Enzyme Exploitation: Manipulating Enzyme Function for Therapy, Synthesis and Natural Product Modification

DISSERTATION

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By

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Abstract

Enzymes are effective targets for therapy, research, and synthesis, as well as magnificent tools for natural product modification. In the development of cancer therapies, rebeccamycin and granulatimides serve as starting point for therapeutic inhibitor design due to their ability to inhibit the enzymes, respectively topoisomerase I and checkpoint kinase I, which contribute to the control of the cell cycle. The design and synthetic progress of hybrid products is reported herein.

In the study of cell-surface oligosaccharide function, glycosidase inhibitors have assisted in the understanding of glycan processing and recognition. Inactivators of glycosidases have also been utilized in the treatment of diabetes, flu and HIV. The design and synthesis of a novel glycosidase inhibitor is discussed.

Glycosyl transferases have proven to be useful instruments in the chemoenzymatic synthesis of glycopeptides and glycoproteins, contributing to the study of their structure and functional relationships. Efforts to use PNGase for glycopeptide synthesis and progress toward the determination of peptide sequence specificity of PgL are detailed.

Finally, the enzyme transglutaminase which has been employed for improved quality of meats and other foods is investigated for its potential use in the enhancement of the properties of soy protein toward the production of valuable end products.
Dedication

Dedicated to my daughter, D’Shayln, for her patience and assistance during this trial
Acknowledgments

I must thank my advisor, Dr. Peng George Wang, for allowing me to explore a variety of projects, providing me with exposure to a broad range of research issues and methods.

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2. McKittrick BA (Original Commentary); O'Neil CL, Wang PG (First Update):
   & Sons, Ltd.; 2008.

Fields of Study

Major Field: Chemistry
Table of Contents

Abstract ........................................................................................................................................ ii

Dedication ...................................................................................................................................... iii

Acknowledgments ........................................................................................................................ iv

Vita .............................................................................................................................................. v

List of Tables ................................................................................................................................. x

List of Figures .............................................................................................................................. xi

List of Schemes ............................................................................................................................ xvi

Introduction ................................................................................................................................... 1

Chapter 1: Synthesis of glycosylated granulatimide analogs mediated by a “click” reaction followed by direct arylation ................................................................. 1

Chapter 2: Design and synthesis of a glycosidase inhibitor ......................................................... 1

Chapter 3.1: Exploring Yeast Peptide: N-Glycanase as a Tool for Glycoprotein Synthesis ......................................................................................................................... 2

Chapter 3.2: PglL sequence specificity investigations ................................................................ 3

Chapter 4: Transglutaminase-mediated modification of soy protein ........................................... 3
1 Chapter 1: Design and synthesis of a rebeccamycin/ granulatimide anti-cancer derivative

1.1 Introduction ................................................................................................. 5
1.2 Targeting the Cell Cycle ............................................................................ 8
1.3 Rebeccamycin Analogues as Topoisomerase I Inhibitors......................... 13
1.4 Other Rebeccamycin Analogues ................................................................. 22
1.5 Derivatization of Indolocarbazole Compounds with Uncommon Sugars .... 29
1.6 Pyrrolocarbazoles as Checkpoint 1 Kinase Inhibitors ............................ 36
1.7 Design of Click Rebeccamycin / Granulatimide Derivative....................... 43
1.8 Conclusion ................................................................................................. 56
1.9 Chapter 1 Experimental.............................................................................. 58

2 Chapter 2: Design and Synthesis of a Mechanistic Glycosidase Inhibitor .... 70

2.1 Introduction ............................................................................................... 70
2.2 Applications of Glycosidase Inhibitors..................................................... 71
2.2.1 Inhibitors as Therapeutics .................................................................... 71
2.3 Inhibitor Review ....................................................................................... 75
2.3.1 Noncovalent Inhibitors ....................................................................... 75
2.3.2 Covalent Inhibitors ............................................................................ 79
2.4 Novel Inhibitor Design ............................................................................. 82
2.4.1 Design ......................................................................................................... 82
2.4.2 Mechanism .................................................................................................. 83
2.5 Synthesis ......................................................................................................... 85
  2.5.1 Selective protection ..................................................................................... 85
  2.5.2 Glycal to Mannose Derivative .................................................................... 86
2.6 Conclusion ......................................................................................................... 92
2.7 Chapter 2 Experimental ...................................................................................... 94
3 Chapter 3: Chemoenzymatic synthesis of glycopeptides ......................... 106
  3.1 Introduction ...................................................................................................... 106
    3.1.1 Glycosylation ............................................................................................ 106
    3.1.2 Fmoc Solid-Phase Peptide Synthesis ........................................................ 106
  3.2 N-glycosylation ............................................................................................... 110
    3.2.1 Background ............................................................................................... 110
    3.2.2 Peptide:N-glycanase ................................................................................. 112
    3.2.3 Synthetic Peptide Targets ......................................................................... 116
    3.2.4 Conclusion ................................................................................................ 119
    3.2.5 Experimental: Peptide Synthesis for N-Glycosylation ......................... 119
  3.3 O-Glycosylation ............................................................................................... 124
    3.3.1 Background ............................................................................................... 124
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3.2</td>
<td>PglL</td>
<td>126</td>
</tr>
<tr>
<td>3.3.3</td>
<td>Synthetic Peptides for Sequence Specificity Study</td>
<td>128</td>
</tr>
<tr>
<td>3.3.4</td>
<td>Conclusion</td>
<td>133</td>
</tr>
<tr>
<td>3.3.5</td>
<td>Experimental</td>
<td>133</td>
</tr>
<tr>
<td>4</td>
<td>Chapter 4: Soy Protein Modification Using Transglutaminase</td>
<td>141</td>
</tr>
<tr>
<td>4.1</td>
<td>Introduction</td>
<td>141</td>
</tr>
<tr>
<td>4.2</td>
<td>Transglutaminase</td>
<td>142</td>
</tr>
<tr>
<td>4.3</td>
<td>Soy Protein Experiments</td>
<td>144</td>
</tr>
<tr>
<td>4.4</td>
<td>Conclusion</td>
<td>151</td>
</tr>
<tr>
<td>4.5</td>
<td>Chapter 4 Experimental</td>
<td>153</td>
</tr>
<tr>
<td>5</td>
<td>References</td>
<td>163</td>
</tr>
<tr>
<td>Appendix A: Copyright Permissions</td>
<td>176</td>
<td></td>
</tr>
<tr>
<td>APPENDIX B: NMR Data</td>
<td>188</td>
<td></td>
</tr>
</tbody>
</table>
List of Tables

Table 1.1: Inhibitory Potencies of Compounds 1-19 Toward Topoisomerase I. Antiproliferative Activities In Vitro Against Murine P388 Leukemia Cells and B16 Melanoma Cells ................................................................................................................ 17

Table 1.2: Inhibitory Potencies of Rebeccamycin Analogues Toward PKC and Topoisomerase I. Antiproliferative Activities In Vitro Against Murine B16 Melanoma and P388 Leukemia Cells ................................................................................................................ 19

Table 1.3: Amino-substituted Cytotoxicity and Topo I Inhibition Properties .............................................. 21

Table 1.4: In Vitro Antiproliferative Activities against Nine Tumor Cell Lines .............. 29

Table 1.5: Anticancer Activity (IC$_{50}$) of Synthesized Compounds in Two Cancer Cell Lines (µM) ........................................................................................................................ 32

Table 2.1: $K_i$ Values of the Gluconolactone and Piperidines .............................................. 77

Table 2.2: $K_i$ Values [µM] of the Amidine Analogs ............................................................... 79

Table 4.1: Experiment Set-up ......................................................................................... 149

Table 4.2: Tgase/BSA Experiment Set-up............................................................................ 154

Table 4.3: Set-up for Tgase Catalyzed Conjugation of Maltoheptaose and PON1 ....... 160

Table 4.4: Repeated Experiments ..................................................................................... 161
List of Figures

Figure 1.1: Natural G1 checkpoint / Topo I inhibitor .......................................................... 6
Figure 1.2: G2 checkpoint / Chk1 inhibitors ..................................................................... 7
Figure 1.3: Novel monosaccharide derivatives ................................................................. 7
Figure 1.4: p53 Pathway .................................................................................................. 9
Figure 1.5: Function of Topoisomerase in DNA Replication ........................................... 10
Figure 1.6: Topo I Mechanism ..................................................................................... 11
Figure 1.7: Function of Chk1 ........................................................................................ 12
Figure 1.8: Camptothecin and Derivatives .................................................................... 13
Figure 1.9: Natural Indolocarbazole Topo I Inhibitor ..................................................... 14
Figure 1.10: Rebeccamycin Derivative .......................................................................... 16
Figure 1.11: SAR rebeccamycin series ........................................................................ 17
Figure 1.12: Natural sugar conjugated rebeccamycin analog library ............................. 18
Figure 1.13: Glucosamino-Reb derivative ..................................................................... 21
Figure 1.14: Fluoro- & Hydroxy-substituted Indolocarbazoles ....................................... 23
Figure 1.15: Rebeccamycin Dimers .............................................................................. 24
Figure 1.16: Rebeccamycin/Staurosporine Analogs ..................................................... 25
Figure 1.17: Inverted Reb Derivatives .......................................................................... 26
Figure 1.18: Aza-rebeccamycin analogs ..................................................................... 27
Figure 1.19: Rebeccamycin derivative library ............................................................... 30
Figure 1.20: Glycosylation of indolocarbazole core ................................................................. 31
Figure 1.21: Indolocarbazole vs. bis(indolyl)maleimide glycosylation ................................. 32
Figure 1.22: Standard ICT assay in HeLa cells comparing camptothecin (CPT) with
Rebeccamycin-sugar derivatives 1.41, 1.44, and 1.47 .......................................................... 35
Figure 1.23: G2 Checkpoint Inhibitors .................................................................................. 36
Figure 1.24: Granulatimide analogs ....................................................................................... 38
Figure 1.25: Structural basis for Chk1 inhibition by isogranulatimide. .............................. 40
Figure 1.26: Glycosylated Granulatimide Derivatives ............................................................ 42
Figure 1.27 ............................................................................................................................ 43
Figure 1.28: Novel Reb/Gra Click Derivatives ..................................................................... 44
Figure 1.29: Photochemical Oxidation Will Not Yield Cyclized Derivative ...................... 47
Figure 1.30: Mechanism of Formation of Linked Intermediate ......................................... 50
Figure 1.31: Singly-substituted Maleimide ......................................................................... 54
Figure 2.1: Glycosidase mechanism ...................................................................................... 71
Figure 2.2: Natural Glycosidase Substrates ....................................................................... 72
Figure 2.3: Glycosidase Inhibitors ....................................................................................... 73
Figure 2.4: Cancer-related Glycosidase Inhibitors ............................................................... 75
Figure 2.5: Oxycarbenium Ion Intermediate and Gluconolactone Inhibitor ...................... 76
Figure 2.6: Naturally Occurring Piperidine Inhibitors .......................................................... 77
Figure 2.7: Lactone/Piperidine Analogs .............................................................................. 77
Figure 2.8: Corrected Structure of the Hydroximolactam and Its Analogous Lactone ..... 79
Figure 2.9: Mechanism-based Inactivators with Reactive Aglycons ................................... 80
Figure 3.15: Synthetic Glycopeptide

Figure 3.16: MS analysis of Synthetic Glycopeptide.

Figure 3.17: Triton-X Detergent

Figure 3.18: Triton-X in MS Analysis

Figure 3.19: Native Sequence

Figure 3.20: HPLC Purity Scan of Native Sequence

Figure 3.21: -2P Sequence

Figure 3.22: HPLC Purity Scan of -2P Sequence

Figure 3.23: +1R Sequence

Figure 3.24: HPLC Purity Scan of +1R Sequence

Figure 3.25: +2E Sequence

Figure 3.26: HPLC Purity Scan of +2E Sequence

Figure 4.1: Tgase Reaction. A) Forms cross-linked protein, B) Deamidation in absence of 1° amine.

Figure 4.2: Proposed Transglutaminase Mechanism

Figure 4.3: Initial Cross-linking Experiments

Figure 4.4: Initial Cross-linking Experiment Samples

Figure 4.5: Temperature experiments

Figure 4.6: Synthetic Peptide Linkers

Figure 4.7: SDS-PAGE for Concentration and Linker Experiments

Figure 4.8: Peptide Linker Experiment Samples

Figure 4.9: Tgase activity verification w/ BSA
Figure 4.10: SPI Control & Reaction Samples after 4 d @ rt........................................ 155
Figure 4.11: Temperature Experiments at ~1 wk. ........................................................... 157
Figure 4.12: Maltoheptaose w/ linker ............................................................................. 159
Figure 4.13: SDS-PAGE for PON1/ Maltoheptaose Experiments ................................. 161
Figure 4.14: MALDI on PON1, Control (3) and Reaction (5) ....................................... 162
Figure B.1: $^1$H NMR for Compound 1.53 ................................................................. 188
Figure B.2: $^1$H NMR for Compound 1.54 ................................................................. 189
Figure B.3: $^{13}$C NMR for Compound 1.54 ............................................................... 190
Figure B.4: $^1$H NMR for Compound 1.55 ................................................................. 191
Figure B.5: $^{13}$C NMR for Compound 1.55 ............................................................... 192
Figure B.6: 1H NMR for Compound 2.26 ................................................................. 193
Figure B.7: $^{13}$C NMR for Compound 2.26 ............................................................... 194
Figure B.8: $^1$H NMR for Compound 2.29 ................................................................. 195
Figure B.9: $^{13}$C NMR for Compound 2.29 ............................................................... 196
Figure B.10: $^1$H NMR for Compound 2.30 ............................................................. 197
Figure B.11: $^{13}$C NMR for Compound 2.30 ............................................................. 198
<table>
<thead>
<tr>
<th>Scheme</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Synthesis of open-chain derivatives</td>
<td>45</td>
</tr>
<tr>
<td>1.2</td>
<td>Synthesis of 1', 6'-linked derivative</td>
<td>46</td>
</tr>
<tr>
<td>1.3</td>
<td>Theoretical Ring Closure Plan</td>
<td>48</td>
</tr>
<tr>
<td>1.4</td>
<td>Copper-Catalyzed “Click” Reaction/Direct Arylation Sequence</td>
<td>49</td>
</tr>
<tr>
<td>1.5</td>
<td>Cyclization Plan</td>
<td>52</td>
</tr>
<tr>
<td>1.6</td>
<td>Attempt at Single Substitution Under Sonogashira Conditions</td>
<td>53</td>
</tr>
<tr>
<td>1.7</td>
<td>Attempt at Organoindium Methodology</td>
<td>54</td>
</tr>
<tr>
<td>1.8</td>
<td>Coupling of iodoindole forms diacetylated derivative</td>
<td>55</td>
</tr>
<tr>
<td>1.9</td>
<td>Attempt to Add Iodide Just Prior to Click Reaction</td>
<td>56</td>
</tr>
<tr>
<td>2.1</td>
<td>Inhibition Mechanism</td>
<td>84</td>
</tr>
<tr>
<td>2.2</td>
<td>Non-selective, Bulky Protection of Mannose</td>
<td>86</td>
</tr>
<tr>
<td>2.3</td>
<td>2-deoxy-2-I-mannose from glycal</td>
<td>88</td>
</tr>
<tr>
<td>2.4</td>
<td></td>
<td>88</td>
</tr>
<tr>
<td>2.5</td>
<td>Elimination Product</td>
<td>89</td>
</tr>
<tr>
<td>2.6</td>
<td>Substitution on Benzyl-protected Mannosyl-2-iodide</td>
<td>90</td>
</tr>
<tr>
<td>2.7</td>
<td>Completion of Desired Substitution at C2</td>
<td>91</td>
</tr>
<tr>
<td>2.8</td>
<td>2-Hydrazinyl-Glucoside</td>
<td>92</td>
</tr>
<tr>
<td>3.1</td>
<td>SPPS</td>
<td>109</td>
</tr>
</tbody>
</table>
Introduction

Chapter 1: Synthesis of glycosylated granulatimide analogs mediated by a “click” reaction followed by direct arylation

Rebeccamycin (Reb), a β-glucosylated indolocarbazole, is an effective anti-tumor agent which exerts its effects via the formation of a ternary structure with DNA and topoisomerase. Its aglycone moiety intercalates into the base pairs of DNA, while the glycan moiety reinforces the stability of the Reb-DNA-Topo ternary structure. Granulatimide (Gra), which has no sugar moiety, is a kinase (Chk1) inhibitor. We have designed novel monosaccharide analogs of Gra, which should be promising candidates for anti-cancer activity, due to their structural similarity to the natural antibiotics Reb and Gra. It would be especially interesting to explore the biological target profile of these new analogs, since Reb is the inhibitor of topoisomerase and Gra is the inhibitor of kinase Chk1. Besides the structural novelty, the chief merit of this design is the convenience of the synthesis via application of click chemistry in combination with direct arylation of the resulting triazole-intermediate.

Chapter 2: Design and synthesis of a glycosidase inhibitor

Glycosidases catalyze the hydrolysis of glycosidic linkages. Their function is essential in digestion as well as for the processing of cell surface oligosaccharides, which mediate cell-cell recognition during infection, immune response and tumor metastasis.
Glycosidase inhibitors, therefore, have the potential to be effective therapeutic agents in cancer and infections. Modulation of glycoside activity could also be essential in the understanding of cell surface glycan structure and function. We have designed a mechanism-based glycosidase inhibitor, which has potential application to the study of a variety of glycoside-based systems.

Chapter 3.1: Exploring Yeast Peptide: N-Glycanase as a Tool for Glycoprotein Synthesis

The goal of the proposed research is to explore the utilization of yeast peptide:N-glycanase for efficient N-linked glycopeptide and glycoprotein synthesis. N-linked glycosylation is one of the most ubiquitous posttranslational modifications of proteins in eukaryotes. Glycoproteins are involved in many important biological events such as cell adhesion, tumor metastasis, pathogen infection, and immune response. However, a clear understanding of glycoproteins’ structure and function is often hampered by their structural micro-heterogeneity. To tackle this problem, various chemical and chemoenzymatic methods have been developed to synthesize structurally defined glycopeptides and glycoproteins.\(^1\) Particularly, the introduction of enzymatic methods, e.g., using glycosyltransferases and endoglycosidases, has significantly broadened our synthetic repertoire.\(^2,3\) But each method has its own limitations, and the construction of large, homogeneous glycopeptides and glycoproteins is still a challenging task. The yeast enzyme peptide:N-glycanase (PNGase) was functionally identified and shown that it can efficiently release intact N-glycans from misfolded glycoproteins. PNGase belongs to transglutaminase superfamily. Transglutaminases catalyze the crosslinking of two proteins utilizing the Cys-His-Asp triad for catalysis and amines such as lysine as the
nucleophile. Based on the mechanistic similarity, by manipulations of reaction conditions, PNGase might work “in reverse” as an enzyme to synthesize glycoproteins.

Chapter 3.2: PglL sequence specificity investigations

Bacterial infections constitute one of the major health problems worldwide. Polysaccharides, forming a thick capsule that surrounds the bacterial pathogen, represent a major determinant of pathogenicity. With the increasing emergence of resistance toward major antibiotics, development of polysaccharide-based vaccines provides an attractive approach for fighting the infectious diseases.\(^4,5\) The objective of this proposal is to explore a recently established bacterial protein glycosylation system to obtain polysaccharide conjugate vaccines in a facile, efficient, and easily applicable manner. This bacterial glycosylation system comprises two highly promiscuous proteins (PglB for N-glycosylation and PglL for O-glycosylation), which catalyze the transfer of polysaccharides from a diphospho-lipid donor to target proteins.\(^6,7\) The importance of the lipid moiety for PglL recognition has recently been investigated in this group. Currently, the peptide primary sequence specificity of the enzyme is being explored to obtain substrate requirements for PglL.

Chapter 4: Transglutaminase-mediated modification of soy protein

Protein-based polymers have attracted more attention as desirable alternatives to petroleum-based polymers due to the natural limitation of petroleum resources, and environmental concerns. Soybean protein, the by-product of soybean oil production, is one of the most abundant natural polymers in the US. Utilization of soy protein in the
production of biodegradable adhesives, plastics or other materials will help to alleviate environmental issues, in addition to adding value to soy crops.

The major drawbacks of natural soy protein are low protein strength and poor water resistance. Chemical modification has been used to modify soybean proteins to improve their structures and characteristics\textsuperscript{8,9}. However, the use of enzymes in the processing of agricultural products is viewed as an environmentally safe procedure; similar results are achieved without exposure to harsh chemicals.

Transglutaminase catalyzes an acyl transfer reaction between the $\gamma$-carboxyamide group of a peptide-bound glutaminyl residue and a variety of primary amines, including the amino group of lysine. Reaction with a protein-bound lysine creates a cross-linked protein with increased protein strength and water resistance, rendering soy protein more useful for industrial applications.
1 Chapter 1: Design and synthesis of a rebeccamycin/granulatimide anti-cancer derivative

1.1 Introduction

Most cancer cells have an inoperative G1 checkpoint due to inactivation of the p53 tumor suppressor gene but a functioning G2 checkpoint. It has been proposed that treatment with radiotherapy or DNA-damaging chemotherapeutic agents in combination with drugs that inhibit the G2 checkpoint might promote the selective killing of tumors bearing p53 mutations, thereby providing therapeutic benefit.

Several naturally existing antibiotics have been found to act as antitumor agents and inhibitors of topoisomerase and/or kinases. Rebeccamycin (Reb) (Figure 1.1), a microbial metabolite isolated from cultures of *Saccharothrix aerocolonigenes*, is a well-known topoisomerase I inhibitor, introducing breaks in eukaryotic DNA. Research has indicated that the indolocarbazole chromophore intercalates into DNA and the sugar moiety reinforces the affinity for DNA by interacting with the grooves.
Granulatimide (Gra) and isogranulatimide (Figure 1.2), isolated from the ascidian *Didemnum granulatum*, have been shown to act as G2 specific cell cycle checkpoint inhibitors. Studies have demonstrated that the imide nitrogen and a basic nitrogen at position 14 or 15 in the imidazole ring are important for checkpoint inhibition. These compounds show structural resemblance to the aglycon of rebeccamycin and related derivatives, such as UCN-01, a potent bisindolemaleimide inhibitor of protein kinase Cβ and of the checkpoint kinase Chk1. Research has indicated that, like UCN-01 (Figure 1.2), isogranulatimide binds in the ATP-binding pocket of Chk1 and hydrogen bonds with the backbone carbonyl oxygen of Glu and the amide nitrogen of Cys. The basic N15 of isogranulatimide interacts with Glu, causing a conformation change in the kinase glycine-rich loop that may contribute importantly to inhibition.
We have developed a Reb/Gra hybrid analog (Figure 1.3), which could potentially demonstrate DNA binding capability, similar to that of rebeccamycin. Its aglycon shows high similarity to that of the granulatimides, which indicates the potential for inhibition of G2 checkpoint kinases.
1.2 Targeting the Cell Cycle

Cancer therapy has recently been directed at elements of the mammalian cell cycle. The cell cycle consists of several phases which lead to the successful duplication of the entire cell. In the first growth phase (G1), the cell undergoes normal biosynthesis and metabolism; particularly important is the production of enzymes required for DNA synthesis. The synthesis (S) phase follows, during which DNA replication results in duplication of the entire genome in preparation for cellular division. Upon completion, the cell enters the second growth phase (G2) wherein microtubules and other biomolecules necessary for mitosis are produced.21,22

The cell cycle is controlled by sets of cascading signals called checkpoints, which either halt the cycle or allow it to proceed to the next phase. The G1 checkpoint, or restriction point, is responsible for determining whether the cell proceeds toward division, or enters a resting stage, as is the case with most human cells. The G1 checkpoint ensures that cells are large enough and contain sufficient nutrients to support daughter cells. The G2 checkpoint ensures that DNA synthesis in S-phase has been completed successfully and without error.22,23

An important function in the restriction point is mediated by the p53 tumor suppressor gene. Upon DNA damage, p53 is activated by the dissociation of the p53 from its negative regulator mdm2. Once activated, p53 will either induce a cell cycle arrest to allow for repair and survival of the cell, or apoptosis to discard the damaged cell. (Figure 1.4)
One enzyme that is operative in the p53 pathway is topoisomerase. Topoisomerases assist in the relaxation of supercoiled DNA so that replication and transcription can occur. Topoisomerase I (Topo I) functions by creating a nick in one strand of DNA, then resealing the lesion after the double helix has rotated. (Figure 1.5)
The mechanism (Figure 1.6) proceeds through the formation of a covalent intermediate, the “cleavable complex” between a tyrosine residue in the active site of Topo I and the 3’ DNA terminus. Reverse trans-esterification reseals the cleavage after the passage of the other strand through the “cleavable complex”.14,24,25
In the cell cycle, checkpoint kinase 1 (Chk1) is activated by a complex of checkpoint proteins which recognize unreplicated or damaged DNA. Activated Chk1 then phosphorylates and inhibits the cdc25 protein phosphatase, which prevents the removal of inhibitory phosphates from the Maturation Promoting Factor (MPF) complex, thereby stalling the cell in the G2 phase until errors can be corrected. (Figure 1.7)
Many of the cancer cells with an inoperative G1 checkpoint due to inactivity of the p53 tumor suppressor gene still retain a functioning G2 checkpoint. Treatment of such cells with DNA damaging agents should activate the G2 checkpoint cascade; however, when inhibitors of the G2 checkpoint are also administered, abrogation of the G2 checkpoint might promote the selective killing of these tumors which already have a non-functional G1 checkpoint by forcing them into a premature & lethal mitosis, termed “mitotic catastrophe”.\textsuperscript{10,11} As a note, inhibitors of the G2 checkpoint when used alone should have no effect on normal or cancer cells. Even used in combination with a DNA-damaging
1.3 Rebeccamycin Analogues as Topoisomerase I Inhibitors

DNA topoisomerase I is a nuclear enzyme that controls the topological state of DNA and is implicated in various vital processes such as replication, transcription, recombination, chromosome condensation / opening and mitosis.\textsuperscript{26,27} Topoisomerase I catalyzes unwinding of supercoiled DNA by nicking and rejoining a single strand of DNA.\textsuperscript{25,28} Eukaryotic topoisomerase I cleaves the DNA by becoming covalently linked via a tyrosine residue (Y723) to the 3' DNA terminus (\textbf{Figure 1.6}). The covalent intermediate called “cleavable complex” allows the passage of the other strand through the break after which reverse trans-esterification reseals the cleavage.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figures/camptothecin.png}
\caption{Camptothecin and Derivatives}
\end{figure}
A well-known family of topoisomerase I poisons consists of camptothecin and its derivatives (Figure 1.8).\textsuperscript{29-31} Topotecan and irinotecan currently administered as chemotherapeutic agents against resistant cancers.\textsuperscript{32-35}

![Chemical structure of Topotecan](image)

**BE-13793C**

Figure 1.9: Natural Indolocarbazole Topo I Inhibitor

Interest in indolocarbazole compounds appeared with the discovery of the ability of BE-13793C to inhibit topoisomerase I (Figure 1.9).\textsuperscript{36} This compound showed inhibitory activity toward topoisomerase I and topoisomerase II with an IC\textsubscript{50} value of 2 µM for both enzymes. Rebeccamycin, isolated from cultures of *Saccharotrix aerocolonigenes*, possesses an indolocarbazole framework linked to a carbohydrate moiety via one of the indole nitrogens.\textsuperscript{12,13} Rebeccamycin was also found to inhibit topoisomerase I with an IC\textsubscript{50} value of 1.75 µM; however, it was inactive toward topoisomerase II and PKC (IC\textsubscript{50} >100 µM).\textsuperscript{37}
Comparison of the topoisomerase I-mediated DNA single-strand breaks revealed by gel electrophoresis in the absence and presence of the drug indicates that, like camptothecin (Figure 1.8), another Topo I inhibitor, rebeccamycin inhibits the activity of topoisomerase I by stabilizing the DNA-topoisomerase I "cleavable complex". The presence of the sugar moiety is critical for the drug ability to interfere with topoisomerase I since the corresponding aglycon without the carbohydrate unit, contrary to that observed for BE-13793C, has no effect on the activity of the enzyme.

To improve the solubility of rebeccamycin for therapeutic purposes, water soluble derivatives have been prepared by introducing an aminoalkyl group at different sites on the bacterial metabolite. Becatecarin (Figure 1.10) is a diethylaminoethyl analog of rebeccamycin. Although becatecarin had undergone several clinical trials, recent research shows that the rebeccamycin derivative was found to lead to increased drug resistance. Robey and coworkers found becatecarin to be a weak substrate for ABCG2 and, at high concentrations, becatecarin was found to act as an ABCG2 inhibitor. Their results suggest that becatecarin may induce ABCG2 in tumors leading to increased resistance to other ABCG2 substrate drugs.
A large number of rebeccamycin derivatives have been prepared in order to elucidate the structure-activity relationships, a sample of which is shown in Figure 1.11 and Table 1.1.\textsuperscript{14} In the aglycon series as well as for the compounds with the sugar unit, the presence of the chlorine atoms on the indole residues proved to be detrimental to topoisomerase I inhibition. Compared with the corresponding aglycons, the presence of the sugar moiety enhanced the activity of the compounds toward topoisomerase I as well as the relative cytotoxicities. The sugar residue attached to the indolocarbazole chromophore proved essential for efficacy against the activity of topoisomerase I as well as for the formation of intercalation complexes.
Table 1.1: Inhibitory Potencies of Compounds 1-19 Toward Topoisomerase I. Antiproliferative Activities In Vitro Against Murine P388 Leukemia Cells and B16 Melanoma Cells

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Topoisomerase I MIC µM</th>
<th>P388 leukemia IC_{50} µM</th>
<th>B16 melanoma IC_{50} µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>1.75</td>
<td>1.22</td>
<td>0.48</td>
</tr>
<tr>
<td>1.2</td>
<td>0.17</td>
<td>0.17</td>
<td>0.3</td>
</tr>
<tr>
<td>1.3</td>
<td>0.51</td>
<td>0.85</td>
<td>0.7</td>
</tr>
<tr>
<td>1.4</td>
<td>1.63</td>
<td>0.49</td>
<td>nd</td>
</tr>
<tr>
<td>1.5</td>
<td>0.59</td>
<td>0.69</td>
<td>0.90</td>
</tr>
<tr>
<td>1.6</td>
<td>0.19</td>
<td>0.58</td>
<td>4.1</td>
</tr>
<tr>
<td>1.7</td>
<td>0.19</td>
<td>5.79</td>
<td>5.8</td>
</tr>
<tr>
<td>1.8</td>
<td>0.18</td>
<td>0.55</td>
<td>nd</td>
</tr>
<tr>
<td>1.9</td>
<td>1.80</td>
<td>5.4</td>
<td>&gt;125</td>
</tr>
<tr>
<td>1.10</td>
<td>0.02</td>
<td>6.1</td>
<td>nd</td>
</tr>
<tr>
<td>1.11</td>
<td>32</td>
<td>12</td>
<td>4.8</td>
</tr>
<tr>
<td>1.12</td>
<td>&gt;26</td>
<td>9.2</td>
<td>nd</td>
</tr>
<tr>
<td>1.13</td>
<td>25</td>
<td>&gt;25</td>
<td>24</td>
</tr>
<tr>
<td>1.14</td>
<td>31</td>
<td>12</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

(Continued)
(Table 1.1 Continued)

<table>
<thead>
<tr>
<th>1.15</th>
<th>29</th>
<th>&gt;29</th>
<th>&gt;250</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.16</td>
<td>&gt;29</td>
<td>&gt;29</td>
<td>4.6</td>
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<tr>
<td>1.17</td>
<td>2.7</td>
<td>9.5</td>
<td>&gt;100</td>
</tr>
<tr>
<td>1.18</td>
<td>&gt;24</td>
<td>&gt;24</td>
<td>17</td>
</tr>
<tr>
<td>1.19</td>
<td>&gt;23</td>
<td>&gt;23</td>
<td>nd</td>
</tr>
</tbody>
</table>

In order to investigate the role of the sugar moiety, Prudhomme and colleagues synthesized rebeccamycin analogues possessing various sugar units and compared their biological activities with the parent Reb\textsuperscript{41}. Since N-methylation at the imide nitrogen was found to be consistent with topoisomerase I inhibition, N-methylated aglycon was used for coupling with various natural carbohydrate units via α or β-N-glycosidic bonds (Figure 1.12, Table 1.2).

![Figure 1.12: Natural sugar conjugated rebeccamycin analog library](image)

Figure 1.12: Natural sugar conjugated rebeccamycin analog library
Table 1.2: Inhibitory Potencies of Rebeccamycin Analogues Toward PKC and Topoisomerase I. Antiproliferative Activities In Vitro Against Murine B16 Melanoma and P388 Leukemia Cells

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Topoisomerase I MIC µM</th>
<th>IC$_{50}$ B16 melanoma µM</th>
<th>IC$_{50}$ P388 leukemia µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reb</td>
<td>1.75</td>
<td>0.48</td>
<td>1.22</td>
</tr>
<tr>
<td>1.20</td>
<td>0.6</td>
<td>1.06</td>
<td>0.7</td>
</tr>
<tr>
<td>1.21</td>
<td>&gt;20</td>
<td>0.52</td>
<td>6.0</td>
</tr>
<tr>
<td>1.22</td>
<td>2.0</td>
<td>3.3</td>
<td>6.0</td>
</tr>
<tr>
<td>1.23</td>
<td>6.0</td>
<td>nd</td>
<td>6.0</td>
</tr>
<tr>
<td>1.24</td>
<td>0.6</td>
<td>5.4</td>
<td>6.0</td>
</tr>
<tr>
<td>1.25</td>
<td>&gt;20</td>
<td>4.75</td>
<td>6.2</td>
</tr>
<tr>
<td>1.26</td>
<td>2.0</td>
<td>3.75</td>
<td>6.2</td>
</tr>
</tbody>
</table>


For their DNA unwinding studies, the lab treated closed circular DNA with topoisomerase I either in the absence or presence of the derivatives at 30 µM. In the absence of any Reb analog, supercoiled DNA was relaxed by Topo I, as evidenced by agarose gel electrophoresis. In the presence of compounds 1.22 and 1.24 containing β-linked sugars, the relaxation was totally inhibited; whereas the relaxation was much less affected by the corresponding α-anomers 1.21 and 1.23. Neither of the fucose derivatives had a strong effect; it was noted that the β-anomer 1.26 had a very slight inhibitive effect on the relaxation whereas the α-anomer 1.25 had no effect at all. This group also found the presence of chlorine atoms to be detrimental to Topo I activity. Rebeccamycin and its N-methyl-maleimide derivative (not shown), both of which contain chloro groups on the indolocarbazole ring, have little effect on the Topo I-mediated DNA relaxation. In contrast, the analogue 1.20, lacking the chlorine atoms, completely inhibited the relaxation of supercoiled DNA.
Additionally, for each compound, the minimum drug concentration at which topoisomerase I-mediated DNA cleavage could be detected was determined. In agreement with the unwinding experiments, compounds bearing a β-N-glycosidic bond are more potent topoisomerase I inhibitors than the α-anomers. However the results show very little differences in terms of cytotoxicities, suggesting that topoisomerase I inhibition may not be the only factor in the cytotoxicity of these compounds. A linear DNA ligation assay was used to characterize the effect of intercalating agents. This assay is based on the circularization of the linear pAT DNA (cut with EcoRI) in the presence of DNA ligase; this enables the determination of the extent of interference of the various indolocarbazoles on the rate of formation of circular DNA molecules and multimers. This data also confirmed that the β-L-fucosyl derivative 1.26 is considerably less effective than its glucosyl and galactosyl counterparts, suggesting that L-fucose derivatives do not intercalate efficiently into DNA. This may be due to the influence of the C6 hydroxyl group on the sugar unit; this position could play a key role in the interaction with DNA. The effect of the 4-O-methyl-β-glucose derivative 1.20 on DNA unwinding and ligation is significantly less pronounced than that of the β-glucose derivative 1.22 indicating that intercalation of the methoxy derivative may not be as complete as it is with the hydroxy derivative. From this data, it can be concluded that the α-configuration in these analogs precludes intercalative binding of the drug to DNA.

In another study, Bailly, et al. reported the analysis of a rebeccamycin analog conjugated with a 2’-glucosamine (1.28, Figure 1.13). The research concluded that, although the presence of the amino group contributed to tighter binding with DNA, the alteration did
not confer enhanced topoisomerase poisoning or cytotoxicity. The binding affinity may
have been enhanced simply by the addition of a positively charged group, which need not
participate in any specific molecular interactions. Additionally, the presence of this group
may have resulted in decreased cellular uptake, thereby reducing potential cytotoxicity.
Upon further study, it was also found that the presence of formaldehyde allowed for the
formation of a covalent drug-DNA complex.\textsuperscript{42}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{glucosamino-reb-derivative.png}
\caption{Glucosamino-Reb derivative}
\end{figure}

<table>
<thead>
<tr>
<th>Compound</th>
<th>Topo I MIC</th>
<th>P388 μM IC\textsubscript{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.27</td>
<td>0.191</td>
<td>0.57</td>
</tr>
<tr>
<td>1.28</td>
<td>0.186</td>
<td>0.84</td>
</tr>
</tbody>
</table>

1.4 Other Rebeccamycin Analogues

Several different families of rebeccamycin analogues have been studied over the years.\textsuperscript{43}

The variety of alterations include:

- modifications at the imide heterocycle\textsuperscript{14,37,39,41}
- Fluoro-\textsuperscript{44} and hydroxy-\textsuperscript{36,45} substitutions on the indole moieties
- construction of dimers\textsuperscript{46}
- coupling the sugar unit to the second indole nitrogen\textsuperscript{47}
- "inverting" the indolo[2,3-a]carbazole skeleton to indolo[2,3-c ]carbazole\textsuperscript{47}
- replacing one or both indole moieties by 7-azaindole units\textsuperscript{48,49}

Modifications of the imide portion of the molecule included various substitutions on the imide nitrogen of both hydrophilic & hydrophobic moieties. These were found to have little effect on the activity of the compound.\textsuperscript{50} When the imide function was replaced by an amide function, a non-negligible inhibition of PKC was observed.

Fluoro substituents were introduced in the various positions by feeding experiments with addition of 6-fluoro, 5-fluoro or 4-fluorotryptophan to the fermentation broths, leading to the formation of 2, 10-difluoro, 3, 9-difluoro and 4, 8-difluoro analogs, respectively (Figure 1.14).\textsuperscript{44,51}
Figure 1.14: Fluoro- & Hydroxy-substituted Indolocarbazoles

The most cytotoxic compound in this series was determined to be the 4'-demethylated-3,9-difluoro compound, which demonstrated ~10-fold more potency toward topoisomerase I than camptothecin. The 4'-demethylated-4,8-difluoro analogue was interestingly 10-fold less potent than camptothecin. The 2,10-difluoro-4'-methylated and 4'-demethylated analogues were shown to be equal to camptothecin for inducing Topo I-mediated DNA cleavage. It has been shown that the presence of the methyl group at the 4'-O position (as in natural rebeccamycin) has little effect on potency for inducing Topo I-mediated DNA cleavage; however it did show increased cytotoxicity against P388 murine leukemia cells, again almost 10-fold. As observed for the fluoro-analogs, compounds bearing hydroxy substituents in the 3, 9-positions induced the strongest cytotoxicity and Topo I inhibition.

In an effort to increase the affinity for DNA and further stabilize the ternary complex DNA-Topo I-drug, dechlorinated rebeccamycin dimers were produced. Two potential
bis-intercalating agents were prepared by coupling at the imide nitrogens.\textsuperscript{46} (Figure 1.15) Compared with dechlorinated rebeccamycin, only dimer A exhibited a higher affinity; the more rigid aromatic linker potentially being too short to enable bis-intercalation. Neither dimer, however, led to enhanced topoisomerase I inhibition.

![Figure 1.15: Rebeccamycin Dimers](image)

Coupling of the Reb sugar to the second indole nitrogen leads to an analog with greater similarity to the structure of staurosporine. As reported earlier, staurosporine shows inhibitive activity toward kinases, but not Topo I. Derivatives coupled at the 1’ and 2’ positions were prepared in an effort to elucidate the importance of this connection.\textsuperscript{47} The
imide function was also reduced to an amide to yield staurosporine analogs (Figure 1.16). Not surprisingly, compound 1.29 proved to be the most efficient Topo I inhibitor; it also showed the greatest antiproliferative activity against B16 melanoma and P388 leukemia cells. None of these compounds exhibited activity against protein kinase C, a target of staurosporine.

![Rebeccamycin/Staurosporine Analogs](image)

Figure 1.16: Rebeccamycin/Staurosporine Analogs

To investigate the effect of the orientation of the sugar moiety and of the indolocarbazole framework on the biological activity, "inverted" rebeccamycins possessing an indolo[2,3-c]carbazole chromophore instead of the conventional indolo[2,3-α]carbazole unit were synthesized\(^5\) (Figure 1.17). The experiments indicated that the mono-glycosylated analogue 1.32 retained the capacity to intercalate into DNA, but the structural change was detrimental to Topo I inhibition and antiproliferative activities. The bis-glycosylated derivative 1.33 lost its capacity to form stable complexes with DNA.
Derivatives which had undergone replacement of one or both indole moieties by azaindole units were prepared in order to determine if the shift to a heteroatom would enhance the binding of the drugs to the target macromolecules. In 7-aza-rebeccamycin analogues, the replacement of carbon atoms by nitrogen atoms introduces a basic character into the molecule that can modify the pharmacological properties. These nitrogen atoms may also contribute to the formation of hydrogen bonds within the active site of target enzyme(s) and/or DNA and thereby stabilize or modify the orientation of the drug in the drug-enzyme complexes. Examples are shown in Figure 1.18.
Considering the analogs wherein both indole rings were replaced with azaindole functions, the diaza-analogue 1.35, with the free imide nitrogen, exhibits the strongest cytotoxicity toward murine leukemia (L1210) cells, being slightly more potent than rebeccamycin and dechlorinated rebeccamycin, and about ten times more efficient than the N-methyl bisaza-analog 1.34. To assess the selectivity of these bisaza-compounds, the cytotoxicity profile of 1.34 was compared with those of Reb, dechloro-Reb and the β-glucosylated derivative 1.22. The antiproliferative activities against nine tumor cell lines were examined: murine leukemia (L1210), human leukemia (K-562) and seven human solid tumors: ovarian carcinoma (IGROV1), neuroblastoma (SK-N-MC), small-cell lung carcinoma (H69), colon carcinoma (HT29), non-small cell lung carcinoma (A549), and two epidermoid carcinomas (A431 and KB-3-1). While 1.22 is almost equally potent in all the cell lines tested (IC$_{50}$ range of 0.25–0.88 mM), 1.34 appears highly cytotoxic for
A431 cells, slightly less cytotoxic for SK-NMC, NCI-H69 and inactive in IGROV1 cells (data not reported). Reb was also non selective. This different cytotoxicity profile suggests that the aza-analogues do not share the same mechanism of action, or that additional targets are affected by the non aza-analogues. Topo I inhibition evaluation was not completed on the bis-aza-analogs.

For the analogs containing one azaindole substitution, it was found that the position of the newly incorporated nitrogen atom in the aromatic framework had a major effect on the DNA binding capacity of the drug. Based on melting temperature ($T_m$) experiments, results indicated that when the carbohydrate is linked to the indole moiety and the azaindole is unsubstituted, DNA binding is enhanced. Both compounds 1.36 and 1.37 give higher $\Delta T_m$ values than that of dechlorinated rebeccamycin. When the azaindole bears the sugar, DNA binding capacity is abolished. Compounds 1.38 and 1.39 failed to elevate the melting temperature of DNA, suggesting that they have little, if any, interaction with DNA. Analog 1.37 showed significantly greater Topo I inhibition than analog 1.39. This could be correlated with the higher DNA affinity of 1.37 vs. 1.39, yet previous studies have shown that there is no direct relationship between DNA binding affinities and topoisomerase I inhibition.

In terms of cytotoxicity, the aza-compounds, 1.36-1.39 with one azaindole moiety, were tested against the nine tumor cell lines mentioned above. Table 1.4 shows no marked differences in cytotoxicity toward the murine leukemia cell line (L1210), regardless of the position of the carbohydrate, pointing to the possibility of different biological targets. As with the bis-aza analogs, these compounds exhibited greater selectivity than the
indolocarbazole compounds. Interestingly, almost identical profiles of cytotoxicity were observed with all the aza derivatives, suggesting a common mechanism of action.

Table 1.4: In Vitro Antiproliferative Activities against Nine Tumor Cell Lines

<table>
<thead>
<tr>
<th>Analog</th>
<th>L1210</th>
<th>IgROV</th>
<th>SK-N-MC</th>
<th>HT29</th>
<th>A549</th>
<th>A431</th>
<th>NCI-H69</th>
<th>K-562</th>
<th>KB-3-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reb</td>
<td>0.14</td>
<td>0.25</td>
<td>0.1</td>
<td>0.3</td>
<td>0.3</td>
<td>0.25</td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>d-Reb</td>
<td>0.11</td>
<td>2.6</td>
<td>0.1</td>
<td>2.5</td>
<td>2</td>
<td>3.8</td>
<td>0.1</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>1.22</td>
<td>0.67</td>
<td>0.88</td>
<td>0.25</td>
<td>0.86</td>
<td>0.94</td>
<td>0.84</td>
<td>0.33</td>
<td>ne</td>
<td>0.6</td>
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<td>1.3</td>
<td>ne</td>
<td>ne</td>
<td>17.8</td>
<td>47.2</td>
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<td>ne</td>
</tr>
<tr>
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<td>8.5</td>
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<td>0.7</td>
<td>0.11</td>
</tr>
<tr>
<td>1.39</td>
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<td>100</td>
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</tr>
<tr>
<td>1.37</td>
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<td>ne</td>
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<td>5.3</td>
<td>ne</td>
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<td>ne</td>
<td>ne</td>
</tr>
</tbody>
</table>


1.5 Derivatization of Indolocarbazole Compounds with Uncommon Sugars

Three approaches to the modification of indolocarbazole derivatives have been reported:

(i) replacement of the hydrogen on the imide functionality with other groups, especially a hydrophilic group;

(ii) substitution on the fused aromatic ring or a change of the benzene ring to another hetero-aromatic ring, and

(iii) Replacement of the β-glycoside with other sugar moieties.

Most of the current modifications through the first two approaches work to improve the water solubility of the compounds. A few papers in recent years reported the synthesis and activity of indolocarbazoles with a modified sugar moiety. These studies suggest
that altering glycosylation patterns on indolocarbazole has a high potential for generating novel chemotherapeutics. The discovery of new drug candidates is frequently promoted by the diversification of glycosylated natural products through the strategy called “glycorandomization”.

Rebeccamycin derivatives (primarily β-N-glycosylated) synthesized in the Wang group, employing the glycorandomization strategy, are shown in Figure 1.19.\textsuperscript{56}

![Rebeccamycin derivative library](image)

Figure 1.19: Rebeccamycin derivative library

The synthesis of these derivatives demonstrated the difficulty in glycosylation of the pre-formed indolocarbazole backbone. With such a convergent design, although it is considerably more favorable to form the indolocarbazole core prior to attachment of the
carbohydrate library, it was found that the indolocarbazole acceptor is a weaker nucleophile than the bis(indolyl)maleimide. While an effective method for the β-glycosylation of this framework (Figure 1.20), discovered by Ohkubo\textsuperscript{55}, was used to produce derivative 1.41, this methodology could not be applied to the remainder of the library, which consisted of 2-deoxysugars. The glycosyl-chloride donor in heterogeneous basic media yielded only the α-isomer in this case. Although the Mitsunobu reaction was ineffective for glycosylation of the indolocarbazole core (1.40), it proved to be the most efficient method for the formation of the β-N-glycosyl-bis(indolyl)maleimide (Figure 1.21).

Figure 1.20: Glycosylation of indolocarbazole core
The cytotoxicities of the rebeccamycin conjugates were tested with MTS assay in two cancer cell lines: colorectal carcinoma cells (SW620) and leukemia cells (K562) (Table 1.5). Rebeccamycin (Reb) and analogues 1.41, 1.44, 1.45, 1.47, 1.48 – all having a
methylated maleimide nitrogen and conjugated with different sugar moieties are much more active than the corresponding aglycon 1.40. Interestingly, compound 1.41 displayed similar activity to Reb, despite the substitution of chlorine atoms at the C1, C11 positions and methyl substitution at the N6 position. These results suggest that the carbohydrate moiety in each of these conjugates is a key element for its cytotoxicity. In comparing α-linked compounds (1.44, 1.47) with the β-linked compounds (1.45, 1.48), no difference in activity was observed. It also appears that the 2-deoxyglucose compounds (1.47, 1.48) show slightly higher activity than the corresponding 2,6-dideoxyglucose compounds (1.44, 1.45). Compound 1.49, conjugated with a 2,3,6-trideoxy-3-benzylamino glucose, showed the least cytotoxicity, with activity similar to that of the aglycon 1.40. The above results indicate that hydroxyl groups at the 2-, 3-, and 6-positions in the carbohydrate moiety could play a very important role in the anticancer activity. The large drop in activity in compound 1.49 suggests that the 6-position may be of particular importance, as previously noted with the activity studies of the fucosyl-derivatives 1.25 & 1.26.

In view of the substitution at the N6 position of the imide, data shows that compound 1.43, with propylimidazole substitution showed identical activity to that of Reb and 1.41, which indicates that the sugar moiety has a stronger effect on activity than N6 substitution; a hypothesis which was confirmed by the activity of compound 1.42. With a benzylated sugar moiety, the compound was devoid of anticancer activity (IC₅₀ > 50 µM). Compound 1.46, with NH₂ attached at N6 of the imide, did not rescue the loss of activity demonstrated by with 2,6-deoxyglucose conjugates (compare to 1.44), rather, compound 1.46 also shows complete loss of activity (IC₅₀ > 100 µM).
Comparing compounds 1.49 and 1.50, the anhydride function does not appear to confer higher activity than the amide function. For compounds 1.50 and 1.51, deprotection of the sugar C3-NH₂ did not confer greater anticancer activity, indicating either that the C3-OH in the sugar moiety is critical, or perhaps the polarity of this position is not a key element for its activity. Further validation and analysis with different compounds would need to be performed for further clarification.

The conjugate library was also tested for inhibition of topoisomerase I, using in vivo complex of topoisomerase (ICT) bioassay in HeLa cells. Formation of the covalent complex (topoisomerase/DNA) is essential for the cytotoxic action of the topoisomerase poisons. The unique nature of topoisomerase/DNA interaction (viz., formation of the covalent intermediate) can be exploited to quantify topoisomerase-mediated DNA damage in the intact cell. In the presence of Topo I poisons, the DNA breaks are stabilized and a covalent Topo I-DNA complex can be trapped upon addition of protein denaturants. The standard ICT assay was performed comparing camptothecin (CPT) with three rebeccamycin analogues (1.41, 1.44, and 1.47) that differ only in the carbohydrate side chains; compound 1.41 was linked with glucose, compound 1.47 with 2-deoxyglucose, and compound 1.44 with 2,6-deoxyglucose. To ensure uniformity between six different treatments, a range of DNA concentrations was probed from 0.01 to 5 µg; thus, the signals can be directly compared between each culture. Very surprisingly, their topoisomerase inhibition is in agreement with the anticancer activity (Figure 1.22).
Figure 1.22: Standard ICT assay in HeLa cells comparing camptothecin (CPT) with Rebeccamycin-sugar derivatives 1.41, 1.44, and 1.47. Exponentially growing HeLa cells were incubated with the compounds indicated to the right of the blot for 30 min at 37 °C, followed by sarkosyl lysis and a standard ICT assay (CsCl gradient separation of DNA, recovery of DNA and spotting from 0.01 to 5 µg of DNA on the blot). The blot was probed with anti-Topo I antibody. Signals were developed using ECL and a short (1 min) exposure. (Reprinted with permission from Zhang, G., et al., Syntheses and Biological Activities of Rebeccamycin Analogues with Uncommon Sugars. Journal of Medicinal Chemistry, 2005. 48(7): p. 2600. Copyright 2005 American Chemical Society)

Compound 1.41 with the glucose moiety showed strong topoisomerase inhibition similar to that of camptothecin (positive control) at the same molar concentration, indicated by the formation of Topo I-DNA complex in the immunoblot. However, compound 1.47
with the 2-deoxy-D-glucose moiety gave a very weak (but detectable on longer exposure) signal while compound 1.44 with 2,6-dideoxy glucose moiety was inactive. Clearly, the 2-OH and 6-OH of the sugar moiety in rebeccamycin analogues strongly modulate drug activity in topoisomerase I inhibition in vivo. This was regarded as surprising since the N6 region of rebeccamycin was previously thought to be involved in topoisomerase inhibition while the sugar moiety was involved in DNA interaction. The results indicated that the sugar moiety also modulated the topoisomerase I activity, a theory deemed worthy of further investigation with various modifications in sugar moiety.

1.6 Pyrrolocarbazoles as Checkpoint 1 Kinase Inhibitors

The first pyrrolocarbazoles identified as G2 checkpoint inhibitors were granulatimide and isogranulatimide, natural compounds isolated from the ascidian Didemnum granulatum (Figure 1.23).18,19 These compounds were found to inhibit the G2 checkpoint with IC\textsubscript{50} values of 2-3 µM.20

Figure 1.23: G2 Checkpoint Inhibitors
To assess the importance of the imide nitrogen, 10-methylisogranulatimide (Figure 1.24) was prepared by alkylation of isogranulatimide. This N-methylated compound proved to be about 15-fold less potent than isogranulatimide on the G2 checkpoint assay\textsuperscript{20} suggesting that the imide NH participates in hydrogen bonding with its target. By analogy with the prototypical Chk1 inhibitor indolocarbazole UCN-01, bearing a hydroxylactam moiety, 9-hydroxyisogranulatimide was prepared (Figure 1.24).\textsuperscript{20} However, 9-hydroxyisogranulatimide did not exhibit significant activity in the G2 checkpoint assay. Synthetic derivatives, isogranulatimides B and C, inhibited the G2 checkpoint with IC50 values of 1 and 6 µM, respectively.\textsuperscript{58}
Of these compounds, those displaying the most efficient inhibition of the G2 checkpoint were evaluated for their activity toward Chk1. In comparison with the inhibitory activity of UCN-01 (IC$_{50}$ = 7 nM), the activities of granulatimide analogues were weaker (IC$_{50}$ values: granulatimide 0.25 µM, isogranulatimide 0.1 µM, isogranulatimide B 2.3 µM and isogranulatimide C 0.65 µM). To elucidate the structural basis for the inhibition of Chk1 by isogranulatimides, the crystal structure of an isogranulatimide-Chk1 complex was determined and compared to that of the UCN-01-Chk1 complex (Figure 1.25). Two fundamental hydrogen bonds are conserved between both compounds and the ATP-
binding pocket of Chk1: between the maleimide NH and the carbonyl oxygen of Glu$^{85}$, and between the carbonyl oxygen on the left of the maleimide and the amide NH of Cys$^{97}$. The supplementary hydrogen bonds observed in the UCN-01-Chk1 complex are partially responsible for the better inhibitory activity of the glycosylated indolocarbazole compound.
Figure 1.25: Structural basis for Chk1 inhibition by isogranulatimide.
A: binding of isogranulatimide in the active site of Chk1: Chk1 atoms involved in binding to isogranulatimide or to UCN-01 binding. Dashed lines, hydrogen bonds between isogranulatimide and Chk1 (in Å); gray, inhibitor carbons; beige, enzyme carbons; red, oxygens; blue, nitrogens. B: binding of UCN-01 in the active site of Chk1. C: conformation change in the Chk1 glycine-rich loop induced by isogranulatimide binding. Green, glycine-rich loop in the absence of isogranulatimide (IGR); blue, glycine-rich loop in the presence of isogranulatimide. Only the side chain of Glu17 is displayed. (Reprinted from Jiang, X., et al., *Inhibition of Chk1 by the G2 DNA damage checkpoint inhibitor isogranulatimide*. Mol. Cancer Ther., 2004. 3(10): p. 1221-1227. Copyright 2004 American Association For Cancer Research)
To enhance the affinity for the ATP-binding pocket of Chk1, various glycosylated granulatimides structurally related to UCN-01 were designed. The sugar moiety in one set of compounds was attached to both indole and imidazole nitrogens to give a structure most similar to that of UCN-01. Another set of glycosylated granulatimides and isogranulatimides were synthesized in which the sugar part was attached either to the indole nitrogen as in rebeccamycin, or to the imidazole nitrogen (Figure 1.26). Neither the G2 checkpoint nor the Chk1 inhibitory properties of the glycosylated granulatimide derivatives were reported in the patent. Glycosylated aza-derivatives of granulatimide were also prepared; however, likely due to the presence of a methyl group on the maleimide portion of the molecule, poor Chk1 inhibitory activity was observed (Figure 1.27).
Figure 1.26: Glycosylated Granulatimides

UNC-01 related Glycosylated Granulatimides

Rebeccamycin-related Glycosylated (Iso)Granulatimides

Figure 1.26: Glycosylated Granulatimide Derivatives
1.7 Design of Click Rebeccamycin / Granulatimide Derivative

Based on the broad scope of previous research on Reb and Gra analogs, the varied functionality of such similar compounds piqued our interest. In an effort to obtain an analog with the desired effects, we have designed a model structure which contains elements of both rebeccamycin and granulatimide, giving the novel structure the potential to effect both the DNA damage and the G2 checkpoint inhibition necessary for the proposed therapeutic benefit, targeting tumors with an inactive G1 checkpoint/ restriction point. Glucose-conjugated examples are shown in Figure 1.28.
The design of the proposed structure incorporates a 1,2,3-triazole functionality, easily achievable through Cu-catalyzed azide-alkyne cycloaddition chemistry,\textsuperscript{60-62} to increase the simplicity of synthesis. The original synthetic plan was meant to be simple and straightforward, based on the synthetic schemes of many rebeccamycin derivatives. The method shown in Scheme 1.1 was effective for the production of the un-cyclized analogs, having the 1’-attachment to the carbohydrate moiety.
The synthesis began with Grignard activation of the indole for coupling with the dibromomaleimide to yield 1.52, followed by a Sonogashira coupling with TMS-acetylene to provide the protected alkyne, 1.53. After deprotection with cesium fluoride, the terminal alkyne was reacted with the commercially available protected azido-glucose in a copper-mediated alkyne-azide cycloaddition to provide the protected compound 1.54. Removal of the acetate groups in the presence of NaOMe in methanol yielded the desired derivative 1.55. Basic hydrolysis of the imide gave the anhydro-derivative 1.56.
Following deprotection of the carbohydrate moiety of 1.54, an initial attempt at formation of the indolyl-6'-glycosyl linkage in compound 1.58 under Mitsunobu conditions was unsuccessful (Scheme 1.2). The plan to tosylate the 6-OH position of the unprotected carbohydrate followed by peracetylation (1.57) should allow for the desired connection to be formed after activation of the indole NH by sodium hydride. A final deprotection should yield the desired product 1.58.

We hit an interesting challenge when it came to the formation of the closed-ring, aromatic derivatives. In the synthesis of rebeccamycin derivatives, photochemical oxidative cyclization is frequently used to form the indolocarbazole core. Such a method, however, could promote the breakdown of these triazoles, with the release of molecular
nitrogen. It was considered necessary, therefore, to plan an alternate route to the completion of this structure (Figure 1.29).

![Figure 1.29: Photochemical Oxidation Will Not Yield Cyclized Derivative](image)

The idea for the ring-closure was to take advantage of the mechanism of the “click” reaction by placement of an activating group at the proper point of the indole moiety. In theory, the synthesis would start with a Grignard addition of a 2-Y-indole, where Y is a protected functional group. After formation of intermediate B, the functional group, Y would be deprotected/activated to X (C) in order to act as a leaving group for coupling with Cu-aryl click reaction intermediate to form the desired C-C bond (Scheme 1.3).
In the search for previous work done in this area, a stellar example was found in the work reported by Ackermann. In their studies on the use of copper complexes for C-H bond functionalization, the researchers discovered that a single copper catalyst was able to promote “click” reactions as well as perform C–H bond functionalizations of the resulting 1,2,3-triazoles, allowing for the syntheses of fully substituted 1,2,3-triazoles (Scheme 1.4).
Scheme 1.4: Copper-Catalyzed “Click” Reaction/Direct Arylation Sequence

(Adapted with permission from Ackermann, L., et al., Copper-Catalyzed “Click” Reaction/Direct Arylation Sequence: Modular Syntheses of 1,2,3-Triazoles. Organic Letters, 2008. 10(14): p. 3081. Copyright 2008 American Chemical Society.)

The theory behind this idea is depicted in Figure 1.30. In the “click” mechanism shown, the terminal alkyne (green) is activated by the copper complex for cycloaddition with the azide (orange). The click intermediate is then coupled with the aryl iodide (black), to yield compound 1.60, with the desired C-C bond pre-formed. Activation of the 3-position of the indole for coupling with the bromomaleimide portion of the molecule, should yield the fully-cyclized product.
Figure 1.30: Mechanism of Formation of Linked Intermediate.
The reaction scheme based on this idea is shown in Scheme 1.5. The 2-I-indole was a simple derivative that could potentially give the desired coupling under click conditions. The challenge with using this methodology then became the necessary formation of the alkynyl-maleimide structure D. Single-substitution on the dibromomaleimide was unsuccessful under Sonogshira conditions (Scheme 1.6). It is highly possibly that the formation of the singly-substituted maleimide provided a stronger binding site for the Pd catalyst, which would explain the presence of only doubly-substituted product, regardless of reactant ratios employed.
Scheme 1.5: Cyclization Plan
Scheme 1.6: Attempt at Single Substitution Under Sonogashira Conditions

A second attempt at the formation on compound 1.61 was based on a reported selective cross-coupling reactions using indium organometallics. The work of Sarandeses, Sestelo and colleagues represents the first report of a general and convenient method for the synthesis of 3-halo-4-substituted maleimides utilizing cross-coupling reactions. Indeed, a similar structure to the desired was achieved in 40% yield using the reported methodology (Figure 1.31).
The formation of the tri-acetylindium was completed according to the reported procedure; however, its formation was not able to be confirmed by MS or NMR. The subsequent coupling reaction was performed following the reported procedure, however, none of the singly-substituted derivative could be detected by MS. (Scheme 1.7) The formation of the doubly-substituted maleimide, however, was detected.

Scheme 1.7: Attempt at Organoisindium Methodology
After the unsuccessful attempts at forming the 3-bromo-4-ethynylmaleimide, attempts were made to form the activated indole derivative, prior to coupling with the dibromomaleimide (Scheme 1.8). The 2-I-indole was prepared by a well-known method. Though the Grignard addition to form compound 1.63 was successful, further manipulation to add the acetylene function yielded only the di-ethynyl compound (1.65).

![Scheme 1.8: Coupling of iodoindole forms diacetylated derivative](image)

The next effort at achieving the desired substitution at position 2 of the indole (Scheme 1.9) involved the application of Boc protection to the indole nitrogen following the Grignard addition to the dibromomaleimide to yield 1.66. After addition of the TMS-alkyne, installation of the iodide was attempted using t-BuLi and 1,2-diiodoethane. This reaction, however, did not yield the desired product, 1.68.
Scheme 1.9: Attempt to Add Iodide Just Prior to Click Reaction

Continued efforts toward the fully cyclized derivative continue. Pre-installation of a more benign functional group, such as TMS, in the 2-position of the indole, which would allow further functionalization could lead to the successful synthesis of this structure.

1.8 Conclusion

Synthetic efforts toward completion of structural types III & IV will continue. The future plan for 1.58 (type III) will begin with that depicted in Scheme 1.2. Efforts toward structures of type IV will require a bit more effort and ingenuity. It has been suggested that placement of a silyl group at the 2-position of the indole might provide a useful handle for modifiable reactivity of this position.

Upon completion of samples of each of the four structural types, the compounds will undergo biological evaluation. Cell growth inhibition assays on several cell lines will be conducted, potentially including MCF-7, K562, MCF-7/DOX, K562/DOX, CPTv10c22 and m-AMSA. Combination treatment of the new analogs with the P-gp inhibitor cyclosporine A (CsA) on P-gp over-expression cell lines (MCF-7/DOX, K562/DOX) will also be performed. These experiments will not only evaluate the anti-tumor efficacies of
the synthetic analogs, but also determine whether the anti-tumor effect is correlated with their P-gp and/or topoisomerase inhibitory capacities. To further confirm the P-gp inhibition results, the analog uptake and efflux in the K562/DOX cell line will be determined in the presence or absence of the P-gp inhibitor CsA by FACS (fluorescent-activated cell sorting). To further confirm the topoisomerase inhibition results, the modified ITC (in vivo complex of topoisomerase) assay in the MCF-7 cell line will be performed. The top cell-growth inhibitors will be submitted to the NIH for the free NCI-60-Cell-Line screening. Additionally, the IC\textsubscript{50} values of these analogs on selected kinases (~10) will be determined.
1.9 Chapter 1 Experimental

3-bromo-4-(1H-indol-3-yl)-1-methyl-1H-pyrrole-2,5-dione: A solution of ethylmagnesium bromide was prepared from Mg (412.9 mg, 17 mmol) and C₂H₃Br (1.269 mL, 17 mmol) in THF (7.5 mL). The mixture was stirred for 1 h at room temperature and then indole (2.001 g, 17 mmol) in toluene (60 mL) was added dropwise. The mixture was stirred at room temperature for 1.5 h and then a solution of N-methyl-2,3-dibromomaleimide (1.195 g, 4.4 mmol) in toluene (60 mL) was added dropwise. After stirring for 20 min, dichloromethane (90 mL) was added, then the mixture was stirred for 65 h at rt. Saturated aqueous NH₄Cl was added. After extraction with EtOAc, the organic phase was dried over MgSO₄, the solvent was removed and the residue purified by flash chromatography (eluent hexane/EtOAc, 3:1) to give **1.52** (1.14 g, 3.73 mmol, 84% yield) as a rusty-orange solid.

MS, m/z [M+Na]⁺ : 326.9482 / 328.9438
3-(1H-Indol-3-yl)-1-methyl-4-trimethylsilanylethynyl-pyrrole-2,5-dione:

Pd(PPh₃)₂Cl₂ (37.3 mg, 0.053 mmol) and CuI (27.1 mg, 0.142 mmol) were added to a solution of 1.52 (299.9 mg, 0.983 mmol) in Et₃N (30 mL) and the resulting mixture was degassed with argon. Trimethylsilylacetylene (0.28 mL, 1.967 mmol) was added and the reaction stirred for 1 h. The mixture was poured into sat’d NH₄Cl (50 mL) and extracted with ether (3 x 50 mL). The combined organic layers were washed with brine, dried over Na₂SO₄, and concentrated under vacuum. The crude product was purified by flash chromatography (Hexanes: EtOAc 3:1) to yield 1.53 (271.8 mg, 85.7 %) as an orange solid.

MS (crude material), m/z [M+Na⁺]: 345.0766.

¹H NMR (500 MHz, CHLOROFORM-d) δ ppm 0.27 (s, 9 H) 3.13 (s, 3 H) 7.22 - 7.27 (m, 1 H) 7.28 - 7.34 (m, 1 H) 7.44 (d, J=8.20 Hz, 1 H) 8.29 (d, J=3.15 Hz, 1 H) 8.47 (d, J=7.88 Hz, 1 H) 8.91 (br. s., 1 H)
(2R,3R,4S,5R,6R)-2-(4-((1H-indol-3-yl)-1-methyl-2,5-dioxo-2,5-dihydro-1H-pyrrol-3-yl)-1H-1,2,3-triazol-1-yl)-6-(acetoxymethyl)tetrahydro-2H-pyran-3,4,5-triyl triacetate: To the solution of the 1.53 (51.9 mg, 0.16 mmol) in THF (10 mL) & MeOH (10 mL), cesium fluoride (26.7 mg, 0.18 mmol) was added. The reaction was monitored by TLC analysis (Hex: EtOAc 1:1). Upon the disappearance of the silylated starting material, glucosyl azide (63.0 mg, 0.16 mmol) was added, followed by sodium ascorbate (70.9 mg, 0.36 mmol) and CuSO₄·5H₂O (12.7 mg, 0.05 mmol). The mixture was stirred vigorously for 20.5 h, although TLC analysis did not indicate complete consumption of the alkyne intermediate. The reaction mixture was poured onto ice water, and extracted with EtOAc, dried with Na₂SO₄. The solvent was removed in vacuo and the crude product was purified by flash chromatography (5→75% EtOAc in hexanes)

MS, m/z [M+Na]+:646.1537, 413.1964(unidentified contaminant)

1H NMR (500 MHz, CHLOROFORM-d) δ ppm 1.88 (s, 3 H) 2.02 (s, 3 H) 2.05 (s, 3 H) 2.09 (s, 3 H) 3.16 (s, 3 H) 4.00 (ddd, J=10.09, 4.73, 1.89 Hz, 1 H) 4.17 (dd, J=12.61, 1.89 Hz, 1 H) 4.30 (dd, J=12.77, 4.89 Hz, 1 H) 5.26 (t, J=9.62 Hz, 1 H) 5.42 (dd, J=9.46, 9.10 Hz, 1 H) 5.49 (dd, J=9.46, 9.10 Hz, 1 H) 5.88 (d, J=9.14 Hz, 1 H) 6.97 (t, J=7.50 Hz, 1
H) 7.02 (d, $J=7.90$ Hz, 1 H) 7.12 (t, $J=7.60$ Hz, 1 H) 7.27 (d, $J=8.20$ Hz, 1 H) 8.00 (d, $J=2.84$ Hz, 1 H) 8.48 (s, 1 H) 9.13 (br. s., 1 H)

$^{13}$C NMR (126 MHz, CHLOROFORM-$d$) δ ppm 20.43, 20.74, 20.93, 24.53, 61.74, 67.90, 70.80, 72.83, 75.48, 86.15, 105.97, 111.99, 119.58, 121.05, 122.29, 123.07, 123.74, 125.31, 131.66, 132.91, 136.70, 138.83, 169.05, 169.53, 170.20, 170.79, 171.36, 171.51
3-(1H-indol-3-yl)-1-methyl-4-(1-((2R,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)-1H-1,2,3-triazol-4-yl)-1H-pyrrole-2,5-dione: To pentaacetate derivative 1.54 (25 mg, 0.040 mmol) in methanol (5 mL) at room temperature was added NaOMe solution (20 µL, 25 wt% in MEOH). The reaction turned from a yellow suspension to a dark brown solution within 20 min; it was allowed to stir at rt until completion (~1 h) (TLC 1:1 Hexanes: EtOAc & 10:1 EtOAc:MeOH). Acidic ion-exchange resin was added to achieve pH 7. The solution was filtered (over additional resin) and then the volatiles were removed in vacuo to give the unprotected product. The crude product was passed thru a small column to remove a contaminating product to yield the desired product 1.55 (16.6 mg, 91.2 %) as an orange solid.

\[^{1}H\] NMR (500 MHz, CHLOROFORM-\(d\)) \(\delta\):ppm 3.08 (s, 3 H) 3.45 - 3.59 (m, 3 H) 3.70 (dd, \(J=12.30, 5.36\) Hz, 1 H) 3.84 - 3.93 (m, 2 H) 5.62 (d, \(J=9.14\) Hz, 1 H) 6.77 (d, \(J=8.20\) Hz, 1 H) 6.89 (t, \(J=7.57\) Hz, 1 H) 7.10 (t, \(J=7.57\) Hz, 1 H) 7.38 (d, \(J=8.20\) Hz, 1 H) 8.11 (s, 1 H) 8.43 (s, 1 H)
$^{13}$C NMR (126 MHz, CHLOROFORM-d) $\delta$: ppm 24.48, 62.57, 71.04, 74.18, 78.70, 81.38, 89.79, 106.65, 113.04, 119.81, 121.77, 123.26, 123.67, 126.40, 126.43, 133.21, 134.93, 138.57, 139.21, 172.72, 172.97
2-Iodoindole. Butyllithium (5.3 mL, 2.0 M in cyclohexane) was added dropwise to a solution of indole (1.17 g, 10.0 mmol) in dry THF (20 mL) at -70 °C. The suspension that formed after 30 min stirring was kept at -70 °C for 20 additional min. CO₂ (g) was then bubbled through the mixture for 10 min, and the clear solution was allowed to stand for 10 min. The butane was allowed to evaporate under vacuum (0 °C), and the remaining solution was cooled to -70 °C. t-Butyllithium (6.2 mL, 1.7 M pentane) was added dropwise, resulting in a yellow solution, which was stirred at -70 °C for 1 h, after which 1,2-diiodoethane (2.82 g, 10.0 mmol) dissolved in THF was added, forming a suspension within 5 min. After keeping the reaction suspension at -70 °C for 1 h, water (1 mL) was added, resulting in a light yellow solution with clumpy white precipitate, and the reaction was allowed to reach room temperature. The dark orange gelatinous solution was then poured into NH₄Cl (saturated aqueous, 50 mL) under stirring, and formed a precipitate. Ether (50 mL) was then added, forming a light orange mixture upon stirring for several min. The organic phase was separated, washed with brine, dried (MgSO₄), and evaporated. The orange syrupy residue was stored in freezer overnight. LCI-MS on crude product showed many masses, including the desired, m/z [M+H]+: 243.9571. Orange syrup was dried to a light brown powder (2.2480g, 92.5 %). 100 mg of the crude was
purified by flash chromatography (hexane/ether, 7:1→4:1), leading to complete loss of product. Remainder of product was used without further purification.
3,4-Dibromo-2,5-dihydro-l-(phenylmethyl)-lH-pyrrole-2,S-dione. To a solution of 3,4-dibromomaleimide (1.002 g, 3.93 mmol) in 10 mL of acetone were added anhydrous potassium carbonate (0.556 g, 4.02 mmol), turning the solution yellow, and benzyl bromide (0.475 mL, 0.684 g, 4.00 mmol). The reaction mixture was stirred at room temperature, forming an orange suspension. After stirring overnight, the reaction suspension had become brown and water was added and the mixture was extracted with ethyl acetate. The organic extract was washed with brine, dried over anhyd. Na₂SO₄, and concentrated under vacuum. The residue was passed through a short column of silica gel, eluting with ethyl acetate. After solvent removal, the brown crystals were washed with MeOH to yield tan crystals. MS, m/z [M+Na]⁺ (relative intensity): 365.8749 (44), 367.8723 (100), 369.8700 (22); ¹H NMR (500 MHz, CDCl₃) δ 4.73 (2H, s), δ 7.3 (5H, m)
1-benzyl-3-bromo-4-(2-iodo-1H-indol-3-yl)-1H-pyrrole-2,5-dione: A solution of ethylmagnesium bromide was prepared from Mg (73.1 mg, 3.01 mmol) and C$_2$H$_5$Br (224 µL, 3.00 mmol) in THF (2.5 mL). The mixture was stirred for 1 h at room temperature then 2-iodoindole (738.1 mg, 3.04 mmol) in toluene (20 mL) was added dropwise. The mixture was stirred at room temperature for 1.5 h, and then a solution of N-methyl-2,3-dibromomaleimide (349.1 mg, 1.01 mmol) in toluene (20 mL) was added dropwise (2:15 PM), resulting in a dark violet solution. After stirring for 20 min, dichloromethane (30 mL) was added then the mixture was stirred for 5 d at rt. Saturated aqueous NH$_4$Cl was added. After extraction with EtOAc, the organic phase was dried over MgSO$_4$, the solvent was removed and the residue purified by flash chromatography (5% EtOAc in hexanes) to give the desired product (457.8 mg, 89 %yield) as a bright orange solid.

MS, m/z [M+Na]$^+$ : 528.8899, 530.8848

$^1$H NMR (500MHz, CDCl$_3$) 8.61 (1H, s, NH), 7.45–7.26 (7H, m, arom), 7.18 (1H, t, J=7.5 Hz), 7.13 (1H, t, J=7.6 Hz), 4.82 (2H, s, N-CH$_2$).

$^{13}$C NMR (125 MHz, CDCl$_3$) 42.96, 81.00, 110.92, 112.01, 120.06, 121.51, 123.72, 124.76, 127.06, 128.27, 128.93, 129.02, 136.13, 138.40, 139.02, 165.67, 167.80.
3-(4-Bromo-1-methyl-2,5-dioxo-2,5-dihydro-1H-pyrrol-3-yl)-indole-1-carboxylic acid tert-butyl ester: To a stirred solution of the indolylmaleimide (306.4 mg, 1.00 mmol), MeCN (6 mL), and DMAP (24 mg, 0.2 mmol, 20 mol %) was added Boc₂O (0.382 g, 1.75 mmol, min 1.5 equiv). solution became dark red then yellow within 2 min. TLC (Hex:EtOAc 2:1) at 1.5h showed presence of charged species remaining. After stirring overnight, the solution was diluted with Et₂O and washed with H₂O (50 mL), aq sat. NaHCO₃ (50 mL), brine (50 mL), and dried (MgSO₄). Aqueous layer was still quite orange, so it was extracted with DCM until the majority of the color was absent. The DCM layers were dried over Na₂SO₄ and concentrated separately from the ether extract.

MS, m/z [M+Na]⁺: 426.9978, 428.9977

Ether extract (330 mg, 81 %) was used without further purification.
3-(1-Methyl-2,5-dioxo-4-trimethylsilanylethynyl-2,5-dihydro-1H-pyrrol-3-yl)-indole-1-carboxylic acid tert-butyl ester: Pd(PPh₃)₂Cl₂ (32.5 mg, 0.046 mmol, 5 mol%) and CuI (19.5 mg, 0.102 mmol, 10 mol%) were added to a solution of 1 (330.0 mg, 0.814 mmol) in Et₃N (30 mL) and the resulting mixture was degassed with argon. Trimethylsilylacetylene (0.28 mL, 2.00 mmol) was added and the reaction stirred for 2.5 h. sat’d NH₄Cl (50 mL) was added and allowed to stir vigorously for 5 min before extracting with ether (3 x 50 mL). The combined organic layers were washed with sat’d Cu₂SO₄, water, and brine, then dried over MgSO₄, and concentrated under vacuum. MS on crude material, m/z [M+Na]⁺: 445.1279. Crude material (~115 mg) was used without further purification, the remainder was purified by column chromatography (5 → 10% EtOAc in Hexanes) to yield the desired product (200.3 mg, ~90% total yield) as a yellow-orange powder.
Chapter 2: Design and Synthesis of a Mechanistic Glycosidase Inhibitor

2.1 Introduction

Glycosidases are enzymes which catalyze the hydrolysis of glycosidic bonds. They function in the digestion of oligosaccharides, as well as in the processing of cell surface carbohydrates which are important for cell to cell recognition during infections, metastasis and immune responses. Inhibitors of glycosidases could potentially have therapeutic effects in some of these cases. “Retaining” glycosidases operate via a double displacement mechanism wherein the glycosyl substrate is activated for substitution by an acid/base residue (Asp, Glu) in the active site of the enzyme; a covalent glycosyl-enzyme intermediate is then formed by attack on the substrate by a nucleophilic active site residue. After activation of water by the general acid/base residue, the covalent intermediate is hydrolyzed, leaving the free enzyme and sugar. The “retaining” mechanism transformations occur via two oxycarbenium-like transition states (not shown). In “inverting” glycosidases, both the general acid/base and the nucleophilic residues operate in the active site; however, no covalent intermediate is formed. The glycosidic substrate is activated for substitution while the water is simultaneously activated for hydrolysis of the glycosidic bond (Figure 2.1).
2.2 Applications of Glycosidase Inhibitors

2.2.1 Inhibitors as Therapeutics

In digestion, α-glucosidases such as amylase and maltase act in the small intestines to break down starch to glucose, which then enters the bloodstream. The insulin released by
the pancreas escorts the glucose into the cells where it can be used for energy. For type II diabetics, sufficient insulin is produced; however the cells are not receptive to it, leading to a postprandial hyperglycemic condition. This elevated glucose level can promote glycation of proteins through the Maillard reaction leading to the accumulation of advanced glycation end products (AGEs), which contribute to blood vessel damage. Inhibitors of α-glucosidases in the gut have been employed to slow the rise in blood glucose by suppressing the breakdown of starch (Figure 2.2). The reduced release of glucose into the system also has the effect of suppressing AGE accumulation. Acarbose (Precose) & miglitol (glyset) are examples of inhibitors currently used in the treatment of type II diabetes (Figure 2.3).
Glycosidase inhibitors are also potentially useful for treatment of HIV and flu infections, as well as for the study of lysosomal storage disorders, in which defective glucosidases result in the unhealthy accumulation of glycosyl sphingolipids (gangliosides) (Figure 2.2). Glucosylceramide is the only glycosphingolipid common to plants, fungi and animals. It is usually considered to be the principal glycosphingolipid in plants. It is a major component of the outer layer of the plasma membrane. These structures occur mostly in nonneuronal tissue and accumulate abnormally in Gaucher’s disease, where glucocerebrosidase is absent or nonfunctional.

Figure 2.3: Glycosidase Inhibitors
Zanamivir and oseltamivir (Figure 2.3) are glycosidase inhibitors that are effective against viral sialidase. Influenza viruses express two envelope glycoproteins, hemagglutinin and sialidase. The hemagglutinin binds to sialic acids on the non-reducing ends of cell surface oligosaccharides. The sialidase frees the virus from the cell after replication. These sialidase inhibitors help to prevent replication of the influenza virus by inhibiting the cleavage of the terminal sialosides through which the replicated viruses are connected to the cell surface.

Glycosidase inhibitors can also serve as useful tools in the study of glycan structure and function. There is a distinct difference in carbohydrate profiles between normal and tumor tissues. It has been realized that glycans expressed by diseased cells are either displayed at different levels, or with different structures than in normal cells. Glycans regulate tumor proliferation, differentiation, invasion, metastasis, immune surveillance and angiogenesis. Altered glycosylation results in alterations in the expression of cell surface oligosaccharides, or altered protein folding and stabilization of tertiary structure, resulting in defects in glycoprotein recognition and elimination by the cell quality control mechanisms.

The characteristic carbohydrate expression associated with malignant transformation is caused by “aberrant glycosylation” catalyzed by specific glycosyltransferases and glycosidases. The aberrant glycosylation of tumors defines the stage, direction, and fate of tumor progression, and the expression of specific carbohydrate epitopes in certain tumors affects their invasive and metastatic potential. The expression of core 2 branched O-glycans is closely correlated with the malignant potential of colorectal cancer.
In the study of cancer therapeutics, α-glucosidases & mannosidases may be particularly effective targets for inhibition due to their important functions in post-translational modifications on glycoproteins. The α-glucosidase inhibitors castanospermine and N-methyldeoxynojirimycin (N-MDNJ) have demonstrated ability to reduce angiogenesis in murine tumor cells. The α-mannosidase inhibitor swainsonine effected a reduction in metastasis in murine cancer models (Figure 2.4).68

![Cancer-related Glycosidase Inhibitors](image)

**Figure 2.4: Cancer-related Glycosidase Inhibitors**

2.3 Inhibitor Review

2.3.1 Noncovalent Inhibitors70

Both shape and charge have reportedly contributed to the efficacy of non-covalent inhibitors. Lactone inhibitors were observed to be relatively strong inhibitors of β-glycosidases,71-75 with their activity attributed, at least in part, to their structural similarity
to the oxycarbenium transition state. The polarity of the lactone function was also highlighted for its potential charge similarity with the transition state (Figure 2.5). Modeling studies have suggested that an enzyme carboxylate group assists in the stabilization of the oxycarbenium ion intermediate without the formation of a glycosyl ester. An analogous interaction may be responsible for the inhibition activity of the lactone.

![Oxycarbenium ion TS\(^+\) intermediate](image)

![Gluconolactone 2.1](image)

Figure 2.5: Oxycarbenium Ion Intermediate and Gluconolactone Inhibitor

These charge-charge interactions are implicated in the inhibition by nojirimycin and similar structures. The protonated form of these naturally occurring basic inhibitors (Figure 2.6) does not share the half-chair shape of the oxycarbenium ion intermediate, yet they demonstrated effective inhibition of both \(\alpha\)- and \(\beta\)-glycosidases (Table 2.1).
Figure 2.6: Naturally Occurring Piperidine Inhibitors.

Table 2.1: $K_i$ Values of the Glucosylactone and Piperidines

<table>
<thead>
<tr>
<th>Enzyme (source)</th>
<th>2.1</th>
<th>2.2</th>
<th>2.3</th>
<th>2.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-glucosidase (brewer’s yeast)</td>
<td>2000</td>
<td>330</td>
<td>14.6</td>
<td>&gt;1500</td>
</tr>
<tr>
<td>$\beta$-glucosidase (almonds)</td>
<td>400</td>
<td>2.8</td>
<td>18</td>
<td>1.5</td>
</tr>
<tr>
<td>$\beta$-glucosidase (Agrobacterium faecalis)</td>
<td>1.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>


The question of the importance of inhibitor shape vs. charge interaction led to the design of inactivators which incorporated both the sp$^2$-hybridized anomeric carbon as well as the positive charge of the piperidines (Figure 2.7). These analogs were synthesized and evaluated by the Ganem group$^{86-88}$, and with further analysis featuring $^{15}$N labels completed by Vasella and coworkers.$^{89}$

Figure 2.7: Lactone/Piperidine Analogs
All three lactone analogues 2.5-2.7 proved to be micromolar inhibitors of several α- and β-glycosidases (Table 2.2), leading Ganem et al. to call them "broad spectrum transition state analogues", based on the proposed structures displayed in Figure 2.7. The structures shown would have the benefit of possessing an endocyclic double bond, providing the half-chair conformation (as opposed to the flattened-chair conformation of gluconolactone), resembling the transition state more closely. This report concluded that the shape of the inhibitor was more important than charge interaction (proton transfer from the enzyme to the inhibitor). Vasella and coworkers decided to challenge these conclusions by synthesizing 2.7 and its two monolabeled 15N isotopomers to verify the structure.89,90

1H and 15N NMR spectroscopy and X-ray structure analysis of 2.7 clearly demonstrated that the double bond is exocyclic, and the ring a flattened chair (2.8, Figure 2.8). Upon protonation, 2.8 undergoes a conformational change to a half-chair, suggesting that protonation by the glycosidase is an important factor in its binding. The weaker inhibition of the hydroximolactone 2.9 as compared with that of the hydroximolactam 2.8 would then reflect its lower basicity. In keeping with this hypothesis, 2.9 proved a stronger inhibitor at pH 4.5 than at pH 6.8 (Table 2.2). This group concluded that the binding of basic glyconoamidine 2.5 and the lactam hydrazone 2.6 was likely driven by electrostatic interactions, as with the piperidines.
Figure 2.8: Corrected Structure of the Hydroximolactam and Its Analogous Lactone

Table 2.2: $K_i$ Values [μM] of the Amidine Analogs

<table>
<thead>
<tr>
<th>Enzyme (source)</th>
<th>2.9</th>
<th>2.5</th>
<th>2.6</th>
<th>2.7/2.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-glucosidase (brewer’s yeast)</td>
<td>6800</td>
<td>-</td>
<td>-</td>
<td>2.9</td>
</tr>
<tr>
<td>$\beta$-glucosidase (almonds) at pH 6.8</td>
<td>4300</td>
<td>10</td>
<td>8.4</td>
<td>14</td>
</tr>
<tr>
<td>$\beta$-glucosidase (almonds) at pH 4.5</td>
<td>98</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$\beta$-glucosidase (Agrobacterium faecalis)</td>
<td>30</td>
<td>-</td>
<td>-</td>
<td>0.6</td>
</tr>
<tr>
<td>$\beta$-galactosidase (bovine liver)</td>
<td>-</td>
<td>-</td>
<td>19</td>
<td>-</td>
</tr>
<tr>
<td>$\alpha$-mannosidase (jackbeans)</td>
<td>-</td>
<td>9.0</td>
<td>3.1</td>
<td>-</td>
</tr>
</tbody>
</table>


2.3.2 Covalent Inhibitors

Covalent inhibitors of glycosidases are able to ablate enzyme activity through the formation of a covalent bond between the enzyme and some portion of the active site. The bond is typically formed by the attack of a nucleophilic portion of the enzyme on an electrophilic portion of the inhibitor molecule, leading to loss of activity, either by steric blockage of the active site, or by alteration of a critical active site residue.91

Some mechanism-based inhibitors were designed with reactive aglycons, which upon hydrolytic cleavage by the enzyme, are able to generate a highly reactive species. This
species can the inhibit enzyme activity by reaction with a nucleophilic residue in the active site (Figure 2.9). The disadvantage with these types of inhibitors lies in the understanding that once the reactive aglycon has been released, it has no inherent affinity for the active site and may react nonselectively at other sites.

Aziridines and epoxides have also been used to label the catalytic nucleophile in retaining glycosidases. Inhibitors containing these functionalities and a general mechanism are shown in Figure 2.10. Though the aziridine inactivators potentially have the theoretical advantage of a higher initial non-covalent affinity for the negatively charged active site, the spiro-aziridines depicted were shown to be very weak inhibitors of a single β-glucosidase and a single α-glucosidase, respectively. The epoxide inhibitors, conduritol-B-epoxide especially, have undergone considerably more study than the aziridine derivatives. CBE was used to label and identify both active sites of mammalian sucrose-isomaltase due to its effective inactivation of enzyme activity. CBE was also instrumental in the study of the biochemical basis for some forms of Gaucher’s disease, as is was shown to be a selective inactivator of human glucocerebrosidase, not affecting
the activity of other mammalian β-glycosidases. An X-ray crystal structure of CBE covalently bound to the glucocerebrosidase was reported in 2005.

Fluorinated glycosidase inhibitors have served as the greatest inspiration for the current design. Glycosidase inactivation by fluorinated glycosides occurs specifically through the formation of a covalent bond between the enzyme nucleophile and the anomeric position of the inactivator. This species can be long-lived, up to months, though enzyme activity can be restored through hydrolysis of the covalent intermediate, or by transglycosylation onto an acceptor substrate. The 2-deoxy-2-fluoro substitution in compound 2.10 leads

Figure 2.10: Aziridine and Epoxide Inhibitor Examples and Inactivation Mechanism (X=O or NH)
to destabilization of transition states in both the glycosylation and deglycosylation mechanisms such that formation and subsequent hydrolysis of the covalent enzyme-inactivator intermediate is slow. This destabilization is attributed to the inductive effects of fluorine, as well as the lack of hydrogen-bonding interactions ordinarily formed with the hydroxyl group in position 2.\textsuperscript{100,101} The addition of activated leaving groups, such as fluoride or di- and trinitrophenolates, can be used to accelerate the glycosylation reaction, leading to an accumulation of covalent intermediates (Figure 2.11). Due to the presence of the 2-hydroxy group in the 5-F-glycosides (2.11), they tend to have higher rates of both glycosylation and deglycosylation compared with the 2-F-glycosides.\textsuperscript{98}

![Figure 2.11: Activated Fluorinated Glycosides](image)

2.4 Novel Inhibitor Design

2.4.1 Design

The inhibitor design is based on the success of the 2-deoxy-2-F-glycosides, which have been shown to be competitive inhibitors of glycosidases.\textsuperscript{100-102} Since these glycosides have allowed the elucidation of the glycosidase mechanism via the formation of a long-lived covalent enzyme-inhibitor complex, the novel inhibitor design incorporates
modifications on the 2-position of glucose to produce β-glucosidase inhibitors (Figure 2.12). Although the modifications made in these inhibitors do not confer special electronegativity as in the inductive effect of the 2-deoxy-2-F-glycosides, it is expected that the inhibitors will be able to form the covalent glycosyl-enzyme intermediate, which is necessary for their inhibitive activity as described in the following section.

![2-F-glucoside, 2-aminoxy-glucoside, 2-hydrazinyl-glucoside, 2-amino-glucoside](image)

Figure 2.12: Inhibitor design

2.4.2 Mechanism

The inhibitor design is directed toward retaining glycosidases. Its efficacy depends on the formation of the covalent enzyme-substrate complex (Scheme 2.1)
Scheme 2.1: Inhibition Mechanism

In this design, it is possible for aminolysis to proceed through a 6-membered transition state. With the added advantage of the intramolecular nature of the interaction, the ester aminolysis should occur more readily than intermolecular hydrolysis. Furthermore, this mechanism could potentially benefit from substrate-assisted catalysis such as that seen in the catalysis of peptide bond formation on the ribosome (Figure 2.13)\textsuperscript{103-106} Several studies have been conducted to elucidate the role of the adenosine 2’-OH, removal of which dramatically decreases the rate of peptide bond formation. In this inhibitor model it is possible that the 3-OH could act as a proton shuttle in much the same manner as the adenosine 2’-OH.
The P- and A- site tRNA substrates are black, ribosome residues and ordered water molecules that stabilize the developing charges are gray. The attack of the α-NH group on the ester carbon results in a six-membered transition state, where the 2’-OH group donates its proton to the adjacent 3’-oxygen while simultaneously receiving one of the amino protons. Alternatively, the water molecule indicated by an asterisk could be used for a proton shuttle. (Rodnina, M.V., M. Beringer, and W. Wintermeyer, *Mechanism of peptide bond formation on the ribosome*. Quarterly Reviews of Biophysics, 2006. 39(03): p. 203-225. © Cambridge Journals, reproduced with permission.)

2.5 Synthesis

The synthetic efforts toward the 2-aminoxy-glucoside and the 2-hydrazinyl-glucoside

(Figure 2.12) follow.

2.5.1 Selective protection

Selective protection of galactose proceeds at low temperatures, with the axial group at the 4-position left unprotected due at least in part to steric hindrance. This thought was applied to the selective protection of mannose in an attempt to produce compound 2.13,
however, it was found that the order of reactivity due to steric does not cleanly mimic the protection of galactose, yielding an inseparable mixture of products (Scheme 2.2). Additionally, the use of a bulky donor, HOBt-pivaloyl ester was attempted based on the report of selective benzylation of diols under mild conditions. This reagent, however, did not yield the desired protection pattern (2.14), again resulting in an inseparable mixture (Scheme 2.2).

![Scheme 2.2: Non-selective, Bulky Protection of Mannose](image)

2.5.2 Glycal to Mannose Derivative

With the unsuccessful use of bulky protecting groups for the selective protection of mannose, we then turned to the use of a glycal as a starting material for the generation of the desired selectively-protected mannose. Our initial efforts were based on the work reported regarding C2-hydroxyglycosylation to generate C2-hydroxy-α-mannopyranosides (2.15) from glycal donors with the aid of dibenzothiophene 5-oxide (DBTO) as the oxygen donor. The DBTO was prepared by oxidation of
dibenzothiophene with H$_2$O$_2$.\textsuperscript{110} With the C2-hydroxyglycosylation procedure, however, we were only able to see the starting glycal, and possibly an intermediate epoxide (Figure 2.14).

![Figure 2.14: C2-hydroxyglycosylation method](image)

After the disappointing turnout of this somewhat complex reaction, it was discovered that a protected mannosyl-2-iodide could be easily achieved from the reaction of a peracetylated glycal and NIS in the presence of AcOH (Scheme 2.3). This reaction proceeded readily, yielding the desired scaffold (2.16); however, the subsequent step, substitution at the 2-position with N-hydroxypythalimide\textsuperscript{111} did not provide the desired compound (2.17). MS analysis of the reaction suggested that instead of substitution, elimination had occurred to yield a product with the mass of the starting glycal. The reaction was attempted with the addition of a silver salt, with and without the presence of base, to potentially activate the iodide toward S$_N$2 reaction, to no avail.
Scheme 2.3: 2-deoxy-2-I-mannose from glycal

An attempt to directly install the anomeric p-nitrophenol in the reaction of the glycal with NIS also led only to the recovery of the starting glycal. The use of MeOH as the nucleophile led to the desired product (2.19); however, both reactions, upon workup, yielded a derivative with an unprotected anomeric hydroxyl group (2.20, Scheme 2.4).

Scheme 2.4
When the substitution reaction was performed on compound 2.19, the methoxy mannoside, a portion of the starting material was recovered, additionally, an interesting elimination was observed yet no desired product was detected by MS. With compound 2.20 as the starting material, the same mass was detected indicating the formation of the elimination product (2.23) instead of the desired product 2.22 (Scheme 2.5).

In an attempt to promote the nucleophilic substitution, the starting glycal protected with electron donating benzyl ethers was used as the starting material. With this protecting group, we were able to achieve the desired substitution at C2 (Scheme 2.6). Starting with commercially available 3,4,6-tribenzyl glucal, iodination at C2 proceeded smoothly to
provide compound 2.24. Substitution on this derivative in the presence of cesium carbonate gave the desired product 2.25 as detected by MS, with no evidence of elimination as seen with the acetylated derivatives 2.19 and 2.20.

Scheme 2.6: Substitution on Benzyl-protected Mannosyl-2-iodide

A theoretical problem arose when considering the deprotection of the final compound as to whether the aminoxyl N-O bond would survive the hydrogenation for removal of the benzyl ethers. A shift to p-methoxybenzyl protecting groups, because of the ability to be removed under oxidative conditions, should allow for the completion of the desired derivative (Scheme 2.7). Deprotection of the acetylated glycal using sodium methoxide in methanol gave the unprotected glucal, which was then p-methoxybenzylated in the presence of NaH to give glucal 2.26. When iodinated in the presence of MeOH as a nucleophile, intermediate 2.27 was obtained; this was unable to form the substitution product 2.28, presumably because of steric hindrance by the methoxy group. NIS iodination in the presence of water gave compound 2.29, which was able to be converted, although in low yield, to the desired substitution product 2.30.
Scheme 2.7: Completion of Desired Substitution at C2

For the synthesis of the 2-hydrazinyl-glucoside inhibitor (Figure 2.10), the tri-acetylated 2-l-mannose compound 2.19 was treated with aqueous hydrazine. Employment of 1.2 equivalents of hydrazine yielded recovered starting material, along with singly- and doubly-deprotected derivatives of 2.19, but no substitution was detected. MS analysis on the crude material following the use of a large excess of hydrazine showed masses representing potential complete deprotection and C2-substitution to provide the desired derivative (Scheme 2.8).
The purification of **2.31** proved to be tedious and ineffective, leaving a sample too impure for NMR analysis. An effective method for the removal of excess hydrazine from such a compound will need to be employed for further analysis.

### 2.6 Conclusion

Upon completion of the oxylamino and hydrazine inhibitors, activity testing will be completed. After evidence of successful inhibition of glycosidases, proof of covalent linkage would lend veracity to the mechanism of the enzymes. Radiolabeled inhibitors can be synthesized, and the subsequently inactivated enzyme analyzed for radioactivity. This linkage could also be analyzed by inhibition by inhibitors containing 13C; NMR analysis could be used to confirm covalent linkage.

The success of this inhibitor design had great implications for future research, therapy and even agriculture. Natural sugar derivatives of this inhibitor type could be instrumental for the study of glycoprotein processing in cancer and other maladies. Cancer cell-targeted derivatives could be developed for the inhibition of carbohydrate processing, as in the inhibition of viral sialidases. To increase the value and utility of
agricultural byproducts such as Camelina meal, inhibitors of myrosinase, a plant glucosidase could prevent the release of poisonous glucosinolate hydrolysis products, yielding a meal suitable for livestock feed.
2.7 Chapter 2 Experimental

**1,3,4,6-tetra-O-acetyl, 2-deoxy-2-I-mannose:** Glucal(OAc)$_3$ (500.3 mg, 1.84 mmol) was dissolved in dry CH$_2$Cl$_2$ (25 mL) under argon in a foil-covered flask. To the resulting solution at rt was added AcOH (0.21 mL, 3.68 mmol, 2 eq) followed by NIS (627.2 mg, 2.79 mmol, 1.5 eq). After 1h, there seemed to be no further consumption of starting material; added additional NIS (0.229 g, 1.02 mmol) and allowed to stir for 30 additional min. The reaction was then quenched by the addition of 20% Na$_2$S$_2$O$_3$ (50 mL) and the reaction mixture was stirred vigorously for 5 min. The mixture was diluted with EtOAc (100 mL), washed with 20% Na$_2$S$_2$O$_3$ (50 mL), sat’d NaCl, and the organic layer dried over Na$_2$SO$_4$. The solvent was removed and the residue was purified by flash chrom.

(Hexanes: EtOAc 7:1$\rightarrow$ 7:2). MS, m/z [M+Na]$^+$ (relative intensity): 480.9838 (100)$^1$H NMR (CDCl$_3$): d 6.36 (d, 1H, J = 1.6 Hz, H-1), 5.42 (dd, 1H, J = 9.7, 9.7 Hz, H-4), 4.56 (dd, 1H, J = 4.4, 9.7 Hz, H-3), 4.50 (dd, 1H, J = 1.6, 4.4 Hz, H-2), 4.20 (dd, 1H, J = 4.5, 12.4 Hz, H-6a), 4.13 (dd, 1H, J = 2.4, 12.4 Hz, H-6b), 4.10 (dddd, 1H, J = 9.8, 4.4, 4.4, 2.4 Hz, H-5), 2.14, 2.09, 2.08, 2.04 (4s, 12H, CH$_3$COO); $^{13}$C NMR (CDCl$_3$): d 170.78, 170.02, 169.43, 168.28 (CH$_3$COO), 94.85 (C-1), 71.58 (C-5), 68.79 (C-3), 67.20 (C-4), 61.99 (C-6), 27.34 (C-2), 21.04, 20.97, 20.85, 20.75 (CH$_3$COO).
(2R,3R,4S,5S,6S)-2-(acetoxymethyl)-5-iodo-6-methoxytetrahydro-2H-pyran-3,4-diyl diacetate: Glucal(OAc)₃ (1.0279 g, 3.78 mmol) was dissolved in dry CH₂Cl₂ (25 mL) under argon in a foil-covered flask. To the resulting solution at rt NIS (1.7255 g, 7.67 mmol, 2 eq) in ACN, MeOH (30 mL, 735 mmol, 200 eq) was added to the mixture. After 5 h, the reaction was then quenched by the addition of sat’d Na₂S₂O₃ (50 mL) and the reaction mixture was stirred vigorously. The mixture was diluted with EtOAc (100 mL) and washed with water and sat’d NaCl. The organic layer was dried over Na₂SO₄. The solvent was removed under vacuum and the residue was purified by flash chromatography (10-15% EtOAc in Hexanes) to yield the desired product (1.375 g, 84.7 %) as a light syrup.

MS, m/z [M+Na]⁺: 452.9797. (No NMR Data)
Glucal(OBn)$_3$ (1.0423 g, 2.50 mmol) was dissolved in dry CH$_2$Cl$_2$ (25 mL) under argon in a foil-covered flask and p-nitrophenol (3.5474 g, 25.5 mmol, 10 eq) in ACN was added. To the resulting solution at rt was added NIS (1.6323 g, 7.25 mmol, 3 eq) in ACN with a small amt of DMF to aid dissolution.

The reaction was then quenched by the addition of 20% Na$_2$S$_2$O$_3$ (50 mL) and the reaction mixture was stirred vigorously for 5 min. The mixture was diluted with EtOAc (100 mL), washed with 20% Na$_2$S$_2$O$_3$ (50 mL), sat’d NaCl, and the organic layer dried over Na$_2$SO$_4$. The solvent was removed under vacuum and the residue was purified by flash chrom. (0→10 % EtOAc in Hexanes—extra side products formed on column). Hydrolyzed product seen in fraction 2.

MS, m/z [M+Na]$^+$: (1) 704.0733; (2) 583.0692

(No NMR data)
2-(((2S,3R,4S,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-2-hydroxytetrahydro-2H-pyran-3-yl)oxy)isoindoline-1,3-dione: To a solution of the protected 2-I-Man (100.6 mg, 0.180 mmol) and N-hydroxy-phthalimide (36.9 mg, 0.226 mmol, 1.2 eq) in DMF (12 mL) was added cesium carbonate (145.3 mg, 0.446 mmol, 2.5 eq). The resulting dark orange solution was heated to 90 °C for 4 h, then allowed to cool to ambient temperature. The mixture was diluted with EtOAc (10 mL) and washed with saturated NH₄Cl (30 mL), water (30 mL), NaHCO₃ (30 mL) and brine (30 mL). The organic extracts were dried over MgSO₄, filtered, and the solvent was removed by rotary evaporation to provide a dark syrup. Purification by flash chromatography (1:1 hexanes/EtOAc) yielded a mixture. Dry loaded a second column and purified by flash chromatography (7:1 → 1:1 hexanes/EtOAc) MS, $m/z$ [M+Na]$^+$ :618.2027, 650.2311(methanolized phth), 636.2130(hydrolized phth). (No NMR Data due to impurity of products)
(2R,3S,4S)-3,4-bis((4-methoxybenzyl)oxy)-2-(((4-methoxybenzyl)oxy)methyl)-3,4-dihydro-2H-pyran: To tri-O-acetyl-D-glucal (2.008 g, 7.34 mmol) in methanol (15 mL) at room temperature was added NaOMe (502.3 mg, 8.8 mmol). The reaction was allowed to stir at rt until completion (~5 h) (TLC 1:1 Hexanes: EtOAc). Acidic ion-exchange resin was added to achieve pH 7. The solution was filtered (over additional resin) and then the volatiles were removed in vacuo to give the unprotected product, which was used without further purification.

To a stirred solution of D-glucal (4.012 g, 27.371 mmol) in DMF (200 mL) was added 4-methoxybenzyl chloride (~15.0 mL, 109.483 mmol), followed by careful addition of NaH (60% dispersion in oil, 19.850 g, 496.242 mmol) at 0 °C. Stirring was continued for 30 min at 0 °C and then allowed to warm to rt. TLC analysis shows no remaining starting glycal. The reaction suspension was cooled to 0 °C, methanol (50 mL) followed by water (200 mL) were cautiously added; and the resulting solution was extracted with ethyl acetate (3 × 250 mL). The combined organic layers were dried over Na$_2$SO$_4$, filtered and concentrated in vacuo. The residue was diluted with EtOAC and washed with water (5 x 100 mL) to remove residual DMF. The organic extract was dried over Na$_2$SO$_4$, filtered
and concentrated in vacuo. Column chromatography (hexanes : EtOAc 1:0, then 8:1, then 3:1) provided the desired glycal (12.204 g, 87.8 %) as an amber syrup. MS, m/z [M+Na]+ : 529.2014

$^1$H NMR (500 MHz, CHLOROFORM-d) δ ppm 3.69 - 3.73 (m, 2 H) 3.78 (d, $J$=3.78 Hz, 9 H) 4.00 (ddd, $J$=8.51, 5.04, 2.84 Hz, 1 H) 4.15 (d, $J$=5.67 Hz, 1 H) 4.43 - 4.57 (m, 5 H) 4.72 (d, $J$=11.03 Hz, 1 H) 4.82 (dd, $J$=6.31, 2.52 Hz, 1 H) 6.39 (d, $J$=5.67 Hz, 1 H) 6.82 (d, $J$=8.83 Hz, 2 H) 6.85 (d, $J$=7.57 Hz, 4 H) 7.13 (d, $J$=8.51 Hz, 2 H) 7.24 (d, $J$=7.88 Hz, 4 H)

$^{13}$C NMR (126 MHz, CHLOROFORM-d) δ ppm 55.45, 55.48, 68.42, 70.40, 73.34, 73.57, 74.32, 75.75, 100.31, 113.99, 114.04, 129.55, 129.67, 129.77, 130.33, 130.58, 130.73, 144.82, 159.42, 159.45, 159.48
(2S,3S,4S,5R,6R)-3-iodo-2-methoxy-4,5-bis((4-methoxybenzyl)oxy)-6-(((4-methoxybenzyl)oxy)methyl)tetrahydro-2H-pyran: Glucal(OPMB)₃ (1.1255 g, 2.22 mmol) was dissolved in dry CH₂Cl₂ (25 mL) under argon in a foil-covered flask and methanol (79.8 μL, 19.7 mmol, 10 eq) was added, followed by the addition of NIS (1.40 g, 6.22 mmol, 3 eq) in ACN (20 mL). TLC after 3 days appeared to show presence of majority of starting material*. Additional NIS (1.345 g, 5.98 mmol, 3 eq) and methanol (2 mL, 48.80 mmol, 22 eq) was added to the reaction flask and continued to stir at rt. After 24 hours, based on TLC analysis, additional methanol (~ 12 mL) was added to the reaction flask and continued to stir at rt. After 48 h, the reaction was quenched by the addition of 20% Na₂S₂O₃ (50 mL) and the reaction mixture was stirred vigorously for 5 min. The mixture was diluted with EtOAc (100 mL), washed with 20% Na₂S₂O₃ (50 mL), sat’d NaCl, and the organic layer dried over Na₂SO₄. The solvent was removed under vacuum, leaving a white crystalline product in an amber syrup (1.5948 g). The aqueous layer was re-extracted with ether, which was dried & evaporated to leave an off-white, crystalline powder (73.7 mg). The residue was purified by flash chrom. (0→10 % EtOAc in Hexanes), leaving an amber syrup which contains desired product and unreacted side-product from previous step. (1.1044 g, 75 %)

MS, m/z [M+Na]⁺: 687.0813.
(2S,3S,4S,5R,6R)-3-iodo-4,5-bis((4-methoxybenzyl)oxy)-6-(((4-methoxybenzyl)oxy)methyl)tetrahydro-2H-pyrano-2-ol: Glucal(OPMB)₃ (2.0660 g, 4.07 mmol) was dissolved in CH₂Cl₂ (25 mL) under argon in a foil-covered flask and water (1.0 mL) was added, followed by the addition of NIS (2.66 g, 12.34 mmol, 3 eq) in ACN (15 mL). After 1 h, the reaction was quenched by the addition of 20% Na₂S₂O₃ (50 mL) and the reaction mixture was stirred vigorously for 5 min. The mixture was diluted with EtOAc (100 mL), washed with 20% Na₂S₂O₃ (50 mL), sat’d NaCl, and the organic layer dried over Na₂SO₄. The solvent was removed under vacuum, leaving an amber syrup. The residue was purified by flash chrom. (0-30% EtOAc in Hexanes), leaving an off-white, syrupy solid. (2.5996 g, 97.9 %)

MS, (crude) m/z [M+Na]⁺: 673.1105

¹H NMR (500 MHz, CHLOROFORM-d) δ ppm 3.06 (dd, J=8.67, 3.94 Hz, 0 H) 3.22 (d, J=3.15 Hz, 1 H) 3.30 (dd, J=8.51, 4.10 Hz, 1 H) 3.78 (d, J=4.73 Hz, 9 H) 4.03 - 4.08 (m, 1 H) 4.10 (q, J=7.04 Hz, 1 H) 4.33 - 4.39 (m, 1 H) 4.39 - 4.46 (m, 2 H) 4.47 - 4.54 (m, 1 H) 4.55 - 4.66 (m, 1 H) 4.68 - 4.76 (m, 1 H) 4.76 - 4.80 (m, 1 H) 4.82 - 4.89 (m, 1 H) 5.39 (t, J=2.84 Hz, 1 H) 5.55 (d, J=2.21 Hz, 1 H) 6.76 - 6.81 (m, 2 H) 6.81 - 6.89 (m, 4 H) 7.01 - 7.08 (m, 2 H) 7.22 (d, J=8.51 Hz, 1 H) 7.26 (t, J=4.26 Hz, 1 H) 7.28 - 7.33 (m, 1 H) 7.33 - 7.37 (m, 1 H)
$^{13}$C NMR (126 MHz, CHLOROFORM-$d$) $\delta$ ppm 55.45, 55.48, 68.06 - 68.16, 69.20, 70.86, 72.23, 73.22, 74.94, 75.36, 76.04, 76.36, 92.04, 96.35, 113.95, 113.98, 114.06, 114.17, 129.78, 129.81, 129.85, 129.87, 129.93, 159.41 - 159.46, 159.45, 159.46 - 159.49, 159.51 - 159.54, 159.56,
2-(((2S,3R,4S,5R,6R)-2-hydroxy-4,5-bis((4-methoxybenzyl)oxy)-6-(((4-methoxybenzyl)oxy)methyl)tetrahydro-2H-pyran-3-yl)oxy)isoindoline-1,3-dione: To a solution of the protected 2.29 (1.002 g, 1.5411 mmol) was added a mixture of cesium carbonate (1.287 mg, 3.9500 mmol, 2.5 eq) and \textit{N}-hydroxy-phthalimide (0.322 mg, 1.974 mmol, 1.2 eq) in DMF (24 mL). The resulting dark orange solution was heated to 90 °C. After 2 h, reaction was allowed to cool to ambient temperature. The mixture was diluted with EtOAc (100 mL) and washed with saturated NH\textsubscript{4}Cl (50 mL), water (50 mL), NaHCO\textsubscript{3} (2 x 50 mL) and brine (50 mL). The organic extracts were dried over Na\textsubscript{2}SO\textsubscript{4}, filtered, and the solvent was removed by rotary evaporation to provide an orange syrup, which was filtered through silica gel and evaporated to leave an orange syrup. Purified crude by flash chromatography (9:1→0:1 hexanes/EtOAc).

MS, \textit{m/z} [M+Na]\textsuperscript{+}: 708.2146, 740.2470(methanolized phth).

\textsuperscript{1}H NMR (500 MHz, CHLOROFORM-\textit{d}) δ ppm 3.63 (ddd, \textit{J}=16.08, 11.03, 1.89 Hz, 1 H) 3.75 (s, 3 H) 3.80 (s, 6 H) 3.83 - 3.92 (m, 2 H) 3.92 - 4.01 (m, 1 H) 4.31 - 4.47 (m, 3 H) 4.56 - 4.67 (m, 2 H) 4.67 (s, 1 H) 4.74 (dd, \textit{J}=11.98, 10.72 Hz, 1 H) 4.81 - 4.90 (m, 1 H)
5.45 (d, J=3.15 Hz, 1 H) 5.51 (s, 1 H) 6.79 - 6.84 (m, 4 H) 6.85 - 6.90 (m, 2 H) 7.08 (dd, J=8.51, 6.62 Hz, 2 H) 7.22 - 7.25 (m, 2 H) 7.31 (dd, J=11.35, 8.51 Hz, 2 H) 7.75 (ddd, J=8.67, 5.52, 3.15 Hz, 2 H) 7.83 (ddd, J=12.45, 5.52, 3.15 Hz, 2 H)

$^{13}$C NMR (126 MHz, CHLOROFORM-d) δ ppm 51.20, 55.55, 55.63, 67.10, 67.81, 68.14, 72.37, 72.75, 73.03, 73.33, 73.45, 73.49, 74.97, 75.02, 75.48, 79.56, 82.32, 105.33, 105.94, 114.03, 114.07, 114.12, 114.26, 114.35, 123.93, 124.06, 129.81, 129.83, 129.86, 129.95, 130.00, 130.08, 134.83, 134.96, 159.65, 163.56
(2R,3S,4R,5R,6S)-5-hydrazinyl-2-(hydroxymethyl)-6-methoxytetrahydro-2H-pyran-3,4-diol: To a suspension of the protected 2-l-Man (51.6 mg, 0.120 mmol) in DMF (6 mL) was added hydrazine hydrate (134 µL, 2.32 mmol, 20 eq). The resulting solution was allowed to stir at rt. TLC (1:1 Hex : EtOAc) @ 1 h indicated nearly complete reaction. After 3 h at rt, the starting material was not seen by TLC, however, the major product matches the doubly deprotected product mass. An additional 20 eq of hydrazine was added. After 5 h, reaction showed progress toward most polar product and cesium carbonate (97.7 mg, 0.3 mmol, 2.5 eq) was added to promote substitution reaction. TLC at 22h showed disappearance of spot representing doubly deprotected product; only most polar products remain. Solvent was removed under vacuum. The residue was loaded on silica gel and purified by column chrom. (0→ 5 % MeOH in DCM), and the fractions analyzed by MS.

MS (fractions), m/z [M+Na]^+: 326.9498; 255.0854, 326.9511, 241.0699
3 Chapter 3: Chemoenzymatic synthesis of glycopeptides

3.1 Introduction

3.1.1 Glycosylation

Glycosylation is one of the most important and ubiquitous post-translational modifications in protein synthesis. Glycosylation of proteins often assists protein folding, increases resistance to proteolytic degradation and generates unique epitopes for molecular recognition. Glycoproteins are involved in a variety of essential biological processes such as cell-cell interaction, cell differentiation and immune regulation.\(^2,113-115\) Furthermore, abnormal glycosylation of proteins has often been correlated with a number of human diseases.\(^116\) Despite their important role in biological systems, our understanding of the structure-function relationship of glycoproteins is still underdeveloped. Naturally occurring glycoproteins are universally heterogeneous. Obtaining structurally-defined homogeneous glycoproteins is thus becoming one of the highest priorities in glycobiology.

3.1.2 Fmoc Solid-Phase Peptide Synthesis

A convenient tool for the production of synthetic glycopeptides and glycoproteins is chemical peptide synthesis on a solid phase resin. The method for solid phase peptide synthesis (SPPS) was developed by Robert Bruce Merrifield, for which he was presented
the Nobel Prize in Chemistry 1984. The principle involved the stepwise addition of protected amino acids to a growing peptide chain which was bound by a covalent bond to a solid resin particle. In this procedure, reagents and by-products were easily removed by filtration, and purification of intermediates was unnecessary. The advantages of this method were reportedly “speed and simplicity of operation”\textsuperscript{117}. In addition to elimination of tedious purification steps associated with solution phase synthesis, Merrifield’s SPPS also permitted the development of automation and the extensive range of robotic instrumentation now available\textsuperscript{118}. Additionally, coupling reactions can be carried out rapidly and nearly to completion using an excess of the activated amino acid derivative, which is removed at the end of the reaction by simple washing operations\textsuperscript{119}. The shift in amino-protection to the base-sensitive Fmoc-protecting group developed by Carpino in 1970\textsuperscript{120}, from the acid-labile Boc group allowed the entire process of SPPS to be carried out using milder chemistry\textsuperscript{121}. The process of SPPS used for our synthetic peptides is diagrammed in Scheme 3.1. The Fmoc-N-protected C-terminal amino-acid residue is anchored via its carboxyl group to an amino resin to yield an amide-linked peptide that will ultimately produce a C-terminal amide peptide. After loading the first amino acid, the desired peptide sequence is assembled in a linear fashion from the C-terminus to the N-terminus by repetitive cycles of Fmoc-deprotection and amino acid coupling reactions. Fmoc deprotection was carried out in the presence of morpholine, and the subsequent amino acid for coupling is activated by O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) and N-methyl morpholine (NMM) to form an amino acyl 1-hydroxy-
benzotriazolyl ester (HOBt). After this activated ester is then coupled with the unprotected amino terminus of the growing chain on the resin, the Fmoc deprotection is completed and the entire process repeats until the peptide sequence is complete. Treatment of the resin with trifluoroacetic acid (TFA) in the presence of cation scavengers, such as triisopropyl silane (TIS) and water, yields the fully deprotected peptide product.
Scheme 3.1: SPPS
3.2 N-glycosylation

3.2.1 Background

Over the past several decades, there has been tremendous progress in both chemical and enzymatic synthesis of glycoproteins. However, none of the existing methods can efficiently transfer fairly complex oligosaccharide chains (such as N-glycans) onto deglycosylated peptide or protein backbone in a single step.

Four major synthetic strategies exist for N-glycopeptide assembly (Figure 3.1). The Glyco-amino acid building block approach (A) has been successful for preparing glycopeptides carrying relative small oligosaccharides. This method becomes problematic when a large oligosaccharide is pre-attached, due to its susceptibility to strong acidic conditions (e.g., TFA or HF) required for de-protection and releasing of the peptide. The convergent coupling method (B) facilitates the coupling of a glycosylamine to a protected peptide containing a free aspartyl side chain. With this process, preparation of selectively protected full-size peptide requires extra-steps and the yield of final coupling might be low due to intramolecular aspartimide formation. Chemoselective ligation (C) allows for unnaturally linked glycopeptide synthesis. This native chemical ligation methodology, involving peptide thioester and N-cysteine-containing peptide/protein, was also applied for ligating glycopeptide fragments. Finally, chemoenzymatic synthesis (D) involves enzyme-catalyzed glycosylation, which normally proceeds with 100% control of the stereochemistry of the newly formed glycosidic bond, with high or absolute regioselectivity and does not require glycosyl protecting groups. The oligosaccharyl transferases (OT) responsible for oligosaccharide transfer in
glycoprotein biosynthesis also has the potential for in vitro synthetic purposes, but the complexity of the system is still an obstacle to its practical application.\textsuperscript{148-151}

Figure 3.1: Approaches for N-glycopeptide/ glycoprotein synthesis:
A) Glyco-amino acid building block, B) convergent coupling, C) chemoselective ligation and D) chemoenzymatic approach.

The incorporation of enzymes in a chemical synthetic scheme greatly enhances the overall efficiency of the synthesis. A unique advantage of this method is that an enzyme such as an endoglycosidase could have the ability to transfer a large oligosaccharide chain to a pre-assembled GlcNAc-peptide acceptor in a single step\textsuperscript{152-161}, thus allowing a highly convergent synthesis.
3.2.2 Peptide:N-glycanase

A yeast enzyme peptide:N-glycanase (PNGase) was functionally identified and shown that it can efficiently release intact N-glycans from misfolded glycoproteins.\textsuperscript{162} The enzyme hydrolyzes the $\beta$-aspartylglycosylamine bond of asparagine-linked glycopeptides and glycoproteins.\textsuperscript{163,164} Misfolded glycoproteins are initially retained in the endoplasmic reticulum and are then dislocated to the cytosol where they undergo ubiquitination, deglycosylation, and degradation by proteasomes.\textsuperscript{165-170} Based on sequence homology, PNGase is classified as a member of the transglutaminase superfamily.\textsuperscript{163,171} Enzymes in this family are known to catalyze the formation of amides between the side-chain amine of peptide-bound lysine residues and the carboxamide group of peptide-bound glutamine residues;\textsuperscript{172} some members also assist in the cleavage of peptide bonds. Sequence alignment and mutational studies reveal that PNGase shares a conserved catalytic mechanism with the transglutaminase and cysteine protease family. The catalytic mechanism (\textbf{Figure 3.2}) shows that the active site contains the catalytic triad of Cys, His, and Asp. Cys-191 acts as a nucleophilic residue to attack the amide bond between GlcNAc and asparagines.\textsuperscript{173} His-218 functions as a catalytic base to facilitate the nucleophilic attack. The carboxylate in the aspartic acid is important to maintain the proper orientation of the histidine residue in the triad.
Given the varied activity of the family to which PNGase belongs, it is reasonable to ascertain whether this enzyme could potentially function “in reverse” for the synthesis of N-linked glycopeptides and glycoproteins. Desirable yield of protease-catalyzed syntheses often requires altered reaction conditions such as “low-water” reaction media or solid phase conditions to shift the preference for synthesis over hydrolysis. The aspartic acid residues in the peptide can be activated with esters to help influence this
preference. Since the first step in hydrolysis reaction is a similar nucleophilic attack in the amide bond by thiol-group, replacing the amide bond with ester bond will increase the reactivity of the carbonyl group, thus facilitating the nucleophilic addition.

In the proposed mechanism (Figure 3.3), the Cys-His-Asp catalytic triad aligns the Cys to undergo nucleophilic attack to the carboxyl group of the aspartic ester, forming the tetrahedral intermediate and transforming into a thioester linkage. The amino group on the anomeric carbon of the GlcNAc containing glycan would then serve as a nucleophile to attack the thioester, displacing the Cys residue and resulting in the formation of β-aspartylglycosylamine linkage. This “reverse” synthetic mechanism is theoretically quite plausible. Further exploration of this possibility may eventually yield an invaluable enzyme tool for glycoprotein synthesis.

As mentioned previously, to push the reaction toward synthesis, water-free conditions may be necessary. The rationale for using enzymes in non-aqueous solvent is that the structure of the enzyme is not dependent of the nature of the solvent, implying that the intrinsic catalytic activity is usually not compromised in the organic solvent. Several techniques have been developed for using a great variety of enzymes in organic solvents. Inorganic salt pretreatment and lipid-coating are particularly useful for two classes of enzymes, proteases and glycosidases. Several common organic solvents can be employed such as ethanol, acetonitrile, isopropyl ether, benzene, DMSO and chloroform. Widely-used inorganic salts including sodium, potassium, calcium and lithium salts can be added for activation. In the event of enzyme instability in the presence of high concentrations of
organic solvents, a covalent enzyme-lipid (detergent) complex might confer added stability to the enzyme structure and function.

Figure 3.3: Proposed "Reverse" Mechanism
3.2.3 Synthetic Peptide Targets

Ribonuclease A (RNase A) is an endonuclease that cleaves single-stranded RNA. RNase A was the model protein used to work out many spectroscopic methods for assaying protein structure, including absorbance, circular dichroism/optical rotary dispersion, Raman, EPR and NMR spectroscopy. RNase A was also the first model protein for the development of several chemical structural methods, such as limited proteolysis of disordered segments, chemical modification of exposed side chains, and antigenic recognition. RNase A, and to a greater extent its oligomers and some homologs (such as onconase from frogs), have cytotoxic and cytostatic effects, particularly on cancer cells. This has led to the development of onconase as a cancer therapeutic, particularly for external use against skin cancers. As with many protein drugs, the internal use of non-human ribonucleases such as onconase is limited by the patient's immune response. The short glycosylation sequence chosen was M-M-K-S-R-D(OMe)-L-T-K-D-K-D-R-NH₂ (Figure 3.4).

![Chemical structure of a synthetic peptide substrate](image)

Figure 3.4: RNAse A-14. Synthetic Peptide Substrate.
Erythropoietin or EPO is a glycoprotein hormone that is a cytokine for erythrocyte precursors in the bone marrow. Also called hematopoietin or hemopoietin, it is produced by the kidney, and is the hormone regulating red blood cell production. Erythropoietin is available as a therapeutic agent produced by recombinant DNA technology in mammalian cell culture. It is used in treating anemia resulting from chronic renal failure or from cancer chemotherapy. Its use is also believed to be common as a blood doping agent in endurance sports such as bicycle racing, triathlons and marathon running. The sequence chosen for glycosylation testing was Ac-G-Q-A-L-L-V-D(OMe)-S-S-Q-P-W-E-P-L-NH₂ (Figure 3.5). The glycosylation motif is highlighted.

![Figure 3.5: EPO-15. Synthetic Peptide Substrate](image)

Analysis of a shorter synthetic peptide, an 8-amino acid portion of the EPO target sequence, showed the presence of a slightly lower mass, presumably due to possible aspartimide formation (Figure 3.6). Aspartimide formation is a well-documented problem in the synthesis of aspartic acid-containing peptides and proteins. This reaction can occur under both basic and acidic conditions. The resulting imide ring is
susceptible to opening by nucleophilic attack on either of the carbonyl carbons, resulting in the generation of peptide side products, such as β-peptides, α- and β-peptide esters, and racemization of the desired product as well as the side products. The potential for aspartimide formation of the EPO-15 derivative is likely increased by the presence of the methyl ester on the side-chain of aspartic acid. Normally, bulky protecting groups such as t-buty1 esters are used to inhibit attack on the carboxyl function by the preceding amide nitrogen; the use of the methyl ester, ironically for the purpose of activation of the aspartyl side-chain toward nucleophilic attack by PNGase, may also encourage formation of this undesired imide. Formation of this cycle may have also contributed to the lack of results seen in initial glycosylation experiments (not reported).

Figure 3.6: EPO-8, Aspartimide Formation
3.2.4 Conclusion

While it may be possibly to employ peptide:N-glycanases in the chemoenzymatic synthesis of complex glycopeptides, further study will need to be conducted prior to its realization. The mere fact that the natural substrate for these enzymes is a complex oligosaccharide-linked glycoprotein could bring into question the binding affinity of the enzyme for any un-glycosylated peptide substrate. Affinity studies on various peptide substrates may provide clarity in this matter. Additionally, due to the potential for generation of undesired products as a result of aspartimide formation, the use of backbone amide-protected amino acids just prior to aspartyl coupling could significantly reduce or eliminate the yield of these side products. In either case, confirmation of binding and nucleophilic attack by the enzyme would provide great impetus for the continuation of this study.

3.2.5 Experimental: Peptide Synthesis for N-Glycosylation

**General Synthetic Procedure**

178 Deprotection of rink amide resin

Rink Amide AM resin (loading 0.63 mmol/g, 252.8 mg, 0.16mm effective) was soaked in DMF for 2 h, filtered and washed with DMF. It was then treated (N₂ bubbled through reaction vessel) with DMF:morpholine (1:1, 6mL) for 1 h, filtered and washed with DMF.

Fmoc removals
Resin bound peptide intermediates were treated with DMF:morpholine (1:1, 6mL) for 1 h, filtered and washed with DMF.

**Couplings**

The resin was treated with a 5-fold excess of Fmoc-amino acids and a 1.1-fold excess of glycosylated amino acids dissolved in DMF, with the addition of 1.5 equiv of HOBr, 2.0 equiv of NMM and 1.0 equiv of HBTU per equivalent of Fmoc-amino acid. Coupling times were at least 6 h for Fmoc-amino acids and at least 18 h for glycosylated amino acids. The excess reactants were then removed by filtration and the support washed with DMF. Following the final Deprotection, the resin was washed with DMF, CH$_2$Cl$_2$, and MeOH; it was then dried *in vacuo*.

**Cleavage**

The dried resin was treated with TFA:TIS:H$_2$O (95:2.5:2.5) under N$_2$ for 1-2 h at rt. The mixture was filtered and the resin washed with TFA, CH$_2$Cl$_2$ and MeOH. The filtrate was evaporated and the glassy residue was washed (5 x 20 mL), followed by trituration with ether, giving the peptide as a white crude powder.

**Purification**

Analytical and semi-prep HPLC was performed on a Shimadzu HPLC system with an LC20 pump system and an LC20 UV detector, using TSK-GEL ODS-120T columns (4.6 mm x 25.0 cm and 7.8 mm x 30.0 cm, TOSOH BIOSCIENCE). Purified fractions were analyzed on Shimadzu ESI-MS.
Samples were chromatographed using a mobile phase system of 0.1% TFA in water and 0.1% TFA in ACN with a gradient of 0-45% ACN over 60 min. Peak detection was at 220 nm. Pure samples were pooled and lyophilized to yield white fluffy powder.

EPO-15:

![Chemical Structure](image)

Chemical Formula: C$_{77}$H$_{119}$N$_{19}$O$_{24}$

Exact Mass: 1693.8675
Molecular Weight: 1694.8817

MS, $m/z$ [M+2H]$^{2+}$: 848.65

Figure 3.7: HPLC Purity Scan of EPO-15
EPO-8:

Chemical Formula: C_{46}H_{67}N_{11}O_{16}
Exact Mass: 1029.4767
Molecular Weight: 1030.0883

Purification: Cleavage attempted on 27.4 mg resin using 95% TFA, 2.5% H₂O, 2.5% TIS for 2 h

HPLC: (MP A: 0.1% TFA in H₂O, MP B: 0.1% TFA in ACN, UV detection 220 nm)

MS, m/z [M+H]^+: 1030.60; [M+Na]^+: 1052.60
Figure 3.8: HPLC Purity Scan of EPO-8
3.3 O-Glycosylation

3.3.1 Background

The development of a facile and widely applicable platform for the generation of polysaccharide (PS) conjugated anti-bacterial vaccines would be a major advancement for the benefit of pharmaceutical health. Polysaccharides, forming a thick capsule that surrounds the bacterial pathogen, represent a major determinant of pathogenicity.\textsuperscript{179,180} With the increasing emergence of resistance toward major antibiotics, development of polysaccharide-based vaccines provides an attractive approach for fighting the infectious diseases.\textsuperscript{4,5,181}

The traditional chemical approach has enabled the production of several highly successful conjugated vaccines in clinical use.\textsuperscript{182,183} However, the traditional approach suffers from multiple fermentations, purification steps, low yields, non-specific chemical conjugation, and therefore, ultimately leading to the high cost of vaccine production. The objective of this proposal is to explore a recently established bacterial protein glycosylation system to obtain polysaccharide conjugate vaccines in a facile, efficient, and easily applicable manner.

This bacterial glycosylation system comprises two highly promiscuous proteins (PglB for N-glycosylation and PglL for O-glycosylation), which catalyze the transfer of polysaccharides from a diphospho-lipid donor to target proteins.\textsuperscript{7,184-186} The peptide preference in the context of both primary sequence and local secondary structure of proteins is being explored to determine PglL substrate sequence specificity with the aim
of establishing a facile system for \textit{in vivo} synthesis of PS conjugated vaccines in engineered \textit{E. coli} strains based on the PglB and PglL glycosylation pathway. Compared with the traditional approach for glycocojugate vaccine production, the proposed approach has several advantages. The most obvious advantage is that such a biotechnology approach has the potential to lower the cost of production in comparison with the traditional chemical coupling approach. The traditional approach requires production and extraction of PS, expression and purification of a carrier protein, and chemical coupling and separation of glycoconjugates. Besides the complexity of the process, the natural PSs from the pathogens are required and culture of the pathogen is difficult and costly. For our fermentation method, the production and separation steps will be simplified once the \textit{E. coli} strains harboring the PS gene cluster, carrier protein and glycosylation enzyme genes are constructed (\textbf{Figure 3.7}).
3.3.2 PglL

PglL is an oligosaccharyltransferase involved in O-glycosylation of proteins in *Neisseria meningitidis*. It has demonstrated ability to transfer virtually any glycan from UndPP & FarPP lipid carriers (Figures 3.8 & 3.9) to meningococcal pilin. The oligosaccharyl transferase activity of PglL was detected in *E. coli* cells in the absence of any other neisserial protein except for the pilin substrate, indicating its ability to be applied to exogenous systems.
Figure 3.10: PgL Substrates: Glycan donors.

Figure 3.11: PgL Reaction.
3.3.3 Synthetic Peptides for Sequence Specificity Study

Sequences were chosen to incorporate structural, steric and charge specificity issues (Figure 3.10).
Initial results do not indicate the formation of the desired glycopeptide. (Figure 3.11)
Additional study showed the presence of a new product by HPLC; however, mass spec analysis was inconclusive due to the detergent signal (Figure 3.12).
Due the PEGylated detergent, no glycopeptide was able to be detected by LC/MS. Additionally, confidence in these experiments is low since PgIL activity had not been verified prior to these experiments. However, in order to confirm the obliteration of any positive results, a synthetic glycopeptide (Figure 3.13) was prepared to determine the efficiency of mass spectral analysis in the presence of detergent. Figure 3.14 shows the results of this analysis. The signal for the glycopeptide is completely absent in the presence of the detergent.
Figure 3.15: Synthetic Glycopeptide

Glycopeptide

Glycopeptide with detergent

Figure 3.16: MS analysis of Synthetic Glycopeptide.
3.3.4 Conclusion

Improved analytical methods will enable efficient continuation into the investigation of the peptide sequence specificity of PglL. Systematic determination of enzyme activity in the presence of ionic or zwitterionic detergents could open access to greatly enhanced separation of product for more reliable analysis by MS. Such a determination would also provide verification of PglL activity by efficient glycosylation of natural substrate, pilin. Analysis would also be improved by the utilization of radioactive carbohydrate lipid donors, as in the peptide specificity studies of PglB.\textsuperscript{187} The analysis of the systematically altered peptide sequences synthesized herein can then be completed for glycosylation using verified lipid donors.

3.3.5 Experimental

**General Synthetic Procedure**

**Synthesis**

Syntheses of the target peptides were performed using Fmoc-SPPS on Rink Amide AM resin (0.68 mmol/g, 100 mg, ~70 µmol) using peptide synthesis vessels (Chemglass) with nitrogen bubbling. Prior to each coupling reaction, the resin was deprotected using 50% morpholine in DMF (dimethylformamide) for 3 min. Deprotection was performed twice for each cycle. The resin was filtered and washed with DMF for 30 sec (repeated 3 times), and a solution of 4.4 equiv of the amino acid (0.30 mmol), 4 equiv of HBTU (2-\textit{(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate}, 0.28 mmol)
and 5 eq of NMM (N-methylmorpholine, 0.35 mmol) was added to the resin and allowed to couple for 45 min. The coupling process was repeated when the Kaiser test indicated incomplete coupling. The reaction was then filtered and washed with DMF (3 X 30 sec) prior to deprotection. Following the final Deprotection, the resin was washed with DMF, CH₂Cl₂, and MeOH; it was then dried in vacuo.

**Cleavage**

The dried resin was treated with TFA:TIS:H₂O (95:2.5:2.5) under N₂ for 1.5 h at rt. The mixture was filtered and the resin washed with TFA, CH₂Cl₂ and MeOH. The filtrate was evaporated and the glassy residue was washed (5 x 20 mL), followed by trituration with ether, giving the peptide as a white crude powder.

**Purification**

Analytical and semi-prep HPLC was performed on a Shimadzu HPLC system with an LC20 pump system and an LC20 UV detector, using TSK-GEL ODS-120T columns (4.6 mm x 25.0 cm and 7.8 mm x 30.0 cm, TOSOH BIOSCIENCE). Purified fractions were analyzed on Shimadzu ESI-MS.

Samples were chromatographed using a mobile phase system of 0.1% TFA in water and 0.1% TFA in ACN with a gradient of 5-15% ACN over 30 min. Peak detection was at 220 nm. Pure samples were pooled and lyophilized to yield white fluffy powder.

**Enzyme reaction**

**Solution 1:**

- 10 µL of 20 mM peptide
- 20 µL of 0.1 M Tris-HCl (pH 7.5)
- 10 µL of 0.1 M MnCl₂ (Mn²⁺)
Solution 2:

- 0.5 mg of GlcNAc-PP-Undecaprenol
- 8 µL of Triton-X-100 (14% soln)

Solution 3:

- 40 µL of PglL

Solution 3 was added to Solution 1, followed by the addition of Solution 2. After the reaction was quenched and extracted, analysis was performed by HPLC and LC/MS. The starting peptide was identified, but product was not. There is the possibility that the product analysis was obliterated by the attachment of detergent to the saccharide portion of the glycopeptide.

Triton X-100, a non-ionic detergent (Figure 3.15) was used in the activity analysis of PglL. It gives a MS scan with a pattern characteristic of PEGylated entities (Figure 3.16).

\[
\text{Figure 3.17: Triton-X Detergent}
\]
This pattern was detected by LC/MS for some peaks during the analysis of the reaction mixture. It is plausible that any glycopeptide formed would not be detected in the presence of detergent due to the formation of complexes. Determination of an efficient method for removal of detergent is necessary.
Peptides

Native:

\[
\text{Chemical Formula: C}_{36}\text{H}_{54}\text{N}_{12}\text{O}_{15} \\
\text{Exact Mass: 894.3832} \\
\text{Molecular Weight: 894.8854}
\]

Figure 3.19: Native Sequence

MS, \( m/z \ [M+Na]^+ : 917.55 \)

Figure 3.20: HPLC Purity Scan of Native Sequence
Figure 3.21: -2P Sequence

MS, $m/z$ [M+Na]$^+$: 794.25; [M+H]$^+$: 775.25

Figure 3.22: HPLC Purity Scan of -2P Sequence
**+1R:**

Chemical Formula: $C_{33}H_{59}N_{15}O_{13}$

Exact Mass: 873.4417

Molecular Weight: 873.9143

Figure 3.23: +1R Sequence

**Mass Spectrometry (MS), m/z [M+H]$^+$**: 874.45

Figure 3.24: HPLC Purity Scan of +1R Sequence
+2E:

![Chemical Structure of +2E]

**Figure 3.25: +2E Sequence**

**MS, m/z [M+H]**: 861.45, 747.25 (impurity)

**Figure 3.26: HPLC Purity Scan of +2E Sequence**
4 Chapter 4: Soy Protein Modification Using Transglutaminase

4.1 Introduction

Improved utilization of the proteinaceous co-products from industrial processing of current and future crops will improve the value of these crops in new and existing markets. The use of soybeans as a source of biodiesel is quickly growing. When the use of soy as a source of bio-diesel grows further, then the need will increase for finding higher value markets beyond those based on the nutritional value of these proteinaceous materials.

In order to be used in industrial markets, these proteins will need to be physically or chemically modified to impart valued properties required for the desired applications. Overall, this project will lead to new well characterized proteins with improved value so that they can be used to replace products made from non-renewable sources. Our research will reduce dependence on non-renewable materials and produce higher value products that will benefit a large segment of our economy through the chemical modification of proteinaceous co-products of cereal and soy processing. Some anticipated end-uses include bio-based and biodegradable plastics, packaging materials, films, fibers, and an improved understanding of processing of proteinaceous materials.
Soybean is well known to contain a high amount of protein. Soybean meal contains approximately 50% crude protein and is used primarily as protein source in livestock feed. Soy flour is similar to soybean meal; however, care has been taken during processing to minimize protein denaturation. Further purification provides soy protein concentrate (~70% protein) and soy protein isolate (~90% protein).

The industrial use of soybean proteins dates back to the 1930s and 1940s and major areas of interest included wood adhesives, plastics, fibers, foams, and films or coatings. However, the emergence of cheaper, synthetic alternatives relegated soybean meal to feed use.

Current applications for soybean protein include food (baby formula, protein shakes, textured vegetable protein, etc.); edible films which can be engineered to reduce fat uptake in fried foods, or be impregnated with antimicrobial agents, fiber for the production of thermal underwear, household textiles, exercise clothes; and adhesives made from chemically cross-linked or modified protein.

4.2 Transglutaminase

Transglutaminase (Tgase) is an enzyme which catalyzes the formation of a covalent bond between a free amine group and the γ-carboxamide group of protein- or peptide-bound glutamine. The bonds formed exhibit high resistance to proteolytic degradation. In nature the function of the enzyme promotes the formation of extensively cross-linked and generally insoluble protein polymers in order to create barriers and stable structures, in blood clots (coagulation factor XIII) and skin and hair, for example.
The Tgase catalytic reaction is generally viewed as being irreversible. For the purpose of this project, it will be utilized to increase the strength and water-resistance of soy protein for industrial applications. The transglutaminase modified proteins will then be processed to provide electrospun fibers, films and standard melt samples where properties such as tensile strength, elongation, and solubility will be evaluated.

Figure 4.1: Tgase Reaction. A) Forms cross-linked protein, B) Deamidation in absence of 1° amine.

The mechanism of the reaction is shown in Figure 4.2.207
4.3 Soy Protein Experiments

Our Tgase crosslinking experiments were run on ADM, Inc. soy protein isolate samples obtained from the Bong group at The Ohio State University. 250 µL enzyme suspension (0.2 mg/mL enzyme) was added to 500 µL SPI suspension (10 mg/mL SPI) and allowed to shake 4 d at rt; control samples contained no enzyme. SDS-PAGE analysis showed
definite crosslinking of the proteins when compared to control samples (Figure 4.3). It is easy to conclude that the reaction lead to the formation of larger (cross-linked) proteins as evidenced by the lighter reaction lanes as opposed to the dark control lanes. The cross-linked proteins are more easily seen in viewing reaction and control samples (Figure 4.4).
Figure 4.3: Initial Cross-linking Experiments
The qualitative observation that the control samples smelled spoiled after several days at rt, along with the notice that they were yellowish in color when compared to the white reaction samples confirms the additional stability conferred by the Tgase catalyzed cross linkage.

Transglutaminase activity measured at a lower concentration still showed evidence of cross linkage by SDS-PAGE. Additionally, experiments performed at both rt and 37 °C revealed that the rt experiments are just as efficient as physiological temperature, allowing for simplicity in the application of the enzyme for industrial purposes (Figure 4.5).
The final set of experiments was designed to test the efficiency of the cross-linking reaction with different concentrations of Tgase, as well as with various SPI concentrations (Table 4.1). Additionally, glutamine and lysine peptide linkers (Figure 4.6) were synthesized to evaluate cross-linking reaction in the presence of each.

![Figure 4.5: Temperature experiments](image)

![Figure 4.6: Synthetic Peptide Linkers](image)
Table 4.1: Experiment Set-up

<table>
<thead>
<tr>
<th>Lane</th>
<th>Soy (µL)</th>
<th>Tgase (µL)</th>
<th>Lys (mg)</th>
<th>Gln (mg)</th>
<th>Water (µL)</th>
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<td>450</td>
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- **Lane 2** serves as a control for all experiments
- **Lanes 3 & 4** compare the cross-linking reaction for 50 mg/mL protein using 0.01 mg/mL & 0.1 mg/mL Tgase respectively
- **Lanes 4, 5 & 6** compare the reaction of 0.1 mg/mL Tgase with varying concentrations of soy protein isolate (50 mg/mL, 5 mg/mL & 0.5 mg/mL) respectively
- **Lane 5** serves as the control for the remaining experiments
- **Lanes 7 & 8** assess the potential increase in cross-linkage when a Lys-rich peptide linker is added to the reaction.
  - Peptide concentrations: 0.5 mg/mL & 1 mg/mL respectively
  - Soy protein concentration: 5 mg/mL
  - Tgase concentration: 0.1 mg/mL
- **Lanes 9 & 10** assess the potential increase in cross-linkage when a Gln-rich peptide linker is added to the reaction.
  - Peptide concentrations: 0.5 mg/mL & 1 mg/mL respectively
  - Soy protein concentration: 5 mg/mL
  - Tgase concentration: 0.1 mg/mL
Referring to the SDS-PAGE results (Figure 4.7), lanes 3 & 4 show that the cross-linking reaction for 50 mg/mL protein is significantly faster with 0.1 mg/mL Tgase, as compared to 0.01 mg/mL. Lanes 4, 5 & 6 show a trend in the development / disappearance of two proteins at ~188 and ~30 kDa with varying concentrations of soy protein isolate. Note that at the high concentration, very little of the 30 kDa protein is seen, however at the low concentration, very little of the 188 kDa protein is produced. Lanes 7-10 are not indicative of any cross-linkage enhancement as compared to Lane 5; however, the Lys peptide linker shows a marked increase in the size of protein polymer upon visual inspection (Figure 4.8). The control (5) and Gln peptide samples (9 & 10) have less
sediment and no polymer on the sides of the vials, presumably the result of the positively charged Lys peptide linker.

Figure 4.8: Peptide Linker Experiment Samples

4.4 Conclusion

Treatment of soy-protein with transglutaminase can alter the proteins stability for use in beneficial applications such as the production of biodegradable packaging. Though the experiments completed are very preliminary to the project, we were able to demonstrate through the peptide-linker experiments the promiscuity of the enzyme in terms of acceptable amino substrates, whereas the glutamine substrate specificity is as yet undetermined. Though previous work on soy protein films has included the use of Tgase, none of the reports indicate the formation and analysis of films produced from previously cross-linked proteins. This is an avenue worth evaluation. Additionally, the covalent
cross-linking of plasticizers with the protein prior to film formation is also worthy of research.
4.5 Chapter 4 Experimental

Materials

Soy samples were obtained from Dennis Bong’s group (Jo Marie Bacusmo).
ADM, Inc. Soy protein isolates from the Pro-Fam Series- 781, 825, 875, 880, 891, 974
Transglutaminase was obtained from Ajinomoto Food Ingredients LLC.

Experimental Procedures

Transglutaminase activity was verified using Bovine Serum Albumin

Enzyme Stock (Activa Tgase enzyme pak): Enzyme (0.2000g) was suspended in 10 mL water to yield a concentration of 20 mg/mL (0.2 mg/mL enzyme).
BSA Stock: previously prepared to a concentration of 10 mg/mL protein.
Set up 500 µL reactions as indicated in Table 4.2 (results shown in Figure 4.9).

1       2       3       4

Figure 4.9: Tgase activity verification w/ BSA
Table 4.2: Tgase/BSA Experiment Set-up

<table>
<thead>
<tr>
<th>Lane</th>
<th>BSA (µL)</th>
<th>Tgase (µL)</th>
<th>Water (µL)</th>
<th>Buffer (µL)</th>
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</tr>
<tr>
<td>4</td>
<td>100</td>
<td>-</td>
<td>400</td>
<td>-</td>
</tr>
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</table>

Loading: After ~48 h, mixed 1 portion sample with 1 portion 2X loading buffer (source unknown), boiled for 3-5 min to denature, and loaded on gel. Ran gel @ 120 V.

Results: Clear indication of similar Tgase activity in each sample. Experiments on soy protein isolates will be run in water to simplify future applications.

**Transglutaminase activity with Soy protein isolates**

**Enzyme Stock** (Activa Tgase enzyme pak):

Enzyme (0.2000g) was suspended in 10 mL water to yield a concentration of 20 mg/mL (0.2 mg/mL enzyme).

**Soy protein stocks** (Soy-T-5)

Protein (~0.1g) was suspended in 10 mL water to yield a concentration of ~10 mg/mL.

Set up 1 mL reactions using 500 µL each of protein stock, 250 µL enzyme stock and 250 µL water (500 µL for control samples with no enzyme). Reactions were placed on orbital shaker at rt. Visible coagulation of all samples after 4 days.
**Loading**: After ~4 days, mixed 10 µL of each sample & control with 30 µL 4X loading buffer (Novagen 70607), boiled for 3-5 min to denature, and loaded 10-15 µL on gel. Ran gel @ 120 V. First 3 samples were too light to view.

![Image](image_url)

Figure 4.10: SPI Control & Reaction Samples after 4 d @ rt.

**Repeated loading:**

After 11 days, mixed 30 µL of each sample & control with 10 µL 4X loading buffer (Novagen 70607), boiled for 3-5 min to denature, and loaded 20 µL on gel. Ran gel @ 100 V. *(Figure 4.3)*

**Observation:**

Visible coagulation of all samples after 4 days *(Figure 4.10)*. SDS PAGE after 11 days reaction time *(Figure 4.3)* demonstrates a high incidence of crosslinking in all samples tested. In all cases, the vast majority of the protein was of MW >> 188 kDa, as visible protein could be seen in all reaction wells after completion of gel analysis.
Control samples smell spoiled after several days and are still yellowish (as are stocks), the reaction samples have no malodor & are more whitish in color, indicating a stabilization of aqueous mixtures upon cross-linkage of proteins.

**Lower concentration transglutaminase activity with soy protein isolates**

**Enzyme Stock** (Activa Tgase enzyme pak) (Soy-T-7): Enzyme (0.203g) was suspended in 10 mL water to yield a concentration of ~20 mg/mL (0.2 mg/mL enzyme).

**Soy protein stocks**

Previously prepared Soy-T-5

Set up 500 µL reactions using 100 µL each of protein stock, 100 µL enzyme stock and 300 µL water (400 µL for control samples with no enzyme). Reactions were placed on rotator at rt.
**Temperature experiments**

Purpose: Compare reactivity of T-gase at rt and 37°C

![Temperature Experiments at ~1 wk.](image)

Repeated temperature experiments with 891 only

Purpose: Compare reactivity of T-gase at rt and 37°C

**Enzyme Stock:** Enzyme (0.203g) was suspended in 10 mL water to yield a concentration of ~20 mg/mL (0.2 mg/mL enzyme).

**Soy protein stocks:** Protein (~1 g) was suspended in 10 mL water to yield a concentration of ~100 mg/mL.

Set up 1 mL reactions using 500 µL each of protein stock, 250 µL enzyme stock and 250 µL water for reaction samples and 500 µL each of protein stock with 500 µL water for control samples. Reactions were placed on stir plates either at rt or in 37-40 °C oil bath.

**Observations:**
SDS-PAGE analysis shows no detectable difference in reactivity of enzyme at rt and 37°C. (Figure 4.5)

Concentration & Peptide linker experiments (Soy-T-17)

Purpose:

1. Re-test cross-linking reaction with different concentrations of Tgase

2. Test cross-linking reaction with varying concentrations of soy protein

3. Test cross-linking reaction with lysine peptide linker

4. Test cross-linking reaction with glutamine peptide linker

Set up experiments according to Table 4.1.
Tgase for Glycoprotein Conjugation

Materials

PON1 was obtained from Edwin Motari: 3.1 mg/mL G3C9 in 15 mM Tris, pH 7.5

Maltoheptaose w/ linker (Figure 4.12) was obtained from T.J. Styslinger (MW 1299.19)

~8 mg

Transglutaminase was obtained from Ajinomoto Food Ingredients LLC.

![Figure 4.12: Maltoheptaose w/ linker](image-url)

Experimental Procedures
PON1 was diluted to 0.3 mg/mL

Maltoheptaose solution was prepared in water to a concentration of 2 mg/mL

Enzyme (0.2000g) was suspended in 10 mL water to yield a concentration of 20 mg/mL (0.2 mg/mL enzyme).

Set up 500 µL reactions as indicated in Table 4.3 (results not displayed).

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<th>Vial #</th>
<th>Rxn</th>
<th>SPI (µL)</th>
<th>PON (µL)</th>
<th>Sugar (µL)</th>
<th>Tgase (µL)</th>
<th>Water (µL)</th>
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<td>-</td>
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Loading: After ~23 h, mixed 60 µL sample and 20 µL loading buffer, boiled for 3-5 min to denature, and loaded on gel. Ran SDS-PAGE gel @ 120 V.

Results: Samples were too dilute to be seen on gel. 200 µL of each sample was reduced to ~20 µL, to which 10 µL loading buffer was added for analysis by SDS-PAGE. Results were inconclusive.
The experiment was repeated with higher concentrations of protein & sugar as shown in Table 4.4 (results shown in Figure 4.13).

Table 4.4: Repeated Experiments

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<th>Vial #</th>
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<th>Sugar (µL)</th>
<th>Tgase (µL)</th>
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Figure 4.13: SDS-PAGE for PON1/ Maltoheptaose Experiments

**Loading:** After ~23 h, mixed 60 µL sample and 20 µL loading buffer, boiled for 3-5 min to denature, and loaded on gel. Ran SDS-PAGE gel @ 120 V.
Results: On the gel, samples 3-5 look very similar, but different from sample 6, which contains no sugar. Conjugation cannot be determined by this experiment. Kaarina Lokko was kind enough to run MALDI on samples 3 & 5 (control vs. rxn). The results are displayed in Figure 4.14. MALDI analysis did not present evidence of conjugation.

Figure 4.14: MALDI on PON1, Control (3) and Reaction (5)
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175
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Figure 1.4

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Scheme 1.14

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Figure B.2: $^1$H NMR for Compound 1.54
Figure B.3: $^{13}$C NMR for Compound 1,54
Figure B.4: $^1$H NMR for Compound 1.55
Figure B.5: $^{13}$C NMR for Compound 1.55
Figure B.6: 1H NMR for Compound 2.26
Figure B.7: $^{13}$C NMR for Compound 2.26
Figure B.8: $^1$H NMR for Compound 2.29
Figure B.9: $^{13}$C NMR for Compound 2.29
Figure B.10: $^1$H NMR for Compound 2.30
Figure B.11: $^{13}$C NMR for Compound 2.30