorous stirring in a capped vial. The vial was removed from heat to room temperature after a transparent solution was obtained and a DMF solution (0.5 mL) of activated Boc-Cys(Trt)-PEG$_8$-COOH was added dropwise with constant stirring. The reaction was monitored by TLC (95:5:0.1/$CHCl_3$:MeOH:AcOH). After 16 hours, there was still unreacted H_2N-DPPE in the reaction mixture, and solution was cloudy. The mixture was concentrated under vacuum and redissolved in $CHCl_3$. Silica gel column chromatography in a 5% to 10% MeOH gradient with 0.05% AcOH in $CHCl_3$ yielded pure Boc-Cys(Trt)-PEG$_8$-DPPE in 63% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.41-7.20 (m, 15H), δ 6.5 (bs, 1H), δ 5.22 (m, 1H), δ 4.92 (d, 1H, J = 8.0 Hz), δ 4.32 (dd, 1H, J_1 = 4.08 Hz, J_2 = 11.97 Hz), δ 4.16-4.07 (m, 3H), δ 3.90 (m, 1H), δ 3.73 (m, 2H), δ 3.67-3.55 (m, 28H), δ 3.48 (Ψt, 2H, J = 4.7 Hz), δ 3.38 (Ψt, 2H, J = 4.84 Hz), δ 2.70 (m, 1H), δ 2.59 (Ψt, 2H, J = 6.47 Hz), δ 2.48 (m, 1H), δ 2.30 (m, 2H), δ 1.39 (s, 9H), δ 1.23 (m, 12H), δ 0.90 (t, 6H, J = 6.49 Hz).

Synthesis of H_2N-Cys-PEG$_n$-DPPE (n = 8): Boc-Cys(Trt)-PEG$_8$-DPPE (160 mg) was dissolved in a minimal amount of CH_2Cl_2. 50% TFA/CH_2Cl_2 including 1% H_2O and 1% TIS were added to the clear solution to a final volume 100ml. Constant stirring for 1 hr at rt resulted in H_2N-Cys-PEG$_8$-DPPE in 100% yield as assessed by its molecular weight. The compound was then lyophilized to

be used for native chemical ligation without further purification. MALDI-TOF MS $C_{59}H_{116}N_3O_{18}PS$ *m/z* calcd (M) 1217.8, obsd 1219.2 ($M+H^+$).

2.7.2 Synthesis of GLP-1 Peptide with C-terminus Thioester:

Synthesis of Boc-Ala-COSR Linkers (R: 3-mercaptopropionic acid=MPA, or thiolactic acid=TLA): Boc-Ala-OSu (3g, 10 mmol) was combined with either 3-mercaptopropionic acid (910 μL, 10 mmol) or thiolactic acid (934 μL, 10mmol) in 20 mL of dry DMF. To the clear solution, was added DIEA (1.75 mL, 10 mmol) and the reaction was followed by TLC (50:50:0.1/EtOAc:Hexanes:AcOH). Consumption of the starting materials was complete in 2 h. The solvent was evaporated and the orange dry solid was purified in a 0% to 40% EtOAc gradient in hexanes using silica gel chromatography. Pure Boc-Ala-COS-MPA-COOH was obtained in 83% yield and pure Boc-Ala-COS-TLA-COOH was obtained in 68% yield . Boc-Ala-COS-MPA-COOH (mixture of conformers) ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.34 (d, 0.3H^a, *J* = 6.42 Hz), δ 4.98 (d, 0.7H^a, *J* = 7.97 Hz), δ 4.37 (m, 0.7H^b), δ 4.15 (m, 0.3H^b), δ 3.10 (t, 2H, *J* = 6.95 Hz), δ 2.66 (t, 2H, *J* = 6.81 Hz), δ 1.44 (s, 9H), δ 1.36 (d, 3H, *J* = 7.23 Hz). Boc-Ala-COS-TLA-COOH (mixture of rotamers) ^{1}H NMR (300MHz, $CDCl_3$) δ 6.57 (bs, 0.45H^c), δ 5.12 (d, 0.55H^c, *J* = 7.77 Hz), δ 4.38 (m, 0.9H^d), δ 4.13 (m, 1.1H^d) , δ 1.48 (d, 3H, *J* = 7.31 Hz), δ 1.40 (s, 9H), δ 1.34 (d, 3H, *J* = 7.31 Hz).

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Synthesis of GLP-1 peptide thioesters:

GLP-1 was synthesized by manual *t*-Boc SPPS on MBHA resin on a 0.3 mmol scale. The first residue was 3-mercaptopropionic acid thioester of Boc-Alanine (MPAL) in order to obtain C-terminal thioester upon HF cleavage. MPAL was double coupled for 15 min (×2). The resin was split in half after the 17^{th} residue and the synthesis was resumed till the 30^{th} residue, with checks with Kaiser test after each coupling. Cleavage of the peptide from resin was done with anhydrous HF, using p-cresol as scavenger. Crude peptide was obtained in 63% yield (78 mg). Purification with C18 preparative RP column (Vydac 218TP54, 5μm) yielded 7 mg of pure peptide using CH_3CN/H_2O/0.1%TFA as mobile phase. MALDI-TOF MS *m/z* calcd ($M+H^+$) 3653.9, obsd 3653.7 ($M+H^+$).

2.7.3 Native Chemical Ligation of GLP-1 Peptide and PEGylated DPPE:

H2N-Cys-PEG8-DPPE (5 mg) was suspended in 200 μL of 5% SDS in H_2O with sonication. To this suspension, 400 μL of 100 mM MOPS pH = 8.3 was added. Final pH of the lipid suspension was 7.9 and addition of lyophilized 1 mg of purified GLP-1 peptide thioester further decreases the pH of the solution to 7.7. The pH was not adjusted further. The reaction was initiated with 5 μL of benzylmercaptan and 5μL of thiophenol. Vigorous stirring was employed to get the mixture agitated. Progress of the ligation was monitored by taking samples every 1 hr for MALDI-TOF analysis. After the GLP-1 thioester peptide was consumed (24 h), reaction was quenched by the addition of 1 mL of 5% TFA. Excess thiophenol and benzylmercaptan were removed by repetitive Et_2O extractions. The aqueous layer is then lyophilized for purification. Purifications

were made either with preperative VydacC4 or analytical (Vydac 214TP54, 5μm) reverse phase column using IPA/water/ 0.1%TFA solvent system with a flow rate of 6.0 mL/min or 1.0 mL/min respectively. MALDI-TOF MS *m/z* calcd ($M+H^+$) 4603.9, obsd 4605.1 ($M+H^+$).

2.7.4 Synthesis of Other GLP-1 Analogues:

GLP-1 analogues with β-amino acids were synthesized by manual *t*-Boc SPPS on MBHA (0.2mmol/g) resin on a 0.9 mmol scale. The resin was split 3× before residue 28 for incorporation of β-Glu, β-Leu or β-HFL; and the synthesis was continued till the 30th residue. Cleavage of the peptide from resin was done with anhydrous HF, using p-cresol as scavenger.

The GLP-1βGlu analogue was recovered with the highest crude yield of 84%. Purification first with C18Semiprep (Vydac 218TP54, 5μm) yielded 6.3 mg of pure peptide. MALDI-TOF MS *m/z* calcd ($M+H^+$) 3312.7, obsd 3313.3 ($M+H^+$).

GLP-1βLeu analogue had a crude yield of 70%. Purification with first C18Prep, then with C18Semiprep (Vydac 218TP54, 5μm) yielded 3.2 mg of pure peptide. MALDI-TOF MS *m/z* calcd ($M+H^+$) 3298.0, obsd 3297.7 ($M+H^+$).

GLP-1βHFL had crude yield of 52% and was purified the same way to yield 2.1 mg of pure peptide. MALDI-TOF MS *m/z* calcd ($M+H^+$) 3406.5, obsd 3406.2 ($M+H^+$).

2.7.5 Biological Assays:

Luciferase Coupled cAMP Accumulation Assays:

Luciferase coupled cAMP accumulation assays were carried out by M.Beinborn, Tufts Medical Center.

HEK293 cells were transfected by hGLP-1R cDNA and the cAMP responsive luciferase reporter gene construct (CRE6x-luc) which contains a multimerized cAMP responsive element ligated upstream of the luciferase coding region. After 24 h post infection, cells were incubated with ligands in various concentrations at 37 °C, 6- or 18-hours. After receptor activation, cells were lysed and the luciferase activity was analyzed. Each experiment was done in triplicate.

Protease Stability Assays:

Degradation of peptides by DPP-IV was followed using RP–HPLC and individual stabilities were determined. Specific activity of DPP-IV enzyme ($\Delta\varepsilon_{410nm}$ = 8800 $M^{-1}cm^{-1}$) was assessed using chromogenic substrate *Gly-Pro-p-nitroanilide,* in 100 mM Tris·HCl, pH 8.0. Calibrated enzyme (20 U/L) was incubated separately with peptides (10 μM) in 50 mM Tris.HCl, 1 mM EDTA, pH 7.6, at 37 °C for 200 mins. Reactions were quenched with 600 μL of 0.2% TFA at 10 min. time intervals and stored at -20°C until injection. RP-C_{18} analytical column (Vydac 218TP54, 5μm, 250mm) was used for quantifying intact and digested peptides monitored at 230 nm using CH_3CN/H_2O/0.1%TFA as the mobile phase. First order rate constants were obtained as the fitted value ± standard deviation using equation: $\ln[A] = -k.t + \ln[A]_0$ where A is the

concentration of peptides; k the first order rate constant; t the reaction time in min; and $[A]_0$ the initial concentration of peptides.

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CHAPTER 3 SYNTHESIS OF L-β-HEXAFLUOROLEUCINE AND ITS USE IN PEPTIDE SYNTHESIS

The functions of proteins are dictated by their unique structures. Proteins adopt a bewildering array of conformations that render them active or inactive toward their targets. On/off mechanisms of ion channels and active/inactive forms of an enzyme are determined by the changes in overall structure. Folding of a polypeptide into secondary, tertiary and quaternary structure is crucial to find the delicate balance that endows their specific action. Protein folding has been an intriguing subject of research for many years however dynamics of the protein-folding is not entirely understood. Many models for elucidating the cascade of folding steps have somewhat explained folding of certain set of peptides; however, general rules for protein folding do not exist.

Among living things, most proteins are solely made of L-α-amino acids, while D-amino acids and amino acids with extended backbones (β-, γ- and etc., Figure1) are not utilized by the translational machinery. Many non-canonical amino acids exist in nature and there are organisms that utilize a broader palette of amino acids within their polypeptides.[1] These are generally bacteria, or other life forms, that have made use of unusual amino acids because of evolutionary pressures. β-amino acids are among the non-conventional amino acids that are often seen in microbial products, but are less common as organisms become more complex. Recent developments in β-amino acid synthesis and their utilization in peptide synthesis provide interesting opportunities for peptide and protein design.[2] This chapter focuses on β-amino acids, their characteristics and structures as

alternative peptide/protein building blocks; followed by the synthesis of a fluorinated β-amino acid and its use in peptide synthesis.

3.1 β-AMINO ACIDS:

3.1.1 Properties of β- amino acids:

β-amino acids differ from α-amino acids by their extended backbone by an extra methylene group. β-amino acids may contain side chains on either 2- or 3- (α- or β-) positions, or both. The increased combinations of substituents on the backbone allows for a greater variety β-building blocks.

The nomenclature of β-amino acids has been evolving. Nevertheless, in the literature three subclasses have been classified with simple names and also widely used for β-peptides: β^2, β^3, and $\beta^{2,3}$-amino acids.[3] The numbers indicate where the substituents are attached, and the stereochemistry is indicated following IUPAC rules. (Figure2)

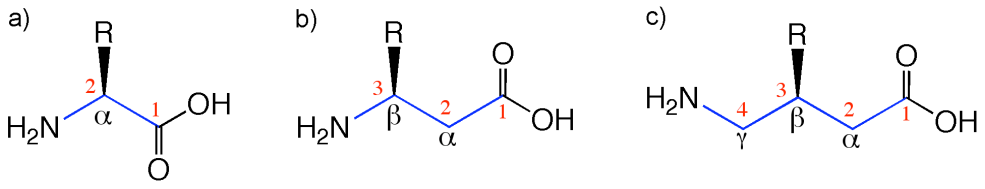


Figure 1. **Examples of a) An α-amino acid b) A β3-amino acid c) A γ3-amino acid. Sigma bonds of backbones are shown in blue.**

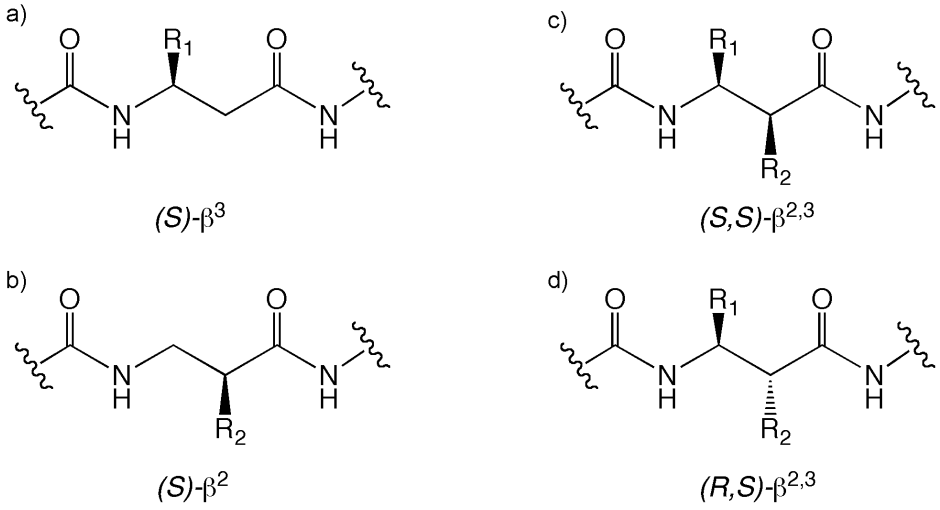


Figure 2. **β-aminoacid substitution patterns. Four more stereoisomers are possible with (R)- conformations. Only (S)-isomers that are relevant to peptide synthesis were shown.**

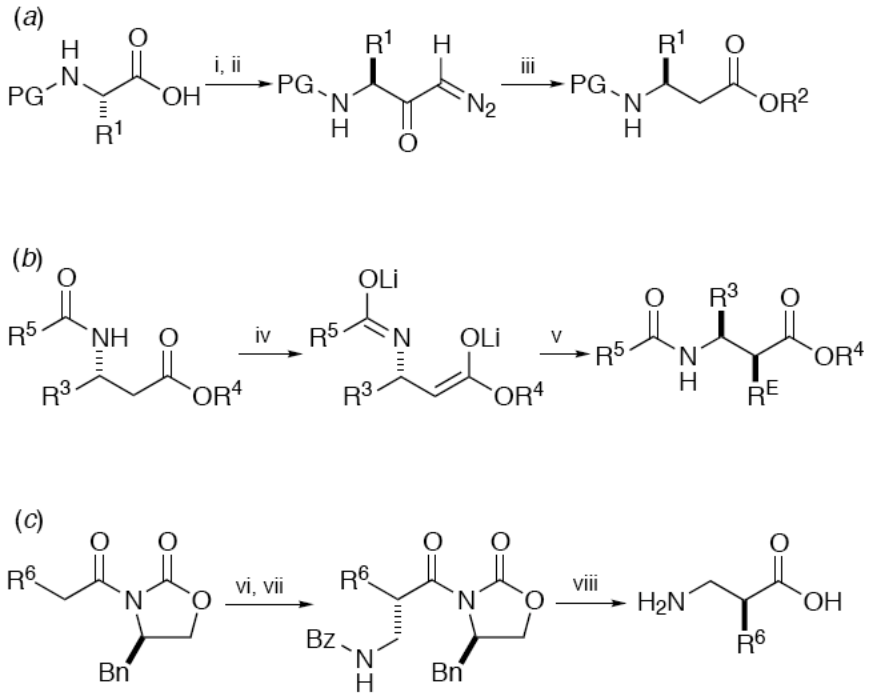
3.1.2 Synthesis of β-amino acids and Their Occurrence in Nature:

3.1.2.1 β^3-amino acids:

The first chemically synthesized β^3-amino acids were derived from their corresponding α-amino acids via Arndt-Eistert homologation,[4] where the substituents are on 3-position of the β-amino acid. This route provides access to the so-called β^3-amino acids.[5] (Scheme1a)

Occurrence of β^3-amino acids in Mammalian Metabolism:

Nature utilizes many β^3-amino acids, some of which are also present in mammalian metabolism. The most common one is β-Alanine (also named as β-hGly), which is one of the catabolites of nucleobase thymine. β-Alanine is not incorporated into proteins but rather circulates as carnosine; a dipeptide of β-Alanine and L-Histidine. Both carnosine and free β-Alanine play a role in neurotransmission. β-Leucine (or β^3-hVal), is a product of L-Valine metabolism and holds a key role as a precursor for its α-counterpart, L-Leucine. (Figure 3)



Scheme 1. **a) Synthesis of β^3-amino acids via Arndt-Eistert homologation, b) Synthesis of $\beta^{2,3}$-amino acids from β^3-amino acids, c) Synthesis of β^2-amino acids through Evan's chiral auxiliary method. Reagents and conditions: i, ClCO2Et, NEt3, THF; ii, CH2N2, Et2O; iii, Ag+, NEt3, R2OH; iv, LiNR2, LiCl; v, electrophile (REX); vi, TiCl4, NEt3; vii, BzNHCH2Cl; viii, hydrolysis. (PG denotes a protecting group of Z, Boc, or Fmoc) (reproduced with permission[5])**

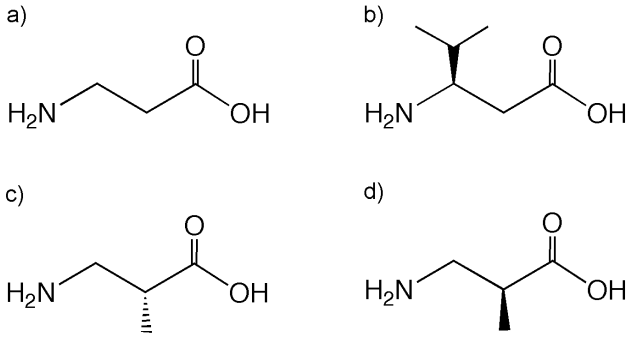


Figure 3. Depiction of a) β-Alanine (β-homoGlycine), b) β-Leucine (β3-homoValine), c) R-β-Aminoisobutyric acid (R-β^2-homoAlanine), d) S-β-Aminoisobutyric acid (S-β^2-homoAlanine).

3.1.2.2 β^2-amino acids:

Soon after the reported synthesis of β^3-amino acids, Seebach and co-workers reported syntheses of β^2-amino acids using an enantioselective Mannich reaction or a Curtius degradation through Evan's method. (Scheme1b) There are various examples of enantioselective preparation of certain β^2-amino acids in the literature in addition to these reactions.[6] However, there is no generalized strategy yet to obtain all proteinogenic side chains by a single approach. Methodologies developed for β^2amino acids have recently been discussed in an extensive review.[7,8]

Occurrence of β^2-amino acids in Mammalian Metabolism:

It is synthetically challenging to obtain β^2-amino acids since they cannot be easily homologated from naturally existing α-amino acids. Nature, on the other hand, produces a variety of monomeric β^2-amino acids. Mammalian metabolism includes only two such examples: R-β-Aminoisobutyric acid (R-β-AiB or R-β^2-hAla) and S-β-Aminoisobutyric acid (S-β-AiB or S-β^2-hAla). (Figure 3)

R-β-Aminoisobutyrate is a common β-amino acid in mammals and is produced via uracil catabolism. Abundance depends on the pyrimidine catabolism-rate of the body.[6] Accordingly, in certain neoplastic states, excretion of (R)-β-AiB increases, which makes this molecule a diagnostic tool for some cancer types. Its isomer, (S)-β-Aminoisobutyrate is a product of L-Valine

decomposition that has a much lower plasma concentration (1-4μM) compared to the (R)-isomer, and it is not used as a determinant of any metabolic state.

Similar to β-Alanine and β-Leucine, none of the β^2-amino acids are found in mammalian proteins. In organisms that make use of β-amino acids, incorporation of β^2-amino acids into natural products is also less common than their β^3-counterparts. β^2 amino acids that are found in nature are generally isolated in monomeric form rather than being included in a complex product.[6]

3.1.2.3 $\beta^{2,3}$-amino acids:

The first examples of $\beta^{2,3}$-substituted amino acids that have been used in β-peptide synthesis are Gellman's cyclopentyl and cyclohexyl containing β-amino acids. In these amino acids, C2 and C3 carbons are included in five or six membered ring structures that result in conformationally fixed dihedral angles. (Figure 8) Enantiomerically pure synthesis of two such derivatives, trans-2-aminocyclopentane carboxylic acid and trans-2-aminocyclohexane carboxylic acid, have been reported by Gellman and co-workers.[9] [10] Homopeptides of these cyclic-$\beta^{2,3}$-amino acids were shown to adopt stable secondary structures that can be controlled by the size of the cyclic ring on the side chain.[10] Later, Seebach and co-workers reported the synthesis of acyclic $\beta^{2,3}$-amino acids where they installed acyl side chains at the C2 position of the homologated β^3-amino acids. These types of amino acids can also be prepared by Davies' method from enolate esters.[11] (Scheme1c)

3.1.3 Structural Features of β-amino acids :

Due to the extra methylene group in the backbone of β-amino acids, they are more flexible than their α-analouges, hence it is reasonable to expect that the β-peptides would be less likely to have defined secondary structures. Astonishingly, the very first study of Seebach and co-workers showed six to seven residue-long β^3-peptides could form helical structures in organic solvents.[12] In addition to their structural features, those peptides were stable at higher temperatures. Such short sequences of polypeptides with high structural stability cannot be achieved with α-amino acid monomers. This work had been the milestone for many structures later discovered containing β-amino acids.[13,14,15] Today, the field has unique examples of β-proteins that are delicately designed and have shown to have excellent stabilities.[9,16,17] It would not be unreasonable to expect the disclosure of a *de novo* system based on β-proteins that have catalytic function similar to naturally occurring α-counterparts.

Understanding and designing systems for β-peptides require a chemical level investigation of β-backbone. How is it that these molecules that are thought to be even more flexible than the α-amino acids can fold into even sturdier constructs?

An α-amino acid is also an inherently flexible molecule with two σ-bonds that can freely rotate; yet many polypeptides that are made of α-amino acids fold into compact structures that are thermodynamically and kinetically stable. β-amino acids consists of three σ-bonds that further increase the conformational flexibility of the molecule (Figure1 in blue). The only restriction of rotation in

either case is around the amide bonds that are almost planar due their partial double bond character. In solution, the mobile backbone of a β-amino acid is expected to provide more rotational freedom and disturb any secondary structure. Before Seebach's experimental work, Dado and Gellman had argued against this view. They speculated that "α-peptides were able to adopt helical structures because of their incapacity to make short-range hydrogen bonds in solution".[18] Among the molecules they analyzed were monomers and dimers of α-amino acids, as well as single or dimerized β- and γ-amino acids for investigating the hydrogen-bonding capacity of extended amino acid backbones at short range. Among all the non-covalent forces that hold together a protein backbone in a folded structure, hydrogen bonds seem to be the major contributor to many well-known secondary structures such as α-helices and β-turns. Peptides are made of continuous amide linkages, which contain both hydrogen-bond acceptors (C=O) and donors (N-H) on the same functional group. In α-peptides, spacing between each amide group is too short to have any favorable hydrogen bonding interactions because a hydrogen bond is strongest when the angle between the donor and the acceptor is linear or close to linear. The hypothetical five-membered ring that could form via hydrogen-bonding between consecutive amides would have a constrained angle (<100°). (Figure 4) On the other hand, hydrogen bonding between the one or two residue distant neighbor is viable, since the angles approach a linear arrangement (Figure 4).

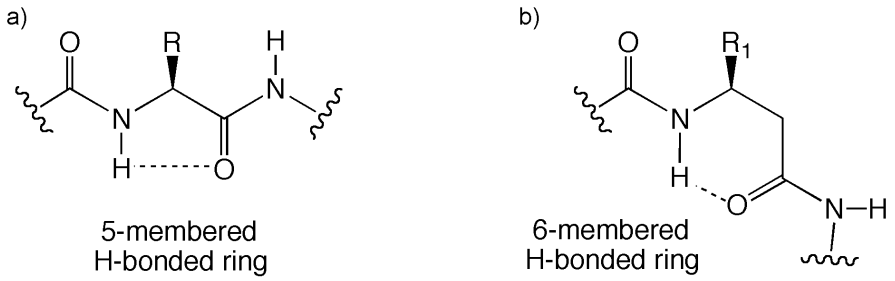
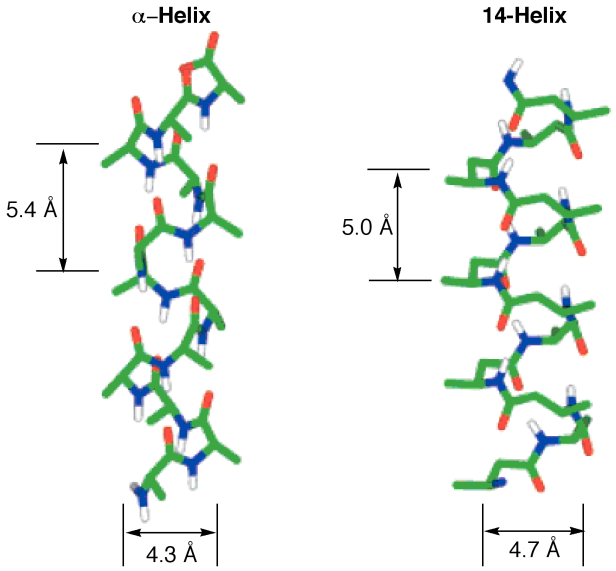


Figure 4. Hypothetical hydrogen bonds between the nearest neighbor amide groups in a) α-amino acid polymers, b) β^3-amino acid polymers.

α-Peptides commonly form 10- and 13-membered rings that consists of two and three residues respectively.[19] β-turns and 3-10 helices are made up of 10-membered hydrogen bonding chains. The most common secondary structure in proteins, the α-helix, is a H-bonded, 13-membered ring (Figure 5). The inability of the amide groups to interact with their nearest amides leads to construction of long-range and repetitive H-bonds that confer specific folds. Would extended backbones of β-aminoacids change this pattern due to their spatial freedom and make hydrogen bonds that the α-backbone cannot? Studies on β-dipeptides revealed that H-bonding interactions between adjacent amides is unfavorable in the β-dimer as well, in which case the authors suggested that it is conceivable for β-peptides to obtain a long-range, intramolecular H-bonded secondary structures. At the time of this study, a 14-helix structure (Figure 5) of β-peptides had not yet been described; but the prediction of the authors was borne out. They emphasized the importance of hydrogen bonding patterns of the amide groups on the secondary structure, regardless of the flexibility around σ-bonds.



residues per turn	3.6	3
rise per residue (Å)	1.5	1.6
size of hydrogen bonded ring	13	14

Figure 5. Comparison of the characteristics of the α-helix to a β-helix (14-helix). Backbone is shown in green, backbone carbonyls are shown in red, and hydrogen bonding amide hydrgens are shown in white. (reproduced with permission[5])

Another supportive argument for β-peptides to adopt defined structures can be made when L-Asparagine's conformational behavior is considered. In addition to its α-carbonyl group, L-Asparagine (Asn) contains a β-amide carbonyl that can hydrogen bond to other H-bond acceptors within a polypeptide/protein. (Figure 6) When α-Asn is incorporated at the N-terminus of an α-helix, it forms the "Asx N-Cap" motif (Asx= Asn or L-Aspartic acid) where the β-carbonyl group can interact with amide hydrogens of the preceding amino acids in the peptide (Wan-Milner-White1999JMolBiol).[20] The side chain of Asn mimics the main chain hydrogen bonding, and brings the helix to termination.[21] Although the α-carbonyl should equally form these hydrogen bonding interactions and provide helix-continuation, the N-cap motif is favored by 1.0-2.0kcal/mol.[20] [21] So, even though β-carbonyl H-bonding (i.e., N-cap conformation) seems entropically unfavorable because it requires fixing three σ bonds fixed at predetermined dihedral angles, it is energetically preferred over α-hydrogen bonding where only two σ-bonds would be restricted. These observations raise the expectation for pre-organization of β-backbones into defined secondary structures via hydrogen bonding interactions.[22]

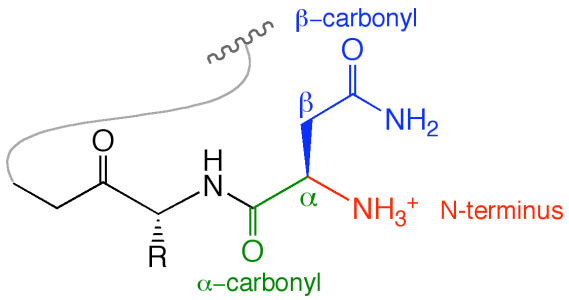


Figure 6. **L-Asparagine residue at the N-terminus (shown in red) of a peptide chain. Theoretically, both α-carbonyl (shown in green) and side chain carbonyl (or β-carbonyl, shown in blue) have an equal chance to hydrogen bond with the peptide residues. These interactions are mutually exclusive.**

Following these initial studies, both experimental and computational research expanded our understanding of β-peptide structures and has led to discovery of numerous secondary structures that β-amino acids–derived peptides can achieve.[23,24] Highlighted below are some representative structures of β-peptides and principles behind their design.[25]

3.2. β-PEPTIDES AND THEIR PROPERTIES:

3.2.1 Structural Features of β-peptides:

3.2.1.1 The 14-Helix:

3.2.1.1.1 Evolution of the 14-Helix into a Water-Soluble Scaffold:

A 14-helix is formed by contiguous H-bonds between amide groups of the i^{th} residue and the carbonyl groups of the $(i+2)^{th}$ residue (Figure 7).[26] β^3-peptides, β^2-peptides and $\beta^{2,3}$-peptides all adopt 14-helices when the residues are placed accordingly.[5,27,28,29,30] Peptides made only of β^3-amino acids are conformationally more stable than the ones made only of β^2-amino acids. In either case six to seven residue-long chains were sufficient to obtain a stable structure in MeOH. In addition, Gellman and co-workers used a modified cyclohexyl side chain, trans-2-aminocyclohexanecarboxylic acid (ACHC), within β-amino acid backbones to create homo-oligomers that can adopt a single conformer with high stability.[31 32] Due to restricted tortional angles of the α and β carbons, the polypeptide backbone is forced into a 14-helix conformer and could attain a secondary structure with as few as four residues. Although structural stability was superior and the number of residues was small in comparison to Seebach's peptides, cyclic sidechains are non-proteinogenic and may, therefore, have limited use beyond structural models.[9,33] Another limitation of these early discovered structures of β-peptides is their limited water solubility either with proteinogenic or with unnatural side chains. Characterization via NMR and CD were performed in methanol or other organic solvents that may not be relevant in a biological context. In order to overcome this problem, the Gellman group synthesized

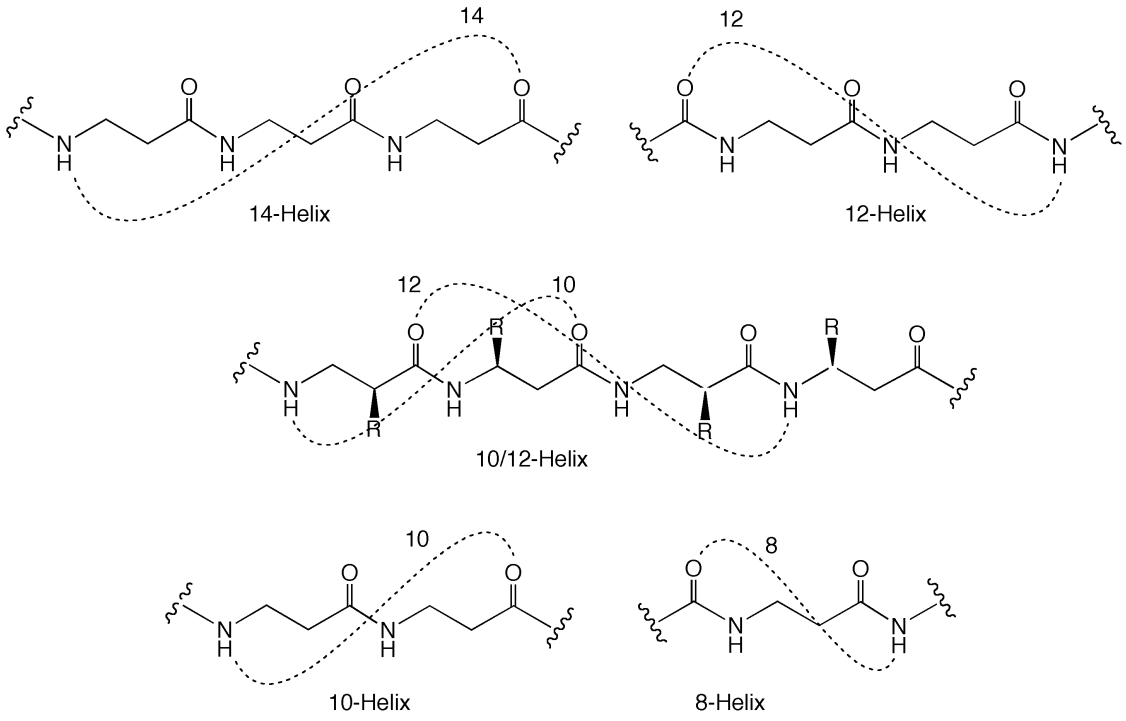


Figure 7. Illustration of several structures of β-peptides adopt. Each structure is named by the number of atoms in the hydrogen-bonded ring. Arrows show the H-bonding donor and the acceptor.

diaminocyclohexanecarboxylic acid (DCHC) as a hydrophilic equivalent of the ACHC side chain (Figure 8).[34,31,35] The resulting six-residue DCHC peptide is the first water soluble 14-helix that has been characterized by high resolution X-ray diffraction method. Following Gellman's work, the Seebach and DeGrado groups published consecutive studies using proteinogenic side chains. They used intramolecular salt bridges to stabilize the 14-helix.[28,36] Placement of positively and negatively charged residues on two out of three faces produced hydrophilic, stable helices formed in both water and methanol. An improved design came from the Schepartz group; they used macrodipole-stabilizing residues along with the intramolecular salt bridges.[37] Similar to an α-helical peptide, a 14-helix also bears a macrodipole due to uni-directional positioning of hydrogen bonds along the helix. The macrodipole of the 14-helix places a partial positive charge on the C-terminus and a partial negative charge on the N-terminus of the helix. (Figure 9a) This is contrary to an α-helix that bears a negative charge on the C-terminus and a positive charge on the N-terminus. Stabilization of α-helices is reinforced by capping the termini by placing positively charged residues on the C-terminus and negatively charged residues on the N-terminus. Using these design principles, a water-soluble peptide was synthesized with free carboxylic and amine ends in order to stabilize the macrodipole of the 14-helix.[38] Moreover, using hydrophilic and charged residues only on one side of the helix was enough to obtain a water-soluble version of the 14-helix with excellent stability.[37] While the second side of the helix contained hydrophobic residues for structural integrity, the remaining face was free to accept possible modifications (Figure 8b).

A modifiable surface on a newly discovered 14-helix has been an advantage when searching for relationships between different side chains[39] and 14-helix stability in water.[40,41] Modification of those side chains to mimic protein-protein interactions allowed exploration of possible higher oligomerization states.[42,43] A detailed investigation of the 14-helix uncovered principles of β-peptide design and elucidated the differences from the conventional α-peptides.[44,45] A comparison of the 14-helix and the α-helix are shown in Figure 5.

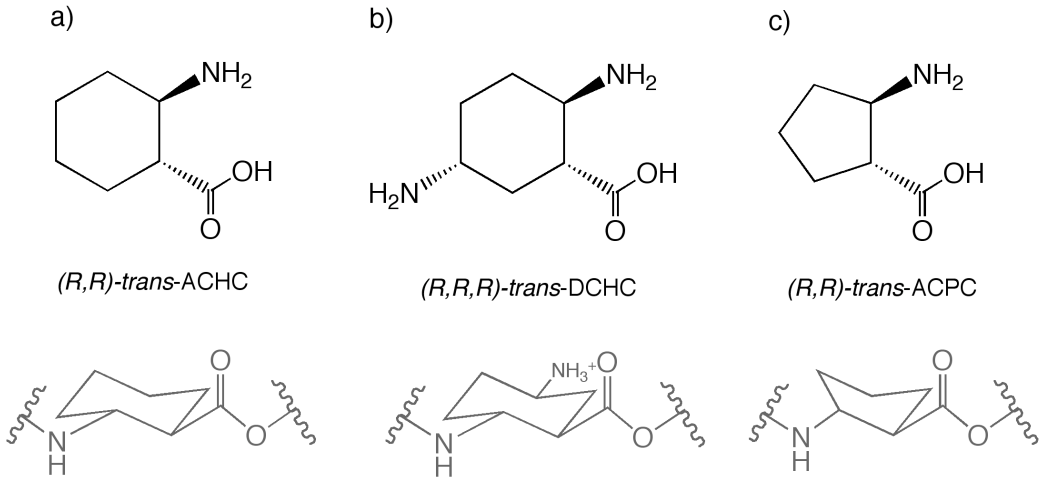


Figure 8. **Torsionally restricted cyclic β-amino acids a) (R,R)-trans-2-aminocyclohexanecarboxylic acid (ACHC), b) (R,R,R)-trans-2-diaminocyclohexanecarboxylic acid (DCHC), c) trans-2-aminocyclopentane carboxylic acid (ACPC). Proposed conformations of the cyclic side chains within a polypeptide chain is shown in gray.**

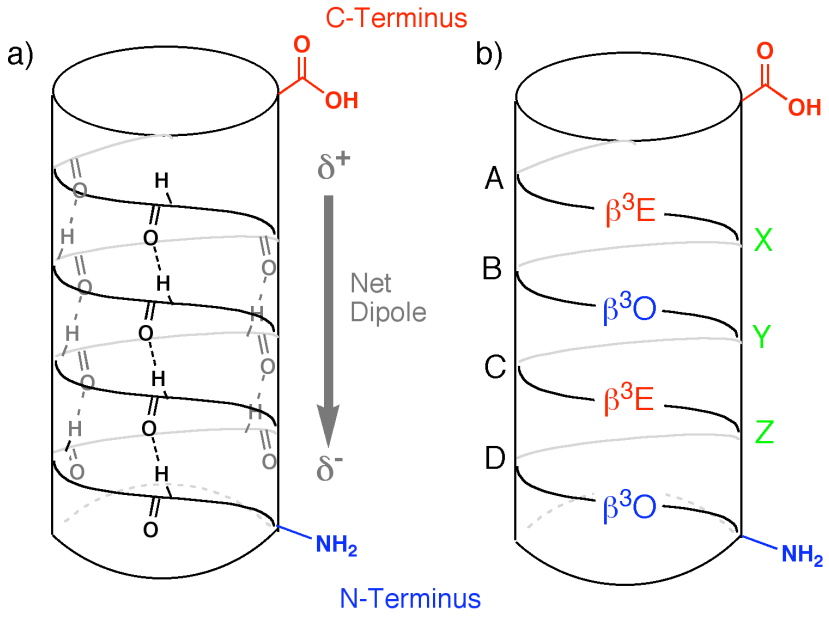


Figure 9. a) Hydrogen bonding pattern on a 14-helix and the direction of the macrodipole b) Microdipole-stabilized 14-helix. X, Y and Z are replacable with any residue, where A, B, C and D are hydrophobic residues. β^3E is β^3-Glutamic acid and β^3O is β^3-Ornithine.

3.2.1.2 The 12-Helix:

A 12-helix that hydrogen bonds between the carbonyl group of i^{th} residue and the amide group of the $(i+2)^{th}$ residue is sterically unfavorable. (Figure 10) The Gellman group used trans-2-aminocyclopentane carboxylic acid (ACPC) (Figure 9) homopeptide to prepare a stable 12-helix. This construct has a dihedral angle between the β2 and β3 substituents fixed at 30º. The same 12-helix structure was also observed in a trans-3-amino-pyrrolicine-4-carboxylic acid (APC)-bearing peptide, which also contains 5-membered cyclic residues that restrict the torsional freedom and provided water-solubility to the construct. [46] Recently, the Gellman group engineered similar structures by inserting α-amino acids into β-peptide chains without compromising the desired 12-helix structure. [10,31,47,48]

3.2.1.3 The 10/12-Helix:

An alternating pattern of hydrogen bonded rings that contain ten- and twelve- members have also been reported by the Seebach group.[33,49] A peptide consisting of mixed monomers of β^2 and β^3 amino acids (i.e, β^2/β^3-peptides) of the same configuration adopts 10/12-helix structure. Although this type of β-conformer has not found as much application as the 14-helices, structural features are interesting and worth discussing. Different from 12- and 14-helices, which have all the carbonyl groups oriented in the same direction along the helix, the 10/12-helical structure consists of carbonyl groups that are alternating in opposite directions (Figure 10). The resulting 10/12 helix, therefore, possesses a far smaller

dipole moment. Non-polar properties of the 10/12 helix stem from the absent microdipole, and make it possible to dissolve these peptides in organic solvents such as methylene chloride and ethyl acetate. This is especially helpful in preparation of these peptides using fragment coupling in solution.[6] β^2/β^3-peptides with terminal protections are more stable 10/12 helices than the ones without. When the 12-membered ring structure of the 10/12 helix is compared to that of 14-membered ring in the 14-helix, the hydrogen bonding distance is smaller and the angle between carbonyl oxygen and the amide hydrogen (O---H(N)) is larger. Although the 10-membered ring possesses a smaller dihedral angle than a 14-helix, in overall structure of the 10/12 helix, the hydrogen bonding pattern seems to be stronger than a 14-helix as suggested by theoretical models and MD simulations. [26]

Segments of shorter β^2/β^3-peptides that are two to four residues long can also adopt turn structures which can accurately mimic those of α-peptidic turns, specifically Zn fingers.[50]

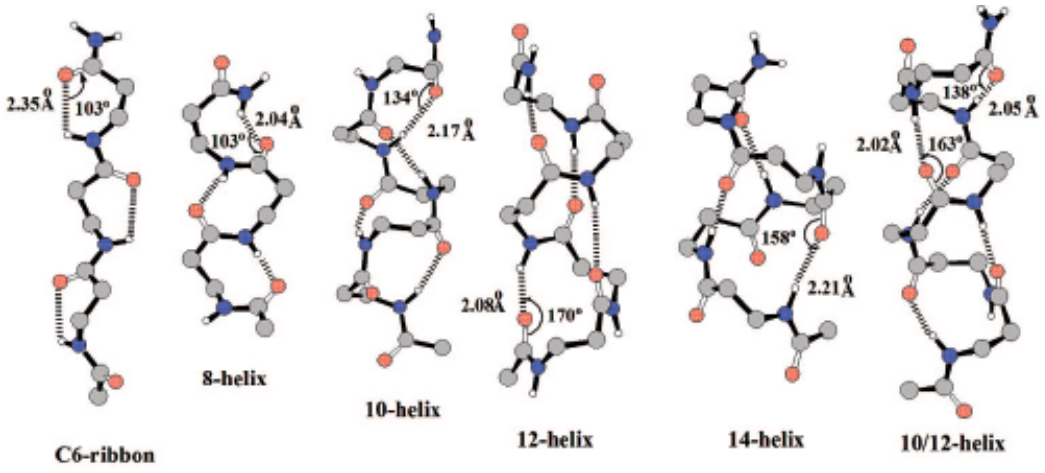


Figure 10. **Hydrogen bonded diagrams of different β- secondary structures. (reproduced with permission[26])**

3.2.1.4 The 10-Helix, the 8-Helix and the 6-Helix:

There are additional structures that have been either suggested by theoretical models or discovered experimentally by use of rigid β-building blocks.

A 10-helix is observed with restrained ring structures on the side chains of β-monomers, where the dihedral angle is smaller than 60°.[135] Angles smaller than 60^{o} and resulting structures can only be attained with four-membered cyclic side chain. Claridge et al. have synthesized β-peptides bearing oxetane side chains and observed 10-membered structure in organic solvents by NMR.

Only theoretical studies have suggested the possibility of an 8-helix, where a long polypeptide of β-amino acids with a cyclopropane ring was proposed to form 8-membered hydrogen-bonded rings.[27] The Proposed structure spans just two residues per turn and requires a non-linear hydrogen bonding pattern.

The 6-Helix (or a C_6-ribbon) is an experimentally non-viable structure where the hydrogen bonding is supposed to occur between the amide and the carbonyl groups of the same β-amino acid.[26] Formation of the six-membered ring is highly unfavorable due to the reasons discussed in section 2.1 of this chapter.

3.2.1.5 Sheet-like Conformations of β-amino acids:

Just as α-pepides adopt "β-sheet" structures in solution, β-peptides do the same. It was hypothesized that they can adopt two distinct sheet structures.[51] The first is a β-peptide chain that adopts an anti conformation around the C2-C3 bond. In this case, backbone carbonyls orient in the same direction resulting in a net dipole of the sheet structure. (Figure11a) In a second structural motif, the C2-C3 torsion angle is gauche; so every other carbonyl unit is therefore oppositely

directed along the backbone, neutralizing the net dipole. (Figure11b) The Seebach[52] and Gellman[53] groups have designed and characterized a series of sheet-like structures and these have been reviewed recently.[45]

3.2.1.6 Substituent Effects on β-peptide Structure:

In addition to the substitution position of the side chains on either the α- or β- carbon of the β-amino acids, the nature of the substituents also affects secondary structure.[54] Inter-residue electrostatic or steric interactions have an impact on the conformational preference of the peptide.[39] For instance, hydrophobic or salt bridge interactions between i^{th} and $(i+3)^{th}$ positions of a 14 helix are important for the stability of the secondary structure.[55] On the other hand, geminal disubstitutions in β-peptides completely destroys the helical structure.[5] Interplay between the side chain of a single residue on a dodecapeptide can be critical to the oligomerization state in the quaternary structure.[56,57] For stabilization of the 14-helix structure, β-branched β-amino acids such as β3-Val and β3-Leu were superior compared to other hydrophobic residues.[58] In Figure 8, a summary of structures that can be obtained by certain conformers/sidechains of β-amino acids is shown.

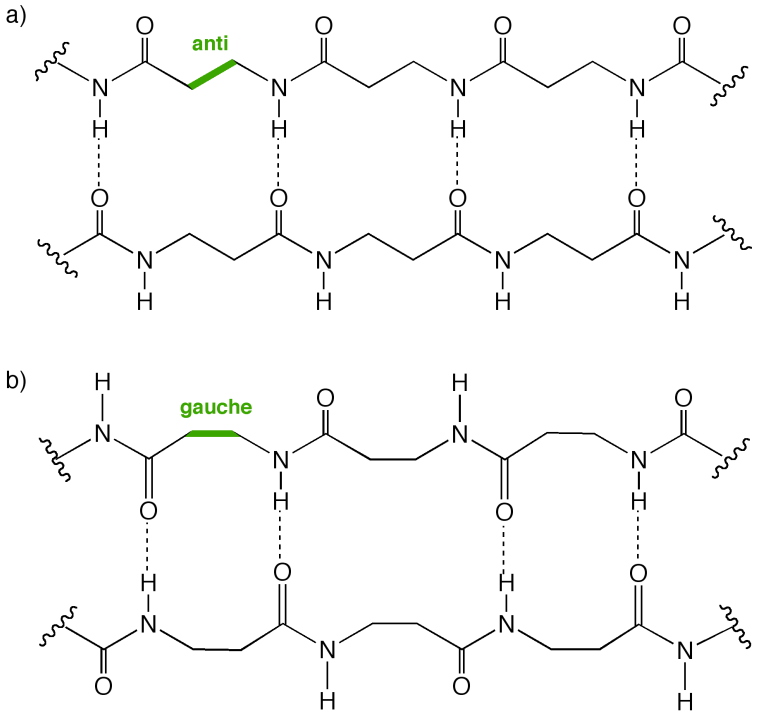


Figure 11. **Sheet-like structures of β-peptides with their monomers either in a) anti or b) gauche conformation.**

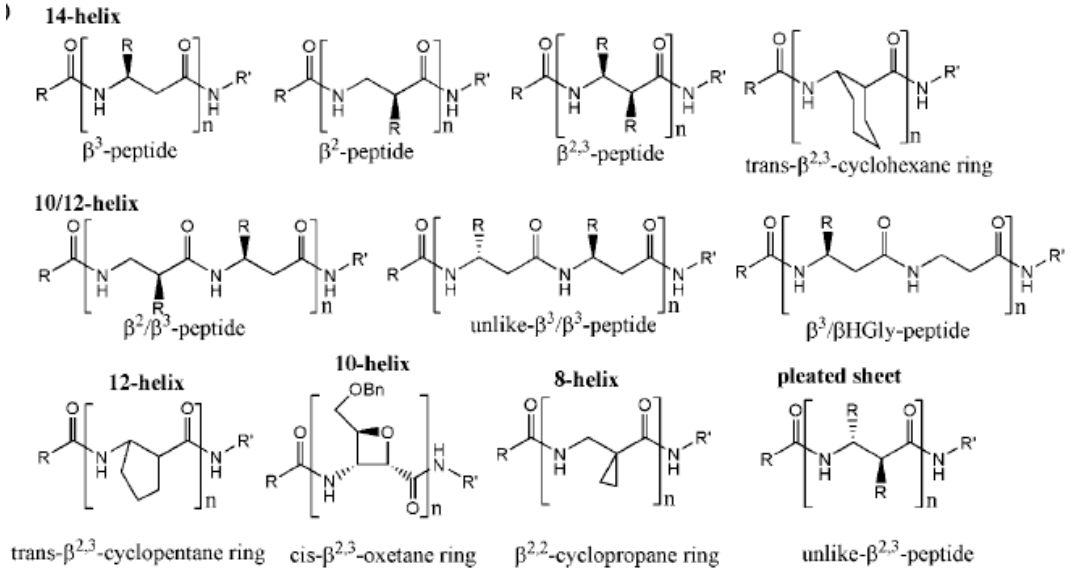


Figure 12. Structures of β-peptides correlated with the choice of the monomeric β-amino acids. (reproduced with permission[26])

Properties of side chains, substitution patterns and their effect on the overall structure of β-peptides have been investigated both experimentally and by computational methods.[42,44,26] Outcomes of these studies have been utilized in designing well-defined polymers. β-amino acids have been incorporated into many physiological peptides and/or made into peptide mimics since the secondary structures of β-peptides can be reasonably predicted and manipulated.[59,60]

3.2.1.7 Length Requirements:

The minimum length requirement for a stable secondary structure in α-peptides is about 15 residues in mixed organic solvents and 20 residues in aqueous solutions. β-peptides, however, can form stable structures with as few as six to seven residues. If non-proteinogenic side chains are used, this number drops to four residues - the minimum number required for one full turn of a 14-helix. Furthermore, certain β-peptides with proteinogenic side chains preserve their secondary structures at high temperatures, as assessed by NMR studies.[61] In contrast, α-peptides exhibit thermal denaturation at lower temperatures than their β-counterparts. A smaller number of residues in a stable structure becomes advantageous when therapeutic options are sought, as lower molecular weight molecules generally possess superior phamacokinetics.

3.2.1.8 Protease Resistance:

β-peptides, even with proteinogenic side chains, do not serve as substrates of any extant enzymes.[62] α-Proteins do not seem to recognize any of the β-peptides unless the β-peptide is deliberately and specifically designed as a substrate. Many degradation studies with known proteases and metabolic enzymes proved that β-backbones are resilient against enzyme digestion and therefore hold promise for designing peptide mimics that are stable *in vivo*. Examples of proteolytically stable antibiotics are discussed below.

3.2.2 Peptidomimetic β-Peptides

Soon after the discovery of stable β-peptides, Seebach and coworkers designed a six-residue β-peptide to mimic a MHC-binding peptide. This designer β-peptide successfully inhibited the natural killer cell toxicity to the same extent as a 9-residue α-peptide that it was designed to mimic.[63] In this study, specific side chains from the α-peptide were installed on an unstructured β-peptide in order to recapture the 3-D display of functional groups.

Mimicking a conventional α -helix with β-peptide helices has also been successfully demonstrated. Inhibition of the lipid transport protein SR-B1 was accomplished by creating an amphiphatic helical structure with a β-helix that resembles natural ligands.[64] Homology between the natural peptide and a designed β-peptide was sufficient to create an efficient inhibitor. Similarly, appending an amphiphatic α/β-peptide in place of the C-terminal helix of

interleukin-8 (IL-8) had comparable affinity towards its receptor as the original IL-8.[59]

A more specific peptidic design was prepared for inhibition of oncoprotein hDM2.[65] Protein-protein interactions between hDM2 and its partner p53 strictly depend on the correct arrangement of side chain residues. Positioning of binding site residues in p53 on a 14-helix with the correct spatial alignment created a small β-peptide inhibitor (β53-1) with high affinity (368 $\pm$ 76 nM to 583 $\pm$ 88 nM) and specificity towards hDM2.[42] An analogous α-peptide inhibitor[66] derived from p53 binding site binds only 1.6 to 2.5-fold stronger (233 $\pm$ 32 nM) to the target, and can readily be displaced by β53-1 with an IC_{50} value of 94.5 $\pm$ 4.4 μM. This is the first work that stable tertiary structure of a β-peptide had been utilized successfully for interfering with protein-protein interactions, and for specific recognition of the protein target.[43,67] Later, β53-1 was further engineered[68] to acquire stronger binding affinity to hDM2[69] and to have better cell-penetration properties.[70,71]

Inhibition of HIVgp41 fusion protein has been pursued in a similar fashion. Taking advantage of the α-helical region within the hydrophobic pocket of gp41 protein, several β-peptide epitopes were designed to display the required residues.[72,73] The resulting β-helices prevented oligomerization of the gp41 proteins with each other, displaying IC_{50} values in the μM range that are much less potent than the peptide drug Fuseon® (IC_{50} = 0.1 nM). The redeeming quality of the β-peptide inhibitors is superior protease stability.

The Gellman group has designed a series of 12-helix structures to tackle human cytomegalovirus (HCMV) entry to mammalian cells. β-peptides made of 13-residues successfully imitated the glycoprotein pattern of α-helical gβ proteins on HCMV and inhibited virus-cell membrane assembly.[74]

Mixtures of β- and α/β-peptides have been systematically investigated for the inhibition of the anti-apoptotic protein Bcl-x_L.[75,76,77] Scanning experiments of the peptide library led to an optimal chimeric construct for binding Bcl-x_L homology domain. Not only was this peptide able to prevent BH3 binding to its ligand, but was also specific to its target among the other Bcl family members.[78]

Short β^2/β^3-peptides containing four residues have been tested as mimics of the somatostatin hormone that consists of a turn structure in its recognition motif.[79,80] The interaction domain of the designed β^2/β^3-peptide was a one-to-one match to the natural somatostatin hairpin region and displayed powerful agonism toward the somatostatin receptor[81]. Studies in rats also demonstrated oral bioavailability; metabolic stability; and slow, but complete, excretion.

Cyclic β-peptides were synthesized and shown to stack efficiently on top of each other forming tube-like tertiary structures.[82,83] One such cyclic β-tripeptide was shown to possess antiproliferative activity *in vitro* in a range of human cancers.[84]

Successful DNA and RNA mimetics have also been created with β-peptides and reviewed elsewhere.[59,85,86]

3.2.3 Antimicrobial β-Peptides:

De novo design of the first anti-microbial β-peptides were reported by DeGrado et al.[87] A series of peptides have been synthesized in varying lengths from 6 to 18 residues and tested for bactericidal activity. However these peptides were also highly hemolytic due to their enhanced hydrophobicity. Similar to antimicrobial α-peptides, β-peptides also need to be designed in a way to achieve balance between hydrophobic and hydrophilic residues.[10] Several groups have published work geared towards decreasing the non-polar residues on β-peptides.[88] In several cases, this resulted in improved selectivity for *E. coli* relative to erythrocytes.[89,90]

β^2/β^3 peptides, as well as α/β peptides, were also shown to have antibiotic properties against a panel of bacteria and showed minimal toxicity to red blood cells.[91]

Gellman and co-workers designed a 17-mer β-peptide that is effective on two drug-resistant bacteria and is completely non-hemolytic.[92] Further studies created analogues of mixed α/β-peptides with the same enhanced properties. Interactions of these peptides with the lipid membranes were investigated in detail and led to design of bacteria-specific antibiotics.[93]

Interestingly, recent studies on antimicrobial α/β peptides revealed that a stable secondary structure may not be necessary for the activity in all cases. Regardless of the sequence or the random fold of the α/β-peptide, constructs seem to behave as well as the well-folded antimicrobials. It is contrary to what has been thought as the primary component of antibiotic peptide design so far. Although

potencies of α and β-peptides corrolate well with conformational stability[94], α/β peptides have been shown to display a different behavior within the context of antimicrobial peptide design.[95,96]

3.3 Design of a β-Protein

3.3.1 Design Principles and Advances:

As the field has grown and more is understood about the principles of β-peptide folding, a breakthrough design came from the Schepartz lab, where they designed and synthesized a bundle of β-peptides made out of twelve residues that can homo-oligomerize in water.[97] Preceding work reported a β-peptide pair that cooperatively folds in an equimolar ratio into a quaternary structure.[98] In the light of the previous study, dodecameric peptides have been prepared with helix-stabilizing salt-bridges on one face, at positions 3, 6, 9 and 12. (Figure 13) In order to assure the interhelical, hydrophobic interactions, leucine residues at positions 2, 5, 8, and 11 formed a leucine hydrophobic face and the two phenylalanine side chains on positions 4 and 7 constituted an aromatic face. Positions 1 and 10 were replaced with a complementary charge pair in order to maintain homo-oligomerization. CD experiments revealed that the new oligomer underwent a concentration-dependent transition in solution from being unstructured at lower micromolar concentrations to having strong helical features at higher concentrations (a few hundred micromolar). Sedimentation equilibrium experiments also suggested that a defined stoichiometry was present in the final structure, where eight peptides are involved in a quaternary fold. X-ray crystallography finally proved that an octameric bundle was indeed the only species formed by this zwitterionic β-peptide. Assembly of the peptides was unique: Each peptide formed a parallel dimer with another, and the resulting dimers associated with each other in an anti-parallel fashion to form a slightly

curved, but sheet-like tetrameric bundle. Tetramers then associated in a perpendicular arrangement. (Figure 13) β-Homoleucine residues were in the middle of this structure, forming a relatively large hydrophobic core that is solvent excluded. Examination of crystal structure reveals details of the assembly; the parallel dimers are held such that the aromatic face of one helix stacks up against the salt bridge face of a second. There are two different interactions that hold the two helices in this conformation. As the primary force, Glu1 and Orn10 of the aromatic face interact with Orn3 and Glu12 of the salt-bridge face respectively. Meanwhile aromatic groups seem to nest within the side chain hydrocarbons of the Asp6 and Orn9 on the apposing helix. Each parallel dimer still displays a free salt bridge face that can interact with another the salt bridge face of another dimer when aggregating in an antiparallel fasion. This forms strong electrostatic interactions that hold together the tetramer. Both in parallel and antiparallel conformations, leucine side chains are always packed next to each other. Finally, when two tetramers interact, all thirty-two leucine side chains are buried in the middle of the structure and create a hydrophobic core with a total buried area of 2386 $Å^2$ that is comparable to small globular proteins.[99]

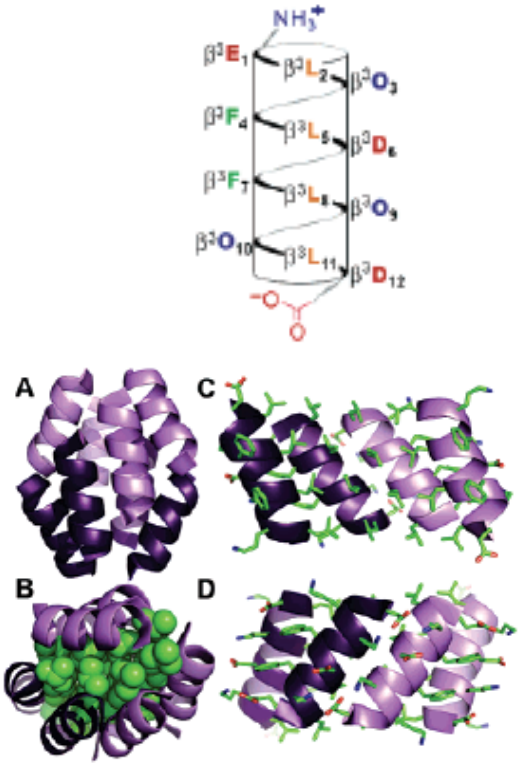


Figure 13. Design of an oligomeric β-protein: a) primary structure of each peptide b) crystal structure of the octameric bundle (reproduced with permission[97])

3.3.2 Engineering the Miniature β-Protein:

A modified version of the first octameric bundle provided a more elaborate picture of the intramolecular interactions of the structure.[100 101] In place of the phenylalanine residues in the zwitterionic dodecapeptide, tyrosine residues were used at positions 4 and 7. Positions 1 and 10 were both replaced with glutamic acid.[97] The overall structure was similar to the parent octameric bundle, with leucines packed in the core and excluded from water. A comparison of calorimetric measurements revealed that the newly designed bundle with Tyr residues on the aromatic face was thermodynamically and kinetically more stable than parent peptide in terms of its folding. The authors suggest that this may be due to hydrogen bonding that may occur between hydroxyl groups of the Tyr residue and the carbonyl group of Glu10, which is the nearest possible H-bond acceptor. Although the distance between these groups is quite long (~4 Å) for a hydrogen bond to form, crystallographic data suggests that a water network within the 4 Å distance is not only helping with the hydrogen bond formation but also contributing to solvation of the entire assembly. The previously used phenylalanine residue lacks the capacity to form a hydrogen bonded water network and it exposes the hydrophobic benzyl side chain to water, which further destabilizes the bundle.

The Schepartz group also showed that oligomerization states of the β-peptides can be controlled via side chain modifications.[56] Replacement of some of the leucine residues of the octamer with valine led to tetrameric bundles. It is remarkable that not only β-peptides were able to accommodate the changes in the

core without compromising the structure, but also were able to form discrete assemblies.

3.3.3 A Fluorous β-Protein

3.2.4 Properties of Organic Fluorine:

The carbon-bound fluorine has unique features since it is usually hydrophobic yet possesses a very high dipole moment due to its electronegativity. The strong electron withdrawing ability of fluorine makes the C-F chemical bond rather short (1.3-1.4Å)[102], and strong (105.4 kcal mol^{-1}).[103] The electron density of the C-F pair is mostly concentrated on fluorine; but at the same time, these electrons are tightly held around the nucleus of fluorine. As a consequence, organic fluorine cannot participate in hydrogen bonding even though the C-F bond is polarized ($C^{\delta+}$-$F^{\delta-}$).[104] [105] Overall polarizability of the diatomic unit (C-F) by an external hydrogen donating system is low, which should not be confused with the inherent dipole moment. This dual chemistry gives organic fluorine its "polar hydrophobic" character,[106] effects of which have also been investigated in peptide/protein design and engineering.[107,108]

3.2.5 Fluorine in Peptide Design and Synthesis:

In several initial studies, decoration of α-peptide surfaces with fluorinated residues has been shown to improve thermodynamic and chemical stability of the peptide assemblies compared to their hydrocarbon equivalents.[109,110,111,112] The stabilizing effect of fluorine due to its hydrophobic character was confirmed and the concept was applied to synthesis of "self-segregating" peptides in an aqueous environment[111,113] and then in non-polar membrane environments.[114] Design and

organization of membrane-proteins is especially challenging compared to water-soluble proteins where hydrophobic residues are embedded into a solvent-excluded protein cavity and hydrophilic side chains are solvated outside the structure. Differences between the hydrophobicities of the hydrocarbon side chains of amino acids are much less pronounced in an already highly hydrophobic environment such as lipid bilayers. Unless there is a reasonable driving force for the system[115,116,117,118] hydrocarbon-hydrocarbon interactions between peptide and long acyl chains of the membrane would be as favorable as the ones among the peptides, thus making the quaternary structure unlikely to form. An exclusive transmembrane protein would need to be embedded in a membrane without losing the driving force that holds together protein conformation. With the insights gained from the phase separation properties of fluorinated residues, Bilgicer and Kumar have shown that fluorinated surfaces on the transmembrane helices drive bundle formation within lipid bilayers. Fluorinated polypeptides are orthogonal to components of the natural membranes, and they present an important tool for modifying and investigating membrane proteins.

3.2.6 Flourinated β-Bundle:

Very recently, the Schepartz lab has expanded their work on octameric β-bundles in order to study the effects of fluorinated residues on β-peptide folding. Fluorinated β-peptides were designed with the same principles as the hydrocarbon equivalents explained in section 3.1. and 3.1.2. Fluorines were introduced via L-β-Hexafluoroleucine as the building block.[119] Replacement of selected β-Leucine residues with L-β-Hexafluoroleucine resulted in a stable construct. Design of the

fluorinated core was first tested by molecular modeling in order to determine the positions where fluorinated residues would be introduced, and to prevent molecular crowding. The hexafluoroleucine side chain is larger than isopropyl group of leucine, and the β-bundle cannot accommodate all the fluorocarbons if all of the original thirty-two leucines in the core of the octameric bundle were to be replaced. Incorporation of only one hexafluoroleucine residue per peptide at position 8 created an octameric bundle where all the fluorine groups were buried in the solvent excluded core. Temperature dependent CD experiments revealed that the melting temperature of the fluorinated analogue is 82 °C, while the hydrocarbon equivalent template melts at 85 °C. X-ray structure of the assembly shows a continuous electron density over tightly packed fluorines between neighboring peptides. In addition, observation of cold denaturation of the bundle at high temperatures further confirms the formation of a "fluorous sub-phase" within the hydrophobic pocket. This data is consistent with the previously studied fluorinated coiled coils, which also display similar melting characteristics.[110,113]

The fact that bulky flurorinated side chains could be tolerated in the mini β-protein is promising for further research that may include synthesis of fluorous transmembrane β-helices. Such a structure can self-assemble within a lipid bilayer and would be orthogonal to natural molecules with its β-backbone.

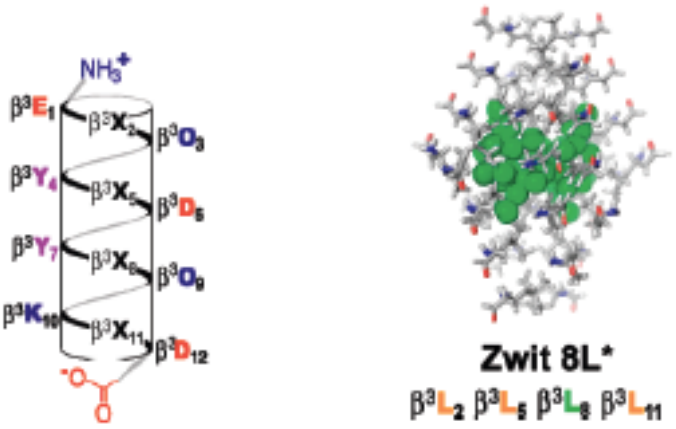


Figure 14. **Design of a fluorinated β-protein: a) primary structure monomeric peptide. Fluorinated side chains can be in any positions depicted with X, b) crystal structure of the octameric bundle. (reproduced with permission [119])**

3.4 SYNTHETIC STRATEGIES FOR S-β-HEXAFLUOROLEUCINE AND ITS USE IN PEPTIDE SYNTHESIS:

3.4.1 S-β-Hexafluoroleucine:

In order to explore the effects of a S-β-Hexafluoroleucine mutation in Glucagon-Like-Peptide-1, we have devised an efficient synthesis of S-β-Hexafluoroleucine from L-β-homoserine and/or D-α-Aspartic acid. S-β-Hexafluoroleucine has already been utilized in a β-peptide system previously, for investigating the effects of the fluorinated side chain on the structure of a mini β-protein,[119] where S-β-Hexafluoroleucine residue was obtained by Arndt-Eistert homologation of L-α-Hexafluoroleucine. Here we present an alternative and simpler method for large scale synthesis of S-β-Hexafluoroleucine.

3.4.2 Practical Synthesis of S-β-Hexafluoroleucine:

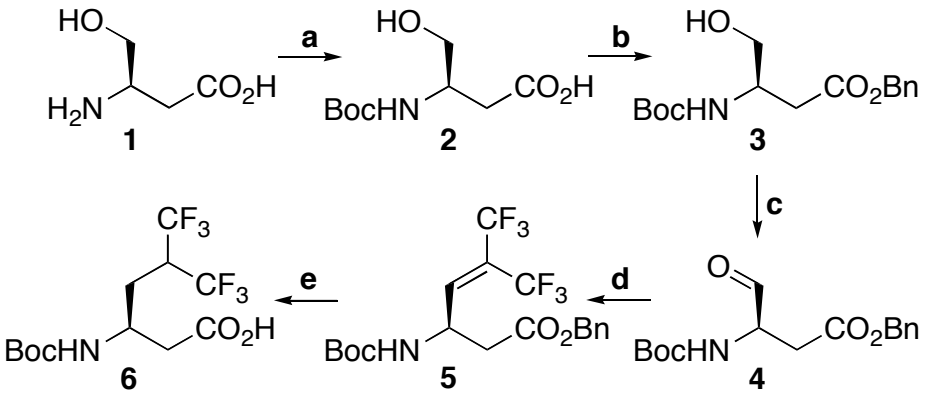
After the first synthesis of L-α-Hexafluoroleucine in our lab,[120] we intended to devise a route for access to the β equivalent of this amino acid. Such a building block could be used in peptide synthesis of protease resistant analogues of therapeutic peptides. We employed a novel strategy to obtain enantiomerically pure S-β-hexafluoroleucine and envisioned two routes from readily-available resources. Initially, we started with commercially available L-β-homoserine and synthesized S-β-Hexafluoroleucine in five steps with 40% overall yield. For a large scale synthesis we used D-α-Aspartic acid as the starting material. Aspartic acid, either in L- or D- form, is the only amino acid that possesses α– and β–structure at the same time. Taking advantage of this property, L-β-homoserine

can be reached with simple redox reactions. S-β-Hexafluoroleucine may then be prepared from L-β-homoserine in three additional steps. Scheme 2a

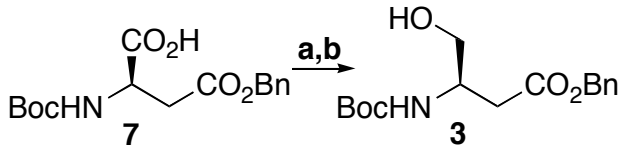
Here we describe the enantiomerically pure synthesis of S-β-(Boc)Hexafluoroleucine from L-β-homoserine and D-α-(OBzl)Aspartic acid-OH. [Scheme2]

The key intermediate (4) for S-β-hexafluoro leucine is prepared from L-β-homoserine. After N-t-Boc and C-OBzl protections,[121] side chain hydroxyl of L-Boc-β-homoserine-OBzl was oxidized by a modified Dess-Martin reagent.[122,123] The resulting aldehyde (**4)** was converted into L-β-hexafluoroleucine olefin (**5)** in a Wittig-type reaction using procedures that were previously developed in our laboratory.[120] Catalytic hydrogenation of **(5)** reduced the olefin and simultaneously removed the benzyl group on the carboxylic moiety, resulting in N-Boc protected S-β-hexafluoroleucine **(6)**. Characterization of the compound is further supported by X-ray crystallographic structure determination. (Figure 15)

Either D-α-Aspartic acid-OH or commercially available, side-chain protected D-α-(OBzl)Aspartic acid-OH was used for large scale starting material. Following N-t-Boc protection, free carboxylic acid of D-Boc-α-Asp(OBzl)-OH **(7)** was reduced with HBTU/$NaBH_4$ to give **(3)**.[124] (Scheme 3) Subsequent conversion of **(3)** to **(6)** followed the exact same procedures in Scheme 2, from (c) to (f).



Scheme 2. **Synthesis of Boc-L-β-hexafluoroleucine. Reagents and Conditions: (a) $(Boc)_2O$, THF, 0 ^oC , 99%; (b) $CsCO_3$, benzyl alcohol in DMF, 98% ; (c) DessMartin periodinane in CH_2Cl_2, 89%; (d) PPh_3, 2,2,4,4-tetrakis(trifluoromethyl)-1,3-dithietane, Et_2O, –78^oC, Ar, 58%; (e) Pd/C in MeOH, rt, 90%.**



Scheme 3. **Conversion of D-Boc-α-Asp(OBzl)-OH to L-Boc-β-homoserine-OBzl. Reagents and conditions: (a) HBTU in 2% of DMF in THF; (b) $NaBH_4$, 0°C, 90%.**

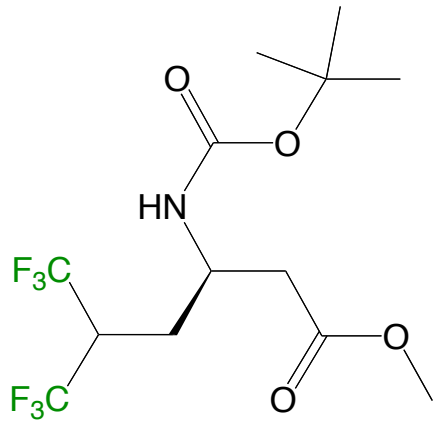


Figure 15. Chemical structure of N-Boc-S-β-hexafluoroleucine-OMe. Trifluoromethyl groups are shown in green.

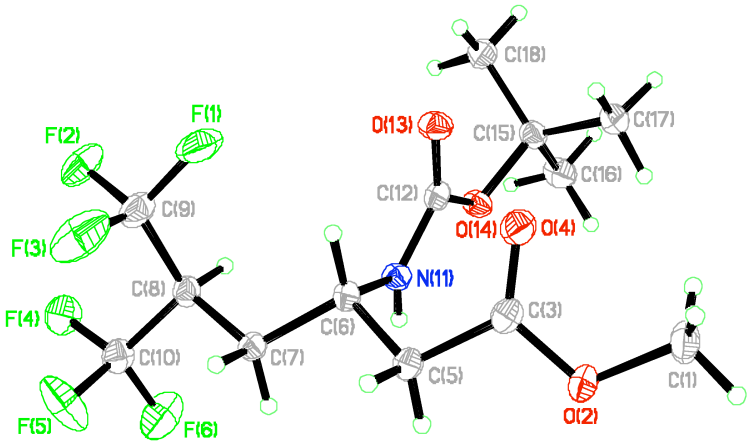


Figure 16. ORTEP diagram of X-ray diffraction structure of N-Boc-S-β-hexafluoroleucine-OMe obtained by X-ray diffraction. Fluorines are shown in green, oxygens in red, and nitrogen in blue.

Enantiomeric purity of the final product was determined by Mosher's method.[125] Analysis of the ^{19}F spectrum of MTPA derivatives showed a single diastereisomer demonstrating that the synthesis produces N-protected L-β-hexafluoroleucine in >98% enantiomeric excess. (Appendix)

3.4.3 Use of S-β-Hexafluoroleucine in Peptide Synthesis: A Fluorinated GLP-1 Analouge with β-amino acid Substitution

Enantiomerically pure S-β-Hexafluoroleucine was used in synthesis of a fluorinated GLP-1 analogue. GLP-1 is an incretin peptide responsible for insulin secretion from pancreas after feedings upon interacting with its receptor GLP-1R. A broad overview of this incretin hormone is included in Chapter 1. Analogues of GLP-1 is being investigated for diabetes treatment, although rapid proteolytic degradation of the peptide is a major obstacle. The N-terminus of GLP-1 includes the site for degradation by dipeptidyl peptidase IV (DPP-IV). (Figure 16) Previous studies from our lab has shown that replacement of the glutamic acid at position 9 in GLP-1 peptide with L-Hexafluoroleucine provides resistance towards (DPP-IV), yet retains the agonistic properties towards GLP-1.[126] Our aim in this study is to investigate effects of β-amino acid substitution at position 9 by replacing L-Glutamic acid with either L-β-Glutamic acid, L-β-Leucine, or L-β-Hexafluoroleucine. Because β-backbone structures are not recognized by any of the conventional proteases, resistance of these analogues to degradation is assured, however, effects of the extended backbone have not been investigated before.

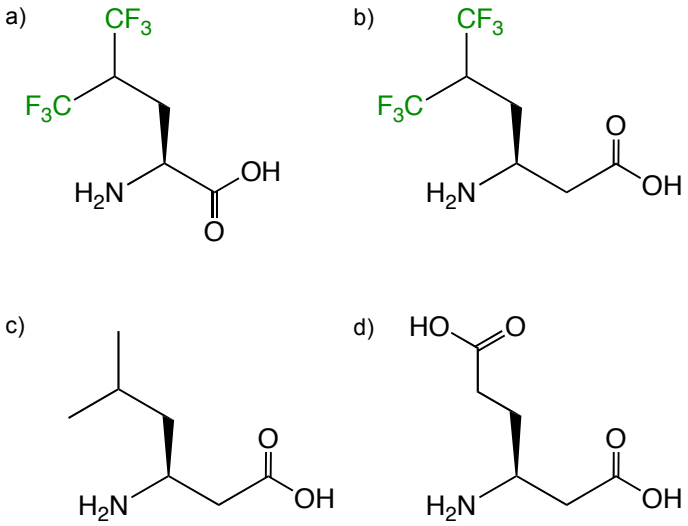


Figure 17. Structures of a) L-Hexafluoroleucine (HFL), b) L-β-Hexafluoroleucine (βHFL), c) L-β-Leucine (βLeu) and d) L-β-Glutamic acid (βGlu).

Figure 18. Primary sequences of GLP-1 analogues with β-amino acid substitutions at position 9. The DPP-IV cleavage site is shown with the arrow.

In addition to proteinogenic side chains (of β-Glu and β-Leu), L-βHexafluoroleucine provides an unnatural side chain with unique electronic properties.

When α-amino acid substitutions are analyzed, wild type GLP-1 with glutamic acid at 9th position is found to have a binding affinity of 0.18 nM[127] to 1.9 nM[126] in IC_{50} values as assessed by two different studies. Leu9 substitution diminishes binding by two-fold (IC_{50} = 0.4 nM with respect to 0.18 nM from Calas et al.[127]) and HFL9 follows it with 2.5-fold decrease (IC_{50} = 5.1 nM with respect to 1.9 nM from Kumar et al. [126]). The fact that uncharged isopropyl and hexafluroisopropyl groups at the 9 position display similar binding affinity as wild type and activation of the receptor only a few fold less, suggests that large hydrophobic groups are tolerable at this position. It has been also hypothesized that polar-hydrophobic fluorine atoms may mimic the glutamic acid side chain due to their high electron density and create a similar environment as a carboxylate moiety. We expected a similar pattern of binding and activation of GLP-1R with β-amino acid containing analogues, unless backbone manipulation causes a large decrease in recognition and binding of these molecules.

3.4.4 Receptor Activation Assays and Results:

Luciferase coupled cAMP accumulation experiments revealed that three analogues with β-amino acid substitution at position 9 have same efficacy as the wild type GLP-1. However, dose-response curves show that the activation of receptor is much smaller than the native peptide. EC_{50} for βHFL-GLP1 is 8.2×10^{-8} M, EC_{50} for βLeu-GLP1 is 2.5×10^{-8} M and EC_{50} for βGlu-GLP1 is 4.7×10^{-8}

M, compared to native GLP-1 ($EC_{50} = 3.3 \times 10^{-12}$ M) (graph not shown). Lower activation capacity of these peptides may be because of possible disruption of N-terminus structure within the receptor active site. Due to presence of the extra methylene group in β-amino acids, the backbone is extended by one more carbon unit compared to the wild type, which, in turn may cause repositioning of the side chains. This may also alter secondary structure that the peptide adopts when it is bound to its receptor. Without any crystal structure data it is not possible to know whether the peptide is only partially disturbed around substituted β-amino acid, or the whole structure is affected. Because the N-terminus region includes crucial residues for activity and the indispensable N-terminus histidine residue is proximal to the modification site, it is not surprising that a backbone modification in this region decreases the activity of the analogues.

Biological assays and analysis relating to these peptides are detailed in Chapter 2.

3.5 CONCLUSION

Nature had evolved to use α-amino acids as building blocks for proteins that eventually gave rise to systems and organisms that rely solely on these building blocks. It is intriguing to consider if this is due to the relative abundance of α-isomers. These questions have led scientists to create conditions in laboratory experiments that are meant to mimic those of earth before life had emerged. The Miller experiment mimicked the composition of the atmosphere and the components of earth billion years ago successfully gave clues about reactions that may have occurred. This provides some insight into the first molecules that may have emerged. Among the many products of the experiment, Miller identified a few β-amino acids in addition to the various α-amino acids. This suggests that resources for producing β-peptides and proteins were also present in primordial times. Today we also now know that β-peptides can form stable structures that are very much protein-like, yet there are no known enzymes made out of β-amino acids in nature. In principle, if given the chance of billion years to evolve, efficient systems made of β-amino acids could be born. Moreover, higher order oligomerization states has been shown within β-peptides that could lead to assembly of protein clusters and machinery. It is curious what evolutionary advantages might have been afforded by an α-backbone. Miller, in his later work, reasons that this is due to the availability of a richer variety of α-amino acids,[128] while there may have been a more limited variety of β-aminoacids. Miller's experiments were only capable of producing minimally-substituted β-amino

acids. This may be due to the higher energy expenditure that is required for synthesis of an extended β-backbone compared to an α-amino acid backbone.

In addition, Schepartz comments on the slight differences in the energetic cost during synthesis and folding of β-peptides and proteins.[101] Gathering the extended backbone of these β-amino acids into a polypeptide chain may also have incremental energy differences that may had an impact on the very first polypeptide assemblies. Once a stable, self-assembling structure is established with an α-backbone, the remaining β and γ amino acids may have been forever set aside from the evolution of natural systems, since they will be outnumbered by already existing self-replicating molecules. Over millions of years, α-proteins thrived and brought about the variety of living things on the earth. It will be interesting to see whether research on β-amino acids and proteins would advance in a way to develop as many functions and assemblies as α-proteins have.[129]

What does the future hold for β-peptides? They have already shown promise in antimicrobial peptide design[130], inhibitor[131] of protein-protein interactions and design of β-proteins with controllable oligomerization states.[132] A logical next step could be creation of functional β-peptides that may function as orthogonal enzymes in an α-protein world or in the design of protease resistant, structured trans-membrane entities for probing membrane proteins. It seems that we have not yet seen the full potential of β-peptides.[133,134]

3.6 EXPERIMENTAL

Materials and Methods:

Reactions were monitored by thin-layer chromatography with visualization by UV light or staining with ninhydrin. Silica gel used in flash chromatography had 230-400 mesh and 60 Å pore size. NMR spectra were obtained on either Bruker Avance 300 MHz or Bruker Avance III 500 MHz multinuclear spectrometers at Tufts University Chemistry Department. ^{13}C and ^{19}F NMR spectra were both broadband proton-decoupled, and ^{19}F spectra were referenced against an external standard CF_3CO_2H (–78.5 ppm). Mass spectra were obtained either on a Thermo LTQ ESI mass spectrometer or Bruker MALDI-TOF spectrometer. High resolution mass spectra utilized ionization modes as indicated.

(*R*)-3-(*tert*-butoxycarbonylamino)-4-hydroxybutanoic acid (2): H-L-β-homoserine-OH **(1)** (0.948 g, 8 mmol) was dissolved in 80 mL MeOH and Boc-anhydride (1.921 g, 8.8 mmol) was added. The suspension was sonicated in an ice bath for 3 h. The reaction was followed by TLC (90%EtOAc/10%MeOH/0.1%AcOH). After complete conversion, the reaction mixture was concentrated and 100 mL of water was added. Aqueous solution was titrated with 1N $KHSO_4$ to pH=2.0. The clear solution was extracted with Et_2O, washed once with H_2O and the organic layer was dried over $MgSO_4$. Following removal of solvent in vacuo, pure Boc-N-β-homoserine-OH **(5)** was obtained as a clear oil in 95% yield.^1H NMR (300 MHz, CD_3OD) δ 4.91 (s, 1H), 3.94 (m, 1H),

3.56 (dd, 1H, *J* = 5.28 Hz, 5.28 Hz), 3.49 (dd, 1H, *J* = 5.98 Hz, 6.00 Hz), 2.58 (dd, 1H, *J* = 5.96 Hz, 5.98 Hz), 2.42 (dd, 1H, *J* = 7.46 Hz, 7.51 Hz), 1.43 (s, 9H); ^{13}C NMR (75.5 MHz, CD_3OD) δ 174.3, 156.7, 79.0, 63.7, 49.9, 35.7, 27.7.

(*R*)-benzyl 3-(*tert*-butoxycarbonylamino)-4-hydroxybutanoate (3): Boc-N-β-homoserine-OH **(2)** (1.007 g, 4.6 mmol) was dissolved in 22.5 mL MeOH and 2.25 mL H_2O was added. The solution was titrated to pH=7.0 with 20% aqueous solution of Cs_2CO_3 (0.75 g, 2.3 mmol). After evaporation to dryness, the resulting white solid was concentrated twice from dry DMF and further dried under vacuum. Dried cesium salt was re-dissolved in dry DMF (50 mL), then benzyl bromide (575 uL, 4.83 mmol) was added drop-wise while stirring. The reaction was completed in 5 hours leaving a fine white precipitate in solution. The mixture was concentrated and 100 mL of H_2O was added into flask. The white precipitate was extracted with ethyl acetate. Combined ethyl acetate layers were washed once with H_2O, dried over $NaSO_4$ and concentrated in vacuo. Boc-N-β-homoserine-OBn **(4)** was obtained as pure, colorless oil in 98% yield.^{1}H NMR (300 MHz, $CDCl_3$) δ 7.34 (m, 5H), 5.45 (d, 1H, *J* = 8.59 Hz), 5.12 (s, 2H), 4.04 (b, 1H), 3.65 (m, 3H), 2.66 (d, 2H, *J* = 6.24 Hz), 1.43 (s, 9H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 172.1, 156.3, 136.1, 129.0, 128.7, 128.6, 80.1, 67.0, 64.4, 39.8, 34.4, 28.7.

(*R*)-benzyl 3-(*tert*-butoxycarbonylamino)-4-oxobutanoate (4): Water saturated CH_2Cl_2 was prepared by vigorously stirring CH_2Cl_2 (200 mL) while adding water (200 uL, 11 mmol) to yield 1ul H_2O/ ml CH_2Cl_2. Dess Martin periodinane (35 mL, 15% wt in CH_2Cl_2) was prehydrolyzed by dropwise addition of water saturated in methylene chloride to obtain a translucent solution. Translucent Dess-Martin reagent (5.5 g, 13 mmol, 235 mL) was poured on to Boc-N-β-homoserine-OBn **(3)** (1 g, 3.24 mmoles) dissolved in CH_2Cl_2 (30 mL). The white precipitate formed immediately after addition of Dess-Martin reagent. The reaction was followed by TLC (ethyl acetate), and complete conversion of **(4)** to aldehyde **(3)** was observed within 25 minutes. The volume of the reaction was reduced by half after vacuum-evaporation and then diluted with 200mL Et_2O. Sodium thiosulfate (5.12g, 32.4 mmol) in 80% saturated aqueous sodium bicarbonate was added, and the mixture was stirred until two phases were clear. Organic layer was collected and the remaining aqueous layer was washed twice with Et_2O. Combined organic layers were washed twice with saturated aqueous sodium bicarbonate solution, twice with H_2O, and twice with brine, then dried over $MgSO_4$ and concentrated in vacuo. Pure Boc-N-β-homoserine(aldehyde)-OBn **(4)** was obtained as yellow oil with 89% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.68 (s, 1H), 7.40-7.36 (m, 5H), 5.67 (d, 1H, J = 7.82 Hz), 5.16 (s, 2H), 4.44-4.38 (m, 1H), 3.08 (dd, 1H, J = 4.87 Hz, 4.86 Hz), 2.91 (dd, 1H, J = 4.87 Hz, 4.89 Hz), 1.49 (s, 9H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 199.6, 171.5, 156.0, 135.7, 129.1, 129.0, 128.7, 81.0, 67.5, 56.5, 35.0, 28.8; $[\alpha]^{20}_D$ = -25.3 (c –1.9, $CHCl_3$); HRMS (FAB) calcd for $C_{16}H_{21}NO_5$ 308.1498, found 308.1498 (M+H).

(*R*)-benzyl-3-(*tert*-butoxycarbonylamino)-6,6,6-trifluoro-5-(trifluoromethyl) hex-4-enoate (5): A solution of PPh_3 was prepared by dissolving 11.46 g of solid (43.6 mmol) in 50 mL dry ether, and cooled down to –78°C. 2,2,4,4-tetrakis(trifluoromethyl)-1,3-dithietane (4 g, 11 mmol) was added to the stirring solution of PPh_3 under Ar and in dry Et_2O. Solution turned light yellow after complete addition of reagent, and left for stirring for one hour. Vacuum dried Boc-N -β-homoserine(aldehyde)-OBn **(4)** (0.837 g, 2.73 mmol) was dissolved in dry Et_2O (20 mL) and slowly added into the stirring mixture at –78°C. The mixture was left in dry ice-acetone bath overnight and slowly warmed up to room temperature over 16 hrs. A more intense yellow color and a slight precipitate were observed within first 24 h. The reaction was mixed under an argon atmosphere, at r.t. for 4 days during which the white precipitate got heavier and the solution became bright orange. The precipitate was filtered and the filtrate was concentrated. The resulting orange oil was dissolved once again in n-pentane to remove insoluble impurities. Further purification was done by flash column chromatography (silica gel) using CH_2Cl_2. Boc-N-β-hexafluoroleucine(olefin)-OBn **(5)** was obtained in 58% yield in yellow oil form. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.43-7.36 (m, 5H), 6.91 (d, 1H, J = 8.78 Hz), 5.51 (b, 1H), 5.18 (s, 2H), 5.05 (m, 1H), 2.84 (m, 2H), 1.45 (s, 9H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 170.8, 155.0, 146.0, 135.5, 132.8, 129.1, 129.0, 128.7, 123.0, 119.3, 81.0, 67.6, 45.3, 39.2, 28.6; ^{19}F NMR (282.6 MHz, $CDCl_3$) δ -58.95 (s, 3F), -64.88 (m, 3F); HRMS (ESI) calcd for $C_{19}H_{21}F_6NO_4$ 464.1267, found 464.1289 (M+Na).

(*S*)-3-(*tert*-butoxycarbonylamino)-6,6,6-trifluoro-5-(trifluoromethyl)hexanoic acid (6): Boc-N-β-hexafluoroleucine(olefin)-OBn **(5)** (0.5 g, 1.1 mmol) was dissolved in 20 mL of MeOH and 4.6 g of Pd/C (4.4 mmol) was added. The suspension is stirred under H_2 for 2 h and both the double bond and the benzyl protection group on the carboxylic acid were removed. Pd/C was removed by filtration and Boc-N -β-hexafluoroleucine-OH **(6)** was obtained in pure form with 90% yield, after purified with flash column chromatography (silica gel, $CHCl_3$) several times. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.46 (s, 9H), 2.05 (m, 2H), 2.65 (m, 2H), 3.2 (dd, J=7.8 Hz, 1H), 4.11 (m, 1H), 5.19 (d, J=8.5 Hz, 1H) ^{13}C NMR (300 MHz, $CDCl_3$) δ 171.9, 156.0, 80.1, 60.8, 52.4, 46.2, 39.1, 29.1, 28.6. ^{19}F NMR (300 MHz, $CDCl_3$) δ -71.51 (d, J-8.11 Hz, 3F), -70.98 (d, J=7.55 Hz, 3F).

Preparation of MTPA derivatives of (6): Boc-N-β-hexafluoroleucine-OH was deprotected by stirring 1.4 μmol (0.5 mg) of amino acid in 50% TFA/CH_2Cl_2 for 10 minutes. TFA/CH_2Cl_2 was removed under vacuum and H_2N-β-hexafluoroleucine-OH was redissolved in dry DMF. 2.8 μmol (0.4μL) TEA was added to the solution and stirred. Either 1.4 μmol (0.5 μL) (+) MTPA or 1.4 μmol (0.5 μL) (-) MTPA was delivered to the reaction mixtures with 10 uL syringe and reactions was stirred for 30 minutes. Removal of solvents under vacuum and analysis of the crude mixture with ^{19}F NMR confirmed the presence of the MTPA derivatives and further revealed enantiomeric purity, since none of the

diasteromeric shifts that could belong to the other enantiomer were present in spectra.

3.7 REFERENCES

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CHAPTER 4 CONLCUSIONS AND FUTURE PROSPECTS

Chapter 4 is dedicated to possible improvements for the GLP-1 analouges described in previous chapters. In this thesis, we aimed to pursue unprecedented modifications on GLP-1 that could, in principle, improve the therapeutic properties of this peptide.

4.1 A Lipidated GLP-1 Analogue

4.1.1 Conclusions

In chapter 2, we proposed a design of a macromolecule in order to anchor GLP-1 to the cell membranes. The anchoring unit consists of a dipalmitoyl fatty acid chain that is attached to the C-terminus end of the GLP-1 peptide by a short, flexible PEG linker. Proximity of the lipidated GLP-1 to its receptor is expected to result in improved binding and stimulation of downstream signals.

Experimental results revealed that the lipidated construct had the same affinity as the native GLP-1 to the cognate receptor, whereas activation was 6800-fold less than GLP-1. The decrease in activity may be due to either the inability of the lipid component to integrate into the cell membranes during incubation, or the conformational disturbance of the ligand by the large C-terminal attachment, or both. Additional experiments, as suggested in chapter 2, section 2.4.2.1 are required to understand the underlying problems.

A similar study was performed recently using recombinant methods by expressing a membrane anchoring GLP-1 ligand that can localize on the human

embryonic kidney 293 (HEK293) cell membranes.[1] GLP-1 peptide is attached to the N-terminus of a transmembrane peptide with a poly(asparagine-glycine) linker. The C-terminus of the transmembrane peptide is intracellular and provides tethering to the cell membrane. Constructs were shown to bind and activate GLP-1R comparably to soluble GLP-1, as assessed by luciferase coupled cAMP accumulation experiments. Design of the tethered ligand helped to investigate the GLP-1R and GLP-1 physiology in vivo, as well as providing a useful tool for probing GPCR functions in general. From our research perspective, this study also supportes the hypothesis that anchoring of the GLP-1 ligand to cell membrane would benefit the cAMP signaling pathway. [1]

4.1.2 Future directions

Although our lipidated GLP-1 construct is not as potent as the best GLP-1R agonists such as GLP-1 peptide or Exenedin, our experimental results can be used as a basis to improve the molecule. Most importantly, incorporation of the lipidated molecule into bilayers should be analyzed by either using ELISA assays on non-chromogenic ligand, or by synthesizing its chromogenic analogs and visualizing via fluorescence microscopy. Moreover, there are still alternative modifications that can be made to a GLP-1 construct for better binding and signaling properties. First, PEG linkers with varying lengths ranging from four to fifteen unit long oligomers may be used in order to search for the optimum distance between the peptide and the lipid. While very short linkers may completely abolish binding due to restrictions in distance, very long ones may

impose an entropic penalty. Long PEG linkers may also interfere with the extracellular domain of the receptor, which may alter signaling. As a second option, a variety of membrane anchoring molecules can be used to carry GLP-1. Cholesterol, farnesol and different phospholipids with varying fatty acid chains are other cell membrane components that can be used to attach the ligand. The different hydrophobic natures of these molecules may help localizing GLP-1 at different sites at the cell surface and may help concentrate it around the receptor. For instance, a recent report indicates the presence of GLP-1R within cholesterol-rich lipid rafts.[2] Certainly, we are aware that even an optimized construct may not be as flexible and/or spatially available as the soluble form. Therefore, the search for more effective GLP-1R agonists is still a challenge for our group.

4.2 GLP-1 Analogues with β-aminoacid Substitutions

4.2.1 Conclusions

In Chapter 3, synthesis of a set of GLP-1 analogues that contained β-aminoacids at the protease-sensitive site of GLP-1 have been presented and results have been discussed in chapter 2. Overall data show that these analogues that contain β-backbone have the same efficacy as the wild type GLP-1, but are less potent by a factor of 100 to 1000-fold. Lower activation capacity of these peptides is probably due to disruption of N-terminus structure within the receptor active site. It is a common drawback for GLP-1 derivatives that are modified on the N-terminal residues, which bears the indispensable residues for receptor activation, but is also prone to degradation by DPP-IV.[3] Protease stability assays

show that GLP-1 analogues with β-Leu and β-Glu at 9th position of GLP-1 maintained their stabilities longer than control GLP-1 peptide by 9.6 and 5.8-fold respectively. This may be a winning advantage of these compounds if they could exhibit longer plasma lives in vivo and result in glucose lowering effect comparable to GLP-1. There are examples in literature reinforce this hypothesis, where GLP-1 analogues with lower activity, but with better protease resistance compared to wild type show excellent insulinotropic activities in vivo.[4 5 6 7] Next step for β-aminoacid substituted GLP-1 analogues (as suggested in chapter 2, section 2.4.2.2) is to test them in vivo, in order to see whether they will be able to exhibit glucose-lowering effect without causing toxicity to subject animals.

As a part of Chapter 3, a novel synthesis of Boc-L-β-hexafluoroleucine is also described. Efficient and straightforward synthesis of this amino acid presents a practical method in creating fluorinated version of β-leucine for peptide synthesis. β-peptides are recently finding use as peptidomimetics, structural models and antimicrobial peptides, where there is a vast opportunity to investigate the effects of fluorinated side chains and take advantage of its unique properties in further designs. Indeed, Schepartz and co-workers have recently utilized Fmoc protected verion of this amino acid in synthesis of 14-helix β-peptides for searching the effects of fluorine in the structure-function of the mini-β-protein. In our lab, incorporation of this compound into GLP-1 has allowed us to investigate two different aspects. First, it is a comparison compound for HFL9-GLP-1, a fluorinated, protease stable, and active analogue of GLP-1 that has previously been synthesized in our lab. Difference between these two analogues gives reveals

the changes introduced by a β-backbone. Second, it gave us the opportunity to compare it within the group of other β-substituted GLP-1s, which would reveal the effects of the fluorinated chain.

In our experience, incorporation of this amino acid during peptide synthesis did not require different methods or solvents compared to α–amino acids. Manual Boc-SPPS was performed through conventional methods, using DMF as the solvent and HBTU for activating amino acids. Coupling efficiencies were not diminished as assessed by Kaiser test and no extra couplings were required.

As a final note, the strategies used for creating the β-backbone of β-L-hexafluoroleucine can be adapted to synthesize fluorinated γ-analogue of L-hexafluoroleucine.

4.2.2 Future directions

Due to advances in β-peptide design, it is also possible now to design peptidomimetics for naturally occurring secondary structures. Among the recent examples are the hDM2 inhibitors[8] and somastatin agonist[9]. Even with the new discoveries in the ligand structures of GLP-1R, it is still far away to redesign GLP-1 on an artificial β-backbone or even on a *de novo* α-helix. Crystal structures of exenedin[10], then GLP-1[11] complexed with the GLP-1R extracellular domain showed that the secondary structure of the GLP-1R antagonists are α-helical. This was a predicted outcome that had been suggested in many previous studies[12 13] and is consistent with class B, G-protein coupled receptor (GPCR) ligands in general. Crystal data show that exenedin forms a more continuous helix

when compared to GLP-1, which has a slight break at the middle of the helical structure. Both of these ligands have high affinity towards receptors and activate downstream signals efficiently. This suggests that the extracellular domain of the GLP-1R is able to accommodate small structural differences in ligands. Since 14-helix structures of β-peptides were proven to replace and imitate α-helical domains successfully, a 14-helix design of a GLP-1 mimic could be a new challenge.

In chapter 3, the crystal structure of GLP-1 bound to its receptor's extracellular domain has been discussed in detail. The residues of interest for a βGLP-1 mimic are the ones that are in close contact with the receptor, and the conserved residues -especially on the N-terminus- since they impact the activation of the receptor. Both of these regions are structurally challenging to imitate. Crystal structure of GLP-1 shows that C-terminus residues are the main contact points with receptor's extracellular domain, however these residues are not perfectly aligned on one face of the helix.[11] Another obstacle in designing β-mimic of the GLP-1 is positioning the N-terminal residues that are crucial for activity. The N-terminus of the peptide is random coil in solution and is proposed to adopt helical structure upon interacting with the transmembrane component of the GLP-1R. Crystal structure data for this part of the peptide is not resolved because only the extracellular domain of the receptor is available for X-ray crystallography. Nevertheless, strong evidence for helical structure of this region is a premise for a starting point for future GLP-1 mimics.

4.3 REFERENCES

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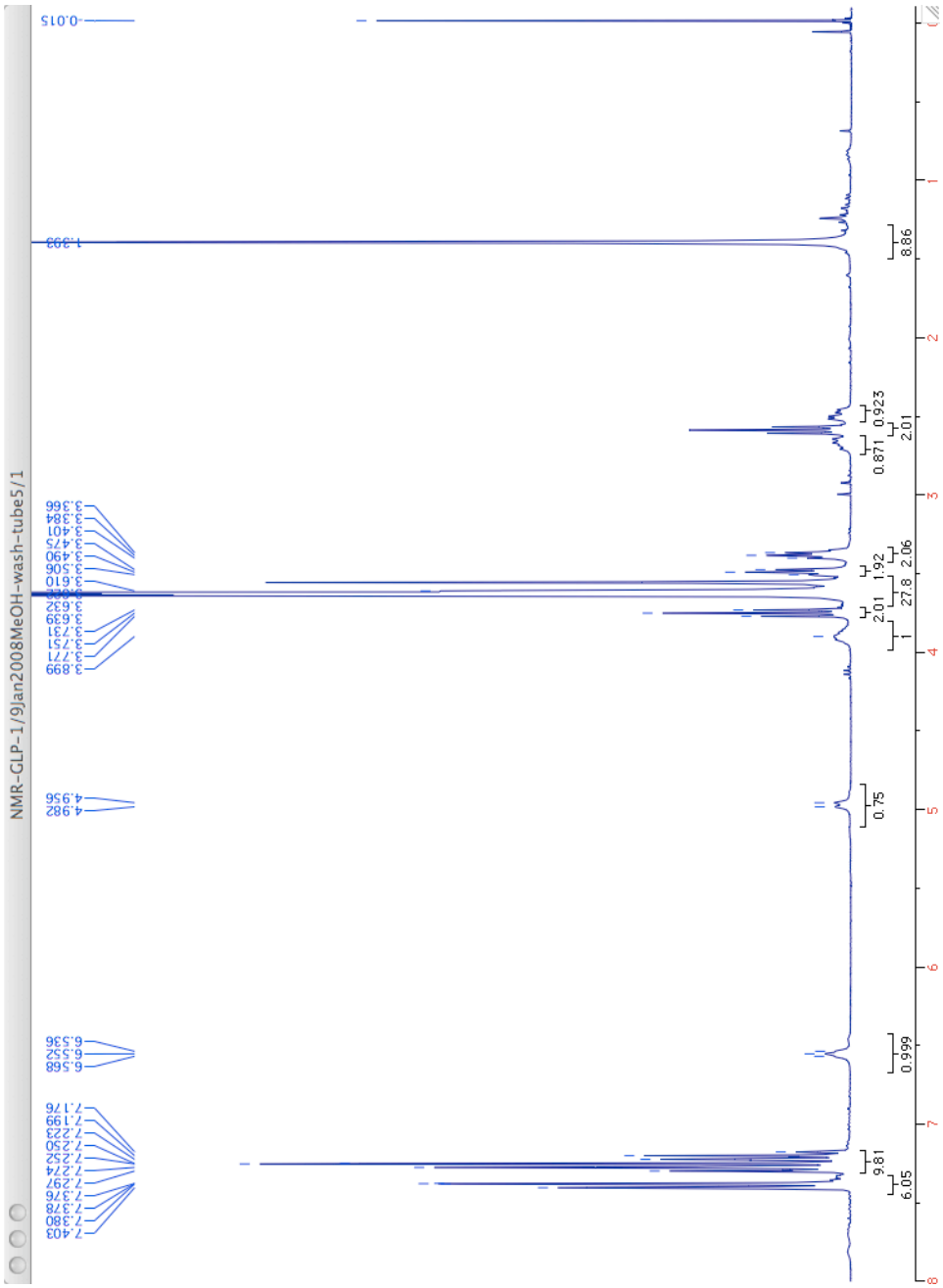
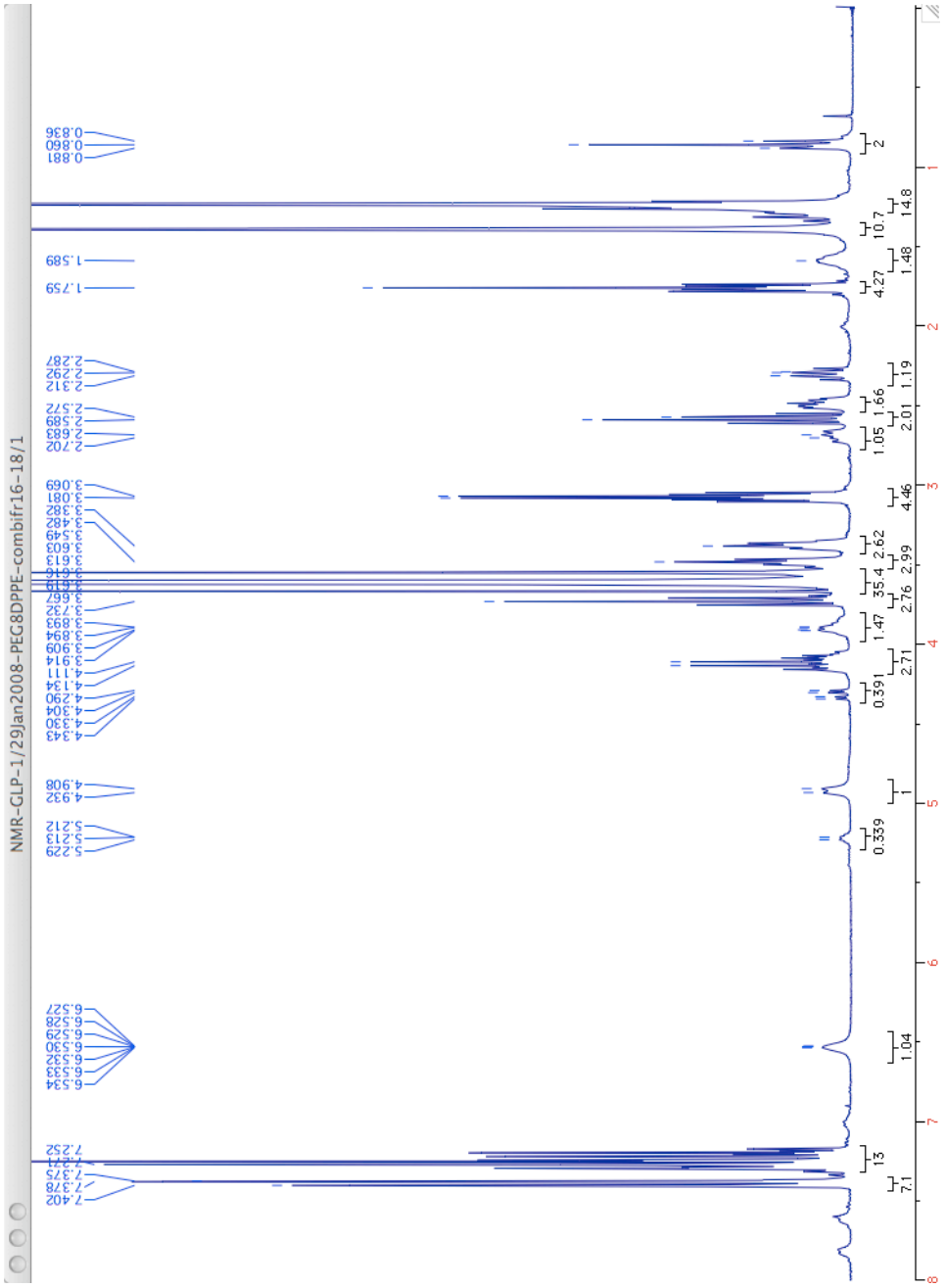
APPENDIX-CHAPTER 2

^{1}H NMR (300 MHz, $CDCl_3$) for **Boc-Cys(Trt)-PEG$_n$-COOH (n = 8)**



^{1}H NMR (300 MHz, $CDCl_3$) for **Boc-Cys(Trt)-PEG$_n$-DPPE (n=8)**



MALDI-TOF MS for **H_2N-Cys-PEG_n-DPPE (n = 8)**

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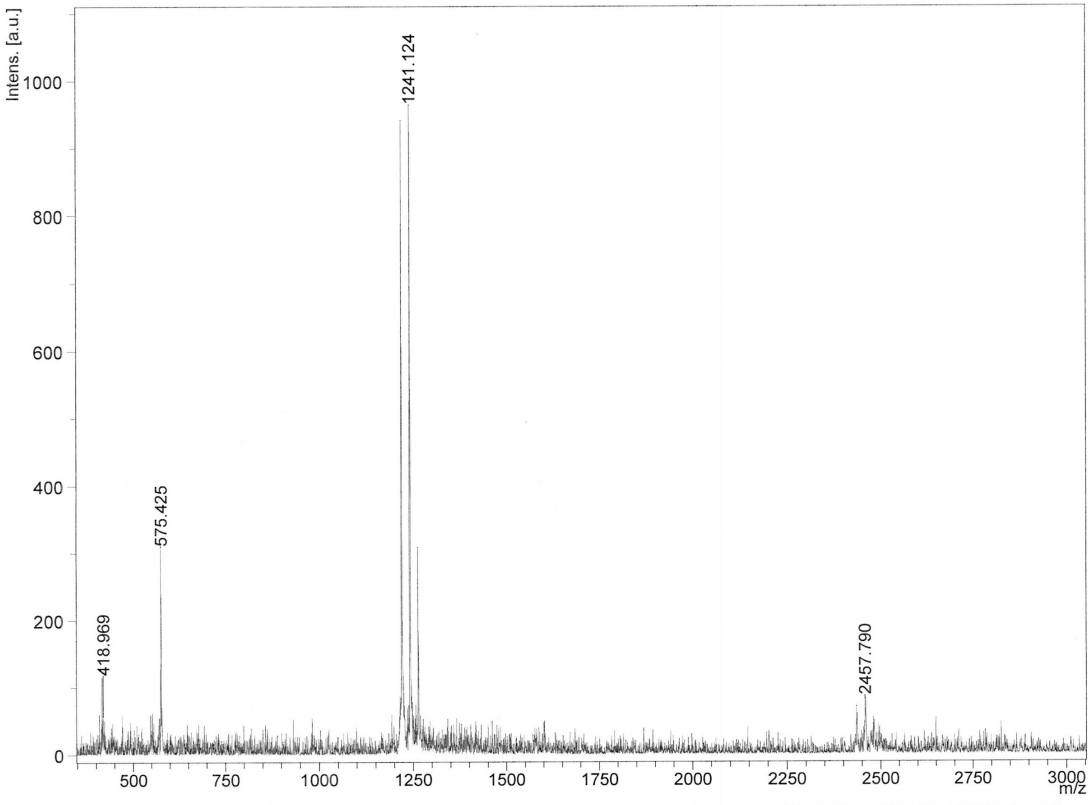
Comment 2



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1241.124				961.00	
1263.155				306.00	
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2457.790				78.00	
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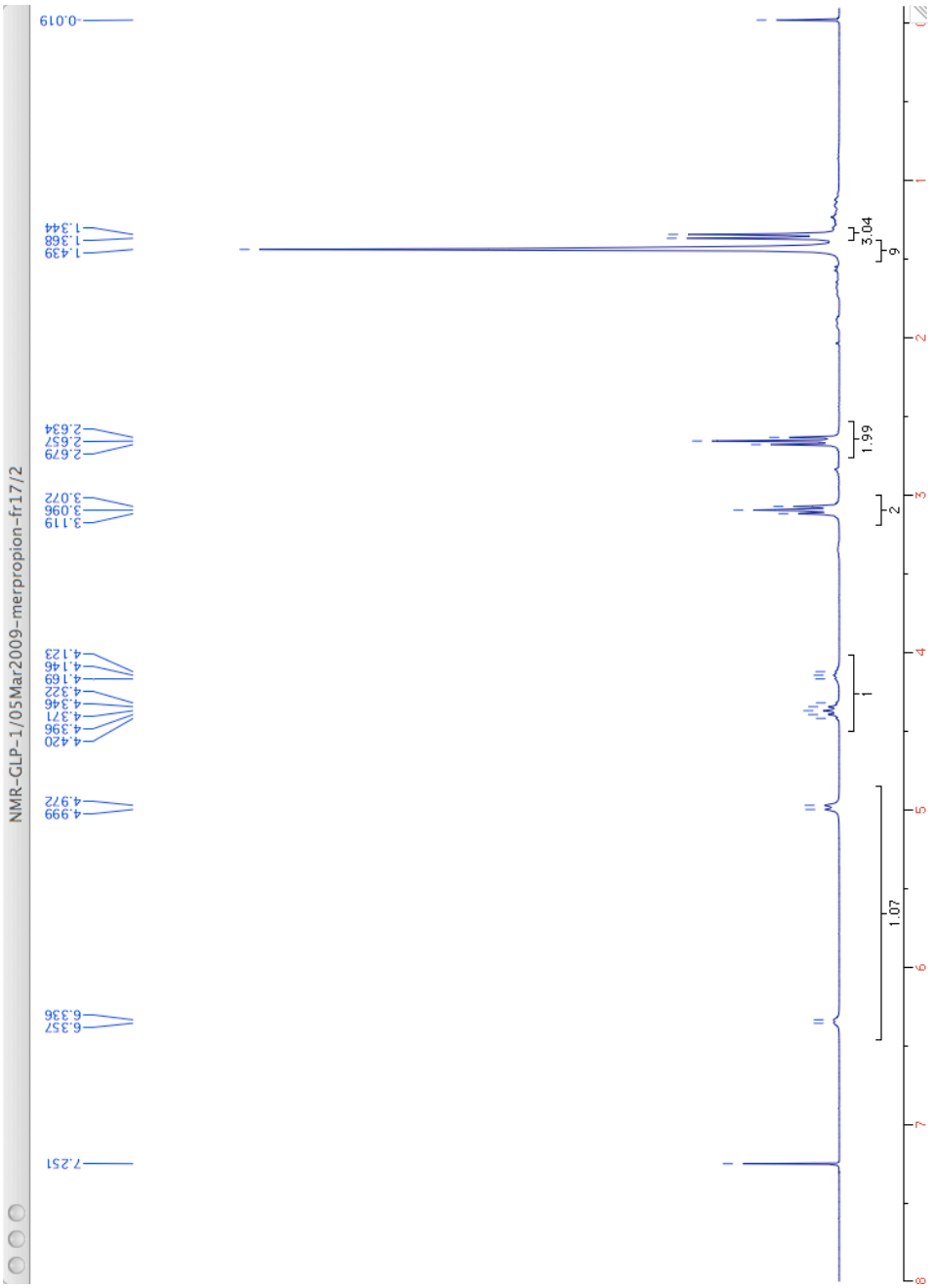
^{1}H NMR (300 MHz, $CDCl_3$) for **Boc-Ala-COSR (R: 3-mercaptopropionic acid=MPA)**



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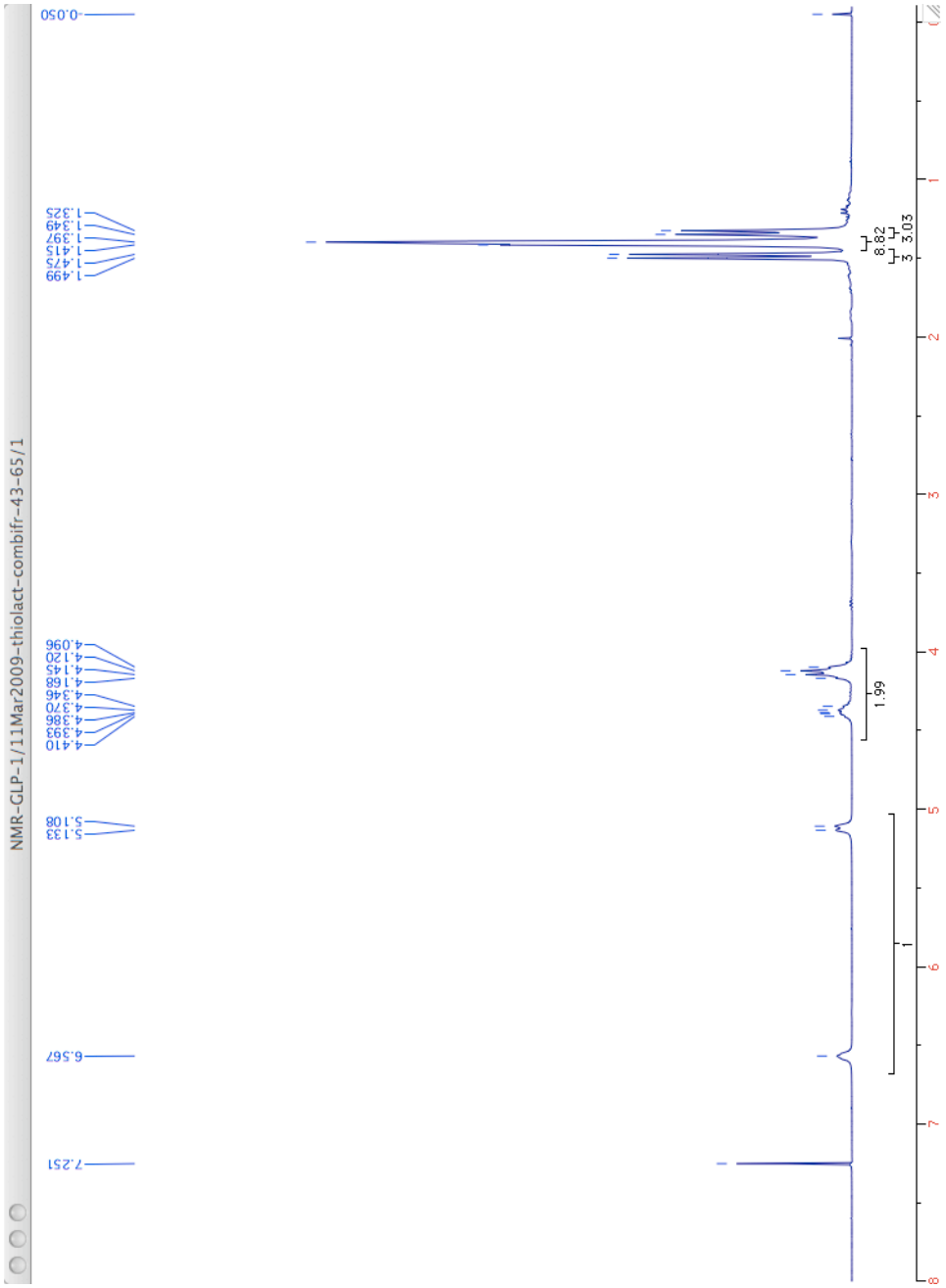

MALDI-TOF MS for **GLP-1 peptide thioester (GLP-1-COSR (R: 3-mercaptopropionic acid=MPA))**

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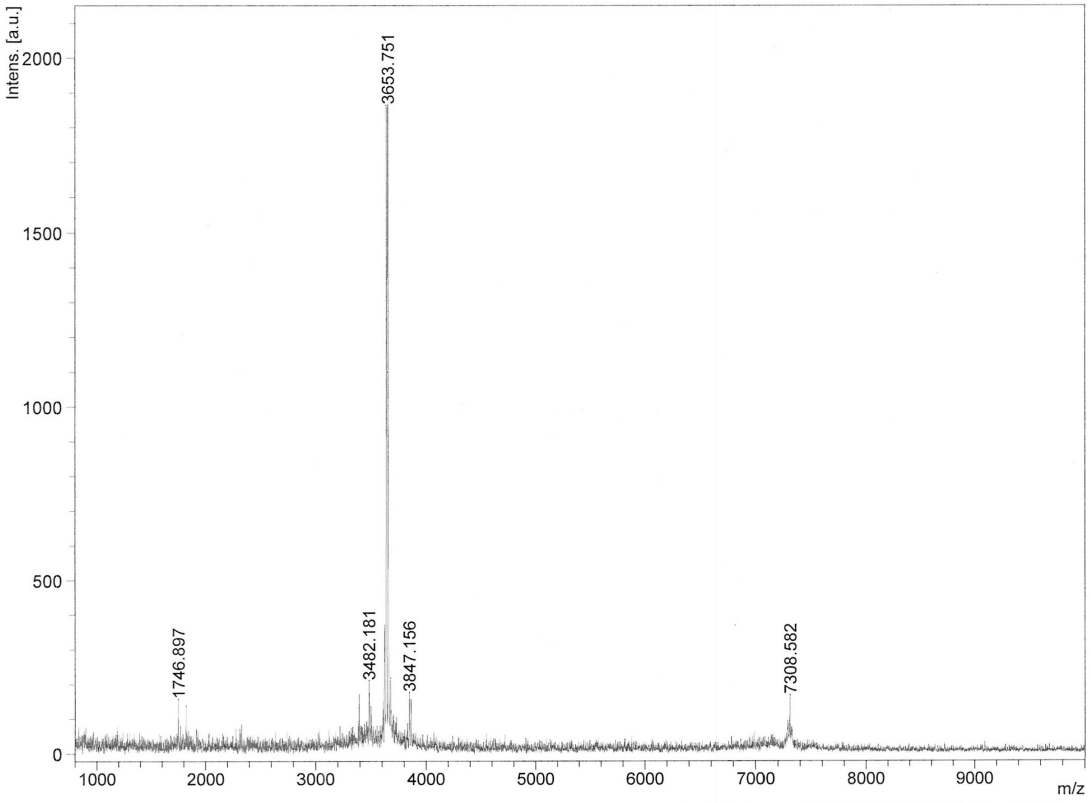

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3482.181				205.00	
3624.088				366.00	
3635.961	44.6	11047	1179	336.53	5016
3651.599	42.0	12292	1287	261.10	3808
3653.751				1243.00	
3677.854				212.00	
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MALDI-TOF MS for **GLP1-PEG$_n$-DPPE (n = 8)**

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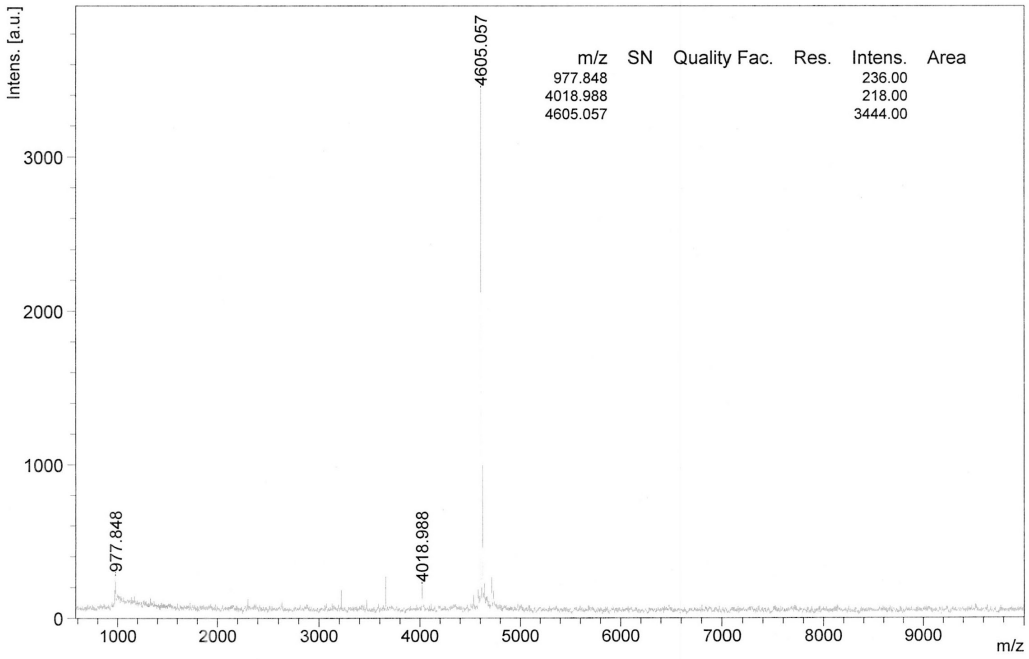

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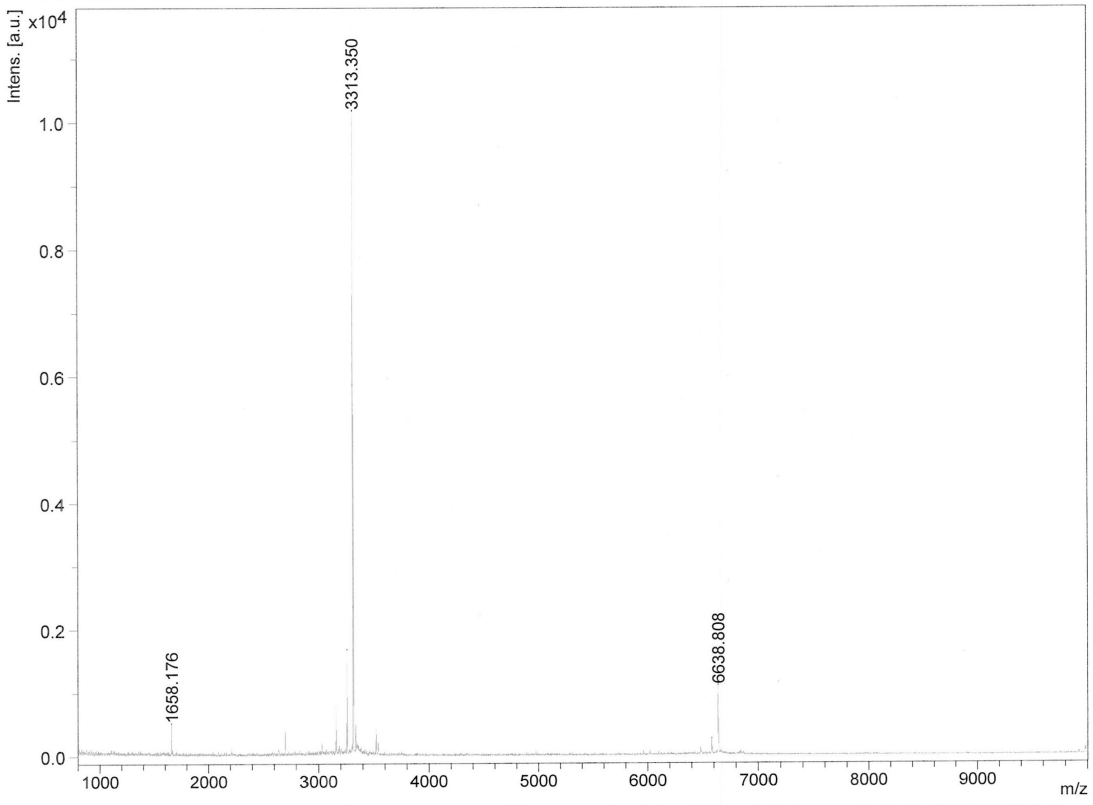

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MALDI-TOF MS for **βLeu9GLP1**

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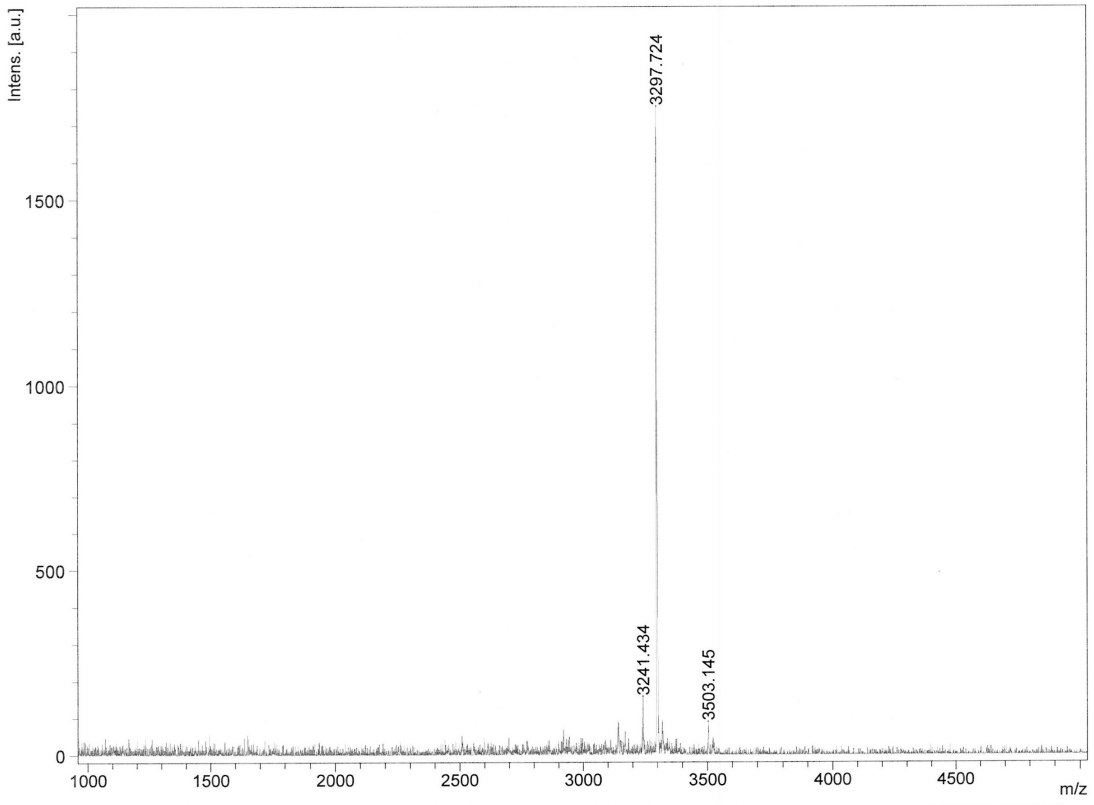
Comment 2



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MALDI-TOF MS for **βHFL9GLP1**

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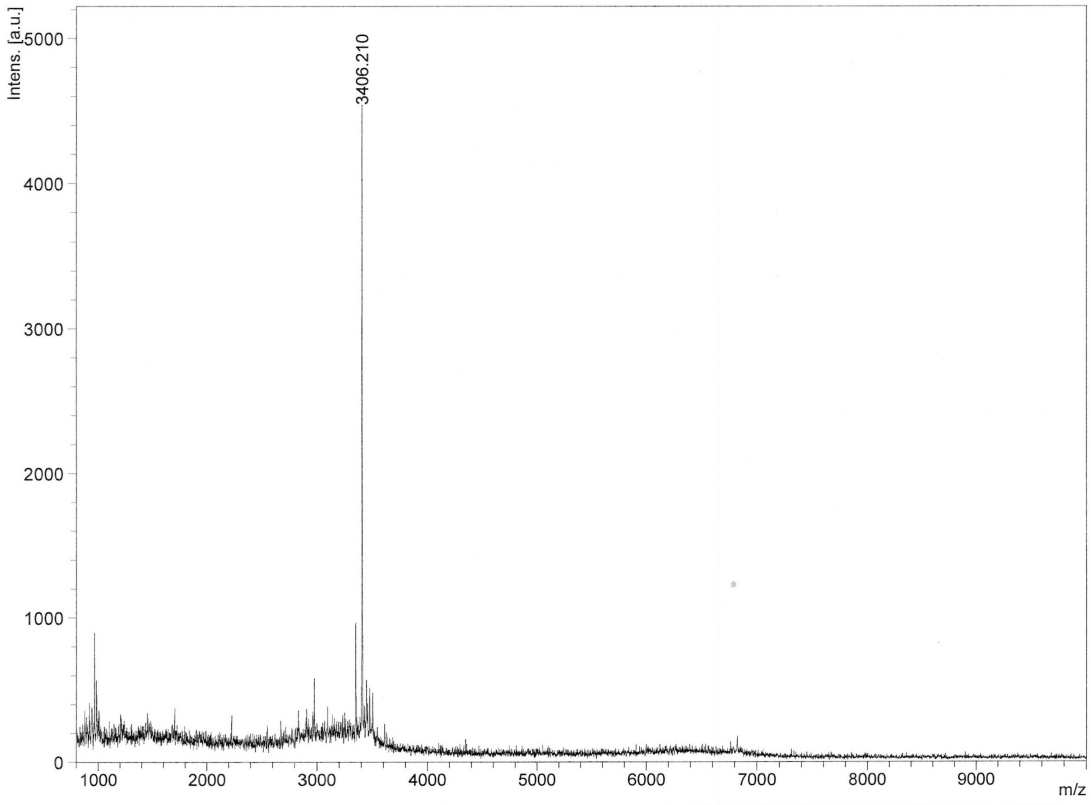

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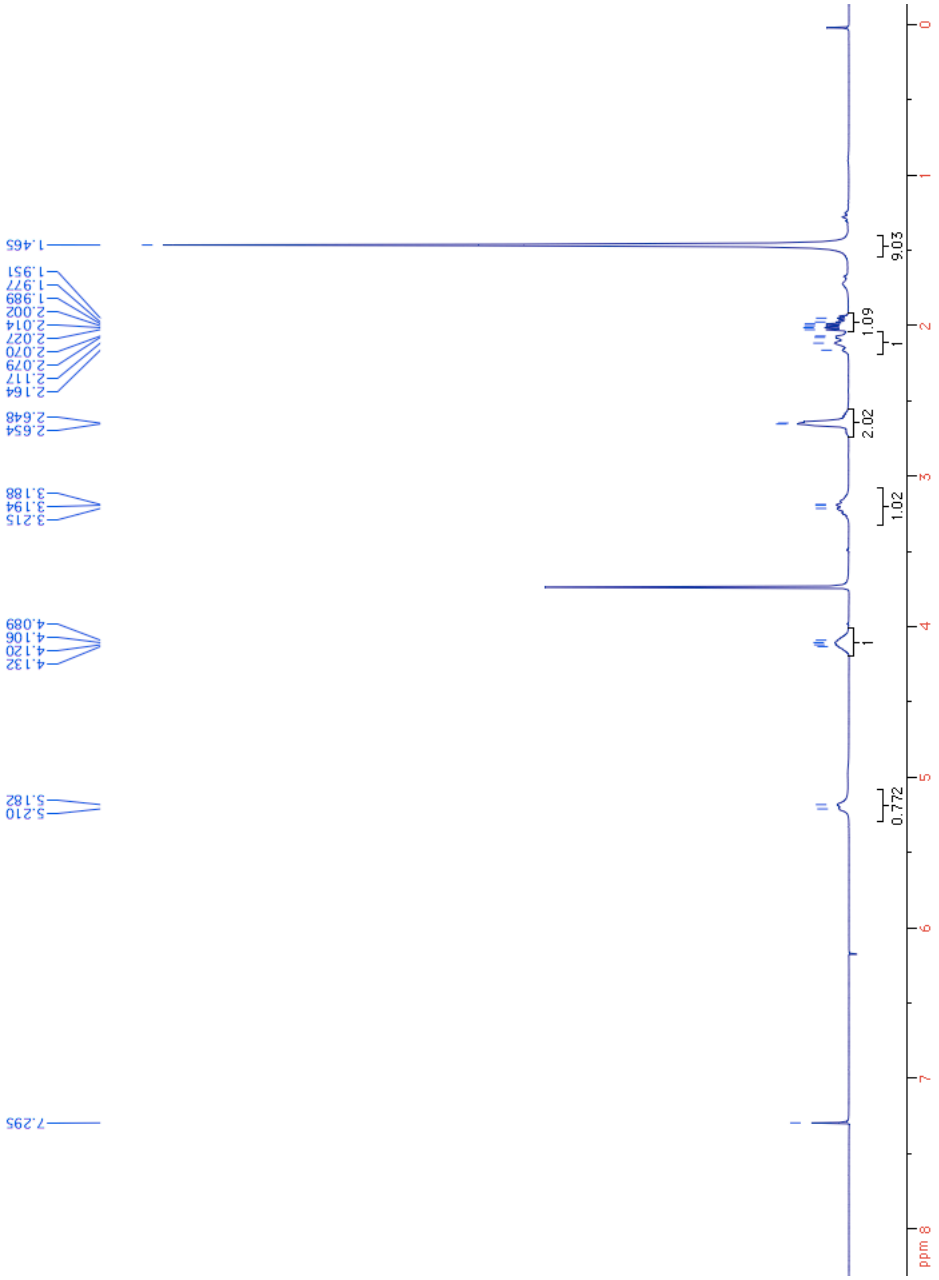
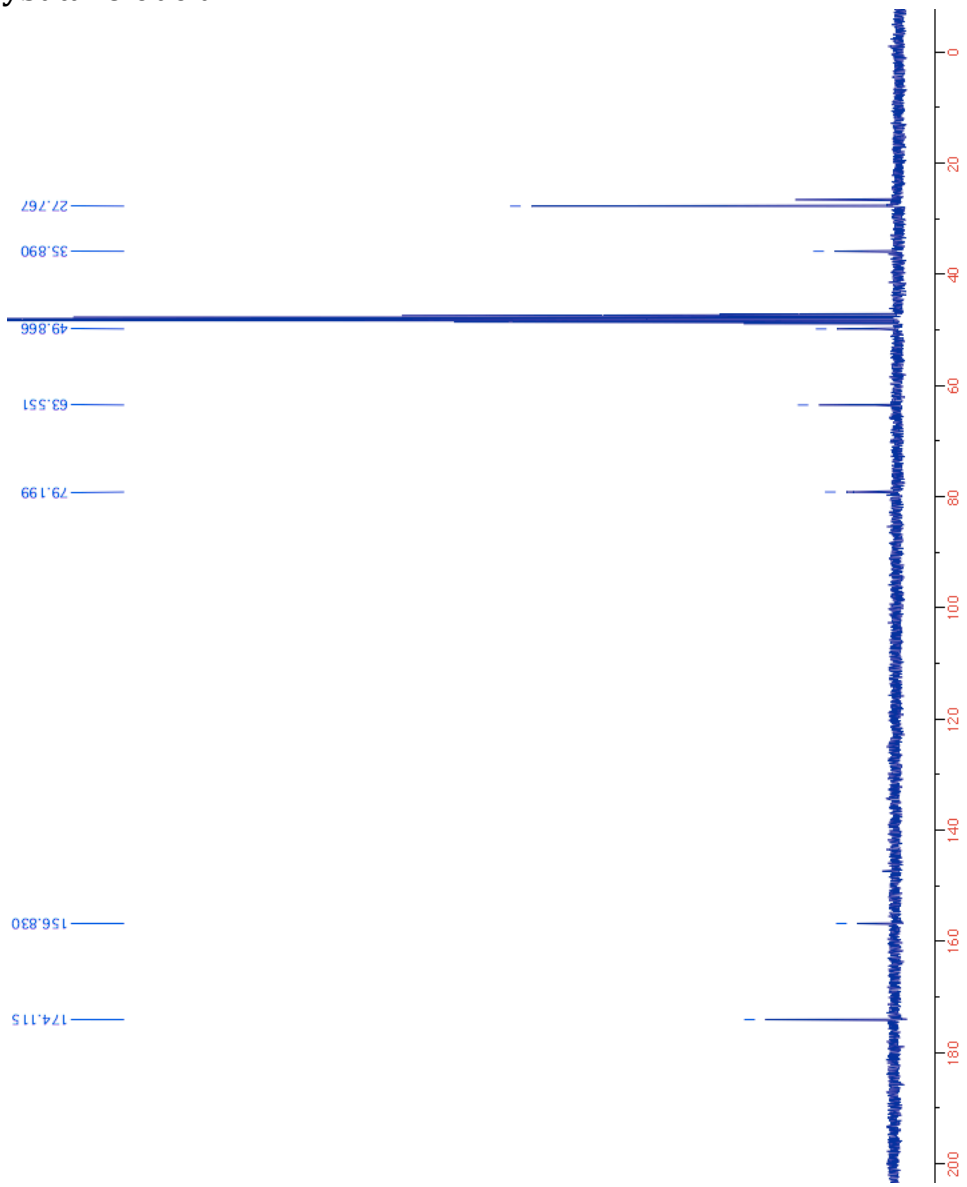
APPENDIX-CHAPTER 3

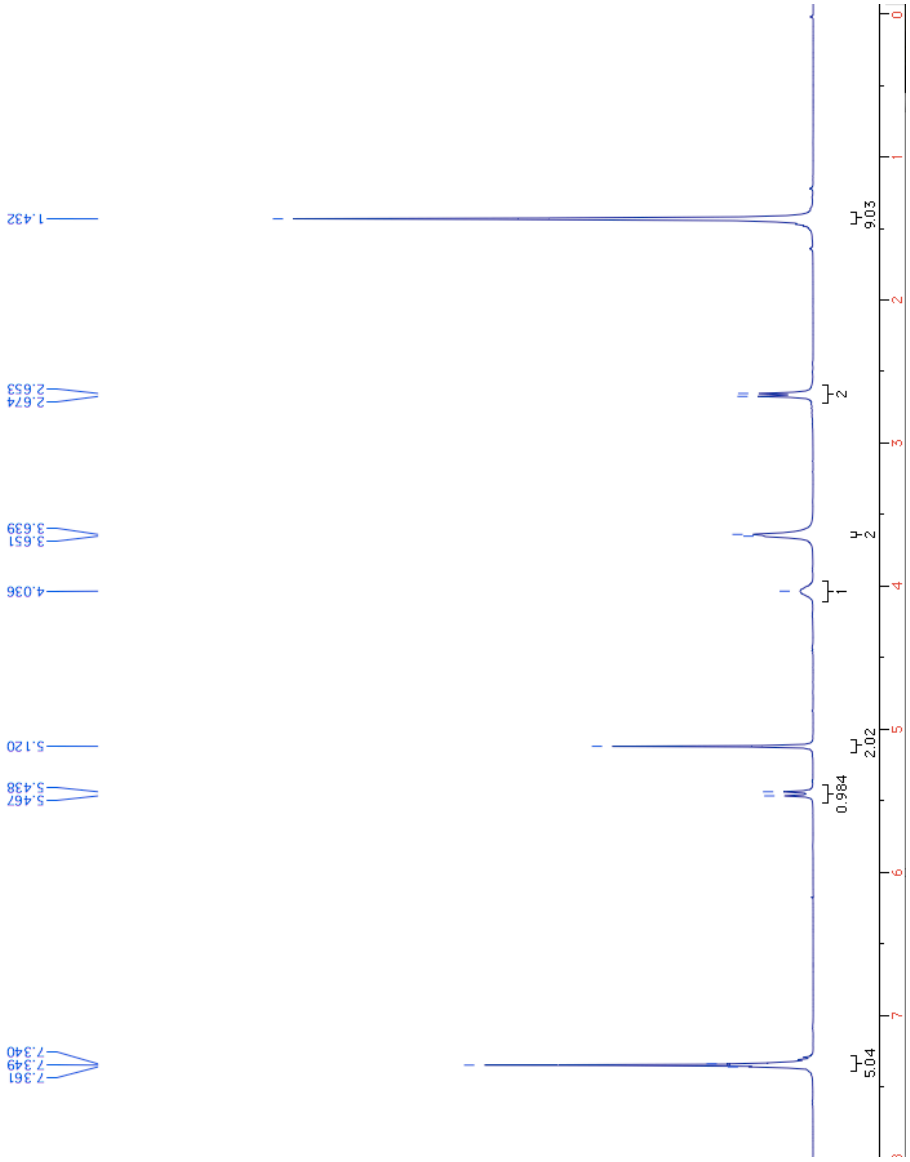
^{1}H NMR (300 MHz, $CDCl_3$) **(*R*)-3-(*tert*-butoxycarbonylamino)-4-hydroxybutanoic acid**



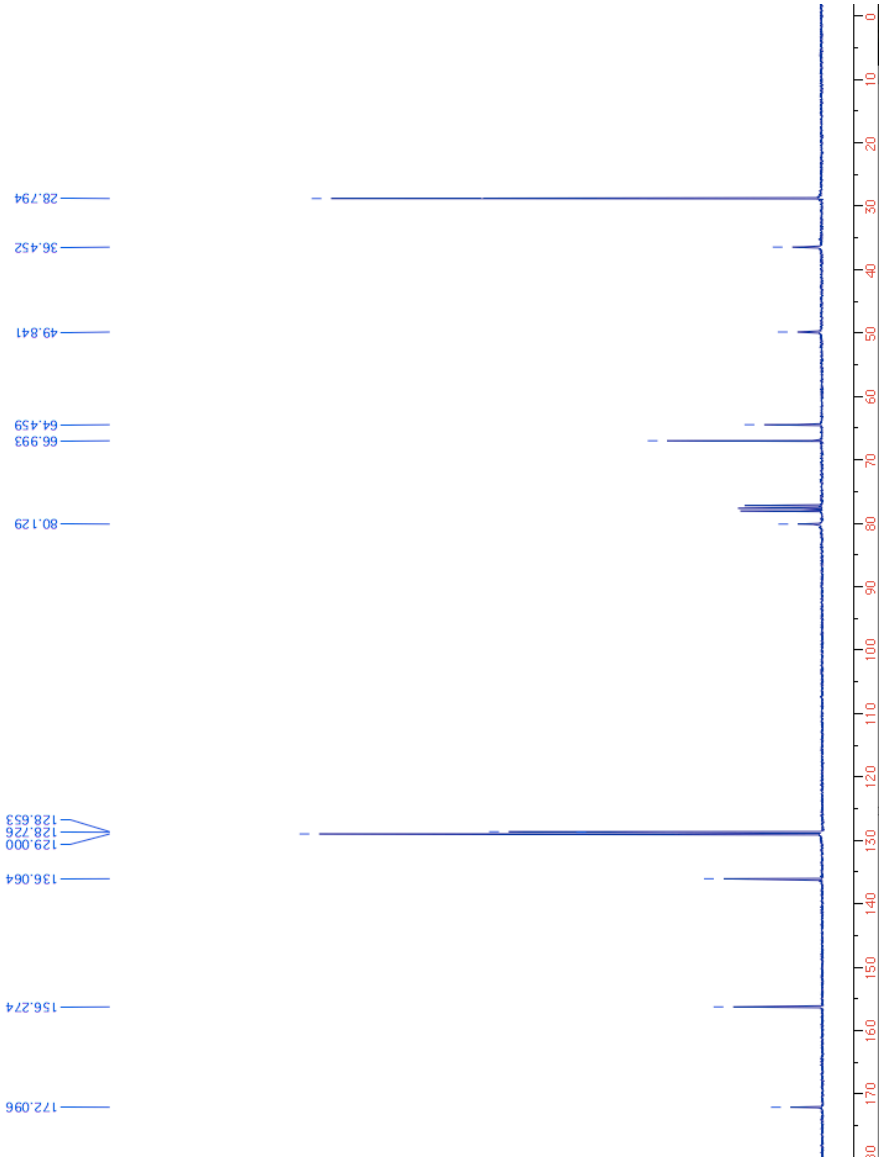
^{13}C NMR (75.5 MHz, CD_3OD) **(*R*)-3-(*tert*-butoxycarbonylamino)-4-hydroxybutanoic acid**



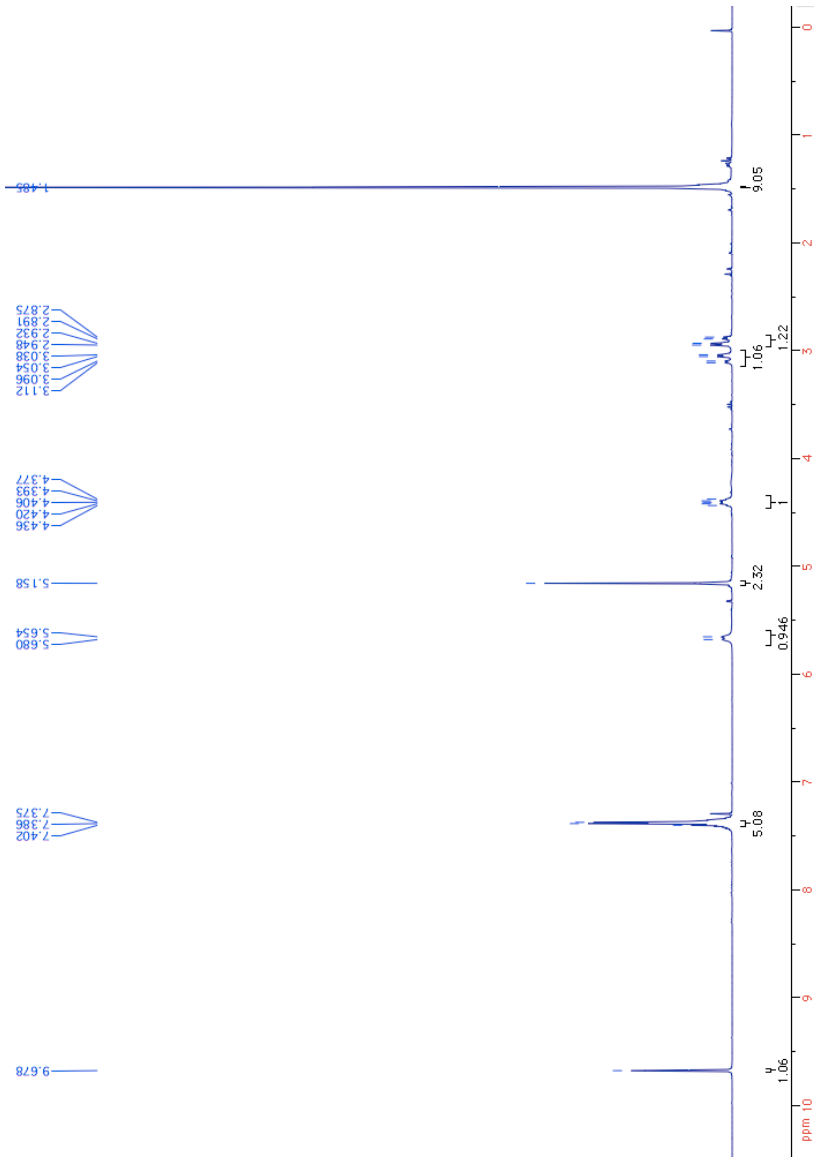
^{1}H NMR (300 MHz, $CDCl_3$) **(*R*)-benzyl 3-(*tert*-butoxycarbonylamino)-4-hydroxybutanoate**



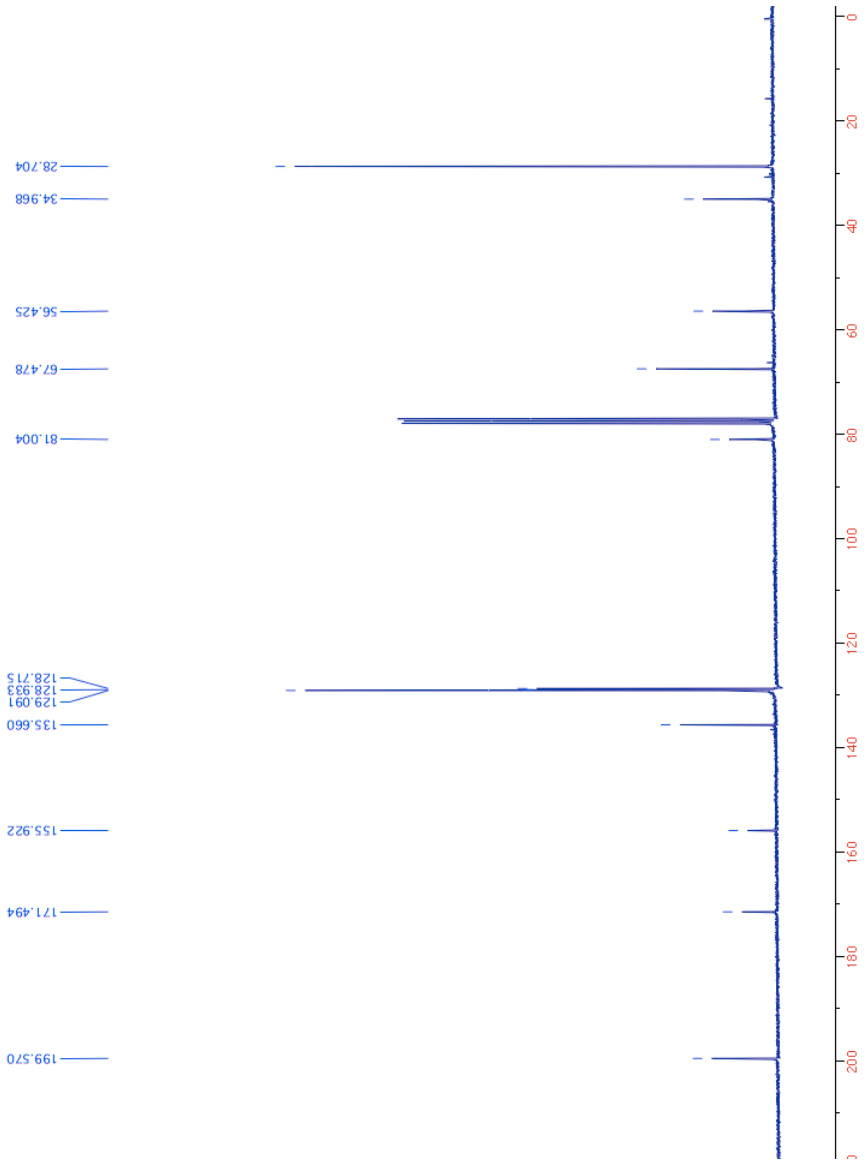
^{13}C NMR (75.5 MHz, CD_3OD) **(*R*)-benzyl 3-(*tert*-butoxycarbonylamino)-4-hydroxybutanoate**



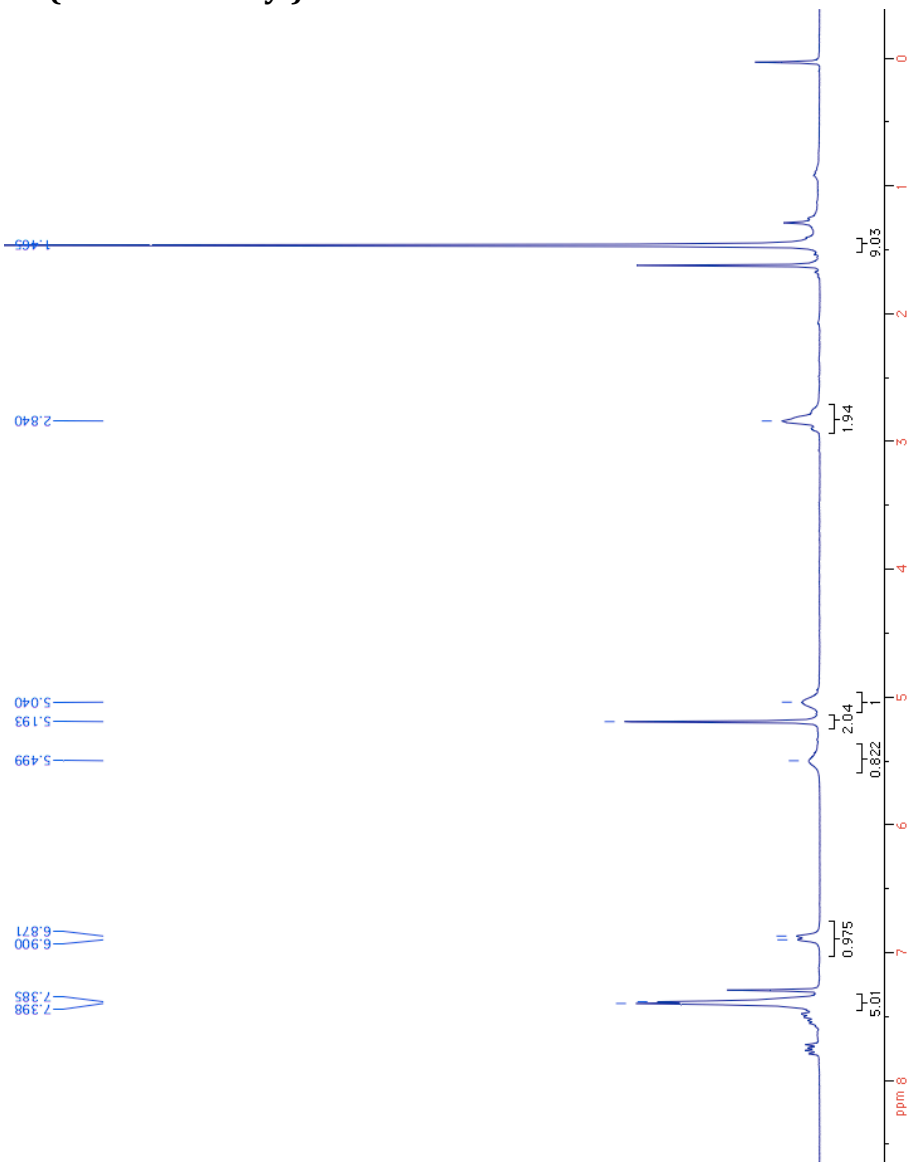
^{1}H NMR (300 MHz, $CDCl_3$) **(*R*)-benzyl 3-(*tert*-butoxycarbonylamino)-4-oxobutanoate**



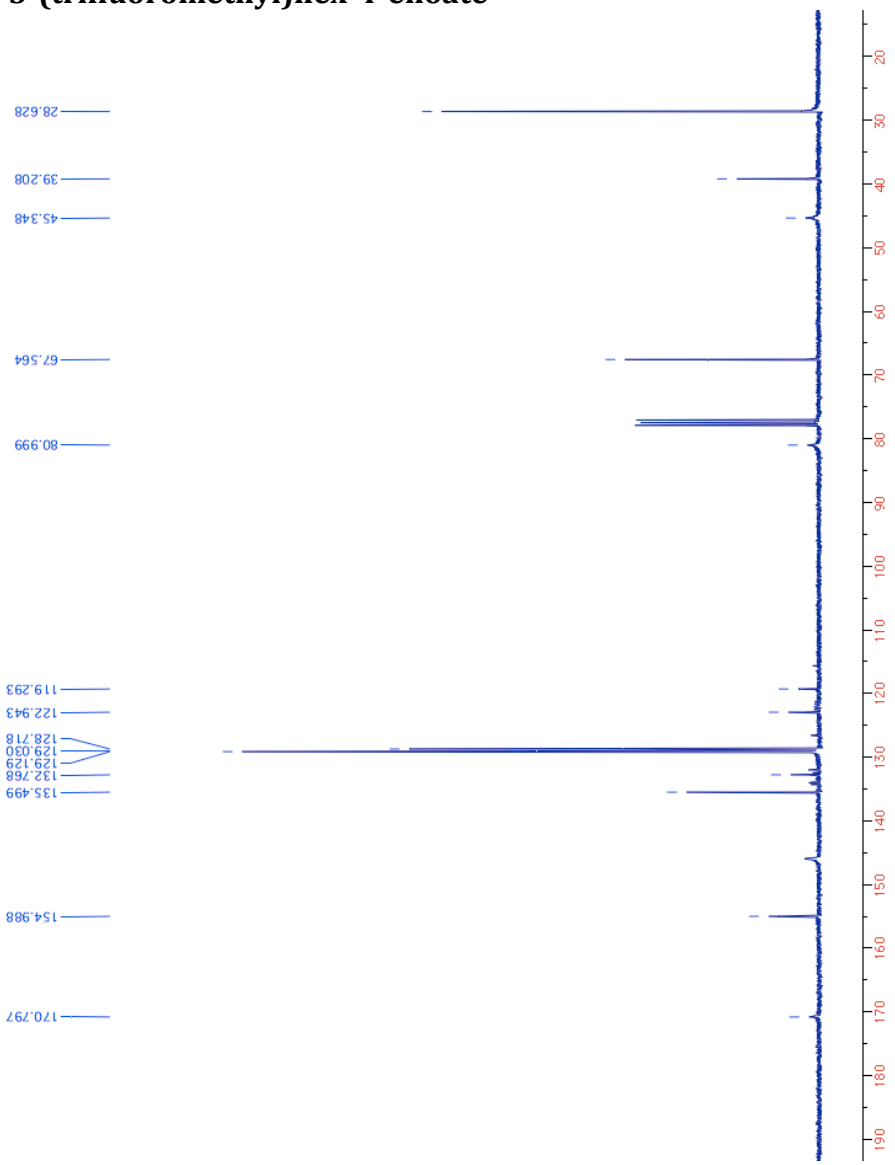
^{13}C NMR (75.5 MHz, CD_3OD) **(*R*)-benzyl 3-(*tert*-butoxycarbonylamino)-4-oxobutanoate**



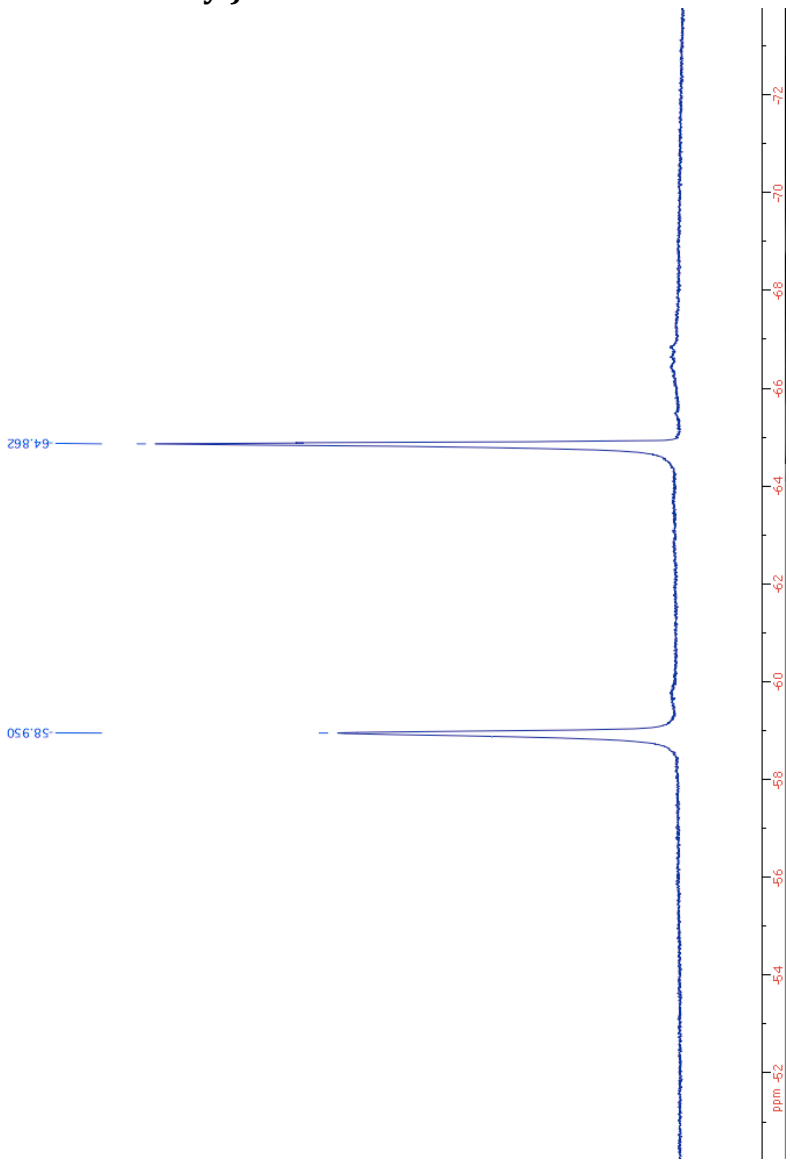
^{1}H NMR (300 MHz, $CDCl_3$) **(*R*)-benzyl 3-(*tert*-butoxycarbonylamino)-6,6,6-trifluoro-5-(trifluoromethyl)hex-4-enoate**



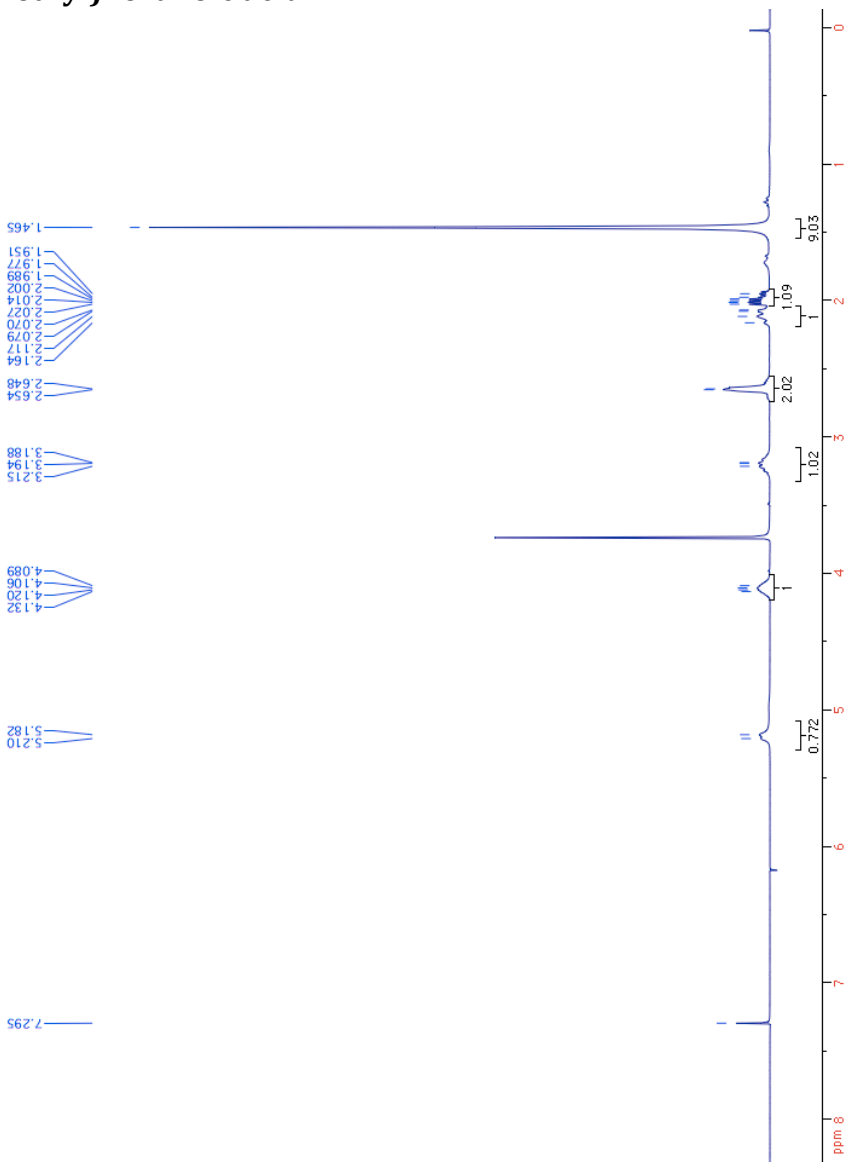
^{13}C NMR (75.5 MHz, CD_3OD) **(*R*)-benzyl 3-(*tert*-butoxycarbonylamino)-6,6,6-trifluoro-5-(trifluoromethyl)hex-4-enoate**



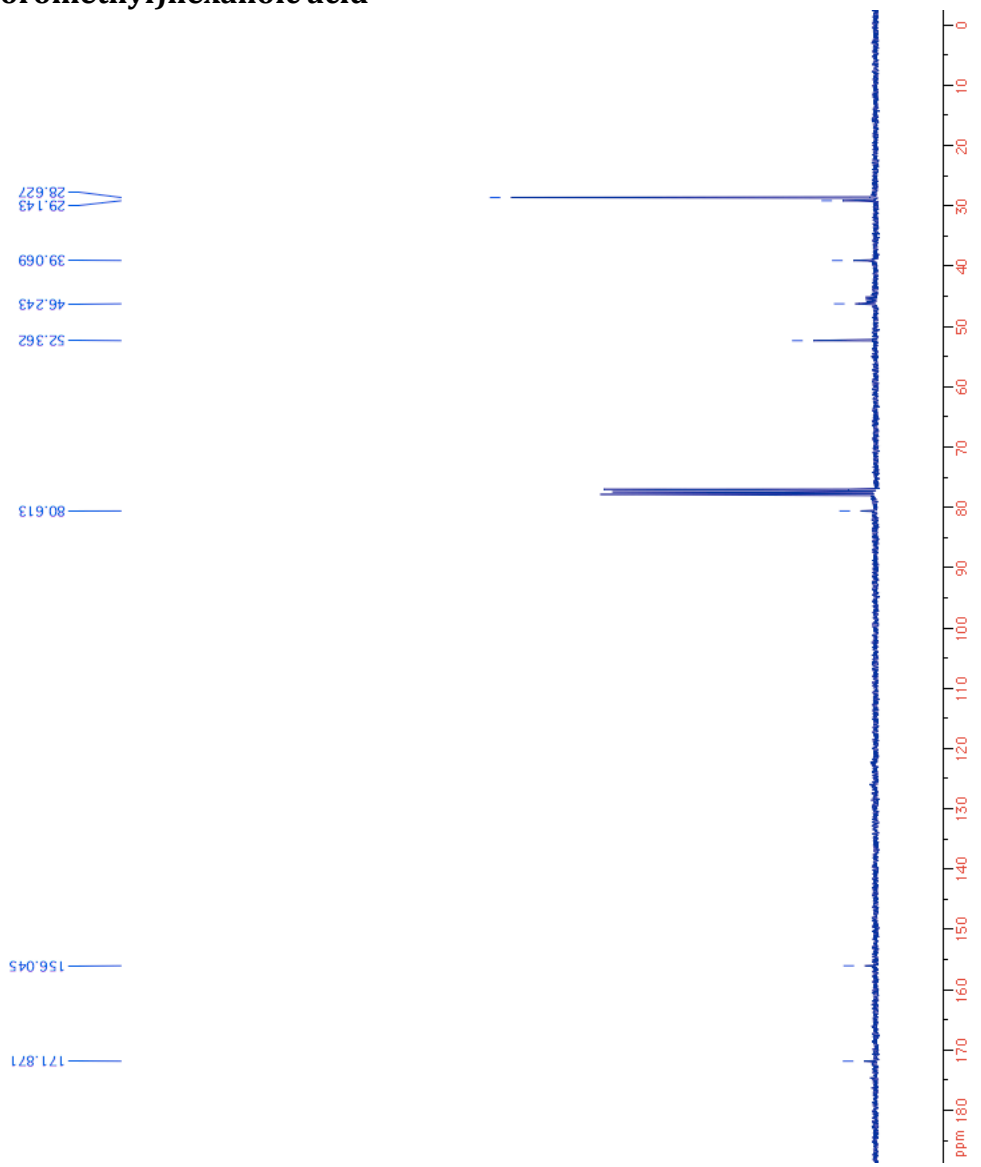
^{19}F NMR (282.6 MHz, $CDCl_3$) **(*R*)-benzyl 3-(*tert*-butoxycarbonylamino)-6,6,6-trifluoro-5-(trifluoromethyl)hex-4-enoate**



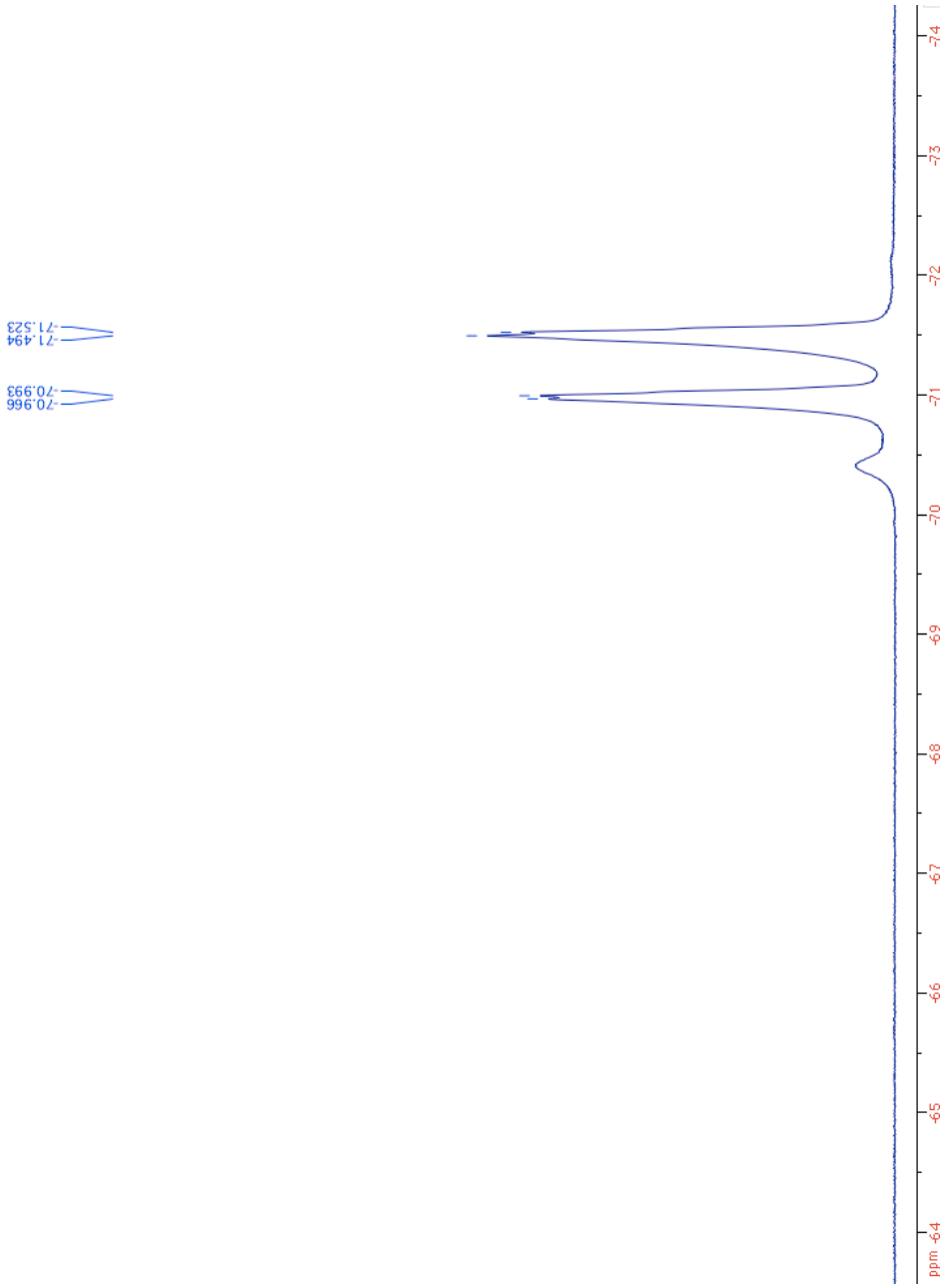
^{1}H NMR (300 MHz, $CDCl_3$)**(*S*)-3-(*tert*-butoxycarbonylamino)-6,6,6-trifluoro-5-(trifluoromethyl)hexanoic acid**



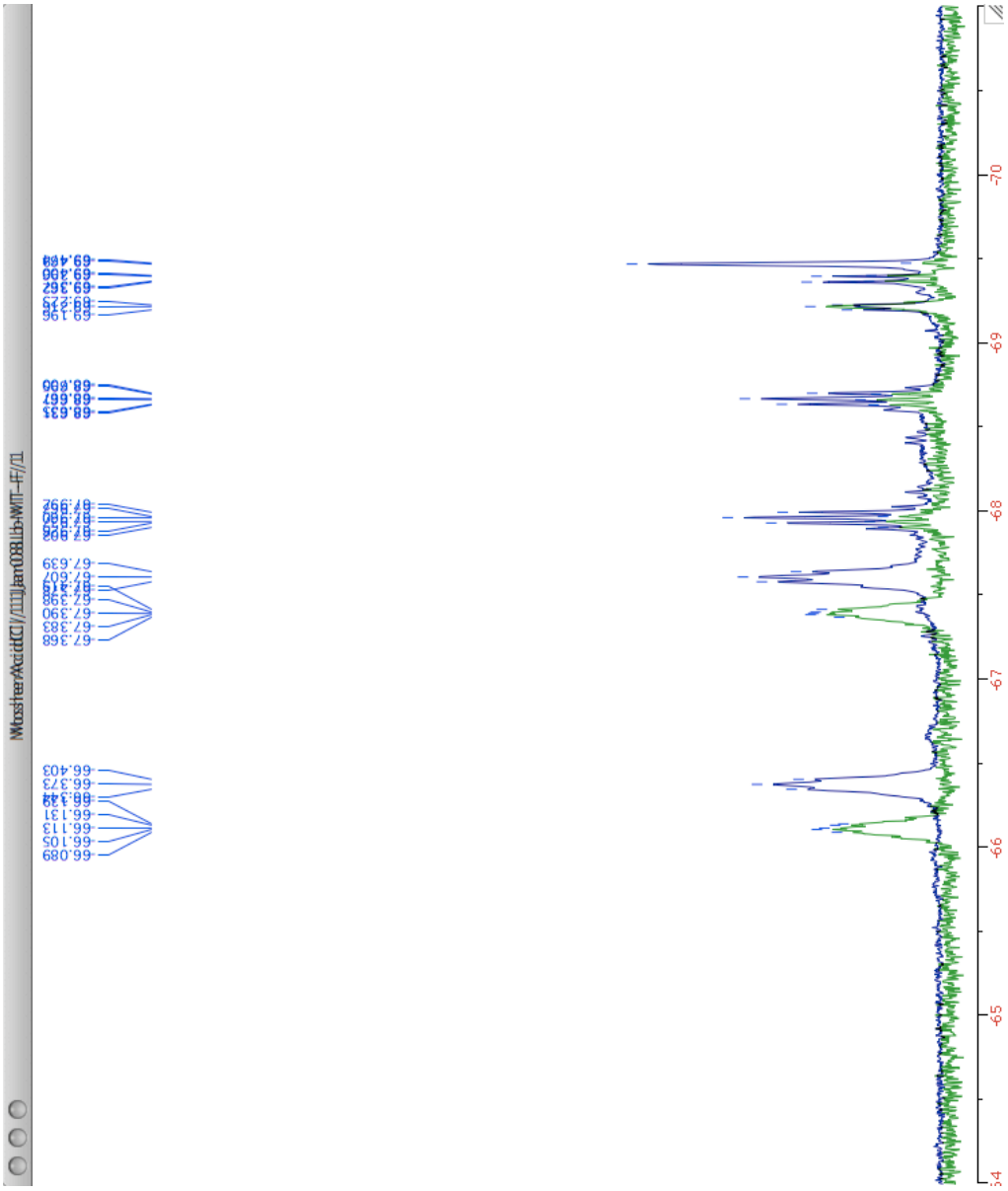
^{13}C NMR (75.5 MHz, CD_3OD) **(*S*)-3-(*tert*-butoxycarbonylamino)-6,6,6-trifluoro-5-(trifluoromethyl)hexanoic acid**



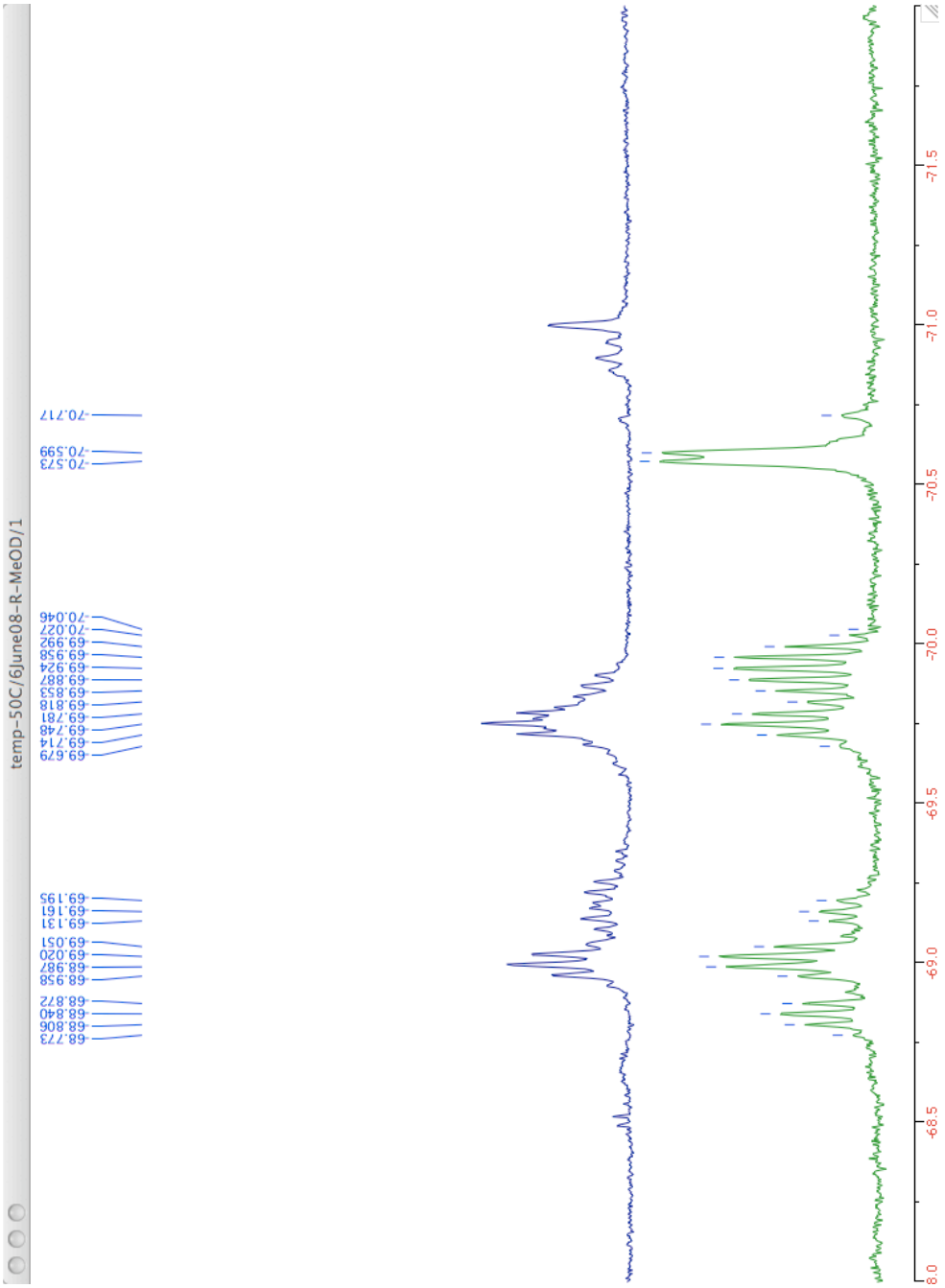
^{19}F NMR (282.6 MHz, $CDCl_3$) **(*S*)-3-(*tert*-butoxycarbonylamino)-6,6,6-trifluoro-5-(trifluoromethyl)hexanoic acid**



^{19}F NMR (282.6 MHz, $CDCl_3$) R-(+)-Mosher Amide of **(*S*)-3-amino-6,6,6-trifluoro-5-(trifluoromethyl)hexanoic acid (green)**overlaid **on** S-(-)-Mosher Amide of **(*S*)-3-amino-6,6,6-trifluoro-5-(trifluoromethyl)hexanoic acid (blue)**



Detail of ^{19}F NMR (282.6 MHz, $CDCl_3$) R-(+)-Mosher Amide of **(*S*)-3-amino-6,6,6-trifluoro-5-(trifluoromethyl)hexanoic acid (green)**overlaid **on** S-(-)-Mosher Amide of **(*S*)-3-amino-6,6,6-trifluoro-5-(trifluoromethyl)hexanoic acid (blue)** (AT ROOM TEMPERATURE)



^{19}F NMR (282.6 MHz, $CDCl_3$) R-(+)-Mosher Amide of **(*S*)-3-amino-6,6,6-trifluoro-5-(trifluoromethyl)hexanoic acid (green)**overlaid **on** S-(-)-Mosher Amide of **(*S*)-3-amino-6,6,6-trifluoro-5-(trifluoromethyl)hexanoic acid (blue)**
(AT 50°C)