Exploring Novel Human Pancreatic Alpha-Amylase Inhibitors: A Departure from Carbohydrate-Based Therapeutics

by

Christina Rose Tysoe

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Abstract

Human pancreatic $\alpha$-amylase (HPA) catalyzes a key step in the degradation of ingested starch. Accordingly HPA activity has been positively correlated to post-prandial blood glucose levels and has been identified as a viable target for inhibition and the development of therapeutics towards the treatment of diabetes and obesity. This work directs the hunt away from traditional saccharide-based inhibitors, which represent all carbohydrate metabolism therapeutics currently in use, to novel inhibitors with improved selectivity and potency. In Chapter 2, the synthesis of flavonol-based HPA inhibitors based upon the structure of Montbretin A, a complex flavonol glycoside, is explored. Through the synthesis of a library of Montbretin A analogues we were able to identify an inhibitor of HPA with a $K_i$ of 44 nM that formed new interactions within the amylase active site. Chapter 3 details work on the peptide-based HPA inhibitor helianthamide previously isolated from the Caribbean Sea anemone *Stichodactyla helianthus*. Recombinant expression of helianthamide as a fusion peptide was achieved in *Escherichia coli* and *Pichia pastoris*. Kinetic analysis indicated that recombinant helianthamide is one of the most potent HPA inhibitors known to date, with a $K_i$ of 0.01 nM. Structural analysis of the recombinant material indicated that it contained three disulfide bonds in a 1-5, 2-4, 3-6 pattern. Site-directed mutagenesis of helianthamide indicated that disruption of disulfide bonds led to a large decrease in potency, while alanine variants of residues forming polar contacts with HPA’s active site residues led to smaller decreases in potency, indicating that the intact tertiary structure of helianthamide is necessary for blockage of the amylase active site. Small peptides were
synthesized based on the sequence of helianthamide, but most showed modest or no inhibition of HPA.
Lay Summary

This thesis details the pursuit of novel therapeutics for the management of type II diabetes. Human pancreatic α-amylase is an enzyme involved in starch digestion and presents a promising target for the mediation of blood glucose levels. In the first project, Montbretin A, a natural product extracted from a plant, acts as inspiration for the synthesis of new HPA inhibitors. While Montbretin A showed promise as a potential therapeutic, its complex structure and low yielding biosynthesis limit its large-scale production. By synthesizing simpler versions of Montbretin A we were able to produce an HPA inhibitor with similar potency. In the second project another HPA inhibitor named helianthamide is studied. This inhibitor is a protein isolated from the sea anemone *Stichodactyla helianthus*. We were able to produce helianthamide by engineering *E. coli* and analysis of the material indicated that it was a very potent inhibitor of HPA and remarkably stable.
Preface

Chapter 2 is based on work conducted in Professor Stephen Withers’ laboratory at UBC. I was responsible for the synthesis of all Montbretin A analogues other than compounds M02 and M05 which were synthesized by Dr. Anna Win-Mason. I performed kinetic analysis on all compounds. Dr. Sami Caner of Professor Gary Brayer’s laboratory crystallized M07 and M10 in complex with HPA and solved the resulting structures. Some work contained in Chapter 2 has been published in *Nature Chemical Biology* (2015) 11(9): 691-696 with the title “The amylase inhibitor montbretin A reveals a new glycosidase inhibitor motif” by Williams, L.K.; Zhang, X.H.; Caner, S.; Tysoe, C.; Nguyen, N.T.; Wicki, J.; Williams, D.E.; Coleman, J.; McNeill, J.H.; Yuen, V.; Andersen, R.J.; Withers, S.G. and Brayer, G.D. In this work I was responsible for the synthesis and kinetic analysis of MbA analogue M01. X.H.Z. performed chemoenzymatic degradation of MbA and analysis of resulting derivatives. N.T.N crystallized the MbA/HPA complex. L.K.W. and S.C. solved and analyzed its structure. J.W. performed kinetic analysis of MbA variants. D.E.W. performed ROESY NMR studies on MbA. J.C., J.M., R.J.A., S.G.W. and G.D.B. designed and supervised the project. A second publication containing work detailed in Chapter 2 is in progress.

Chapter 3 is based on work conducted in Professor Stephen Withers’ laboratory at UBC. I was responsible for the recombinant expression of helianthamide and subsequent kinetic and structural analysis. I was also responsible for the synthesis of the small-helianthamide inspired peptides. Work contained in Chapter 3 has been published in *ACS Central Science* (2016) 2: 154-161 with the title “Potent Human α-Amylase Inhibition by the β-Defensin-like Protein Helianthamide” by Tysoe, C.; Williams,
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List of Abbreviations

- $[E]$: Enzyme concentration
- $[E]_0$: Initial enzyme concentration
- $[EI]$: Enzyme-inhibitor complex concentration
- $[I]$: Inhibitor concentration
- $[I]_T$: Total inhibitor concentration
- $[S]$: Substrate concentration
- Abg: *Agrobacterium* sp. β-glucosidase
- AcOH: Acetic acid
- Bar': Barnase'
- Bar'-Hel: Barnase'-Helianthamide
- BLAST: Basic Local Alignment Search Tool
- BMGY: Buffered glycerol-complex medium
- BMMY: Buffered methanol-complex medium
- BnBr: Benzyl bromide
- Boc: tert-Butyloxycarbonyl
- BSA: Bovine serum albumin
- CD: Circular dichroism
- CNPG3: 2-Chloro-p-nitrophenvl α-d-maltotrioside
- DCM: Dichloromethane
- DMF: Dimethylformamide
- DTNB: 5,5'-dithiobis-(2-nitrobenzoic acid)
• EDTA: Ethylenediaminetetraacetic acid
• ESI-TOF: Electrospray ionization – time of flight
• EtOAc: Ethyl acetate
• EtOH: Ethanol
• Fmoc: Fluorenylmethyloxycarbonyl chloride
• GH: Glycoside hydrolase
• GuHCl: Guanidine hydrochloride
• HBTU: (2-(1H-benzotriazolyl)-1,1,3,3-tetramethyluronium hexafluorophosphate)
• Hel: Helianthamide
• HMBC: Heteronuclear Multiple Bond Correlation
• HPA: Human pancreatic $\alpha$-Amylase
• HPLC: High performance liquid chromatography
• HSQC: Heteronuclear single quantum coherence
• IUBMB: International Union of Biochemistry and Molecular Biology
• $K_I$: Inhibition constant
  • $K_{I\text{-app}}$: Apparent inhibition constant
• $K_M$: Michaelis-Menten constant
• LC: Liquid chromatography
• MALDI-TOF: Matrix-assisted laser desorption/ionization – time of flight
• MbA: Montbretin A
• MeOH: Methanol
• MGAM: Maltase-glucoamylase
• MS: Mass spectrometry
• NMR: Nuclear magnetic resonance
• ORF: Open reading frame
• Pet Ether: Petroleum ether
• PFP TFA: Pentafluorophenyl trifluoroacetate
• PhoA: Alkaline phosphatase
• PPA: Porcine pancreatic α-amylase
• Pyr: Pyridine
• RB: Round bottom
• RBI: Ragi bifunctional inhibitor
• RP-HPLC: Reversed phase high performance liquid chromatography
• RPM: Rotations per minute
• RT: Room temperature
• SDS-PAGE: Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
• SI: Sucrase-isomaltase
• t-BuOH: tert-Butanol
• TCEP: (Tris(2-carboxyethyl)phosphine)
• TFA: Trifluoroacetic acid
• THF: Tetrahydrofuran
• TLC: Thin layer chromatography
• TMA: Yellow meal worm α-amylase
• TNP: Trinitrophenol
• UV/Vis: Ultaviolet-visible
• V: Initial reaction rate
• $V_{\text{Max}}$: Maximal velocity
• $V_0$: Uninhibited initial reaction rate
• ZDF: Zucker diabetic fatty
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To my parents, John and Suzanne.
Chapter 1. Introduction

1.1 Glycosidases

Scientists have long marveled at the chemical diversity accessible through carbohydrate-based compounds. With their ability to form branching structures through differing points of attachment and varying stereochemistries, the assemblies accessible to a small carbohydrate chain border on the unimaginable. Coupling carbohydrates with other functional groups, such as proteins, fatty acids or small molecules, further expands the diversity of these compounds. As one can imagine, carbohydrate-processing enzymes encompass an enormous range of substrate specificity and resulting biological functions. Among such enzymes, glycosidases, or glycoside hydrolases, act to hydrolyze glycosidic linkages, between two carbohydrate groups or between a carbohydrate and aglycone unit.

Counter to the impressive diversity of their substrates, glycoside hydrolase reactions are grouped into two general classes – retaining, or inverting – designated on the basis of whether they retain or invert the stereochemistry of the anomeric position of the released glycone unit (Figure 1.1)\(^1\). Retaining glycosidases act through a double displacement mechanism in which the nucleophilic residue forms a covalent bond with the anomeric position of the glycone unit, displacing the aglycone, whose departure is facilitated through protonation by the acid/base residue. The newly deprotonated acid/base residue can then deprotonate the hydrolytic water as it attacks the anomeric center of the glycone, breaking its bond with the nucleophilic residue and creating a new
hemiacetal with the same stereochemistry as the substrate. Inverting glycosidases have a one-step mechanism, involving separate acid and base residues (except in cases when the aglycone is a good enough leaving group to not necessitate protonation by an acid residue). This allows for the simultaneous deprotonation of the attacking nucleophilic water and protonation of the leaving aglycone, to create a new bond with differing stereochemistry to substrate.

**Figure 1.1. General mechanisms of retaining (top) and inverting (bottom) glycosidases.**

From these two general types of mechanism, a large array of chemical functions can arise. Differences in activity and substrate specificity are achieved through the
unique architecture of a glycosidase active site, identity of catalytic residues, and the potential use of cofactors. IUBMB nomenclature classifies enzymes based on substrate specificity, and while useful for a number of applications, it fails to accurately represent glycosidases that can act on many different substrates. To address the limitations of traditional enzyme classification, the Glycoside Hydrolase (GH) Family database was constructed to group glycosidases based on their sequence, and by extension, structural similarities. This allows enzymes to be categorized in a way that teases out meaningful evolutionary relationships and mechanistic similarities, and allows for the prediction of function for new enzymes based on their sequence. There are currently 145 different GH families.

Given the multitude and diversity of biological roles that glycosidases play, the specific inhibition of these enzymes is commonly sought to control their activities. Many glycosidase inhibitors have arisen as a means of mediating endogenous glycosidase activities, or as a way of thwarting the activity of an enzyme from a pathogenic source. The specific inhibition of glycosidases has been pursued towards the treatment of many diseases, such as Influenza, HIV, and as pharmacological chaperones for a number of lysosomal storage disorders. In this body of work we will explore the inhibition of a glycosidase involved in carbohydrate metabolism and its applications for the treatment of diabetes and obesity.

1.2 Inhibiting Carbohydrate Metabolism for the Management of Diabetes

Diabetes mellitus is a metabolic disorder resulting from the inability to either produce adequate levels of insulin or effectively respond to any insulin that is present.
The insulin hormone promotes the absorption of glucose from the bloodstream and inhibits gluconeogenesis in the liver. As a result, the ultimate consequence of diabetes is a dysregulation of blood glucose levels, which can lead to a number of serious consequences over time, including but not limited to: nerve and blood vessel damage, heart disease, kidney disease, stroke, and blindness\textsuperscript{10-14}. Type II diabetes accounts for 90-95\% of all diabetes cases and has become increasingly common in the industrialized world\textsuperscript{15,16}. This form of the disease manifests as pancreatic β-cell impairment, which leads to the gradual loss of insulin production and cellular responsiveness to insulin\textsuperscript{17}. Genetic susceptibility has been shown to play a role in the onset of type II diabetes. A number of genes have been associated with this disease and are thought to confer variability in glucose metabolism or insulin resistance between different populations\textsuperscript{15}. While genetic factors can dispose individuals to type II diabetes, lifestyle choices have been shown to play the predominant role in its ultimate onset. There is a strong correlation between obesity and type II diabetes, and approximately 80-87\% of type II diabetics are overweight or obese\textsuperscript{15,18,19}. Perhaps related to the link between diabetes and obesity is an additional association with a high nutritional intake of simple sugars and saturated fats as well as a lack of physical activity\textsuperscript{20}.

High levels of therapeutic insulin have also been linked to obesity\textsuperscript{21}. As a result, therapeutic interventions that lower blood glucose levels in type II diabetics independently of insulin are of value. This can be accomplished by controlling the influx of glucose into the bloodstream from gluconeogenesis in the liver or carbohydrate catabolism in the gastrointestinal tract. Metformin is one of the most widely prescribed anti-diabetics and is often the first line of treatment for type II diabetics. Metformin
prevents hyperglycemia through the inhibition of gluconeogenesis\textsuperscript{22}. The mode of action of metformin is not fully understood and it appears to act through many different pathways, such as mild inhibition of mitochondrial respiratory chain complex 1, inhibition of mitochondrial glycerophosphate dehydrogenase, and through AMPK activation\textsuperscript{23,24}. Despite the success of metformin, it is not an appropriate line of action for some patients due to its association with certain side effects. Patients with liver, lung, cardiac and kidney impairment can experience a number of risks upon metformin usage\textsuperscript{25,26}. Metformin has been linked to an increased risk of lactic acidosis, especially upon renal impairment\textsuperscript{27,28}. As renal damage can result from unmanaged diabetes, this has disqualified many diabetic individuals from use of this drug. As a result, therapeutics that act through differing modes of action and can be used in populations with hepatic and renal dysfunction are of value.

As an alternative approach, therapeutic interventions directed at the influx of glucose from the diet have been explored\textsuperscript{29}. This approach offers a dual function as it can also be used as a means of promoting weight loss in obese and pre-diabetic individuals to prevent the onset of diabetes. The use of carbohydrate metabolism inhibitors can also avoid potential hypoglycemia associated with insulin or metformin overdose.

The ability of different foods to affect blood glucose levels can be described by their glycemic index. Foods rich in starch typically have a high glycemic index, as one starch molecule is composed of thousands of individual glucose units. As such, starch degradation is a good target for the development of anti-diabetic therapeutics. The digestion of starch involves a number of enzymes and begins in the oral cavity with the
hydrolysis of insoluble starch polymers into shorter oligomers by salivary $\alpha$-amylase$^{30}$. Upon reaching the small intestine, pancreatic $\alpha$-amylase provides a more extensive hydrolysis, cleaving the starches into a mixture of maltose, maltotriose and other small branched oligosaccharides$^{31}$. The resulting mixture then passes into the brush border of the small intestine where it is processed into glucose by maltase-glucoamylase and sucrose-isomaltase$^{32,33}$. Inhibiting the enzymes involved in this pathway offer a means of preventing a potentially large and harmful influx of glucose into the bloodstream.

Miglitol, voglibose and acarbose (Figure 1.2) are three inhibitors of carbohydrate metabolism currently in medical use. Miglitol and voglibose are small pseudo-monomosaccharides that inhibit the $\alpha$-glucosidases of the brush border of the small intestine. This allows them to also prevent the cleavage and subsequent absorption of ingested disaccharides such as sucrose, in addition to the maltose and maltotriose produced from starch degradation. While effective at mediating post-prandial blood glucose levels, these inhibitors are associated with a number of side effects$^{34-37}$. This may in part be due to their small size leading to systemic absorption and activity on other $\alpha$-glucosidases. Acarbose inhibits pancreatic $\alpha$-amylase in addition to the brush border $\alpha$-glucosidases$^{38}$. Acarbose is more widely used than miglitol and voglibose, and has especially witnessed a surge in usage in the Asia-Pacific region$^{39-41}$. It appears that while acarbose is effective in both Caucasian and Asian populations, South East and East Asians experience greater effects from this drug$^{42-44}$. Unfortunately, acarbose also produces unwanted side effects and has been linked to hepatotoxicity in limited cases$^{45}$. While acarbose itself is not absorbed from the intestine, it can be processed by $\alpha$-amylase and the derivatives of this hydrolysis could be potentially absorbed$^{46}$. 
While the inhibition of the brush border α-glucosidases allows these therapeutics to prevent the metabolism and absorption of disaccharides such as sucrose, there is a consequence to the resulting displacement of di- and tri-saccharides into the lower gut. Small saccharides exert a strong osmotic effect within the large intestine causing water to be drawn into the bowels resulting in diarrhea, nausea, and abdominal discomfort. In addition, these displaced disaccharides are rapidly processed by bacteria in the lower gut resulting in flatulence and bloating, which can cause additional gastrointestinal discomfort\textsuperscript{36,47,48}. An inhibitor of carbohydrate metabolism that avoids these side effects while allowing for the control of post-prandial blood glucose levels is of value.
1.3 Targeting Human Pancreatic $\alpha$-Amylase

Human pancreatic $\alpha$-amylase (HPA) catalyzes the endo-hydrolysis of $\alpha$(1-4)-d-glucosidic linkages in starch (Figure 1.3). It is part of the sequence-related GH13 Family of glycosidases, which are unified by their retaining mechanisms and hydrolysis or transglycosylation of substrates with $\alpha$-glucoside linkages. The architecture of the HPA active site accommodates (1-4)-$\alpha$-linked D-glucosyl oligosaccharides, allowing this enzyme to hydrolyze not only starch, but also glycogen and a number of other poly- and oligo- saccharides. There are five major subsites, S-3 to S+2, in the amylase active site, as well as one minor subsite, S+3, used for binding longer substrates. Three active site residues, D197, E233, and D300, have been highlighted as necessary for catalysis through a classical double-displacement mechanism. D197 is the catalytic nucleophile, which forms a covalent bond with the glucosyl unit positioned in the S-1 subsite. E233 acts as the acid base residue, which first protonates the leaving group in S+1, then deprotonates the incoming nucleophilic water in the second half of the double displacement mechanism. Finally, D300 is thought to be important for coordinating the substrate and positioning the catalytic water in the active site for efficient hydrolysis, and while not directly involved, it has been shown that mutating this residue leads to a significant decrease in enzyme activity.
Figure 1.3. Human Pancreatic α-Amylase. (a) Structure of HPA with maltohexaose substrate (yellow) bound within the active site cleft (PDB: 5TD4). (b) Close up of HPA active site. Side chains of catalytic residues drawn in magenta. (c) Binding of maltohexaose across the HPA subsites with hydrolysis occurring between S-1 and S+1.

The specific inhibition of HPA has been highlighted as an effective means of managing post-prandial blood glucose and could present benefits over general carbohydrate metabolism inhibition. The activity of pancreatic α-amylase activity is positively correlated with post-prandial blood glucose levels, and preclinical studies with HPA inhibitors have shown a decrease in fasted plasma glucose and post-prandial plasma glucose\(^ {53,54,55}\). Specific inhibition of HPA over the brush border α-glucosidases would also lead to the displacement of longer chain carbohydrates rather than disaccharides into the lower gut. These larger carbohydrates are hypothesized to exert a smaller osmotic effect, and would instead act more like fiber. While specific inhibition of HPA would still allow for sucrose to be metabolized and absorbed, inhibition of starch degradation should be adequate to prevent hyperglycemia due to the high glycemic
index of this energy source. Since HPA acts within the lumen of the small intestine, inhibitors can have, and are actually preferred to have, a poor bioavailability so as to remain within the gastrointestinal tract. Minimal absorption into the bloodstream could prevent side effects associated with inhibition of off-target enzymes or metabolic processing. This opens up the use of structures previously considered unfavorable in drug design. These combined factors compel the search for a potent and specific inhibitor of human pancreatic α-amylase. Luckily, as we will explore, there is no shortage of α-amylase inhibitors in nature, offering an abundance of compounds to study for potential application.

Amylases and their ability to hydrolyze starch are found across all kingdoms of life. Plants, which synthesize and use starch for energy storage, use amylases for the redistribution of glucose to their seeds and budding regions. Meanwhile, species that consume plant material use this class of enzyme to access this valuable source of glucose. Many plants have evolved the ability to produce amylase inhibitors as a defense against predators or as a means of modulating their own enzymes. Amylase inhibitors have also been found in many prokaryotic organisms, secreted as an offense against competitors. The structures and specificity of these inhibitors vary widely. Some, like acarbose, are secondary metabolites that closely mimic the structure of the natural substrate; others like the protein-based inhibitors can instead exploit their relatively large size to clog the enzyme active site. Some of these inhibitors act on proteases and α-glucosidases in addition to α-amylase, further highlighting the diverse anti-feedant strategies of these organisms. Many of these α-amylase inhibitors are specific for
insect or plant enzymes and show no activity towards mammalian $\alpha$-amylases. Here we will focus on inhibitors that show activity towards HPA.

### 1.4 Carbohydrate-Based Inhibitors of HPA

Carbohydrate-based inhibitors are perhaps the most well-studied class of glycosidase inhibitors, with many examples of both natural and synthetic inhibitors. These molecules are thought to encourage tight binding by mimicking the structure of the enzyme’s substrate or transition state. Glycosidase reactions proceed through an oxocarbenium ion-like transition state, which is characterized by the development of a positive charge and partial planarity. A common tactic in the design of glycosidase inhibitors is the incorporation of a nitrogen atom into a substrate-like sugar. The positive charge of the nitrogen is thought to mimic that of the transition state and can develop additional polar interactions within the active site. Another approach is the incorporation of a double bond into the saccharide structure to mirror the partial planarity of the transition state.

Despite the prevalence of carbohydrate-based glycosidase inhibitors, there are relatively few examples of potent HPA inhibitors. The accumulation of interactions spanning the six subsites of the HPA active site is what confers appreciable affinity for its substrate. Studies have shown that $\alpha$-amylase can cleave substrates as small as maltotriose, however it does so with much lower catalytic efficiency than towards longer chain substrates. As a result, HPA inhibitors typically require longer saccharide chains to effectively bind when compared to inhibitors of other $\alpha$-glucosidases.

The anti-diabetic therapeutic acarbose is a competitive inhibitor of HPA with a $K_i$ of approximately 2 - 20 $\mu$M. It is produced as a secondary metabolite in
Actinoplanes sp. and Streptomyces sp. Acarbose is a pseudo-tetrasaccharide composed of maltose and the pseudo-disaccharide acarviosine (Figure 1.4a). The unsaturated valienamine of the acarviosine mimics the partial planarity of the oxocarbenium ion-like transition state to encourage tight binding of this moiety in subsite S-1. The exocyclic amine of the acarviosine interacts with the acid-base residue to further promote binding\textsuperscript{54}. While the acarviosine is resistant to hydrolysis, there are other potential sites of hydrolysis in acarbose. X-ray crystallographic analysis of HPA in complex with acarbose showed the enzyme binding a modified version of the inhibitor (Figure 1.4b), which was hypothesized to result from enzyme-catalyzed hydrolysis and transglycosylation\textsuperscript{46,54}. Evidence of amylase-catalyzed transglycosylation of acarbose has also been observed during X-ray crystallographic analysis of complexes with porcine and bacterial $\alpha$-amylases\textsuperscript{64-66}. It was shown that this transglycosylation product binds HPA more tightly than acarbose\textsuperscript{46}. However the HPA-mediated processing of acarbose may also release smaller derivatives of this inhibitor, which may be systemically absorbed and produce undesirable side effects.

Acarbose biosynthesis is carried out within the same pathway as the antibiotics, Validamycin A and B. These compounds also contain the planar valienamine group but show no inhibitory activity towards HPA\textsuperscript{67-69}. Streptomyces sp. produces other acarviosine-containing secondary metabolites that show inhibitory activity towards HPA. These inhibitors are closely related to acarbose and exhibit more potent inhibition towards pancreatic $\alpha$-amylase with $K_i$’s ranging from 0.008 to 0.3 $\mu$M, likely due to their longer chain lengths\textsuperscript{61}. 
Figure 1.4. Acarviosine-based HPA inhibitors. (a) Acarbose (b) HPA-modified acarbose

HPA exhibits low affinity for mono- and di-saccharides, and as a result, is not inhibited by the majority of known pseudo-monosaccharide α-glucosidase inhibitors. However, these molecules can be elongated to produce effective inhibitors of HPA. One such example is the elongation of the α-glucosidase inhibitor gluconohydroximino-1,5-lactam. This compound has low affinity towards HPA with a $K_i = 18$ mM. However upon enzymatic elongation into a pseudo-trisaccharide the resulting compound showed a significant increase in potency to a new $K_i$ of 25 µM.

Inhibitor elongation has also been used in the development of mechanism-based inhibitors of HPA. Mechanism-based inhibitors are substrate mimics that exploit the catalytic mechanisms of enzymes to trap them in a covalent complex. Retaining glycosidases are susceptible to specific targeting by mechanism-based inhibitors as
they form covalent contact with their substrate during hydrolysis. The installation of a good leaving group at the anomeric center of the inhibitor encourages attack by the enzyme’s catalytic nucleophile. The presence of an electron-withdrawing group, such as fluorine, at C-2 or C-5 then destabilizes formation of the oxocarbenium ion-like transition state of the subsequent hydrolysis step, effectively trapping the enzyme in a covalent complex. Mechanism-based inhibitors of HPA can be used for structural and mechanistic analysis, such as characterization of active site residues. One such example is trinitrophenyl 2,2-difluromaltoside, which was the first mechanism-based inhibitor of HPA to be synthesized (Figure 1.5a). Mechanism-based inhibitors of HPA have also been produced by in situ elongation, analogous to the in situ transglycosylation and elongation of acarbose. One example is the use of 5-fluoro idosyl fluoride with maltosyl fluoride donors, which were shown to be converted into a new elongated inhibitor upon incubation with HPA (Figure 1.5b). Cyclophellitol derivatives have also shown to be mechanism-based inhibitors of HPA and have been used in structural analysis of HPA towards study of surface starch binding sites (Figure 1.5c). These compounds act in similar ways to the fluorinated inhibitors; the epoxide provides a reactive center for nucleophilic attack and the absence of a ring oxygen prevents formation of the oxocarbenium ion-like transition state necessary for subsequent hydrolysis.
**Figure 1.5. Mechanism-based inhibitors of HPA.** (a) Mechanism of HPA inactivation by trinitrophenyl 2,2-difluoromaltoside. Trinitrophenol (TNP) acts as a good leaving group to encourage attack by the nucleophilic residue. Placement of two fluorides at C2 destabilizes development of the oxocarbenium ion-like transition state of the second step of the double displacement mechanism, leading to slow hydrolysis. (b) Structure of the oligosaccharyl 5-fluoroglycosyl fluoride, produced by *in situ* elongation. (c) Structure of glucosyl α-epi-cyclophellitol.

### 1.5 Flavonoids and Other Phenolic Compounds as HPA Inhibitors

Flavonoids are a class of secondary metabolites that are ubiquitous throughout the plant and fungal kingdoms. These phenolic molecules contain a 15-carbon frame consisting of two phenyl groups joined through a central heterocycle. Structurally, flavonoids are divided into six subclasses: anthocyanidins, flavanones, flavones, flavonols, flavandiols, and isoflavones (Figure 1.6a). Their conjugated π-electrons systems can confer a number of chemical functions, such as UV/Vis absorbance and the ability to act as reducing agents. Further chemical modification, often through
methylation, glycosylation or linkage to other phenolic groups, produces a plethora of
derivatives of varying biological activities. Flavonoid derivatives have been shown to
carry out a number of roles in their host organisms including pollinator attraction,
predator defense, UV protection, radical scavenging, and chemical messaging\textsuperscript{75-78}.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{flavonoid_subclasses.png}
\caption{(a) Flavonoid Subclasses. (b) Cinnamic Acid. (c) Gallic Acid.}
\end{figure}

The consumption of flavonoids has been touted with a number of health benefits,
from cancer prevention to the promotion of cardiovascular health\textsuperscript{78}. Notably, they have
also been promoted as supplements for weight loss. Evidence has arisen to show that
many plant extracts rich in flavonoid derivatives inhibit digestive $\alpha$-glucosidases and $\alpha$-
A number of flavonoid constituents have been isolated from such extracts and have been shown to moderately inhibit salivary and pancreatic $\alpha$-amylase. $\alpha$-Amylases increase binding affinity for their starch substrate through the formation of $\pi$-CH interactions between aromatic residues in the enzyme and the starch’s glucosyl rings. Flavonoid-based inhibitors can take advantage of this by forming $\pi$-$\pi$ interactions with these residues. Meanwhile, the hydroxyls of flavonoids can form polar interactions with the catalytic Glu and Asp residues.

Of the six flavonoid subclasses, flavonols and flavones were shown to have the highest potency towards $\alpha$-amylase. These two subclasses contain both a C4 carbonyl and C2-C3 double bond within their pyrone nucleus. This is thought to stabilize binding in the enzyme active site due to increased electron density and extensive electron delocalization. The flavone scutellarein and related flavonol, quercetagtein, were the most potent of these two subclasses (Figure 1.7), exhibiting IC$_{50}$’s of 10 $\mu$M against human salivary $\alpha$-amylase. Certain flavonols have been tested for their effects on hyperglycemia and glycogen metabolism in diabetic rats and were shown to produce a 50% decrease in hyperglycemia when used at a concentration of 3 mg / 12 hrs.

![Figure 1.7. Structures of quercetagetin and scutellarein.](image-url)
Cinnamic acid derivatives were also enriched in many of the extracts showing inhibitory activity towards HPA\textsuperscript{75,79,80,87}. These compounds are important precursors for flavonoid biosynthesis and are commonly found together with flavonoids in many plant extracts (Figure 1.6b). Use of extracts rich in cinnamic acid derivatives led to a significant decrease in amylase activity. However upon isolation, individual cinnamic acid derivatives such as caffeic acid and chlorogenic acid showed a lower potency than most flavonoids, acting as noncompetitive inhibitors with IC\textsubscript{50}'s around 1 - 12 mM\textsuperscript{81,87,88}. Gallic acid derivatives were also found in \(\alpha\)-amylase-inhibiting plant extracts (Figure 1.6c)\textsuperscript{89}. Tested gallotannins, polymers of gallic acid and glucose, show mixed non-competitive inhibition towards salivary \(\alpha\)-amylase with inhibition constants of 1 - 9 \(\mu\text{M}\textsuperscript{90,91}. Gallic acid was also found to functionalize a number of inhibitory flavonoid derivatives. Theaflavin gallates are flavonoid-based pseudotannins which show IC\textsubscript{50} values as low as 3 \(\mu\text{M} against human salivary \(\alpha\)-amylase\textsuperscript{84,92}.

None of these individual phenolic compounds showed particularly potent inhibition towards \(\alpha\)-amylase until recently. Montbretin A, a complex flavonol glycoside was recently isolated from the methanolic extract of Crocosmia crocosmiiflora and exhibited high affinity towards HPA with an inhibition constant of 8 nM\textsuperscript{93}. As will be explored further in Chapter 2, Montbretin A has shown promise as a potential anti-diabetic therapeutic and has provided inspiration for the development of additional flavonoid-based HPA inhibitors.

1.6 Peptide Inhibitors of Pancreatic \(\alpha\)-Amylase

A number of plant and microbial species produce peptide-based inhibitors of \(\alpha\)-amylases as a means of defense and competition. Several different classes of protein-
based α-amylase inhibitors have been elucidated. Many of these classes, however, show no activity towards mammalian enzymes and instead target endogenous, insect, and/or bacterial α-amylases. Protein inhibitors of α-amylases can exhibit astonishingly high potencies towards their target enzyme when compared to the phenolic or carbohydrate-based inhibitors. The accumulation and positioning of the various functional groups along their long peptide chains allows them to form numerous polar and other contacts within and around the enzyme active site. On the other hand, their size and three-dimensional structure can also allow these proteins to act simply by occluding the enzyme active site; many show potent inhibition of their target α-amylase while maintaining few to no polar interactions with the enzyme. Here we will cover previously studied peptide classes that inhibit mammalian α-amylases. Protein-based inhibitors of α-amylase that do not inhibit mammalian enzymes include knottins, thionin-like, Kunitz-type, and thaumatin-like peptides, and will not be discussed in this section 56.

1.6.1 Streptomyces Peptides

Perhaps the most potent known inhibitor of any α-amylase is the peptide tendamistat (Figure 1.8), produced by Streptomyces tendae. Tendamistat is a 74-residue protein with an estimated $K_i$ of 0.009 – 0.2 nM against HPA 94. This protein is composed of a β-sandwich fold stabilized by two disulfide bonds 95,96. Crystallographic analysis indicated that the peptide forms contacts with the enzyme over four discontinuous regions of its sequence. Of particular note are residues W18, R19, and Y20 of tendamistat, which are observed protruding into the enzyme active site. Only R19 forms interactions with the enzyme’s catalytic residues, with a hydrogen bond with acid-base residue E233 95.
This WRY motif is conserved amongst tendamistat’s structural relatives also produced in *Streptomyces* sp. These peptides share approximately 30% sequence identity to tendamistat and display similarly high potency against pancreatic $\alpha$-amylase with estimated inhibition constants from 0.028 to 0.250 nM $^{56,97,98}$. Examples of these are HAIM, PAIM, Parvulustat and AAI-CC5. Tendamistat was initially explored as a therapeutic agent for the management of diabetes, and was shown to decrease blood plasma glucose levels during clinical trials$^{99,100}$. However its vulnerability to degradation and pronounced immunogenicity, thought to be due to its structural resemblance to T-cell receptors, eventually led to its abandonment in the clinic$^{94,101}$.

### 1.6.2 Cereal-Type Inhibitors

The cereal-type $\alpha$-amylase inhibitors are produced in barley, wheat, ragi and rye, and show activity towards insect, bacterial, bird and mammalian $\alpha$-amylases$^{57}$. Cereal-type inhibitors are larger than the *Streptomyces* peptides, ranging from 120 - 160 residues and consisting of an $\alpha$-helical-rich structure stabilized by five disulfide bonds.
(Figure 1.9)$^{57}$. In some cases, these peptides may further assemble as homodimers or heterooligomers$^{57,102,103}$. The cereal-type structural class also includes trypsin protease inhibitors as well as double-headed inhibitors that inhibit both $\alpha$-amylase and trypsin$^{56}$.

![Image](Figure 1.9. Ragi bifunctional inhibitor. (PDB: 1B1U))

Ragi bifunctional inhibitor (RBI) from Indian finger millet is a monomeric competitive inhibitor of yellow mealworm $\alpha$-amylase (TMA) and pancreatic $\alpha$-amylase with an approximate inhibition constant of 15 nM against porcine pancreatic $\alpha$-amylase (PPA)$^{104}$. An X-ray crystal structure was solved for this protein in complex with TMA$^{102}$. RBI forms contact with the enzyme active site cleft over 26 residues of its sequence. Notably, S1 and S5 of RBI protrude into the active site to form hydrogen bonds with the three catalytic residues of TMA. RBI also shows inhibitory activity towards trypsin, with the capability of forming a ternary 1:1:1 complex with trypsin and $\alpha$-amylase$^{104}$. The trypsin-binding loop is located on the opposite end of the peptide from the $\alpha$-amylase binding site, involving residues R34 and L35. Another example of the cereal-type $\alpha$-amylase inhibitors is inhibitor 0.19 which is active towards mammalian, bird, insect and
bacterial $\alpha$-amylases. Inhibitor 0.19 is reported to have a $K_i$ of 0.29 nM against human salivary $\alpha$-amylase$^{105}$. Unfortunately many cereal-type inhibitors have been linked to wheat- and rye-related allergenicity and dermatitis, preventing clinical application$^{106,107}$.

### 1.6.3 Lectin-Like Inhibitors

The lectin-like $\alpha$-amylase inhibitors are produced in red, white and black kidney beans. Members of this class predominantly inhibit insect $\alpha$-amylases, however one member, $\alpha$AI1 (Figure 1.10), also shows activity towards mammalian $\alpha$-amylases$^{108}$. These inhibitors are expressed in legumes as part of a lectin-arcelin-$\alpha$AI1 supergene family as a multipronged defense against predators$^{109,110}$. While structurally homologous to the legume lectins, these $\alpha$-amylase inhibitors are missing two loops essential for sugar binding while having additional binding loops for $\alpha$-amylase inhibition$^{111}$.

![Figure 1.10. $\alpha$-Amylase Inhibitor 1 ($\alpha$AI1). Monomer unit of the homodimer $\alpha$AI1 inhibitor observed bound to two $\alpha$-amylase molecules (PDB: 1VIW). $\beta$-Strands shown in orange, $\alpha$-helices shown in red, loops in blue.](image)

These inhibitors were shown to exist as heterotetramers composed of two $\alpha$- and two $\beta$-subunits$^{112}$. These heterotetramers can bind to $\alpha$-amylase in a 1:2
inhibitor/enzyme stoichiometry, allowing for inhibition of two enzyme molecules at once\textsuperscript{113}. α-Al1 was shown to inhibit mammalian pancreatic α-amylase through slow onset mixed non-competitive inhibition with an overall $K_i$ of 0.030 – 0.1 nM\textsuperscript{112-115}. Despite evidence for assembly as a α2β2 heterodimer, X-ray crystallographic analysis of αAl1 in complex with PPA showed the inhibitor bound to two amylase molecules as a homodimer. In this structure, two hairpin loops from each inhibitor monomer were observed extending into the enzyme active site clefts to form hydrogen bonds between catalytic residues and Y37, Y186, and Y190 of the αAl1\textsuperscript{116}.

1.6.4 Designer Peptide Inhibitors

In addition to naturally occurring protein inhibitors of HPA, a number of synthetic peptides have also been explored. Using a combinatorial chemistry approach, one group was able to synthesize a novel octapeptide inhibitor with an IC\textsubscript{50} of 0.5 μM against PPA\textsuperscript{117}. The lead sequence, GHWYYRCW, was isolated through successive screening rounds and sub-library creations. Further modification of the arginine with a tosyl group increased potency. Similarities between the binding motifs of the lead peptide and the conserved WRY region of tendamistat were noted. However unlike the rigid structures of the \textit{Streptomyces} peptides, this peptide was only active in its flexible acyclic form. More recently, a family of highly potent peptide inhibitors of HPA ($K_i = 1 - 7$ nM) were identified during a library screen using the Random Non-Standard Peptides Integrated Discovery (RaPID) approach\textsuperscript{118}. Large libraries of randomized DNA and corresponding peptides ($\sim 10^{13}$) could be screened through this approach. Translated peptides were displayed on their encoding mRNAs and their binding capabilities towards the HPA target were tested. Using this approach, a conserved sequence was
identified as the lead structure. This nonapeptide contained lariat cyclization between the N-terminus and internal cysteine residue (Figure 1.11). This peptide was co-crystallized with HPA, revealing that two tyrosine residues from the inhibitor appear to be responsible for the majority of the interactions within the active site.

![Figure 1.11. Structure and sequence of piHA. (PDB: 5KEZ)](image)

Other approaches have been taken towards the creation of novel non-natural peptide inhibitors of HPA. Larger carbohydrate binding domains have been used as scaffolds in phage display screens to produce peptides capable of binding in the PPA active site\(^{119}\). Anti-PPA camelid antibodies have also been produced, and were potent inhibitors of the enzyme with a \(K_i\) of 10 nM\(^{120}\).

### 1.7 The Search For New HPA Inhibitors

The gentle but unforgiving pressures of evolution have created and refined an abundance of chemical solutions for the challenges that living organisms face. Many of these chemical compounds have provided humanity with invaluable therapeutics and tools, despite potentially arising under different evolutionary pressures. Originally designed as a way to ward off starch-hungry predators, \(\alpha\)-amylase inhibitors are now
being explored as a means of controlling blood sugar levels in the face of diabetes’ increasing prominence in the industrialized world.

This body of work sets to pay homage to some of Nature’s ingenious chemical tools and their potential use towards the production of novel anti-diabetic therapeutics. In Chapter 2, a complex flavonol glycoside isolated from *Crocosmia crocosmiiflora* acts as inspiration for the design and synthesis of novel HPA inhibitors. In Chapter 3, the structure and mode of inhibition of a novel peptide inhibitor of HPA originally isolated from *Stichodactyla helianthus* is explored. Through these two projects, we aim to better understand the structural requirements of $\alpha$-amylase inhibition and highlight novel lead structures for the development of anti-diabetic therapeutics that can offer benefits over the carbohydrate-based therapeutics currently in use.

**1.8 Aims of this Thesis**

**1. Design and Synthesis of Montbretin A Analogues**

Montbretin A is a potent and specific inhibitor of human pancreatic $\alpha$-amylase, however its complex structure limits its large-scale production for therapeutic usage. The synthesis of simple Montbretin A analogues and their subsequent kinetic analysis will highlight functionalities necessary for potent HPA inhibition and potentially provide viable therapeutic leads.

**Specific Aims:** Design and synthesize a library of Montbretin A analogues. Subject each molecule to kinetic analysis and select lead candidates for further applications and analysis.
2. Structural Analysis of Helianthamide

Helianthamide was discovered in a high-throughput screen of marine extracts. Initial analysis indicated that it could be a potent peptide inhibitor of HPA with a completely different structure from other α-amylase inhibitors. However insufficient material was available for adequate analysis. The recombinant expression of helianthamide can provide a viable means of producing this inhibitor as well as a way of producing variant structures. Upon expression, analysis of helianthamide can be conducted to provide further insight into its structure, specificity and mode of inhibition. These lessons can then be applied to the production of medically relevant HPA inhibitors.

**Specific Aims:** Recombinantly express helianthamide and perform kinetic analysis, mutagenesis studies, and other structural analyses. Synthesize small peptides based on helianthamide’s sequence to explore their potential as HPA inhibitors.
Chapter 2. Design and Synthesis of Montbretin A Analogues

2.1 Introduction

2.1.1 Discovery of Montbretin A, a Potent and Specific HPA Inhibitor

In previous work from the Withers laboratory, a library of crude marine and terrestrial biological extracts from the National Cancer Institute was screened for novel HPA inhibitors via a high-throughput plate-based assay\(^93\). During this screen one sample was an immediate frontrunner for HPA inhibition, practically eliminating all enzyme activity during the assay. This inhibitory material was the methanolic extract of the iris *Crocosmia crocosmiiflora*. Subsequent activity-based isolation yielded a complex acyl flavonol glycoside as the compound largely responsible for the observed inhibition. This compound, Montbretin A (MbA), contained a myricetin flavonol core, which was glycosylated at the 3- and 4’- positions. The 3-OH was adorned with an \(\alpha\)-linked D-glucopyranosyl-(\(\beta\)1\(\rightarrow\)2)-D-glucopyranosyl-(\(\beta\)1\(\rightarrow\)2)-L-rhamnopyranoside trisaccharide. On this trisaccharide, a 6-O-caffeic ester was attached to the central D-glucosyl group. The 4’-OH of myricetin bore a \(\beta\)-linked L-rhamnopyranosyl-(\(\alpha\)-1\(\rightarrow\)4)-D-xyloside (Figure 2.1). Kinetic analysis indicated that MbA had an inhibition constant of 8 nM towards HPA and showed no activity towards human maltase-glucoamylase or sucrase-isomaltase, suggesting its use could avoid the side effects associated with general \(\alpha\)-glucosidase inhibition.
Along with MbA, two related molecules were also isolated from the *C. crocosmiiflora* extract. These compounds, Montbretin B and C, were identical to Montbretin A other than the substitutions on their cinnamic acyl moiety. Loss of the free hydroxyl from the 3-position of the caffeic ester produced compounds with inhibition constants of 3.6 and 6.1 µM. An X-ray crystal structure of MbA in complex with HPA was elucidated, showing π-stacking between the flavonol and caffeic ester moieties within the amylase active site\textsuperscript{121} (Figure 2.2). In this structure a number of interactions were observed between the phenolic groups of MbA and the active site residues. Most notably, the catechol group of the caffeic ester was seen to hydrogen bond with acid-base residue E233 and nucleophilic residue D197. The 7-OH of the myricetin A-ring was also seen to interact with D197. Interestingly, not many interactions were seen

![Chemical structure of Montbretins A, B, and C](image)

<table>
<thead>
<tr>
<th>Montbretin</th>
<th>R\textsuperscript{1}</th>
<th>K\textsubscript{i} (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>OH</td>
<td>8</td>
</tr>
<tr>
<td>B</td>
<td>H</td>
<td>3600</td>
</tr>
<tr>
<td>C</td>
<td>OMe</td>
<td>6100</td>
</tr>
</tbody>
</table>

**Figure 2.1. Structure of Montbretins A, B and C.**
between MbA’s extensive glycosidic appendages and the enzyme. Only the xylose of the 4’-disaccharide formed hydrogen bonding with enzymatic residues. NMR analysis of MbA indicated that the A-ring of myricetin and catechol of the caffeic ester participated in π-stacking in solution, allowing MbA to exist in a pre-stacked confirmation\textsuperscript{121}.

**Figure 2.2. Structure of HPA/MbA complex.** (PDB: 4W93)

In the same study, a step-wise chemoenzymatic degradation of MbA was also carried out. Through this experiment a ‘minimum inhibitory structure’ was identified. This structure was missing all carbohydrate appendages other than the D-glucopyranosyl-(β1→2)-L-rhamnopyranose disaccharide that linked the myricetin and caffeic ester moieties. This compound was labeled MbA-GRX (Figure 2.3) and exhibited a \( K_I \) of 90 nM against HPA, only a 10-fold decrease in potency compared to the parent compound, yet possessed a much simpler structure.
Montbretin A was also administered to ZDF diabetic rats and was shown to produce significant decreases in fed and fasted blood plasma glucose levels when used at a dose of 7.5 mg/kg per day\textsuperscript{55}. The extent of effects on plasma glucose levels was similar to that seen with acarbose at a roughly equal dose. Given that, unlike acarbose, Montbretin A shows no inhibition towards the digestive $\alpha$-glucosidases, these results illustrate MbA’s potency and suggest that it can achieve similar outcomes to clinically used inhibitors with fewer side effects.

2.1.2 Design and Synthesis of Montbretin A Analogues

These studies have highlighted Montbretin A as a promising lead for the therapeutic reduction of post-prandial blood glucose levels. However, production of this compound on a large scale faces a number of challenges. The extraction and purification of Montbretin A from the corms of \textit{C. crocosmiiflora} involves many steps and requires the processing of a large amount of biomass. It has also been difficult to separate Montbretin A from other phenolic relatives and secondary metabolites when
working on a large scale. To combat this, researchers in the Bohlmann lab at UBC are currently exploring the details of Montbretin A’s biosynthesis to uncover the enzymes involved in its production to potentially export the biosynthetic apparatus to a recombinant host.

An alternative approach is the design and synthesis of inhibitors based upon Montbretin A’s structure in hopes of finding chemically accessible compounds with similar potency and specificity. The use of synthetic compounds could also circumvent issues potentially associated with a flavonol glycoside-based therapeutic. There is evidence that flavonol glycosides undergo degradation by microbial enzymes within the lower intestine\textsuperscript{122-125}. Given the location of the desired enzymatic target within the small intestine, such bacterial degradation shouldn’t impede activity. However, there has been some evidence of flavonol glycoside hydrolysis and absorption in the small intestine\textsuperscript{126-129}. Whether or not MbA would be subjected to such processes is currently unclear. Regardless, it is worth pursuing synthetic analogues from a production standpoint and to also further understand structural requirements for $\alpha$-amylase inhibition.

The step-wise chemoenzymatic degradation of MbA highlighted a simplified inhibitory structure, suggesting that an inhibitor containing similar flavonol and caffeic acyl moieties could achieve equivalent levels of potency. The catechol moiety of MbA’s caffeic ester was shown to be necessary for potent inhibition. However, one point of variation could be in replacing the ester linkage with an amide to improve stability (Figure 2.4). This then leaves us with two other regions for alteration. The first is the identity of the flavonol core.
Figure 2.4. Designing synthetic MbA analogues. There were three regions of potential alteration in the design and synthesis of our MbA analogues. The first is the identity of the flavonoid core (highlighted in blue). The second is the substitution of an amide linkage in the caffeate moiety (highlighted in red). The third is the length and chemical identity of the linker, which would join the two phenolic moieties.

Montbretin A possesses a myricetin core. Myricetin and related flavonol quercetin showed comparable binding affinities against PPA and salivary α-amylase (Figure 2.5). In the HPA/MbA crystal structure there were minimal points of contact between the myricetin B-ring and the HPA active site. This portion of the flavonol instead pointed out into solution, decorated with the L-rhamnopyranosyl-(α-1→4)-D-xyloside disaccharide. This suggests little impact in using a flavonol with differing B-ring substitution for our synthesis. In terms of the effects of hydroxylation of the flavonol A-ring, while fisetin and quercetin showed similar levels of potency, quercetagetin proved twice as potent towards α-amylase.
Figure 2.5. Flavonol substructure. Ring and position labeling of the flavonol core are shown as well as differing substitutions of various flavonols. The maximum inhibition (%) of each flavonol against salivary α-amylase is also indicated.  

Ultimately quercetin was selected as the flavonol base for our analogue synthesis. Quercetin and its glycosylated derivatives are some of the most abundant flavonols in nature and are found in many common plant materials. As a result, quercetin-based compounds are substantially cheaper than those of other flavonols. For illustration, 10 g of 95% pure quercetin retails for $50 CAD while just 25 mg of 96% pure myricetin costs approximately $150 CAD (Sigma-Aldrich® 2017). Meanwhile 5 mg of quercetagetin retails for $278.00 USD (Santa Cruz Biotechnology® 2017), and while this flavonol could potentially provide an increase in potency compared to myricetin, its high cost is prohibitive. Not only is quercetin substantially less expensive than myricetin and
quercetagetin, but its simpler structure would require less protecting groups during synthesis. Quercetin had a similar inhibition constant with HPA ($K_i \sim 100 \mu M$) to what was previously determined with myricetin$^{93}$.

During structural analysis of Montbretin A, there appeared to be no interactions between the D-glucopyranosyl-(β1→2)-D-glucopyranosyl-(β1→2)-L-rhamnopyranoside and HPA. It seemed that the disaccharide present within MbA-GRX simply acts as a bridge to join and orient the two active moieties. Due to its lack of direct interactions with the enzyme, a number of different chemical structures can be explored for this linker region during our analogue synthesis. Requirements of linker length and rigidity can be assessed. The possibility of incorporating new functionalities to create new favorable interactions with the enzyme can also be explored.

### 2.2 Specific Aims

The ultimate aim of this project is the design and synthesis of chemically accessible Montbretin A analogues that would provide similar potency and specificity towards HPA. Construction of a library of related compounds will also allow for assessment of structural requirements for HPA inhibition. This library will be screened for inhibition against HPA and lead compounds will be selected for further analysis and application.
Results and Discussion

2.3 Synthesis and Kinetic Analysis of MbA Analogues with Simple Alkyl Linkers

The goal for the first MbA analogue was to simply link the flavonol and caffeic acyl moieties together to assess whether simply joining these two groups resulted in an increase in potency compared to the lone constituents. For this first synthesis quercetin hydrate was used as the starting material (Scheme 2.1).

Scheme 2.1. Synthesis of M01

The 3-OH of quercetin was the desired site of alkylation in this synthesis. The catechol group of quercetin’s B-ring could be protected through installation of a diphenylketal, leaving the 3-OH, 5-OH and 7-OH free\textsuperscript{131}. It was hypothesized that the 5-
OH of quercetin would be relatively inert due to H-bonding with the nearby C-4 carbonyl and would not interfere with alkylation of the 3-OH. However, while the relative acidity of quercetin’s hydroxyls is somewhat debated, most studies indicate that the 7-OH is more acidic than the 3-OH. As a result, the 7-OH presented a potential site for unwanted alkylation during this synthesis. Fortunately, previous work using partially protected derivatives of quercetin showed preferential attachment at 3-OH.

Alkylation of the partially protected material was carried out with 1-bromo-6-chlorohexane and K$_2$CO$_3$. This reaction resulted in mono- and di- alkylated material, which could be easily separated but resulted in a lowered yield of only 35% for the desired mono-alkylated product. This mono-alkylated material was analyzed by HMBC to determine the site of alkylation (Figure 2.6). A cross peak was observed between the signal at 4.08 ppm (t, 2H) corresponding to -OCH$_2$- of the hexyl chain (which experienced slight overlap with peaks from residual ethyl acetate in this spectrum) and the signal at 138.7 ppm, which corresponded to C3 of the flavonol core. The signal from C3 also experienced a change in chemical shift upon alkylation, from 137.1 to 138.7 ppm when compared to the starting material. Conversely, the carbon at the alternative site of alkylation (C7; 165.0 ppm) did not exhibit any cross peaks with protons on the hexyl chain and does not experience a change in chemical shift upon alkylation.

These data confirmed that alkylation primarily occurred at the 3-OH as desired. The alkyl chloride group could then be converted to an azide through treatment with sodium azide.

Caffeic acid was first activated with pentafluorophenyl trifluoroacetate, and then converted to an appropriate co-reactant for click chemistry through treatment with
propargyl amine. A click reaction was then carried out with the azido alkyl quercetin \textbf{01e} to join the flavonol and caffeic amide. Finally, deprotection of the diphenylketal was achieved by reflux in aqueous acetic acid.

![Chemical structure of 01d]

**Figure 2.6. HMBC spectrum of 01d.** NMR analysis indicated that alkylation primarily occurred at the 3-OH of the partially protected quercetin. The cross peak between protons of the -OCH$_2$- group of the hexyl chain and C3 of quercetin is circled in red.
The resulting analogue **M01** was tested as an inhibitor of HPA and exhibited an inhibition constant of 14 µM. This represents an approximate 10-fold increase in potency compared to lone flavonol \( (K_i \sim 100 \, \mu\text{M}) \) and a 100-fold higher potency than ethyl caffeate, which has a \( K_i \) of 1.3 mM\(^93\). However this first analogue is still approximately 1000-fold less potent than MbA.

While the diphenylketal protection scheme allowed for the synthesis of the first MbA analogue, the incomplete protection of quercetin led to lowered yields during alkylation. This could also potentially lead to the formation of additional side products and further lowered yields when constructing analogues with more complex linkers. A new protection scheme was sought for the synthesis of future analogues. Quercetin-3-O-rutinoside, or rutin, is a common glycosidic derivative of quercetin found in many plant materials. Rutin hydrate is similarly affordable to quercetin, with 50 g retailing for $147.50 CAD (Sigma-Aldrich®, 2017). Use of this 3-O-glycoside also presented an opportunity for a new protection strategy based on the work of Huang and coworkers (Scheme 2.2)\(^{139}\). Rutin was first benzylated with benzyl bromide in the presence of \( K_2\text{CO}_3 \). This was followed by cleavage of the 3-O-glycoside by treatment with HCl in ethanol, freeing the 3-OH for subsequent modification. Benzylation under these conditions resulted in the protection of the 7-OH, 3’-OH, and 4’-OH. Benzylation of the relatively inert 5-OH could be forced by use of an excess of potassium carbonate and benzyl bromide, however this material proved to be less soluble, an undesirable trait given the already poor solubility of **02b**.
Scheme 2.2. Synthesis of MbA analogues with simple alkyl linkers

This material was then used for the synthesis of three new MbA analogues containing simple alkyl linkers (Figure 2.7). Linker lengths were chosen on the basis that the disaccharide linker of MbA-GRX forms a bridge of 7 atoms between the two polyphenolic groups. To probe optimal linker length, propyl, pentyl, and heptyl linkers were used. Benzyl quercetin 02b was alkylated with 1-bromo-3-chloropropane, 1-bromo-5-chloropentane, or 1-bromo-7-chloroheptane. Tetrabutylammonium azide was used to convert the alkyl chloride to an azide, which was then reduced with trimethylphosphine. Installation of the azide onto the alkyl linker could also be achieved prior to attachment to the flavonol. However the azide displacement reaction of 02c was high yielding and often required no additional purification before use in the subsequent
step. Meanwhile, pre-installation of the azide generally led to lowered yields during alkylation (60% vs. 76%). Coupling with caffeic acid was achieved through reaction with activated pentafluorophenyl caffeic ester 01a. Final deprotection of the protecting groups was then achieved with boron tribromide. Post-doctoral fellow Dr. Anna Win-Mason was responsible for the synthesis of analogue M02, which contained a heptyl linker, as well as an additional analogue M05, which contained an 8-atom polyethylene glycol-based linker.

Figure 2.7. MbA Analogues with simple linkers.
These four compounds were tested as inhibitors of HPA (Table 2.1). Analogues M02 and M03 showed similar potency to M01, both with inhibition constants of 4 µM. The kinetic results of analogues M01 to M03 indicate that joining the flavonol and caffeic acid groups through a simple linker increased potency by approximately 10 - 25 times compared to the lone flavonol ($K_i \sim 100 \mu M$). These analogues were still substantially less potent than MbA and MbA-GRX and instead were of similar potency to Montbretins B and C. It is likely that these structures do not exist in the pre-stacked confirmation in solution, as observed with MbA, and pay an entropic cost to adopt this confirmation upon binding in the α-amylase active site.

<table>
<thead>
<tr>
<th>MbA Analogue</th>
<th>$K_i$ against HPA (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M01</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>M02</td>
<td>4.2 ± 0.8</td>
</tr>
<tr>
<td>M03</td>
<td>4.0 ± 0.4</td>
</tr>
<tr>
<td>M04</td>
<td>111 ± 12</td>
</tr>
<tr>
<td>M05</td>
<td>86 ± 17</td>
</tr>
</tbody>
</table>

Error determined through fit errors produced by Grafit

The similarity in inhibition constants between M02 and M03 indicated no cost in potency from shortening the analogue linker to five atoms. On the other hand, M04, which contained a propyl linker, exhibited an inhibition constant of 111 µM, suggesting that its linker was too short to allow for π-stacking and proper orientation of the caffeic acyl and flavonol within the active site. M05, which contained the eight-atom...
polyethylene glycol-based linker, with a $K_i$ of 86 µM, also failed to offer a significant increase in potency over the lone flavonol. The fact that this analogue performs much worse than those with simple alkyl-based linkers suggests that M01 to M03 may benefit from the hydrophobic effect when adopting the active $\pi$-stacking conformation, allowing them to offset some entropic costs. Additionally or alternatively, conformational constraints on the chain imposed by the linking oxygen atoms, or hydrogen bonds formed by solvent water with these oxygens, that are not formed in the complex, may limit binding.

2.4 Synthesis and Kinetic Analysis of MbA Analogues with Amino Acid-Based Linkers

Analogues M01 to M05 showed that joining the caffeic acyl and flavonol groups with simple alkyl linkers could increase potency relative to the individual phenolic moieties. However these compounds were significantly less potent than MbA-GRX, despite having the potential to achieve all of the same interactions within the amylase active site. It was hypothesized that their comparatively low potency was due to a lack of rigidity in their linkers an resulting entropic cost in achieving the active $\pi$-stacking conformation upon binding in the active site. More rigid linkers could increase potency as long as an active conformation was a major component of the ground state structure. Amino acids were considered as a means of introducing said rigidity into the analogue linkers while potentially also creating new interactions within the enzyme active site.

A series of analogues with peptide-based linkers was synthesized (Scheme 2.3). These analogues contained a linker composed of a single amino acid linked to quercetin’s 3-OH through a propyl chain. Quercetin-3-O-propyl amine, 03b, was
synthesized under the same conditions used in the synthesis of M04. An Fmoc-L-amino acid could then be coupled to the amine either through activation with PFP TFA or HBTU. Eight different amino acids were used for this series of analogues. The Fmoc group could be deprotected with 20% piperidine in DCM. A second coupling with pentafluorophenyl caffeic ester 01a, followed. Deprotection of the benzyl ethers could be achieved as before through treatment with BBr₃.

Scheme 2.3. Synthesis of MbA analogues with amino acid based linkers

Yields of the final BBr₃ deprotection step were disappointingly low throughout our analogue synthesis, averaging around 15 - 20%. The final deprotection step of the catechol diphenylketal during the synthesis of M01 was much higher yielding. However, lack of deprotection on the 7-OH of quercetin would likely lead to significant side product formation and lowered yields during couplings with the Fmoc-L-amino acids and caffeic acid. Deprotection of the benzyl protecting groups via hydrogenation would be
ideal however the conjugated alkene of the caffeic amide would likely not survive these conditions.

Through this procedure a series of eight MbA analogues could be synthesized (Figure 2.8). The amino acid residues of initial interest for linker construction were glycine, proline and tyrosine. Glycine, with its lack of side chain, would act as a parent compound for this series. Proline was pursued in hopes that its constrained side chain would introduce the needed rigidity into the analogue linker. It was hoped that proline’s limited conformational range would favor the active $\pi$-stacking conformation, stabilizing this state and avoiding entropic costs upon amylase binding. Tyrosine was chosen due to its potential to introduce new interactions within the active site. Other L-amino acids were later included to further explore the effects of modifying the linker functional groups. The results of the kinetic analysis of the eight inhibitors against HPA are shown in Table 2.2.

The glycine-containing parent analogue $\text{M06}$ had an inhibition constant of $K_i = 61$ $\mu$M against HPA, displaying a level of potency similar to that of $\text{M05}$ or the lone flavonol. The presence of the amide functionality within its otherwise hydrophobic linker likely prevents this analogue from benefiting from the hydrophobic effect towards stabilization of the active phenolic stacking orientation. Comparison of $\text{M06}$ to the more potent analogue $\text{M01}$, which also has a polar functional group in its otherwise hydrophobic linker, indicates that the length of the preceding alkyl chain and relative placement of the polar functionality affects the analogues’ ability to achieve active conformations.
Figure 2.8. Analogues synthesized containing a single amino acid residue.
### Table 2.2. Kinetic analysis of analogues M06 to M13 against HPA

<table>
<thead>
<tr>
<th>MbA Analogue</th>
<th>Amino Acid Substituent</th>
<th>$K_i$ against HPA ($\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M06</td>
<td>Gly</td>
<td>61 ± 12</td>
</tr>
<tr>
<td>M07</td>
<td>Pro</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>M08</td>
<td>Thr</td>
<td>51 ± 10</td>
</tr>
<tr>
<td>M09</td>
<td>Glu</td>
<td>34 ± 5</td>
</tr>
<tr>
<td>M10</td>
<td>Tyr</td>
<td>0.044 ± 0.011</td>
</tr>
<tr>
<td>M11</td>
<td>Phe</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>M12</td>
<td>L-DOPA</td>
<td>0.063 ± 0.027</td>
</tr>
<tr>
<td>M13</td>
<td>Trp</td>
<td>3.8 ± 0.7</td>
</tr>
</tbody>
</table>

*Error determined through fit errors produced by Grafit*

Analogue M07, which contained a proline residue in its linker, had an inhibition constant of $K_i = 1 \, \mu$M, making it 60 times more potent than M06. This analogue also bound 4 times more tightly than the heptyl and pentyl linked analogues M02 and M03. Introduction of a proline into the linker appears to provide some rigidification and favoring of the active stacking orientation of the phenolic moieties. However, as M07 is still 11 times less potent than MbA-GRX, it seems that its single proline residue falls short compared to MbA-GRX’s disaccharide in terms of stabilizing the active orientation of phenolic groups.

Analogue M08, which contained a threonine within the linker, exhibited a similar inhibition constant to M06, suggesting that the polar groups of its linker do not form any additional interactions within the active site and instead cause it to suffer similar entropic costs as M06. M09, with its glutamate-derived linker, showed a modest increase in
potency compared to M06 and M08 with an inhibition constant of 34 \( \mu \text{M} \). The glutamate side chain may be long enough to form additional polar interactions in the active site. However, it appears that the presence of this polar functional group with its linker still leads to an overall loss of potency compared to the hydrophobic linkers of M02 and M03.

Analogue M10, which contained a tyrosine residue within its linker, was initially pursued as a means of introducing new interactions between the analogue and enzyme. The resulting inhibitor proved to be very potent against HPA with a \( K_I = 44 \text{ nM} \). This was a substantial increase in potency compared to the previous analogues of this series. Compared to the glycine-containing parent compound M06, inclusion of a tyrosine residue at this position led to an approximately 1400-fold increase in potency. M09, which had the potential of forming similar polar interactions in the active site with its glutamate side chain, bound in the active site almost 800-times less tightly than this tyrosine-containing analogue. It seems that the presence of the tyrosine’s aromatic ring and relative placement of the phenolic hydroxyl allowed for the formation of a number of favorable interactions within the active site and resulting stabilization of the active stacking conformation of the analogue’s flavonol and caffeic amide moieties.

Analogue M11, M12 and M13 were synthesized to further explore the success of this functionality. M11 contained a phenylalanine in place of the tyrosine. This analogue had an inhibition constant of 2.8 \( \mu \text{M} \), which represents a 64-fold decrease in potency compared to M10. It appears that inclusion of the tyrosine hydroxyl and resulting presence of a hydrogen bonding partner at this site contributes 2.5 kcal·mol\(^{-1}\) to the binding affinity of M10. M11 was 22 times more potent than the glycine-containing
analogue M06, suggesting that its aromatic side chain can create favorable π –interactions in the active site, corresponding to a 1.8 kcal·mol⁻¹ contribution to binding affinity. A comparison of ΔG_{binding} of the amino acid containing analogues to that of M06 is shown in Table 2.3.

M12 contained a L-DOPA residue in its linker and was made to assess if an additional hydroxyl group could create more interactions within the active site, and increase potency compared to M10. Kinetic analysis indicated that its catechol group seemed to afford no additional increase in binding affinity since this analogue had a similar potency to M10 with an inhibition constant of 63 nM. M13 with a tryptophan incorporated into its linker, showed similar potency to M11, indicating that its indole group offers no additional interactions other than those produced by M11’s phenylalanine.

Table 2.3 Comparison of ΔG_{binding} of amino acid containing MbA analogues to that of parent compound M06.

<table>
<thead>
<tr>
<th>MbA Analogue</th>
<th>Amino Acid Substituent</th>
<th>ΔΔG_{binding to M06} (kcal·mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M06</td>
<td>Gly</td>
<td>--</td>
</tr>
<tr>
<td>M07</td>
<td>Pro</td>
<td>- 2.47</td>
</tr>
<tr>
<td>M08</td>
<td>Thr</td>
<td>- 0.11</td>
</tr>
<tr>
<td>M09</td>
<td>Glu</td>
<td>- 0.35</td>
</tr>
<tr>
<td>M10</td>
<td>Tyr</td>
<td>- 4.34</td>
</tr>
<tr>
<td>M11</td>
<td>Phe</td>
<td>- 1.84</td>
</tr>
<tr>
<td>M12</td>
<td>L-DOPA</td>
<td>- 4.12</td>
</tr>
<tr>
<td>M13</td>
<td>Trp</td>
<td>- 1.66</td>
</tr>
</tbody>
</table>
2.5 X-ray Crystallographic Analysis of Analogues in Complex with HPA

In order to gain further insight into the interactions that these analogues formed in the HPA active site, X-ray crystallographic analysis of analogues M07 and M10 in complex with HPA was pursued. Dr. Sami Caner of the Brayer Lab conducted soaking trials, X-ray diffraction data collection and data processing for this work. The two resulting X-ray crystal structures showed the two analogues in non-covalent complex within the enzyme active site. An overlay of both structures with a previous structure of the MbA-GRX/HPA complex, also elucidated by Dr. Sami Caner showed significant overlap of the flavonol and caffeic acyl moieties of the MbA analogues with those of MbA-GRX (Figure 2.9).

Figure 2.9. Overlays of synthetic analogues and MbA-GRX. (A) Overlay of MbA-GRX (yellow) with M07 (purple). (B) Overlay of MbA-GRX (yellow) and M10 (pink).
Figure 2.10 Polar contacts formed in the HPA active site with (a) MbA-GRX (b) M07 and (c) M10. Hydrogen bonds are shown as grey dashed lines; hydrogen bond distances in Å are indicated above the dashed lines. Enzymatic residues involved in hydrogen bonding with the inhibitor are shown as colored spheres.
Analogue M07 created all of the same polar contacts within the HPA active site as seen with MbA-GRX (Figure 2.10 and Figure 2.11). Hydrogen bonding occurred between the catechol of the caffeic acyl group and R195, D197 and E233 of the enzyme. The 7-OH of the flavonol formed hydrogen bonds with D197 and H201. A hydrogen bond was also observed between the C4 carbonyl of the flavonol and the side chain of T163. Meanwhile, the 3'-OH of the flavonol formed a hydrogen bond with the side chain of H201, as is seen with MbA-GRX, indicating that use of the more cost-effective quercetin starting material is sufficient to form the necessary interactions within the active site. The 4'-OH of MbA is glycosylated. The absence of this 4'-O-disaccharide in MbA-GRX opens this position up for hydrogen bonding with K200 in the active site, an interaction that is also seen with M07.

Figure 2.11. X-ray crystal structure of M07 in complex with HPA. M07 is shown in purple, residues of the enzyme are shown in grey, hydrogen bonding is shown as grey dashes.
In addition to these polar contacts, there also seems to be a $\pi$–CH stacking interaction between the proline side chain of M07 and W59 of the enzyme. This $\pi$–interaction likely contributes to some of the $\Delta G_{\text{binding}}$ associated with the increase in binding potency when compared to the M06 parent compound. Nonetheless, despite its high degree of alignment and reproducibility of polar contacts, M07 is 11 times less potent than MbA-GRX. This highlights the importance of MbA’s glycosidic appendages in stabilization of its pre-stacked conformation.

Analogue M10 also formed all of the same hydrogen bonds within the amylase active site as were observed with MbA-GRX and M07 (Figure 2.10 and 2.12). In addition to these previously mentioned interactions, the tyrosine of M10’s linker formed a number of other contacts in the active site. Hydrogen bonding occurred between M10’s tyrosine hydroxyl and the main chain carbonyl and side chain hydroxyl of T163. The side chain of HPA’s T163 was rotated relative to its orientation in the M07 crystal structure in order to form this hydrogen bond with M10's tyrosine. This reorientation weakened the hydrogen bond between the T163 side chain and the C4 carbonyl of M10’s flavonol, from 3.1 Å to a new distance of 3.6 Å. There also appeared to be a number of $\pi$–interactions between M10’s tyrosine side chain and residues W59, Q63, and L165 of the enzyme. The formation of these new interactions with the enzyme active site allows M10 to bind more tightly than does MbA-GRX, despite lacking the rigidity of its disaccharide linker.
2.6 Specificity Analysis of MbA Analogues

It was quite possible that our simplified MbA analogues could inhibit a number of these enzymes in addition to HPA. To assess this, analogues M02 and M10 were tested for inhibition against not only metabolically relevant α-glucosidases but also a selection of other unrelated glycosidases. It was thought that the simplicity and flexibility of M02 with its undecorated heptyl linker could lead to increased inhibitory promiscuity and activity towards previously untested glycosidases. It was also of interest to investigate the inhibitory specificity of our lead compound M10 to determine whether inclusion of its tyrosyl residue would confer increased specificity towards HPA or perhaps lead to
increased binding affinity for other glycosidases as well. These two compounds were
tested against 10 glycosidases in concentrations up to 500 µM to screen for inhibition.

The results of this survey are compared to previous studies with MbA and MbA-GRX
and shown in Table 2.4.

### Table 2.4. Inhibitory specificity of M10 and M02 and comparisons to MbA-GRX
and MbA.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>IC$_{50}$ with M10 (µM)</th>
<th>IC$_{50}$ with M02 (µM)</th>
<th>IC$_{50}$ with MbA-GRX (µM)$^{121}$</th>
<th>K$_i$ with MbA (µM)$^{93,121}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPA (GH13)</td>
<td>$K_i = 0.044 \pm 0.01$</td>
<td>$K_i = 4.2 \pm 1.4$</td>
<td>$K_i = 0.093 \pm 0.008$</td>
<td>0.008 ± 0.0004</td>
</tr>
<tr>
<td><em>Roseburia inulivorans</em> Amylase A (GH13)</td>
<td>NI$^a$</td>
<td>NI$^a$</td>
<td>NI$^b$</td>
<td>NI$^a$</td>
</tr>
<tr>
<td><em>Butyrivibrio fibrisolvens</em> Amylase B (GH13)</td>
<td>NI$^a$</td>
<td>63 ± 7.0</td>
<td>ND</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td><em>Homo sapiens</em> ntMGAM (GH31)</td>
<td>NI$^a$</td>
<td>NI$^a$</td>
<td>NI$^b$</td>
<td>NI$^a$</td>
</tr>
<tr>
<td><em>H. sapiens</em> ctMGAM (GH31)</td>
<td>NI$^a$</td>
<td>NI$^a$</td>
<td>75.5 ± 2.5</td>
<td>NI$^a$</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em> α-glucosidase (GH13)</td>
<td>10.5 ± 0.7</td>
<td>28.2 ± 0.5</td>
<td>446</td>
<td>NI$^a$</td>
</tr>
<tr>
<td><em>Agrobacterium</em> β-glucosidase (GH1)</td>
<td>35.4 ± 5.7</td>
<td>NI$^a$</td>
<td>ND</td>
<td>NI$^c$</td>
</tr>
<tr>
<td>Green coffee bean α-galactosidase (GH27)</td>
<td>NI$^a$</td>
<td>NI$^a$</td>
<td>ND</td>
<td>NI$^c$</td>
</tr>
<tr>
<td><em>E. coli</em> β-galactosidase (GH2)</td>
<td>NI$^a$</td>
<td>NI$^a$</td>
<td>ND</td>
<td>NI$^c$</td>
</tr>
<tr>
<td>Jack bean α-mannosidase (GH38)</td>
<td>NI$^a$</td>
<td>NI$^a$</td>
<td>ND</td>
<td>NI$^c$</td>
</tr>
</tbody>
</table>

NI$^a$ = no inhibition observed at inhibitor concentrations up to 500 µM.
NI$^b$ = no inhibition observed at inhibitor concentrations up to 100 µM.
NI$^c$ = no inhibition observed at inhibitor concentrations up to 0.1 µM.
ND = not determined.

Previous studies showed that MbA was selective for HPA over the intestinal
brush border α-glucosidases sucrase-isomaltase (SI) and maltase-glucoamylase.
MbA did show some inhibition towards the intestinal bacterial enzyme \textit{B. fibrisolvens} amylase B, however this inhibition was much less profound than with HPA. Besides this, MbA showed no activity towards any of the other glycosidases it was screened against. It appeared that cleavage of MbA’s 4’-O-disaccharide led to some loss in inhibitory specificity, as MbA-GRX displayed additional but mild inhibition towards sucrase-isomaltase and \textit{S. cerevisiae} \(\alpha\)-glucosidase.

As with MbA, synthetic analogue \textbf{M02} showed no inhibitory activity towards the intestinal brush border \(\alpha\)-glucosidases. This compound did inhibit \textit{B. fibrisolvens} amylase B, as was also the case with MbA, though did so to a lesser extent. \textbf{M02} also inhibited \textit{S. cerevisiae} \(\alpha\)-glucosidase with an IC\textsubscript{50} of 28 \(\mu\)M.

Tyrosine-containing analogue \textbf{M10} also showed no inhibition against the intestinal brush border \(\alpha\)-glucosidases. However, unlike MbA and \textbf{M02}, \textbf{M10} also did not inhibit the \textit{B. fibrisolvens} \(\alpha\)-amylase B. In this regard, \textbf{M10} was more selective for HPA over other medically relevant \(\alpha\)-glucosidases when compared to \textbf{M02}, MbA and MbA-GRX. On the other hand, \textbf{M10} did show inhibitory activity against \textit{S. cerevisiae} \(\alpha\)-glucosidase as was seen with \textbf{M02} and MbA-GRX. \textbf{M10} also inhibited \textit{Agrobacterium} \(\beta\)-glucosidase with an IC\textsubscript{50} of 35 \(\mu\)M.

The emergence of inhibitory activity with these compounds against the \textit{S. cerevisiae} \(\alpha\)-glucosidase is likely due to the fact that this enzyme is a GH13 family \(\alpha\)-glucosidase, much like HPA. On the other hand, the MGAM and ctSI \(\alpha\)-glucosidases are GH31 family glycosidases. While \textbf{M02} and \textbf{M11} inhibited the yeast \(\alpha\)-glucosidase, their binding affinities were actually lower than that of lone quercetin, which has been screened against this enzyme in the past and showed an IC\textsubscript{50} of 7 \(\mu\)M\textsuperscript{130}. If anything,
these data demonstrate that functionalization of quercetin towards the production of MbA-like structures decreases activity towards the yeast α-glucosidase.

2.7 Glycosynthase-Derived MbA Analogues

While there was success in producing simple MbA analogues that functioned as good HPA inhibitors, these analogues ultimately lacked the rigidity of MbA-GRX’s disaccharide linker. As a result, any improvements in potency were achieved through the introduction of new contacts within the active site cleft. Analogues with carbohydrate-based linkers were of interest to potentially introduce desired rigidity. The incorporation of carbohydrate-based linkers presents a number of synthetic challenges, which largely prevents an easy chemical synthesis of MbA or MbA-GRX for commercial use.

The use of enzymes is one way to circumvent some of the challenges of carbohydrate chemistry. Carbohydrate-processing enzymes have evolved remarkable selectivity for their substrates, allowing for the precise positioning of select functionalities within their active sites to ensure that chemical transformation takes place only at the target location. Glycosynthases are glycosidases that have been engineered to catalyze the formation of new glycosidic linkages. These engineered enzymes are typically produced by mutation of the catalytic nucleophile of retaining glycosidases into Ser, Gly, or Ala. This allows for specific binding of carbohydrate substrates while preventing hydrolysis\(^\text{140}\). Instead, when incubated with an activated glycosyl fluoride donor sugar of opposite anomeric configuration to that of the natural substrate and an appropriate acceptor molecule, these enzymes instead facilitate the formation of a new glycosidic bond (Figure 2.13).
One such enzyme, Abg2F6, has the ability to form β-1,4-glucosidic linkages. Abg2F6 has been evolved to exhibit high catalytic efficiency as well as a high degree of promiscuity towards both acceptor and donor substrates\textsuperscript{141,142}. The tolerance of Abg2F6 to a wide variety of acceptor groups could allow for use of a flavonol-based acceptor. Abg2F6 has also been shown to accommodate various amino sugar donors, which could simplify functionalization of a flavonol derivative for subsequent coupling with caffeic acid. Through this approach, we aimed to synthesize a new series of three MbA analogues containing disaccharyl linkers.

Rutin could be converted to quercetin-3-O-glucoside through treatment with the rhamnosidase, naringinase from \textit{Penicillium decumbens}. The quercetin-3-O-glucoside product could then act as an acceptor for Abg2F6 (Scheme 2.4).
Scheme 2.4. Chemoenzymatic synthesis of MbA analogues

The flavonol glucoside was incubated with Abg2F6 and either 4-aminoglucosyl fluoride, 6-aminoglucosyl fluoride, or 4-aminogalactosyl fluoride, previously synthesized by Dr. Hong-Ming Chen. This allowed the installation of a second carbohydrate moiety.
onto the flavonol and functionalization of the intermediate with an amine for subsequent coupling. Coupling with caffeic acid was then carried out with HBTU to yield three new MbA analogues.

![Chemical structures of MbA analogues](image)

**Figure 2.14. MbA analogues containing disaccharide linkers**

This two-step chemoenzymatic synthesis proved to be a quick way of producing MbA analogues with disaccharide linkers (Figure 2.14). Unfortunately limited amounts of the amino sugar donors limited the scale of this synthesis and yields of the caffeic acid coupling step were low. Perhaps more importantly, kinetic analysis of the resulting compounds indicated that none of these analogues provided a significant increase in potency compared to lone quercetin (Table 2.5). **M15** showed no inhibition against the enzyme, indicating that the stereochemistry at the site of caffeic acid attachment likely led to unfavorable steric clashing in the enzyme active site. **M14** and **M16** showed inhibitory potency similar to that of the lone flavonol, both with IC₅₀’s of approximately ∼100 µM.
Table 2.5 Kinetic analysis of MbA analogues M14 to M16 against HPA

<table>
<thead>
<tr>
<th>MbA Analogue</th>
<th>IC$_{50}$ against HPA (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M14</td>
<td>134 ± 1.2</td>
</tr>
<tr>
<td>M15</td>
<td>NI</td>
</tr>
<tr>
<td>M16</td>
<td>101 ± 20</td>
</tr>
</tbody>
</table>

NI = no inhibition observed at inhibitor concentrations up to 500 µM.

While the disaccharide linkers of these analogues provided rigidity, these linkers create a much wider turn than what is seen in MbA due to the $\beta$-linkage of the quercetin-3-O-glucoside of the rutin starting material and $\beta$-1,4 linkage produced by the Abg2F6 glycosynthase reaction (Figure 2.15). Compared to the tight turn created by the $\alpha$-linked D-glucopyranosyl-(β1→2)-L-rhamnopyranoside of MbA-GRX, the disaccharide linkers of M14 to M16 would likely not allow for $\pi$-stacking and proper orientation of the phenolic groups.

Figure 2.15. Comparison of glycosidic linkages of (a) MbA-GRX and (b) Isoquercetin. The point of attachment of the glycoside introduced through the Abg2F6 reaction is circled in red in section (b). As shown the $\alpha$-linked disaccharide of MbA-GRX creates a much tighter turned compared to that formed through our chemoenzymatic synthesis.
2.8 Future Directions – Polymer Functionalization

While intact flavonol glycosides, such as MbA, exhibit minimal to no absorption from the intestines, there is some evidence of their cleavage and subsequent absorption of the aglycone. Due to their low solubility and the rapid metabolism within the liver, the absorption of lone flavonols produces little to no toxicity\textsuperscript{78,143,144}. The chemical modification of flavonoids has been shown to increase solubility and metabolic stability in certain cases\textsuperscript{145,146}. This has been useful in some cases, such as for the production of hydroxyethylrutosides for treatment of hypertension\textsuperscript{147}. However for compounds such as our synthetic MbA analogues, increased bioavailability and metabolic stability could lead to undesired outcomes.

The lead compounds of this study are derivatives of quercetin that contain an alkyl-based linkage at the 3-position. It is possible that the alkyl nature of the analogue linkers could promote absorption from the intestine and confer new biological activities on these compounds. Increased absorption from the intestine would also remove the inhibitor from its targeted site of action. To minimize possible absorption of these alkylated flavonol derivatives, the installation of these inhibitors onto polymers is of interest. Functionalization onto an appropriate polymer could help localize the inhibitors in the digestive system, avoid degradation, and increase solubility.

Polymer functionalization would necessitate the installation of an additional functional group within the linker of the MbA analogues to act as a point of attachment. Analogues, based on the lead compound M\textsuperscript{10}, with dipeptide-based linkers containing a lysine and tyrosine, were pursued for this purpose. The first compound synthesized, M\textsuperscript{17} (Figure 2.16), contained an ethyl linker before the dipeptide to allow for a similar
synthetic route to that used for the previous analogues (Scheme 2.5). Unfortunately, kinetic analysis showed no inhibition of HPA by this compound. The relative placement of the functional groups within the linker of this analogue likely led to steric clashes with the HPA active site residues.

Figure 2.16. Candidate compounds for polymer functionalization.

Next direct attachment of the dipeptide linker onto the 3-position of the flavonol towards the production of M18 was pursued. Attachment of Fmoc-L-lysine to the 3-OH of 02b was successful, however the resulting ester linkage was cleaved upon subsequent treatment with piperidine for Fmoc deprotection. This synthesis was abandoned. Even if this linkage could be built to avoid piperidine exposure it would likely be too labile to survive BBr₃ deprotection or even within the digestive tract. In order to avoid this labile ester linkage, a similar compound M19 was designed using lysinol in place of the lysine.
Scheme 2.5. Pursued synthetic routes towards MbA analogues for polymer functionalization.

The synthesis of this analogue has been started. The lysinol was first activated for attachment to the flavonol by conversion of the free hydroxyl of the Fmoc-lysinol-Boc
starting material to a bromide using a Hanessian reaction \((\text{Br}_2/\text{PPh}_3)\). This could then be coupled to 02b with \(\text{K}_2\text{CO}_3\) as seen in previous syntheses. Future coupling with Fmoc-tyrosine and then caffeic acid as previously described could then yield compound M19. It is hoped that this compound prove to retain good inhibitory activity against HPA and that the lysinol functional group will provide a suitable point of attachment for future polymer functionalization.

### 2.9 Conclusion

The results of the kinetic analysis of MbA analogues M01 to M13 provide new insight into the requirements for HPA inhibition. Inhibitors with hydrophobic or aromatic based linkers between the flavonol and caffeic acyl moieties performed substantially better than those with polar functional groups. The binding of the starch substrate in the amylase active site relies on a number of hydrophobic \(\pi\) –stacking interactions in addition to the polar interactions formed with the sugar hydroxyls. Taking advantage of this, M10 and M12, with their phenol-containing linkers, were 1.5 – 2 times more potent inhibitors than MbA-GRX, which has a \(K_i\) of 90 nM against HPA. The structures of M10 and M12 mirror those of many of the protein inhibitors of \(\alpha\)-amylases, which often rely on aromatic residues to form interactions within the amylase active site. The results of this study also highlight the importance of MbA's pre-stacked conformation of the flavonol and caffeic acid moieties, afforded by its rigid disaccharide linker. Analogues with flexible linkers that could not offset the entropic cost of reorienting their phenolic moieties, either through the hydrophobic effect or by forming new interactions in the active site, performed no better as HPA inhibitors than lone quercetin. Meanwhile,
analogues M14 to M16, with rigid linkers that did not provide proper orientation of the phenolic moieties, also performed poorly.

Optimization of MbA analogue synthesis is still needed, especially in terms of the selection of the protecting groups for the flavonol core and subsequent deprotection strategy. The relative reactivity of the 7-OH of quercetin can lead to side products and low yields if this group is left free, as was seen with the alkylation reaction during the synthesis of M01. On the other hand, protection of the 7-OH, 3'-OH and 4'-OH of quercetin through installation of benzyl protecting groups necessitated a harsh low-yielding BBr$_3$ deprotection, as the caffeic acyl group would not survive hydrogenation. The use of benzoyl protecting groups could avoid the low yields associated with BBr$_3$ deprotection. There is however a chance that benzoyl protecting groups would not survive basic or acidic conditions during the synthesis, such as the use of 20% piperidine, as was seen for the phenolic ester linkage in the attempted synthesis of M18. However this could potentially be avoided by synthesis of the complete linker before its attachment onto the flavonol.

Nonetheless, this study provides new lead structures for the development of novel HPA inhibitors. A lead compound, with a $K_i$ of 44 nM and greater specificity than MbA for HPA over the other medically relevant glycosidases, was identified. Pharmacokinetic studies with this compound are to be pursued in the future. Functionalization of its structure onto an FDA-approved polymer for therapeutic administration is also of interest and has been started, as detailed in Section 2.8.
Chapter 3. Potent HPA Inhibition by the β-Defensin Homologue, Helianthamide

3.1 Introduction

Around the time of Montbretin A’s initial discovery, a similar high-throughput screen was run on UBC’s Marine Natural Product Library, also in search of novel HPA inhibitors. This library, assembled by Professor Raymond Andersen’s laboratory, contains 10,000 natural product extracts of marine origin. Initially during this screen, none of the marine extracts appeared very potent, with only a handful of samples resulting in an ~20% decrease in HPA activity. Nonetheless, a couple of extracts were selected for activity-guided isolation. It was during the enrichment process that one sample quickly became a front-runner for HPA inhibition. This material was a methanolic extract of the Caribbean Sea anemone *Stichodactyla helianthus*. Activity-guided isolation was performed on a 154 g anemone specimen to yield 2 mg of active material. Preliminary kinetic analysis suggested that the material was a potent competitive inhibitor of HPA with estimates of a low nM-range inhibition constant. NMR analysis indicated that the inhibitor was peptidic in nature and sequencing was pursued through a combination of Edman degradation and LC/MS-MS analysis of proteolytic fragments. The result was a 44-residue peptide of the following sequence, bearing six cysteine residues:

```
ESGNACIYHGVSGLCKASCAEDKAMAGMGVCEGHLCYKTPW
```

BLAST and PSI-BLAST analysis of this sequence revealed no significant homologues within the non-redundant protein sequence database at the time of its
discovery. MALDI-TOF mass spectrometric analysis before and after treatment with dithiothreitol and iodoacetamide indicated the presence of three disulfide bridges.

This peptide would eventually be named helianthamide after the organism of its isolation. Once the original supply of helianthamide had been used up attempts were made to collect more material from a second S. helianthus specimen, however no helianthamide could be detected or retrieved. To continue analysis, a synthetic version of the peptide was purchased for X-ray crystallography studies. Prior to incubation with the enzyme, the peptide was shown to be linear with all of its cysteines in reduced form, with no significant secondary structural elements observed through CD spectroscopy. The synthetic peptide was co-crystallized with porcine pancreatic \( \alpha \)-amylase. The resulting crystal structure displayed the two molecules in a non-covalent complex, with helianthamide fitting neatly into the enzyme's active site cleft (Figure 3.1).

**Figure 3.1. Helianthamide-PPA Complex.** X-ray crystallographic analysis showed the two proteins in a non-covalent complex. PPA is shown in white and helianthamide is shown in blue, as sticks on the left and spheres on the right (PDB: 4X0N)\textsuperscript{148}. 
Upon closer inspection, helianthamide’s previously reduced cysteines were all seen to be oxidized into disulfide bridges in a 1-5, 2-4, 3-6 topology. Analysis of the 3D structure with the software DALI revealed helianthamide to be structurally homologous to the β-defensins. The β-defensins are a family of small disulfide-rich antimicrobial peptides characterized by their cationic and amphipathic nature, 1-5, 2-4, 3-6 disulfide topology, and anti-parallel β-sheet core149.

Small disulfide-rich peptides have proven to be successful and widely employed evolutionary scaffolds. The disulfide-rich core provides a stable, versatile structure for a number of different applications, from enzyme and ion channel inhibition to antimicrobial activity. These peptides can adopt a number of different folds and disulfide topologies150. Knottins, for example, have a 1-4, 2-5, 3-6 disulfide topology and are particularly prevalent throughout Nature. There are even a number of knottins that inhibit insect α-amylases58,151. The β-defensin fold, on the other hand, is relatively rare amongst small disulfide-rich peptides, and is usually not seen outside its traditional role in antimicrobial activity.

While rare, structural homologues of the β-defensins have been uncovered in the past (Figure 3.2). Many of these homologues are potent toxins that act through ion channel inhibition or membrane disruption152. One such toxin, ShI, was also isolated from S. helianthus. ShI acts to block voltage-gated Na⁺ channels through interactions formed with various acidic and basic regions on its surface153. Indeed, in the case of the β-defensins and many of its toxic structural relatives, a specific distribution of charged residues on the peptide surface is required for their activities. The cationic and amphipathic nature of β-defensins allows these peptides to form pores in microbial
Figure 3.2. Structural and sequence comparisons of helianthamide with other disulfide rich peptides. (a) Structural comparison of helianthamide (PDB: 4X0N) to other disulfide rich peptides, hβD-1 (PDB: 2NLS), Shl (PDB: 1SH1), and AAI (PDB: 1CLV). β-sheets depicted in orange, helices in red, loops in blue, and disulfide bonds in yellow. (b) Sequence alignment of helianthamide with two β-defensins, and comparisons to Shl, and representatives from the α-defensin and knottin families. Disulfide connectivities are color coded where bonding partners share the same color.
membrane bilayers\textsuperscript{154-156}. While it appeared that helianthamide emulates these peptides in its tertiary structural elements, its sequence is predominantly uncharged, suggesting it would have no antimicrobial or neuro-/cardio-toxic activities.

The unsuccessful second attempt to isolate helianthamide from \textit{S. helianthus} raised some questions about the production of this peptide. There was initially some doubt if this inhibitor was actually a product of \textit{S. helianthus}, or if it was instead from an algal or prokaryotic species associated with the original extract. However, at the time of writing this thesis, a second $\alpha$-amylase inhibitor homologous to the $\beta$-defensins was discovered in a different sea anemone, \textit{Heteractis magnifica}\textsuperscript{157}. The fact that \textit{S. helianthus} also produces one of the other known examples of $\beta$-defensin structural homologues further supports the notion that this organism has evolved the ability to use this structural scaffold for defensive/competitive purposes. It is possible that expression of helianthamide results from certain environmental stimuli that were not triggered during the second collection attempt. Nonetheless, helianthamide was scarce even in the original \textit{S. helianthus} specimen, with a 2 mg yield from a 154 g anemone. Even if more material were to be recovered from \textit{S. helianthus}, extraction of helianthamide from this source would not be a practical means of production.

In order to continue analysis of helianthamide, more active material was needed. While use of a synthetic peptide allowed for X-ray crystallographic analysis, synthesis of a peptide of helianthamide's size and sequence through SPPS can present a number of challenges and limitations in terms of cost of reagents, yield and quality control, potentially making it difficult to access on a larger scale\textsuperscript{158,159}. Recombinant expression provides an alternative approach. The utilization of well-honed cellular machinery
towards the construction of active proteins could provide a relatively inexpensive and high-yielding means of helianthamide production.

3.2 Specific Aims

Upon consumption of the original supply of helianthamide, more active material was needed for further analysis. This chapter explores the recombinant expression of helianthamide and its subsequent structural and kinetic analysis. Importantly we must confirm whether the structure of the recombinant material is consistent with the crystal structure derived from the synthetic material and the limited data on the natural material. The contributions of individual amino acid residues in helianthamide to inhibitor potency will also be explored through site-directed mutagenesis and kinetic analysis. Finally, solid phase peptide synthesis will also be explored for the production of smaller peptides based on helianthamide’s sequence to further determine the basis for this inhibitor’s incredible potency and to potentially synthesize new inhibitors with similar modes of activity.
Results and Discussion

3.3 Recombinant Expression of Helianthamide

Recombinant expression of helianthamide was pursued in *Escherichia coli*. The cytoplasm of the *E. coli* cell is maintained as a reducing environment and is generally not conducive to expression of disulfide-rich peptides. Expression attempts were instead directed to the *E. coli* periplasm via the SEC pathway by incorporation of an N-terminal phoA signal sequence\(^{160,161}\). The *E. coli* SEC pathway is a post-translational secretory system, employing a number of chaperone and oxidoreductase proteins to guide proper folding and release of secreted proteins\(^{162,163}\).

Two constructs were explored for lone helianthamide expression, one with an N-terminal His6tag and one without. These constructs were trialed in 25 mL cultures. Unfortunately no trace of helianthamide expression was observed. The expression of small peptides can present a number of challenges. Small peptides are particularly susceptible to targeting by cellular proteases and are prone to low-level expression and difficult recovery\(^{164-166}\). In a few cases small disulfide-rich peptides have been successfully expressed by incorporation of specific signal sequences and pre- and pro-peptide regions into the expression construct\(^{151,167}\). However no information pertaining to possible pre- or pro-peptide regions of helianthamide is available. An alternative approach for small peptide expression is the incorporation of a fusion partner. Expression of target peptides as fusion proteins can increase yield by conferring protection against proteolytic degradation, increasing solubility, and/or mimicking the peptide’s precursor structure\(^{168}\). A number of β-defensins and other small disulfide-rich
peptides have been expressed in *E. coli* with high yields through incorporation of a fusion partner\textsuperscript{169-172}.

Fusion expression of helianthamide using an inactive form of the bacterial ribonuclease barnase (bar') as a fusion partner was pursued. Barnase is a 110-residue peptide naturally secreted by the bacterium *Bacillus amyloliquefaciens*. It has been shown to exhibit robust and reversible folding and its interactions with chaperone proteins of the SEC pathway have been well-documented\textsuperscript{162,173,174}. It also contains no cysteines in its sequence. These qualities have allowed barnase to successfully promote folding of fusion partners in the past and make it especially suitable for the expression of a small disulfide rich peptide such as helianthamide\textsuperscript{175}.

![Figure 3.3. N-terminal region of helianthamide.](image)

**Figure 3.3. N-terminal region of helianthamide.** X-ray crystallographic analysis of the helianthamide-PPA complex indicated that the N-terminal region of helianthamide extended out from the peptide core suggesting that introduction of an N-terminal Ser residue after TEV protease cleavage would not adversely affect binding. Helianthamide is shown as blue sticks, PPA is shown as grey spheres (PDB: 4X0N).
The construct of the barnase’-helianthamide fusion peptide initially contained a Factor Xa protease site between the two units, which would leave a native N-terminus on helianthamide after cleavage. While expression of this fusion was successful, cleavage with Factor Xa protease proved to be incredibly inefficient, preventing successful isolation of free helianthamide. A TEV protease cleavage site was inserted in place of the Factor Xa site. This new protease system proved much more effective. The TEV protease cleavage sequence is ENLYFQ | S, which would install an additional Ser residue onto the N-terminus of the isolated peptide. The crystal structure of the PPA/Helianthamide complex suggested that the N-terminus of Helianthamide extends out into solution (Figure 3.3). On this basis, it was hypothesized that an additional N-terminal Ser would not affect adversely inhibitor binding. An illustration of the final bar’-hel fusion construct is displayed in Figure 3.4.

Expression of the bar’-hel fusion in E. coli was successful, providing moderate yields of 20 mg/L. The peptide was removed from the culture medium through precipitation by treatment with 60% ammonium sulfate and could be purified by immobilized metal affinity chromatography (IMAC). After cleavage of the fusion with TEV protease, helianthamide could be separated from the rest of the reaction mixture.
by treatment with 50% methanol. This caused all other peptide species to precipitate out of solution allowing for simple isolation of helianthamide.

Unfortunately, helianthamide only accounts for around 25% of the length of the bar'-hel fusion peptide. Further, after TEV protease cleavage, methanol treatment, and final HPLC purification, usually only an average of 1 mg of helianthamide per 1 L expression culture was obtained. While this expression system allowed the production of active material for kinetic and structural analyses, the overall yield would likely be insufficient for the production of material for animal studies.

Expression of helianthamide in *Pichia pastoris* was pursued in attempts to increase yield. Small Ubiquitin-like Modifier (SUMO) protein was used as a fusion partner in this case. Expression of the SUMO-hel fusion in *P. pastoris* was successful, however expression yields were no better than that of the bar'-hel expression in *E. coli*. Preliminary trials of lone helianthamide expression in *P. pastoris* were also attempted relying upon the α-factor secretion signal peptide of the pPic9K vector for directed expression. However no expression of helianthamide was observed during preliminary trials. Further optimization is needed to increase the yield of helianthamide, which may necessitate the use of additional expression hosts, fusion partners, or incorporation of new pre- and pro-peptide regions.

### 3.4 Kinetic Analysis of Recombinant Helianthamide

Preliminary analysis of the helianthamide sample extracted from *S. helianthus* suggested that the peptide had high affinity towards HPA. Tight-binding inhibitors can be difficult to analyze through standard Michaelis-Menten kinetics. The Michaelis-Menten model employs a number of approximations for its effective use. One
approximation is that \([I] = [I]_T\) throughout the course of the reaction. That is to say, \([EI]\) is much less than \([I]_T\) due to \([E]\) being much smaller than \(K_i\). However due to the near stoichiometric binding of tight-binding inhibitors, this is often not the case, and a large portion of the inhibitor population exists as enzyme-inhibitor complex\(^{176}\). Normally, reaction conditions where \([I]_T \geq 10[E]_T\) are employed, allowing for the approximations of the Michaelis-Menten model to be met. However with tight-binding inhibitors such conditions require very low \([E]\), often producing reaction rates that are below a measurable level when using standard spectroscopic-based assay procedures. To circumvent this, other models can be used that take into account the change in inhibitor concentration as the result of high affinity\(^{177}\). The Morrison model is one such case. The Morrison equation (Equation 3.1) describes the fractional initial velocity of an enzymatic reaction as a function of \([I]\), using fixed values for \([E]\) and \([S]\). Solving this quadratic function allows \(K_{i-app}\) (the apparent inhibition constant) to be determined, which can then be used to solve for \(K_i\). The relationship of these two values for a competitive inhibitor is described in Equation 3.2\(^{176,178}\).

**Equation 3.1.**

\[
v = v_o (1 - ([E]_0 + [I] + K_{1-app}) - \frac{\sqrt{([E]_0 + [I] + K_{1-app})^2 - 4[E]_0[I]}}{2[E]_0})
\]

**Equation 3.2.**

\[
K_{1-app} = K_i (1 + \frac{[S]}{K_M})
\]
Helianthamide was tested as an inhibitor of HPA using the following conditions: 

\[ [S] = 4 \text{ mM (} \sim K_M); 0.1 \text{ nM} \leq [I] \leq 25 \text{ nM}; 1 \text{ nM} \leq [E] \leq 20 \text{ nM}. \]

The resulting kinetic data and their subsequent fitting to the Morrison equation are shown in Figure 3.5. A value for \( K_{i\text{-app}} \) was solved for each enzyme concentration, the five values were averaged, and the average value was used to solve for \( K_i \). Through this method we determined a \( K_i \) of 0.01 ± 0.004 nM against HPA.

**Figure 3.5. Kinetic analysis of recombinant helianthamide versus HPA.** Dose response curves of \( \frac{v}{v_o} \) versus \([I]\) were constructed for various enzyme concentrations at \([\text{CNP-G3}] = 5 \text{ mM}. \) The curves were fit to the Morrison equation of tight-binding inhibition (Equation 3.1) via a least mean squares method to give values for \( K_{i\text{-app}} \) which were averaged and used to calculate \( K_i \) by Equation 3.2, which is the relationship between these two values for competitive inhibitors.
This inhibition constant makes helianthamide one of, if not the most potent $\alpha$-amylase inhibitor known to date, rivaling tendamistat ($K_i = 0.009 - 0.2$ nM) for this title. The bar'-hel fusion also exhibited potent inhibition towards HPA with a $K_i$ of 0.5 nM, making it only 50-fold weaker than free helianthamide. This is consistent with the X-ray crystal structure of the helianthamide/PPA complex that showed the N-terminus of helianthamide protruding into solution.

Helianthamide was also tested as an inhibitor of 10 other glycosidases, including the human intestinal $\alpha$-glucosidase maltase-glucoamylase and three bacterial $\alpha$-amylases (Table 3.1). The inhibitor showed specificity towards pancreatic $\alpha$-amylases when tested at a concentration of 1 $\mu$M. Due to the remarkable potency of this peptide towards HPA, extremely low concentrations of the inhibitor can be used, and as a result any mild inhibition of other enzymes would be inconsequential.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porcine pancreatic $\alpha$-amylase</td>
<td>0.1 nM</td>
</tr>
<tr>
<td><em>H. sapien</em> maltase-glucoamylase</td>
<td>NI</td>
</tr>
<tr>
<td><em>R. inulinivorans</em> $\alpha$-amylase A</td>
<td>NI</td>
</tr>
<tr>
<td><em>B. fibrisolvens</em> $\alpha$-amylase B</td>
<td>NI</td>
</tr>
<tr>
<td><em>B. licheniformis</em> $\alpha$-amylase</td>
<td>NI</td>
</tr>
<tr>
<td>Bovine liver $\beta$-galactosidase</td>
<td>NI</td>
</tr>
<tr>
<td>Green coffee bean $\alpha$-galactosidase</td>
<td>NI</td>
</tr>
<tr>
<td>Jack bean $\alpha$-mannosidase</td>
<td>NI</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> $\alpha$-glucosidase</td>
<td>NI</td>
</tr>
<tr>
<td>Almond $\beta$-glucosidase</td>
<td>NI</td>
</tr>
</tbody>
</table>

NI = no inhibition observed at a concentration of 1 $\mu$M.
In order to test whether helianthamide, as a β-defensin homologue, had any antibacterial activity, preliminary antimicrobial assays with helianthamide were carried out with *E. coli*. No antimicrobial activity was observed in this case, an unsurprising result due to the relative lack of cationic residues in the helianthamide sequence.

### 3.5 Structural Analysis of Recombinant Material

It was important to verify that the recombinantly expressed helianthamide had similar structural properties to the native material isolated from *S. helianthus* in addition to what was observed in the crystal structure with the synthetic peptide. Attempts at crystallizing the recombinant material on its own or in complex with HPA were carried out by our collaborators in the Brayer lab, however none of the crystallization attempts proved successful. Preliminary attempts at NMR analysis were also pursued. Indeed, N15-labeled peptide was expressed in minimal media, however low yields limited this pursuit.

#### 3.5.1 CD Spectroscopy

A CD spectrum was acquired for the helianthamide material isolated from *S. helianthus* by previous Withers lab researcher Dr. Jacqueline Wicki. Collaborators in the Brayer lab ran a similar experiment with the synthetic linear peptide prior to its use in crystallography. This allowed a comparison of our recombinantly expressed peptide with the native and synthetic materials as well as determination of the proportion of different secondary structural elements. The results (Figure 3.6 and Table 3.2) showed that the linear synthetic peptide was predominantly disordered, while the recombinant and natural peptides possessed a similar composition of secondary structural elements.
Figure 3.6. CD spectrophotometric analysis of natural (blue) and synthetic (green) and recombinant (red) helianthamide.

Table 3.2. Secondary structural analysis of the CD spectra of the natural, synthetic, and recombinant helianthamide

<table>
<thead>
<tr>
<th></th>
<th>Proportion of each secondary structural estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α-helix</td>
</tr>
<tr>
<td>Synthetic</td>
<td>0.06</td>
</tr>
<tr>
<td>Natural</td>
<td>0.19</td>
</tr>
<tr>
<td>Recombinant</td>
<td>0.11</td>
</tr>
</tbody>
</table>
3.5.2 Thiol Titration

The X-ray crystal structure of synthetic helianthamide in complex with PPA showed the inhibitor possessing 3 disulfide bonds in a 1-5, 2-4, 3-6 topology. In order to independently probe the oxidation states of the recombinant peptide’s cysteine residues the content of free thiols in the sample was measured using 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). This reagent reacts with free thiol groups in a stoichiometric manner to release 2-nitro-thiobenzoate, the release of which can be monitored at 412 nm. Excess DTNB was incubated with a series of concentrations of recombinant helianthamide for 30 minutes in a spectrophotometer. Since DTNB is also prone to spontaneous hydrolysis, control reactions were also monitored. BSA, which possesses one free thiol, was used as a positive control. Analysis with this method indicated no free thiol groups in the recombinant helianthamide, suggesting all cysteines present were oxidized.

3.5.3 LC-MS/MS Disulfide Analysis

It was also important to determine the disulfide topology of the recombinantly expressed helianthamide to compare with the crystal structure of the synthetic peptide. To do so, a number of disulfide connectivity determination protocols were attempted, however helianthamide proved to be remarkably stable to proteolysis, and was more or less resistant to attempts to cleanly reduce individual disulfide bonds for analysis. The original attempt was a partial reduction and alkylation protocol in which the recombinant material was treated with 10 - 25 mM TCEP for 0.5 - 1 hour, followed by addition of excess N-ethylmaleimide. It was hoped that the brief incubation in reducing agent would reduce only one or two of the disulfide bonds, allowing for specific alkylation of the free
cysteines. The differentially reduced and alkylated peptides would then be subjected to complete reduction and alkylation with iodoacetamide followed by enzymatic digestion and analysis by LC-MS/MS\textsuperscript{181,182}. Attempts to carry out this protocol with helianthamide encountered immediate difficulties. Helianthamide appeared resistant to attempts at partial reduction. Incubation time, [TCEP], [GuHCl], and temperature were increased. However in no case was partial reduction and alkylation observed. It was later discovered that there have been previous accounts of β-defensin peptides being reduced in an all-or-nothing fashion, which could explain why helianthamide was resistant to partial reduction\textsuperscript{183}. Additionally, such protocols risk disulfide scrambling and can produce misleading results\textsuperscript{184,185}. As such, procedures utilizing peptide with intact disulfide bonds were preferred.

Next a protease-based disulfide determination procedure was attempted. The aim was to treat helianthamide with various proteases such as proteinase K, trypsin and chymotrypsin to cleave the peptide into fragments. The helianthamide fragment that contained the two adjacent cysteines would then be subjected to one round of Edman degradation to separate these two adjacent disulfide bonds for mass spectrometric analysis\textsuperscript{186}. Unfortunately, helianthamide remained recalcitrant to cleavage by these proteases. Increasingly harsh conditions were trialed as proteinase K can withstand exposure to GuHCl and detergents\textsuperscript{187}. However no release of fragments was observed under any of the trialed conditions.

In the end we resorted to a messy brute force analysis in which recombinant helianthamide was incubated in 11 M HCl for 4 days at 37°C, followed by analysis of resulting hydrolytic fragments by LC-MS/MS\textsuperscript{188}. 
### Table 3.3 Select LC-MS/MS peaks and their corresponding sequences for disulfide assignment

<table>
<thead>
<tr>
<th>Mass</th>
<th>Parent peak</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1198.42</td>
<td>600.21 [M+2H]²⁺</td>
<td>YHGVS\textsubscript{G}IC \textsubscript{C}E</td>
</tr>
<tr>
<td>1279.58</td>
<td>640.79 [M+2H]²⁺</td>
<td>HGV\textsubscript{S}G\textsubscript{I}CKA MGV\textsubscript{C}</td>
</tr>
<tr>
<td>1286.70</td>
<td>429.90 [M+3H]³⁺</td>
<td>G\textsubscript{I}CK KAM\textsubscript{A}MG\textsubscript{V}C</td>
</tr>
<tr>
<td>1311.70</td>
<td>656.85 [M+2H]²⁺</td>
<td>CY\textsubscript{I}YHG\textsubscript{V} L\textsubscript{CC} C</td>
</tr>
<tr>
<td>1327.68</td>
<td>443.56 [M+3H]³⁺</td>
<td>V\textsubscript{C} I\textsubscript{C}K\textsubscript{A}S\textsubscript{C}AE \textsubscript{C}Y</td>
</tr>
<tr>
<td>1347.66</td>
<td>449.22 [M+3H]³⁺</td>
<td>NS\textsubscript{C}Y\textsubscript{I} L\textsubscript{CC}YK C</td>
</tr>
<tr>
<td>1416.68</td>
<td>709.34 [M+2H]²⁺</td>
<td>G\textsubscript{I}C EKAM\textsubscript{A}MG\textsubscript{V}C\textsubscript{E}</td>
</tr>
<tr>
<td>1439.60</td>
<td>480.88 [M+3H]³⁺</td>
<td>SC E\textsubscript{G}H\textsubscript{L}CC\textsubscript{Y} S\textsubscript{C}AE</td>
</tr>
<tr>
<td>1534.56</td>
<td>768.28 [M+2H]²⁺</td>
<td>E\textsubscript{S}G\textsubscript{N}SC G\textsubscript{H}\textsubscript{L}CC S\textsubscript{C}AE</td>
</tr>
<tr>
<td>1551.70</td>
<td>776.85 [M+2H]²⁺</td>
<td>G\textsubscript{N}SC G\textsubscript{H}\textsubscript{L}CC\textsubscript{Y} K\textsubscript{A}S\textsubscript{C}A</td>
</tr>
<tr>
<td>1606.82</td>
<td>804.41 [M+2H]²⁺</td>
<td>CY G\textsubscript{V}\textsubscript{C}E\textsubscript{G}H\textsubscript{L}C S\textsubscript{G}\textsubscript{I}CK</td>
</tr>
<tr>
<td>1631.78</td>
<td>816.89 [M+2H]²⁺</td>
<td>S\textsubscript{E}S\textsubscript{G}N\textsubscript{S}C L\textsubscript{CC}YK C\textsubscript{A}E</td>
</tr>
<tr>
<td>1698.81</td>
<td>566.27 [M+3H]³⁺</td>
<td>S\textsubscript{G}N\textsubscript{S}C L\textsubscript{CC} C\textsubscript{A}EDEK\textsubscript{A}M</td>
</tr>
<tr>
<td>1760.80</td>
<td>881.40 [M+2H]²⁺</td>
<td>G\textsubscript{N}SC L\textsubscript{CC}YK T S\textsubscript{C}AEDE</td>
</tr>
<tr>
<td>1818.97</td>
<td>607.32 [M+3H]³⁺</td>
<td>S\textsubscript{E}S\textsubscript{G}N\textsubscript{S}C\textsubscript{C}Y\textsubscript{I}Y\textsubscript{H}G\textsubscript{C} CC S\textsubscript{C}A</td>
</tr>
</tbody>
</table>
Figure 3.7. MS/MS data of selected peaks and their corresponding sequences for disulfide analysis of helianthamide. The data agree with the 1-5,2-4,3-6 connectivity seen in the crystal structure.

It should be noted that, even after this 4-day incubation period, the vast majority of the peptide remained intact based on subsequent HPLC analysis. A script was written by Dr. William Walters to predict the masses of all possible peptide fragments resulting from the helianthamide sequence. A number of peaks from the LC-MS/MS
analysis were selected for confirmation of the disulfide topology based on their MS/MS fragmentation spectra (Table 3.3). The several fragments that were analyzed appeared to agree with the previously observed 1-5, 2-4, 3-6 topology. A depiction of two of the MS/MS fragmentation spectra is shown in Figure 3.7.

3.6 Site-Directed Mutagenesis of Helianthamide

3.6.1 Mutagenesis to Disrupt Polar Contacts between Helianthamide and HPA

The X-ray crystal structure of PPA with synthetic helianthamide showed the two molecules in a non-covalent complex, with one third of helianthamide’s surface area buried at an interface with the enzyme. Within the active site, residues Y7, I8, Y9 and H10 of helianthamide formed the majority of the polar contacts with the enzyme (Figure 3.8a). The side chain of Y7 formed a hydrogen bond with D300 of the enzyme. The main chain carbonyl of I8 was seen to hydrogen bond with Y151 on the border of the active site. Y9 interacted with nucleophilic residue D197 as well as Y62 and H101. Finally H10 formed interactions with both D197 and D300. The interactions of the YIYH sequence with the enzyme's active site residues highlighted this region of the inhibitor as a new potential amylase inhibitory motif, analogous to the conserved WRY sequence of tendamistat and the other *Streptomyces* peptide inhibitors. To probe the contributions of individual residue side chains of helianthamide towards HPA binding affinity, site-directed mutagenesis of the recombinant barnase’-helianthamide fusion was pursued. As mentioned previously the bar’-hel fusion is also a potent inhibitor of HPA, with a $K_i$ of 0.5 nM. Given the difficulty of measuring inhibition constants of tight binding inhibitors, use of the weaker-binding (but still potent) bar’-hel fusion as the base for site-directed mutagenesis allowed for easier measurement and comparison of binding affinities. To
this end, alanine variants of the bar'-hel fusion were made for Y7, Y9, and H10. Phenylalanine variants were also made for Y7 and Y9.

Figure 3.8. Highlighted areas of contact between helianthamide and PPA. Helianthamide shown in blue, PPA shown in grey. (a) Interactions between the YIYH motif of helianthamide and residues within the amylase active site. (b) Interactions between serines of helianthamide and the loop of high thermal motion on PPA. (c) Hydrophobic interface between helianthamide and V163 of PPA. (PDB: 4X0N)

Outside of this prominent four-residue stretch, interactions were also observed between the side chains S2, S5, and S13 of helianthamide and residues H305, G308, and S310 of the enzyme, which are contained on a loop of high thermal motion near the active site (Figure 3.8b). Alanine variants were made for S5 and S13. The main chain atoms of residues A28, G29, and G31 also formed hydrogen bonds with enzyme residues Y151, N152, and E240. However since these are main chain interactions they cannot be readily probed through site-directed mutagenesis studies.
The seven bar'-hel fusion mutants were expressed in *E. coli* and purified by IMAC, cation exchange chromatography, and hydrophobic exchange chromatography. Unlike the expression of lone helianthamide, which only required IMAC purification before TEV protease cleavage and subsequent purification by methanol, the barnase’-helianthamide fusion required three different chromatographic techniques to reach suitable purity as observed by SDS-PAGE.

### Table 3.4. Kinetic analysis of barnase'-helianthamide fusions against wild-type HPA and a T163R HPA mutant

<table>
<thead>
<tr>
<th>Helianthamide Type</th>
<th>HPA $K_i$ (nM)</th>
<th>T163R HPA $K_i$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bar'-hel</td>
<td>0.5 ± 0.1</td>
<td>20 ± 7</td>
</tr>
<tr>
<td>Bar'-hel-Y7A</td>
<td>2.5 ± 0.03</td>
<td>107 ± 9</td>
</tr>
<tr>
<td>Bar'-hel-Y9A</td>
<td>23 ± 0.25</td>
<td>960 ± 88</td>
</tr>
<tr>
<td>Bar'-hel-H10A</td>
<td>9 ± 0.4</td>
<td>321 ± 57</td>
</tr>
</tbody>
</table>

*Error determined through least-mean squares method or through fit errors produced by Grafit*

The bar'-hel mutants were tested as inhibitors of HPA and results were analyzed using the Morrison method (Table 3.4). The effects of individual mutations were small, ranging from a 5-fold decrease for Y7A to 50-fold for Y9A when compared to the potency of the wild-type bar'-hel. All of the tested mutants exhibited nM-range inhibition constants. Cumulatively, the interactions of the Y7, Y9 and H10 side chains only contributed approximately 6 kcal·mol$^{-1}$ to binding affinity, suggesting that other protein-ligand interactions must be making important contributions to inhibitor potency. This may however be an underestimate since it could be the case that the loss of
interactions from a single residue mutation is compensated for by neighboring residues. Both Y9 and H10 hydrogen bond with D197, while H10 and Y7 hydrogen bond with D300. Y9 forms additional interactions with Y62 and H101, which cannot be replaced in its absence and may account for the larger decrease in potency experienced by the Y9A mutant.

The Y7F and Y9F variants showed an even smaller change in potency than the alanine variants. Y9F exhibited a $K_i$ of 2.2 nM and the YF7 variant bound with the same affinity to that of wild-type bar’-hel ($K_i = 0.5$ nM). The S5A and S13A variants also bound with the same affinity as wild-type bar’-hel. The lack of discrimination seen among these serine variants could be due a consequence of the use of the fusion peptides. While the fusion is still very potent with a $K_i$ of 0.5 nM, the installation of the barnase’ on the N-terminus of helianthamide produces a 50-fold decrease in potency and its presence could have already impacted the interactions at Ser5 and Ser13 as these residues reside near or on the N-terminal strand.

In addition to the polar contacts mentioned above, there appears to be a major hydrophobic interface between the inhibitor and enzyme, contributing to helianthamide’s large buried surface area as shown in Figure 3.8c. Residues 162-165 of PPA consist of the hydrophobic sequence LVGL. This region formed a point of contact with I8 and V12 as well as the C-terminal region of helianthamide, namely T42 and the aromatic rings of Y40 and W44. Helianthamide forms a hydrophobic pocket around residue 163 of the enzyme, which is Val in PPA and Thr in HPA. We wanted to examine the effects of disrupting this region and thereby perturbing helianthamide-HPA interactions in a global sense. A T163R mutant of HPA was expressed for this purpose to ensure a significant
effect upon binding. Kinetic analysis of T163R HPA with CNPG3 revealed near identical $k_{\text{cat}}$ and $K_M$ values for this substrate as seen with the wild-type enzyme ($K_M = 2.76 \pm 0.34 \text{ mM}; \ k_{\text{cat}} = 1.0 \times 10^3 \text{ min}^{-1}$), indicating that this replacement does not lead to significant perturbations in the HPA active site, at least when acting upon the substrate CNPG3. However, there could well be a difference in binding affinity for starch or maltohexaose substrates as T163 is shown to interact with glucosyl units in the S-3 subsite of the active site (Figure 3.9).

**Figure 3.9. Binding of maltohexaose in the HPA active site and relative placement of T163.** Analysis of the X-ray crystal structure of maltohexaose bound within the HPA active site indicates that T163 interacts with substrate units bound in the S-3 subsite (PDB: 5TD4).

The T163R variant of HPA bound the bar'-hel fusion 40-fold more weakly than did wild-type HPA, with a new $K_i$ of 20 nM. This loss of affinity allowed a second kinetic analysis of the helianthamide variants to be performed via the more commonly used
Michaelis-Menten model in order to confirm whether the relative binding affects previously measured were meaningful. Quantification of inhibition of HPA-T163R by the Y7A, Y9A, and H10A bar'-hel variants under these more traditional conditions revealed the same 40-fold decrease in affinity in each case (Table 3.4). This reinforced the trends observed in the original data set and validates the Morrison method of absolute $K_i$ determination for sub-nM enzyme inhibitors.

Site-directed mutagenesis analyses of the interactions between $\alpha$-amylases and their proteinaceous inhibitors have only been conducted in a few other cases. A study of the lectin-type inhibitor $\alpha$AI1 showed that mutagenesis of R74, W188, and Y190 led to complete elimination of inhibitory activity. This led to the initial conclusion that $\alpha$AI1 had a similar WRY motif to tendamistat, however eventual structural analysis indicated that only Y190 formed contact with the enzyme’s catalytic residues, while W188 forms stacking interactions in the active site cleft$^{116,189}$. In the case of the cereal-type 0.19 inhibitor, catalytic mutants of $\alpha$-amylase that showed no hydrolytic activity lost affinity for the inhibitor but were still able to bind substrate molecules$^{190}$. However, unlike helianthamide, neither of these peptides formed an extensive hydrophobic interface with the enzyme and instead appear to rely much more heavily on the formation of individual polar contacts.

3.6.2 Site-Directed Mutagenesis of Helianthamide’s Cysteines

Alanine variants of the six cysteine residues of helianthamide were also made and expressed in E. coli. The resulting bar'-hel fusion mutants were tested as inhibitors of HPA. Inhibitors that seemed of high potency (nM-range inhibition constants) were analyzed via the Morrison Method. Inhibitors that showed µM-range inhibition constants
were analyzed under both Morrison Method and Michaelis-Menten conditions (Table 3.5).

Table 3.5. Kinetic analysis of cysteine bar’-hel fusion mutants against HPA

<table>
<thead>
<tr>
<th>Helianthamide Type</th>
<th>Morrison Method $K_i$ (µM)</th>
<th>Michaelis-Menten $K_i$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bar’-hel</td>
<td>(0.5 ± 0.1) x 10^{-3}</td>
<td>--</td>
</tr>
<tr>
<td>Bar’-hel-C6A</td>
<td>4.0 ± 0.3</td>
<td>5.3 ± 1.0</td>
</tr>
<tr>
<td>Bar’-hel-C16A</td>
<td>(5 ± 0.6) x 10^{-3}</td>
<td>--</td>
</tr>
<tr>
<td>Bar’-hel-C20A</td>
<td>3.8 ± 0.6</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>Bar’-hel-C33A</td>
<td>(8 ± 0.4) x 10^{-3}</td>
<td>--</td>
</tr>
<tr>
<td>Bar’-hel-C38A</td>
<td>3.5 ± 0.3</td>
<td>2.4 ± 0.6</td>
</tr>
<tr>
<td>Bar’-hel-C39A</td>
<td>6.0 ± 0.6</td>
<td>5.9 ± 2.0</td>
</tr>
</tbody>
</table>

Error determined through least-mean squares method or through fit errors produced by Grafit.

On average, replacing the cysteines of helianthamide reduced binding affinities much more than the disruption of individual polar contacts. The C6A, C20A, C38A and C39A mutants all had inhibition constants in the µM range, showing a 7000 - 13,000 fold decrease in potency compared to the wild-type. This substantial loss of potency associated with the disruption of disulfide bonding highlights the importance of helianthamide’s rigid three-dimensional structure for its potent HPA inhibition. On the other hand, C16A and C33A mutants were still nM-range inhibitors showing only a 10 – 16 fold decrease in potency, similar in scale to the changes seen for the Y7A, Y9A, and H10A mutants. This support the results of the LC-MS/MS disulfide determination.
experiment, suggesting that C16 and C33 participate in disulfide bonding with each other, due to the similarly small relative loss of potency of their alanine mutants. The results of this kinetic analysis also suggest that the C16-C33 disulfide bond plays a smaller supporting role in the stabilization of the tertiary structure compared to the other two disulfides (Figure 3.10).

![Figure 3.10. X-ray crystal structure of helianthamide’s disulfide bonds. (PDB: 4X0N)](image)

### 3.7 Synthesis of Small Helianthamide-Derived Peptides

Issues associated with production, stability, immunogenicity, and/or bioavailability can often impede the therapeutic use of a protein. This may compel the synthesis of smaller related peptides in hopes of finding similarly active structures. The synthesis of small peptides based upon a larger inhibitor’s structure can also be used for structural analysis to evaluate the importance of specific sequence regions. A number of small
peptides have been synthesized based upon structures of proteinaceous α-amylase inhibitors. Tendamistat, with its remarkable potency and unwanted immunogenicity, has been a particularly popular model, and a number of peptide libraries have been inspired by its sequence. Most of the small synthetic peptides designed from tendamistat’s sequence exhibited μM-range inhibition constants against HPA. However in one study researchers were able to synthesize a peptide with an inhibition constant of 270 nM against HPA. This peptide contained the sequence CYQSWRYSQAC, and showed similar inhibition in both its linear and cyclic forms owing to the inherent β-hairpin turn of this sequence. Small peptides have also been synthesized based on the sequence of the cereal-type α-amylase inhibitor RBI. Crystallographic analysis indicated that the N-terminal region of RBI formed prominent interactions in the amylase active site and small peptides based on this region showed similarly high potency towards α-amylase.

To further probe the mode of helianthamide’s inhibition and to potentially identify accessible sequences of similar potency, a library of helianthamide-inspired peptides was pursued. These peptides were based upon the sequence of helianthamide and the crystal structure of its complex with PPA. Both linear and cyclic peptides were pursued in this synthesis. The cyclization of small peptides can introduce rigidity into the structures to potentially increase binding affinity. However it may also perturb the orientation of residues and prevent binding. In this study, cyclization of the helianthamide-inspired peptides would occur through disulfide bonding by incorporation and oxidation of a cysteine pair in the peptide sequences.
The first batch of peptides was based on residues 1 - 16 of helianthamide (Figure 3.11a). As discussed in Section 3.6, the X-ray crystal structure of the helianthamide/PPA complex showed a number of interactions between residues 2 - 13 of helianthamide and the enzyme, with the most notable being in the aforementioned YIYH motif. The inclusion of residues I8 and V12 in our small peptides, which created hydrophobic interactions with the enzyme (Figure 3.11b), could potentially replicate the hydrophobic interface between PPA and helianthamide. Within the region of interest the C6-C38 and C16-C33 disulfide bonds reside only a few angstroms apart. It was hypothesized that C6 and C16 could act as disulfide bonding partners in the first batch of small peptides to potentially achieve a similar organization of residues to that seen in the helianthamide/PPA complex.

**Figure 3.11. Region of interest for the synthesis of the first batch of helianthamide-inspired peptides.** (a) Residues 1 - 16 (PDB: 4X0N) shown in magenta sticks with the distance between C6 and C16 shown in grey dashes as 4 Å. (b) Residues 1 – 16 of helianthamide in complex with PPA.
Table 3.6. Sequences of the first batch of small helianthamide-based peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>YIYH</td>
</tr>
<tr>
<td>2</td>
<td>YIYHGV</td>
</tr>
<tr>
<td>3</td>
<td>YIYHGVS</td>
</tr>
<tr>
<td>4</td>
<td>YIYHVSGI</td>
</tr>
<tr>
<td>5</td>
<td>CYIYHGVSIC</td>
</tr>
<tr>
<td>6</td>
<td>ESGNSCYIYHGVSIC</td>
</tr>
<tr>
<td>7</td>
<td>SYIYHGVS</td>
</tr>
<tr>
<td>8</td>
<td>CYIYHGVS</td>
</tr>
<tr>
<td>9</td>
<td>CYIYHGVS</td>
</tr>
<tr>
<td>10</td>
<td>ESGNSCYIYHGVS</td>
</tr>
<tr>
<td>11</td>
<td>ESGNSCYIYHGVS</td>
</tr>
</tbody>
</table>

Solid phase peptide synthesis was carried out to produce eleven peptides based on this region (Table 3.6). Synthetic peptides 1 to 6 were based directly upon the sequence of helianthamide, with varying N- and C-terminal positions. Peptides 7 to 11 deviated somewhat from helianthamide’s sequence to potentially reduce ring size or to substitute in a serine for cysteine where no disulfide bonding would be formed in any case. Cyclization via disulfide bond formation was carried out by overnight incubation in 0.1 M NaHCO₃, 3 mM cysteine, 0.3 mM cystine, 1 M GuHCl, pH 8.1. These peptides were purified by RP-HPLC, characterized by MALDI-TOF and tested in their linear and cyclized (oxidized) forms where applicable. None of the eleven peptides showed any
inhibition towards HPA in their cyclized or linear forms. It appears that the sequence of the N-terminal region of helianthamide does not produce any inhibition on its own despite being responsible for the majority of polar interactions in the intact peptide.

Despite these unpromising results, a second batch of small helianthamide-inspired peptides was pursued, this time attempting to include portions of the C-terminus (Figure 3.12a). The C-terminus of helianthamide also formed interactions with the enzyme in the X-ray crystal structure. W44 formed hydrogen bonds with residue N53 of the amylase, and its indole side chain interacted with V163, which borders the enzyme active site cleft. The C-terminus of helianthamide also seemed to contribute to the hydrophobic interface between the inhibitor and enzyme (Figure 3.12b). In helianthamide the C-terminal region is in close spatial proximity to residues 7 - 13 and a covalent bridge between these two regions is formed in helianthamide through the C6-C38 disulfide bond. It was thought that this disulfide bond could be replaced with a peptide bond to join the C terminal residues to the YIYHVSGI sequence of residues 7 - 14. Incorporation of a C-terminal cysteine would allow disulfide formation with a cysteine representative of C39. Two peptides, 12 and 13, were made through this strategy.

In addition to this it was noted that the distance between Cα of I15 and C38 was 5.5 Å in the PPA-helianthamide crystal structure (3.12a). This distance could be achieved by the insertion of one glycine residue, as also highlighted in the loop of HGV of residues 10-12 in Figure 3.12a. It was thought that the C-terminal region of helianthamide could be attached to the N-terminal YIYHVSGI sequence through a glycine, proline or diglycine. From this point a disulfide bond could be formed between cysteine representatives of residues C6 and C38 as is seen in the native structure, for
added rigidity. There were some variations to be trialed with this derived sequence. The fate of what was once C39, which no longer had a disulfide-bonding partner in the small peptide derivatives, was tested with a variety of amino acid replacements such as serine, alanine, and glycine. This strategy resulted in peptides 14 to 19.

Figure 3.12. Region of interest for the synthesis of the second batch of helianthamide-inspired peptides. (a) The second batch of small peptides included elements of helianthamide's C-terminus as well as the N-terminal region. Residues of interest are colored in magenta within the helianthamide sequence. Due to the close spatial proximity of these regions (through the C6-C8 disulfide bond or distance of 5.5 Å between I15 and C38), they could be joined by one or two residues. (b) Residues of interest in complex with HPA, showing hydrophobic contacts with W44 and other C-terminal residues.
Table 3.7. Sequences of the second batch of small helianthamide-based peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>WPTKYCGGYIYHGVSGC</td>
</tr>
<tr>
<td>13</td>
<td>WPTKYCGYIYHGVSGC</td>
</tr>
<tr>
<td>14</td>
<td>CYIYHGVSIGCSYKTPW</td>
</tr>
<tr>
<td>15</td>
<td>CYIYHGVSIGCAYKTPW</td>
</tr>
<tr>
<td>16</td>
<td>CYIYHGVSIPCSYKTPW</td>
</tr>
<tr>
<td>17</td>
<td>CYIYHGVSICAYKTPW</td>
</tr>
<tr>
<td>18</td>
<td>CYIYHGVSIGGCSYKTPW</td>
</tr>
<tr>
<td>19</td>
<td>CYIYHGVSIGGCAYKTPW</td>
</tr>
</tbody>
</table>

Peptides from this second batch were tested in their oxidized cyclic forms. Only two of these peptides showed inhibitory activity towards HPA. Peptides 12 and 13 had IC\textsubscript{50}’s of 155 \(\mu\)M and 396 \(\mu\)M towards HPA, respectively (Table 3.8). These two peptides differed from the other cyclic peptides of this study in that they contained at least one residue separating the first cysteine and the YIYH motif. For all other cyclic peptides of this project, the disulfide bond in front of the YIYH motif likely did not adequately resemble the conformation within the native structure and prevented appropriate orientation and placement of the subsequent residues. Increasing the distance between the YIYH motif and the disulfide bond in peptides 12 and 13, allowed some interactions to form with the enzyme. However these two peptides are still nowhere near as potent as helianthamide.
### Table 3.8. Kinetic analysis of active peptides against HPA.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>IC$_{50}$ against HPA (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>WPTKYCGGYIYHGVSGC (oxidized)</td>
<td>141 ± 20</td>
</tr>
<tr>
<td>13</td>
<td>WPTKYCGYIYHGVSGC (oxidized)</td>
<td>396 ± 19</td>
</tr>
</tbody>
</table>

Despite synthesizing peptides that contained the majority of the residues of helianthamide that formed polar contacts with the enzyme, almost none of these sequences proved to be significantly inhibitory. The lack of activity seen for almost all of these peptides further highlights the importance of helianthamide’s rigid three-dimensional structure and its ability to form an extensive interface with the enzyme.

### 3.8 Conclusion and Future Directions

The initial isolation of helianthamide from *S. helianthus* highlighted this peptide as a potential new lead for potent HPA inhibition. To gain further understanding on the mode of inhibition of this novel β-defensin homologue, a reliable source of active material was required. We were able to produce active helianthamide for structural and kinetic analysis through recombinant expression as a barnase'-based fusion. Using the recombinant material it was demonstrated that helianthamide has an inhibition constant of 10 pM against HPA, making it one of the most potent α-amylase inhibitors known to date. The small disulfide-rich structure of helianthamide proved remarkably stable, withstanding prolonged exposure to acids, solvents, and proteases. While hindering some structural analysis, these characteristics suggest that it would hold up well as an oral therapeutic in the harsh environment of the digestive tract.
Structural analysis of helianthamide indicated the importance of its intact three-dimensional structure and proper disulfide bonding for potent HPA binding. Loss of single cysteine residues from otherwise intact helianthamide during the cysteine site-directed mutagenesis study resulted in a substantial loss of potency. The formation of individual polar contacts does contribute to helianthamide’s potency, as observed through the results of the site-directed mutagenesis of the YIYH motif and the mild activity of some of the small helianthamide-inspired peptides. However, the accumulation of polar contacts pales in comparison to the importance of the rigid three-dimensional structure for forming an interface with HPA.

Due to the relatively low overall yield of the barnase’-helianthamide expression system there is a need for an improved means of production to explore helianthamide’s full potential as an inhibitor of carbohydrate metabolism in pharmacokinetic studies and subsequent preclinical work. While preliminary attempts to express helianthamide without a fusion partner were unsuccessful, other expression systems and hosts should be explored towards the improved production of this highly potent inhibitor.
Chapter 4. Concluding Remarks

The accumulation of interactions across the six subsites of the HPA active site cleft is necessary for its appreciable affinity for the starch substrate. Consequently, the enzyme shows little affinity for shorter chain carbohydrates, such as maltosides and maltotriosides. This preference extends to the structures of inhibitors that can also act on HPA. Despite there being an abundance of previously characterized $\alpha$-glucosidase inhibitors, many of these compounds do not inhibit HPA due to their small size. Elongated versions of these inhibitors have been shown to inhibit $\alpha$-amylases, however they are vulnerable to HPA-catalyzed hydrolysis and transglycosylation. Many of these inhibitors also show activity towards the brush border $\alpha$-glucosidases, leading to unwanted gastrointestinal side effects during clinical usage. As a result, there has been motivation to find specific inhibitors of HPA that are not carbohydrate-based, which provides the basis of this thesis.

HPA binds the starch substrate through two general types of interactions: polar-based interactions with the starch polymer’s hydroxyls, and $\pi$-CH interactions between the starch’s glucosyl rings and aromatic residues within the active site. These $\pi$-based interactions allow certain aromatic moieties to also inhibit HPA. Mild inhibition of $\alpha$-amylases by various flavonoids has been observed, however until the discovery of a flavonol acyl glycoside called Montbretin A, none of these compounds produced potent inhibition. Unlike other flavonoids, which exhibited $\mu$M-range inhibition constants against HPA, Montbretin A competitively inhibited the enzyme with a $K_i = 8$ nM. This inhibitor was also shown to be selective for HPA over the brush border $\alpha$-glucosidases,
suggesting that its use would avoid side effects associated with the general carbohydrate metabolism of carbohydrate-based inhibitors currently in clinical use\textsuperscript{93}. A minimum inhibitory structure (MbA-GRX) was identified during a chemoenzymatic degradation study of MbA and was used as a basis for the design and synthesis of MbA analogues for this thesis work. Sixteen analogues of MbA were synthesized. Many of these analogues were substantially less potent than MbA-GRX despite their potential to form all of the same interactions in the active site, highlighting the importance of MbA’s pre-stacked structure for its potent inhibition of HPA. On the other hand, two of the synthetic analogues exhibited nM-level inhibition constants towards HPA and were more potent than MbA-GRX. These two compounds, containing tyrosine and L-DOPA in their linkers, allowed for additional hydrogen bonding and π-interactions to occur within the active site, and could achieve potent inhibition of HPA despite lacking the pre-stacked conformation of MbA and MbA-GRX.

The isolation of helianthamide from \textit{Stichodactyla helianthus} represented the discovery of a new structural class of protein-based HPA inhibitor. Helianthamide was shown to be structurally homologous to the antimicrobial β-defensins, a fold not previously seen in α-amylase inhibitors. Recombinant helianthamide was shown to have a $K_{i} = 0.01$ nM with HPA\textsuperscript{148}. Previously, tendamistat, a peptide-based inhibitor of HPA produced in \textit{Streptomyces} sp., was considered to be the most potent inhibitor of HPA with a $K_{i} = 0.009 – 0.2$ nM. However helianthamide now rivals tendamistat for this title.

Like, MbA, helianthamide was initially thought to also heavily rely on its phenolic functional groups for potent inhibition of HPA. During X-ray crystallographic analysis of helianthamide in complex with PPA, a potential YIYH inhibitory motif was highlighted as
forming the majority of polar contacts with the enzyme. An overlay of this YIYH motif with MbA (Figure 4.1) showed similar placement of Y9 of helianthamide with the A-ring of MbA’s myricetin and H10 of helianthamide with the catechol of MbA’s caffeic acid.

![Overlay of Montbretin A and Helianthamide’s YIYH motif in complex with HPA.](image)

**Figure 4.1 Overlay of Montbretin A and Helianthamide’s YIYH motif in complex with HPA.** Montbretin A is shown as yellow sticks, helianthamide is shown as marine blue sticks, and the catalytic residues of α-amylase are shown as green and turquoise sticks.

Despite this interesting overlap of aromatic moieties between helianthamide and MbA, site-directed mutagenesis studies on helianthamide and the production of small helianthamide inspired peptides instead suggested that helianthamide relies on its rigid disulfide-rich 3D structure and resulting ability to form a robust hydrophobic interface with the enzyme for its potent inhibition. This contrasts with a number of protein-based
inhibitors of HPA, such as the cereal-type inhibitors and lectin-like inhibitors, which rely more heavily on the formation of interactions with the enzyme’s catalytic residues.

This thesis presents two differing but promising approaches for the future development of HPA inhibitors. The synthesis of MbA analogue M10 with a $K_i = 44$ nM provides a chemically accessible route to continue the exploration of this inhibitory motif for preclinical and clinical usage. Meanwhile, the highly potent inhibition and rigid structure of helianthamide suggests that this peptide would perform well as an oral therapeutic for the mediation of carbohydrate metabolism. These two inhibitors provide alternative routes to the traditional carbohydrate-based inhibitors of starch metabolism and can be explored as an effective means of controlling blood sugar.
Chapter 5. Experimental Procedures

5.1 Synthesis of Montbretin A Analogues

5.1.1 General Materials

All reagents and solvents were purchased from commercial suppliers (Sigma-Aldrich® or Thermo Fisher Scientific®) unless otherwise specified. 6-Aminoglucosyl fluoride, 4-aminoglucosyl fluoride and 4-aminogalactosyl fluoride were synthesized by Dr. Hong-Ming Chen. Dichloromethane could be dried by distillation with calcium hydride. Deionized water was prepared using a Millipore-Direct QTM 5 Ultrapure Water System. Naringinase from *Penicillium decumbens* was purchased from Sigma-Aldrich. Abg2F6 was expressed as previously reported

Analytical thin layer chromatography (TLC) was performed on Merck pre-coated 0.2 mm aluminum-backed sheets of Silica gel 60F254. TLC plates were visualized with UV light (254 nm) and stained with 1% ferric chloride in 50% water 50% methanol, or with 10% ammonium molybdate in 2 M H₂SO₄, followed by heating. Flash chromatography was performed with silica gel (pore size 60 Å, 220-440 mesh particle size) from Sigma-Aldrich. High performance liquid chromatography (HPLC) was performed on an Agilent 1260 Infinity Bio-inert Quaternary LC using an Agilent Eclipse XDB-C18 column (9.4 x 250 mm, 5 µm) at room temperature using acetonitrile and water with a flow rate of 4 mL/min. Elution of material was monitored by UV/Vis at 210, 280, and 350 nm.

¹H NMR, ¹³C NMR, HMBC, and HSQC spectra were acquired on a Bruker 300 MHz or 600 MHz spectrometer. Low resolution mass spectra were acquired on a
Waters ZQ Mass Detector equipped with an ESCI ion source and Waters 2695 HPLC. High resolution mass spectra were acquired on a Waters/Micromass LCT ESI-TOF.

5.1.2 General Procedures

(E)-Perfluorophenyl 3-(3,4-dihydroxyphenyl)acrylate (01a). Caffeic acid (0.798 g, 4.38 mmol) was dissolved in 10 mL of DMF. Pyridine (0.57 mL, 7.05 mmol) was added, followed by 1.21 mL (7.05 mmol) of pentafluorophenyl trifluoroacetate. The reaction mixture was stirred at room temperature for 2 hours then diluted with DCM and washed 4x with 1 M HCl. The material was dried over MgSO_4 and concentrated in vacuo. The crude material was purified by silica gel chromatography using an eluent system of pet. ether/EtOAc (6:4). Pentafluorocaffeic ester (01a) was isolated as a pale yellow powder upon evaporation of collected fractions (1.41 g, 4.08 mmol, 93% yield); ¹H NMR (300 MHz, acetone-­d₆) δ 8.59 (s, 1H), 8.41 (s, 1H), 7.88 (d, J = 15.9 Hz, 1H), 7.30 (d, J = 1.9 Hz, 1H), 7.21 (dd, J = 8.1 Hz, J = 2.0 Hz, 1H), 6.93 (d, J = 8.2 Hz, 1H), 6.60 (d, J = 15.9 Hz, 1H); ¹⁹F NMR (300 MHz, acetone-­d₆) δ -155.56 (d, J = 18.4 Hz, 2F), -161.33 (t, 1F), -165.51 (m, 2F); ¹³C NMR (150MHz, acetone-­d₆) δ 163.7, 151.0, 150.1, 146.4, 143.1, 141.5, 141.0, 139.7, 139.4, 138.0, 126.9, 123.9, 116.5, 115.8, 111.0; ESI-­MS: m/z: 345 [M-­H]⁻
(E)-3-(3,4-Dihydroxyphenyl)-N-(prop-2-yn-1-yl)acrylamide (01b). Ester 01a (0.33 g, 0.94 mmol) was dissolved in 10 mL of CH$_3$Cl and 5 mL of DMF then 0.6 mL (0.94 mmol) of propargylamine and 0.13 mL (0.94 mmol) of triethylamine were added. The reaction mixture was stirred at rt for 4 hours and then diluted with DCM and washed 5x with 1 M HCl. The material was dried over MgSO$_4$ and concentrated in vacuo. The resulting material was recrystallized with ethanol/pet. ether yielding amide 01b as pale orange crystals (0.2 g, 0.92 mmol, 97% yield); $^1$H NMR (300 MHz, acetone-d$_6$) δ 8.63 (s, 1H), 8.49 (s, 1H), 7.94 (d, J = 15.3 Hz, 1H), 7.42 (d, J = 2.1 Hz, 1H), 7.25 (dd, J = 8.2 Hz, J = 1.8 Hz, 1H), 6.95 (d, J = 8.5 Hz, 1H), 6.71 (d, J = 15.5 Hz, 1H), 5.57 (br, 1H), 3.65 (m, 2H), 3.55 (t, 1H); $^{13}$C NMR (100 MHz, acetone-d$_6$) δ 165.8, 147.3, 145.5, 140.8, 127.4, 121.0, 117.9, 115.6, 114.1, 80.6, 71.4, 28.4; ESI-MS: m/z: 216 [M-H]$^-$

2-(2,2-Diphenylbenzo[d][1,3]dioxol-5-yl)-3,5,7-trihydroxy-4H-chromen-4-one (01c). This synthesis is based on the procedure of Li et al.$^{131}$ Quercetin hydrate (6.15 g, 18.2 mmol) was dried at 110°C under high vacuum for 1 hour then 250 mL of diphenyl ether was added, along with 5.4 mL (27.3 mmol) of Ph$_2$CCl$_2$. The mixture was heated to 180°C and stirred for 3 hours. Product was precipitated by adding 800 mL of pet ether, then
filtered and purified by silica gel chromatography with 5% EtOAc/toluene. The material was further purified by crystallization from chloroform, producing **01c** as a yellow powder (6.02 g, 12.91 mmol, 71% yield). ¹H NMR (300 MHz, CDCl₃); δ 12.10 (s, 1H), 9.70 (br, 1H), 8.22 (br, 1H), 7.34 (m, 12H), 6.96 (d, J = 6.9 Hz, 1H), 6.33 (d, J = 1.8 Hz, 1H), 6.26 (d, J = 1.7 Hz, 1H); ¹³C NMR (150MHz, acetone-d₆) δ 176.6, 165.0, 162.3, 157.7, 149.3, 148.2, 145.9, 140.8, 137.1, 130.2, 129.3, 126.9, 126.2, 123.9, 118.4, 109.5, 108.7, 104.1, 99.1, 94.5; ESI-MS: m/z: 467 [M+H]^+.

![Chemical structure of 01c](image)

3-((6-Chlorohexyl)oxy)-2-(2,2-diphenylbenzo[d][1,3]dioxol-5-yl)-5,7-dihydroxy-4H-chromen-4-one (**01d**). Ketal **01c** (1.44 g, 3.07 mmol) was dissolved in 40 mL of DMF then 0.55 g (4.0 mmol) of K₂CO₃ and 0.46 mL (3.07 mmol) of 1-bromo-6-chlorohexane were added, and the reaction was stirred at rt overnight. The mixture was then diluted with DCM, washed 6x with 1 M HCl, dried over MgSO₄, and concentrated in vacuo. The material was purified by silica gel chromatography with 5% EtOAc/toluene to give chloride **01d** as a yellow oil (0.63 g, 1.07 mmol, 35% yield). ¹H NMR (300 MHz, acetone-d₆) δ 12.76 (s, 1H), 9.72 (br, 1H), 7.72 (d, J = 2.0 Hz, 1H), 7.63 (dd, J = 7.4 Hz, J = 1.8 Hz, 1H), 7.45 (m, 8H), 7.17 (m, 3H), 6.50 (d, J = 1.9 Hz, 1H), 6.26 (d, J = 1.9 Hz, 1H), 4.08 (t, 2H), 3.50 (t, 2H), 1.67 (m, 4H), 1.39 (m, 4H); ¹³C NMR (100MHz, acetone-d₆) δ 179.5, 164.9, 163.2, 157.8, 156.2, 149.9, 148.1, 140.8, 138.7, 130.2, 129.3, 126.9,
ESI-MS: m/z: 585 [M+H]^+, 607 [M+Na]^+

3-((6-Azidohexyl)oxy)-2-(2,2-diphenylbenzo[d][1,3]dioxol-5-yl)-5,7-dihydroxy-4H-chromen-4-one (01e). Chloride 01d (0.628 g, 1.07 mmol) was dissolved in 25 mL of DMF and 0.14 g (2.14 mmol) of sodium azide was added. The mixture was heated to 40°C and stirred overnight, then diluted with DCM and washed 6x with 1 M HCl, dried over MgSO₄, and concentrated in vacuo. The product was purified by silica gel chromatography with 30% EtOAc/toluene, giving azide 01e as a yellow oil (0.54 g, 0.91 mmol, 85% yield). H NMR (300 MHz, acetone-d₆) δ 12.77 (s, 1H), 9.68 (s, 1H), 7.72 (d, J = 3.1 Hz, 1H), 7.63 (dd, J = 7.6 Hz, 1.7 Hz, 1H), 7.43 (m, 6H), 7.20 (m, 5H), 6.50 (d, J = 2.0 Hz, 1H), 6.26 (d, J = 1.7 Hz, 1H), 3.50 (t, 2H), 3.22 (t, 2H), 1.67 (m, 4H), 1.37 (m, 4H); C NMR (100MHz, acetone-d₆) δ 179.5, 164.9, 163.2, 157.8, 156.2, 149.9, 148.1, 140.8, 138.7, 130.2, 129.3, 126.9, 125.6, 124.8, 118.6, 109.6, 109.3, 104.8, 99.4, 94.5, 73.1, 51.8, 30.5, 29.3, 27.0, 26.2; ESI-MS: m/z: 592 [M+H]^+, 614 [M+Na]^+
(E)-3-(3,4-Dihydroxyphenyl)-N-((1-(6-((2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4H-chromen-3-yl)oxy)hexyl)-1H-1,2,3-triazol-4-yl)methyl)acrylamide (M01). This click reaction was performed under conditions previously described by Lee et al.\textsuperscript{197}. 0.11 g (0.18 mmol) of (01f) and 0.04 g (0.18 mmol) of (01b) were added to a 25 mL RB flask and 4 mL of t-BuOH and 2 mL of DCM were added to dissolve the material. Copper sulfate (0.007 g, 0.03 mmol) and sodium ascorbate (0.016 g, 0.08 mmol) were dissolved in 2 mL of H$_2$O and the resulting solution was added to the reaction flask then the mixture was stirred vigorously overnight. The DCM was evaporated \textit{in vacuo} and the solution was diluted with water. The product was extracted with EtOAc, dried over anhydrous magnesium sulfate, and concentrated \textit{in vacuo}. The material was purified using silica gel chromatography with 50% EtOAc/toluene to yield 0.077 g (0.09 mmol) of a yellow solid. This material was then dissolved in 4 mL of acetic acid and 1 mL of water, and the solution was refluxed for 2 hours. The reaction was cooled, diluted with water and the product was extracted with EtOAc, dried over anhydrous magnesium sulfate, and concentrated \textit{in vacuo}. The product was purified using silica gel chromatography with 50% EtOAc/toluene yielding M01 as a yellow film (0.046 g, 0.07 mmol, 40% yield). $^1$H NMR (300 MHz, DMSO-d$_6$) $\delta$ 8.54 (s, 1H), 7.83 (d, J = 15.7 Hz, 1H), 7.54 (d, J = 15.7 Hz, 1H), 7.31 (m, 2H), 7.12 (d, J = 15.7 Hz, 1H), 6.97 (d, J = 15.7 Hz, 1H), 6.89 (d, J = 15.7 Hz, 1H), 6.84 (m, 1H), 5.86 (d, J = 15.7 Hz, 1H), 4.30 (t, J = 6.8 Hz, 2H), 3.67 (s, 3H), 3.15 (t, J = 6.8 Hz, 2H), 2.82 (s, 3H), 2.70 (m, 2H), 2.47 (s, 3H), 2.28 (s, 3H), 2.10 (s, 3H), 1.97 (s, 3H), 1.85 (s, 3H), 1.61 (s, 3H), 1.40 (s, 3H), 1.20 (s, 3H), 0.88 (t, J = 6.8 Hz, 3H).
1H), 7.70 (d, J = 2.0 Hz, 1H), 7.56 (dd, J = 8.1 Hz, J = 1.9 Hz, 1H), 7.44 (d, J = 2.0 Hz, 1H), 7.21 (dd, J = 8.0 Hz, J = 2.0 Hz, 1H), 7.02 (d, J = 8.2 Hz, 1H), 6.87 (d, J = 8.0 Hz, 1H), 6.65 (d, J = 15.5 Hz, 1H), 6.47 (d, J = 1.7 Hz, 1H), 6.21 (d, J = 1.9 Hz, 1H), 5.45 (br, 1H), 4.10 (t, 2H), 3.57 (t, 2H), 2.85 (m, 2H), 1.75 (m, 4H), 1.44 (m, 4H); ¹³C NMR (150 MHz, DMSO-d₆) δ 178.2, 172.3, 164.9, 163.5, 157.6, 156.0, 149.2, 148.2, 147.5, 145.6, 140.8, 138.1, 130.8, 129.8, 123.3, 122.8, 122.5, 121.7, 118.4, 118.0, 116.1, 114.6, 104.5, 99.1, 94.5, 75.1, 52.8, 32.6, 30.9, 28.5, 27.2, 26.2; ESI-MS: m/z: 667 [M+Na]⁺; HRMS (ESI-TOF): m/z calc’d for C_{33}H_{32}N_{4}O_{10}Na: 667.2011 [M+Na]⁺; found: 667.2009

7-(Benzyloxy)-2-(3,4-bis(benzyloxy)phenyl)-3,5-dihydroxy-4H-chromen-4-one (02b)

This protocol was based on work described by Huang et al.¹³⁹. Rutin hydrate (20 g, 32.7 mmol) was added to a 500 mL RB flask, dissolved in 150 mL of DMF and 31 g (224 mmol) of K₂CO₃ added. The mixture was stirred and heated to 70°C, 27 mL (224 mmol) of BnBr was added and the reaction was stirred vigorously at 70°C for 18 hours. The reaction mixture was then cooled to room temperature and 10% AcOH was added until pH 5 was achieved, causing the benzyalted intermediate to precipitate out of solution as a thick brown oil. The thick orangey brown oil was collected and redissolved in 200 mL EtOH. HCl (30 mL) was added and the mixture was stirred at 70°C for two hours when a bright yellow precipitate had formed. The reaction mixture was cooled to room
temperature and the yellow precipitate was collected and washed with cold EtOH. The product could be purified by recrystallization with EtOH yielding 02b as a yellow powder (13 g, 22.9 mmol, 70% yield). $^1$H NMR (300 MHz, DMSO-d$_6$) $\delta$ 7.85 (m, 2H), 7.43 (m, 15H), 7.27 (d, $J$ = 9.0 Hz, 1H), 6.57 (m, 2H), 5.24 (s, 4H), 5.21 (s, 2H); ESI-MS: m/z: 573 [M+H]$^+$, 595 [M+Na]$^+$, 571 [M-H]$^-$. 7-(Benzyloxy)-2-(3,4-bis(benzyloxy)phenyl)-3-(3-chloropropoxy)-5-hydroxy-4H-chromen-4-one (03a). Alcohol 02b (10 g, 17.5 mmol) was added to a 250 mL RB flask then dissolved in 50 mL of DMF and 25 mL of THF. K$_2$CO$_3$ (3.6 g, 26.25 mmol) was added and the mixture was stirred while 2.6 mL (26.25 mmol) of 1-bromo-3-chloropropane was added, then stirred at rt overnight. The mixture was then diluted with DCM and washed 4x with 1 M HCl, dried over MgSO$_4$, and concentrated in vacuo. The material was purified by silica gel chromatography with 5% EtOAc/toluene yielding chloride 03a as a yellow crystalline powder (8.6 g, 13.3 mmol, 76% yield). $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.64 (m, 2H), 7.42 (m, 15H), 7.03 (d, $J$ = 9.0 Hz, 1H), 6.44 (m, 2H), 5.26 (s, 2H), 5.24 (s, 2H), 5.11 (s, 2H), 4.14 (t, $J$ = 6.0 Hz, 2H), 3.62 (t, $J$ = 6.0 Hz, 2H), 2.08 (p, $J$ = 6.0 Hz, 4H); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 178.7, 164.8, 162.6, 157.0, 156.3, 151.4, 148.7, 138.0, 137.2, 136.6, 136.0, 128.9, 128.8, 128.7, 128.5 128.2, 128.1 (x2), 127.6, 127.5, 127.4, 127.3, 123.4, 123.1 (x2), 115.3, 114.5, 114.0, 113.7, 106.4, 98.5, 93.1 (x2), 71.7, 71.4, 71.0, 69.6, 52.2, 36.5; ESI-MS: m/z: 649 [M+H]$^+$, 671 [M+Na]$^+$
3-(3-azidopropoxy)-7-(benzyloxy)-2-(3,4-bis(benzyloxy)phenyl)-5-hydroxy-4H-chromen-4-one (03b). A solution of NaN₃ (5.2 g, 79.8 mmol) in water (10 mL) was added to tetrabutylammonium hydroxide (40% aqueous solution, 10.4 g, 39.9 mmol). After one minute, dichloromethane (50 mL) was added, and the organic layer was separated. The aqueous layer was extracted three times with dichloromethane. The organic layers were dried over MgSO₄, and concentrated in vacuo. The resulting colourless oil was then immediately added to a 250 mL RB flask containing of chloride 03a (8.6 g, 13.3 mmol) dissolved in DMF (70 mL). The reaction mixture was stirred vigorously at 50°C overnight. The mixture was then cooled to rt, diluted with DCM and washed 4x with 1 M HCl, dried over MgSO₄, and concentrated in vacuo. The material was purified by silica gel chromatography with 5% EtOAc/toluene yielding azide 03b as a yellow crystalline solid (7.9 g, 12.0 mmol, 90% yield). \(^{1}\)H NMR (300 MHz, CDCl₃) \(\delta\) 7.64 (m, 2H), 7.39 (m, 15H), 7.03 (d, J = 8.2 Hz, 1H), 6.44 (m, 2H), 5.26 (s, 2H), 5.23 (s, 2H), 5.12 (s, 2H), 4.07 (t, J = 6.0 Hz, 2H), 3.61 (t, J = 6.0 Hz, 2H), 2.08 (p, J = 6.0 Hz, 2H); \(^{13}\)C NMR (75 MHz, CDCl₃) \(\delta\) 178.5, 164.6, 162.2, 156.8, 156.2, 151.4, 148.6, 138.0, 137.1, 136.7, 135.9, 128.9, 128.8, 128.7, 128.5 128.2 (x2), 128.1, 127.6, 127.5, 127.4, 127.3, 123.4, 123.0 (x2), 115.3, 114.5, 113.9, 113.7, 106.3, 98.7, 93.2 (x2), 71.6, 71.0, 70.6, 69.6, 66.2, 41.7; ESI-MS: m/z: 656 [M+H]⁺, 678 [M+Na]⁺
3-(3-aminopropoxy)-7-(benzyloxy)-2-(3,4-bis(benzyloxy)phenyl)-5-hydroxy-4H-chromen-4-one (03c). Azide 03b (7.9 g, 12.0 mmol) was added to a 250 mL RB flask then dissolved in THF (60 mL). 0.05 M Aqueous NaOH (12 mL) was added. 1 M PMe₃ in THF (36 mL, 36.0 mmol) was added, then stirred at rt overnight. The reaction mixture was then concentrated in vacuo. The material was purified by silica gel chromatography with a gradient of 78:10:10:2 to 30:30:30:10 DCM/Acetone/MeOH/H₂O yielding amine 03c as a yellow solid (4.5 g, 7.2 mmol, 60% yield). ¹H NMR (300 MHz, CDCl₃) δ 7.61 (m, 2H), 7.35 (m, 15H), 6.98 (d, J = 9.0 Hz, 1H), 6.33 (m, 2H), 5.19 (s, 2H), 5.18 (s, 2H), 4.97 (s, 2H), 3.85 (t, J = 6.0 Hz, 2H), 3.28 (t, J = 6.0 Hz, 2H), 2.05 (p, J = 6.0 Hz, 4H); ¹³C NMR (75 MHz, CDCl₃) δ 178.3, 164.7, 161.4, 156.5, 156.1, 151.9, 148.5, 137.4, 137.1, 136.6, 135.7, 128.9, 128.7 (x3), 128.4, 128.1, 128.0, 127.7, 127.6, 127.4 (x2), 123.2, 123.1, 122.4, 115.9, 114.5, 113.5, 113.0, 105.5, 98.8, 93.1 (x2), 71.5, 70.8, 70.4, 69.9, 53.7, 38.5; ESI-MS: m/z: 630 [M+H]+

(E)-3-(3,4-dihydroxyphenyl)-N-(5-((2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4H-chromen-3-yl)oxy)pentyl)acrylamide (M03). Pentylic amine 02e (108 mg, 0.165 mmol)
was added to a 5 mL RB flask. DCM (2 mL) was added. Pyridine (20 µL, 0.247 mmol) was added. Pentafluorocaffeic ester 01a (63 mg, 0.181 mmol) in THF (1 mL) was added. The reaction mixed was stirred at rt for 23 hours. The reaction was diluted with DCM and the reaction was washed with 3 x 1 M HCl, dried over MgSO₄, and concentrated in vacuo. The material was purified by silica gel chromatography with 78:10:10:2 DCM/Acetone/MeOH/H₂O to yield (02f penty1) as a yellow solid (100 mg, 0.122 mmol, 74% yield). This material was then dissolved in anhydrous DCM and added to a 5 mL RB flask kept under nitrogen. The mixture was cooled to -78°C in an acetone dry ice bath. 488 µL (0.488 mmol) of 1M BBr₃ in DCM was added. After 2.5 hours another 488 µL aliquot of 1M BBr₃ was added for a total of 0.976 mmol. The reaction was stirred for 2.5 hours before being warmed to rt. The reaction was quenched with sat. NaHCO₃. The mixture was extracted 3 times with EtOAC, dried over MgSO₄, and concentrated in vacuo. The material was first purified by silica gel chromatography with a gradient of 78:10:10:2 to 30:30:30:10 DCM/Acetone/MeOH/H₂O. This was followed by purification by HPLC with a reversed phase C-18 column and gradient of 5% ACN/H₂O to 95% ACN/ H₂O over 50 minutes to yield M03 as yellow powder (11 mg, 0.02 mmol, 16% yield). ¹H NMR (600 MHz, MeOH-d₄) δ 7.59 (d, J = 2.2 Hz, 1H), 7.50 (dd, J = 8.4 Hz, J = 2.2 Hz, 1H), 7.38 (d, J = 15.7 Hz, 1H), 7.00 (d, J = 2.2 Hz, 1H), 6.90 (d, J = 8.5 Hz, 1H), 6.89 (dd, J = 8.3 Hz, J = 2.1 Hz, 1H), 6.75 (d, J = 8.2, 1H), 6.37 (d, J = 2.0, 1H), 6.36 (d, J = 15.7 Hz, 1H), 6.18 (d, J = 2.0 Hz, 1H), 3.93 (t, J = 6.2 Hz, 2H), 3.19 (m, 2H), 1.76 (p, J = 7.2 Hz, 2H), 1.56 (p, J = 7.4 Hz, 2H), 1.48 (p, J = 7.2 Hz, 2H); ¹³C NMR (HSQC, 600 MHz, MeOH-d₄) δ 142.2 122.4, 122.0, 118.2, 116.7, 116.3, 116.2, 114.8, 99.8, 94.7, 73.7, 40.2, 30.7, 30.0, 24.6; ESI-MS: m/z: 572 [M+Na]⁺,
(E)-3-(3,4-dihydroxyphenyl)-N-(3-((2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4H-chromen-3-yl)oxy)propyl)acrylamide (M04). (150 mg, 0.238 mmol) of Propyl amine 03c was added to a 10 mL RB flask. DCM (2 mL) was added. Pyridine (29 µL, 0.358 mmol) was added. Pentafluorocaffeic ester 01a (91 mg, 0.262 mmol) in THF (1 mL) was added to this solution, then stirred at rt for 23 hours. The reaction was diluted with DCM and the reaction was washed with 3 x 1 M HCl, dried over MgSO₄, and concentrated in vacuo. The material was purified by silica gel chromatography with 78:10:10:2 DCM/Acetone/MeOH/H₂O to give (02f propyl) as a yellow solid (122 mg, 0.154 mmol, 65% yield). This material was then dissolved in anhydrous DCM and added to a 5 mL RB flask kept under nitrogen. The mixture was cooled to –78°C in an acetone dry ice bath. 616 µL (0.616 mmol) of BBr₃ in 1 M DCM was added. After 2.5 hours another 616 µL aliquot of 1 M BBr₃ was added for a total of 1.232 mmol. The reaction was stirred for 2.5 hours before being warmed to rt. The reaction was quenched with sat. NaHCO₃. The mixture was extracted 3 times with EtOAC, dried over MgSO₄, and concentrated in vacuo. The material was first purified by silica gel chromatography with a gradient of 78:10:10:2 to 30:30:30:10 DCM/Acetone/MeOH/H₂O. This was followed by purification.
by HPLC with a reversed phase C-18 column and gradient of 5% ACN/H$_2$O to 95% ACN/ H$_2$O over 50 minutes to give of **M04** as yellow powder (10 mg, 0.019 mmol, 12% yield). NMR (600 MHz, MeOH-d$_4$) $\delta$ 7.58 (d, J = 2.0 Hz, 1H), 7.47 (dd, J = 8.4 Hz, J = 2.2 Hz, 1H), 7.38 (d, J = 15.2 Hz, 1H), 7.03 (d, J = 2.2 Hz, 1H), 6.91 (d, J = 8.5 Hz, 1H), 6.87 (dd, J = 8.4 Hz, J = 2.1 Hz, 1H), 6.75 (d, J = 8.2, 1H), 6.37 (d, J = 2.0, 1H), 6.34 (d, J = 15.7 Hz, 1H), 6.17 (d, J = 2.0 Hz, 1H), 3.93 (m, 2H), 3.19 (m, 2H), 1.79 (p, J = 7.2 Hz, 2H); $^{13}$C NMR (HSQC, 600 MHz, MeOH-d$_4$) $\delta$ 142.5, 122.2, 122.0, 118.2, 116.7, 116.3 (x2), 114.8, 99.8, 94.7, 73.7, 40.9, 36.7; ESI-MS: m/z: 544 [M+Na]$^+$, 520 [M-H]; HRMS (ESI-TOF): m/z calc’d for C$_{27}$H$_{23}$NO$_{10}$Na: 544.1214 [M+Na]$^+$; found: 544.1236

**General procedure for synthesis of (03d) via HBTU activation.** Fmoc-L-Xaa (1.586 mmol) and HBTU (601 mg, 1.568 mmol) were dissolved in DMF (7 mL). 4-Methylmorpholine (172 µL, 1.568 mmol) was added. The mixture was stirred at room temperature for 1 hour. Propyl amine **03c** (0.5 g, 0.793 mmol) was dissolved in DMF (2 mL) and added to the mixture. The reaction was stirred vigorously at rt overnight. The mixture was diluted with DCM and washed with 3 x 1M HCl, dried over MgSO$_4$, and concentrated in vacuo. The material was purified by silica gel column chromatography using an eluent system of 78:2:10:10 DCM/H$_2$O/Acetone/MeOH. An average of 0.714 mmol (90% yield) of **03d** was isolated as a yellow solid.

**General procedure for synthesis of (03d) via use of PFP activated amino acids.** Propyl amine **03c** (0.5 g, 0.793mmol) was added to a 10 mL RB flask and dissolved in DCM (5 mL). Pyridine (128 µL, 1.586 mmol) was added. Fmoc-L-Xaa-PFP (0.872
mmol) was dissolved in DCM (1 mL) and this solution was added to the reaction. The mixture was stirred at room temperature overnight. The reaction was then washed with 3 x 1 M HCl, dried over MgSO₄, and concentrated in vacuo. The material was purified by silica gel column chromatography using an eluent system of 78:2:10:10 DCM/H₂O/Acetone/MeOH. An average of 0.674 mmol (85% yield) of 03d was isolated as a yellow solid.

**General Procedure for synthesis of (03e).** Fmoc deprotection of 03d: 0.5 mmol of 03d was dissolved in DCM (2 mL). Piperidine (0.5 mL) was added. The mixture was stirred for 1.5 hr at room temperature. The reaction was quenched with 1 M HCl and concentrated in vacuo. This material was then dissolved in DCM (4 mL) and added to a 10 mL RB flask. Pyridine (60 µL, 0.75 mmol) was added. Pentafluorocaffeic ester 01a (190 mg, 0.55 mmol) was dissolved in THF (1 mL) and was added to the reaction. The reaction was stirred overnight at room temperature. The reaction was then washed with 3 x 1 M HCl, dried over MgSO₄, and concentrated in vacuo. The material was purified by silica gel column chromatography using an eluent system of 78:2:10:10 DCM/H₂O/Acetone/MeOH. An average of 0.325 mmol (65% yield) of 03e was isolated as a yellow solid.
(E)-3-(3,4-dihydroxyphenyl)-N-(2-((3-((2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4H-chromen-3-yl)oxy)propyl)amino)-2-oxoethyl)acrylamide (M06). **03e Gly** (152 mg, 0.179 mmol) was dissolved in anhydrous DCM and added to a 5 mL RB flask kept under nitrogen. The mixture was cooled to -78°C in an acetone dry ice bath. 717 µL (0.717 mmol) of 1 M BBr₃ in DCM was added. After 2.5 hours another 717 µL aliquot of BBr₃ was added for a total of 1.433 mmol. The reaction was stirred for 2.5 hours before being warmed to rt. The reaction was quenched with sat. NaHCO₃. The mixture was extracted 3 times with EtOAC, dried over MgSO₄, and concentrated *in vacuo*. The material was first purified by silica gel chromatography with a gradient of 78:10:10:2 to 30:30:30:10 DCM/Acetone/MeOH/H₂O. This was followed by purification by HPLC with a reversed phase C-18 column and gradient of 5% ACN/H₂O to 95% ACN/ H₂O over 50 minutes to give M06 as yellow powder (5.2 mg, 0.0089 mmol, 5% yield). **¹H NMR** (600 MHz, MeOH-d₄) δ 7.85 (d, J = 2.2 Hz, 1H), 7.72 (dd, J = 8.8 Hz, J = 2.1 Hz, 1H), 7.38 (d, J = 15.7, 1H), 7.03 (d, J = 2.1 Hz, 1H), 6.78 (d, J = 8.2 Hz, 1H), 6.73 (d, J = 7.9 Hz, 1H), 6.72 (dd, J = 8.0 Hz, J = 2.7 Hz, 1H), 6.38 (d, J = 15.7 Hz, 1H), 6.37 (d, J = 1.8 Hz, 1H), 6.15 (d, J = 1.9 Hz, 1H), 3.95 (s, 2H), 3.49 (t, J = 6.6 Hz, 2H), 3.00 (t, J = 6.1 Hz, 1H), 1.30 (m, 2H); **¹³C NMR** (HSQC, 600 MHz, MeOH-d₄) δ 142.8, 122.3, 122.1, 118.5, 116.5, 116.3 (x2), 115.0, 99.8, 94.8, 72.5, 47.1, 39.2, 32.0; **ESI-MS**: m/z: 601 [M+Na]⁺
577 [M-H]⁻; HRMS (ESI-TOF): m/z calc'd for C_{29}H_{26}N_{2}O_{11}Na: 601.1429 [M+Na]⁺; found: 601.1447

(S,E)-N-(3-((2-(3,4-dihydroxyphenyl))-5,7-dihydroxy-4-oxo-4H-chromen-3-yl)oxy)propyl)-1-(3-(3,4-dihydroxyphenyl)acryloyl)pyrrolidine-2-carboxamide (M07). 03e Pro (67 mg, 0.075 mmol) was dissolved in anhydrous DCM and added to a 5 mL RB flask kept under nitrogen. The mixture was cooled to -78°C in an acetone dry ice bath. 302 µL (0.302 mmol) of 1 M BBr₃ in DCM was added. After 2.5 hours another 302 µL aliquot of 1 M BBr₃ was added for a total of 0.604 mmol. The reaction was stirred for 2.5 hours before being warmed to rt. The reaction was quenched with sat. NaHCO₃. The mixture was extracted 3 times with EtOAC, dried over MgSO₄, and concentrated in vacuo. The material was first purified by silica gel chromatography with a gradient of 78:10:10:2 to 30:30:30:10 DCM/Acetone/MeOH/H₂O. This was followed by purification by HPLC with a reversed phase C-18 column and gradient of 5% ACN/H₂O to 95% ACN/ H₂O over 50 minutes to give of M07 as yellow powder (9.3 mg, 0.015 mmol, 20% yield). ¹H NMR (600 MHz, MeOH-d₄) δ 8.55 (s, 1H), 7.60 (d, J = 2.2 Hz, 1H), 7.52 (d, J = 8.5 Hz, 1H), 7.45 (dd, J = 8.3 Hz, J = 2.0 Hz, 1H), 7.26 (d, J = 15.4 Hz, 1H), 6.87 (dd, J = 8.4 Hz, J = 1.4 Hz, 1H), 6.79 (d, J = 8.3 Hz, 1H), 6.61 (d, J = 2.4 Hz, 2H), 6.50 (d, J = 15.1 Hz),
6.10 (d, J = 2.0 Hz, 1H); 4.51 (dd, J = 8.7 Hz, J = 3.2 Hz, 1H), 3.90 (m, 2H), 3.86 (m, 2H), 3.70 (m, 1H), 3.59 (m, 1H), 2.18 (m, 2H), 2.07 (m, 2H), 1.61 (m 2H); $^{13}$C NMR (HSQC, 600 MHz, MeOH-d$_4$) δ 142.3, 122.2, 122.0, 118.1, 116.5, 116.2, 116.1, 114.9, 99.8, 94.7, 73.7, 62.0, 47.3, 40.9, 32.0, 30.5, 25.7; ESI-MS: m/z: 641 [M+Na]$^+$, 617 [M-H]$^-$; HRMS (ESI-TOF): m/z calc'd for C$_{32}$H$_{30}$N$_2$O$_{11}$Na: 641.1742 [M+Na]$^+$; found: 641.1747

(2S,3R)-N-(3-((2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4H-chromen-3-yl)oxy)propyl)-2-((E)-3-(3,4-dihydroxyphenyl)acrylamido)-3-hydroxybutanamide (M08).

03e Thr (36 mg, 0.038 mmol) was dissolved in anhydrous DCM and added to a 5 mL RB flask kept under nitrogen. The mixture was cooled to -78°C in an acetone dry ice bath. 152 µL (0.152 mmol) of 1 M BBr$_3$ in DCM was added. After 2.5 hours another 152 µL aliquot of 1 M BBr$_3$ was added for a total of 0.304 mmol. The reaction was stirred for 2.5 hours before being warmed to rt. The reaction was quenched with sat. NaHCO$_3$. The mixture was extracted 3 times with EtOAC, dried over MgSO$_4$, and concentrated in vacuo. The material was first purified by silica gel chromatography with a gradient of 78:10:10:2 to 30:30:30:10 DCM/Acetone/MeOH/H$_2$O. This was followed by purification by HPLC with a reversed phase C-18 column and gradient of 5% ACN/H$_2$O to 95% ACN/ H$_2$O over 50 minutes to give M08 as yellow powder (3.5 mg, 0.0057 mmol, 15%
yield). $^1$H NMR (600 MHz, MeOH-d$_4$) $\delta$ 8.55 (s, 1H), 7.58 (d, $J$ = 2.4 Hz, 1H), 7.48 (dd, $J$ = 9.0 Hz, $J$ = 2.0 Hz, 1H) 7.42 (d, $J$ = 15.4 Hz, 1H), 7.03 (d, $J$ = 2.2 Hz, 1H), 6.96 (dd, $J$ = 8.5 Hz, $J$ = 2.4 Hz, 1H), 6.77 (d, $J$ = 8.0 Hz, 1H), 6.71 (d, $J$ = 7.9 Hz, 1H), 6.65 (d, $J$ = 2.0 Hz, 1H), 6.26 (d, $J$ = 15.1 Hz, 1H), 6.17 (d, $J$ = 2.1 Hz, 1H), 4.32 (d, $J$ = 10.0 Hz, 1H), 4.20 (m, 1H), 3.67 (m, 2H), 3.39 (t, $J$ = 5.5 Hz, 2H), 1.72 (m, 2H), 1.43 (d, $J$ = 5.5 Hz, 3H); $^{13}$C NMR (HSQC, 600 MHz, MeOH-d$_4$) $\delta$ 142.5, 122.0 (x2), 118.5, 116.7, 116.3 (x2), 116.1, 99.8, 94.8, 78.5, 71.5, 59.2, 37.9, 32.0, 20.5; ESI-MS: m/z: 645 [M+Na]$^+$, 621 [M-H]$^-$; HRMS (ESI-TOF): m/z calc’d for C$_{31}$H$_{30}$N$_2$O$_{12}$Na: 645.1691 [M+Na]$^+$; found: 645.1680

(S,E)-5-((3-((2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4H-chromen-3-yl)oxy)propyl)amino)-4-(3-(3,4-dihydroxyphenyl)acrylamido)-5-oxopentanoic acid (M09).

**03e Glu** (50 mg, 0.051 mmol) was dissolved in anhydrous DCM and added to a 5 mL RB flask kept under nitrogen. The mixture was cooled to -78°C in an acetone dry ice bath. 204 $\mu$L (0.204 mmol) of 1 M BBr$_3$ in DCM was added. After 2.5 hours another 204 $\mu$L aliquot of 1 M BBr$_3$ was added for a total of 0.408 mmol. The reaction was stirred for 2.5 hours before being warmed to rt. The reaction was quenched with sat. NaHCO$_3$. The mixture was extracted 3 times with EtOAC, dried over MgSO$_4$, and concentrated in

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vacuo. The material was first purified by silica gel chromatography with a gradient of 78:10:10:2 to 30:30:30:10 DCM/Acetone/MeOH/H$_2$O. This was followed by purification by HPLC with a reversed phase C-18 column and gradient of 5% ACN/H$_2$O to 95% ACN/ H$_2$O over 50 minutes to give of M09 was obtained as yellow powder (5.2 mg, 0.008 mmol, 16% yield). $^1$H NMR (600 MHz, MeOH-d$_4$) δ 7.85 (d, J = 2.2 Hz, 1H), 7.72 (dd, J = 8.8 Hz, J = 2.1 Hz, 1H), 7.38 (d, J = 15.7, 1H), 7.03 (d, J = 2.1 Hz, 1H), 6.87 (d, J = 8.2 Hz, 1H), 6.73 (d, J = 8.0 Hz, 1H), 6.71 (dd, J = 8.4 Hz, J = 2.1 Hz, 1H), 6.50 (d, J = 1.8 Hz, 1H), 6.38 (d, J = 15.6 Hz, 1H), 6.17 (d, J = 2.1 Hz, 1H), 4.32 (dd, J = 8.0 Hz, J = 5.5 Hz, 1H), 3.72 (m, 2H), 3.52 (m, 2H), 2.54 (m, 2H), 2.09 (m, 2H), 1.72 (m, 2H); $^{13}$C NMR (HSQC, 600 MHz, MeOH-d$_4$) δ 142.8, 122.3, 122.1, 118.5, 116.5, 116.3 (x2), 115.0, 99.8, 94.8, 72.5, 52.1, 41.9, 36.0, 30.2, 27.6; ESI-MS: m/z: 673 [M+Na$^+$], 649 [M-H$^-$]; HRMS (ESI-TOF): m/z calc’d for C$_{32}$H$_{30}$N$_2$O$_x$Na: 673.1640 [M+Na$^+$]; found: 673.1621

(S,E)-3-(3,4-dihydroxyphenyl)-N-(1-((3-((2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4H-chromen-3-yl)oxy)propyl)amino)-3-(4-hydroxyphenyl)-1-oxopropan-2-yl)acrylamide (M10). 03e Tyr (56 mg, 0.055 mmol) was dissolved in anhydrous DCM and added to a 5 mL RB flask kept under nitrogen. The mixture was cooled to -78°C in an acetone dry
ice bath. 220 µL (0.22 mmol) of 1 M BBr₃ in DCM was added. After 2.5 hours another 220 µL aliquot of 1 M BBr₃ was added for a total of 0.44 mmol. The reaction was stirred for 2.5 hours before being warmed to rt. The reaction was quenched with sat. NaHCO₃. The mixture was extracted 3 times with EtOAC, dried over MgSO₄, and concentrated in vacuo. The material was first purified by silica gel chromatography with a gradient of 78:10:10:2 to 30:30:30:10 DCM/Acetone/MeOH/H₂O. This was followed by purification by HPLC with a reversed phase C-18 column and gradient of 5% ACN/H₂O to 95% ACN/ H₂O over 50 minutes to give M10 as yellow powder (11 mg, 0.016 mmol, 29% yield). ¹H NMR (600 MHz, MeOH-d₄) δ 7.59 (d, J = 2.2 Hz, 1H), 7.47 (dd, J = 8.1 Hz, J = 1.8 Hz, 1H), 7.22 (d, J = 15.6 Hz, 1H), 7.06 (d, J = 8.4 Hz, 2H), 6.89 (d, J = 8.4 Hz, 1H), 6.81 (d, J = 2.1 Hz, 1H), 6.69 (dd, J = 7.8 Hz, J = 2.4 Hz, 1H), 6.64 (d, J = 8.4 Hz, 1H), 6.63 (d, J = 8.4 Hz, 2H), 6.30 (d, J = 15.6 Hz, 1H), 6.26 (d, J = 2.2 Hz, 1H) 6.16 (d, J = 2.1 Hz, 1H), 4.62 (dd, J= 8.4, J = 6.6 Hz, 1H), 3.73 (p, J = 5.2 Hz, 2H), 3.60 (m, 2H), 3.09 (dd, J = 13.6 Hz, J = 6.6 Hz, 1H), 2.89 (dd, J = 13.6 Hz, J = 8.4 Hz, 1H), 1.79 (m, 2H); ¹³C NMR (HSQC, 600 MHz, MeOH-d₄) δ 142.8, 131.1 (x2), 122.4, 122.1, 117.5, 116.5, 116.3 (x2), 116.2, 116.1, 115.0, 99.8, 94.8, 71.5, 57.1, 38.2, 37.9, 30.0; ESI-MS: m/z: 707 [M+Na]⁺, 683 [M-H]⁻; HRMS (ESI-TOF): m/z calc’d for C₃₆H₃₂N₂O₁₂Na: 707.1847 [M+Na]⁺; found: 707.1841
(S,E)-3-(3,4-dihydroxyphenyl)-N-(1-((3-((2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4H-chromen-3-yl)oxy)propyl)amino)-1-oxo-3-phenylpropan-2-yl)acrylamide (M11). 03e Phe (44 mg, 0.047 mmol) was dissolved in anhydrous DCM and added to a 5 mL RB flask kept under nitrogen. The mixture was cooled to -78°C in an acetone dry ice bath. 187 µL (0.187 mmol) of 1 M BBr₃ in DCM was added. After 2.5 hours another 187 µL aliquot of 1 M BBr₃ was added for a total of 0.375 mmol. The reaction was stirred for 2.5 hours before being warmed to rt. The reaction was quenched with sat. NaHCO₃. The mixture was extracted 3 times with EtOAC, dried over MgSO₄, and concentrated in vacuo. The material was first purified by silica gel chromatography with a gradient of 78:10:10:2 to 30:30:30:10 DCM/Acetone/MeOH/H₂O. This was followed by purification by HPLC with a reversed phase C-18 column and gradient of 5% ACN/H₂O to 95% ACN/ H₂O over 50 minutes to give M11 as yellow powder (7.5 mg, 0.0113 mmol, 24% yield). ¹H NMR (600 MHz, MeOH-d₄) δ 7.58 (d, J = 2.2 Hz, 1H), 7.46 (dd, J = 8.4 Hz, J = 2.2 Hz, 1H), 7.25 (m, 2H), 7.21 (m, 3H), 7.13 (m, 1H), 6.87 (d, J = 8.5, 1H), 6.80 (d, J = 2.1 Hz, 1H), 6.69 (dd, J = 8.3 Hz, J = 2.1 Hz, 1H), 6.64 (d, J = 8.2 Hz, 1H) 6.29 (d, J = 15.7 Hz, 1H), 6.21 (d, J = 2.0 Hz, 1H), 6.13 (d, J = 2.0 Hz, 1H), 4.69 (dd, J = 8.6 Hz, J = 6.4 Hz, 1H), 3.73 (m, 2H), 3.60 (m, 2H), 3.19 (dd, J = 13.0 Hz, J = 6.6 Hz, 1H), 2.97 (dd, J = 12.8 Hz, J = 8.6 Hz, 1H), 1.80 (m, 2H); ¹³C NMR (HSQC, 600 MHz, MeOH-d₄) δ
142.7, 131.1 (x2), 129.9 (x2), 127.5, 122.4, 122.1, 117.5, 116.5, 116.2, 116.1, 115.0, 99.8, 95.0, 71.5, 57.2, 38.2, 37.8, 29.7; ESI-MS: m/z: 691 [M+Na]^+, 667 [M-H]^-; HRMS (ESI-TOF): m/z calc’d for C_{36}H_{32}N_{2}O_{11}Na: 691.1898 [M+Na]^+; found: 691.1898

(S,E)-3-(3,4-dihydroxyphenyl)-N-(3-(3,4-dihydroxyphenyl)-1-((3-(2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4H-chromen-3-yl)oxy)propyl)amino)-1-oxopropan-2-yl)acrylamide (M12). 03e Dopa (34 mg, 0.034 mmol) was dissolved in anhydrous DCM and added to a 5 mL RB flask kept under nitrogen. The mixture was cooled to -78°C in an acetone dry ice bath. 134 µL (0.134 mmol) of 1 M BBr₃ in DCM was added. After 2.5 hours another 134 µL aliquot of 1 M BBr₃ was added for a total of 0.268 mmol. The reaction was stirred for 2.5 hours before being warmed to rt. The reaction was quenched with sat. NaHCO₃. The mixture was extracted 3 times with EtOAC, dried over MgSO₄, and concentrated in vacuo. The acetonide protecting group of the L-DOPA was removed by 1 hr treatment in 10% TFA/DCM. The material was first purified by silica gel chromatography with a gradient of 78:10:10:2 to 30:30:30:10 DCM/Acetone/MeOH/H₂O. This was followed by purification by HPLC with a reversed phase C-18 column and gradient of 5% ACN/H₂O to 95% ACN/ H₂O over 50 minutes to
give M12 as yellow powder (4.8 mg, 0.007 mmol, 20% yield). $^1$H NMR (600 MHz, MeOH-d$_4$) δ 7.58 (d, J = 2.2 Hz, 1H), 7.47 (dd, J = 8.6 Hz, J = 2.2 Hz, 1H), 7.22 (d, J = 15.7 Hz, 1H), 6.88 (d, J = 8.5 Hz, 1H), 6.82 (d, J = 2.0 Hz, 1H), 6.68 (dd, J = 9.0 Hz, J = 1.8 Hz, 1H), 6.67 (d, J = 2.0 Hz, 1H), 6.64 (d, J = 8.2 Hz, 1H), 6.59 (m, 2H), 6.30 (d, J = 15.7 Hz, 1H), 6.24 (d, J = 2.0 Hz, 1H), 6.14 (d, J = 2.2 Hz, 1H), 4.61 (m, 1H), 3.74 (m, 2H), 3.62 (m, 2H), 3.02 (dd, J = 14.0 Hz, J = 6.5 Hz, 1H), 2.85 (dd, J = 14.0 Hz, J = 6.5 Hz, 1H), 1.80 (m, 2H); $^{13}$C NMR (HSQC, 600 MHz, MeOH-d$_4$) δ 143.0, 131.0 (x2), 122.5, 122.2, 117.5, 116.5, 116.3, 116.2, 116.1, 115.0, 100.1, 94.8, 71.2, 57.0, 38.5, 37.9, 30.0; ESI-MS: m/z: 723 [M+Na]$^+$, 699 [M-H]$^-$; HRMS (ESI-TOF): m/z calc’d for C$_{36}$H$_{32}$N$_2$O$_{13}$Na: 723.1797 [M+Na]$^+$; found: 723.1799

(S,E)-3-(3,4-dihydroxyphenyl)-N-(1-((3-((2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4H-chromen-3-yl)oxy)propyl)amino)-3-(1H-indol-3-yl)-1-oxopropan-2-yl)acrylamide (M13). 03e Trp (46 mg, 0.043 mmol) was dissolved in anhydrous DCM and added to a 5 mL RB flask kept under nitrogen. The mixture was cooled to -78°C in an acetone dry ice bath. 171 µL (171 mmol) of 1 M BBr$_3$ in DCM was added. After 2.5 hours another 171 µL aliquot of BBr$_3$ was added for a total of 0.342 mmol. The reaction was stirred for
2.5 hours before being warmed to rt. The reaction was quenched with sat. NaHCO₃. The mixture was extracted 3 times with EtOAC, dried over MgSO₄, and concentrated in vacuo. The material was first purified by silica gel chromatography with a gradient of 78:10:10:2 to 30:30:30:10 DCM/Acetone/MeOH/H₂O. This was followed by purification by HPLC with a reversed phase C-18 column and gradient of 5% ACN/H₂O to 95% ACN/ H₂O over 50 minutes to give M13 as yellow powder (2.4 mg, 0.003 mmol, 8% yield). ¹H NMR (600 MHz, MeOH-d₄) δ 7.42 (d, J = 15.3 Hz, 1H), 7.14 (d, J = 7.6 Hz, 1H), 7.41 (s, 1H), 7.40 (d, J = 2.2 Hz, 1H), 7.36 (dd, J = 8.4 Hz, J = 2.2 Hz, 1H) 7.03 (d, J = 2.2 Hz, 2H), 7.02 (d, J = 2.2 Hz, 1H), 6.95 (m, 2H), 6.81 (d, J = 8.2 Hz, 1H), 6.77 (dd, J = 8.2 Hz, J = 3.4 Hz, 2H), 6.70 (d, J = 2.0, 1H), 6.26 (d, J = 15.9 Hz, 1H), 4.63 (m, 1H), 3.74 (m, 2H), 3.65 (m, 2H), 3.17 (dd, J = 13.0 Hz, J = 7.0 Hz, 1H), 2.66 (dd, J = 12.6 Hz, J = 7.6 Hz, 1H), 1.72 (m, 2H); ¹³C NMR (HSQC, 600 MHz, MeOH-d₄) δ 144.9, 134.7, 125.0, 122.8 (x2), 122.2 (x2), 116.4 (x3), 114.8 (x3), 99.8, 95.6, 72.1, 60.0, 44.3, 38.5, 28.2; ESI-MS: m/z: 730 [M+Na]⁺, 706 [M-H]; HRMS (ESI-TOF): m/z calc’d for C₃₈H₃₃N₃O₁₁Na: 730.2007 [M+Na]⁺; found: 730.2020

Enzymatic hydrolysis of rutin to isoquercetin. Rutin hydrate (1.0 g, 1.64 mmol) was dissolved in 325 mL of 20 mM citrate solution (pH 3.4) in a 1 L RB flask. 12.5 mg of
Naringinase from *Penicillium decumbens* was suspended in 250 μL of 20 mM sodium acetate (pH 4). This solution was heated at 81°C for 7.5 minutes. The enzymatic solution was added to the solution of rutin and this was heated to 60°C and stirred vigorously for 6 hours. The solution was then left at 4°C overnight, causing the isoquercetin product to precipitate out of solution. The material could then be recrystallized from ethanol and water to yield isoquercetin (654 mg, 1.41 mmol, 86% yield). \(^1^H\) NMR (300 MHz, DMSO-\(d_6\)) \(\delta\) 7.78 (d, \(J = 2.2\) Hz, 1H), 7.72 (dd, \(J = 8.5\) Hz, \(J = 1.9\), 1H), 7.49 (d, \(J = 2.1\) Hz, 1H), 6.85 (d, \(J = 8.5\) Hz, 1H), 6.51 (d, \(J = 1.9\) Hz, 1H), 5.14 (d, \(J = 8.3\) Hz, 1H), 5.33 (d, \(J = 7.0\) Hz, 1H), 3.62 – 4.12 (m, 6H); ESI-MS: m/z: 465 [M+H]^+, 487 [M+Na]^+, 463 [M-H]^–.

(04a). Isoquercetin (9.0 mg, 0.019 mmol) and 6-aminoglucosyl fluoride (9.0 mg, 0.050 mmol) were added to a vial. 1 mL of 50 mM sodium phosphate buffer (pH 7) was added. 4 mg/mL of Abg2F6 was used in the reaction. The reaction was stirred at rt for 23 hours. Abg2F6 was precipitated out of solution by treatment with methanol and the reaction was purified by C18 reversed-phased HPLC to give 04a as a yellow powder (5.0 mg, 0.008 mmol, 42% yield). (600 MHz, MeOH-\(d_4\)) \(\delta\) 8.51 (s, 1H), 7.70 (d, \(J = 2.2\) Hz, 1H),
7.50 (dd, J = 8.2 Hz, J = 2.0, 1H), 7.48 (d, J = 2.1 Hz, 1H), 6.85 (d, J = 8.5 Hz, 1H), 6.32 (d, J = 1.9 Hz, 1H), 5.13 (d, J = 8.3 Hz, 2H), 3.74 – 3.65 (m, 6H), 3.62 – 3.50 (m, 6H); ¹³C NMR (HSQC, 600 MHz, MeOH-d₄) δ 123.0, 117.3, 116.0, 109.9, 104.9 (x2), 95.5, 78.1 (x2), 75.8 (x2), 71.2, 62.5 (x4), 57.3; ESI-MS: m/z: 723 [M+Na]⁺, 706 [M-H]⁻  

(04b). Isoquercetin (9.0 mg, 0.019 mol) and 4-aminoglucosyl fluoride (9.0 mg, 0.050 mmol) were added to a vial. 1 mL of 50 mM sodium phosphate buffer (pH 7) was added. 4 mg/mL of Abg2F6 was then used in the reaction. The reaction was stirred at rt for 23 hours. Abg2F6 was precipitated out of solution by treatment with methanol and the reaction was purified by C18 reversed-phased HPLC to give 04b as a yellow powder (7.1 mg, 0.011 mmol, 60% yield). (600 MHz, MeOH-d₄) δ 8.51 (s, 1H), 7.71 (d, J = 2.2 Hz, 1H), 7.56 (dd, J = 8.4 Hz, J = 1.9, 1H), 7.36 (d, J = 2.1 Hz, 1H), 6.89 (d, J = 8.4 Hz, 1H), 6.26 (d, J = 1.8 Hz, 1H), 5.14 (d, J = 7.7 Hz, 1H), 5.11 (d, J = 7.5 Hz, 1H), 3.74 – 3.62 (m, 6H), 3.60 – 3.47 (m, 6H); ¹³C NMR (HSQC, 600 MHz, MeOH-d₄) δ 123.0, 117.5, 116.0, 109.8, 104.8 (x2), 95.5, 78.1 (x2), 75.8 (x2), 71.0 (x2), 62.6 (x2), 62.5 (x2); ESI-MS: m/z: 648 [M+Na]⁺, 624 [M-H]⁻
Isoquercetin (9.0 mg, 0.019 mmol) and 4-aminogalactosyl fluoride (9.0 mg, 0.050 mmol) were added to a vial. 1 mL of 50 mM sodium phosphate buffer (pH 7) was added. 4 mg/mL of Abg2F6 was then used in the reaction. The reaction was stirred at rt for 23 hours. Abg2F6 was precipitated out of solution by treatment with methanol and the reaction was purified by C18 reversed-phased HPLC to give 04c as a yellow powder (4.7 mg, 0.0076 mmol, 40% yield). (600 MHz, MeOH-d₄) δ 8.55 (s, 1H), 7.71 (d, J = 2.2 Hz, 1H), 7.56 (dd, J = 8.5 Hz, J = 1.9, 1H), 7.49 (d, J = 2.1 Hz, 1H), 6.85 (d, J = 8.5 Hz, 1H), 6.32 (d, J = 1.9 Hz, 1H), 5.14 (d, J = 8.3 Hz, 1H), 5.12 (d, J = 8.2 Hz, 1H), 3.74 – 3.61 (m, 6H), 3.60 – 3.52 (m, 4H), 3.48 (m, 2H); ¹³C NMR (HSQC, 600 MHz, MeOH-d₄) δ 122.9, 117.3, 116.0, 109.9, 104.9 (x2), 95.5, 78.1 (x2), 75.8 (x2), 71.0 (x2), 62.6 (x2), 62.5 (x2); ESI-MS: m/z: 648 [M+Na]⁺, 624 [M-H]⁻
(M14). Caffeic acid (1.4 mg, 0.008 mmol) and HBTU (3.0 mg, 0.008 mmol) were added to a vial. They were dissolved in DMF (0.5 mL). 4-Methylmorpholine (1 μL, 0.008 mmol) was added. The mixture was stirred at rt for 1 hour. 04a (5.0 mg, 0.008 mmol) was dissolved in 100 μL of DMF and added to the vial. This mixture was stirred at rt overnight. The mixture was diluted in ethyl acetate and washed with 3 x HCl. Ethyl acetate was evaporated and the reaction was redissolved in 100 μL of 50% water/ACN and purified by RP-HPLC to give M14 as a yellow film (~ 1 mg, 0.0013 mmol, 16% yield). (600 MHz, MeOH-d₄) δ 7.59 (d, J = 2.2 Hz, 1H), 7.47 (dd, J = 8.0 Hz, J = 2.2, 1H), 7.40 (d, J = 2.1 Hz, 1H), 7.32 (d, J = 15.6 Hz, 1H), 7.07 (d, J = 2.2 Hz, 1H), 6.93 (dd, J = 8.2 Hz, J = 2.0 Hz, 1H), 6.84 (d, J = 8.2 Hz, 1H), 6.75 (d, J = 8.4 Hz, 1H), 6.40 (d, J = 15.6 Hz, 1H), 6.32 (d, J = 1.9 Hz, 1H), 5.12 (m, 2H), 3.74 – 3.45 (m, 12H); ¹³C NMR (HSQC, 600 MHz, MeOH-d₄) δ 142.5, 122.4, 122.0, 117.7, 116.5, 116.0 (x2), 115.2, 100.9 (x2), 100.1, 95.5, 78.1 (x2), 75.8 (x2), 71.5, 62.6 (x2), 62.5 (x2), 57.6; ESI-MS: m/z: 810 [M+Na]⁺, 786 [M-H]⁻
(M15). Caffeic acid (1.9 mg, 0.011 mmol) and HBTU (4.1 mg, 0.011 mmol) were added to a vial. They were dissolved in DMF (0.5 mL). 4-Methylmorpholine (1.5 µL, 0.011 mmol) was added. The mixture was stirred at rt for 1 hour. 04b (7.1 mg, 0.011 mmol) was dissolved in 100 µL of DMF and added to the vial. This mixture was stirred at rt overnight. The mixture was diluted in ethyl acetate and washed with 3 x HCl. Ethyl acetate was evaporated and the reaction was redissolved in 100 µL of 50% water/ACN and purified by RP-HPLC to give M15 as a yellow film (~ 1 mg, 0.0013 mmol, 12% yield). (600 MHz, MeOH-d$_4$) δ 7.59 (d, J = 2.2 Hz, 1H), 7.46 (dd, J = 8.4 Hz, J = 2.0, 1H), 7.42 (d, J = 2.0 Hz, 1H), 7.22 (d, J = 15.6 Hz, 1H), 7.06 (d, J = 2.0 Hz, 1H), 6.89 (dd, J = 8.4 Hz, J = 2.0 Hz, 1H), 6.81 (d, J = 8.4 Hz, 1H), 6.61 (d, J = 8.4 Hz, 1H), 6.36 (d, J = 15.7 Hz, 1H), 6.32 (d, J = 1.8 Hz, 1H), 5.14 (m, 1H), 5.10 (m, 1H), 3.70 – 3.61 (m, 6H), 3.59 – 3.52 (m, 6H); $^{13}$C NMR (HSQC, 600 MHz, MeOH-d$_4$) δ 144.5, 122.5, 122.0, 117.7, 116.3, 116.1, 116.0, 115.2, 99.8 (x2), 99.6, 95.5, 76.1 (x2), 72.8 (x2), 70.0 (x2), 64.5 (x2), 62.5 (x2); ESI-MS: m/z: 810 [M+Na]$^+$, 786 [M-H]$^-$.
(M16). Caffeic acid (1.3 mg, 0.0076 mmol) and HBTU (2.9 mg, 0.0076 mmol) were added to a vial. They were dissolved in DMF (0.5 mL). 4-Methylmorpholine (1 µL, 0.0076 mmol) was then added. The mixture was stirred at rt for 1 hour. 04c (4.7 mg, 0.0076 mmol) was dissolved in 100 µL of DMF and added to the vial. This mixture was stirred at rt overnight. The mixture was diluted in ethyl acetate and washed with 3 x HCl. Ethyl acetate was evaporated and the reaction was redissolved in 100 µL of 50% water/ACN and purified by RP-HPLC to give M16 as a yellow film (~ 1 mg, 0.0013 mmol, 17% yield). (600 MHz, MeOH-d₄) δ 7.61 (d, J = 2.2 Hz, 1H), 7.50 (dd, J = 8.5 Hz, J = 2.0, 1H), 7.49 (d, J = 2.1 Hz, 1H), 7.36 (d, J = 15.4 Hz, 1H), 7.03 (d, J = 2.0 Hz, 1H), 6.89 (dd, J = 8.4 Hz, J = 2.0 Hz, 1H), 6.83 (d, J = 8.5 Hz, 1H), 6.75 (d, J = 8.4 Hz, 1H), 6.40 (d, J = 15.7 Hz, 1H), 6.32 (d, J = 1.9 Hz, 1H), 5.12 (m, 2H), 3.74 – 3.69 (m, 6H), 3.60 – 3.42 (m, 6H); ¹³C NMR (HSQC, 600 MHz, MeOH-d₄) δ 142.5, 122.4, 122.1, 117.7, 116.4, 116.3 (x2), 115.2, 100.9 (x2), 99.1, 95.5, 79.1 (x2), 74.7 (x2), 72.0 (x2), 64.4 (x2), 63.5 (x2); ESI-MS: m/z: 810 [M+Na]⁺, 786 [M-H]⁻
5.2 Enzyme Kinetics

5.2.1 General Materials and Methods

2-Chloro-4-nitrophenyl α-maltotrioside (CNPG3) was purchased from Sekisui Enzymes. All other chromogenic substrates were purchased from Sigma-Aldrich. Wild-type HPA was expressed by Emily Kwan. *R. inulinivorans* α-amylase A, *B. fibrisolvens* α-amylase B and *Agrobacterium* β-glucosidase were expressed in house. N-terminal maltase-glucoamylase, C-terminal maltase-glucoamylase and C-terminal sucrose-isomaltase were generous gifts from Prof. David Rose’s lab at the University of Waterloo. Porcine pancreatic α-amylase, Green coffee bean α-galactosidase, Bovine liver β-galactosidase, Jack bean α-mannosidase, *S. cerevisiae* α-glucosidase, Almond β-glucosidase, and *E. coli* β-galactosidase were purchased from Sigma-Aldrich.

The release of 2-chloro-nitrophenol or 4-nitrophenol resulting from glycosidase-catalyzed hydrolysis of chromogenic substrates was monitored at 400 nm. All assays were performed on a Varian Cary 300 UV/Vis spectrophotometer. 200 µL reactions were monitored in blackout quartz cuvettes. Reactions were monitored over 5 minutes to measure the initial reaction rate.

5.2.2 Determination of IC_{50} Values for HPA Inhibitors

Reactions were run at 30°C in 50 mM sodium phosphate, 100 mM sodium chloride (pH 7.0) with [CNPG3] = 4 mM (~K_m). At least 5 different inhibitor concentrations were used for each IC_{50} determination. For each reaction HPA was first incubated with varying [I] at 30°C for 1 minute. The reaction was then initiated by
addition of CNGP3. Initial reaction rates ($v_o$) were plotted in GraFit 7.0 software to yield values of IC$_{50}$.

**5.2.3 Determination of $K_i$ Values for HPA Inhibitors Using Michaelis-Menten Conditions**

Reactions were run at 30°C in 50 mM sodium phosphate, 100 mM sodium chloride (pH 7.0). Reactions were run using two or three different [CNPG3] (2 mM and 4 mM or 2 mM, 4 mM, and 6 mM) for each range of inhibitor concentrations. Inhibitor concentrations generally ranging from 1/7 to 7x $K_i$ were used. For each reaction, HPA was incubated with varying [I] at 30°C for 1 minute. The reaction was then initiated by addition of CNGP3. These data were fit to a competitive inhibition model using nonlinear regression analysis, as performed by the GraFit 7.0 program to provide a value for $K_i$. Dixon and Lineweaver-Burke plots of each data set validated the use of a competitive inhibition model. $\Delta G_{binding}$ was calculated using the equation $\Delta G_{binding} = RT\ln(K_i)$, where $RT = 0.592$ kcal mol$^{-1}$.

**5.2.4 Determination of $K_i$ Values for HPA Inhibitors Using the Morrison Method**

For helianthamide and its tight-binding variants, inhibition constants were calculated using the Morrison equation for tight-binding inhibition. Reactions were run with a final [CNPG3] = 4 mM. Typically six different inhibitor concentrations were used for each enzyme concentration. A total of 1 - 5 different enzyme concentrations were used, ranging from 1 nM to 10 nM. $v/v_o$ was plotted against [I] for each enzyme concentration to form a series of dose response curves. These data sets were then fitted to the Morrison equation using a least mean squares method to determine a value of $K_{i-app}$ for each enzyme concentration. $K_{i-app}$ was then used to calculate $K_i$. 
5.2.5 Measurement of Inhibitors Specificity with Other Glycosidases

Kinetic studies were performed at 37°C in an appropriate buffer (specific conditions listed below). The enzyme was incubated with different concentrations of inhibitor for two minutes before initiating the reaction by the addition of substrate. Initial reaction rates were measured by monitoring ΔA₄₀₀nm over the course of five minutes. If inhibition was observed at the highest [I] tested an IC₅₀ analysis was carried out using [S] ~ K_M.

_Agrobacterium_ β-glucosidase (Abg)¹⁴¹: 50 mM sodium phosphate buffer (pH 7). PNP β-Gal, K_M = 4.1 mM.

_E.coli_ β-galactosidase²⁰⁰: 50 mM sodium phosphate, 1.0 mM MgCl₂ (pH 7). PNP β-Gal, K_M = 60 mM.

_Bovine liver_ β-galactosidase²⁰¹: 50 mM sodium phosphate buffer (pH 7). PNP β-Gal, K_M = 0.65 mM.

_S.cerevisiae_ α-glucosidase²⁰²: 50 mM sodium phosphate buffer (pH 7). PNP α-Glc, K_M = 0.75 mM.

_R. inulinivorans_ α-amylase A²⁰³: 50 mM sodium phosphate, 100 mM sodium chloride (pH 7). CNPG3,

_B. fibrisolvens_ α-amylase B²⁰⁴: 50 mM sodium phosphate, 100 mM sodium chloride (pH 7). CNPG3,

_N-terminal maltase-glucoamylase³³: 50 mM sodium phosphate buffer (pH 7). PNP α-Glc, K_M = 12 mM.

_C-terminal maltase-glucoamylase³²: 50 mM sodium phosphate buffer (pH 7). PNP α-Glc, 2 mM.

_C-terminal sucrose-isomaltase³³: 50 mM sodium phosphate buffer (pH 7). PNP α-Glc, 4 mM.

_Porcine pancreatic_ α-amylase²⁰⁵: 50 mM sodium phosphate, 100 mM sodium chloride (pH 7). CNPG3, K_M ~ 1 mM.
Green coffee bean $\alpha$-galactosidase$^{206}$: 50 mM sodium phosphate buffer (pH 7). PNP $\alpha$-Gal, $K_M \sim 0.5$ mM.

Jack bean $\alpha$-mannosidase$^{207}$: 50 mM sodium phosphate buffer (pH 7). PNP $\alpha$-mann, $K_M = 2.5$ mM.

Almond $\beta$-glucosidase$^{208}$: 50 mM sodium phosphate buffer (pH 7). PNP $\beta$-Gal, $K_M = 1.7$ mM.

5.2.6 Determination of Michaelis-Menten Parameters for Hydrolysis of CNPG3 by T163R HPA

Initial reaction rates were determined for reactions using seven different [CNPG3] with 10 nM T163R HPA. Reaction were initiated by addition of enzyme and monitored for 5 minutes at 30°C. Data were fit to the Michaelis-Menten equation via nonlinear regression in GraFit to obtain $V_{\text{max}}$ and $K_M$ values. A value of $14.6 \times 10^3$ M$^{-1}$ cm$^{-1}$ was used as a molar extinction coefficient for 2-chloro-4-nitrophenol in order to determine $k_{\text{cat}}$.\textsuperscript{209}

5.3 Protein Expression

5.3.1 General Materials and Methods

Commercially available vectors, restriction enzymes, T4 DNA ligase, fast alkaline phosphatase, $P$. pastoris strains and $E$. coli were purchased from Thermo Fisher Scientific\textsuperscript{®} or New England BioLabs\textsuperscript{®}. Chromatographic resins and columns were purchased from GE Healthcare\textsuperscript{®}. DNA primers and gene blocks were acquired from IDT\textsuperscript{®} Canada through the Nucleic Acid and Protein Services Units at the University of British Columbia. DNA sequencing was carried out by Genewiz\textsuperscript{®}.
5.3.2 Expression of the Barnase’-Helianthamide Fusion in Escherichia coli

A synthetic gene of the desired helianthamide construct was ordered as a pUC-57 plasmid from BioBasic® Canada. The gene was subcloned into a pET-29b+ vector and single clones were sequenced to verify correct ORF. The desired plasmid was transformed into electrocompetent BL21* (DE3) E. coli, which were screened on Luria-Bertani agarose plates containing 50 µg/mL kanamycin. Starter cultures were made by incubating colonies containing the desired plasmid in 5 mL of LB medium containing 50 µg/mL kanamycin overnight at 37°C. Starter cultures were added to 500 mL of LBE-5052 autoinduction media. The expression cultures were incubated at 25°C (230 RPM) for 24 hours. The resulting culture supernatant was treated with 60% ammonium sulfate. The ammonium sulfate solution was stirred at 4°C for one hour before centrifugation to isolate the precipitated proteins. The proteins were re-suspended in His-trap binding buffer (20 mM sodium phosphate, 500 mM sodium chloride, 5 mM imidazole, pH 8) and applied to a HisTrap™ FF Ni-NTA agarose column. The protein of interest was eluted over a gradient of increasing [imidazole]. The crude fusion was then cleaved using 100 units of TurboTEV protease (Accelagen) per milligram of fusion at 30°C for 16 hours. Helianthamide was purified from the reaction mixture by addition of an equal volume of methanol, leading to precipitation of TEV protease, bar’, and bar’-hel. This material was exchanged back into water and purified with a HPLC reversed phase-C18 column using a gradient of 5% ACN (0.1 % TFA) to 95% ACN (0.1 % TFA).

5.4.2 Site-Directed Mutagenesis of Barnase’-Helianthamide

Site-directed mutagenesis was performed via the four-primer method. Two primers ("T7" and "T7-term") that were complementary to regions on the pET-29b+
flanking the gene of interest were used. Two internal primers, which were complementary to each other and contained the desired mutation (“forward” and “reverse”), were also designed and used for each site-directed mutant. In the first round of PCR, two PCR reactions were run to create two new oligos from the DNA template. In one reaction the “T7” and “reverse” primers were used. In the second, the “T7-term” and “forward” primers were used. Each PCR reaction was performed with a total volume of 50 µL in Phusion HF buffer (Fermentas) containing 10 ng of the pET-29b+ helianthamide-barnase’ template pair of primers (20 pmol each), deoxynucleotides (20 pmol), and Pfu polymerase (1.25 units). The reaction was cycled 25 times in a Perkin-Elmer GeneAmp PCR 2400 system. The resulting oligos were purified with a Qiagen QIAquick gel extraction kit. A third PCR reaction was run using these two oligo products as the DNA template and “T7” and “T7-term” primers. The resulting DNA fragment was gel-purified and digested with restriction enzymes HindIII and Ndel. The pET-29b+ vector was also treated with restriction enzymes HindIII and Ndel, followed by treatment with fast alkaline phosphatase. Ligation between the gene fragment and pET-29b+ vector was carried with T4 DNA ligase. The mutated pET-29b+ helianthamide-barnase’ plasmids were then transformed into BL21*(DE3) E. coli via electroporation and screened on Luria-Bertani agarose plates containing 50 µg/mL kanamycin. A number of clones were sequenced to verify correct ORF. Following expression in 500 mL of LBE-5052 autoinduction media, the resulting bar’-hel fusion mutant peptides were purified with HisTrap™ FF Ni-NTA agarose, HiTrap™ SP FF cation-exchange, and HiTrap™ Phenyl FF hydrophobic-exchange columns.
5.3.3 Expression of SUMO-Helianthamide Fusion in *Pichia pastoris*

A SUMO-helianthamide gene block was ordered from IDT Canada. The gene block was treated with *NotI* and *EcoRI* restriction enzymes. The pPic9k vector was also treated with restriction enzymes *NotI* and *EcoRI*, followed by treatment with fast alkaline phosphatase. Ligation between the SUMO-helianthamide gene block and pPic9k vector was then carried out with T4 DNA ligase. The resulting pPIC9K - T163R HPA plasmid was transformed into *E. coli* (TOP10) by electroporation. The transformed cells were screened on Luria-Bertani plates containing 100 µg/mL ampicillin. Plasmids were isolated from *E. coli* using the Qiagen QIAprep Spin Miniprep kit for sequencing. The pPIC9K - T163R HPA plasmid (linearized with *Sacl*) was then transformed into electrically competent *P. pastoris* GS-115. Transformed cells were plated on Minimal Dextrose plates and grown at 30°C for two days. Fifty transformants were screened on minimal methanol, minimal dextrose, and YPD plates. Twenty colonies that grew on all three plates were selected and grown in 3 mL buffered glycerol complex medium (BMGY) for two days at 30°C. The cells were washed and induced in 1 mL of buffered methanol complex medium (BMMY) for two days at 30°C. The cultures were concentrated by Centricon and analyzed for expression by SDS-PAGE. Four of the colonies were taken forward for 500 mL test expressions. Colonies were grown in 60 mL BMGY media at 30°C 200 RPM. 20 mL of the overnight cultures were added to 600 mL BMGY. After 16 hours the cells were transferred to 300 mL BMMY. After one day 2 mL of 50% methanol was added. The cultures were left overnight. The culture supernatants were then collected and purified via HisTrap™ FF Ni-NTA agarose column and expression yield was determined.
5.4.3 Site Directed Mutagenesis of HPA

A pPic9K-HPA plasmid previously produced in house by Emily Kwan was used as a template for site-directed mutagenesis of HPA. Site directed mutagenesis of HPA was performed by the four-primer method as described above, this time using the AOX 5’ and AOX 3’ regions of the pPic9K plasmid to design flanking primers. The gene fragment was treated with SacI and NotI restriction enzymes and ligated into pPIC9K. The resulting pPIC9K - T163R HPA plasmid was transformed into E. coli (TOP10) by electroporation. The transformed cells were screened on Luria-Bertani plates containing 100 µg/mL ampicillin. Plasmids were isolated from E. coli using the Qiagen QIAprep Spin Miniprep kit for sequencing. The pPIC9K - T163R HPA plasmid (linearized with SacI) was then transformed into electrically competent P. pastoris GS-115. Transformed cells were plated on Minimal Dextrose plates and grown at 30°C for two days. Colonies were screened on MM, MD, and YPD plates as described above.

5.3.4 Expression of T163R HPA in Pichia pastoris

A detailed protocol for the expression and purification of HPA can be found in the work done by Rydberg et al. Expression was carried out in P. pastoris. Colonies were grown in 60 mL BMGY media at 30°C 200 RPM. 20 mL of the overnight culture was added to 600 mL BMGY. After 16 hours the cells were transferred to 300 mL BMMY. After 24 hrs 2 mL of 50% methanol was added. The culture was left overnight. The culture supernatant was collected and purified via Phenyl Sepharose and Hitrap™ Q columns.
5.4 Structural Analysis of Helianthamide

5.4.1 CD Spectroscopy

Analysis was conducted on a Jasco J-815 CD spectrophotometer. Samples of synthetic, natural, and recombinant helianthamide were diluted in distilled water. Analysis was run from 260 to 190 nm for the natural and synthetic material, and 250 to 190 nm for the recombinant material. The resulting data were analyzed on the Dichroweb server\(^2\). All three data sets were analyzed with CONTIN software using reference sets 4, 7, and SP175\(^{213,214}\). The data of the natural and synthetic material were also analyzed using CDSSTR software with reference sets 4, 7, and SP175. The secondary structural estimates were averaged and presented in Table 3.2.

5.4.2 Thiol Titration Assay

A solution of thiol titration buffer was prepared (0.2 mM 5,5’-dithiobis-(2-nitrobenzoic acid), 6 M guanidinium chloride, 20 mM HEPES, 1 mM EDTA, pH 7.4). Helianthamide was added to the solution in final protein concentrations ranging from 2 – 6 \(\mu\)M\(^{215}\). The release of 2-nitro-5-thiobenzoate was monitored at 412 nm at 25°C over the course of 30 minutes. During each measurement, \(\Delta A_{412}\) was also measured for the thiol titration buffer to take into account the rate of spontaneous hydrolysis of the DTNB reagent. The experiment was also conducted with BSA, which has one free thiol group, as a positive control.

5.4.3 Disulfide Assignment of Recombinant Material

Intact helianthamide was incubated in 11 M hydrochloric acid at 37°C for 4 days. The sample was diluted and neutralized before desalting by C18 reverse phase
chromatography. The peptide mixture was injected into an Agilent 6460 QQQ LC/MS mass spectrometer. Elution and MS/MS analysis was carried out over a 40 minute period. A script was written by Dr. William Walters to predict all possible fragments for the helianthamide sequence in the event of random hydrolysis and was used to analyze the resulting MS and MS/MS peaks.

5.5 Solid Phase Peptide Synthesis of Small Helianthamide Peptides

5.5.1 General Materials and Methods

Fmoc L-amino acids were purchased from Millipore Sigma®. The following side chain protections were used: Arg (Pbf), Asn (Trt), Asp (OtBu), Gln (Trt), Glu (OtBu), His (Trt), Lys (Boc), Ser (tBu), Thr (tBu), Trp (Boc), Tyr (tBu) and Cys (Trt). All solvents and reagents were purchased from Sigma-Aldrich or Thermo Fisher Scientific. MALDI-TOF analysis was run on Bruker Autoflex MALDI-TOF.

5.5.2 Solid Phase Peptide Synthesis

Solid phase peptide synthesis was carried out on an Intavis AG Multipep peptide synthesizer. Peptides were synthesized via Fmoc chemistry at a scale of 5 µmol in a 96-well plate. 25 µmol of Rink Amide resin was used for each synthesis. 25 µmol of Fmoc L-amino acids, 25 µmol of HBTU-HOBt, and 25 µmol of N-methylmorpholine were used in each round of coupling. 150 µL x 2 of 20% piperidine in DMF were used for each deprotection step. After stepwise chain assembly, the peptide could be deprotected and cleaved with TFA/phenol/water/TIPS (85/5/5/2). Peptides were precipitated and washed in cold diethyl ether and re-suspended in 50% water/ACN 0.1% TFA. They were then
purified by reversed phase HPLC, and the mass of each peptide was analyzed by MALDI-TOF.

5.5.3 Peptide Oxidation for Disulfide Bond Formation

Peptides were first dissolved in 6 M GuHCl, and then diluted 6-fold into a buffer containing 0.1 M NaHCO₃, 3 mM cysteine, 0.3 mM cystine, pH 8.1 to a concentration of 1 mg/mL of peptide²¹⁶. The reaction was incubated at rt overnight with gentle stirring. Peptides were then purified by RP-HPLC and analyzed by MALDI-TOF.
References


Appendices

Appendix A: Kinetic Data of HPA Inhibition by MbA Analogues

Figure AA1. M01 vs HPA $K_i$ Dixon Plot

Figure AA2. M02 vs HPA $K_i$ Dixon Plot
Figure AA3. M03 vs HPA $K_i$ Dixon Plot

Figure AA4. M04 vs HPA $K_i$ Dixon Plot
Figure A5. M05 vs HPA $K_i$ Dixon Plot

Figure A6. M06 vs HPA $K_i$ Dixon Plot
Figure AA7. M07 vs HPA $K_i$ Dixon Plot

Figure AA8. M08 vs HPA $K_i$ Dixon Plot
Figure AA9. M09 vs HPA $K_i$ Dixon Plot

Figure AA10. M10 vs HPA $K_i$ Dixon Plot
Figure AA11. M11 vs HPA $K_i$ Dixon Plot

Figure AA12. M12 vs HPA $K_i$ Dixon Plot
Figure AA13. M13 vs HPA $K_i$ Dixon Plot

Figure AA14. M14 vs HPA $IC_{50}$
Figure AA15. M16 vs HPA IC$_{50}$
Appendix B: Helianthamide Expression Data

Sequence of original barnase'-helianthamide gene optimized for expression in E. coli. Contains a Factor Xa cleavage site which was later mutated to a TEV protease cleavage site.

ATG AAA CAA TCC ACC ATC GCA CTG GCC CTG CTG CCG CTG CTG
TTC ACG CCG GTT ACA AAG CCG CTG GTG CAT CAT CAT CAT CAT
CAC TCG AGT GGC GCA CAG GTT ATT AAC ACC TTT GAT GGT GTT
GCT GAC TAT CTG CAA ACG TAC CAT AAA CTG CCG GAT AAT TAT
ATC ACC AAA TCA GAA GCA CAG GCC CTG GGT TGG GTC GCA TCG
AAA GGT AAC CTG GCA GAT GTG GCT CCG GGC AAA AGT ATT GGC
GGT GAC ATC TTC TCC AAT CGT GAA GGT AAA CTG CCG GGC AA
GTT CGT ACC TGG CGC GAA GCG GAT ATT AAC TAT ACG TCA
GCC TTT CGT AAT TCG GAT CGC ATT CTG TAC AGC TCT GAC TGG
CTG ATC TAT AAA ACC ACG GCC TAC CAA ACC TTC ACG AAA
ATT CGT ATC GAA GGC CGC AAG AGT GGT AAC TCC TGC TAT ATT
TAC CAC GGC GTT AGC GGT ATC TGC AAA GCG TCT TGT GCC GA
GAT GAA AAA GCA ATG GCA GCC ATG GGC GTG TGT GAA GGT CAT
CTG TGT TGT TAC AAA ACC CCG TGG TGA TAG
**Peptide sequence of the complete barnase'-helianthamide fusion protein.** His-tag is highlighted in green. The active site mutation of barnase is highlighted in pink. The TEV protease recognition site is highlighted in yellow.

```
10  20  30  40  50
MKQSTIALAL LPLLFTPVTK AAGHHHHHH SGAQVINTFD GVADYLQTYH
60  70  80  90  100
KLPDNYITK5 EAQLGWMVAS KGNLADVAPG KSIGGDIFSN REGKLPGKS5
110 120 130 140 150
RTWREADINY SSGFRNDSR1 LYSSDWLIYK TTDAYQTFTK IRENLYFQSE
160 170 180 190
SGNSCYIYHG VSGICKASC5 EDEKAMAGMG VCEGHLCYK TPW
```

**Primers used in site-directed mutagenesis of helianthamide and HPA.** Bolded sequences indicate the site of mutagenesis.

**TEV Protease Site Introduction**

**Forward**
5’ AAA ATT CGT **GAA AAC CTG TAT TTT CAG AGC** GAA AGT GGT AAC TCC TGC TA **3’**

**Reverse**
5’ ACC ACT TTC **GCT CTG AAA TAT CAG GTT TTC** ACG AAT TTT CGT GAA GGT TT (**3’**)

**S5A**

**Forward**
5’ AA GGC CGC GAA AGT GGT **AAC GCA** TGC TAT ATT TAC CAC GGC GT **3’**

**Reverse**
5’ AC GCC GTG GTA AAT ATA GCA **TGC** GTT ACC ACT TTC GCG GCC **TT 5’**

**C6A**

**Forward**
5’ AG AGC GAA AGT GGT **AAC TCC GCA** TAT ATT TAC CAC GGC GTT **AG 3’**

**Reverse**
5’ CT AAC GCC GTG GTA AAT ATA **TGC** GGA GTT ACC ACT TTC GCT **CT 3’**

**Helianthamide Y7A**

**Forward**
5’ GC GAA AGT GGT **AAC TCC TGC GCA** ATT TAC CAC GGC GTT **AGC GG 3’**

**Reverse**
5’ CC GCT AAC GCC GTG GTA AAT **TGC** GCA GGA GTT ACC ACT TTC **GC 3’**
Y7F
Forward
5’ GC GAA AGT GGT AAC TCC TGC TTT ATT TAC CAC GGC GTT AGC GG 3’
Reverse
5’ CC GCT AAC GCC GTG GTA AAT AAA GCA GGA GTT ACC ACT TTC GC 3’

Helianthamide Y9A
Forward
5’ GT GGT AAC TCC TGC TAT ATT GCA CAC GGC GTT AGC GGT ATC TG 3’
Reverse
5’ CA GAT ACC GCT AAC GCC GTG TGC AAT ATA GCA GGA GTT ACC AC 3’

Y9F
Forward
5’ GT GGT AAC TCC TGC TAT ATT TTT CAC GGC GTT AGC GGT ATC TG 3’
Reverse
5’ CA GAT ACC GCT AAC GCC GTG AAA AAT ATA GCA GGA GTT ACC AC 3’

Helianthamide H10A
Forward
5’ GT AAC TCC TGC TAT ATT TAC GCA GGC GTT AGC GGT ATC TGC AA 3’
Reverse
5’ TT GCA GAT ACC GCT AAC GCC TGC GTA AAT ATA GCA GGA GTT AC 3’

C16A
Forward
5’ AC CAC GGC GTT AGC GGT ATC GCA AAA GCG TCT TGT GCC GAA GA 3’
Reverse
5’ TC TTC GGC ACA AGA CGC TTT TGC GAT ACC GCT AAC GCC GTG GT 3’

C20A
Forward
5’ GC GGT ATC TGC AAA GCG TCT GCA GCC GAA GAT GAA AAA GCA AT 3’
Reverse
5’ AT TGC TTT TTC ATC TTC GGC TGC AGA CGC TTT GCA GAT ACC GC 3’

C33A
Forward
5’ CA ATG GCA GGC ATG GGC GTG GCA GAA GGT CAT CTG TGT TGT TA 3’
Reverse
5’ TA ACA ACA CAG ATG ACC TTC TGC CAC GCC CAT GCC TGC CAT TG 3’
C38A
Forward
5’ GC GTG TGT GAA GGT CAT CTG GCA TGT TAC AAA ACC CCG TGG TG 3’
Reverse
5’ CA CCA CGG GGT TTT GTA ACA TGC CAG ATG ACC TTC ACA CAC GC 3’

C39A
Forward
5’ TG TGT GAA GGT CAT CTG TGT GCA TAC AAA ACC CCG TGG TGA TA 3’
Reverse
5’ TA TCA CCA CGG GGT TTT GTA TGC ACA CAG ATG ACC TTC ACA CA 3’

HPA T163R
Forward
5’ AG GTC AGA GAT TGT CGT CTG AGA GGT CTT CTT GAT CTT GCA CT 3’
Reverse
5’ AG TGC AAG ATC AAG AAG ACC TCT CAG ACG ACA ATC TCT GAC CT 3’

Bar’-hel after HiTrap™ SP
Bar’-hel after HiTrap™ Phenyl

Figure AB1. Barnase’-helianthamide purification
Figure AB2. Barnase'-helianthamide TEV cleavage reaction.

Figure AB3. HPLC purification of helianthamide after methanol treatment.
Figure AB4. SUMO-helianthamide expression in *P. pastoris*.
Appendix C: Kinetic Analysis of Helianthamide Fusion Peptides

Figure AC1. Hel-bar’ vs HPA $K_i$ determination via the Morrison Method

Figure AC2. Y7A hel-bar’ vs HPA $K_i$ determination via the Morrison Method
Figure AC3. Y9A hel-bar' vs HPA $K_i$ determination via the Morrison Method

Figure AC4. H10A hel-bar' vs HPA $K_i$ determination via the Morrison Method
Figure AC5. C6A hel-bar' vs HPA $K_i$ Dixon plot

Figure AC6. C16A hel-bar' vs HPA $K_i$ determination via the Morrison Method
Figure AC7. C20A hel-bar' vs HPA $K_i$ Dixon plot

Figure AC8. C33A hel-bar' vs HPA $K_i$ determination via the Morrison Method
Figure AC9. C38A hel-bar' vs HPA $K_i$ Dixon plot

Figure AC10. C39A hel-bar' vs HPA $K_i$ Dixon plot
Figure A11. Determination of Michaelis-Menten parameters for the T163R HPA mutant

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Appendix D: Synthesis and Kinetic Analysis of Small Peptides

Figure AD1. MALDI-TOF spectrum of peptide 12 in oxidized form. Highest peak at 1889 m/z ([M+H]^+).

Figure AD2. MALDI-TOF spectrum of peptide 13 in oxidized form. This spectrum was taken months after use of this peptide for kinetics with mild degradation occurring since. Highest peak at 1853 m/z ([M+Na]^+).
Figure AD3. Peptide 12 vs. HPA IC$_{50}$

Figure AD4. Peptide 13 vs. HPA IC$_{50}$