T-0632 Amine Analogs as Agonists for GLP-1 Receptor

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T-0632 Amine Analogs as Agonists for GLP-1 Receptor

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Submitted in Partial Fulfillment
of the
Prerequisite for Honors
in Biochemistry
under the advisement of David Haines

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ABSTRACT

The glucagon-like peptide-1 receptor (GLP-1R) is implicated in the pancreatic signaling pathway necessary for insulin secretion. As such, selective stimulation of GLP-1R may mitigate the hyperglycemic effects of type 2 diabetes mellitus (T2DM). Previous studies demonstrate that the non-peptide small molecule T-0632 (T-06) binds GLP-1R without generating a downstream response. We intend to improve the current understanding of GLP-1R binding and signal propagation by chemically modifying T-06 to increase the extent of GLP-1R active site interaction. To do this, we produced an analog of T-06 in which an amine group was inserted at the standard carboxylic acid locus. In addition to altering enzyme-substrate interactions in its own right, this amine group also served as an attachment site for a His-Gly-Glu tripeptide, which was assembled via solution-phase peptide synthesis. This tripeptide is believed to be of particular physiological import in terms of binding and activating GLP-1R in vivo. We expect this T-06 analog to interact with the GLP-1R binding site in a way that mimics binding and activation events between GLP-1R and its natural substrate, thereby reproducing the conditions necessary for GLP-1R activation. Future studies will determine whether these small molecule ligand mimetics have pharmacological potential as type 2 diabetes therapeutics.
INTRODUCTION

Type 2 Diabetes

Type 2 diabetes mellitus (T2DM) is a global health concern, affecting an estimated 371 million people worldwide. According to the Center for Disease Control, diabetes-associated complications were the seventh leading cause of death in 2010. Although the mechanisms of T2DM onset and progression span several organ systems, the primary symptom associated with T2DM is hyperglycemia, or elevated levels of blood-borne glucose. If left untreated, however, the long-term effects of T2DM include blindness, heart disease, and kidney failure. Although healthy diet and exercise are somewhat effective in managing hyperglycemia, additional pharmacological treatments are necessary in order for diabetic patients to fully manage their symptoms. As such, T2DM is a topic of intense investigation in academic, medical, and pharmaceutical institutions.

In a healthy individual, food is converted to glucose through digestive processes and released into the bloodstream through cellular channels in the small intestine. This triggers the release of the digestive hormones gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) from intestinal K- and L-cells, respectively. GIP and GLP-1 localize to the membranes of pancreatic β-cells, where they bind and activate associated transmembrane receptors. Activation of these receptors generates a molecular signaling cascade that results in the production and secretion of insulin into the bloodstream. Once introduced to the bloodstream, insulin promotes glucose uptake into tissue cells throughout the body for storage or metabolic processing (Figure 1).
Figure 1. Schematic of Type 2 Diabetes: Physiological Effects. The mechanisms of type 2 diabetes mellitus are shown. (1) Digestion produces glucose from ingested food. (2) Glucose passes through the small intestine into the bloodstream. (3) In response, the pancreas releases hormones to stimulate insulin production. (4) The quantity of insulin produced is insufficient for glucose absorption by somatic tissues as a result of insulin insensitivity. (5) Glucose remains in the bloodstream, causing hyperglycemia and its downstream effects. Adapted from Rother, et al. 3

This process, known as the incretin effect, is impaired in diabetic individuals. 5 A number of factors, possibly acting in concert, could result in a compromised incretin system. For instance, the mechanisms of insulin production or secretion could be damaged or defective, resulting in reduced concentrations of blood-borne insulin. 6 Additionally, tissue cells may not be responsive to the concentration of insulin produced, a phenomenon known as insulin insensitivity. 3, 6 Regardless of the cause, the body attempts to compensate for its defective incretin effect by upregulating the activity of the pancreatic β-cells. This in turn promotes β-cell
burnout and apoptosis. With fewer available β-cells, the hyperglycemic effects are augmented, expediting the progression of T2DM and its associated effects.¹ ⁴

Current pharmacological research has approached T2DM treatment with respect to insulin production. In an effort to circumvent insulin insensitivity, many proposed treatment methods involve generating sufficiently high insulin concentrations to facilitate glucose uptake in the body's tissues.³ ⁵ Insulin injection, a standard diabetes treatment, is one such way to accomplish this.⁵ However, insulin has been observed to cause weight gain and high blood pressure in patients, both of which amplify the risk of heart disease in an already susceptible population.⁷⁻⁸ Moreover, a treatment regimen of 2-3 insulin injections per day reduces patient compliance.⁸ Alternative means of insulin upregulation have been explored; a broad branch of modern pharmacological study has focused on the administration of recombinant proteins, namely incretin hormones, in order to combat hyperglycemia.⁶

GLP-1 and Analogs

As an essential incretin hormone, GLP-1 and its associated receptor, GLP-1R, have been extensively studied for their potential in T2DM therapeutics. GLP-1 is a 30-residue polypeptide encoded by the proglucagon gene.⁹ Following translation, the first 6 residues of the N-terminus are cleaved to yield GLP-1 in its mature form, (commonly referred to as GLP-1₁⁻³⁶).⁹ Its structure consists of an N-terminal loop containing primarily polar residues and an amphipathic α-helix (Figure 2A,B).¹⁰⁻¹¹ Manufactured in the intestinal L-cells, GLP-1 is secreted into the bloodstream in response to carbohydrate and lipid ingestion.⁹ Due to its role in the incretin effect, GLP-1 is characterized as an insulin inducer.⁹⁻¹⁰ Studies by Toft-Nelsen and Kjems revealed that the injection of GLP-1 hormone immediately following a meal significantly decreased blood glucose levels and increased satiety among diabetic patients.⁷ ¹² However, these effects are short-lived;
native GLP-1 has a half-life of approximately 1 minute, as it is rapidly hydrolyzed by dipeptidyl peptidase 4 (DPP-IV). DPP-IV cleaves GLP-1 between its second and third residues, rendering GLP-1 ineffective in binding and activating GLP-1R. Due to its short half-life, very little GLP-1 arrives at the pancreatic β-cell intact.

DPP-IV degradation makes GLP-1 an inefficient treatment method in its own right. However, small modifications to the molecular structure of GLP-1 have generated an entire field of pharmacological therapies to treat T2DM. For instance, the marketed pharmaceutical exenatide (brand names, Byetta and Bydurean), is a synthetic version of exendin-4 (Ex-4), a polypeptide extracted from gila monster saliva. Sequence alignment revealed that Ex-4 shares 53% sequence homology with GLP-1 (Figure 2A, C). An important distinction between Ex-4 and its homolog is the alanine-to-glycine substitution at the second residue, thus preventing DPP-IV recognition and digestion (Figure 2A). Exenatide has a similar pharmacological identity to GLP-1. As a matter of fact, evidence suggests that exendin-4 may bind the GLP-1 receptor with higher affinity that GLP-1 itself. According to clinical trials by Iltz and colleagues, exenatide reduced fasting plasma glucose concentrations in diabetic patients. Dose-dependent weight loss was also observed in these patients. Although exendin-4 and other GLP-1 mimetics are effective long-term treatment options for T2DM, there is substantial room for improvement. In particular, the relatively short half-life of GLP-1 mimetics requires that individuals with diabetes receive multiple injections throughout the day. Like insulin, GLP-1 mimetic injections are associated with limited patient compliance. Therefore, small-molecule pharmaceuticals pose a potential solution as an orally-administered drug. Such alternative treatments require a more nuanced understanding of the GLP-1 receptor's binding parameters, however.
GLP-1R

GLP-1R is a transmembrane protein expressed on the surface of pancreatic β-cells. When bound to the GLP-1 ligand, GLP-1R induces the downstream production of cyclic adenosine monophosphate (cAMP), a secondary messenger in the incretin signaling pathway. Analysis of the GLP-1R structure indicates its membership in the B family of G-protein coupled receptors (GPCRs). As is common among B family GPCRs, GLP-1R has a long, extracellular N-terminus including three disulfide bridges (Figure 3). Additionally, GLP-1R possesses a 7-pass transmembrane domain known as the integral membrane core domain (7TM),
and an intracellular C terminus (Figure 3).\textsuperscript{22} Class B GPCRs including GLP-1R are proposed to activate according to a two-step model (Figure 4).\textsuperscript{18, 25-26} First, the C-terminal region of GLP-1 is proposed to bind to the extracellular N-terminus of GLP-1R (Figure 4A).\textsuperscript{18}

This localizes the N-terminus of GLP-1 to the 7TM domain in order to promote an interaction (Figure 4B).\textsuperscript{26} The second binding event causes a conformational change in the intracellular portion of the molecule, activating GLP-1R and triggering a signal cascade that ultimately releases insulin from the pancreatic \( \beta \)-cells (Figure C, D).\textsuperscript{25}
Evidence from previous studies has demonstrated that GLP-1R likely binds ligand according to this two-step model. For instance, Mann and colleagues isolated the extracellular N-terminal domain of GLP-1R and found through subsequent binding studies that both exendin-4 and GLP-1 bound to the truncated receptor with the same affinities observed in the wild type receptor.\textsuperscript{27} According to research by Gong and colleagues, ligand binding to the ECD is primarily controlled by the amphipathic α-helix domain of the ligand.\textsuperscript{14} For instance, the binding affinity between GLP-1R and Ex-4 was not affected if residues 1-8 of the N-terminus of Ex-4 were deleted.\textsuperscript{14, 28} However, it is worth noting that truncated Ex-4 (9-39) behaved as an antagonist, whereas native Ex-4 is an agonist.\textsuperscript{19, 28-29}

Additional study of the C-terminal region in both GLP-1 and Ex-4 demonstrated that the hydrophobic face of the α-helical domain was primarily involved in docking the ligand to the receptor without conferring receptor activation. For instance, Swedberg and colleagues revealed that chimeric proteins consisting of the N-terminal region of either Ex-4 or GLP-1 and an arbitrary α-helix at the C-terminal end could bind and activate GLP-1R without any significant
decrease in signal intensity. These results show that the initial ECD binding event on GLP-1R has little effect on subsequent receptor activation. On the other hand, data from this study and others indicate that the ligand's N-terminus must be localized to the binding site in order for such activation to occur. The amphipathic nature of the α-helix allows for both hydrophilic and hydrophobic interactions between ligand and receptor; however, a disproportionately high number of these interactions occur between hydrophobic residues. Crystal structures of the GLP-1R EDC docked with GLP-1 project hydrophobic interactions between Phe^{28*}, Ile^{29*}, Trp^{31*}, Leu^{32*}, and Val^{33*} of GLP-1 with Leu^{32}, Trp^{39}, and Leu^{123} of GLP-1R. Lys^{26*} is the only polar residue in the GLP-1 α-helix that is suspected to engage in polar interactions with the ECD: specifically with Glu^{128}. Consequently, it appears that nonpolar interactions in the ECD of GLP-1R localize the ligand to the receptor for subsequent interactions to promote binding.

Figure 5. Docking Studies Show Representative Interactions Between N-Terminal Residues of GLP-1 and GLP-1R Binding Pocket. Non-covalent interactions between GLP-1 (yellow, etc.) and the GLP-1R binding cleft (cyan) are shown between transmembrane regions 1 and 2 (A), as well as 5 and 6 (B). Residues of interest within the ligand are highlighted with various colors, while salient residues within the binding pocket itself are denoted as green (A) or red (B) sticks. Adapted from Dods & Donnelly.
As discussed above, prior research suggested that removal of the first 8 N-terminal residues changed the pharmacological identity of Ex-4 from that of an agonist to an antagonist.\textsuperscript{16, 27} Likewise, removal of the initial N-terminal residues of GLP-1 yielded a prohibitive impact on receptor activity.\textsuperscript{11, 18, 20} Thus, the N-terminal region of GLP-1 was implicated in activating GLP-1R. Through a series of site-directed mutagenesis experiments, Dods and Donnelly predicted a model for ligand-mediated activation of GLP-1R (Figure 5).\textsuperscript{20} Within this model, the hydrophilic N-terminal residues are integrated into the 7TM region, disrupting the existing network of polar interactions at the base of the binding pocket.\textsuperscript{20} This, in turn, causes the conformational change associated with GLP-1R activation. Among these polar interactions, His\textsuperscript{7}\textsuperscript{*} is suspected of interacting with Arg\textsuperscript{310}, Arg\textsuperscript{190}, and Tyr\textsuperscript{152} (Figure 5). His\textsuperscript{7}\textsuperscript{*} is also projected to engage in electrostatic interactions with Glu\textsuperscript{364}, and Glu\textsuperscript{387} (Figure 5).\textsuperscript{20} Kirkpatrick and colleagues generated similar predictions for polar interactions with His\textsuperscript{7}\textsuperscript{*}; however, they also indicated potential cation-pi interactions with Thr\textsuperscript{149}, Phe\textsuperscript{387}, and Thr\textsuperscript{391} (Table 1).\textsuperscript{21} Additionally, charge-charge interactions were hypothesized to occur between Glu\textsuperscript{9}\textsuperscript{*} and Arg\textsuperscript{90}, Lys\textsuperscript{97}, Lys\textsuperscript{197}, Lys\textsuperscript{288} and Lys\textsuperscript{383}. Gly\textsuperscript{10}\textsuperscript{*}, Phe\textsuperscript{12}\textsuperscript{*}, Thr\textsuperscript{13}\textsuperscript{*}, and Asp\textsuperscript{15}\textsuperscript{*} are also implicated in GP-1R activation.\textsuperscript{19, 21, 29} However, correlational models of GLP-1 residues and the extent of GLP-1R activation demonstrate that His\textsuperscript{7}, Glu\textsuperscript{9}, and Asp\textsuperscript{15} have the most significant impact on EC50/IC50 values (Table 1).\textsuperscript{21}
T-0632: A small-molecule antagonist of GLP-1R

In a clinical setting, peptide-based GLP-1 analogs must be administered to diabetic patients several times a day because they are degraded or excreted in a matter of hours. These drugs are susceptible to hydrolysis in the digestive system and must be administered via injection. Such treatments are met with limited patient compliance due to discomfort and inconvenience. Therefore, engineering a small-molecule agonist to GLP-1R has distinct appeal, as it could be administered orally and has the potential for a longer half-life \textit{in vivo}.\footnote{4} \footnote{5} \footnote{32} Unfortunately, very few small-molecules are known to interact with GLP-1R.\footnote{32} Among the existing classes of small-molecule GLP-1R substrates, (S)-3-[1-(2-fluorophenyl)-2,3-dihydro-3-[(3-isoquinolinyl-carbonyl)amino]-6-methoxy-2-oxo-1H-indole] propanoate, (hereetofore referred to as T-0632, or T-06), has received attention as a potential scaffold from which a GLP-1R agonist could be developed (Figure 6).\footnote{32}
Figure 6. T-0632 Molecular Structure. The molecular structure of T-0632 (T-06) is shown. Generated with ChemDraw software.\textsuperscript{33}

T-0632 is a man-made organic molecule that was initially synthesized to serve as an antagonist to the cholecystokinin receptor 1 (CCK\textsubscript{A}) in order to treat pancreatitis (Figure 6).\textsuperscript{32} However, Tibaduiza and colleagues found that T-06 not only inhibited CCK\textsubscript{A} receptor activity: it also reduced the activity of the incretin effect.\textsuperscript{34} Further investigations revealed this unwanted side effect to be the result of T-06 interaction with GLP-1R.\textsuperscript{34} Due to the diminished prevalence of cAMP, a downstream product of GLP-1R activation, in the presence of T-06, this molecule was classified as an antagonist.\textsuperscript{32} Although its effects on GLP-1R activity are significant, its binding affinity is relatively low; whereas T-06 binds the CCK\textsubscript{A} receptor on a sub-nanomolar scale, its affinity for GLP-1R is on a micromolar scale.\textsuperscript{32} As such, T-0632 is useful as an investigative tool for GLP-1R binding, but measures of its biological activity are more efficiently observed in systems expressing the CCK\textsubscript{A} receptor.\textsuperscript{13}

Site-directed mutagenic studies of GLP-1R in the presence of T-06 revealed that the recognition and binding of GLP-1R by T-06 is mediated by Trp\textsuperscript{33}.\textsuperscript{34} Situated in the extracellular domain of GLP-1R, Trp\textsuperscript{33} and T-06 are suspected to interact via pi-stacking between the aromatic functional groups in each molecule (Figure 7).\textsuperscript{34} These interactions may stabilize the inactivated or "closed" state of GLP-1R, hence the reduction in activity.\textsuperscript{32} Bound to Trp\textsuperscript{33}, T-06 is separated from the 7TM domain by approximately 10 Angstroms.\textsuperscript{32,35} Further investigation of
the interactions between T-06 and GLP-1R suggest that the carboxylic acid moiety of T-06 is localized to the 7TM binding cleft (Figure 7). For example, this acidic functional group is projected to engage in charge-charge interactions with Lys$^{288}$ of the fourth transmembrane domain (TM4), among other residues. Such polar interactions in the binding cleft are reminiscent of those observed between GLP-1R and the N-terminal residues of GLP-1. Thus, the projected binding orientation of T-06 in GLP-1R provides a favorable platform for generating a small-molecule agonist to GLP-1R.

**Figure 7. Proposed Interactions Between T-06 and GLP-1R.** Predicted noncovalent interactions between T-06 and GLP-1R are shown; this includes pi-stacking interactions between the isoquinoline group of T-06 and tryptophan 33 (W33), as well as the potential electrostatic interactions between the carboxylic acid moiety of T-06 and basic residues in the GLP-1R binding cleft. The R-groups of relevant GLP-1R residues are shown, (not to scale). Adapted from Wilson$^{13}$ and Kim.$^{35}$

Previously, T-0632 analogs have been generated in order to achieve alternate structural characteristics.$^{13,35-36}$ Among these analogs, Wilson generated a propyl amine form of T-0632 (known as T-06 amine), in which the carboxylic acid moiety was substituted with that of a primary amine moiety.$^{13}$ Not only does this T-06 analog possess possible alternative interactions with GLP-1R; its primary amine functional group has potential to serve as a C-terminal handle to
an amino acid sequence. The purpose of this study is to produce and investigate two T-06 analogs, including a potential GLP-1 mimetic containing an N-terminal tripeptide (Figure 8). The biological activity of these compounds will be compared with that of the original T-0632 molecule in vitro. Biological studies could improve insight into the spatial relationships between T-06 and the GLP-1 and CCK₄ receptors. Such studies would also illuminate the role of the His-Gly-Glu tripeptide in GLP-1R binding and activation.

Figure 8. Simplified Synthetic Mechanism. The molecular structures for the amine T-06 precursor, T-06 nitrile (A), amine T-06 (B), and His-Gly-Glu-amine T-06 (C) are shown. Generated with ChemDraw Software.³³

Present Research

This study intends to generate an analog of T-0632 in which the carboxylic acid moiety is replaced with a primary amine (Figure 8B). In order to do this, amine T-06 will be generated through the reduction of its molecular precursor, T-0632 with a nitrile adduct. As of yet, the impact of the amine functional group on T-06 binding and activation of GLP-1R is unknown. Therefore, we will subject this compound to biological testing. The binding orientation of T-06 amine is predicted to be different from that of T-06, as this basic functional group may disrupt the charge interactions between T-06 and the 7TM domain, particularly at Lys²⁸⁸. This disruption may impact the activity of GLP-1R, promoting a conformational change that stimulates
downstream cAMP production. Conversely, novel hydrostatic repulsions in this region could alter the orientation with which amine T-06 interacts with the receptor overall, reducing ligand-receptor affinity.

In addition to amine T-06, an alternative T-06 analog and potential GLP-1 mimetic will be produced (Figure 8C). To accomplish this, a His-Gly-Glu tripeptide is coupled to the primary amine moiety of amine T-06 using solution-phase peptide synthesis. The His-Gly-Glu tripeptide was selected based on previous studies in which these GLP-1 residues were found to have the most significant impact on activation of the GLP-1 receptor (Table 1). Because solution-phase peptide synthesis can be carried out in mild conditions and is commonly associated with high yields, we hypothesize that peptide coupling to the amine T-06 analog will be a stable and efficient synthetic process.

In order to assay the biological activity of the amine T-06 and His-Gly-Glu-amine T-06 analogs relative to the standard T-06 molecule, cell studies will be carried out in both GLP-1 and CCK1A receptors. In vitro assays will be conducted in HEK cell lines, stably expressing one of the above mentioned GPCRs. Biological activity will be quantified in terms of downstream cAMP production by these cellular receptors. These studies will be conducted at a collaborating laboratory in Mayo Clinic’s Scottsdale, Arizona campus. If a change in the cAMP response is observed among the T-06 analogs, these results could bear pharmacological significance for T2DM treatment studies using small molecule drugs. For example, these experiments could provide insights into the structure-function relationship of GLP-1R, including the mechanisms of its binding and activation. Moreover, the relative biological activity of the proposed T-06 analogs may support the study of small molecule antagonists as potential T2DM drug candidates.
RESULTS AND DISCUSSION

Reduction of T-0632 Nitrile

Amine T-0632 was produced from T-0632 nitrile through a nitrile reduction mechanism (Figure 9). The presence of unsaturated functional groups throughout the molecular structure of T-0632, including carbonyls, isoquinoline, and benzene rings, require sufficiently mild reducing conditions to prevent unwanted hydrogenation. Sodium borohydride is generally not a potent reducing agent against nitriles, amides, or other nitro compounds. However, in the presence of transition metal salts, sodium borohydride efficiently reduces nitrile to a primary amine through a mechanism that has not fully been elucidated. It is postulated that nitrile forms a complex with the metal boride surface, which in turn activates the nitrile and allows hydride addition to occur. The transition metals are hypothesized to behave as catalysts, generating boride salts and increasing the rate and yield of these hydrogenation reactions.

Synthesis of the propyl amine analog of T-0632 was approached using two separate techniques (Figure 10). In each case, the T-0632 nitrile analog was obtained from a member of the Haines laboratory. According to the first synthetic scheme designed by Wilson, (2014), sodium borohydride and cobaltous chloride were used to mediate reduction of the nitrile functional group. Previous syntheses of T-0632 amine using these methods were reported to produce the desired product at an experimental yield of 50%. However, repeated attempts of this protocol failed to convert the nitrile functional group to the amine. It has been noted throughout the relevant literature that nitrile reduction may have vastly different outcomes depending on small adjustments to the experimental conditions. For example, alternative transition metals have previously been used in nitrile reduction reactions: nickel(II) being among
the best and most characterized of these alternatives.\textsuperscript{38-39} Additionally, it is possible that nitrile reduction is facilitated by specific experimental conditions, such as an anhydrous environment or continuous exposure to hydrogen gas over the course of the reaction; this will be discussed in more detail in the subsequent section.\textsuperscript{40}

\textbf{Figure 9. Synthetic schemes of T-0632 nitrile reduction methods.} Reduction using cobalt chloride and sodium borohydride (A), and nickel (II) chloride and sodium borohydride with Boc anhydride added (B).

The second attempted technique, adapted from Caddick and colleagues, utilized sodium borohydride and nickel chloride to catalyze nitrile reduction.\textsuperscript{39} This protocol also incorporated
Boc anhydride to protect the newly formed primary amine from unwanted side reactions: namely dimerization through nucleophilic attack of one primary amine on the intermediate imine of another T-06 molecule, resulting in an aldimine (Figure 10B). The Boc-protected amine product was also more readily distinguishable from its reactants in NMR spectroscopic analysis. Sodium borohydride was added to a stirring solution of T-06 nitrile, nickel chloride, and Boc anhydride over a period of 30 minutes (Figure 9). The reaction was initially conducted at 0°C in order to minimize thermally-driven byproduct formation. After an additional 16 hours of stirring at room temperature, the reaction was halted with ethylenediamine and subsequently diluted in ethyl acetate. The organic layer was washed with saturated sodium bicarbonate and dried with anhydrous sodium sulfate before solvent and reactants with low boiling points were removed under reduced pressure. NMR analysis suggested that nitrile reduction was unsuccessful (Appendix 1). Proposed improvements to the existing nitrile reduction protocol primarily involved isolated changes to the previously utilized reagents. For example, Khurana and colleagues reported that the experimental yields of primary amines through nitrile reduction with nickel chloride could be improved by performing the reaction under anhydrous conditions in ethanol. Moreover, the efficiency of nickel-catalyzed nitrile reduction reactions was improved by applying slightly pressurized hydrogen gas to the system, indicating that it was a hydrogenation reaction that mediated primary amine formation, as opposed to a hydride reduction reaction.

A second iteration of this protocol was conducted, in which initial reactants were added under a steady stream of nitrogen gas and the overnight reaction was carried out in low-pressure catalytic hydrogenation conditions with ethanol as the solvent. The products were purified and a 30% yield of Boc-protected T-06 amine was confirmed with 1-dimensional and 2-dimensional
NMR spectroscopy (Appendix 2; Appendix 3). Because the O-tert-butyl functional group is sensitive to protonation and subsequent dissociation, Boc deprotection was accomplished with trifluoroacetic acid (TFA) and anisole. As a result, a tert-butyl cation dissociates from the Boc system to be subsequently trapped by anisole via electrophilic aromatic substitution. Electronic rearrangement of the remaining carbamic acid releases carbon dioxide from the solution, producing the primary T-06 amine (Mechanism not shown). Trituration of the product in a solution of dichloromethane and hexanes at a 1:9 ratio yielded pure amine T-06 salt (Appendix 4). A portion of this product was stored for subsequent in vivo cell studies.
Figure 10. Mechanism of T-06 amine by-product formation. The anticipated progression of nitrile to imine to primary amine (A), dimerization through aldimine formation (B), and the synthesis of T-06 alcohol (C) are shown.
Synthesis of His-Gly-Glu Tripeptide

Tripeptide synthesis was initially attempted independent of the propyl amine T-0632 analog according to the experimental mechanism proposed by Smith (2008). These reactions were carried out according to a solution-phase peptide synthesis mechanism. Residues were added in a carboxy-to-amino terminal direction, in which each of the subsequent amino acids was protected at its amino terminus with an Fmoc functional group. Therefore, peptide bond formation was limited to the N-terminus of the preceding amino acid and the C-terminus of the subsequent residue.

Fmoc-protected glycine was added to Glu(OtBu)-OMe, in an 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) coupling reaction in which triethylamine, hydroxybenzotriazole (HOBt), and EDC in dichloromethane were added to the amino acid reactants and stirred for three hours at room temperature. Nucleophilic attack of glycine's hydroxyl group on the partially positive carbon of EDC produces a relatively stable leaving group at the C-terminus of glycine. Thus activated, the C-terminal carbonyl of glycine is susceptible to nucleophilic attack by the HOBt hydroxyl group. The resulting activated ester serves as a stable leaving group for subsequent amide bond formation. The primary amine of Glu(OtBu)-OMe is attracted to the partially positive carbonyl of glycine's C-terminus. Glutamic acid subsequently attacks glycine, displacing the HOBt ester and producing the Fmoc-Gly-Glu(OtBu)-OMe dipeptide (Figure 11). This product was isolated from the organic layer after being washed three times with water and dried over sodium sulfate. The product was further isolated following vacuum filtration and purification via flash chromatography. Proton NMR indicated the formation of the protected dipeptide (Appendix 5).
Figure 11. Synthesis of Fmoc-Gly-Glu(OtBu)-OMe via EDC coupling.

Fmoc deprotection of the amino terminus of glycine was attempted by dissolving the dipeptide product in 20% piperidine in DMF and stirring for 15 minutes. The reaction was diluted in dichloromethane and neutralized by three washes with 0.1 M HCl. This low-pH environment was predicted to protonate the deprotected dipeptide, generating a charged, water-soluble form of the compound. Therefore, the aqueous layer was collected and lyophilized in order to remove excess water. However, NMR analysis revealed that the O-methyl functional group of the protected C-terminus of glutamic acid was present at a lower concentration than other functional groups in the spectrum (Appendix 6). This data suggests that the deprotected amino terminus of glycine underwent nucleophilic attack on the C-terminal carbonyl of glutamic acid, generating a 6-membered ring (Figure 12). Smith reported similar instances of cyclization reactions, in which the dipeptide produced a 6-membered ring due to intramolecular nucleophilic
attack of the de-protected N-terminus of glycine on the C-terminus of glutamic acid, causing the covalently-bound methyl ester to dissociate.

Figure 12. Proposed mechanism of Gly-Glu(OtBu)-OMe cyclization following Fmoc deprotection.

A second attempt at tripeptide synthesis was made in which the primary amine of the previously-synthesized T-06 amine was used as a handle for solution-phase peptide synthesis. EDC coupling techniques were used to covalently bind the C-terminus of Fmoc-Glu(OtBu)-OH to the propyl amine functional group of T-06 amine. As previously described, the two species were combined in the presence of triethylamine, HOBT and EDC (Figure 13). Reactants were stirred for 18 hours and extracted three times with water. After treatment with anhydrous sodium sulfate, the organic layer was evaporated under reduced pressure and examined with NMR spectroscopy. Following purification, it was confirmed that Fmoc-Glu(OtBu)- amine T-06 was
produced at a 52% yield (Appendix 7).

Figure 13. Addition (A) and deprotection (B) of Fmoc-Glu(OtBu)-OH to amine T-0632.

Based on the mechanism of Fmoc-Glu(OtBu)-OH addition, as well as spectral indicia from NMR analysis, chirality was expected at glutamic acid’s α-carbon. However, flash chromatography and other available separation techniques lacked sufficient selectivity to isolate these diastereomers; the respective polarities of these isomers may be nearly equivalent. High-performance liquid chromatography (HPLC) may provide the resolution necessary to separate
these species. Of the unreacted T-06 amine, two additional attempts were made to add Fmoc-Glu(OtBu)-OH, neither of which was successful. This failure to react was attributed to the chemical identity of the T-06 analog; during the nitrile reduction reaction, for instance, it is possible that the intermediate imine functional group was hydrolyzed, producing T-06 alcohol rather than T-06 amine (Fig. 10C). These products were not differentiable though spectroscopic analysis due to the relative impurity of the compound, yet would have vast differences in reactivity during peptide bind formation. Additional NMR analysis of the unreactive samples shows a difference in the chemical environment of the T-06 alkyl chain; the downfield shift of these peaks supported the presence of a hydroxyl group at the primary amine locus of amine T-0632 (Appendix 8).

Fmoc-deprotection of the glutamic acid was accomplished by a 15-minute reaction with 20% piperidine in DMF. The reaction was halted by dissolution in water, followed by the removal of solvent and reactants via lyophilization. Due to mechanical failure of the NMR machine, no data is available for the glutamic acid deprotection event. Glutamic acid deprotection was immediately followed by a coupling reaction with Fmoc-Gly-OH, in which the EDC coupling protocol described above was used to link Fmoc-Gly-OH to the N-terminus of the glutamic acid handle. Flash chromatography over a solvent gradient of ethyl acetate in hexanes produced Fmoc-Gly-Glu(OtBu)-T-06 amine (Appendix 9).
Figure 14. Addition (A) and deprotection (B) of Fmoc-Gly-OH to Glu(OtBu)-amine T-06.

In order to complete the synthesis of His-Gly-Glu-amine T-0632, another Fmoc-deprotection reaction was carried out by dissolving Fmoc-Gly-Glu(OtBu)-T-06 amine in 20%
piperidine in DMF (Figure 14B). Following dilution with water, the organic layer was extracted with dichloromethane and analyzed with NMR spectroscopy. These results supported glycine deprotection (Appendix 10). Boc-His(Boc)-OH was added into a solution of Gly-Glu(OtBu)-T-06, EDC, HOBT, triethylamine, and dichloromethane (Figure 15). After approximately 3 hours of stirring, the organic layer was washed three times with water and subjected to NMR analysis. Due to its miniscule sample size, this product could not be purified further. However, the spectroscopic data supports the successful addition of Boc-His(Boc)-OH to the existing dipeptide-amine T-06 analog. Additional purification and spectroscopic investigation will confirm the identity of this final synthetic product (Appendix 11).

![Figure 15. Addition of Boc-His(Boc)-OH to Gly-Glu(OtBu)-amine T-06.](image)
CONCLUSIONS

Synthesis of the amine T-0632 analog was achieved through nitrile reduction with sodium borohydride and nickel chloride in mild catalytic hydrogenation conditions. This method had more reproducible synthetic success and roughly equivalent experimental yields when compared to previous T-06 nitrile reduction protocols utilizing sodium borohydride and cobaltous chloride.

T-06 amine served as a suitable synthetic handle for solution phase peptide synthesis. The systematic addition of amino acids necessary to produce the desired His-Gly-Glu tripeptide was achieved without the cyclization or side reactions observed in independent tripeptide synthesis reactions. This synthetic protocol may be applicable to the production of alternative small molecule-peptide hybrid compounds.

Biological testing of T-06 amine and tripeptide-amine T-06 analogs will be conducted at Mayo Clinic in order to assess their respective capacities to bind and activate the GLP-1 receptor. T-06 amine is predicted to cause conformational shifts within the binding pocket due to electrostatic repulsions among the positive transmembrane residues of GLP-1R. The tripeptide-T-06 amine analog is further hypothesized to mimic ligand-receptor dynamics at the GLP-1R active site. Significant differences in the binding affinity or receptor activation protocols of either of these compounds as compared to T-0632 would support their future investigation and optimization as potential pharmaceuticals targeting insulin production in type 2 diabetes mellitus.
EXPERIMENTAL

General

All reagents were purchased from Sigma-Aldrich (St. Louis, MO). Solvent evaporation under reduced pressure was accomplished with a Buchi Rotavapor R-114 with Buchi Waterbath B-480 (Buchi, New Castle, DE). Lyophilization was carried out at 100 mTorr and -85°C on a VirTis BenchTop Pro Freeze Dryer (SP Scientific, Gardiner, NY). $^1$H and 2-dimensional Nuclear Magnetic Resonance Spectroscopy experiments were conducted with a Bruker 500 MHZ NMR Spectrometer (Bruker Instruments, Billerica, MA). All NMR samples were dissolved in deuterated chloroform, with tetramethylsilane (TMS) as the standards.

Thin-layer chromatography was conducted with Merck 60-F254 pre-coated silica gel plates and 250 nm UV light. Flash chromatography was carried out using a Biotage Flash Purification system with detection wavelengths set to 254 nm and 220 nm. Fractions absorbing at 254 nm were collected for subsequent analysis. Samples were loaded onto a Biotage SNAP Ultra 1 g silica samplet and run through a 10g pre-packed silica cartridge.

Reduction of T-0632 Nitrile with Cobaltous Chloride and Sodium Borohydride

In an oven-dried flask, pure T-0632 nitrile (0.030 g, 0.0586 mmol) and cobaltous chloride (0.0279 g, 0.117 mmol) were dissolved in 0.352 mL of methanol, producing a bright blue solution. Tetrahydrofuran (0.75 mL) was added to the reaction and heat was applied to the system. Upon returning to room temperature, 0.222 g (0.586 mmol) sodium borohydride was added over a 5-minute period, during which the solution turned black and emitted heat and hydrogen gas. The system was sealed with a septum cap and stirred at 20°C for one hour. Hydrochloric acid was added to the system until the solution turned pink. Subsequently, 8 mL
concentrated sodium hydroxide was added to the system in order to produce a pH 9 solution; the color transitioned from pink to grey-blue. Dichloromethane was used to extract the organic layer, which was then washed with saturated sodium chloride. The organic layer was subsequently dried with sodium sulfate and filtered. The rose-colored product was then subjected to solvent evaporation under reduced pressure. The resulting NMR indicated that the solid material obtained was predominantly composed of starting material. The aqueous layer from the organic extraction was washed in concentrated sodium hydroxide in order to deprotonate the T-06 amine salt and subjected to a second extraction with dichloromethane. After drying, filtration, and evaporation, the new organic layer was analyzed with NMR spectroscopy; there was no evidence of product in this spectrum. ¹H NMR of T-0632 nitrile in CDCl₃ (Appendix 1): δ2.3 (m, 1H, H of alkyl chain), δ2.5 (t, 2H, H of alkyl chain), δ2.7 (m, 1H, H of alkyl chain), δ3.7 (s, 3H, -OCH₃), δ6.3 (s, 1H, H of m-anisidine), δ6.6 (d, 1H, m-anisidine), δ7.3-8.1 (m, 1H, H of m-anisidine; 4H, H of difluorobenzene; 4H, H of benzene of isoquinoline), δ8.5 (s, 1H, amine) δ8.9 (s, 1H, H of N-containing ring of isoquinoline), δ9.1 (s, 1H, H of N-containing ring of isoquinoline).

**Reduction of T-0632 Nitrile with Nickel Chloride and Sodium Borohydride**

T-0632 nitrile (0.3 g, 0.619 mmol) dissolved in 5 mL methanol was placed in an oven-dried flask under a stream of nitrogen gas. The system was brought to 0°C over ice. To this solution, Boc-anhydride (270.26 mg, 1.24 mmol) and nickel chloride (8.02 mg, 0.0619 mmol) were added, producing a light violet solution. Over a 30-minute period, sodium borohydride (164.08 mg, 4.34 mmol) was stirred into solution. Nitrogen gas was applied to the system at all times between the addition of reagents. The solution was diluted in 10 mL ethanol and subjected to 20 psi under hydrogen gas. This reaction was shaken for 16 hours, during which time a white
precipitate was formed. The precipitate was treated with ethylenediamine (41.4μL, 0.62 mmol) and stirred for 30 minutes before solvent evaporation. Ethyl acetate (15.5 mL) was used to dissolve the evaporated product and the organic layer was subsequently extracted with 3 rinses of saturated sodium bicarbonate. The organic layer was dried with sodium sulfate, filtered, and evaporated under reduced pressure. Purification of the resulting white precipitate was accomplished through flash chromatography: crude product was dissolved in hexanes and loaded into the silica gel samplet. The column was run over a concentration gradient of 35-38% ethyl acetate in hexanes. Unreacted T-06 nitrile began to elute at a concentration of 35% ethyl acetate, while the desired product eluted at an approximate concentration of 37% ethyl acetate. Pure Boc-protected amine T-0632 was confirmed via ¹H NMR. ¹HNMR of Boc-protected amine T-0632 in CDCl₃ (Appendix 2): δ1.47 (s, 9H, Boc), δ1.3-1.8 (m, 3H, H of alkyl chain), δ2.2 (s, 2H, H of alkyl chain), δ2.7 (s, 1H, alkyl chain), δ3.17 (m, 2H, alkyl chain) δ3.7 (s, 3H, -OCH₃), δ4.5 (s, 3H, primary amine), δ6.2 (s, H of m-anisidine, 1H), δ6.6 (d, 1H, H of m-anisidine), δ7.2-8.0 (m; 1H, H of m-anisidine; 4H, H of fluorobenzene; 4H, H of benzene of isoquinoline, 4H), δ8.5 (d, 1H, amine), δ8.9-9.0 (s, 1H, H of N-containing ring of isoquinoline), δ9.1 (s, 1H, H of N-containing ring of isoquinoline). Additionally, COSY NMR spectroscopy was used to identify protons in the alkyl chain to confirm successful nitrile reduction. The most notable interaction was that observed at the crosspeak between 3.17 ppm, representing two hydrogens in the alkyl chain, and the broad peak at 4.5 ppm, which was associated with the primary amine functional group (Appendix 3).

**Synthesis of Amine T-0632 via Boc Deprotection**

Cold trifluoroacetic acid (5mL) and anisole (126μL) were added to pure Boc-protected amine T-0632 (0.11g, 0.193 mmol). The reaction stirred for 1 hour at room temperature.
Subsequently, the brown gel was dissolved in a 1:1 mixture of dichloromethane and hexanes and evaporated under reduced pressure three times. The reaction was then treated with a 1:1 solution of dichloromethane and hexanes and stored for 16 hours at -18°C. Trituration of the product with a 1:9 ratio of dichloromethane and hexanes yielded an off-white, crystalline precipitate. $^1$HNMR analysis confirmed the successful generation of the amine T-06 salt. $^1$H-NMR of amine T-0632 in CDCl3 (Appendix 4): δ1.3-1.8 (m, 3H, H of alkyl chain), δ2.2 (s, 2H, H of alkyl chain), δ2.7 (s, 1H, alkyl chain), δ3.17 (m, 2H, alkyl chain) δ3.7 (s, 3H, -OCH3), δ5.4 (s, 3H, primary amine), δ6.2 (s, H of m-anisidine, 1H), δ6.6 (d, 1H, H of m-anisidine), δ7.2-8.0 (m; 1H, H of m-anisidine; 4H, H of fluorobenzene; 4H, H of benzene of isoquinoline, 4H), δ8.5 (d, 1H, amine), δ8.9-9.0 (s, 1H, H of N-containing ring of isoquinoline), δ9.1 (s, 1H, H of N-containing ring of isoquinoline).

Production of the His-Gly-Glu tripeptide

Synthesis of Fmoc-Gly-Glu(OtBu)-OMe dipeptide

Glu(OtBu)-OMe (0.894 g) and Fmoc-Gly-OH (1.24 g) were mixed together in a solution consisting of triethylamine (0.578 mL), HOBT (0.564 g), EDC (0.80 g), and 178.8 mL of dichloromethane. After three hours of stirring, the solution was rinsed three times with water in order to remove aqueous reagents. The organic layer was then dried with sodium sulfate, filtered, and its solvent evaporated under reduced pressure. The resulting honey-colored gel was analyzed via NMR spectroscopy. $^1$HNMR analysis suggested that the desired product was generated. $^1$H NMR of Fmoc-Gly-Glu(OtBu)-OMe (Appendix 5): δ 1.42 (s, 9H, t-butyl H), δ 1.89-2.00 (m, 1H, $\beta$C$_{Glu}$H$_A$), δ 2.09-2.25 (m, 1H, $\beta$C$_{Glu}$H$_B$), δ 2.3-2.45 (t, 2H, $\gamma$C$_{Glu}$), δ 3.76 (s, 3H, -OMe), δ 3.85 (t, 2H, $\alpha$C$_{Gly}$ H), δ 4.51-4.55 (q, 1H, $\alpha$C$_{Glu}$ H), δ 6.2-6.3 (s, 1H, Gly amide H), δ 6.84 (d, 1H, Glu amide), δ 7.44-7.62 (m, 9H, Fmoc).
Deprotection of Fmoc-Gly-Glu(OtBu)-OMe dipeptide

The existing Fmoc-Gly-Glu(OtBu)-OMe dipeptide was dissolved in 20% piperidine in DMF and stirred for 15 minutes at room temperature. Quenching with three rinses of 0.1 M hydrochloric acid halted the reaction. Aqueous solvent and the polar reactants trapped therein were removed via lyophilization. In this experiment, several rounds of overnight lyophilization were necessary to sufficiently isolate the product from piperidine. Subsequent analysis of the deprotected product via NMR spectroscopy revealed that cyclization had occurred, as evinced by the loss of the O-methyl protecting group at the C-terminus of Glu(OtBu)-OMe. ¹H NMR of cyclized Gly-Glu(OtBu)-OMe (Appendix 6): δ 1.42 (s, 9H, t-butyl H), δ 1.89-2.00 (m, 1H, βC_{Glu}H_A), δ 2.09-2.25 (m, 1H, βC_{Glu}H_B), δ 2.3-2.45 (t, 2H, γC_{Glu}), δ 3.85 (t, 2H, αC_{Gly}H), δ 4.51-4.55 (q, 1H, αC_{Glu}H), δ 6.2-6.3 (s, 1H, Gly amide H), δ 6.6 (d, 1H, Glu amide), δ 7.44-7.62 (m, 9H, Fmoc-- residual dissociated product from deprotection, as indicated by slight changes in the number and integration values of Fmoc-associated peaks).

Attachment of Fmoc-Glu(OtBu)-OH to Amine T-0632

In a dry flask, amine T-0632 salt (0.076 g, 0.162 mmol), Fmoc-Glu(OtBu)-OMe (0.069 g, 0.162 mmol), HOBt (0.025 g, 0.162 mmol), EDC (0.31 g, 0.162 mmol), and triethylamine (49.1 μL, 0.32 mmol) were combined and cooled to 0°C. After 30 minutes of stirring, the reaction was allowed to warm to room temperature and stir for an additional 18 hours. The organic layer was extracted with three rinses of water (approximately 5 mL each). After being dried with sodium sulfate, filtered, and evaporated under reduced pressure, the sample was lyophilized for 24 hours in order to remove excess triethylamine. The sample was subsequently
separated over a concentration gradient of 20-45% ethyl acetate in hexanes via flash chromatography. Unreacted T-06 amine began to elute at a concentration of 37% ethyl acetate. In order to elute the desired product, a second concentration gradient between 45-100% ethyl acetate in hexanes was run for this sample; Fmoc-Glu-amine T-0632 was detected at a concentration of 70% ethyl acetate. The identity of pure Fmoc-Glu-amine T-0632 was confirmed by $^1$H-NMR. $^1$H-NMR of Fmoc-Glu(OtBu)-amine T-0632 in CDCl3 (Appendix 7): δ1.3-1.8 (m, 3H, H of alkyl chain), δ 1.42 (s, 9H, t-butyl H), δ 1.89-2.00 (m, 1H, $\beta$C$_{\text{Glu}A}$), δ 2.09-2.25 (m, 1H, $\beta$C$_{\text{GluB}}$), δ2.2 (s, 2H, H of alkyl chain), δ 2.3-2.45 (t, 2H, $\gamma$C$_{\text{Glu}}$), δ2.7 (s, 1H, alkyl chain), δ3.17 (s, 1H, alkyl chain), δ3.7 (s, 3H, -OCH$_3$), δ 4.51-4.55 (q, 1H, $\alpha$C$_{\text{Glu}}$), δ6.2 (s, H of m-anisidine, 1H), δ 6.6 (d, 1H, Glu amide), δ 7.44-7.62 (m, 9H, Fmoc), δ6.6 (d, 1H, H of m-anisidine), δ7.2-8.0 (m; 1H, H of m-anisidine; 4H, H of fluorobenzene; 4H, H of benzene of isoquinoline, 4H), δ8.5 (d, 1H, amine), δ8.9-9.0 (s, 1H, H of N-containing ring of isoquinoline), δ9.1 (s, 1H, H of N-containing ring of isoquinoline).

Experimental yield for this protocol was approximately 50%. Subsequent attempts to attach Fmoc-Glu(OtBu)-OH to the remaining unreacted T-06 amine using the coupling procedure detailed above were unsuccessful. NMR analysis of the apparently unreactive T-06 amine produced the following results (Appendix 8): δ1.3-1.8 (m, 3H, H of alkyl chain), δ2.2 (s, 2H, H of alkyl chain), δ2.7 (s, 1H, alkyl chain), δ3.48 (s, 2H, alkyl chain) δ3.7 (s, 3H, -OCH$_3$), δ5.4 (s, 3H, primary amine), δ6.2 (s, H of m-anisidine, 1H), δ6.6 (d, 1H, H of m-anisidine), δ7.2-8.0 (m; 1H, H of m-anisidine; 4H, H of fluorobenzene; 4H, H of benzene of isoquinoline, 4H), δ8.5 (d, 1H, amine), δ8.9-9.0 (s, 1H, H of N-containing ring of isoquinoline), δ9.1 (s, 1H, H of N-containing ring of isoquinoline).
Deprotection of Fmoc-Glu(OtBu)-Amine T-0632 and Addition of Fmoc-Gly-OH

Fmoc-Glu-amine T-0632 (0.025 g, 0.028 mmol) was stirred with 20% piperidine (1 mL) for 15 minutes at room temperature. The reaction was then diluted in 20 mL of water and the system was lyophilized for 24 hours in order to remove aqueous solvent and reagents with sufficiently low boiling points. Subsequently, the resulting off-white precipitate was dissolved in 0.5 mL dimethylformamide and combined with Fmoc-Gly-OH (0.025 g, 0.084 mmol), HOBt (0.025 g, 0.084 mmol), EDC (0.016 g, 0.084 mmol), and triethylamine (25.0 μL, 0.168 mmol). The reaction was stirred at 0°C for 30 minutes, followed by 18 hours of stirring at room temperature. The organic layer was extracted with three rinses of water (approximately 5 mL each). After being dried with sodium sulfate, filtered, and evaporated under reduced pressure, the solvent was evaporated under reduced pressure. The sample was subsequently separated over a concentration gradient of 60-80% ethyl acetate in hexanes via flash chromatography. Elution of neither product nor reactant was observed. A second concentration gradient was run for this sample between 0-5% ethanol in ethyl acetate; the chromatogram for this experiment did not demonstrate absorbance at 254 nm at any point. Fractions were systematically collected, their solvents evaporated under reduced pressure, and their contents characterized via NMR spectroscopy. This procedure revealed characteristic T-06 amine peaks in a small subset of the total fractions collected. Due to the low concentration of eluent, a 12,000-scan NMR was conducted in order to elucidate its identity at higher resolution. The structure of Fmoc-Gly-Glu(OtBu)-amine T-06 was confirmed via $^1$H NMR at 12,000 scans. $^1$H-NMR of Fmoc-Gly-Glu(OtBu)-amine T-0632 in CDCl3 (Appendix 9): δ1.3-1.8 (m, 3H, H of alkyl chain), δ 1.42 (s, 9H, t-butyl H), δ 1.89-2.00 (m, 1H, $\beta$C$_{Glu}$H$_A$), δ 2.09-2.25 (m, 1H, $\beta$C$_{Glu}$H$_B$), δ2.2 (s, 2H, H of alkyl chain), δ 2.3-2.45 (t, 2H, $\gamma$C$_{Glu}$), δ2.7 (s, 1H, alkyl chain), δ3.7 (s, 3H, -OCH3), δ 3.85 (t,
2H, αC\textsubscript{Gly} H), δ 4.51-4.55 (q, 1H, αC\textsubscript{Glu} H), δ 6.2-6.3 (s, 1H, Gly amide H), δ6.2 (s, H of m-anisidine, 1H), δ 6.6 (d, 1H, Glu amide), δ6.6 (d, 1H, H of m-anisidine), δ7.2-8.0 (m; 1H, H of m-anisidine; 4H, H of fluorobenzene; 4H, H of benzene of isoquinoline, 4H), δ8.5 (d, 1H, amine), δ8.9-9.0 (s, 1H, H of N-containing ring of isoquinoline).

**Deprotection of Fmoc-Gly-Glu(OtBu)-Amine T-0632**

In order to remove the Fmoc functional group from the amino terminus of glycine, Fmoc-Gly-Glu(OtBu)-amine T-06 (0.0012 g, 0.0013 mmol) was dissolved in 500 μL 20% piperidine in DMF and permitted to stir at room temperature for 15 minutes. The reaction was halted by dilution in 3 mL water. Two 24-hour lyophilization periods were necessary to remove excess piperidine from solution in order to prevent unwanted nucleophilic attacks on the amine T-06 analog. Low product concentration warranted NMR analysis at 24,000 scans to achieve sufficient resolution to determine the identity of deprotected Gly-Glu(OtBu)-amine T-06. \(^1\)H-NMR of Gly-Glu(OtBu)-amine T-0632 in CDCl\(_3\) (Appendix 10): δ1.3-1.8 (m, 3H, H of alkyl chain), δ 1.42 (s, 9H, t-butyl H), δ 1.89-2.00 (m, 1H, βC\textsubscript{Glu}H\textsubscript{A}), δ 2.09-2.25 (m, 1H, βC\textsubscript{Glu}H\textsubscript{B}), δ2.2 (s, 2H, H of alkyl chain), δ 2.3-2.45 (t, 2H, γC\textsubscript{Glu}), δ2.7 (s, 1H, alkyl chain), δ 3.3 (t, 2H, αC\textsubscript{Gly} H), δ3.7 (s, 3H, -OCH\(_3\)), δ 4.51-4.55 (q, 1H, αC\textsubscript{Glu} H), δ 6.2-6.3 (s, 1H, Gly amide H), δ6.2 (s, H of m-anisidine, 1H), δ6.6 (d, 1H, H of m-anisidine), δ 6.6 (d, 1H, Glu amide), δ7.2-8.0 (m; 1H, H of m-anisidine; 4H, H of fluorobenzene; 4H, H of benzene of isoquinoline, 4H), δ8.5 (d, 1H, amine), δ8.9-9.0 (s, 1H, H of N-containing ring of isoquinoline).
Addition of Boc-His(Boc)-OH to Gly-Glu(OtBu)-amine T-06

In order to complete the tripeptide of the His-Gly-Glu(OtBu)-amine T-06 analog, Gly-Glu(OtBu)-amine T-06 (0.0022 g, 0.0031 mmol), Boc-His(Boc)-OH (0.00165 g, 0.00372 mmol), HOBt (0.0125 g, 0.0925 mmol), EDC (0.0006 g, 0.00312 mmol), and triethylamine (10 μL, 0.00725 mmol) were dissolved in 0.5 mL dichloromethane. Initial reaction occurred at 0°C; however, 30 minutes into the reaction, the solution warmed to room temperature and was stirred for an additional 18 hours. Organic extraction with a solution of water and dichloromethane produced a yellow organic layer and a colorless aqueous layer. The organic layer was subsequently dried with anhydrous sodium sulfate, filtered, and evaporated under reduced pressure, producing a dark red glass. $^1$HNMR analysis was conducted for 10,000 scans in order to elucidate the chemical structure of the resulting compound at sufficient resolution to compensate for its low concentration. Spectroscopic evidence supports the production of Boc-His(Boc)-Gly-Glu(OtBu)-amine T-06; however, the strength of this conclusion is mitigated by the low concentration of product relative to the concentration of solvent and other reagents. Unfortunately, the total quantity of product was too small to undergo further purification. $^1$H-NMR of Boc-His(Boc)-Gly-Glu(OtBu)-amine T-0632 in CDCl3 (Appendix 11): δ1.3-1.8 (m, 3H, H of alkyl chain), δ 1.42 (s, 9H, t-butyl H), δ 1.89-2.00 (m, 1H, βC$_{\text{Glu}}$H$_A$), δ 2.09-2.25 (m, 1H, βC$_{\text{Glu}}$H$_B$), δ2.2 (s, 2H, H of alkyl chain), δ 2.3-2.45 (t, 2H, γC$_{\text{Glu}}$), δ2.7 (s, 1H, alkyl chain), δ 3.03-3.22 (m, 2H, βC$_{\text{His}}$ H), δ 3.4 (t, 2H, αC$_{\text{Gly}}$ H), δ3.7 (s, 3H, -OCH$_3$), δ 4.51-4.55 (q, 1H, αC$_{\text{Glu}}$ H), δ 5.6-5.7 (s, 1H, αC$_{\text{His}}$ H), δ 6.2-6.3 (s, 1H, Gly amide H), δ6.2 (s, H of m-anisidine, 1H), δ6.6 (d, 1H, H of m-anisidine), δ 6.6 (d, 1H, Glu amide), δ7.2-8.0 (m; 1H, H of m-anisidine; 4H, H of fluorobenzene; 4H, H of benzene of isoquinoline, 4H), δ8.5 (d, 1H, amine), δ8.9-9.0 (s, 1H, H of N-containing ring of isoquinoline).
REFERENCES

38. Reduction of organic compounds to primary amines. Sodium borohydride.


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