Preparation of Analogs of the GLP-1 Receptor Inverse Agonist, T-0632

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Preparation of analogs of the GLP-1 receptor inverse agonist, T-0632

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Dr. David R. Haines, Advisor

A thesis submitted in partial fulfillment of the requirement for the Bachelor of Arts degree with honors in chemistry from Wellesley College

Wellesley College Chemistry Department
May 2014
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ACKNOWLEDGEMENTS

I would like to offer a huge thanks to Professor Haines for his advice, encouragement, and support in chemistry and beyond.

Thanks to my committee members, Professors Grace Ferris and Don Elmore for supporting my thesis work with helpful feedback and ideas.

A big thanks to the Haines Lab members, who have been excellent resources for help with lab problems and life dilemmas. I'm going to miss you!

And thanks to the other important people (chemistry majors, teammates, fellow students, other professors, faculty, staff, and all of my other friends and family) who helped me throughout this entire process of my thesis work, Wellesley, and life in general! I wouldn't be here without you!
ABSTRACT

T-0632, a non-peptidic, small molecule, acts as an inverse agonist of the glucagon-like-peptide-1 (GLP-1) receptor. The study of T-0632 and its analogs can further the understanding of the structure of the GLP-1 receptor. The carboxylic acid side chain of T-0632 is essential to the biological activity, as shown by the failure of a non-carboxylated analog to inhibit binding to the natural receptor agonist, GLP-1. The work presented here explores additional modifications of the carboxylic side chain, with the goal of further refining our understanding of the role of this functional group in the inhibition of GLP-1 receptor. Modifications will include changing the polarity, charge, and hydrogen bonding capability of the side chain. Preparation of the T-0632 amine analog is described.
INTRODUCTION

Diabetes Background

Diabetes mellitus is a disease that affects the metabolism by limiting the body's ability to process blood glucose levels. During digestion in healthy humans, glucose and other nutrients enter the blood stream, signaling the release of the hormone insulin from the pancreatic β cells. Insulin signals cells to take up glucose, lowering the amount of glucose in the blood stream. Glucose is a necessary nutrient, the primary energy source of cells, and is stored in the body as glycogen. Glucagon, a hormone produced in the pancreas, is responsible for the break down of glycogen so glucose can be utilized for energy production.

In the United States, there are more than 25 million people with diabetes. The Centers for Disease Control and Prevention (CDC) believe that there are 7 million additional, undiagnosed cases. A quarter of those with diabetes are over the age of 65. Diabetes is a leading cause of kidney failure, non-traumatic lower-limb amputations, new cases of blindness, heart disease, stroke, and death. It costs the United States over $174 million in indirect and direct health care needs. Diabetes can be treated through a combination of therapies including diet management, oral pills, and direct, subcutaneous injection of insulin, other hormones, and their analogs.

Diabetes is characterized by high blood glucose levels, known as hyperglycemia. This can be the result of either or both of two factors: insulin resistance or insulin deficiency. Type I diabetes is an autoimmune disorder in which the pancreatic β cells are attacked by the body's immune system and are therefore rendered unable to produce insulin. In type II diabetes, also known as non-insulin dependent diabetes mellitus
(NIDDM) and adult onset diabetes, the pancreatic β cells may have decreased sensitivity or be unresponsive to glucose stimulation or incretin hormones, all of which result in too little or no insulin production. The symptoms can progress to the deterioration of the β cells of the pancreas and the resulting loss of the ability to produce insulin, similar to the biological mechanism responsible for type I diabetes. This makes blood glucose homeostasis difficult to maintain because there is less insulin to interact with less responsive receptors. Type II diabetes accounts for 90-95% of all diabetes cases.

One treatment for diabetes is subcutaneous injection of insulin directly into the blood stream. However, insulin therapy has several shortcomings, including the necessity for subcutaneous injection, a short half-life due to its peptidic nature and requiring injections more than once per day, and the risk of hypoglycemia. Without careful administration, insulin can cause hypoglycemia, depleting the glucose sources in the blood stream. This can have dangerous health effects. The brain needs a relatively constant source of glucose to function properly and going without it for a short time can cause serious brain damage. While insulin injection is currently the only way to maintain normal insulin levels for type I diabetics, there are other options for those with type II diabetes.

**Glucagon-Like-Peptide-1 and Receptor**

Glucagon-like peptide-1 (6/37)-amide (GLP-1) is an incretin hormone produced by humans that plays an essential role in normal regulation of insulin and glucose levels. After eating and during digestion, nutrients signal the release of incretin hormones throughout the intestinal tract. Endocrine cells found in the small intestine (known as L cells) produce
GLP-1 through the cleavage of the 160-amino acid protein proglucagon, initiating the incretin effect.\textsuperscript{4} When blood glucose levels are high, GLP-1 stimulates insulin release from the pancreatic \( \beta \) cells.\textsuperscript{2,5} GLP-1 inhibits glucagon release and improves insulin sensitivity.\textsuperscript{5b} Additionally, GLP-1 encourages satiation by prolonging gastric emptying\textsuperscript{5b} and interacting with neurons in the digestive system that tell the brain it is satisfied.\textsuperscript{2}

By bonding with the GLP-1 receptor, GLP-1 initiates one of the most important parts of the incretin effect: starting the 3'-5' cyclic adenosine monophosphate (cAMP) signal cascade responsible for the stimulation of insulin release. The GLP-1 receptor is a class B, seven transmembrane region (TM1-TM7) G protein coupled receptor (GPCR) found in the pancreatic \( \beta \) cells. The GLP-1 receptor usually couples with \( G\alpha_s \), which activates adenyl cyclases, increasing cellular cAMP.\textsuperscript{6} A distinguishing component of the class B GPCRs, in comparison to the class A GPCRs, is the presence of a large folded globular ectodomain (ECD) at the N-terminus.\textsuperscript{7} Common to class B GPCRs are six cysteine residues located in the ECD.\textsuperscript{8} The entire GLP-1 receptor amino acid sequence has been mapped and important residues are well known.\textsuperscript{9} While the entire amino acid sequence of the GLP-1 receptor is known, a complete high-resolution crystal structure has not yet been obtained (Figure 1).\textsuperscript{7} As a structure with several transmembrane regions, direct X-ray crystallography is not possible.

Important aspects of the receptor can be determined through interaction with the specific ligands. The GLP-1 and GLP-1 receptor interaction is generally thought of as a two-step process (Figure 2). First the N-terminal of the receptor initiates an affinity trap with the C-terminal of the peptide, localizing the N-terminal of the peptide over the core region
Consequently, the C-terminal of the peptide binds to the core region of the receptor causing the conformational change in the receptor initiating the cAMP signal cascade and other incretin effects. It has been shown that the receptor’s N-terminal, TM1, and ECL1 are important for ligand binding and specificity (Figure 1). Specifically, residues W39, K198, R190, and D198 of the receptor are important for binding.
moieties of K202 and D215 are believed to be important to the specificity of the GLP-1 receptor for GLP-1.\textsuperscript{10a} The polar character of the extracellular residue K198 of TM2 is especially important for enhancing the affinity.\textsuperscript{11,12} During the affinity trap, the hydrophobic

![Figure 2. GLP-1 and GLP-1 receptor interaction.](image)

moieties of the peptide are responsible for most of the interactions and are key contributors to ligand-receptor affinity.\textsuperscript{4,13}

After the affinity trap, the ligand is localized over the extracellular loops (ECL) and exposed residues of the TM regions. Interactions here may cause a conformational change resulting in the transmission of signal from the receptor to the G proteins.\textsuperscript{4,8,14} Studies have confirmed that ECL1, adjacent portions of the TM2, and TM4 are essential for GLP-1 reception.\textsuperscript{6,10a,11} The positive charges from residues such as lysine and arginine of the EC1 and TM3 are critical for activation of the GLP-1 receptor. Another important positive charge of the receptor is K288, which contributes to its affinity to the N-terminal of GLP-1.\textsuperscript{11} The moiety is found on the interior face of TM4, which allows for easy access to bond with other nearby residues.\textsuperscript{11} Because K288 is important in the activation of the GLP-1
receptor, it could be an important residue to keep in mind when developing therapeutic approaches to type II diabetes.\textsuperscript{11} Through systematic analysis of each amino acid of GLP-1, Kim Adelhorst confirmed the importance of residues H7, E9 G10, F10, T13, and D15 of the N-terminal of GLP-1 for binding to the core domain of the receptor.\textsuperscript{13, 15}

Intracellular loop (IC) 3 is primarily responsible for transmitting the signal from the agonist to the G proteins.\textsuperscript{8, 14} Particularly key residues are K333 and K351.\textsuperscript{8} The IC3 does not act exclusively as it is believed that IC1 and IC2 act as modulators in the interaction.\textsuperscript{9}

GLP-1 analogs and GLP-1 receptor agonists are some of the most potent therapies for type II diabetes because GLP-1, like insulin, has a diminished presence in diabetic patients and has more specific effects than insulin.\textsuperscript{5a} Presently, it is unclear exactly how much defects of the incretin hormones, such as GLP-1, play a role in diabetes. It has been shown that GLP-1 in type II diabetics is less abundant after eating than in healthy patients.\textsuperscript{5a} GLP-1 as a therapy was first tested for human use in 1987, and more thorough analysis followed in 1992.\textsuperscript{5, 8} The results of these studies indicate that GLP-1 induces insulin production in healthy and diabetic patients.\textsuperscript{14b} One major benefit to using GLP-1 is that it only initiates insulin production and release when blood glucose levels are high, eliminating the danger of hypoglycemia.\textsuperscript{5a, 8} Jens Juul Holst, one of the major researchers in developing GLP-1 therapies for type II diabetes, found that GLP-1 therapy can fully restore insulin response to glucose.\textsuperscript{5a} In addition, when GLP-1 is used as a therapy, it can have trophic effects on pancreatic β cells and inhibitory effects on their degradation, both of which contribute to the recovery of the ability to produce and secrete insulin and improved production and reception of GLP-1.\textsuperscript{5a, 16} Both of these effects are significant for patients
with type II diabetes suffering from an insufficient number of β cells and a lack of functioning β cells. These trophic effects have been clearly shown in animal models.\textsuperscript{16}

GLP-1 was the first non-insulin treatment option and was the starting point for a novel approach to diabetes treatments. A potential drawback to this therapy is that GLP-1, like insulin, is a peptide with a short half-life in the body (less than two minutes).\textsuperscript{2, 17} Additionally, less than one third of natural GLP-1 survives the departure from the gut and the subsequent journey to the pancreatic β cells. When GLP-1 is administered via continuous subcutaneous infusion, only 10\% makes it to the receptors.\textsuperscript{5a} Dipeptidyl peptidase IV (DPP-IV) deactivates GLP-1 through cleavage at the N-terminal at H7-A8 residues and can even turn it into an antagonist of the GLP-1 receptor (Figures 3).\textsuperscript{5a}

Moreover, DPP-IV is the sole enzyme responsible for the degradation of GLP-1.\textsuperscript{18} DPP-IV is found in the endothelial cells lining blood vessels, so upon departure from the gut, GLP-1 is immediately exposed to the possibility of cleavage.\textsuperscript{4}
GLP-1 Receptor Agonists and DPP-IV inhibitor Therapies

Several pharmaceutical companies have developed analogs of GLP-1 with the goal of mimicking its behavior while extending the half-life by establishing a resistance to DPP-IV. This has been done by modifying the important residues of the peptide required for DPP-IV interaction or by adding a large moiety that sterically hinders DPP-IV action. Through modifications at the position sensitive to DPP-IV cleavage (H7-A8), the GLP-1 homology is maintained while breakdown by DPP-IV is resisted.\(^5\) In addition to combating degradation by DPP-IV, an ideal peptidal therapy should also discourage secondary degradation by other endopeptidases and decrease the facility of renal clearance.\(^4\)

One successful GLP-1 analog is Exendin-4 (Figure 4). The compound was originally isolated from a peptide from the venom of a lizard known as the Gila Monster (\textit{Heloderma Suspectum}) and can now be made synthetically. With a glycine residue in the penultimate position of the N-terminus, it has 53\% homology to GLP-1, behaves as an agonist to GLP-1, and is resistant to DPP-IV.\(^4\)\(^-\)\(^5\) This resistance allows exendin-4 to have a half-life of 4 hours. In 2005, exendin-4, under the brand name \textit{Byetta}, was approved by the United States Food

![Figure 4. Amino acid sequence of GLP-1, exendin-4, and liraglutide. Homologous amino acid residues of the peptide to GLP-1 are noted in yellow. GLP-1 is cleaved by DPP-IV as indicated. Exendin-4 is resistant due to its lack of an alanine in the penultimate position. \(^5\) Liraglutide is resistant due to the steric hindrance of its large fatty acid (albumin). \(^4\) Figure adapted from De León.\(^1\)\(]
and Drug Administration (FDA) as the first enteroendocrine-based-therapy for type II diabetes.\textsuperscript{4}

Liraglutide, another GLP-1 analog, is modified by the addition of an acyl group to the K26 position of GLP-1; the acyl-group is then palmitoylated (Figure 4).\textsuperscript{4} The bulky fatty acid moiety sterically inhibits DPP-IV-promoted breakdown. Liraglutide has an 11-15 hour half-life due to its insensitivity to DPP-IV and an innate resistance to renal elimination.\textsuperscript{4,5} Moreover, due to such an effective resistance to breakdown, it can be taken once daily, a definite benefit to liraglutide.\textsuperscript{19} Liraglutide was approved by the FDA in 2010 for therapeutic use for diabetes under the brand name of \textit{Victoza}.

Because of the extreme and exclusive degradation of GLP-1 by DPP-IV, some pharmaceutical companies have focused on synthesizing drugs that inhibit DPP-IV. On animal subjects with glucose intolerance, treatment with DPP-IV inhibitors resulted in an improvement of metabolic and glucose regulation. Even though DPP-IV also interacts with other substrates, its extreme degradation of GLP-1 makes it the ideal target. There is evidence that inhibition of DPP-IV does not have undesired side effects in rats.\textsuperscript{5a} One major advantage to DPP-IV inhibitor treatment is that the drug is administered orally. There are currently four DPP-IV inhibitor-based treatment options available in the United States and the European Union.

In general, GLP-1 receptor agonists are considered more effective due to the fact that they have more specific glycemic control. Moreover, according to treatment satisfaction data, patients are more satisfied with GLP-1 receptor agonist treatment (even via subcutaneous injection) than the DPP-IV inhibitors due to the superior glycemic control.\textsuperscript{19}
Small-Molecules, Including T-0632 and Analogs, as GLP-1 Receptor Agonists and Antagonists

Another option for the treatment of diabetes is a small-molecule agonist of the GLP-1 receptor. Currently there are no small molecule GLP-1 receptor agonist therapies available for oral administration. An ideal drug therapy for type II diabetes would have a larger half-life than peptide GLP-1 receptor agonists, be orally administered, and behave as a high-affinity GLP-1 receptor agonist. There are currently few research groups focusing on the synthesis and effectiveness of small molecules agonists of GLP-1 that are more stable in vivo and easily administered. Only a few small molecules are known to interact with class B GPCRs, let alone behave as agonists. It is difficult to design an effective small-molecule drug because the complete, high-resolution crystal structure of GLP-1 receptors and class B receptors is as of yet undeveloped. While some small molecules have expected pharmaceutical promise, others have been useful in elucidating the class B receptor crystal structure.

Current small-molecule agonists of the GLP-1 receptor, including some quinoxalines, thiopenes, and pyrimidines, are encouraging, but most have several metabolic liabilities and more biological data must be collected before a successful molecule can be used for drug treatment. One group of small molecule agonists, azoanthracenes (1), has particular potency for cAMP activation and has partial agonism (Figure 5). Several compounds from this group are effective in causing insulin secretion in rodents with type II diabetes. In 2012, the leading model from this group was undergoing phase II clinical trials.
There are several low molecular weight antagonists, about which little is known. While it may seem counterintuitive to use antagonists of the GLP-1 receptor to develop drugs for diabetes treatment, these molecules could be useful for further resolution of the crystal structure of the GLP-1 receptor. As antagonists, they show an affinity for the receptor and may elucidate critical residues of the receptor and what molecular requirements are required for a successful drug for type II diabetes. One small molecule, methyl-3-[1[2(fluorophenyl)-3-(isoquinoline-3-carboxyamido)-6-methoxy-3-yl]propanoate (2) (referred to as T-0632), was originally studied during the development of a small-molecule therapy for pancreatitis, but it had an undesired side effect of GLP-1 receptor antagonism (Figure 7, 2). Moreover, there is evidence that suggests that T-0632 reduces the normal activity of the GLP-1 receptor, classifying it as an inverse agonist. T-0632 has a somewhat low affinity for the GLP-1 receptor, possibly making it ineffective for furthering the development of a GLP-1 receptor agonist. However, its effects are nonetheless potent as a tool to learn about the receptor.
The amino acid residue W33, part of the folded globular ECD, is not essential for normal hormonal binding, but it plays an important role in the capacity of T-0632 to interfere with other ligands. This important residue is located only 10 Å from where the hydrophobic parts of GLP-1 usually bond. The T-0632 may bond directly at W33 or it may recruit other receptor residues to create a ligand-binding domain. This rearranging the residues of the receptor offers a possible explanation for the molecule’s activity as an inverse agonist interfering with the binding site conformation for GLP-1 binding. Either way, this position is essential for T-0632 activity. It is believed that the affinity of T-0632 to the receptor is due to stacking of the aromatic rings of T-0632 and W33 residue of the receptor.

The carboxylic acid moiety of T-0632 is hypothesized to specifically bind with K288, of TM4, and several histidine residues found in ECL1 and the exposed extracellular end of TM2. The specific bonding pattern of T-0632 with the ECD and residues of the TM2, ECL1 and TM4 match the mechanism of interaction between GLP-1 and the GLP-1 receptor. These major bonding sites make up the known interaction and positioning between T-0632 and the GLP-1 receptor (Figure 7). Through further study of T-0632, there are huge implications for an improved understanding class B GPCR interactions with non-peptide,
small molecules. Based on the successes of class A GPCR, minor structural modifications may lead to active agonists, even when the original analog was previously inactive.\(^6\)

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**Figure 7. Illustration of bonding between T-0632 and GLP-1 receptor.** The N-terminal folded globular ECD has residue W33, which is thought to bond with the aromatic rings of T-0632. The carboxylic acid bonds with the several histidine residues and K288 in TM2, TM4, and ECL1.\(^{21}\) Figure adapted from Kim.\(^{22}\)

The carboxylic acid moiety of T-0632 is thought to bind in the same core region of the receptor as the N-terminal of GLP-1 is known to bond. It makes sense to modify the moiety to resemble this region of the peptide with the aims of imitating peptide activation of the receptor. The propyl amine analog of T-0632 (3) (known as T-0632-amine) could act as a synthetic handle for adding amino acid residues. The amine as the starting point for the addition of the amino acids allows for physical distance between the main body of T-0632 and the binding region of the molecule. This may allow for closer GLP-1 mimicry, due
to the natural separation in GLP-1 between the residues necessary for interaction with the GLP-1 folded globular ECD and the residues necessary for bonding to the core region of the receptor. Analogs with histidine, glycine, phenylalanine, threonine, aspartic acid, alanine, and glutamic acid moieties would be intelligent choices to explore because these are known to be essential for GLP-1–receptor affinity and activation. The third to last residue, glutamic acid, has similar properties to the carboxylic acid moiety of T-0632. Analyzing the biological activity of an analog with a glutamic acid moiety (5) can help clarify the specific role of the polar and charge character of the ligand in the interaction with the receptor. An analog with all three ultimate residues would be a valuable molecule to explore as well. By adopting a more similar homology to the peptide, amino acid T-0632-amine analogs may also adopt similar behavior and act as agonists to the receptor. Another option is exploring the role of the physical distance between the main body of the molecule and the moiety responsible for receptor agonism. This could be done through the synthesis of 4-oxo-(T-0632-amine)-butanoic acid (6), an analog with a larger alkyl chain between the carboxylic moiety and the main body of T-0632. More research is necessary before conclusions can be drawn.

As mentioned above, T-0632 has a relatively low affinity to the GLP-1 receptor. Yet, it still has benefits in terms of furthering the understanding of GLP-1 receptor and ligand interaction important for small-molecule agonism. An important tool used in the determination of the therapeutic potential of different analogs of T-0632 is the cholecystokinin (CCK) receptor, to which T-0632 has a higher affinity as an antagonist.\(^7\) The CCK receptor is a 7TM GPCR, like the GLP-1 receptor, and modulates many physiologic functions, such as the secretion of gastric acids and pain perception. An important part in
any small-molecule-receptor interaction is the affinity for binding, and then the agonist mechanism. Using the CCK receptor, less T-0632 would be required for determining the initial affinity relationship between analogs and the GLP-1 receptor. If affinity is achieved, the T-0632 analogs would be tested for GLP-1 receptor activity.

Present Research

The research presented here focuses on synthesizing a T-0632 analog with modifications to the carboxylic acid moiety. Combining current knowledge about the important residues involved in binding to the GLP-1 receptor, the known and suspected mechanism of interaction of the receptor with T-0632, and activity of specific analogs, a more sophisticated understanding the GLP-1 receptor and a more efficient type II drug therapy may be achieved. The present goal is the synthesis of T-0632-amine, establishing a molecule with the opposite charge than those previously studied. A positive charge here may repel the positive residues of the ECLs and TM4, such as lysine and histidine. The possible opening caused by this repulsion may lead to a different conformational structure than the change due to the hydrostatic repulsion of T-0632. Using the T-0632 amine, we can explore whether electrostatic interactions play a significant role in conformational changes. These changes may result in changes in receptor activity. After successful synthesis, T-0632-amine would go through biological testing to determine its biological activity. In addition to causing possible changes in the agonist activity, T-0632-amine is a good starting point for the synthesis of analogs with amino acids. Amino acids can be easily added in the direction that mimics the N-terminal of GLP-1.
RESULTS AND DISCUSSION

The synthesis of T-0632-amine (3) has been attempted twice. The entire synthesis of T-0632 amine follows the same procedure as traditional T-0632 through the synthesis of the intermediate T-07 (10) (Figure 8). From T-07, T-0632-amine is synthesized via Michael Addition of acrylonitrile to T-07 followed by a reduction.

![Chemical structure diagram](image)

**Figure 8. Complete synthesis of T-0632-amine (11).** Synthesis of T-0632 is identical to that of T-0632-amine up to the formation of T-07 (10). Two reduction techniques are shown: (A) via catalytic hydrogenation and (B) with sodium borohydride and cobaltous chloride.

**Synthesis of oxime**

Starting with isatin (7) previously synthesized by lab mates, oxime (8) was successfully synthesized using the standard lab technique. Via a condensation reaction, the isatin was reacted with hydroxylamine hydrochloride and sodium carbonate for 24 hours
at room temperature. The product was considered pure based on $^1$H-NMR analysis. Reduction to the amine hydrochloride (9) remained elusive due to mechanical failure of the catalytic hydrogenation instrument.

Extra stores of T-07 were available in lab, allowing synthesis of T-0632 without personal completion of the beginning steps of the total synthesis.

**Michael Addition to form T-0632-nitrile**

The synthesis of T-0632-nitrile (11) was accomplished through a Michael addition. The Michael addition is based on a patent released by Yamada et al, in 1998, using acrylonitrile and potassium carbonate (Figure 9).\(^{20}\) Wellesley College thesis students Kim ('08) and McLoughlin ('14) from the Haines Lab have used various solvents, and, through trial and error, determined that reactions performed in either dimethyl sulfoxide (DMSO) and 1,4-dioxane yield the highest amounts of T-0632 methyl ester (71 and 90-100%, respectively).\(^{21}\) However, with other acrylates, such as acrylonitrile, the yield was not as
high. With DMSO and acrylonitrile to facilitate the Michael addition, $^1$H-NMR analysis of the crude reaction mixture showed almost complete consumption of T-07, resulting in a yield of 70% T-0632-nitrile product.

Another attempt of the Michael addition used 1,4 dioxane as the solvent. The major product of this attempt was not T-0632 nitrile, but possibly a rearranged version of T-07 (12) (Figure 10). Chromatography was attempted several times with extended periods between attempts. As a result less than 10% of pure T-0632 nitrile was isolated.

![Figure 10. Mechanism of rearrangement of T-07 under basic conditions.](image)

Based on analysis of the $^1$H-NMR and $^{13}$C-NMR spectra of the major, undesired product and comparison with $^1$H-NMR and $^{13}$C-NMR spectra of T-07 yielded several conclusions (Appendices 1-4, respectively). One clear conclusion is that the acrylonitrile never bonded, evident by the lack of peaks in the aliphatic region (δ2.2-2.8 ppm). Additionally, the secondary amide α-proton is no longer present, as there are no peaks at δ5.9 ppm in the $^1$H-NMR, where that hydrogen would be expected to appear. The lack of proton signal could be the result of an elimination pathway resulting in a double bond or
perhaps binding between the nucleophilic carbon and some other electrophilic species. However, there are few other shifts in the NMR spectra, most likely eliminating the possibility of an unexpected addition or polymerization. Based on the initial $^1$H-NMR of the reaction mixture, this product was not as abundant because the proportions of those peaks were not so proportionately large. The identity of this product is most likely due to a rearrangement that may have occurred during two stages of the reaction. The rearrangement could have resulted during the first ten minutes of the reaction while the T-07 was under basic conditions and before the acrylonitrile was added (Figure 10). It may have also occurred during period of time the crude reaction mixture remained on the silica gel gravity column and in solution. Silica can trap molecules like T-0632 and analogs due to its acidic nature and this environment could cause the rearrangement via acid catalysis, which has a very similar mechanism as the base catalysis shown in Figure 10. The rearrangement allows for the formation of highly energetically favorable aromatic rings. While we have a predicted structure for the possible rearrangement, none of the predictions exactly agree with the $^1$H- and $^{13}$C-NMR spectra obtained. Based on the large amount of undesired product, future synthetic schemes should eliminate the first time period of basic conditions without the acrylonitrile and the products should be processed in a timely manner and kept at cool temperatures to discourage rearrangement.
Reduction of T-0632-nitrile to T-0632-amine

Two different techniques were used for the reduction of the T-0632-nitrile to T-0632-amine \( (3) \) (Figure 11). From the outset, it was anticipated that the chemoselective reduction would be difficult to accomplish due to the presence of several other unsaturated moieties within the molecule. Additionally, there was the possibility of rearrangement within T-0632-amine: the isoquinoline could shift to the primary amine. Many published techniques were not suitable options because they utilized materials that would reduce carbonyls in the molecule or result in secondary amines and dimers through the formation of aldimines (Figure 12).\(^{22}\) The first attempt of T-0632-amine was done on a 70 mg scale and utilized a hydrogenation technique with a rhodium catalyst.\(^{23}\) Freifelder found that hydrogenation with rhodium catalysts required mild conditions (2-3 atm) compared to those with nickel (90 atm). Furthermore the presence of ammonia in the solvent allows a good yield of primary amines, discouraging the formation of secondary amines and aldimines. Due to scale and instrument restrictions, the reaction was performed under highly diluted conditions. Upon \(^1\)H-NMR analysis, it was determined that there may have
been minor reduced products, but the large majority was recovered T-0632-nitrile.

During the second attempt of the reduction, a technique was used that takes advantage of sodium borohydride and cobaltous chloride (Figure 11). Usually, sodium borohydride is inert to nitriles, but in the presence of transition metal salts, such as cobalt (II) chloride, reduction of nitriles to primary amines can be achieved with good yields.\textsuperscript{24}

\textbf{Figure 12. Mechanism of dimerization through the formation of an aldimine.} (A) The desired reduction of nitrile is shown. (B) The mechanism of dimerization through the aldimine is shown. The undesired dimer can be limited by adding an excess of ammonia.
The complete mechanism is uncertain, but it is clearly an exothermic reaction and hydrogen gas is evolved while black deposits of cobalt-boride (Co₂B) precipitates. Stephen Heinzman and Bruce Ganem experimentally concluded that the Co₂B is responsible for coordinating the functional groups and catalyzing the heterogeneous reduction by NaBH₄. It remains unclear which species is responsible for the actual hydride donation. Purification and handling of this product was done under closely controlled pH and primarily stored in cool conditions to prevent possible rearrangement. Analysis of the crude product via ¹H-NMR suggests about 50% consumption of T-0632-nitrile. After successful separation, it was determined that the two products were indeed T-0632-amine and T-0632-nitrile. Moreover, the splitting of the singlet that represented a hydrogen atom of the N-containing aromatic ring of isoquinoline of T-0632 nitrile (δ8.8 ppm) suggests two conformations in which the primary amine may interact with this hydrogen.
CONCLUSION

The successful synthesis of T-0632-amine was a promising first step to efficient synthesis of T-0632-amine. Both reduction techniques resulted in T-0632 amine, however the reduction via sodium borohydride and cobaltous chloride was more efficient, with approximately 50% yield. There was an insufficient final volume of pure T-0632-amine for biological analysis by the Mayo Clinic in Arizona. Further study is necessary for a larger volume of T-0632-amine, either through larger volume reactions or modified procedures for higher yields. Once an efficient synthesis has been established, the molecule will be tested for biological activity via interaction with the CCK receptor and, if positive, the GLP-1 receptor. Techniques for synthesizing other significant analogs based on known chemistry involved in peptide-receptor interaction of GLP-1 and the GLP-1 receptor will be developed and said analogs will be tested for biological activity. Regardless of the efficacy of these molecules as agonists of the GLP-1 receptor, their behavior will contribute to the knowledge of the important aspects of interaction with the receptor.
EXPERIMENTAL

General

All solvents and reactants were purchased from Sigma-Aldrich or Alfa-Aesar. Catalytic hydrogenation was performed using a Parr Instrument Company Pressure Reaction Apparatus. $^{13}$C- and $^1$H-NMR spectra were obtained using a 300 MHz Bruker WIN-NMR Spectrometer with TMS as an internal reference. Thin layer chromatography (TLC) was performed on Merck 60-F254 pre-coated silica gel plates and analysis was visualized with UV light (254 nm). Flash chromatography was performed using a Biotage Flash Chromatography system with pre-packed Biotage Silica Cartridges. Gravity column chromatography was performed using Sigma-Aldrich Silica gel (0.060-0.2 mm, 70-230 mesh).

Synthesis of Oxime: 1-(2-fluorophenyl)-3-(hydroxyimino)-6-methoxyindolin-2-one (8)

The oxime was synthesized by dissolving isatin (7) (0.400 g, 1.476 mmol), hydroxylamine hydrochloride (0.1088 g, 1.476 mmol), and sodium carbonate (0.1716 g, 1.476 mmol) in 5.2 mL of ethanol. Heat was used to improve the solubility of the isatin in ethanol. The reaction was stirred for 24 hours under ambient conditions. After two hours the reaction was bright yellow. The solution was quenched with water and dried via vacuum filtration. The identity of the oxime (0.300 g, 1.095 mmol, 74% yield) was confirmed via $^1$H-NMR. $^1$H-NMR in CDCl$_3$ (Appendix 5): δ3.8 (s, 3H, -OCH$_3$), δ6.2 (d, 1H, H of m-anisidine), δ6.6 (d, 1H, H of m-anisidine), δ7.11-7.8 (m, 4H, H of fluorobenzene), δ8.2 (d, 1H, H of m-anisidine).
**Synthesis and purification of T-0632 nitrile: 3-[1-(2-fluorophenyl)-3-(isoquinoline-3-carboxyamido)-6-methoxy-2-oxindole-3-yl]propionitrile (11) in DMSO**

The T-0632-nitrile was synthesized by dissolving T-07 (1) (0.100 g, 0.234 mmol) in 1.167 mL of acetone. DMSO (0.038 mL) and potassium carbonate (0.0971 g, 3.51 mmol) were added to the solution and stirred for 10 minutes at room temperature. Six equivalents of acrylonitrile (0.0924 mL, 1.405 mmol) were added. The reaction stirred for 24 hours covered by a septum. After 24 hours, the crude product was magenta. The solution was quenched with 3 mL of water, neutralized to a pH of 4 using hydrochloric acid, washed with dichloromethane, dried with magnesium sulfate, filtered and desolvated. T-0632-nitrile in the crude product was confirmed by $^1$H-NMR.

**Purification of T-0632-nitrile**

The pure T-0632-nitrile was separated from the crude product by flash column chromatography. The crude product was dissolved in dichloromethane and loaded into a 12+M Biotage samplet holder, which was loaded onto a column suspended in mobile phase of 20% ethyl acetate in hexanes. The product was run through the column through a gradient of 20-60% ethyl acetate over 456 mL. The first product eluted (at around 35% ethyl acetate) was the desired product. The identity of T-0632-nitrile was confirmed via $^1$H-NMR. $^1$H-NMR in CDCl$_3$ (Appendix 6): 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, -OCH$_3$), 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 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).
Synthesis and purification of T-0632-nitrile: 3-[1-(2-fluorophenyl)-3-(isoquinoline-3-carboxyamido)-6-methoxy-2-oxindoline-3-yl]propionitrile (11) in 1,4-dioxane

The T-0632-nitrile was synthesized by dissolving T-07 (1) (0.500 g, 1.170 mmol) in 10 mL 1,4 dioxane with potassium carbonate (0.4859 g, 3.51 mmol). After stirring for 15 minutes at room temperature, 6 equivalents of acrylonitrile (0.46 mL, 7.02 mmol) were added. The reaction stirred for 24 hours covered by a septum. The crude product, opaque brown/orange in color, was quenched with 30 mL of water. The organic product, now transparent and yellow, was washed three times with 20 mL of dichloromethane. The crude product was dried using magnesium sulfate and filtered by filter paper. The solvent was evaporated leaving an orange film. The presence of the desired product in the crude product was confirmed by $^1$H-NMR.

Purification of T-0632-nitrile

The T-0632-nitrile was purified via column chromatography. A flash sample was loaded with the crude T-0632-nitrile and loaded onto a 25 g KP-sil – SNAP cartridge suspended in 20% ethyl acetate in hexanes. The column was run with a concentration gradient of 20-40% ethyl acetate in hexanes over 135 mL. Then 90 more mL of 40% ethyl acetate was run through the column. The desired product eluted with 40% ethyl acetate after 205 mL of eluent. Pure T-0632-nitrile (<10% yield) was confirmed via $^1$H-NMR.

$^1$H-NMR in CDCl$_3$ (Appendix 6): δ2.3-2.7 (m, 4H, H of alkyl chain), δ3.7 (s, 3H, -OCH$_3$), δ6.3 (s, H of m-anisidine, 1H), δ6.6 (s, 1H, H of 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, H of amine), δ8.8 (s, 1H, H of N-containing ring of isoquinoline), δ9.1 (s, 1H, H of N-containing ring of isoquinoline).
Synthesis of T-0632-amine: 3-[1-(2-fluorophenyl)-3-(isoquinoline-3-carboxyamido)-6-methoxy-2-oxindoline-3-yl]propylamine (3) via catalytic hydrogenation

T-06-nitrile (0.070 g, 0.136 mmol) was dissolved in 10 mL of ammonia (2M in methanol). The 5% rhodium on alumina powder, reduced (0.0036 g) was added to the mixture and reacted under 2.5 atm for two hours. The solution was filtered with filter paper and desolvated. Upon analysis via ¹H-NMR, there was almost complete recovery of starting materials with trace amounts of T-0632-amine. ¹H-NMR of crude product in CDCl₃ (Appendix 7): δ1.8-1.9 (s, 2H, H of methyl α to primary amine; 3H, H of primary amine – a broad peak), δ2.3-2.8 (m, 4H, 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, H of secondary amine) δ8.9 (s, 1H, H of N-containing ring of isoquinoline), δ9.1 (s, 1H, H of N-containing ring of isoquinoline).

Synthesis and extraction of T-0632-amine: 3-[1-(2-fluorophenyl)-3-(isoquinoline-3-carboxyamido)-6-methoxy-2-oxindoline-3-yl]propylamine (3) via sodium borohydride halide salt

T-0632-nitrile (0.020 g, 0.0391 mmol) and cobaltous chloride hexahydrate (0.0186 g, 0.0781) was dissolved in 0.2344 mL of methanol. To aide solubility of T-0632-nitrile in methanol, approximately 0.5 mL of tetrahydrofuran was added and the reaction flask was heated. The resulting solution was blue. Sodium borohydride (0.0148 g, 0.3906 mmol) was slowly added while stirring at 20°C, which resulted in a black solution that evolved
hydrogen gas for five minutes. The solution was stirred for 1 hour. Hydrochloric acid was added until the black precipitate dissolved, resulting in a yellow organic layer and a rose-colored aqueous layer. Sodium hydroxide was added drop wise in order to create a solution with a pH of 9. The organic layer was extracted with dichloromethane, washed with saturated sodium chloride solution, dried over sodium sulfate, and filtered. The brown, tea-colored solution was desolvated, resulting in a brown gel. The product was dissolved in 20 mL of ethanol and 0.5 mL of hydrochloric acid and then desolvated again. The product, a brown gel, was dissolved in 5 mL of dichloromethane and 1 mL of 5% hydrochloric acid. The aqueous portion was washed three times with dichloromethane. The extracted organic portion was dried, filtered, desolvated, and determined via $^1$H-NMR to be recovered T-0632-nitrile. The aqueous portion was then treated with sodium hydroxide and T-0632-amine was extracted with dichloromethane, dried with sodium sulfate, desolvated, and analyzed via $^1$H-NMR. $^1$H-NMR of in CDCl$_3$ (Appendix 8): $\delta$1.3-1.8 (m, 3H, H of alkyl chain; 3H, H of primary amine – a broad peak), $\delta$2.2 (s, 2H, H of alkyl chain), $\delta$2.7 (s, 1H, alkyl chain), $\delta$3.7 (s, 3H, -OCH$_3$), $\delta$6.2 (s, H of m-anisidine, 1H), $\delta$6.6 (d, 1H, H of m-anisidine), $\delta$7.2-8.0 (m; 1H, H of m-anisidine; 4H, H of fluorobenzene; 4H, H of benzene of isoquinoline, 4H), $\delta$8.5 (d, 1H, amine), $\delta$8.9-9.0 (s, 1H, H of N-containing ring of isoquinoline: 2 singlets each integrating to $\frac{1}{2}$, due to two conformations), $\delta$9.1 (s, 1H, H of N-containing ring of isoquinoline).
Appendices

Appendix 1: $^1$H-NMR of major product (12) from Michael Addition in CDCl$_3$
Appendix 2: $^{13}$C-NMR of major product (12) from Michael Addition in CDCl$_3$
Appendix 3: $^1$H-NMR of T-07 (10) in CDCl$_3$
Appendix 4: $^{13}$C-NMR of T-07 (10) in CDCl$_3$
Appendix 5: $^1$H-NMR of Oxime (8) in CDCl$_3$
Appendix 6: $^1$H-NMR of T-0632-Nitrile (11) in CDCl$_3$
Appendix 7: $^1$H-NMR of crude T-0632-Amine (3) via Catalytic Hydrogenation in CDCl$_3$ in CDCl$_3$
Appendix 8: $^1$H-NMR of T-0632-Amine (3) via Sodium Borohydride Halide Salt Reduction in CDCl$_3$
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References


