Identifying the Mechanism of Action of Bioactive 1,2-Cyclopropyl Carbohydrates

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A thesis submitted to Victoria University of Wellington
in partial fulfillment of the requirements for the degree of
Master in Biomedical Science

2015

Abstract

Cyclopropanes and carbohydrates have long been used in the field of drug development. Previous work has shown that 1,2-cyclopropyl carbohydrates display bioactivity in both HeLa cancer cell lines¹ and in yeast² with a tentatively proposed mechanism of inhibition occurring through an enzymatic cyclopropane ring opening reaction and subsequent formation of a covalent bond with a target enzyme.²

A small library of 1,2-cyclopropyl carbohydrate derivatives were synthesised based on known pharmacophores to examine further the potential mechanism of inhibition of such compounds and confirm the occurrence of enzyme-catalysed cyclopropane ring-opening reactions. Initial synthetic efforts were focused on the synthesis of the 1,2-dichlorocyclopropyl carbohydrate **23**, which, through the optimisation of an essential C-6 detritylation reaction, was achieved in moderate yields of 32% over 7 steps. Following this, the ethoxycarbonyl substituted 1,2-cyclopropyl carbohydrate **54** was synthesised over 7 steps in a 22% yield through a rhodium acetate-catalysed addition of ethyl diazoacetate (**49**) to the glucal substrate **40**. It was envisioned that if enzymatic cyclopropane ring-opening was occurring to form a C-7 carbanion, this would in turn be stabilised through the potential enolate formation of **54**. Use of *N,N*-ditosylhydrazine in the synthesis of propargyl diazoacetate (**58**) followed by a rhodium acetate-catalysed cyclopropanation of **58** with substrate **40** resulted in the successful synthesis of **61** over 7 steps in a total yield of 9%. The incorporation of the propargyl substituent in **61** was introduced as a molecular probe in an attempt to isolate the target protein through an affinity purification procedure. The bioactivity of the propargyl derivative **61** was consistent with the synthesised compounds **23** and **54**. It was proposed that these compounds undergo an enzymatic cyclopropane ring-opening reaction accompanied with a clear diastereoselective preference for the α -stereoisomer of the cyclopropane ring, consistent with a target-based activation of the compounds.

Chemical genetic analysis of the resulting bioactive compounds was undertaken using a deletion mutant array of *Saccharomyces cerevisiae* to elucidate a potential mechanism of action. Analysis of the results showed that, of the 4800 homozygous

deletion strains tested in the high-throughput screens, a total of 122 strains were found following validation to sensitise and 68 to give resistance against **23** and **54**. These sensitive and resistant mutants were subjected to a validation assay. Following validation, genes whose deletion led to sensitivity or resistance were then subjected to gene ontology term enrichment analysis which showed enrichment in the cytosolic ribosome, SNARE complex and SNAP receptor activity for resistant strains and enrichment in endoplasmic reticulum and endomembrane systems was found for the sensitive strain. Genes whose deletion sensitised to both compounds showed strong enrichment in cellular protein localisation, intra-golgi vesicle-mediated transport and the endomembrane system.

Target identification and isolation were attempted through an affinity purification procedure using compound **61** and an azide-modified agarose resin. However, this was without success, either through inaccessibility of the alkyne of the target probe or because the target resides in the membrane-associated fraction which was discarded prior to treatment with the probe.

This study suggests that the 1,2-cyclopropyl carbohydrates synthesised function through a cyclopropane ring-opening reaction, assisted by an enzymatic nucleophile. Chemical genetic analysis showed that the target of these compounds is involved in protein transport and protein localisation most likely relating to the vesicle tethering. Although many aspects of this work still need further investigation, either through the synthesis of new 1,2-cyclopropyl carbohydrates to increase bioactivity and better understand the enzymatic target, or through further biological procedures to better understand the mechanism of action, the use of 1,2-cyclopropyl carbohydrates as a potential pharmaceuticals or probes of protein trafficking shows much promise.

Acknowledgements

I would like to start by acknowledging and thanking my family, for without you none of this would have even been possible. Mum and dad, thank you for all your support, emotionally and financially, which have allowed me to make the decisions that I have regarding my education. Your horrible dad jokes and loving care packages have not gone unnoticed. Yan and Inès, thank you for always being there for a chat and to distract from the stress of non-stop work. Yan, thanks for always being positive, supportive and an amazing friend as well as a brother.

My biggest thank you is to my supervisor AProf. Dr. Paul Teesdale-Spittle, thank you for all your help and patience, with my unrelenting cascade of questions and believing in me when I doubted my abilities. Your positivity and excitement toward both chemistry and biology always inspired me to want to better myself and learn new things. Dr. Joanne Harvey, my secondary supervisor, thank you for your endless energy, positivity and excitement which was definitely enough to keep me excited about chemistry even when everything seems to be going wrong.

To all the ‘breathers’ and ‘ata gee’ that I’m privileged to call my friends although the list is too long to name you know who you are. I hope the ridiculous yarns and the outlandish times continue for many years to come. A special thanks to Mike, Steve, Millie, Marcos, Harley and Kieran you ‘ata gees’ are mean as!

Thank you to all the members, past and present, of the organic synthesis research lab for all the help, suggestions and tips over the past two years. I would have definitely struggled without everyone’s help. I’ve enjoyed working with you all especially when we jam out to Taylor Swift, for arguably far too long. Special thanks to Sarah for putting up with me for almost 6 years, I have absolutely no idea how you did it, amazing! I would also like to thank all the members of the chemical genetics lab, especially Namal, Richard and Bede for introducing me to the exciting and magical world of yeast. Without guys I would have had absolutely no idea what I was doing.

Finally thank you Kristiana for everything! Especially keeping me alive over the past 2 months by bringing me food and proofing all my work. You are amazing.

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Abbreviation list

^1H NMR	Proton nuclear magnetic resonance
^{13}C NMR	Carbon13 nuclear magnetic resonance
δ	Chemical shift (ppm)
Ac	Acetyl
Bn	Benzyl
b.p.	Boiling point
CHCA	Alpha-cyano-4-hydroxycinnamic acid
COG	Conserved oligomeric golgi complex
CID	Collision-induced dissociation
CPD	Cyclopropane-1,1-dicarboxylate
d	Doublet
dd	Doublet of doublets
ddd	Doublet of doublets of doublets
DCM	Dichloromethane
dH ₂ O	Distilled water
DHFR	Dihydrofolate reductase
DMA	Deletion mutant array
DMF	<i>N,N</i> -dimethylformamide
eq	Equivalents
FTMS	Fourier transform mass spectroscopy
GARP	Golgi associated retrograding protein
GFP	Green fluorescent protein
GO	Gene ontology
GSH	Glutathione
HTS	High-throughput screening
IR	Infrared
IUPAC	International Union Of Pure And Applied Chemistry
KARI	Ketol-acid reductoisomerase
LDS	Lithium dodecyl sulfate
LTQ	Liner ion trap
m	Multiplet
m.p.	Melting point

NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser effect
Nu	Nucleophile
o/n	Overnight
ORFs	Open reading frames
Py	Pyridine
q	Quartet
QSAR	Quantitative structure relationship
r.t.	Room Temperature
s	Singlet
SAR	Structure-activity relationship
Sat.	Saturated
SDS	Sodium dodecyl sulfate
t	Triplet
TBAI	Tetrabutylammonium iodide
TBAF	Tetrabutylammonium fluoride
TBDMSCl	<i>tert</i> -butyldimethylsilyl chloride
TBDPSCl	<i>tert</i> -butyl(chloro)diphenylsilyl chloride
TEBAC	Triethylbenzylammonium chloride
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
Tr	Trityl
YGDS	Yeast genome deletion set
YDS	Yeast deletion set

Chapter 1

Introduction

Carbohydrates and cyclopropanes have long been used in the field of pharmacology for their respective reactivity and structural properties. The high stereochemical information contained within carbohydrates and their ability to mimic the structure and function of naturally occurring compounds have made them powerful tools in drug design. Organic chemists have also utilised the ability of cyclopropanes to undergo ring-opening and ring-expansion reactions.³ These investigations have raised the question as to whether cyclopropyl carbohydrates can mimic natural compounds and undergo such reactions via enzymatic processes within cells, which could potentially lead to the discovery of a new covalent bioactive compound.²

This thesis aims to explore these ideas through the synthesis of a variety of potentially bioactive 1,2-cyclopropyl carbohydrate structures. A small library of compounds will be synthesised to undergo both yeast and mammalian cell assays where they will be screened for potential bioactivity. Bioactive compounds will then be subjected to both chemical and genetic techniques in order to identify the molecular target and elucidate their mechanism of action. This will be achieved through the synthesis of a molecular probe to bind any potential targets to an affinity purification resin and through chemical genetic analysis utilising activity screening against yeast deletion set.

1.1 History of drug discovery

The modern era of drug research and drug development as we know it dates back more than a century, when chemical concepts and principles were applied to problems outside the field of chemistry. This application began around the mid-19th century when the periodic table had been established and the atomic hypothesis proposed by Avogadro had been confirmed.⁴ The development of the modern field of chemotherapeutics coincided with the industrial revolution and the move from

biofuels to coal and tar. The use of these new fuels created sizable quantities of by-products that were found to contain large amounts of aromatic and aliphatic compounds. The discovery of the aromatic and aliphatic compounds and their use as dyes in the mid-19th century prompted chemist, Paul Ehrlich, to investigate their affinity with biological tissue. From his work, he postulated the existence of chemo-receptors, and that pathogens contained chemo-receptors with slight structural variation to our own, allowing therapeutic exploitation of these sites.⁴ This became the basis of medicinal chemistry and the birth of modern chemotherapeutics.

Comparatively, the use of plants as herbal medicine far outdates that of medicinal chemistry. The use of plants in traditional Chinese and North American medicine is an ancient practice that is still used currently. Additionally, the isolation and purification of the active compounds from traditional plant-based remedies have also played a role in the advancement of medicinal chemistry in the 19th century. Morphine was first isolated from *Papaver somniferum*, a species of opium poppy plants, in 1815 by F. W. Sertürner.^{4,5,6} The discovery of morphine and its medicinal properties led to an increased pharmaceutical interest in the field of natural products, resulting in the discovery of many more natural bioactive products such as paclitaxel (Taxol), a commonly used anticancer drug.⁷ Later in the 20th century, the accidental contamination of an agar plate containing *Staphylococcus* variants with a white fluffy mould led to the discovery of the natural product penicillin by Alexander Fleming which greatly influenced the field of medicinal chemistry.⁶ Penicillin was found to be a very effective antibiotic and quickly became the most widely used antibiotic due to its lack of toxicity and high efficacy. This led the way to a new era of antibiotics, with the majority of pharmaceutical companies establishing microbiology and fermentation departments in the hope of discovering similar natural products.⁴

1.2 Approaches to drug design

The processes of drug design have come a long way since the major findings of both Paul Ehrlich and Alexander Fleming. Several new approaches have been developed which are more systematic rather than serendipitous, although a certain element of serendipity still remains. These new approaches have been made possible due to the

corresponding advancements made in many other aspects of biological, physical and chemical sciences. The most notable approaches to drug design can be split into three categories.

1.2.1 Structure-based drug design

As the name implies, structure-based drug design relies on the 3D structural knowledge of the biological target. This method of drug design had been very limited until the early 1990s when technological advancements were made in the fields of X-ray crystallography, homology modelling and nuclear magnetic resonance (NMR) spectroscopy, providing methods to better determine the structure of the biological targets.⁸ In 1990, Roberts and colleagues⁹ released the first publication utilising structure-based drug design in the synthesis of small peptide-derived molecules that mimicked the protease activity of the human immunodeficiency virus (HIV). Once a biological target is found and its structure is elucidated, compounds and fragments in a computer data system are typically screened against the target in the search for the best fitting compound, which utilises as many non-covalent interactions as possible. Based on the steric and electronic effects, the compounds of best fit are then synthesised and tested against the target to determine their IC₅₀ concentrations. The lead compounds are normally altered in attempts to heighten their potency by increasing the amount of interactions they can establish.¹⁰ Identifying a biological target can be the most difficult and lengthy process, as extensive research must be carried out to confirm that the target is involved in the pathophysiology of the disease.¹⁰

1.2.2 Ligand-based drug design

The second method of drug design, ligand-based design, is most commonly employed when the 3D structure of the biological target remains unknown. Ligand-based drug design, also known as indirect drug design, relies on the structural knowledge of the compound that produces a desired pharmacological response. This allows for subsequent chemical modification of the lead compound, which will establish the

pharmacophores necessary for the molecular recognition of both the active compound and the biological target.¹¹ This is primarily achieved in two ways: structure-activity relationship (SAR) assays and quantitative structure-activity relationship (QSAR) assays.

SAR assays involve small chemical modifications of the lead compound in an attempt to better utilise the steric and electronic interactions within the biological binding site. This in turn can have both positive and negative effects on the affinity of the compound with the biological binding site. If the structural changes to the ligand cause undesirable steric and/or electronic effects, a decrease in binding affinity will result which will be marked by a reduced potency of the compound (higher IC₅₀). The opposite is true for favourable steric and electronic interactions, leading to a higher binding affinity and in turn a lower IC₅₀.¹² Knowing both the favourable and unfavourable interactions paints a picture of the shape and electronic effects within the binding pocket.

QSAR is similar to SAR assays, though it involves a computational method that finds a quantitative relationship between steric and electronic features of structurally related compounds and their biological activity.¹³ Pharmacophores are identified in a small library of molecules and correlations between the pharmacophores and the biological activity are calculated which then allow for the prediction of the bioactivity of untested compounds, inevitably directing future synthesis. However, for QSAR to be effective, a large amount of structural information is required on previously known molecules that activate the targeted binding site.^{12,14}

1.2.3 Mechanism-based drug design

The term mechanism-based refers to the process of an enzyme catalysing its own inactivation.¹⁵ This typically occurs through an addition reaction, where an active nucleophilic residue located within the enzyme's binding site attacks an electrophilic centre on an inhibitor compound,¹⁶ forming a covalent bond.^{16,17,18} Mechanism-based drug design is therefore the design of a drug that will utilise the enzyme's catalytic mechanism to inactivate itself.¹⁹ Mechanism-based drug design can be approached in

two manners: when the biological targets structure is known, much like structure-based drug design, or when the structural information about the target remains unknown.

The first approach is the synthesis of a covalently binding drug through prior structural knowledge of the target binding site, similar to structure-based drug design. For example, a study conducted by Vavricka and colleagues²⁰ examined the possibilities of designing covalent inhibitors to target the influenza virus based on the knowledge of the active site of the viral neuraminidase (NA) enzyme. They found that the active site of the NA in influenza shared very few similarities with other organisms. Previous research indicated that a conserved tyrosine residue (Tyr406) within the NA active site could play an important role in the influenza NA mechanism.²⁰ Using a fluorescence-based kinetic analysis of wild type influenza and Tyr406-deficient strains, Vavricka and colleagues showed a hundred-fold reduction in k_{cat} (catalytic constant of the conversion from a substrate to a product²¹) indicating that Tyr406 is essential for the catalytic activity in influenza NA. Based on this, they hypothesised that Tyr406 was acting as a catalytic nucleophile and would be able to initiate a nucleophilic attack of a covalent inhibitor. Using previous knowledge that influenza NA is a glycosidase, Vavricka and colleagues utilised structural and mechanism-based knowledge to develop 2 α ,3 α -difluoro-Neu5Ac (**1**) as a carbohydrate-derived covalent inhibitor of influenza NA as shown in scheme 1.1.²⁰ It was envisaged that in order for **1** to undergo a nucleophilic attack at the anomeric carbon within the influenza NA enzyme, there needed to be several essential structural characteristics of the ligand. Firstly, a good leaving group is required at the C-2 position and a highly electronegative group at the C-3 position to prevent the formation of a potential oxocarbenium ion transition state. From the structural analysis of the binding pocket, it was also proposed that the leaving group should ideally be in the α -position and therefore facing the outside of the binding pocket, allowing it to leave more readily.²⁰ This led to a dramatic increase in potency of **1** compared to other influenza NA inhibitors.

Scheme 1.1: Proposed mechanism-based inhibition of influenza NA through the covalent binding drug **1**.²⁰

The second approach is not so well precedent. It involves prior knowledge of how the compound of interest would react when exposed to a nucleophile within an enzymatic-binding site. The expected reaction of the compounds are normally well understood within a chemically controlled environment. The question is then posed, whether it is possible to replicate these reactions within a cell through enzymatic activation, forming a covalent bond between the compound and the enzyme.² This is a concept that will be explored throughout this thesis and covered furthered in section 1.4.1 Cyclopropyl carbohydrates.

1.3 Covalent inhibitors

Covalently binding drugs have long been exploited throughout medicine for many different uses, from analgesics to anti-cancer agents.²² Approximately 30% of drugs currently on the market act through covalent interactions with their target.²² However, more recently, the development of covalent drugs has declined due to the perceived lack of selectivity and off-target reactivity, which led to consequences such as hepatotoxicity and neurotoxicity. These fears arose from several studies undertaken in the 1970's and 80's, which reported that chemically inert drugs were metabolised to form reactive metabolites that covalently bound to a wide array of unrelated proteins

in the liver.²³ The key to overcoming this problem of non-selectivity is to alter the drugs' chemical and physical properties.

As shown in scheme 1.2, the formation of a covalent complex with the target molecule occurs in two steps. Firstly a non-covalent complex is formed involving non-covalent interactions such as hydrogen bonds, hydrophobic interactions and van der Waals forces. This reversible binding positions the ligand in the correct orientation within the binding site. This then allows for the second step to occur, which is the subsequent formation of the covalent bond via attack of the ligand by the enzyme.

$E \cdot I$ = Initial non-covalent complex

$E - I$ = Final covalent complex

Scheme 1.2: General mechanism for covalent binding drugs, E denotes enzyme and I denotes inhibitor.

The selectivity of a covalently binding drug can be greatly increased by altering both the covalent and the non-covalent interactions. A high affinity for the enzyme's binding site, which is indicated by a low dissociation constant, K_i , drives the formation of the non-covalent complex through utilisation of as many non-covalent interactions as possible. The increase in non-covalent interactions better positions the drug within the binding pocket, bringing the electrophile within close proximity of the nucleophile and increasing the residence time of the ligand within the binding pocket. This increased residence time in turn facilitates enzymatic nucleophilic attack. Increases in both the residence time and the amount of non-covalent interactions allow for reduced reactivity of the electrophilic covalent drug. The nucleophilic reaction then occurs due to the close proximity of the electrophile to the nucleophile rather than the presence of highly reactive moieties, thus reducing the chances of off-

target binding and increasing the drugs selectivity.²⁴ Therefore, selectivity is enhanced when the inherent reactivity of the covalent inhibitor is low, ensuring covalent attachment is highly dependent on the long lifetime of the E•I complex (scheme 1.2).

1.4 Cyclopropane chemistry

Cyclopropanes (C_3H_6) are the smallest and simplest ring structures possible, consisting of only three carbon atoms. From the first reported synthesis of cyclopropanes in 1881 by August Freund,²⁵ to their first commercial use as an anesthetic for dogs,²⁶ thousands of cyclopropanated compounds have since been synthesised and discovered. Due to the interesting reactivity of the three-membered carbocycles, cyclopropyl moieties have become an important aspect in many pharmaceutical compounds. So much so that, as of April 2003, there were more than 200 patented pharmaceutical compounds containing cyclopropane functional groups in the literature.²⁷ Cyclopropane-containing compounds have not only been found to have biological activity in humans but also in plants and microorganisms, interacting mainly with enzymes in enzyme-catalysed reactions.

Cyclopropane ring opening is a very important reaction in many active pharmaceutical agents. The physical and chemical properties of the cyclopropane ring greatly aid in its ability to undergo ring opening reactions. As shown in figure 1.0A, cyclopropanes contain bond angles of 60° , which is a substantial deviation from the preferred tetrahedral bond angle of approximately 109° . This decreased bond angle greatly increases the ring strain within the cyclopropane, increasing the cyclopropane's potential energy and providing the driving force of ring opening reactions.²⁸ Additionally, the bonds within the molecule are bent. Although molecular orbital theory describes the cyclopropane's bonds as sp^3 -hybridised, drastic deviation away from the ideal tetrahedral angle results in bent bonds leading to an increased degree of p character²⁹ (figure 1.0B), and consequently increased reactivity. Cyclopropanes also have a high torsional strain due to the eclipsing interactions of vicinal substituents. The combination of both the high ring and torsional strain results in the ability of the cyclopropane to easily undergo ring opening reactions.

Figure 1.0: (A) Cyclopropane showing bond angle at 60°, (B) Molecular orbitals of cyclopropane showing bent bonds.

Cyclopropane moieties are often added as substituents to compounds, which due to their p-like characteristics provides an electrophilic centre, which can be subjected to nucleophilic attack resulting in ring opening or expansion reactions and the formation of a covalent bond. This can lead to irreversible attachment of the nucleophile to the original cyclopropane-containing compound.

Inhibition of dihydrofolate reductase (DHFR) by a pyrimidine (scheme 1.3) occurs in this manner.³⁰ Through a combination of both structure-based and mechanism-based drug design, Haddow and colleagues³⁰ proposed that the mechanism of action of DHFR occurred through a proton transfer from an aspartatic acid residue to dihydrofolate within the active site of the enzyme. From this, they hypothesised that the proton transfer from the aspartatic acid residue could activate a cyclopropyl substituent on pyrimidine (**2**) initiating a nucleophilic attack on the cyclopropane moieties from the enzymatic nucleophile. This in turn would cause a ring opening reaction to occur and, simultaneously, the formation of a covalent bond leading to the inhibition of the enzyme.³⁰ This study showed the first mechanism-based approach to the development of DHFR inhibitors.³⁰

Scheme 1.3: Inhibition of DHFR via a ring-opening mechanism of a spirocyclopropane-containing pyrimidine.³⁰

However, not all bioactive cyclopropyl compounds go through either a ring-opening or ring-expansion reaction to have an effect. Cyclopropanes have been used to constitute novel rigidity to oligopeptide ligands in order to mimic peptide secondary structures, increasing their binding affinity for the enzymes active site, in attempts to outcompete the natural peptide. Although pseudopeptides are commonly designed to mimic the turns and helices of peptides, Martain and colleagues³¹ aimed to incorporate cyclopropanes into an oligopeptide in order to rigidify the structure into a locked β -strand conformation, which was known to be an essential characteristic for the activation of HIV-1 proteases (figure 1.1). Following the synthesis of compounds **6** and **7** it was reported that when the substituents on the cyclopropane were in the trans position, this caused locking of the Φ angle (angle corresponding to the N-C α on the unmodified peptide backbone **5**) at approximately -120° resulting in the locked β -strand conformation. Although the conformation of **6** was only determined in solution it was reported as almost identical to the conformation of **7** of which Martain and colleagues³¹ were able to get an X-ray crystal structure of the ligand (**7**) within the HIV-1 protease binding site. This indicated that the preferred structural conformation

of these compounds in solution closely matches that of the conformation needed in the enzyme's active site.³² Both compounds **6** and **7** were found to be bioactive in the low nanomolar range, indicating potent inhibition of the HIV-1 protease. Therefore, Martain and colleagues³¹ results show compelling evidence that the trans-substituted cyclopropanes introduced into the oliopeptide are able to rigidify the structure locking it into a β -strand conformation.

Figure 1.1: Trans substituents on the cyclopropane provide structural rigidity locking **6** and **7** into a locked β -strand conformation.

1.4.1 Cyclopropyl carbohydrates

Carbohydrates are polyhydroxylated compounds with numerous chiral centres, which have made them an area of great interest in organic chemistry, with the synthesis of novel carbohydrate derivatives being used in different scientific disciplines such as glycobiology, glycomics and chemical synthesis. Cyclopropanes are commonly incorporated into carbohydrates through their installment at the 1,2-position of the sugar. The synthesis of 1,2-cyclopropyl carbohydrates commonly results from the addition of a carbene to a glucal. Though several protected glucals are commercially available, it is also possible to synthesise them from their corresponding parent sugars. The combination of both cyclopropanes and carbohydrates provides an

interesting area of research, with the chemical and physical properties of cyclopropanes such as ring and torsional strain as well as the numerous chiral centres of carbohydrate molecules. For example, a lot of research has been done around pyranoside-based cyclopropanes in ring expansion reactions to form seven-membered oxepines (schemes 1.5 and 1.6).^{33,34,35}

Ring opening reactions of 1,2-cyclopropanated pyranose sugars have been demonstrated to form 2-C-branched pyranosides^{34, 36} with the proposed mechanism involving the selective nucleophilic addition at the anomeric position resulting in the formation of a carbanion, which is stabilised by the electron-withdrawing substituent on C-7 (scheme 1.4). Under basic conditions, C-2 deprotonation occurs, causing the loss of a C-7 substituent formation of the 2-C-branched pyranoside. However, this mechanism has only been shown to occur in the presence of electron-withdrawing substituents on the cyclopropane.^{3,35}

Scheme 1.4: A plausible mechanism for ring opening forming C-2-branched pyranosides.

Formation of oxepines can occur through two different pathways as shown in schemes 1.5 and 1.6. Ring expansion to form the oxepine readily occurs in the presence of a nucleophile under basic conditions or in the presence of a Lewis acid.^{2, 34} Nucleophilic attack at the C-1 position, promoted by a Lewis acid or an electrophile, can result in the ejection of the substituent on either the C-7 position (scheme 1.5) or the C-3 position (scheme 1.6) depending on which position has the superior leaving group. Under the correct conditions, ring opening or expansion (schemes 1.4, 1.5 and 1.6) of 1,2-cyclopropanated sugars have characteristics susceptible to catalysis by enzymes. The enzyme would require a nucleophilic moiety and a correct spatial configuration in its binding site to accommodate the cyclopropanated sugar, along with the ability to stabilise a transition state during the subsequent reaction. Given these properties, nucleophilic attack on the cyclopropanated sugar could lead to the formation of a covalent bond resulting in irreversible binding. Depending on the target this might then result in biological activity, which is the starting process of mechanism-based drug design.

Scheme 1.5: A proposed mechanism for ring expansion through loss of a C-7 leaving group.

Scheme 1.6: A proposed mechanism for ring expansion through the loss of a C-3 substituent.

1.5 Target identification and mechanism of action

The identification of drug targets have been made possible due to the vast advancements made in the research field. To this end, methods for identifying molecular targets can be roughly split into three groups: computational approaches, genetic-based approaches and biochemical approaches. Computational approaches are used to predict drug targets based on the chemical similarities of known inhibitors; genetic-based approaches utilise gene deletion and over-expression sets as a tool to determine pathways, which the compound may act upon;³⁷ and biochemical approaches employ chemical proteomics and affinity purification methods for target identification.

Before target identification can occur, however, the discovery of small bioactive compounds that are able to elicit a desired response either against a cell or organism is required. Whilst cytotoxicity is often perceived as an end-point, addressing the phenotypic response can be carried out through two processes, namely reverse chemical genetics or forward chemical genetics screening, respectively.^{38,39,40,41}

Developments made in the fields of biochemistry and molecular biology makes screening a library of compounds against specifically selected proteins a readily accessible method for identifying new bioactive small molecules. This is known as reverse chemical genetics or target-based screening. The specifically selected proteins are normally involved in the pathophysiology of diseases such as G-protein coupled receptors and kinases. However, target selection and validation can be time-consuming processes. Selection of the target is based on its therapeutic potential and its accessibility in a high-throughput screen (HTS).⁴¹ When the biochemical target is identified, it must first be validated, typically through molecular biological approaches. Once validated, a library of small molecules is then screened to identify functional ligands that are able to induce or suppress the target's activity. When a small bioactive molecule is identified, it is then introduced into a cell or organism in attempts to study its mechanism of action.³⁸ However, although reverse chemical genetics may result in the identification of a highly specific bioactive small molecule, targeting a specific protein may prevent identification of potential off-target effects

that can arise in more complex systems as well as the activation of multiple signalling pathways.⁴² Additionally, targeting a specific protein involved in the pathophysiology of disease can ultimately impede the progress of drug development and discovery as it does not lead to the identification of new targets.⁴⁰

On the other hand, forward chemical genetic screening, also known as phenotype-based screening, screens small molecules against whole cells, allowing for the identification of potential new and unexpected targets and multiple signalling pathways, unlike reverse chemical genetics, which screen against a single isolated protein. This process has become possible due to the recent advances made in assay technology and involves the direct screening of small molecules to evaluate their effects on biological processes.⁴¹ The budding yeast *Saccharomyces cerevisiae* is a commonly used organism in phenotype-based screening due to its extensively studied genetics, rapid doubling time and easy maintenance, making it an ideal organism for HTS. Yeasts are screened against a library of small molecules in an attempt to induce a phenotypic change of interest. If the targeted phenotypic change occurs, the bioactive molecule will be selected for further investigation. This process allows for the rapid identification of bioactive compounds.

The development and use of forward chemical genetic screening has proved of great importance, with 28 of 75 first-in-class drugs being approved between 1999 – 2008 resulting directly from phenotype-based screening compared to only 17 having been developed from target-based screens.³⁹ Although forward chemical genetics allows for the potential discovery of new therapeutic targets, the elucidation of the molecular target remains one of the greatest limitations and challenges of forward chemical genetics. To this end, chemical proteomics methods such as affinity chromatography and chemical genetic analysis can be undertaken to identify the molecular target.⁴³

1.5.1 Affinity-based identification

Affinity purification to identify bioactive small molecules and their targets has proved to be the most direct method for target identification in which whole cell extracts are passed through the affinity matrices and a protein target are isolated.⁴⁴ For example,

the identification of calcineurin as a common target for both immunosuppressant's FK506 and cyclosporin A^{39,38,45} was achieved by subjecting a whole cell lysate to a FK506 and cyclosporin A affi-gel 10 affinity matrix. Affinity purification is considered to be the oldest, yet most commonly used method for target identification.⁴⁶

Affinity purification is commonly achieved through one of two methods. The first method involves the incorporation of an affinity tag such as a fluorescent chromophore to the bioactive molecule, whereas the second method typically requires a chemical modification of the compound so it can be immobilised onto a resin. Resins such as agarose are commonly employed, and the bioactive compounds are attached via a spacing linker group. Ideally, the linker group should be long enough to prevent steric interactions between the resin and the target protein and be mildly hydrophobic therefore preventing auto-aggregation.^{38,46} A reactive terminal on the linker allows it to undergo the appropriate coupling reaction to an appropriate functional group of the bioactive compound (figure 1.2). Alternatively, the bioactive compound can be conjugated onto moieties such as biotin, which interact strongly with avidin, resulting in a high affinity loading of the bioactive compound onto avidin agarose resin.^{38, 39}

However, the modification of bioactive small molecules must be carried out carefully in order to avoid a reduction in the bioactivity. To this end, it is essential that, after derivatisation of the bioactive molecule, it retains its target-recognising properties. This requires some prior knowledge of the interaction with the target, which can be established through SAR assays. The resulting analogue must then be re-evaluated to ensure that biological activity or target affinity is retained.⁴⁷

Figure 1.2: Linker groups attached to agarose or sepharose allow for the attachment of chemically modified bioactive compound. Cell lysate interact with the bioactive compounds allowing for interaction with the target protein. Non-specific proteins are eluted and treatment of the agarose/sepharose matrix with tryptic digest allows isolation of the specific binding proteins.

Following the installment of the bioactive compound to an affinity resin, it is then exposed to its target protein. This is done by passing either whole cell lysates or protein extracts through a column containing the immobilised compound, a process known as affinity pull-down (figure 1.2).^{41,39} This must be done under suitable conditions, as salts and detergents used in the cell lysate process can affect the target-compound interaction. This is normally followed by a series of washes to remove any non-specifically bound proteins. Finally, the tightly bound specific target proteins are removed using a wash or tryptic digest which hydrolyses proteins into peptides that can then be identified by process such as mass spectrometry.^{48,47,49, 42}

1.5.2 Chemical genetics

The process of target identification via genetics or genomic methods relies on the principle of genetic interacting pathways.⁴¹ This is most commonly done by the mutation of yeast genomes through deletion or over-expression of desired genes. The yeast genome was the first eukaryotic genome to be fully sequenced. It consists of 16 chromosomes, with approximately 6400 open reading frames (ORFs), of which approximately 4800 genes are non-essential such that deletion of the gene does not affect the viability of the yeast under standard laboratory conditions. Antibiotic-resistant markers and a synthetic barcode sequence have been integrated individually into the 4800 non-essential genes, allowing for the formation of yeast genome deletion sets (YGDS).^{50,37,51} Small bioactive molecules used in combination with YGDS can be used to help better understand gene activity. This is done through a process much like synthetic lethality. Synthetic lethality occurs when mutations of two non-essential genes lead to cell death, indicating the two pathways have the same essential end point.⁵² Chemical genetic analysis, however, requires the use of small inhibitory molecules to act as the 'second mutation' as shown in figure 1.3. HTS of a YGDS allows for the investigation of these interactions, as the small bioactive molecules act as modulators of gene activity. This high-throughput process is known as a deletion mutant array (DMA).⁵¹ Yeast that become non-viable or show a reduced viability will give insight to pathways and targets that are potentially involved in the bioactivity of the compounds.

Figure 1.3: Chemical genetic approach to identifying drug pathways and interactions. Demonstrating the concept of chemical genetics, showing that cells are not viable with inhibition of the drug target and deletion of the identifiable gene.

1.6 Basis of this research project

Initial interest began in mechanistic studies during the development of an effective synthetic route for the formation of oxepines. The inclusion of oxepines in numerous natural products and heptanoses presented these molecules as challenging synthetic targets. To this end, 1,2-cyclopropyl carbohydrates were chosen as substrates for the formation of oxepines through a cyclopropyl ring opening reaction and subsequent elimination of the C-3 substituent (scheme 1.5).³³ However, later research by Harvey and colleagues utilising 1,2-dibromocyclopropyl carbohydrate for the investigation of synthetic routes to oxepines derivatives, noticed an unexpected ring opening reaction and elimination of the C-7 substituent which took place, leading to the unexpected synthesis of 2-C-branched pyranosides (scheme 1.4).³⁴ These unusual and unexpected ring opening and expansion reactions were further examined in a mechanistic study.⁵³ Once these mechanisms were better understood, the question was posed whether ring opening and ring expansion reactions of 1,2-cyclopropyl carbohydrates could be enzymatically catalysed and, if so, might lead to a mechanism-based inhibition reaction.

An initial project exploring the use of cyclopropanated glucals as covalent inhibitors was conducted by Dylan Davies at Victoria University of Wellington.² He hypothesised that cyclopropanated sugars could undergo enzymatic ring opening or ring expansion reactions resulting in formation of a covalent bond between the cyclopropanated sugars and its target. Should the synthesised cyclopropanated glucals bind covalently to a target it was suggested that this would occur through one of the three mechanisms discussed in section 1.4.1 and shown in schemes 1.4, 1.5 and 1.6.

He synthesised a compound library of 27 glucals with varying substituents at the carbon positions 1-7 (numbering shown in figure 1.4). Of the 27 compounds synthesised, 20 contained a cyclopropane, 12 contained chlorine at the R₄ and/or R₅ position while six contained bromine at the same positions. Substituents in the R₁, R₂ and R₃ position included benzyl, acetyl, trityl, formyl as well as hydroxyl groups. The 27 compounds were subjected to a high-throughput SAR assay to identify the most bioactive compounds in *S. cerevisiae*. The SAR assay was run against the yeast strain *pdr1Δpdr3Δ* (drug efflux pump knockout strain). Of the 27 compounds subjected to

this yeast strain, five were active, with only one also showing activity against the BY4741 wild-type strain. Structural similarities between the five bioactive compounds allowed for the postulation of the essential pharmacophore elements for this series of compounds.

Figure 1.4: IUPAC numbering of carbons on both glucals and cyclopropanated carbohydrates.

The SAR study indicated that the presence of a benzyl group on the R₂ and R₃ positions was essential to the bioactivity of the compound. Lack of this benzyl group resulted in inactivity of the compounds. This indicated that nucleophilic attack of the C-1 carbon, resulting in ring expansion and loss of the C-3 substituent as shown in scheme 1.4 (section 1.4.1), was an unlikely mechanism for the bioactive compound synthesised by Davies as the benzyl group is a poor leaving group.

It was also noted from the SAR results that the compounds containing chloride at the R₄ and R₅ positions resulted in higher bioactivity. As bromine is a substantially better leaving group than chlorine, this greater activity of the chlorine-containing products was proposed to be due to the increased ability of chlorine to stabilise an adjacent negative charge due to its increased electronegativity. Bickelhaupt and colleagues⁵⁴ computationally showed that α -stabilisation of a carbanion by CH₂X is favoured by fluorine and progressively decreases for halogens of lower electronegativity. Therefore this would suggest that the formation of a covalent bond between the cyclopropanated glucals and the target was most likely to proceed by ring opening due to nucleophilic attack at the C-1 position resulting in the formation of a carbanion at the C-7 position as shown in scheme 1.5 (section 1.4.1). This negative charge will then be better stabilised due to the electron-withdrawing effects of the chlorine atoms.

This hypothesis will be further explored in this thesis by synthesising compounds with the ability to better stabilise a carbanion. Of the compounds synthesised by Dylan Davies, **23** was found to be the most active, shown in figure 1.5.

Figure 1.5: Chemical structure of 1,5-anhydro-3,4-di-*O*-benzyl-1,2-*C*-(dichloromethylene)-*D*-glycero-*D*-gulo-hexitol and carbon numbering according to IUPAC.

Davies synthesised a derivative of **23** to act as a molecular probe in order to ascertain whether the target protein could be identified using chemical proteomic affinity purification. This was attempted via the addition of two *p*-azidobenzyl protecting groups at the R₂ and R₃ position with the intention of the azide moiety undergoing a copper-assisted azide-alkyne cycloaddition with an alkyne-substituted sepharose matrix. However, although he was successful in adhering this derivative to the column matrix, no target was obtained and later analysis indicated bioactivity of this product was lost. This result reconfirms the necessity for benzyl groups at the C-3 and C-4 position.

Due to lack of target identification in the affinity purification, deletion set chemical genomic analysis was conducted² in an attempt to identify a mode of action. However although a number of sensitive mutants were identified to **23**, no enrichment through gene ontology (GO) analysis was found. Unusually, there were more deletion mutants that were resistant to the active compound than were sensitive. These resistant strains contained a higher percentage of genes involved in ribosomal translation. Therefore Davies hypothesised that **23** may affect a process that is involved in translation, processing of ribosomal subunits, or protein folding although the exact biological process remains unknown.

1.7 Research hypothesis and objectives

This thesis will further explore principles discussed in the thesis of Dylan Davies and extend his work done on 1,2-cyclopropyl carbohydrates.² It will be divided into two sections, an organic synthesis-based section and a biology-based section. Initial aims of this research will explore the hypothesis that ring opening occurs through nucleophilic attack at the C-1 carbon, leading to the formation of a carbanion. This will be achieved by the synthesis of **23** and variants that contains a carboxyalkyl group at the C-7 position of the cyclopropanated carbohydrate (**24**) instead of the chlorines (figure 1.6). We have hypothesised that the carbonyl will allow for resonance stabilization through the formation of an enolate, providing carbanion stabilisation.⁵⁵ The synthesised compounds will then be subjected to a bioactivity assay to see if bioactivity against *S. cerevisiae* is retained.

Figure 1.6: The proposed structure for a new lead compound, 1,5-anhydro-3,4-di-*O*-benzyl-2-deoxy-1,2-C-(exo-carbmethoxymethylene)-D-glycero-D-gulo-hexitol

The second aspect of this thesis will attempt to identify a mechanism of action and molecular target of **24a** and derivatives if they prove to be bioactive. Chemical genetic analysis using a *pdr1Δpdr3ΔxxxΔ* yeast deletion set library on all bioactive derivatives will be conducted in an attempt to identify a mechanism of action through enrichment via gene ontology. This deletion library contains systematic gene deletion mutants (denoted *xxxΔ*) in a genetic background, which includes deletion of PDR1 and PDR3 genes, both of which encode transcription factors for the pleiotropic drug resistance-related drug efflux pumps. Resistant and sensitive strains will be validated to ensure no false positives.

As previous attempts to isolate a molecular target were unsuccessful through the addition of a molecular probe at the C-3 and C-4 position, our aim is to modify the carboxyl group via the addition of a propargyl group. If bioactivity is retained, cell lysate will be subjected to the compound and a subsequent affinity pull down by a copper-assisted Huisgen azide-alkyne cycloaddition.^{56,57} Any isolated protein will be identified via mass spectrometry.

However, if **24** and its derivatives do not prove to be bioactive, further chemical genetic screening with **23** will be done in order to better identify the mode of action of the dichlorocyclopropane. This will include the use of yeast-based deletion and gene over-expression sets as well as fluorescent protein strains not used in the earlier study by Dylan Davies.

The main objectives of this research will begin with the initial aim to reproduce work done by Davies² in attempts to optimise his synthesis of **23** and produce a true biological replicate. This will be followed by the synthesis of carboxyalkyl derivatives **24**. Synthesised compounds will then need to undergo a bioactivity assay to assess whether they have retained bioactivity. Bioactivity assays will be conducted on *pdr1Δpdr3Δhis3Δ* yeast in liquid media. If bioactivity is retained within the new set of synthesised compounds, modification of the ester to incorporate a linker group (**24b**), allowing for, a chemical proteomic affinity pull-down therefore isolation of the molecular target will be undertaken. If a molecular target is isolated, analysis of the target will be conducted through mass spectrometry. Chemical genetic analysis using the *pdr1Δpdr3ΔxxxΔ* yeast deletion set library will then be conducted on the lead compound and results compares to the chemical genetic screen of **23**, in attempt to identify a mechanism of action and enrichment through GO. Analysis will look at both resistant and sensitive strains.

Chapter 2

Chemical synthesis

2.1 Aims and objective

Aims follow those presented in chapter 1. The aims of the work presented in this chapter are to synthesise target compound **23**, as well as the potential exploration of new C-6 protecting groups on this scaffold. This will be followed by the synthesis of 1,2-cyclopropyl carboxyalkyl derivatives **54** (ethyl) and **61** (propargyl).

Introduction

2.2 Cyclopropane synthesis

Since Simmons and Smith first reported their synthetic procedure for the preparation of cyclopropanes in 1959,⁵⁸ many new approaches have since been developed. The basis of the reactions, however, which is the addition of a carbene or carbene-like reagent across an alkene, remains the same.⁵⁹ Although reaction conditions have been expanded to use a range of different reagents, such as sulfur ylides, ethyl trichloroacetate, sodium trichloroacetates and methyl azides,⁶⁰ the focus of this chapter will be on the more common Simmons-Smith, Mąkosza and diazo-cyclopropanation methodologies.^{60, 61, 62}

2.2.1 Simmons-Smith cyclopropanation

Although the methodology for cyclopropane synthesis had already been established, Simmons and Smith reported the first cyclopropanation reaction that was stereoselective, high yielding and free of any unusual side reactions leading to unwanted by-products.^{63, 3} The synthesis consists of the addition of an alkene to diiodomethane and a zinc-copper couple. Although the exact mechanism of the reaction remains unknown, experimental evidence showed the formation of an

organozinc transition state (**26**), namely iodomethylzinc iodide, which can promote a methylene transfer through π -donation from the alkene, leading to subsequent ejection of zinc iodide and formation of the cyclopropane (scheme 2.1A).^{58, 64,63,62} Despite the known formation of transition state **26**, however, controversy around the exact mechanism still remains. The high stereoselectivity of the Simmons-Smith cyclopropanation system bearing an allylic oxygen atom is said to be due to the coordination of the zinc to the oxygen-containing substituents, such as a hydroxyl or ether group, proximal to the alkene resulting in the *syn*-product (scheme 2.1B).^{62,61} This has been shown to give diastereoselectivities in excess of 250:1.⁶¹

Scheme 2.1: (A) Proposed mechanism for the Simmons-Smith reaction, indicating the proposed transition state through the complexation of the zinc iodide.⁶⁴ (B) Stereoselective addition of the cyclopropane directed through oxygen complexation with organozinc.⁶⁵

2.2.2 Mąkosza *gem*-dihalocyclopropanation

Doering and Hoffmann were the first to report the synthesis of *gem*-dihalocyclopropane, with the synthesis of both dichloro- and dibromocyclopropanes in 1954.⁶⁶ This was achieved through the addition of chloroform to a solution of cyclohexane, potassium *tert*-butoxide and *tert*-butyl alcohol, which proceeds through an α -elimination of hydrochloric acid and formation of the dichlorocarbene.⁶⁶ However, emphasis was put on this reaction needing to occur under anhydrous conditions due to the rapid reaction of dichlorocarbene with water. Mąkosza and Wawrzyniewicz reported an improvement of this synthesis in 1969,⁶⁷ in which dichloro- and dibromocyclopropanes were synthesised from the corresponding haloforms in the presence of concentrated sodium hydroxide and catalytic amounts of triethylbenzylammonium chloride (TEBAC). Although the reaction was carried out under aqueous conditions, it was reported that only a small percentage (approximately 7%) of the dihalocarbene reacted with water. This is due to the use of TEBAC as a quaternary ammonium phase-transfer catalyst.⁶⁷ In this two-phase catalytic system, the haloform is initially deprotonated by the aqueous sodium hydroxide at the interfacial region of the reaction, forming the sodium haloform salt (scheme 2.2A). The haloform anion then complexes with the quaternary ammonium salt at the interfacial region, allowing it to enter the organic phase, where it begins to decompose into the highly reactive carbene (scheme 2.2B). The carbene can then react with the alkene to form the desired cyclopropane (scheme 2.2C).^{67, 60}

Unlike the Simmons-Smith cyclopropanation, addition of the carbene is not directed through the oxygen substituent, instead the addition of the carbene across the alkene is directed through steric interactions, where the carbene is stereoselectively added across the sterically less hindered face.^{68,3} The Mąkosza cyclopropanation provides an effective and high yielding synthesis of *gem*-dihalocyclopropanes due to the biphasic nature of the reaction, preventing the harsh aqueous condition from promoting any undesirable side reactions, such as ring opening reactions, without the need to keep the reaction in absolutely anhydrous conditions as seen in the methodology proposed by Doering and Hoffmann.⁶⁶

Scheme 2.2: Mąkosza cyclopropanation mechanism, indicating phase shifts (Org: organic phase; Int: interphase; Aq: Aqueous); (A) Deprotonation of chloroform; (B) Complexation of the quaternary ammonium cation with the chloroform anion; (C) Decomposition of quaternary ammonium and chloroform, formation of the carbene and synthesis of the cyclopropane.

TEBAC= Benzyltriethylammonium chloride

2.2.3 Diazoester cyclopropanation

The decomposition of diazo compounds into carbenes has been employed for more than a hundred years,⁶⁹ however, the suggestion that the bond formation between the transition-metal catalyst and the diazo compounds promotes the ejection of the dinitrogen species was only proposed some 50 years ago by Peter Yates.⁷⁰ Here, Yates hypothesised that after the ejection of the dinitrogen species the carbene remained bound to the metal catalyst (scheme 2.3).⁷⁰ A milestone in the catalytic use of copper in this manner came many years later when Salomon and Kochi reported the use of copper triflate as an effective metal catalyst in the addition of diazo-compounds to an alkene. The addition caused a reduction of copper (II) to copper (I),

confirming that copper (I) is the active catalyst in the transformation of the carbenoid.⁷¹

Although very effective copper catalysts were still being discovered,⁷¹ the coinciding use of palladium (II) acetate and rhodium (II) acetate by Teyssie and colleagues^{72,73} was proving far more successful. In the case of rhodium acetate, this is due to the thermal and air stability of the catalyst, its inability to undergo π - π interaction with the alkene as well as its coordination unsaturation. As rhodium acetate is a binuclear compound with D_{4h} symmetry and four bridging acetates it possesses only one empty axial coordination site per metal atom.⁶⁹ At the stable oxidation state of +2 with five surrounding ligands (four metal-oxygen bonds and one metal-metal bond), rhodium acetate is a coordinatively unsaturated catalyst as it is lacking two electrons to satisfy the 18 electron rule. This means rhodium acetate does not need to undergo ligand displacement before it becomes a reactive catalyst, making it an effective electrophile, readily undergoing nucleophilic attack by the diazo compounds (scheme 2.3).

The proposed mechanism for the rhodium carboxylate begins with the generation of a metal-carbene species, which adds across the alkene at a later stage to form the cyclopropane, completing the catalytic cycle (scheme 2.4). The suggested formation of the carbenoid occurs through the complexation of the electrophilic α -centre onto the metal catalyst. This in turn leads to the ejection of nitrogen gas to form the carbon-metal double bond (scheme 2.3). Position of the alkene and overall stereochemistry depends on the steric effects of the substituents on both the diazo-compound and the alkene. In the absence of any dominating steric effects *syn*-stereoselectivity is typically observed, with the ratio of *syn/anti* decreasing as the steric bulk of both the alkene and diazo-compound increases.^{74,69} As expected, the addition of the carbene favourably occurs across the sterically less hindered face. Diazoester cyclopropanation provides an effective way to introduce a cyclopropane moiety into a compound, particularly due to the high controllability of the resulting stereochemistry established by metal catalysts such as rhodium acetate.

Scheme 2.3: Mechanism for the formation of a carbenoid species. **35** shows the ejection of the dinitrogen and the formation of the carbenoid species **36**.⁷⁵

Scheme 2.4: Catalytic cycle of carbenoid cyclopropanation.

Results and discussion

2.3 Synthesis of 1,5-Anhydro-3,4-di-*O*-benzyl-2-deoxy-6-*O*-trityl-D-arabino-hex-1-enitol (**40**)

The use of glycals as precursors for the synthesis of 1,2-cyclopropyl carbohydrates is well preceded in literature as reported by Hoberg,^{76,77} Nagarajan⁶⁸ and Harvey.^{3,34} Synthesis of 1,2-cyclopropyl carbohydrates is commonly achieved by the addition of a carbene to the glycal alkene through one of the three methods discussed in section 2.2. Throughout this project the protected glucal 1,5-anhydro-3,4-di-*O*-benzyl-2-deoxy-6-*O*-trityl-D-arabino-hex-1-enitol (**40**) was used as a substrate for the synthesis of 1,2-cyclopropyl carbohydrates, as its derivatives have been shown to facilitate bioactivity.² It can also be readily synthesised from D-glucose (**63**) in five steps (scheme 2.5). Methodology for the synthesis of glucals was initially developed by Karl Zach and Emil Fischer in 1913⁷⁸ and subsequently optimised by others.^{79,80} Synthesis of **39** proceeded with the per-acetylation of D-glucose (**63**) via the addition of acetic anhydride and catalytic amounts of perchloric acid. The resulting peracetylated glucose was subjected to a selective substitution reaction at the C-1 position through the addition of HBr leading to isolation of the α -bromoglycoside (**64**) intermediate as a white solid. Following its isolation, the crude glucosyl halide **64** was then taken up in a solution of H₂O, acetic acid and zinc dust to undergo a reductive elimination reaction,⁸¹ where the anomeric bromide and the C-2 acetyl group are eliminated, resulting in the formation of the 1,2-alkene **37** in a 65% yield over 3 steps. Addition of a reduced amount of HBr compared to literature led to the formation and isolation of the by-product glucose penta-acetate, resulting in the overall moderate yield of **37**.

Scheme 2.5: Synthesis of tri-*O*-acetyl-D-glucal (**37**).

The de-acetylation of **37** is a well-documented reaction with many established procedures. Reaction conditions developed by Czernecki and colleagues⁸² involving methanol and an anion exchange resin resulted in high yields, however, reaction times of approximately 15 hours made the reaction undesirable. Therefore, a modification by Moore and colleagues³⁵ involving Zemplén type conditions, was preferred, with the much shorter reaction time of approximately 15 minutes. Accordingly, the triacetate **37** was dissolved in methanol and treated with catalytic amounts of solid sodium, resulting in the *in situ* formation of sodium methoxide to yield the crude deprotected glucal **38** within 15 minutes. The isolated crude material was then subjected to purification via the successive additions of methanol, acetone and diethyl ether, allowing for precipitation and therefore removal of any impurities, resulting in an overall yield of 99% of the pure triol **38** (scheme 2.6).

Scheme 2.6: Synthesis of 1,5-anhydro-3,4-di-*O*-benzyl-6-*O*-trityl-D-arabino-hex-1-enitol (**40**).

In order to retain the integrity of the cyclopropane that will eventually be installed, an orthogonal protecting group in relation to the highly strained cyclopropane group was required. To this end, trityl chloride was chosen to selectively protect the C-6 OH group due to its mild acid-mediated deprotecting conditions, which would avoid the possibility of base-mediated cyclopropane ring opening reactions or benzyl

deprotection.^{34,68} In a modification of the procedure published by Schmidt and colleagues,⁸³ protection of the primary hydroxyl on the triol **38** was achieved through the addition of trityl chloride to a solution of **38** in a 1:1 mixture of pyridine:dichloromethane (v:v), resulting in high yields of the mono-protected glucal **39**. This then allowed for the protection of both secondary alcohols at the C-3 and C-4 positions. Benzylation was achieved through a modification of the methodology reported by Schmidt and colleagues,⁸⁴ wherein diol **39** was dissolved in DMF followed by the slow addition of NaH and BnBr. Initial benzylation attempts were low yielding due to the inability to keep the reaction under anhydrous conditions. Optimisation of the reaction through the prior co-evaporation of the starting material **39** with DMF to remove traces of water, along with the addition of the TBAI to provide a source of iodide anions for a nucleophilic displacement reaction, proved to be very successful, yielding **40** in high yields (86%). No C-4 mono-benzylated product was isolated as often seen in literature.³⁶

2.4 Synthesis of *gem*-dichlorocyclopropane

Brimacombe and colleagues⁸⁵ reported the first addition of a carbene to an unsaturated sugar in 1967 through a modification of the initial cyclopropanation procedure proposed by Doering and Hoffmann.⁶⁶ However, the essentiality of highly anhydrous conditions made this reaction particularly challenging. To this end, Gross and colleagues⁸⁶ utilised a modification of the methodology proposed by Mąkosza⁶⁷ in the synthesis of cyclopropane-containing compounds from unsaturated sugars, in which a phase transfer catalyst was used, allowing for the use of a 50% (w/w) solution of aqueous sodium hydroxide. Reaction times ranged from 1-6 hours resulting in yields of $\geq 96\%$. This provided the first reported synthesis of a small library of unsaturated cyclopropyl carbohydrates under aqueous conditions. Nonetheless, due to the safety hazards associated with the high concentration of sodium hydroxide utilised in the synthesis proposed by Gross and colleagues, optimisation of the reaction was necessary.

Using a modification of the procedures employed by Gross and colleagues,⁸⁶ the protected glucal **40** was dissolved in chloroform and treated with the phase transfer

catalyst TEBAC. In attempts to reduce the high concentration of sodium hydroxide used by Gross,⁸⁶ the reaction was treated with the dropwise addition of a 33% (w/w) solution of aqueous sodium hydroxide. As expected, the reduction in sodium hydroxide concentration led to a longer reaction time of two days compared to the reported 6 hours. This, however, still successfully yielded both the major (**41**) and the minor (**42**) product in yields of 83% and 13%, respectively (scheme 2.7) which is comparable to those achieved by Gross. Conceivably, the isolated major stereoisomer resulted from the addition of the carbene to the α -face of the glucal due to the steric hindrance exerted by the C-3 benzyl group as well as the bulky C-6 trityl protecting group.

Scheme 2.7: Synthesis of cyclopropane derivatives **41** and **42**.

Separation of the two stereoisomers was achieved through silica column chromatography utilising a 14:1 (v:v) hexanes:ethyl acetate solvent system. Only the major α -stereoisomer **41** was utilised throughout the rest of this thesis as this stereochemistry was shown to be favourable for the compound's bioactivity.²

¹H NMR data for the major product **41** matched those previously reported by Nagarajan and colleagues.⁶⁸ Successful cyclopropanation was marked by the noticeable upfield shift in H-1 from 6.49 ppm in the glucal **40** to 4.05 ppm (doublet, $J_{\text{H1-H2}} = 8$ Hz) and H-2 from 4.88 ppm to 1.89 ppm (doublet of doublets, $J_{\text{H-H}} = 8.1, 4.9$ Hz). The assignment of the spectral data of the minor product **42** was consistent with that proposed by Moore,³⁶ with again a notable shift in H-1 when compared to the glucal starting material **40** from 6.49 ppm to 4.05 ppm (doublet, $J_{\text{H1-H2}} = 8.1$ Hz)

and H-2 from 4.88 ppm to 2.12 ppm (triplet, $J_{\text{H-H}} = 7.8$ Hz) as shown in figure 2.0. Small amounts of inseparable impurities were present in **42**, however, this stereochemistry has been shown to yield little bioactivity as an IC_{50} of 300 μm in *pdr1* Δ *pdr3* Δ *xxx* Δ yeast deletion sets was reported by Davies², therefore no further synthetic efforts were taken with this compound. Successful cyclopropanation was also confirmed by HMBC through the coupling of C-7 (61 ppm) to H-2 (4.88 ppm) and H-3 (4.17 ppm).

Figure 2.0: ^1H NMR of compound **40** (red), **41** (green) and **42** (blue) showing noticeable upfield shifts in both H-1 and H-2.

Acid-mediated deprotection of the C-6 trityl group was demonstrated by Nagarajan and colleagues⁶⁸ via the dropwise addition of formic acid. However, it was later noticed that the unexpected formation of the by-product **43** resulted from the esterification of the primary alcohol on **23** with formic acid under the acidic conditions.³⁶ Reaction conditions were optimised by Davies² in order to reduce the production of the formate ester by-product. Although some of the formate ester by-product was still formed, optimised conditions recommended a reaction time for 2

hours in diethyl ether resulting in a 7:3 ratio in favor of the desired product at a 45% yield, along with the recovery of 20% starting material.² Despite the recovered starting material, this was a substantial improvement from the previous synthesis where an isolated yield of 22% was reported in a 1:2.5 ratio in favor of the formate ester by-product. In the current study, synthetic attempts using the reaction conditions proposed by Davies, led to low yields and a 1:1 ratio of the desired product (**23**) to by-product (**43**). Therefore optimisation of the reaction proposed by Nagarajan and colleagues⁶⁸ was undertaken by slightly increasing the concentration of formic acid and decreasing the amount of solvent, in turn concentrating the reaction. The reaction was then very closely followed by TLC analysis and quenched after 50 min resulting in high yields and a 9:1 ratio of the desired product (**23**) to the formic ester by-product (**43**) (scheme 2.8). Optimisation of the workup proposed by Nagarajan and colleagues⁶⁸ was also undertaken as it was noted by Moore⁵⁹ that the concentration of the reaction, before the dilution with dichloromethane as initially proposed by Nagarajan, resulted in conditions favourable for ester formation. The reaction was therefore quenched with diethyl ether and washed with saturated sodium bicarbonate before the concentration of the crude material. These changes led to production of the desired deprotected alcohol **23** in 81% yield, a substantial improvement from the previously reported 45%.²

Scheme 2.8: Synthesis of **23** and **43**.

2.5 Synthesis of (((2*R*,3*S*,4*R*)-3,4-bis(benzyloxy)-3,4-dihydro-2*H*-pyran-2-yl)methoxy)(*tert*-butyl)diphenylsilane (45)

Before the optimisation of the trityl deprotection reaction (scheme 2.8) was undertaken, alternative primary alcohol protecting groups were explored due to the loss of yield caused by the formation of the formate ester by-product during detritylation.³⁶ A literature search revealed several alternative C-6 protecting groups, however, the decision to use *tert*-butyldiphenylsilyl chloride (TBDPSCl) was made due to two factors. Firstly, selective deprotection of TBDPSCl occurs through the use of tetrabutylammonium fluoride hydrate (TBAF) at very moderately basic conditions, reducing the likelihood of base-mediated cyclopropane ring opening,^{34,68,36} along with orthogonality with benzyl protecting groups.⁸⁷ Secondly, the use of a bulky protecting group would increase the facial steric effects, leading to a higher stereoselectivity for cyclopropane formation on the α -face of the carbohydrate.⁶¹ The literature also indicated no significant reduction in yield during both protection and deprotection using TBDPSCl.^{88,89,90}

The use of TBDPSCl as a primary protecting group on unsaturated carbohydrates has become prevalent in the literature since it was first reported in 1976 by Guthrie and colleagues.⁹¹ In a modification of the procedure written by Lellouche and colleagues,⁹² a solution of the triol **38** and imidazole in DMF was treated with a slight excess of the TBDPSCl at low temperatures (scheme 2.9). It was important to keep the temperature low and the amount of TBDPSCl close to stoichiometric to prevent the undesirable protection of either the C-3 or C-4 hydroxyl groups. Due to the sensitivity of TBDPSCl to water, it was also important to keep the reaction anhydrous. To this end, imidazole was co-evaporated with toluene three times, followed by the co-evaporation of the starting material **38** and imidazole with DMF to remove any traces of water. However, the reaction resulted in disappointing yields, which was accounted for by the poor quality of the TBDPSCl as two spots were seen on the TLC of the reagent and ¹H NMR spectroscopy of the TBDPSCl showed the presence of TBDPSOH, indicating the presence of water in the reagent.

It was also suspected that **44** could undergo silyl migration during the benzylation reaction of the secondary C-3 and C-4 hydroxyl groups as reported in the literature,⁹² therefore assessment of this was first undertaken before attempting to improve the yields of the previous reaction. In an optimisation of the method proposed by Lellouche,⁹² BnBr was added to a solution of the diol **44** in DMF prior to the slow addition of NaH in an attempt to reduce the chances of any silyl migration. No silyl migration was observed, however, the mono-benzylated by-product (**63**) was observed after the reaction in addition to the desired product **45** (scheme 2.9). Benzylation had been expected to occur at the less sterically hindered C-3 hydroxyl group first, but this was contrary to the isolation of **63** in a 30% yield. Whilst waiting for delivery of new, dry TBDPSCl the decision to optimise the detritylation reaction was undertaken resulting in very favourable results (section 2.4, scheme 2.8). It was therefore decided to continue along that synthetic route and further optimisation of TBDPSCl was not undertaken.

Scheme 2.9: TBDPSCl protection and syntheses of compounds **44**, **45** and **63**.

2.6 Synthesis of the ethoxycarbonyl substituted 1,2-cyclopropyl carbohydrates **54** and **55**.

Due to the hypothesis that mechanism-based inhibition of the dichloropropyl derivative **23** occurred through a ring opening reaction and formation of a carbanion (scheme 1.6), the carboxy ether cyclopropane **24** became the new synthetic target. Enolate stabilisation of the carbanion was hypothesised to encourage ring opening reactions. Therefore the synthesis of diazoacetates, in particular ethyl diazoacetate (**49**) was undertaken. In a modification of a procedure reported by Patnaik and colleagues,⁹³ glycine was added to a pre-mixed solution of acetyl chloride and ethanol to undergo a Fischer esterification reaction, yielding **47** quantitatively (scheme 2.11). As shown in scheme 2.10A, the pre-mixing of acetyl chloride and ethanol provides an anhydrous source of HCl. The resulting acidic conditions allow for the protonation of the glycine carbonyl and initiates the Fischer esterification reaction (scheme 2.10B).⁹⁴ This reaction was repeated to synthesise the methyl and butyl derivatives, **46** and **48**, respectively, from the corresponding alcohols in quantitative yields (scheme 2.11). The syntheses of **46**, **47** and **48** as hydrochloride salts provided greater stability of the compounds.⁹⁵

Scheme 2.10: A) *In situ* synthesis of HCl through esterification of acetyl chloride (**63**). B) Fischer esterification of glycine (**66**) for the synthesis of ethyl glycine hydrochloride (**47**).

Since the synthesis of the first diazo compound over 100 years ago,⁹⁶ new versatile and effective methodologies have been developed.⁹⁷ Much effort is focused around creating safer synthetic routes for these explosive compounds^{98,99} or making them more environmentally friendly by removing the necessity for heavy metals.^{97b} To this end, a modification of the well-documented procedure initially published by Searle¹⁰⁰ in 1955 was utilised^{101, 97} where ethyl glycinate hydrochloride **47** was reacted with sodium nitrite at -9 °C. Care was taken to maintain the reaction at -9 °C during the addition of concentrated sulfuric acid as increases in temperature have been reported to result in reduced yields. Due to the explosive nature of the diazo compound **49**, the final product was not concentrated and a yield of 70% was assumed as per the literature (scheme 2.11).¹⁰⁰ Furthermore, due to the specific warning about the especially explosive nature of methyl diazoacetates (**69**),¹⁰⁰ it was decided to discontinue its synthesis.

Scheme 2.11: Syntheses of compounds **46**, **47**, **48** and **49**. Proposed syntheses of **69** and **70** was not undertaken due to safety concerns around the explosive nature of the compounds.

For the syntheses of cyclopropyl carbohydrates **54**, **55**, **56**, several different catalysts and procedures were considered. Although palladium and copper catalysts are known to form metal-alkene pre-complexes which allows for a more directed stereoselective addition of the carbenoid across the alkene,¹⁰² rhodium acetate was selected for the following synthesis due to the higher yields reported in literature and reasons

discussed in section 2.2.3.^{102,72,76} It was also believed that, due to the steric effects of the C-3 and C-4 benzyl groups along with the C-6 trityl protecting group on the diol **40**, steric hindrance would favour selective addition of the carbenoid from the desired α -face of the alkene. Due to the likeness of starting materials, methodology proposed by Timmers and colleagues¹⁰³ was first employed. Although methodology for the cyclopropanation of the substrate triacetyl glucal was well-precedent in the literature, it was believed that the added steric stress of the benzylated glucal utilised in the procedure proposed by Timmer and colleagues¹⁰³ would give a more accurate representation of the substrate **40**. Thus, protected glucal **40** was treated with ethyl diazoacetate (EDA) over 1 hour, however this resulted in a 95% recovery in starting material (table 2.0, entry 1). Accordingly, optimisation of the reaction was undertaken as shown in table 1. Hoberg and colleagues⁷⁶ reported that rapid addition of EDA to unsaturated glucals causes a decomposition of the EDA species, the formation of ethyl fumarates and the recovery of the starting material. The addition of the EDA was thus extended to a dropwise addition over 4 hours according to the procedure proposed by Risseuw and colleagues,^{104,105} however this still led to a disappointing yield and large amount of recovered starting material (entry 2).

Entry	Glucal (equiv.)	Time (hours)	Temperature (°C)	Rhodium Acetate	Rhodium acetate equiv.	Yield
1	40 (1.5)	1	r.t	Sigma (1)	0.5	0% (95% SM)
2	40 (1.5)	4	r.t	Sigma (1)	0.5	7% (90% SM)
3	40 (1.5)	14	r.t	Sigma (1)	0.05	25% (61% SM)
4	38 (3)	14	r.t	Sigma (1)	0.05	0% (60% SM)
5	38 (3)	12	40	Sigma (1)	0.05	3% (81% SM)
6	38 (3)	12	r.t	Compound 50	0.05	21% (37% SM)
7	40 (3)	12	r.t	Sigma (2)	0.05	28% (70% SM)
8	40 (3)	1	r.t	Sigma (2)	0.05	0% (90% SM)
9	40 (3)	14	r.t	Sigma (2)	0.05	58% (21% SM)

Table 2.0: Optimisation of diazoester cyclopropanation reaction. Sigma (1) and Sigma (2) represents the 1st and 2nd batch of rhodium acetate purchased from Sigma respectively. All yields reported are combined yield of both stereoisomers.

A slower addition of EDA of 14 hours improved the yield to 25%, nonetheless an appreciable amount of starting materials were still recovered (entry 3). This was thought to be due to the high steric bulk of the alkene **40** impeding the addition of the carbenoid to the alkene, as increases in the size of the glucal substituents have been shown to cause a reduction in yield.⁷⁶ Therefore the fully unprotected model glucal **38** was used to evaluate the influence of steric hindrance of the starting materials. However, comparable yields to literature⁷⁶ were not obtained even at higher temperatures as shown in entries 4 and 5. The results put doubt on the purity of the rhodium acetate, which prompted the synthesis of fresh rhodium acetate.

The synthesis of rhodium acetate was undertaken by the treatment of hydrated rhodium trichloride with sodium acetate in methanol. Crystallisation of the reaction mixture allowed for the formation of the methanol adduct (Rh(OAc₂)MeOH), where methanol is bound as a ligand, which upon drying yielded the active rhodium acetate (**50**) in a 28% yield. Not only did the use of the newly synthesised rhodium acetate on the triol **38** give higher yields, but more importantly, this gave the lowest amount of recovered starting materials yet (entry 6), indicating a much more reactive catalyst. Thus, a newly purchased batch of rhodium acetate was used with the benzylated glucal **40** under the same conditions to give modest yields similar to those obtained before (entry 7). To confirm the effect given by the speed of EDA addition, the reaction was repeated where EDA was added over an hour, which proved unsuccessful (entry 8). Finally, increasing the time of addition to 14 hours resulted in a favourable yield of 58% (entry 9) for the desired stereoisomers **52** and **53** (scheme 2.12). Separation of the two stereoisomers was attempted, unsuccessfully, therefore the assignment of the stereochemistry was not facile via NMR. However, NMR data indicated the major isomer, which was assumed to be **52** on the basis of steric arguments, was in approximately a 9:1 ratio to the minor isomer **53**. These results were consistent with those reported by Risseuw and colleagues¹⁰⁴ in which separation of the two stereoisomers was not achieved until after the selective deprotection of the C-6 protecting group.

Scheme 2.12: Optimised synthesis of **52** and **53**, leading to an inseparable mixture of both stereoisomers as reported by Risseuw and colleagues.¹⁰⁴

Utilising the aforementioned optimised C-6 trityl deprotection conditions (scheme 2.7),² detritylation of compounds **52** and **53** was achieved with minimal formation of the formate ester by-product (scheme 2.13). Due to the low yields of the formate ester by-product, no formate of the minor isomer was detected. Deprotection of the trityl group allowed for the separation of the stereoisomers through silica gel column chromatography utilising a slow gradient elution of hexane:ethyl acetate of 14:1 to 1:1 (v:v), and allowing for the isolation of the major product in a pleasing 61% yield (scheme 2.13).

Scheme 2.13: Deprotection of the trityl group of the inseparable mixture of stereoisomers **52** and **53**, affording **54**, **55**, and **56**.

HMBC spectroscopy along with correlated 1D and 2D NMR spectroscopy data were used to confirm the formation of the cyclopropane moiety through the interaction between the carbene and the alkene (figure 2.1). As shown in figure 2.1, coupling of both H-2 and H-3 to C-7 indicates the successful addition of the carbene to the glucal alkene.

Figure 2.1: HMBC coupling of **54** (CDCl_3) showing the interactions of H-2 and H-3 with C-7, indicating a successful cyclopropanation.

Although the α -product **52** being the major product was consistent with the hypothesis that carbene addition to the α -face would be more favourable due to the steric hindrance of the pseudo-axial protecting groups on C-6 and C-3, and the opposite being true for the minor product **53**, it was deemed prudent to confirm this via computational modelling of both compounds. To this end, lowest energy conformers of the diastereomeric C-7 ethoxycarbonyl 1,2-cyclopropane carbohydrates **54** and **55**, differing in the configuration at the ring fusion between C-1 and C-2, as well at C-7, were determined by molecular mechanics. Each structure was subjected to exhaustive conformational searching using the mixed torsional/low mode sampling routine as implemented in MacroModel version 9.7 and visualised in Maestro 9.0. A minimum

of 20,000 steps were applied. Structures obtained were minimised using the OPLS_2005 force field using the GB/SA chloroform continuum solvent model and the Polak–Ribiere conjugate gradient (PRCG) conjugate gradients method and terminated on a gradient threshold of $0.05 \text{ kJ mol}^{-1} \text{ \AA}^{-1}$. The simulation was continued until the lowest energy structure reported had been replicated at least 50 times. The global minimum structure of major diastereomer **54** from this calculation was subjected to equilibrium geometry optimisation using the B3LYP hybrid density functional method 23, 24 and the 6-31G** basis set in Spartan '08, resulting in no differences in the key dihedral angles around the pyranose and cyclopropane rings. The lowest energy conformers for both **54** and **55** reported from the molecular modelling are presented in figure 2.2. From the molecular model, the dihedral angles around both the glucose derived ring and the cyclopropane ring were obtained, allowing for the calculation of the expected coupling constants. The dihedral angles obtained from the molecular modeling are shown in table 2.1

Figure 2.2: Lowest energy conformations of **54** and **55**.

Dihedral angles				
	H-1, H-2	H2, H-3	H-1, H-7	H-2, H-7
Compound 54	2.7	104.5	143.8	146.6
Compound 55	4.5	34.3	143.3	146.5

Table 2.1: Calculated dihedral angle of compound **54** and **55** utilising MacroModel version 9.7.

From the calculated dihedral angles, predicted coupling constants were then obtained for the lowest energy conformers through the utilisation of a modified Karplus equation proposed by Haasnoot¹⁰⁶ and implemented by Sweet J computer program.^{107,107b} As shown in table 2.2, the comparison of the calculated coupling constants and the measured coupling constants show a reasonable correlation. Deviation away from the measured coupling constants is most likely to be due to the strain of the cyclopropane ring.

Calculated coupling constants				
	H-1, H-2	H2, H-3	H-1, H-7	H-2, H-7
Compound 54	7.42 Hz	0.90 Hz	6.95 Hz	9.44 Hz
Compound 55	7.39 Hz	7.34 Hz	6.75 Hz	9.44 Hz

Measured coupling constants				
	H-1, H-2	H2, H-3	H-1, H-7	H-2, H-7
Compound 54	7.25 Hz	0 Hz	2.25 Hz	6.25 Hz
Compound 55	2.5 Hz	5 Hz	6.5 Hz	9.5 Hz

Table 2.2: Calculated coupling constants generated by Sweet J from the dihedral angle obtain from MacroModel version 9.7 compared to the measured coupling constants of compound **54** and **55**.

Due to the deviation of the measured coupling constant away from the calculated coupling constant of the H-1, H-7 coupling on **54** and H-1, H-2 coupling on **55**, NOE analyses of both compounds **54** and **55** were undertaken to provide further weight to the stereochemical assignment. NOE analysis of compound **54** through the irradiation of H-7 showed the interactions between H-7 with H-5 and H-3, which confirmed that the addition of the carbene had occurred from the α -face of the alkene with the carboxyethyl group in the *exo*-position to the C-3 and C-2 hydrogen (figure 2.3A). Irradiation of proton H-2 of compound **55** also showed its interactions with H-5, which confirmed the addition of the carbene from the β -face of the alkene as predicted by molecular modeling (figure 2.3B).

Figure 2.3: A) NOE of **54**, irradiation at H-7, NOE correlations shown on compound **54**.

Figure 2.3: B) NOE of **55**, irradiation at H-2, NOE correlations shown on compound **55**.

Full characterisations of compounds **54** and **55** were thus achieved using a combination of both 1D and 2D NMR data, along with molecular modelling and NOE correlations. This allowed for parallel identification of the stereochemistry of both the cyclopropane and the carboxyethyl groups.

2.7 Synthesis of the propargyloxycarbonyl substituted 1,2-cyclopropyl carbohydrates **61** and **62**.

Due to the successfully optimised methodology used in the synthesis of the ethoxycarbonyl substituted 1,2-cyclopropyl carbohydrates **54** and **55**, a similar synthetic route was proposed for the synthesis of propargyloxycarbonyl 1,2-cyclopropyl carbohydrates **61** and **62**. As with the synthesis of the ethyl diazoacetate, initial synthesis of propargyl glycinate hydrochloride was first targeted. This was undertaken through a Fischer esterification of propargyl alcohol with glycine utilising acetyl chloride as a precursor for the *in situ* formation of HCl as proposed by Patnaik and colleagues (scheme 2.14).⁹³ However this resulted in a black viscous solution, which after several attempts at purification yielded no product. The formation of a black by-product was thought to be due to a side reaction with acetyl chloride resulting in polymerisation of the propargyl alcohol. Accordingly, an alternative method reported by Ramapanicker and colleagues¹⁰⁸ was then employed in which they state that no black by-product formed. Their methodology involves first bubbling HCl gas through cold propargyl alcohol to provide a saturated HCl solution before the addition of glycine. HCl gas was readily synthesised via the dropwise addition of concentrated sulfuric acid onto NaCl and, once formed, was then bubbled through concentrated sulfuric acid in order to remove any water before bubbling through the propargyl alcohol. However this methodology, also disappointingly, led to the formation of the black by-product. Purification of the resulting black solution was again unsuccessful and no product was isolated (scheme 2.14).

Scheme 2.14: Unsuccessful syntheses of propargyl glycinate hydrochloride utilising methodology proposed by both Patnaik and colleagues⁹³ and Ramapanicker and colleagues.¹⁰⁸

Due to the unsuccessful synthesis of propargyl glycinate hydrochloride, a new synthetic strategy for propargyl diazoacetate that by-passed the propargyl glycinate hydrochloride altogether was required. A method first reported by Fukuyama and colleagues¹⁰⁹ was selected due to its high yielding synthesis of propargyl diazoacetate and its reduced explosive characteristics. This method involved a two-step process: formation of propargyl bromoacetate, followed by a diazo-transfer reaction with *N,N*-ditosylhydrazine (**57**).¹⁰⁹ The synthesis of *N,N*-ditosylhydrazine (**57**) was undertaken through a nucleophilic attack by the primary amine of *p*-toluenesulfonyl hydrazide on *p*-toluenesulfonyl chloride (TsCl). After recrystallisation, this led to very favourable 81% yield of **57** (scheme 2.15).

Scheme 2.15: Synthesis of **57** and **58**.

As stated by Fukuyama and colleagues *N,N*-ditosylhydrazine proved to be a useful reagent due to its ease of handling, thermal stability and shelf stability.^{109,97a} With hydrazine **57** in hand, the two-step synthesis of the desired diazoacetate **58** was undertaken. Initial treatment of propargyl alcohol (**73**) with bromoacetyl bromide (**74**) led to the formation of propargyl bromoacetate (**75**) via an esterification reaction (scheme 2.16). Without further purification, the resulting ester **75** was subjected to a substitution reaction through nucleophilic attack by the hydrazine **57** in the presence of the bulky base 1,8-diazobicycloundec-7-ene (DBU). This led to the double elimination of toluenesulfinic acid (**78**) resulting in the final propargyl diazoacetate

(**58**) as a orange oil in a 57% yield after purification. A proposed mechanism for the formation of **58** is presented in scheme 2.16 in which double elimination of the toluenesulfinic acid proceeds through the formation of the nitrene species **80**. Moderate yields are typical due to the volatile nature of **58** as noted by Fukuyama and colleagues.¹⁰⁹

Scheme 2.16: Proposed mechanism of the synthesis of **58** noting two-fold elimination of toluenesulfinic acid.

Cyclopropanation of the glucal **40** with the diazoacetate **58** followed the same optimised procedure as with previous diazoacetates (see scheme 2.12), in which a solution of rhodium acetate and glucal **40** in dichloromethane were treated with a solution of **58** in dichloromethane over 14 hours under an argon atmosphere. As mentioned, addition over 14 hours reduced both the undesired formation of fumarates and amount of starting material recovered. Silica gel flash column chromatography was used in an attempt to purify the crude material, utilising both hexanes:ethyl acetate and toluene:ethyl acetate solvent systems, however, although purification was successful, separation of the stereoisomers was not achieved and an inseparable mixture of both stereoisomers **59** and **60** were isolated in a 38% yield (scheme 2.17). Therefore the stereochemistry of the two isomers could not yet be established,

however, it was predicted that the major isomer would result from the sterically-directed addition of the carbenoid to the α -face of the alkene, as seen in the synthesis of the ethyl derivatives **54** and **55**. Despite the inability to separate the stereoisomers, ^1H NMR data indicated the presence of the stereoisomers in a 5:1 ratio. The slightly reduced yield compared to the ethoxycarbonyl derivatives **54** and **55** was thought to be due to partial quenching of the carbenoid by the alkyne. Carbene addition to terminal alkynes undergoing a C-H insertion or cyclopropene formation is preceded in the literature^{110,111} when catalysed by organometallic complexes such as rhodium acetate.¹¹²

Scheme 2.17: Cyclopropanation of **40** with **58** forming an inseparable mixture of stereoisomers **59** and **60**.

As with the separation of the stereoisomers **52** and **53** (scheme 2.13) it was expected that separation of both **59** and **60** would be possible upon removal of the C-6 trityl protecting group. The optimised conditions developed in the selective deprotection of the dichlorocyclopropyl derivative **41** were again utilised in order to reduce the formation of the formate ester by-product. To this end, the mixture of stereoisomers **59** and **60** was treated with formic acid and the reaction quenched after 50 minutes based on closely following the reaction progress by TLC. As stated previously, optimisation of the proposed workup by Nagarajan and colleagues⁶⁸ was undertaken through the addition of NaHCO_3 , as concentration of the reaction mixture before quenching led to the perfect conditions for undesired ester formation.⁵⁹ Separation of the resulting deprotected stereoisomers **61** and **62** proved to be difficult as separation by silica column chromatography utilising both hexanes:ethyl acetate and

toluene:ethyl acetate solvent systems were unsuccessful. The exploration of many different solvent systems to separate the two isomers on TLC seemed futile, however using dichloromethane:ethyl acetate solvent system showed a small amount of promise. A long silica gel column utilising a slow gradient elution of 100:1 $\rightarrow$ 3:1 dichloromethane:ethyl acetate (v:v) was successful, yielding **61** and **62** in a 43% and 10% yield, respectively (scheme 2.18).

Scheme 2.18: Detritylation of the C-6 trityl and separation of stereoisomers **61** and **62**.

With the successful separation of **61** and **62**, HMBC data were then obtained and the outcomes of the cyclopropanation reactions were confirmed. As shown in figure 2.4, analysis of the HMBC spectrum revealed interactions between H-2 and C-7, H-3 and C-7, as well as H-7 and C-2 for compound **61**. Figure 2.5 shows the interactions between H-2 and H-3 to C-7 as well as H-7 to C-2 exhibited in the HMBC spectrum of **62**.

Figure 2.4: HMBC spectrum of **61** showing coupling between H-2 and H-3 to C-7 along with H-7 to C-2.

Figure 2.5: HMBC spectrum of **62** confirming showing between H-2 and H-3 to C-7 as well as H-7 to C-2.

The challenge of determining the stereochemistry was again undertaken in a similar manner to that used for compounds **54** and **55**. As per compounds **54** and **55**, the same methodology utilising the molecular modelling systems with MacroModel version 9.7 was applied in order to identify the lowest energy conformations of both **61** and **62**. The calculated dihedral angles are shown in table 2.3. The dihedral angles determined for compounds **61** and **62** were the same as those reported for compounds **54** and **55**, as shown in table 2.3.

Dihedral angles (degrees)				
	H-1, H-2	H2, H-3	H-1, H-7	H-2, H-7
Compound 61	2.7	104.5	143.8	146.6
Compound 62	4.5	34.3	143.3	146.5

Table 2.3: Calculated dihedral angle of the lowest energy conformers of **61** and **62** as modelled by MacroModel.

The measured and calculated coupling constants are shown in table 2.4. Reasonable correlations were observed as shown in table 2.4, though not without discrepancies, which may be due to the steric strain applied by the cyclopropane ring affecting the position of the Karplus curve.

Calculated coupling constants				
	H-1, H-2	H2, H-3	H-1, H-7	H-2, H-7
Compound 61	7.42 Hz	0.90 Hz	6.95 Hz	9.44 Hz
Compound 62	7.39 Hz	7.34 Hz	6.75 Hz	9.44 Hz

Measured coupling constants				
	H-1, H-2	H2, H-3	H-1, H-7	H-2, H-7
Compound 61	7.25 Hz	0 Hz	2.5 Hz	6.5 Hz
Compound 62	4 Hz	4 Hz	9.25 Hz	6.75 Hz

Table 2.4: Comparison of the calculated and measured coupling constants of **61** and **62**.

Again NOE analyses of both compounds **61** and **62** were undertaken to further confirm the stereochemistry of the two diastereoisomers (figures 2.6 and 2.7). For

compound **61**, irradiation of H-7 indicated clear coupling interactions of H-7 with both H-3 and H-5, indicating that the cyclopropane ring is α to the pyranose ring and carboxyalkyl substituent is in the *exo* position (figure 2.6). Irradiation of H-7 in **62** resulted in the coupling of H-7 solely with H-4 indicating the β -*exo* configuration of the cyclopropane and carboxyalkyl group (figure 2.7). The results are consistent with the lowest energy configuration calculated by the molecular modelling.

Figure 2.6: NOE analysis of the stereoisomer **61** with irradiation at H-7.

Figure 2.7: NOE analysis of the stereoisomer **62** with irradiation at H-7.

Due to the similarities in both molecular modelling and the NOE results, the lowest energy structures of compounds **61** and **62** were visually overlaid in the Spartan software with compounds **54** and **55** to allow for comparison of their conformation. As shown in figure 2.8, the structures overlay very closely in both cases. The deviation of the C-3 benzyl group, seen in figure 2.8A, has a low barrier of activation to the requisite bond rotation. The corresponding propargyl conformation in which the C-3 benzyl group overlays the ethyl conformer is within 1 kJ/mol. This reflects the flexibility of the benzyl substituents under ambient temperatures.

Figure 2.8: Lowest energy conformers of A) **54** and **61**, B) **55** and **62**. Compounds **54** and **55** are indicated in green and red, and compounds **61** and **62** are shown in white and blue.

Summary

Synthesis of the final products **61** and **62** concluded the chemical aspect of this thesis, which included the successful syntheses of the target 1,2-cyclopropyl carbohydrates derivatives **23**, **54**, **55** and **56**. Development of new methodologies and optimisation of reported literature procedures have led to a high yielding synthetic route to 1,2-cyclopropyl carbohydrates. Synthesis of compound **23** was achieved over 7 steps at a modest overall yield of 32%. Similar results were obtained for the carboxyalkyl

derivatives with the isomeric mixtures of **54** and **55**, and **61** and **62** being achieved in overall yields of 22% and 9% respectively over 7 steps. The exploration of a new C-6 TBDPSCl protecting groups was undertaken, however due to low yields and lack of reagent, optimisation of the current trityl protection procedure was the preferred route. This led to very pleasing results during deprotection, leading to a 15% reduction of the formate by-product compared to previously reported syntheses.² All stereochemistry was determined through the use of molecular modeling, analysis of coupling constants and NOE spectroscopy. The target compounds **23**, **54**, **55**, **56**, **61** and **62** will now be subjected to biological analysis to assess their bioactivity. The compounds will be screen against a yeast deletion library in order to identify chemical genetic interactions and elucidate a potential mechanism of action.

Experimental

2.8 General experimental

Unless otherwise stated, all reactions were conducted under nitrogen atmosphere in oven-dried glassware using HPLC grade solvents. CH₂Cl₂, Et₂O and THF were obtained from the PureSolv (Innotive Technologies). Hexanes, ethanol, methanol, ethyl acetate, toluene (Fischer Scientific) and chloroform (Vickers Laboratory) were of HPLC grade and used for reactions and column chromatography as received. DMF (Acros Organics) was stored over molecular sieves. Perchloric acid (Sigma-Aldrich), formic acid (BDH biochemical), D-Glucose (Sigma-Aldrich), NaH (Sigma-Aldrich), TBAI (Honeywell Riedel-de Haën), BnBr (Sigma-Aldrich), TEBAC (Sigma-Aldrich), glycine (BDH bioscience), RhCl₂•3H₂O (Merck & Co.), NaAc•3H₂O (Univar), *p*-toluenesulfonyl hydrazine (Sigma-Aldrich), *p*-toluenesulfonyl chloride (Sigma-Aldrich), propargyl alcohol (Sigma-Aldrich), bromoacetyl bromide (Sigma-Aldrich), and DBU (Merck & Co.) were all used as received. Concentration of solvents *in vacuo* was achieved through the use of a rotary evaporator fitted to a vacuum pump in a water bath of 40 °C.

Reaction progress was monitored using TLC (thin layer chromatography) plates pre-coated in silica DC-Fertigfolien Polygram® SIL G/UV₂₅₄ and visualised by dipping in potassium permanganate in water, anisaldehyde, and phosphomolybdic acid, as well as detection by UV absorption (254 nm). Purification of products was achieved using flash column chromatography. Columns were packed using silica 60 (Pure Science Silica Gel, 40-60 microns) as the stationary phase, using the solvent system indicated. ¹H NMR and ¹³C NMR spectra were recorded on a Varian Unity Inova 500 (operating at 500 MHz for ¹H and 125 MHz for ¹³C) spectrometer, NOSEY spectra were recorded on Varian Directed Drive instrument equipped with an inverse-detected triple resonance HCN probe operating at 25 °C (600 MHz for ¹H). All chemical shifts (δ) were referenced to the solvent peaks (CDCl₃: ¹H - 7.26 ppm; ¹³C - 77.16 ppm, (CD₃)₂SO: ¹H - 2.50 ppm; ¹³C - 39.52 ppm, D₂O: ¹H - 4.79 ppm;). ¹¹³ NMR peaks were assigned based on COSY, HSQC and HMBC 2D experiments. Melting points were obtained using Gallenkamp melting point apparatus and are uncorrected.

Infrared spectra was recorded using thin films on a Bruker Tensor 27 FTIR spectrometer outfitted with an Attenuated Total Reflectance (ATR) sampling accessory. Optical rotation measurements were recorded on a Rudolph research analytical Autopol II automatic polarimeter.

2.9 Chemistry synthesis

Tri-*O*-acetyl-D-glucal (37)

Using a modification of the Kozikowski's procedure⁷⁹, perchloric acid (0.48 mL, 4.5 mmol) was added dropwise to a mechanically stirred solution of D-glucose (72 mg, 0.4 mmol) in acetic anhydride (38.2 mL). Additional D-glucose (9.93 g, 55.1 mmol) was added over a period of 40 minutes at 45 °C, after which the reaction was allowed to cool to room temperature. The solution was then treated with a 33% (w/w) solution of HBr in acetic acid (39 mL, 149 mmol) and left to stir for 1.5 h. After TLC-analysis showed the disappearance of starting materials, the solution was diluted with CH₂Cl₂ (106 mL). The organic layer was then extracted and washed with ice-cold H₂O (3 × 50 mL), followed by sat. aq. NaHCO₃ (6 × 50 mL) before it was dried over anhydrous MgSO₄, which was removed by filtration. The filtrate was then concentrated *in vacuo* to give the crude tetra-*O*-acetyl-D-glucopyranosyl bromide as an off-white solid, which was used without further purification.

A mechanically stirred solution of zinc dust (24 g, 366.3 mmol) in H₂O (76 mL) was cooled to 0 °C before it was diluted with acetic acid (76 mL). The solution was then treated to a dropwise addition of the crude tetra-*O*-acetyl-D-glucopyranosyl bromide (25.06 g, 61.1 mmol) in Et₂O (76 mL) over a period of 1 h. The reaction was allowed to warm to room temperature and left to stir overnight. After TLC-analysis showed the disappearance of starting materials, the solution was filtered removing the zinc dust, then diluted with CH₂Cl₂ (110 mL). The organic layer was collected and washed with H₂O (3 × 50 mL), sat. aq. NaHCO₃ (4 × 50 mL) and brine (60 mL) before it was

dried over anhydrous MgSO_4 . Filtration and concentration *in vacuo* yielded the crude material as a white powder. Recrystallisation from hexanes : Et_2O yielded tri-*O*-acetyl-D-glucal **37** as a white solid (14.9 g, 65%). The spectral data matched those previously reported.^{36,79}

$R_f = 0.26$ (3:1 hexanes : EtOAc , v:v); M.p. 48.5-50.5 °C (lit. 50.0-52.0 °C)¹¹⁴; $[\alpha]_D^{26.6} = -2.0$ (c=1.0, CH_2Cl_2); ^1H NMR (500 MHz, CDCl_3): δ 6.47 (d, $J = 6.4$ Hz, 1H, H-1), 5.34 (m, 1H, H-3), 5.22 (dd, $J = 7.6, 5.9$ Hz, 1H, H-4), 4.84 (dd, $J = 6.3, 3.4$ Hz, 1H, H-2), 4.4 (dd, $J = 12.2, 5.8$ Hz, 1H, H-6a), 4.26 (m, 1H, H-5), 4.20 (dd, $J = 12.2, 3.2$ Hz, 1H, H-6b), 2.09 (s, 3H, CH_3CO), 2.08 (s, 3H, CH_3CO), 2.04 (s, 3H, CH_3CO); ^{13}C NMR (125 MHz, CDCl_3): δ 170.7 (C, CH_3CO), 170.5 (C, CH_3CO), 169.6 (C, CH_3CO), 145.6 (CH, C-1), 99.0 (CH, C-2), 74.0 (CH, C-5), 67.5 (CH, C-3), 67.2 (CH, C-4), 61.4 (CH, C-6), 21.0 (CH_3 , CH_3CO), 20.83 (CH_3 , CH_3CO), 20.77 (CH_3 , CH_3CO); IR (film from CH_2Cl_2): $\nu_{\text{max}} = 2982, 1742, 1650, 1433, 1370, 1221, 1141, 1030, 601 \text{ cm}^{-1}$.

D-Glucal (**38**)

38

To a solution of sodium (0.23 g, 9.84 mmol) in MeOH (300 mL), a solution of **37** (8.87 g, 32.8 mmol) in MeOH (113 mL) was added. The reaction was then left to stir at r.t. for 15 min, after which TLC-analysis deemed the reaction complete. The solution was then concentrated *in vacuo* to provide the crude product. Purification was done through the successive additions of MeOH (7 mL), acetone (50 mL) and Et_2O (100 mL). The solution was then filtered and concentrated *in vacuo* to afford the pure glucal **38** as viscous yellow oil (4.77 g, 99.4%). Spectral data matched those previously reported.³⁶

$R_f = 0.30$ (EtOAc); $[\alpha]_D^{26.7} = +8.0$ (c=1.0, H_2O); ^1H NMR: (500 MHz, D_2O) δ 6.29 (dd, $J = 5.8, 1.3$ Hz, 1H, H-1), 4.68–4.65 (m, 3H, 3 x OH), 4.80 (dd, $J = 6.1, 2.4$ Hz, 1H, H-2), 4.23 (ddd, $J = 7.0, 2.3, 1.7$ Hz, 1H, H-3), 3.92–3.85 (m, 3H, H-5, H-6a, H-6b), 3.67 (dd, $J = 9.0, 7.1$ Hz, 1H, H-4); ^{13}C NMR (125 MHz, D_2O) δ 143.6 (CH, C-

1), 102.7 (CH, C-2), 78.0 (CH, C-5), 68.7 (CH, C-4), 68.2 (CH, C-3), 60.0 (CH, C-6);
 IR (neat, cm^{-1}) ν_{max} = 3293, 28812, 1648, 1410, 1228, 1070, 1011,

1,5-anhydro-2-deoxy-6-*O*-trityl-D-arabino-hex-1-enitol (**39**)

In a modification of the Esswein procedure,⁸⁴ **38** (4.44 g, 32.64 mmol) was dissolved in a solution of CH_2Cl_2 and pyridine (69 mL, 1:1, v:v) before trityl chloride (14.6 g, 52.22 mmol) was added over a period of 3 minutes at r.t. The solution was stirred for 19 hours at r.t. after which, TLC analysis showed completion of the reaction. The reaction was then concentrated *in vacuo* and extracted with CHCl_3 (3×60 mL). The organic layers were then collected and washed with sat. aq. NaHCO_3 (3×60 mL), dried over anhydrous MgSO_4 , filtered and concentrated *in vacuo* to provide a yellow oil. The crude yellow oil was then purified via silica gel column chromatography (1:1 hexanes : EtOAc, v:v) to afford **39** as a white solid (9.87 g, 87%).

R_f = 0.48 (2:1 hexanes : EtOAc, v:v); M.p. 59.0-61.8 °C (lit. 62-64 °C); $[\alpha]_{\text{D}}^{25.4}$ = +40.0 (c=1.0, CH_2Cl_2); ^1H NMR (500 MHz, CDCl_3): δ 7.48 - 7.25 (m, 15H, Ph), 6.39 (dd, J = 6.1, 1.7 Hz, 1H, H-1), 4.76 (dd, J = 6.1, 2.2 Hz, 1H, H-2), 4.24 (d, J = 6.6 Hz, 1H, H-3), 3.9 (dd, J = 9.5, 7.1 Hz, 1H, H-4), 3.84 (dt, J = 13.2, 3.6 Hz, 1H, H-5), 3.57 (dd, J = 10.5, 3.6 Hz, 1H, H-6a), 3.36 (dd, J = 10.5, 3.9 Hz, 1H, H-6b), 2.33 (m, 1H, OH-4); ^{13}C NMR (125 MHz, CDCl_3): δ 144.6 (CH, C-1), 143.4 (C, Ph), 128.5 (CH, Ph), 128.0 (CH, Ph), 127.3 (CH, Ph), 102.5 (CH, C-2), 87.0 (C, C-7), 76.5 (CH, C-5), 71.6 (CH, C-4), 69.4 (CH, C-3), 62.8 (CH, C-6); IR: ν_{max} = 3342, 3058, 2880, 1644, 1489, 1447, 1226, 1000, 745, 697.

1,5-Anhydro-3,4-di-*O*-benzyl-2-deoxy-6-*O*-trityl-D-arabino-hex-1-enitol (**40**)

40

In a slight modification of Schmidt's procedure,⁸⁴ **39** (1.05 g, 2.71 mmol) was co-evaporated *in vacuo* with DMF (18 mL) two times, with an additional evaporation whereupon the volume of DMF (30 mL) was reduced to half to ensure dryness. The solution was then cooled to -5 °C before NaH (0.3 g, 7.3 mmol) was added portion-wise over a period of 3 minutes followed by the addition of TBAI (0.1 g, 0.27 mmol).

The solution was left to stir for 15 min before the dropwise addition of BnBr (0.9 mL, 7.3 mmol), after which it was allowed to warm to room temperature and left to stir overnight. After TLC analysis showed the disappearance of starting materials, the reaction was then quenched with H₂O (8 mL) and extracted with CHCl₃ (3 × 10 mL). The organic fractions were combined, dried over anhydrous MgSO₄, and concentrated under reduced pressure to give the crude product. The crude material was then purified via silica gel column chromatography (14:1 → 1:1 hexanes : EtOAc, v:v) to produce **40** as a white solid (1.32 g, 86%).

R_f = 0.40 (9:1 hexanes : EtOAc); M.p. 114.0-116.0 °C (lit. 116 °C⁸⁴); $[\alpha]_D^{24.2}$ = +0.6 (c=1.0, CH₂Cl₂); ¹H NMR (500 MHz, CDCl₃): δ 7.48 - 7.01 (m, 25H, Ph), 6.49 (d, J = 6 Hz, 1H, H-1), 4.88 (dd, J = 6.0, 4.0 Hz, 1H, H-2), 4.73 (d, J = 11 Hz 1H, PhCH₂), 4.62 (d, J = 11.5 Hz, 1H, PhCH₂), 4.55 (d, J = 11.5 Hz, 1H, PhCH₂), 4.5 (d, J = 11 Hz, 1H, PhCH₂), 4.17 (m, 1H, H-3), 4.0 (m, 2H, H-4, H-5), 3.54 (d, J = 10 Hz, 1H, H-6a), 3.39 (dd, J = 10, 4 Hz, 1H, H-6b); ¹³C NMR (125 MHz, CDCl₃): δ 144.9 (CH, C-1), 143.9 (C, Ph), 138.3 (C, Ph), 138.1 (C, Ph), 128.8 (CH, Ph), 128.4 (CH, Ph), 128.2 (CH, Ph), 127.9 (CH, Ph), 127.8 (CH, Ph), 127.64 (CH, Ph), 127.6 (CH, Ph), 126.9 (CH, Ph), 99.8 (CH, C-2), 86.5 (C, C-7), 77.2 (CH, C-5), 76.1 (CH, C-3), 74.6 (CH, C-4), 73.8 (CH₂, PhCH₂), 70.8 (CH₂, PhCH₂), 62.1 (CH₂, C-6); IR (film from CH₂Cl₂): $\nu_{\max}$ 3060, 3030, 2923, 2874, 1744, 1647, 1492, 1449, 1238, 1099, 1068, 1028, 900, 745, 698 cm⁻¹.

1,5-anhydro-3,4-di-*O*-benzyl-6-*O*-(triphenylcarbinyl)-2-deoxy-1,2-*C*-(dichloromethylene)-D-glycero-D-gulo-hexitol (41) and 1,5-anhydro-3,4-di-*O*-benzyl-6-*O*-(triphenylcarbinyl)-2-deoxy-1,2-*C*-(dichloromethylene)-D-glycero-D-talo-hexitol (42)

In a modification of the procedure conducted by Gross and colleagues,⁸⁶ a solution of the protected glucal **40** (1.29 g, 2.27 mmol) in CHCl_3 (8.2 mL) was treated with TEBAC (16 mg, 0.078 mmol), followed by the dropwise addition of 33% NaOH in H_2O (12 mL). The biphasic solution was stirred rapidly for 48 hours at r.t., after which TLC analysis showed completion of the reaction. The reaction mixture was then quenched with H_2O (60 mL) and extracted with CH_2Cl_2 (3×60 mL). The organic layer was collected, dried over anhydrous MgSO_4 and concentrated *in vacuo* to afford a crude mixture of both stereoisomers **41** and **42**. The crude products were purified using column chromatography (14:1 hexanes : EtOAc, v:v) to afford the major isomer **41** (1.23 g, 83%) as a white solid and the minor product **42** (0.26 g, 13%) as a yellow oil.

Data for **41**: R_f = 0.52 (9:1 hexanes : EtOAc); M.p. 51.5–53.5 °C; $[\alpha]_D^{25.7} = +8.0$ ($c=1.0$, CH_2Cl_2); ^1H NMR (500 MHz, CDCl_3): δ 7.47–7.22 (m, 25H, Ph), 4.81 (d, $J = 3.9$ Hz, 1H, PhCH_2), 4.79 (d, $J = 3.2$ Hz, 1H, PhCH_2), 4.70 (d, $J = 11.5$ Hz, 1H, PhCH_2), 4.26 (d, $J = 10.8$ Hz, 1H, PhCH_2), 4.05 (d, $J = 8$ Hz, 1H, H-1), 3.88–3.84 (m, 2H, H-4, H-5), 3.78 (dd, $J = 9.5, 4.9$ Hz, 1H, H-3), 3.48 (dd, $J = 10.5, 2.4$ Hz, 1H, H-6a), 3.17 (dd, $J = 10.3, 3.0$ Hz, 1H, H-6b), 1.89 (dd, $J = 8.1, 4.9$ Hz, 1H, H-2); ^{13}C NMR (125 MHz, CDCl_3): δ 147.7 (C, Ph), 138.0 (C, Ph), 137.6 (C, Ph), 128.9 (C, Ph), 128.8 (C, Ph), 128.4 (C, Ph), 128.1 (C, Ph), 128.0 (C, Ph), 127.9 (C, Ph), 127.8 (C, Ph), 127.1 (C, Ph), 86.7 (C, C-8), 80.3 (C, C-5), 77.4 (CH, C-4), 75.7 (CH, C-4), 74.8 (CH_2 , PhCH_2), 72.0 (CH_2 , PhCH_2), 63.6 (CH_2 , C-6), 61.6 (C, C-7), 59.2 (CH, C-

1), 34.6 (CH, C-2); IR (film from CH₂Cl₂): $\nu_{\max}$ 3086, 3060, 3030, 2924, 2868, 1491, 1449, 1130, 1093, 1014, 744, 697, 632 cm⁻¹.

Data for **42**: R_f = 0.29 (9:1 hexanes: EtOAc, v:v); $[\alpha]_D^{25.9}$ = -6.0 (c=1.0, CH₂Cl₂); M.p. 49-51 °C; ¹H NMR (500 MHz, CDCl₃): δ 7.50–7.17 (m, 23H, Ph), 6.90 (dd, J = 7.3, 1.4 Hz, 2H, Ph), 4.87 (d, J = 11.7 Hz, 1H, PhCH₂), 4.74 (d, J = 12.0 Hz, 1H, PhCH₂), 4.69 (d, J = 10.5 Hz, 1H, PhCH₂), 4.34 (d, J = 10.7 Hz, 1H, PhCH₂), 4.14–4.09 (m, 2H, H-3, H-4), 4.05 (d, J = 8.1 Hz, 1H, H-1), 3.56 (dd, J = 10.3, 2.0 Hz, 1H, H-6a), 3.41 (m, 1H, H-5), 3.19 (dd, J = 9.3, 2.9 Hz, 1H, H-6b), 2.12 (t, J = 7.8 Hz, 1H, H-2); ¹³C NMR (125 MHz, CDCl₃): δ 143.9 (C, Ph), 138.1 (C, Ph), 137.9 (C, Ph), 128.8 (CH, Ph), 128.6 (CH, Ph), 128.1 (CH, Ph), 128.06 (CH, Ph), 127.93 (CH, Ph), 127.91 (CH, Ph), 127.84 (CH, Ph), 127.79 (CH, Ph), 127.5 (CH, Ph), 86.4 (C, C-8), 78.3 (CH, C-5), 78.1 (CH, C-4), 76.4 (CH, C-3), 74.8 (CH₂, PhCH₂), 71.5 (CH₂, PhCH₂), 62.2 (CH₂, C-6), 62.12 (CH, C-1), 61.9 (CH, C-7), 33.0 (CH, C-2); IR (film from CH₂Cl₂): $\nu_{\max}$ 3380, 3061, 3030, 2932, 2875, 1647, 1491, 1449, 1275, 1091, 1022, 747, 699 cm⁻¹.

1,5-anhydro-3,4-di-*O*-benzyl-2-deoxy-1,2-*C*-(dichloromethylene)-D-glycero-D-gulo-hexitol (23**) and 1,5-anhydro-3,4-di-*O*-benzyl-6-*O*-(formyl)-2-deoxy-1,2-*C*-(dichloromethylene)-D-glycero-D-gulo-hexitol (**42**)**

To a solution of **41** (1.23 g, 1.9 mmol) in Et₂O (11 mL), formic acid (9.05 mL) was added dropwise. The solution was left to stir at r.t. for a period of 50 min before it was diluted with Et₂O (90 mL). The organic layer was collected, washed with sat. aq. NaHCO₃ (4 × 100 mL), dried over anhydrous MgSO₄, and concentrated *in vacuo* to afford the crude products **23** and **43**. The crude products were purified via column chromatography (14:1 → 5:1 hexanes : EtOAc, v:v) to give **23** (0.64 g, 81%) as a white solid and **43** (0.067g, 8%) as clear oil.

Data for **23**: $R_f = 0.53$ (3:1 hexanes : ethyl acetate, v:v); M.p. 84.5-86.0 °C; $[\alpha]_D^{25.5} = +29.0$ (c=1.0, CH₂Cl₂); ¹H NMR (500 MHz, CDCl₃): δ 7.42–7.26 (m, 10H, Ph), 4.95 (d, $J = 11.2$ Hz, 1H, PhCH₂), 4.81 (d, $J = 12.0$ Hz, 1H, PhCH₂), 4.71 (d, $J = 11.8$ Hz, 1H, PhCH₂), 4.67 (d, $J = 11.2$ Hz, 1H, PhCH₂), 3.87 (d, $J = 8.5$ Hz, 1H, H-1), 3.81 (dd, $J = 9.2, 3.9$ Hz, 1H, H-3), 3.73 (dt, $J = 8.3, 3.7$ Hz, 1H, H-5), 3.68 (s, 2H, H-6a,b), 3.59 (t, $J = 8.7$ Hz, 1H, H-4), 1.83 (dd, $J = 8.3, 3.9$ Hz, 1H, H-2), 1.75 (s, 1H, OH); ¹³C NMR (125 MHz, CDCl₃) δ 137.9 (C, Ph), 137.4 (C, Ph), 128.6 (CH, Ph), 128.5 (CH, Ph), 128.2 (CH, Ph), 128.0 (CH, Ph), 127.98 (CH, Ph), 79.4 (CH, C-5), 77.3 (CH, C-3), 74.8 (CH₂, PhCH₂), 74.7 (CH, C-4), 72.0 (CH₂, PhCH₂), 62.7 (CH₂, C-6), 60.9 (C, C-7), 58.8 (CH, C-1), 33.7 (CH, C-2). IR (neat, cm⁻¹) $\nu_{\max} = 3362, 3935, 2954, 2912, 2863, 1496, 1453, 1327, 1108, 989, 885, 820, 731, 694$.

Data for **43**: $R_f = 0.30$ (9:1 hexanes : EtOAc, v:v); $[\alpha]_D^{26.6} = +18.0$ (c=1.0, CH₂Cl₂); ¹H NMR (500MHz, CDCl₃): δ 8.03 (m, 1H, CHO), 7.47–7.26 (m, 10H, Ph), 4.95 (d, $J = 11.5$ Hz, 1H, PhCH₂), 4.81 (d, $J = 11.8$ Hz, 1H, PhCH₂), 4.70 (d, $J = 12$ Hz, 1H, PhCH₂), 4.63 (d, $J = 11.2$ Hz, 1H, PhCH₂), 4.30 (dd, $J = 11.7, 4.8$, 1H, H-6a), 4.15 (dd, $J = 12.2, 2.7$, 1H, H-6b), 3.87 (dddd, $J = 8.3, 5.1, 2.7, 0.7$ Hz, 1H, H-5), 3.86 (d, $J = 7.8$ Hz, 1H, H-1), 3.80 (dd, $J = 9.3, 4.1$ Hz, 1H, H-3), 3.57 (t, $J = 8.6$ Hz, 1H, H-4), 1.82 (dd, $J = 8.0, 4.6$ Hz, 1H, H-2); ¹³C NMR (125 MHz, CDCl₃) δ 160.5 (C, C-8), 137.6 (C, Ph), 137.3 (C, Ph), 128.6 (CH, Ph), 128.5 (CH, Ph), 128.3 (CH, Ph), 128.1 (CH, Ph), 128.0 (CH, Ph), 127.99 (CH, Ph), 77.7 (CH, C-5), 77.3 (CH, C-3), 74.7 (CH₂, PhCH₂), 74.4 (CH, C-4), 71.9 (CH₂, PhCH₂), 63.2 (CH₂, C-6), 60.9 (C, C-7), 58.4 (CH, C-1), 34.0 (CH, C-2); IR (neat, cm⁻¹) $\nu_{\max} = 3031, 2869, 1724, 1497, 1454, 1151, 1087, 733, 696$.

6-*O*-(*tert*-butyldiphenylsilyl)-1,5-anhydro-2-deoxy-arabino-hex-1-enopyranose
(44)

Using a modification of Lellouche and co-workers' procedure,⁹² imidazole (0.45 g, 6.6 mmol) was added to a solution of D-glucal **38** (0.46 g, 3.2 mmol) in DMF (7.5 mL) and then cooled to -18°C using dry ice in a 9:1 ethylene glycol : ethanol (v:v) slurry. TBDPSCl (0.83 mL, 3.2 mmol) was then added dropwise and the reaction was left overnight at -18 °C. After TLC analysis showed the disappearance of starting materials, the solution was quenched by the addition of H₂O (20 mL) and extracted with Et₂O (3 × 8 mL). The organic fractions were combined and washed with H₂O (5 mL), dried over anhydrous MgSO₄, and concentrated *in vacuo* to give the crude product as a viscous yellow oil. Purification by silica gel column chromatography (1:1 hexanes: ethyl acetate, v:v) yielded **44** as a colourless viscous oil (226 mg, 19%). Spectral data matched those previously reported.⁹²

R_f = 0.38 (1:1 hexanes : EtOAc, v:v); $[\alpha]_D^{27.7}$ = +38.0 (c=1.0, CH₂Cl₂); ¹H NMR: (500 MHz, CDCl₃) δ 7.69-7.67 (m, 4H), 7.47-7.39 (m, 6H), 6.32 (dd, J = 6.5, 2 Hz, 1H, H-1), 4.73 (dd, J = 6.0, 2.0 Hz, 1H, H-2), 4.27 (d, J = 7.1, 1H, H-3), 3.99-3.97 (m, 2H, H-6a, H-6b), 3.95-3.79 (m, 2H, H-4, H-5), 2.78 (s, 2.18, 1H, OH), 2.15 (s, 1H, OH), 1.07 (s, 9H); ¹³C NMR (125 MHz, CDCl₃) δ 144.3 (C, C-1), 135.7 (C, Ph), 135.5 (CH, Ph), 132.8 (CH, Ph), 130.0 (CH, Ph), 127.9 (CH, Ph), 102.3 (CH, C-2), 76.7 (CH, C-5), 71.9 (CH, C-4), 69.6 (CH, C-3), 63.8 (CH, C-6), 26.8 (CH₃, SiCH₃), 19.25 (CH₃, SiCH₃); IR (neat, cm⁻¹) ν_{max} = 3367, 3071, 3013, 2930, 2857, 1646, 1472, 1428, 1233, 1107, 1051, 753, 701.

(((2R,3S,4R)-3,4-bis(benzyloxy)-3,4-dihydro-2H-pyran-2-yl)methoxy)(tert-butyl)diphenylsilane (45)

In a slight modification of Schmidt's procedure,⁸⁴ the protected diol **44** (0.217g, 0.6 mmol) was dissolved in DMF (7 mL) and cooled to -5°C. BnBr (0.17 mL, 1.4 mmol) was then added dropwise, followed by the portionwise addition of NaH (0.034 g, 1.4 mmol) over a period of 1 minute. The reaction was then left to warm to r.t. and react overnight. TLC analysis showed that the reaction was not complete therefore addition of catalytic amounts of TBAI (0.02 g, 0.06 mmol) was added to aid in the completion of the reaction. Following completion of the reaction, deemed by TLC analysis, it was quenched by the addition of H₂O (20 mL) and extracted with CHCl₃ (3 × 20 mL). The organic fractions were combined, dried over anhydrous MgSO₄, and concentrated *in vacuo* to give the crude product. The crude material was then purified via silica gel column chromatography (9:1 → 1:1 hexanes : EtOAc, v:v) to give **45** (80 mg, 25%),

R_f = 0.40 (12:1 hexanes : EtOAc, v:v); $[\alpha]_D^{27.7}$ = +24.0 (c=1.0, CH₂Cl₂); ¹H NMR (500 MHz, CDCl₃): δ 7.7-7.66 (m, 5H, Ph), 7.38-7.28 (m, 15H, Ph), 6.40 (dd, J = 6, 1 Hz, 1H, H-1), 4.91 (d, J = 11 Hz, 1H, PhCH₂), 4.85 (dd, J = 6, 2.5 Hz, 1H, H-2), 4.75 (d, J = 11.5 Hz, 1H, PhCH₂), 4.65 (d, J = 11.5 Hz, 1H, PhCH₂), 4.59 (d, J = 11.5 Hz, 1H, PhCH₂), 4.23 (m, 1H, H-3), 4.05-4.01 (m, 2H, H-4, H-5), 3.97-3.93 (m, 2H, H6a, H6b), 1.05 (s, 9H); ¹³C NMR (125 MHz, CDCl₃) δ 154.0 (C, C-1), 135.1 (C, Ph), 135.0 (CH, Ph), 130.2 (CH, Ph), 127.9 (CH, Ph), 127.7 (CH, Ph), 99.9 (CH, C-2), 76.7 (CH, C-5), 75.7 (CH, C-3), 74.4 (CH, C-4), 73.5 (CH₂, PhCH₂), 70.5 (CH₂, PhCH₂), 68.5 (CH, C-6), 26.5 (CH₃, SiCH₃), 19.0 (CH₃, SiCH₃); IR (neat, cm⁻¹) ν_{max} = 3399, 3070, 2930, 2858, 1496, 1427, 1361, 1107, 820, 739, 696, 607, 502.

Methyl glycinate hydrochloride (**46**).

In a modification of Patnaik and colleagues' procedure,⁹³ acetyl chloride (2.5 mL, 34.6 mmol) was added dropwise to MeOH (16 mL) at 0 °C and stirred for a period of 40 minutes before the portion-wise addition of glycine (1.0 g, 13.3 mmol). The solution was then heated to reflux and left to stir for a period of 3 hours. Completion of the reaction was indicated by TLC-analysis, after which the solution was concentrated under reduced pressure to produce **46** as an off-white solid (1.68 g, quant.).

R_f = 0.68 (2:10 Methanol : EtOAc, v:v); M.p. 174.0-175.0 °C; ^1H NMR (500MHz, D_2O): δ 3.92 (s, 2H, H-1a, H-1b), 3.84 (s, 3H, H-3); ^{13}C NMR (125 MHz, D_2O): δ 168.60 (C, C-2), 53.23 (C, C-1), 39.92 (CH_3 , C-3) ; IR (neat, cm^{-1}) ν_{max} = 3011, 2881, 2686, 2632, 1743, 1583, 1495, 1437, 1400, 1256, 1139, 1057, 955, 900, 879.

Ethyl glycinate hydrochloride (**47**)

In a modification of Patnaik and colleagues' procedure,⁹³ acetyl chloride (2.5 mL, 34.6 mmol) was added dropwise to EtOH (16 mL) at 0 °C and stirred for a period of 40 minutes before the portion-wise addition of glycine (1.0 g, 13.3 mmol). The reaction was then heated to reflux and left to stir for a period of 3 hours. After TLC-analysis deemed the reaction complete, the solution was concentrated under reduced pressure to afford **47** as an off white solid (1.82 g, quant.).

R_f = 0.60 (2:10 Methanol : EtOAc, v:v); M.p. 142.6-143.0 °C; ^1H NMR (500MHz, D_2O): δ 4.29 (dd, J = 14.5, 7.5 Hz, 2H, H-1a, H-1b), 3.9 (s, 2H, H-3a, H-3b), 1.28 (t, J = 7.5 Hz, 3H, CH_3); ^{13}C NMR (125 MHz, D_2O): δ 168.09 (C, C-2), 63.21 (C, C-1), 40.07 (C, C-3), 13.07 (CH_3 , C-4); IR (neat, cm^{-1}) ν_{max} = 2975, 2909, 2635, 1742, 1503, 1411, 1244, 1135, 1051, 904, 854.

Butyl glycinate hydrochloride (48)

In a modification of Patnaik, S., and colleagues procedure⁹³ *n*-butanol (16 mL) was cooled to 0 °C followed by the dropwise addition of acetyl chloride (2.5 mL, 34.6 mmol). The solution was stirred at 0 °C for 40 min. Glycine (1 g, 13.3 mmol) was then added portionwise over 1 min. The reaction was then heated to reflux and left to stir for 3 hours. TLC (2:10 Methanol : EtOAc, v:v) analysis showed completion of the reaction. The solution was then concentrated under reduced pressure to afford **48** (2.18 g, Quan. yield) as an off white solid.

R_f = 0.48 (2:10 Methanol : EtOAc, v:v); M.p. 71.0-71.5 °C; ^1H NMR (500MHz, D_2O): δ 4.25 (t, J = 7.2 Hz, 2H, H-1a, H-1b), 3.9 (s, 2H, H-3a, H-3b), 1.65 (t, J = 7.5 Hz, 2H, H-4a, H-4b), 1.36 (dd, J = 15, 7.5 Hz, 2H, H-5a, H-5b), 0.89 (t, J = 7.5 Hz, 3H, H-6a, H-6b, H-6c); ^{13}C NMR (125 MHz, D_2O): δ 168.20 (C, C-2), 66.88 (C, C-1), 40.03 (C, C-3), 29.61 (C, C-4), 18.28 (C, C-5), 12.77 (CH_3 , C-6); IR (neat, cm^{-1}) ν_{max} = 2955, 2873, 2697, 2603, 1740, 1477, 1379, 1238, 1220, 1133, 1062, 917.

Ethyl diazoacetate (49)

In a slight variation for the well-established method by Searle,¹⁰⁰ a solution of ethyl glycinate **47** (0.523 g, 3.7 mmol) in H_2O (1 mL) and CH_2Cl_2 (2.2 mL) was cooled to -5 °C. An ice cold solution of sodium nitrite (0.38 g, 4.4 mmol) and H_2O (1 mL) was then added and the reaction was further cooled to -9 °C. Paying careful attention to retaining the cold temperature, 5% H_2SO_4 (0.01 mL, 0.18 mmol, w/w) in H_2O (0.34 mL) was then added dropwise over a period of 10 minutes. The solution was left to stir for a period of 15 minutes at -9 °C before TLC analysis showed the completion of reaction. The solution was then transferred to an ice-cold separating funnel containing a solution of cold 5% NaHCO_3 in H_2O (1 mL, w/w), followed by extraction with CH_2Cl_2 (3 x 10 mL). The organic layers were then collected, combined, and transferred back into an ice-cold separating

funnel, followed by repetitive washings with NaHCO₃ until the solution is basic as according to indicator paper. The organic layer was again collected, dried over anhydrous NaSO₄, and filtered. The final product was not concentrated due to its explosive nature and a 70% yield of **49** was assumed as stated in the literature.¹⁰⁰

$R_f = 0.50$ (3:1 Hexanes : EtOAc, v:v); M.p. 124.8-126.0 °C; ¹H NMR (500MHz, D₂O): δ 4.19 (dd, $J = 14.0, 7.0$ Hz, 2H, H-1a, H-1b), 3.89 (s, 2H, H-3a, H-3b), 1.27 (t, $J = 7.0$ Hz, 3H, CH₃); ¹³C NMR (125 MHz, D₂O): δ 168.1 (C, C-2), 63.20 (C, C-1), 40.06 (C, C-3), 13.57 (CH₃, C-4); IR (film from CH₂Cl₂): $\nu_{\max}$ 2987, 2113, 1745, 1625, 1244, 1196, 1066, 1025, 767 cm⁻¹.

Rhodium (II) acetate dimer (**50**).

50

Following methodology of Legzdins and colleagues,¹¹⁵ rhodium chloride trihydrate (100 mg, 0.38 mmol) and sodium acetate trihydrate (200 mg, 1.48 mmol) were dissolved in absolute EtOH (2 mL) and acetic acid (2 mL) and heated to reflux for a period of 1 hour. Upon reflux, the solution rapidly turned green and a green precipitate was deposited. Once the solution had cooled to r.t., the solution was filtered and the solid collected and recrystallised from boiling MeOH. The crystals were dried at 45 °C *in vacuo*

for a period of 40 hours to yield the dimer **50** as emerald green crystals (0.048 g, 28.2 %).

M.p. 202 °C; ¹H NMR (500MHz, D₂O): δ 1.90 (s, 12H, CH₃); ¹³C NMR (125 MHz, D₂O): δ 169.12 (C, CO₂), 20.91 (C, CH₃); IR (film from CH₂Cl₂): $\nu_{\max}$ 2970, 2929, 2857, 1738, 1682, 1584, 1415, 1365, 1217, 695 cm⁻¹.

1,5-Anhydro-2-deoxy-1,2-C-(exo-carbmethoxymethylene)-3,4,6-O-acetyl- α -D-arabino-hexitol (51**)**

51

In a modification of the procedure proposed by Hoberg and colleagues,³³ the dimer **50** (0.01 g, 0.02 mmol) was added to a solution of glucal **37** (0.09 g, 0.34 mmol) in dry CH₂Cl₂ (1.5 mL) and stirred vigorously under argon atmosphere. A solution of ethyl diazoacetate (**49**) (0.165 g, 1.36 mmol) in dry CH₂Cl₂ (4.4 mL) was then added dropwise over a period of 14 hours via a syringe pump. After addition of the ethyl diazoacetate solution TLC analysis showed the presence of both starting material and product. The reaction solution was then concentrated *in vacuo* and purified via silica gel column chromatography (5:1 → 1:1 hexanes : EtOAc, v:v) to yield **51** as a pale yellow viscous oil (0.032 g, 27%).

R_f = 0.40 (2:1 hexanes : EtOAc, v:v); $[\alpha]_D^{26.9} +31.0^\circ$ (c=1.0, CH₂Cl₂); ¹H NMR (500MHz, CDCl₃): δ 5.1 (d, J = 4.5 Hz, 1H, H-4), 4.86 (t, J = 4.5 Hz, 1H, H-3), 4.48 (dd, J = 12, 7 Hz, 1H, H-6a), 4.15-4.11 (m, 3H, H-6b, H-9a, H-9b), 3.95 (dd, J = 7.5, 2.0 Hz, 1H, H-1), 3.85 (m, 1H, H-5), 2.1-2.07 (m, 9H, CH₃CO), 2.04 (t, J = 9.5 Hz, 1H, H-7), 1.77 (t, J = ?? Hz, 1H, H-2), 1.3-1.25 (m, 3H, CH₃-Et); ¹³C NMR (125 MHz, CDCl₃): δ 170.7 (C, C-8), 169.5 (C, CH₃CO), 168.5 (C, CH₃CO), 168.1 (C, CH₃CO), 71.9 (CH, C-5), 68.4 (CH, C-4), 66.9 (CH, C-3), 61.8 (CH, C-6), 61.1 (CH₂, C-9), 55.9 (CH, C-1), 24.6 (CH, C-2), 23.1 (CH, C-7), 20.92 (CH₃, CH₃CO), 20.86 (CH₃, CH₃CO), 20.79 (CH₃, CH₃CO), 14.2 (C, C-10); IR (neat, cm⁻¹) ν_{max} = 2983, 1739, 1368, 1214, 1182, 1130, 1030, 914.

1,5-Anhydro-3,4-di-*O*-benzyl-2-deoxy-1,2-*C*-(exo-carbmethoxymethylene)-D-glycero-D-gulo-hexitol (54), 1,5-Anhydro-3,4-di-*O*-benzyl-2-deoxy-1,2-*C*-(exo-carbmethoxymethylene)-D-glycero-D-talo-hexitol (55) and 1,5-Anhydro-3,4-di-*O*-benzyl-6-*O*-(formyl)-2-deoxy-1,2-*C*-(exo-carbmethoxymethylene)-D-glycero-D-gulo-hexitol (56)

In a modification of the procedure reported by Hoberg and Claffey,⁷⁶ the dimer **50** (0.042 g, 0.095 mmol) was added to a solution of the protected glucal **40** (1.09 g, 1.9 mmol) in dry CH₂Cl₂ (8 mL) and the resulting mixture stirred vigorously. A solution of ethyl diazoacetate **49** (4.2 mL, 5.7 mmol) in dry CH₂Cl₂ (27 mL) was then added dropwise over a period of 14 hours (2.3 mL/h) via a syringe pump. According to literature the reaction was quenched after 14 hours. The reaction mixture was then concentrated *in vacuo* and purified via silica gel column chromatography (20:1 → 5:1 hexanes : EtOAc, v:v). However, consistent with the report of Risseuw and colleagues,¹⁰⁴ this led to an inseparable mixture of both stereoisomers **52** and **53** as a clear viscous oil (0.723 g, 58%). The inseparable stereoisomers **52** and **53** (0.545 g, 0.84 mmol) were then dissolved in Et₂O (4.5 mL) and treated with the dropwise addition of formic

acid (3.9 mL, 104 mmol). The reaction was left to stir for 50 minutes, after which TLC analysis showed the presence of both starting material and product. It is important to note that the reaction should not be left for longer in order to avoid the formation of a C6-formyl by-product. The solution was diluted in Et₂O (40 mL), washed with sat. aq. NaHCO₃ (4 x 50 mL), and dried over anhydrous MgSO₄. The solution was filtered and concentrated *in vacuo* to produce the crude product as a viscous yellow oil. Purification via silica gel column chromatography (14:1 → 1:1 hexanes : EtOAc) yielded **54** (208 mg, 61%), **55** (68 mg, 20%), and **56** (7.7 mg, 2%) as pale yellow oils.

Data for **54**: $R_f = 0.40$ (2:1 hexanes : EtOAc, v:v); $[\alpha]_D^{26.7} = +3.0$ (c=1.0, CH₂Cl₂); ¹H NMR (500MHz, CDCl₃): δ 7.35-7.28 (m, 10H, Ph), 4.76 (d, $J = 11.5$ Hz, 2H, PhCH₂), 4.62 (d, $J = 11.5$ Hz, 1H, PhCH₂), 4.59 (d, $J = 12$ Hz, 1H, PhCH₂), 4.16 (m, 2H, CH₂-Et), 3.95 (dd, $J = 7.5, 2.0$ Hz, 1H, H-1), 3.85 (d, $J = 5.0$ Hz, 1H, H-3), 3.79 (m, 1H, H-6a), 3.70 (m, 1H, H-6b), 3.50 (m, 2H, H-4, H-5), 2.02 (dd, $J = 5.5, 2.5$ Hz, 1H, H-7), 1.82 (t, $J = 7.0$ Hz, 1H, H-2), 1.27 (t, $J = 7.0$ Hz, 3H, CH₃-Et); ¹³C NMR (125 MHz, CDCl₃): δ 171.7 (C, C-8), 137.8 (C, Ph), 137.5 (C, Ph), 128.6 (CH, Ph), 128.5 (CH, Ph), 128.1 (CH, Ph), 128.0 (CH, Ph), 127.95 (CH, Ph), 127.93 (CH, Ph), 127.8 (CH, Ph), 76.4 (CH, C-3), 75.2 (CH, C-5), 73.9 (CH, C-4), 73.6 (CH₂, PhCH₂), 71.6 (CH₂, PhCH₂), 62.1 (CH, C-6), 60.8 (CH₂, C-9), 58.1 (CH, C-1), 23.9 (CH, C-2), 23.3 (CH, C-7), 14.2 (CH₃, C-10); IR (neat, cm⁻¹) $\nu_{\max} = 3483, 3031, 2980, 2872, 1715, 1428, 1298, 1089, 853, 735, 696$.

Data for **55**: $R_f = 0.30$ (2:1 hexanes : EtOAc, v:v); $[\alpha]_D^{26.5} = -24.0$ (c=1.0, CH₂Cl₂); ¹H NMR (500MHz, CDCl₃): δ 7.38-7.28 (m, 10H, Ph), 4.98 (d, $J = 11.5$ Hz, 1H, PhCH₂), 4.77 (d, $J = 11.5$ Hz, 1H, PhCH₂), 4.70 (t, $J = 12.5$ Hz, 2H, PhCH₂), 4.49 (dd, $J = 9.5, 4.5$ Hz, 1H, H-3), 4.13 (q, $J = 7.0$ Hz, 2H, H-9a, H-9b), 3.89 (m, 2H, H-1, H-4), 3.66 (m, 2H, H-6a, H-6b), 3.60 (t, $J = 9.0$ Hz, 1H, H-5), 1.75 (t, $J = 6.0$ Hz, 1H, OH), 1.61 (dd, $J = 9.5, 6.5$ Hz, 1H, H-7), 1.48 (m, 1H, H-2), 1.26 (t, $J = 7.5$ Hz, 3H, EtCH₃); ¹³C NMR (125 MHz, CDCl₃): δ 168.8 (C, C-8), 138.4 (C, Ph), 138.3 (C, Ph), 128.4 (CH, Ph), 128.37 (CH, Ph), 128.0 (CH, Ph), 127.8 (CH, Ph), 127.7 (CH, Ph), 127.8 (CH, Ph), 79.8 (CH, C-3), 76.4 (CH, C-4), 74.4 (CH₂, PhCH₂), 71.9 (CH₂, PhCH₂), 63.0 (CH, C-1), 60.5 (CH₂, C-9), 53.6 (CH, C-1), 24.2 (CH, C-2), 22.0 (CH, C-7), 14.3 (CH₃, C-10); IR (neat, cm⁻¹) $\nu_{\max} = 3054, 1724, 1422, 1264, 1072, 896, 732, 704$.

Data for **56**: $R_f = 0.50$ (3:1 hexanes : EtOAc, v:v); $[\alpha]_D^{27.7} = +42.0$ (c=1.0, CH₂Cl₂); ¹H NMR (500MHz, CDCl₃): δ 8.05 (s, 1H, H-11), 7.39-7.28 (m, 10H, Ph), 4.73 (d, $J = 12$ Hz, 1H, PhCH₂), 4.72 (d, $J = 11.5$ Hz, 1H, PhCH₂), 4.58 (d, $J = 11.5$ Hz, 1H, PhCH₂), 4.579 (d, $J = 11.5$ Hz, 1H, PhCH₂), 4.46 (dd, $J = 12.5, 7.5$ Hz, 1H, H-6a), 4.20 (dd, $J = 11.5, 2.0$ Hz, 1H, H-6b), 4.12 (m, 2H, CH₂-Et), 3.93 (dd, $J = 7.0, 2.0$ Hz, 1H, H-1), 3.82 (d, $J = 6.0$ Hz, 1H, H-3), 3.75 (m, 1H, H-5), 3.49 (t, $J = 6.0$ Hz, 1H, H-4), 2.02 (dd, $J = 5.5, 2.0$ Hz, 1H, H-7), 1.83 (t, $J = 7.0$ Hz, 1H, H-2), 1.26 (t, $J = 7.0$ Hz, 3H, CH₃-Et); ¹³C NMR (125 MHz, CDCl₃): δ 168.8 (C, C-8), 138.4 (C, Ph), 138.3 (C, Ph), 128.4 (CH, Ph), 128.37 (CH, Ph), 128.0 (CH, Ph), 127.8 (CH, Ph), 127.7 (CH, Ph), 127.8 (CH, Ph), 79.8 (CH, C-3), 76.4 (CH, C-4), 74.4 (CH₂, PhCH₂), 71.9 (CH₂, PhCH₂), 63.0 (CH, C-1), 60.5 (CH₂, C-9), 53.6 (CH, C-1), 24.2 (CH, C-2), 22.0 (CH, C-7), 14.3 (CH₃, C-10); IR (neat, cm⁻¹) $\nu_{\max} = 3054, 1724, 1422, 1264, 1072, 896, 732, 704$.

= 7.0 Hz, 3H, CH₃-Et); ¹³C NMR (125 MHz, CDCl₃): δ 193.7 (C, C-11), 171.4 (C, C-8), 137.6 (C, Ph), 137.5 (C, Ph), 128.54 (CH, Ph), 128.52 (CH, Ph), 128.0 (CH, Ph), 127.9 (CH, Ph), 127.8 (CH, Ph), 75.4 (CH, C-3), 74.7 (CH₂, PhCH₂), 72.6 (CH, C-5), 71.5 (CH₂, PhCH₂), 62.5 (CH, C-6), 60.8 (CH₂, C-9), 57.1 (CH, C-1), 24.2 (CH, C-2), 24.1 (CH, C-7), 14.2 (CH₂, C-10); IR (neat, cm⁻¹) ν_{max} = 3013, 2872, 1714, 1454, 1367, 1297, 1216, 1180, 1090, 748.

***N,N'*-Ditosylhydrazine (**57**)**

57

p-Toluenesulfonyl hydrazine (5.5 g, 29.6 mmol) and *p*-toluenesulfonyl chloride (8.5 g, 44.3 mmol) were dissolved in CH₂Cl₂ (50 mL) and stirred vigorously at r.t., before it was treated to a dropwise addition of pyridine (3.6 mL). The addition of pyridine caused the solution to become homogenous and after 5 minutes a white precipitate was formed. The reaction mixture was left to stir at r.t. for 1.5 hours before it was cooled to 0 °C, diluted with Et₂O (120 mL) and H₂O (60 mL), and left to stir for a further 15 minutes. The white precipitate was collected via Büchner filtration and washed with Et₂O (100 mL). Purification was achieved through recrystallisation from boiling MeOH (60 mL). The recrystallised product was then collected and washed with cold MeOH (40 mL) and Et₂O (60 mL) to give the final product **57** as white crystals (8.13 g, 81%). Spectral data matches that of Toma and colleagues.¹⁰⁹

R_f = 0.50 (3:1 hexanes : EtOAc, v:v); M.p. 209 °C; ¹H NMR (500MHz, DMSO-d₆): δ 8.73 (s, 1H, SO₂NH), 6.79 (d, *J* = 8 Hz, 2H, Ph), 6.54 (d, *J* = 8 Hz, 2H, Ph), 1.54 (s, 3H, CH₃); ¹³C NMR (125MHz, DMSO-d₆) δ 143.9 (C, Ph), 135.9 (C, Ph), 129.9 (CH, Ph), 128.2 (CH, Ph), 21.5 (C, CH₃); IR (neat, cm⁻¹) ν_{max} = 3232, 3206, 1598, 1200, 1189, 1171, 810.

2-Propynyl 2-diazoacetate (**58**)

In a modification of Fukuyama and colleagues,¹⁰⁹ propargyl alcohol (1.03 mL, 17.8 mmol) and NaHCO₃ (4.5 g, 53.51 mmol) were dissolved in acetonitrile (36 mL) and cooled to 0 °C before bromoacetyl bromide (2.3 mL, 26.7 mmol) was added dropwise over a period of 3 minutes. Addition of bromoacetyl bromide caused the solution to become homogenous and the reaction was left to stir at 0 °C for 15 minutes before it was quenched with H₂O (100 mL). The solution was extracted with CH₂Cl₂ (3 x 40 mL) and the organic layers were collected and washed with brine (3 x 50 mL) before being dried over anhydrous MgSO₄, filtered, and concentrated *in vacuo* to leave a viscous orange oil. The formed bromoacetate product was used without further purification and along with **57** (12g, 35.6 mmol) was dissolved in THF before being cooled to 0 °C. DBU (13.3 mL, 13.3 mmol) was then added dropwise over a period of 5 minutes, causing the solution to turn into a homogenous mixture. The reaction was left to stir for 15 minutes at 0 °C before the reaction was quenched with sat. aq. NaHCO₃ (60 mL). The solution was extracted with Et₂O (3 x 40 mL) and the organic layers were collected, washed with brine (3 x 40 mL), dried over anhydrous MgSO₄ and concentrated *in vacuo* to provide the crude product. Purification by neutralised silica gel column chromatography (3:1 hexanes : EtOAc, v:v) to give **58** as a viscous orange oil (1.3 g, 57%). Spectral data matches that of Toma and colleagues.¹⁰⁹

R_f = 0.50 (3:1 hexanes : EtOAc, v:v); ¹H NMR (500MHz, CDCl₃): δ 4.84-4.76 (m, 3H), 2.50-2.42 (m, 1H); ¹³C NMR (125MHz, CDCl₃): δ 165.8 (C, C-2), 77.6 (C, C-4), 75.0 (CH, C-5), 52.2 (C, C-1), 46.2 (C, C-3); IR (neat, cm⁻¹) ν_{max} = 3286, 3117, 2951, 2114, 1731, 1987, 1387, 1344, 1219, 1165, 1028, 993, 644.

(1*S*,3*R*,4*S*,5*R*,6*S*)-prop-2-yn-1-yl-4,5-bis(benzyloxy)-3-(hydroxymethyl)-2-oxabicyclo[4.1.0]heptane-7-carboxylate (**61**) and (1*R*,3*R*,4*S*,5*R*,6*R*)-prop-2-yn-1-yl-4,5-bis(benzyloxy)-3-(hydroxymethyl)-2-oxabicyclo[4.1.0]heptane-7-carboxylate (**62**)

61

62

Using a modification of the method employed by Hoberg and colleagues,⁷⁶ protected glucal **40** (1.0 g, 1.9 mmol) was dissolved in dry CH₂Cl₂ (8 mL) and stirred under argon. Rhodium acetate (0.047 g, 0.095 mmol) was then added to the reaction mixture, followed by vigorous stirring, before the dropwise addition of diazoacetate **58** (0.7 g, 5.7 mmol) in CH₂Cl₂ (27 mL) via syringe pump over a period of 14 hours (2.2 mL/h). The reaction was then left to stir for a further 3 hours before the solution was concentrated *in vacuo* to yield the crude product. The crude material was then purified via silica gel column chromatography (20:1 → 1:1 hexanes : EtOAc, v:v) to give an inseparable mixture of both stereoisomers **59** and **60** in a combined yield of 38% (447 mg). The isomeric mixture was then dissolved in Et₂O (4.1 mL) and treated with a dropwise addition of formic acid (3.2 mL, 84 mmol). The solution was allowed to stir for 50 minutes at r.t., after which TLC-analysis deemed the reaction complete. The reaction mixture was then diluted with Et₂O (30 mL) and washed with sat. aq. NaHCO₃ (4 x 30 mL). The organic layer was collected, dried over anhydrous MgSO₄, filtered and concentrated *in vacuo* to afford the crude material. Purification by silica gel column chromatography (1:100 → 3:10 EtOAc : CH₂Cl₂, v:v) to provide **61** (123 mg, 43%) and **62** (27 mg, 10%) as clear viscous oils.

Data for **61**: R_f = 0.40 (2:1 hexanes : EtOAc, v:v); $[\alpha]_D^{27.7}$ = +38.0 (c=1.0, CH₂Cl₂); ¹H NMR (500MHz, CDCl₃): δ 7.36-7.28 (m, 10H, Ph), 4.75 (d, J = 12.0 Hz, 2H, PhCH₂), 4.69 (dd, J = 5.0, 2.5 Hz, 2H, H-9a, H-9b), 4.70 (t, J = 12.0 Hz, 2H, PhCH₂), 4.00 (dd, J = 7.5, 2.5 Hz, 1H, H-1), 3.85 (d, J = 4.5 Hz, 1H, H-3), 3.79 (m, 1H, H-6a), 3.70 (m, 1H, H-6b), 3.52 (m, 2H, H-4, H-5), 2.50 (t, J = 2.5 Hz, 1H, H-

11), 2.10 (dd, $J = 6.0, 2.5$ Hz, 1H, H-7), 1.99 (t, $J = 6.0$ Hz, 1H, OH), 1.87 (t, $J = 7.0$ Hz, 1H, H-2); ^{13}C NMR (CDCl_3) δ_c 170.9 (C, C-8), 137.8 (C, Ph), 137.4 (C, Ph), 128.6 (CH, Ph), 128.5 (CH, Ph), 128.0 (CH, Ph), 127.9 (CH, Ph), 127.8 (CH, Ph), 76.0 (CH, C-3), 75.1 (CH, C-4), 75.0 (CH, C-5), 73.9 (CH, C-11), 73.6 (CH_2 , PhCH_2), 71.7 (CH_2 , PhCH_2), 62.0 (CH_2 , C-6), 58.4 (CH, C-1), 52.3 (CH_2 , C-9), 24.5 (CH, C-2), 23.0 (CH, C-7); IR (neat, cm^{-1}) $\nu_{\text{max}} = 3467, 3286, 3062, 2871, 1723, 1427, 1292, 1209, 1117, 1088, 825, 736, 696$.

Data for **62**: $R_f = 0.40$ (2:1 hexanes : EtOAc, v:v); $[\alpha]_D^{27.7} = +59.0$ ($c=1.0$, CH_2Cl_2); ^1H NMR (500MHz, CDCl_3): δ 7.38-7.29 (m, 10H, Ph), 4.99 (d, $J = 11.5$ Hz, 1H, PhCH_2), 4.75-4.61 (m, 5H, CH_2Ph , H-9a, H-9b), 4.44 (dd, $J = 9.5, 4.0$ Hz, 1H, H-3), 3.92 (t, $J = 6.5$ Hz, 1H, H-4), 3.90 (dd, $J = 9.0, 4.0$ Hz, 1H, H-1), 3.66 (m, 2H, H-6a, H-6b), 3.61 (t, $J = 9.0$ Hz, 1H, H-5), 2.49 (t, $J = 2.0$ Hz, 1H, H-11), 1.75 (bs, 1H, OH), 1.65 (dd, $J = 9.5, 6.5$ Hz, 1H, H-7), 1.55 (dd, $J = 7.0, 4.0$ Hz, 1H, H-2); ^{13}C NMR (CDCl_3) δ_c 167.9 (C, C-8), 138.4 (C, Ph), 138.2 (C, Ph), 128.44 (CH, Ph), 128.4 (CH, Ph), 128.0 (CH, Ph), 127.9 (CH, Ph), 127.8 (CH, Ph), 79.8 (CH, C-3), 79.2 (CH, C-4), 74.9 (CH, C-11), 74.5 (CH_2 , PhCH_2), 73.9 (CH, C-5), 72.1 (CH_2 , PhCH_2), 63.0 (CH_2 , C-6), 54.1 (CH, C-1), 51.9 (CH_2 , C-9), 24.9 (CH, C-2), 21.7 (CH, C-7); IR (neat, cm^{-1}) $\nu_{\text{max}} = 3018, 1733, 1411, 1215, 1126, 1099, 747$.

Chapter 3

Biological analysis

3.1 Aims and objectives

This aspect of the thesis focused on the elucidation of the mechanism of action of bioactive 1,2-cyclopropyl carbohydrates. Initial work was done testing the bioactivity of compounds **23**, **54**, **55**, **56**, **61** and **62** through a liquid phase dose-response assays using *pdr1Δpdr3Δhis3Δ* as a proxy wild-type yeast from the *pdr1Δpdr3ΔxxxΔ* yeast genome deletion set. If the synthesised compounds show bioactivity, they will undergo a chemical genetic screen using the *pdr1Δpdr3ΔxxxΔ* library, genetic interactions will be validated and subjected to a gene ontology search in order to identify chemical pathways involved in the mechanism of action of the drug. Finally, target isolation and identification will be attempted with either compound **61** or **62** through affinity purification by way of a copper-assisted azide-alkyne cycloaddition reaction with a modified agarose matrix.

Introduction

3.2 Yeast as a model organism

The budding yeast, *Saccharomyces cerevisiae*, has a long and well-documented history as the first eukaryotic organism to have undergone complete genomic sequencing, which was completed in 1996.¹¹⁶ In 1970, over 600 scientists from North America, Europe and Japan discussed the unique advantages *S. cerevisiae* offers as a model organism for eukaryotic cells as the natural progression from the extensive studies done on prokaryotic cells and phages.¹¹⁶ Accordingly, this led to the completion of the yeast *S. cerevisiae* genome project, which revealed the yeast's genome to be much more compact than initially expected. The genome sequence of

12 million nucleotides encodes approximately 6000 protein-encoding genes, which is about 70% of the total genome. In contrast, the human genome contains approximately 2.85 billion nucleotides that only codes for 20,000-25,000 protein-encoding genes.¹¹⁷ Even compared to other yeast species the *S. cerevisiae* genome is remarkably unique with a much higher ratio of protein-coding genes to genome nucleotides.^{118,116} Coinciding with the completion of the yeast genome project was the first complete proteome of a eukaryotic cell. This led to the classification of yeast proteins into their functions, allowing researchers to see an approximate percentage of proteins involved in each cellular process. For example, a total of 7% of the proteome is involved in the process of intracellular trafficking, whereas 11% of its proteome is responsible in metabolism.¹¹⁶

Since the yeast genome project started four decades ago, the biochemical, genetic and genomic tools available to study this organism have greatly helped in solving many biological problems and questions, more so than any other eukaryotic cells. Accordingly, this has made yeast the most commonly used organism for understanding both vital cellular processes and human diseases.¹¹⁹ A great example of this has been the work on understanding telomeres and telomerase. Work done by Lingner and colleagues¹²⁰ identified two essential genes in *S. cerevisiae* involved in the process of telomere formation, Est2 (reverse transcriptase) and Est1 (accessory proteins). Involvement of the two genes was tested and proven through site-directed mutagenesis in three key conserved amino acids, which led to a complete prevention of telomerase activity.¹²⁰ This allowed for extensive research to be done on the mechanism of telomerase activity in yeast, however, the mechanism of telomerase activity in other eukaryotes remained unknown until an iterative profile search found human homologs of both the Est1 and Est2 genes, with the region of highest homology being the TPR sequence within the Est (figure 3.0).¹²¹ This extensive cross-species homology allowed for the conduction of structure-function analysis of the human homolog of both Est1 (hEST1A, hEST1B, hEST1C) and Est2 (hTERT). Findings in human cell lines confirmed the results seen in *S. cerevisiae* with the human hEST1 and hTERT genes forming both protein-protein interactions in the formation of the telomerase complex, comparable to the Est2 and Est1 genes.¹²²

Figure 3.0: Representation of homology between human and *S. cerevisiae* Est1 gene. The area of highest homology is shown by the shaded area, which all contain TRP (tetratricopeptide). ScEst1 is the yeast genes and the three human homologs associated with it: hEST1A/SMG6, hEST1B/ SMG5 and hEST1C/SMG7. Figure adopted from Sealey and colleagues.¹²² PIN (PILT N-terminal), TRID (hTR-interaction domain), DBD (DNA binding domain).

This cross-species homology combined with the yeast genome project and yeast deletion libraries make the budding yeast one of the most valuable tools in studying eukaryotic life. As with the previous example, studying cellular processes in yeast can identify genes previously unknown in other eukaryotes and help identify their functions. This is also useful in studying human diseases with approximately 285 yeast proteins being homologous to 155 proteins associated in human disease,¹²³ allowing drugs and other inhibitory molecules to be tested through methods such as chemical genetic screens to elucidate a potential mechanism of action.

3.3 The yeast *pdr1Δpdr3ΔxxxΔ* deletion mutant array

Yeast ABC transporters are utilised in many biological processes including the regulation of mitochondrial function and peptide pheromone secretion, however, they are primarily used as xenobiotic efflux pumps conferring pleiotropic drug resistance (PDR).^{124,125} The presence of such effective efflux pumps allows the yeast to quickly adapt to toxic environmental changes as well as aiding in the protection against unwanted endogenous toxic metabolites.¹²⁴ Although this aids in the viability of the yeast, many xenobiotic activities are masked by the pleiotropic drug resistant (PDR) efflux transporters resulting in reduced sensitivity when compared to mammalian cell lines.¹²⁵ This limits the application of yeast in biological screens.

Therefore, the preparation of the *pdr1Δpdr3Δ* deletion library was undertaken by Coorey and colleagues,¹²⁵ which involved the deletion of the genes for the two major transcription factors, *pdr1p* and *pdr3p* resulting in a pleiotropic drug resistant (PDR) network attenuated deletion mutant array. As shown in figure 3.1 many efflux pumps are tightly regulated through the *pdr1p*, *pdr3p* transcription factors. Much research has been conducted using the single deletion of the ABC transporter *PDR5*,^{126,127} however, the deletion of the *pdr1p* and *pdr3p* transcription was found to give a greater hypersensitivity to a larger array of compounds as a reduction in all ABC efflux pumps was observed.¹²⁵

Figure 3.1: The PDR network in yeast are tightly regulated by the *pdr1p* and *pdr3p* transcription factors. Only the PDR ABC transporters are represented in this figure, however, it is worth noting more non-ABC transporters are involved in the PDR network however their functions are not well understood. Arrows indicate positive regulation.

Preparation of the *MATa pdr1Δpdr3ΔxxxΔ* library was completed through the mating of the *MATa pdr1Δpdr3Δ* double mutant with the *MATa* single gene deletion mutant array (DMA) gifted to Coorey and colleagues by Dr Charles Boone (University of Toronto).¹²⁵ The *pdr1Δpdr3ΔxxxΔ* deletion library was then subjected to a range of compounds including known pdr5p substrates. Previous compounds that displayed bioactivity in wild-type yeast were now found to cause hypersensitivity in the *pdr1Δpdr3Δ* deletion library whereas with the known pdr5p substrates bioactivity was now detectable. This indicated that the constructed *pdr1Δpdr3ΔxxxΔ* deletion library was more sensitive to a broad range of xenobiotics, which in turn allowed for the elucidation of the mechanism of action of a wider range of xenobiotic substances than could be achieved with the parental deletion library.¹²⁵

Materials and methods

3.4 Compounds

All 1,2-cyclopropyl carbohydrates used in the biological screens were synthesised in this study (chapter 2), and were purified to >95% as indicated through spectroscopic methods (1D and 2D NMR spectroscopy, IR spectroscopy). Samples were stored at -20 °C under nitrogen atmosphere, and stock solutions were freshly made at a concentration of 100 mM in DMSO. All studies were completed using a single batch unless otherwise stated. Unless otherwise stated, all reagents were purchased from Sigma-Aldrich.

3.5 Yeast strain

Previous bioassays of **23** indicated appreciable potency only against yeast strains containing a *pdr1Δpdr3Δ* background.² Chemical genetic profiling was undertaken using the *pdr1Δpdr3ΔxxxΔ* library, which was obtained from a -80 °C glycerol freezer stock originally prepared by Namal Coorey from the Chemical Genetics Laboratory, within the School of Biological Sciences at Victoria University of Wellington.¹²⁵

Strain	Genotype
<i>pdr1Δpdr3ΔxxxΔ</i>	<i>pdr1Δ::NAT</i> ; <i>pdr3Δ::URA3</i> ; <i>xxxΔ::kanR</i> ; <i>can1Δ::STE2pr-Sp_His5</i> ; <i>Lyp1Δ</i> ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>ura3Δ0</i> ; <i>met15Δ0</i> LYS2

3.6 Yeast culture and growth conditions

The *pdr1Δpdr3ΔxxxΔ* yeast strains were cultured in one of the following three media conditions: synthetic complete media, synthetic drop-out minus URA media and yeast peptone dextrose media, and supplemented with either of two antibiotics: ClonNat at a final concentration of 100 mg/L or geneticin (G418) a final concentration of 200

mg/L. All media was sterilised via autoclave for 21 min at 124 °C and 210 kPa. All yeast were grown under standard protocols as specified by Sherman.¹²⁸

3.6.1 Synthetic complete (SC) media

For 800 mL of SC media, 1.36 g of yeast nitrogen base, without amino acids and ammonium sulfate (Formedium™ ltd), 0.8 g of L-Glutamic acid monosodium salt hydrate (sigma Aldrich) and 1.6 g of the corresponding amino acid mix (see section 3.6.4) was added to 360 mL of distilled water (dH₂O). The mixture was autoclaved at 124 °C for 21 min then allowed to cool before the addition of 40 mL of a 40% glucose solution in dH₂O along with the appropriate antibiotic. dH₂O is then added to make the solution up to 800 mL. For solid SC agar, the same process is followed, with the slight variation where 16 g of agar (Formedium™ Ltd.) in 400 mL dH₂O was autoclaved and added to the SC mixture, making the total volume to 800 mL. The agar must be kept at approximately 65 °C otherwise it will begin to solidify.

3.6.2 Synthetic drop-out (SD) minus URA media

SD media is prepared in the same way as SC, though the amino acid mix used for this media does not contain uracil (URA).

3.6.3 Yeast peptone dextrose (YPD) media

For 800 mL of YPD media, 8 g of Bacto™ yeast extract (BD bioscience), 16 g of peptone (Formedium™ ltd) and 0.096 g of adenine (Sigma-Aldrich) was added to 360 mL of water and autoclaved at 124 °C for 21 min. The solution was then allowed to cool before the addition of 40 mL of a 40% glucose solution in dH₂O, followed by the desired antibiotic. dH₂O was then added to make the resulting solution up to 800 mL. For the solid YPD agar, the same procedure is followed, with the addition of 16 g of agar (Formedium™ ltd) in 400 mL water to the YPD media after autoclaving to bring the total volume to 800 mL. The solid agar was kept at 65 °C to prevent it from solidifying at lower temperatures before pouring plates.

3.6.4 Amino acid mix for SC media

The amino acid mixture for SC media consists of; 3 g adenine, 2 g alanine, 0.2 g *p*-aminobenzoic acid, 2 g asparagine, 2 g aspartic acid, 2 g cysteine, 2 g glutamic acid, 2 g glutamine, 2 g glycine, 2 g histidine, 2 g inositol, 2 g isoleucine, 10 g leucine, 2 g lysine, 2 g methionine, 2 g phenylalanine, 2 g proline, 2 g serine, 2 g threonine, 2 g tryptophan, 2 g tyrosine, 2 g uracil and 2 g valine. As stated above SD amino acid mix does not contain uracil, however everything else remains the same.

3.7 Dose-response assay in *Saccharomyces cerevisiae*

3.7.1 Liquid phase dose-response assay

All dose-response assays were conducted using a single colony of *pdr1Δpdr3Δhis3Δ* yeast cells. Cells were inoculated into 2 mL of YPD and SC media. The inoculated cells were then placed in a rotating drum (Digisystem Laboratory Instruments) at 30 °C at a speed of 45 rpm and left to grow overnight. Cell concentration was calculated using a UV/VIS spectrometer (Unicam 8625) reading at OD₆₆₀. Cells were diluted with the respective media to make a 10 mL solution with a final cell concentration of 5×10^5 cells/mL. Using a multichannel pipette, 99 μL of the cell solution was plated out into flat bottom 96 well plates (Corning Incorporated).

In a separate deep-well 96 well plate (Gene era biotech), a 1.5-fold serial dilution was prepared of compounds **23**, **54**, **55**, **56**, **61** and **62** from the corresponding 100 mM stock solution. Dilutions of the 1,2-cyclopropyl carbohydrates were achieved using DMSO (Sigma-Aldrich).

Subsequently 2 μL of the 1.5-fold drug dilution was added to each well of a 96 well microtiter plate containing the cell solution, making a final DMSO concentration of 2%. A row was treated with 2 μL of DMSO alone to act as a negative control to allow for the calculation of residual growth as well as the inclusion of an individual row containing only media in order to calculate optical density of the growth media. The

plates were then mixed via vortex for 30 seconds at 1000 rpm using an Eppendorf Mix Mate (Krackeler Scientific Inc.) and incubated at 31 °C for 24 hours. After 24 hours, the cells were re-suspended via vortex for 30 seconds at 1000 rpm and the optical density read at 590 nm using a multi-well plate reader (PerkinElmer, Envision Multiplate Reader). Residual growth was calculated as:

$$\frac{OD_{590} \text{ exp} - \text{mean } OD_{590} \text{ background}}{\text{mean } OD_{590} \text{ control} - \text{mean } OD_{590} \text{ background}} \times 100$$

Where mean OD₅₉₀ control is the mean optical density of the DMSO control wells, OD₅₉₀ background is the mean optical density of wells containing only media and OD₅₉₀ is the optical density for each experimental well.

3.7.2 Solid phase dose-response assay

A solid phase dose response was conducted using YPD agar. Based on the results of the liquid IC₅₀ results (see section 3.17.1), seven falcon tubes were prepared for both **23** and **54** with a range of increasing drug concentrations of 0, 20, 50, 100, 200 and 400 µM for **23** and 0, 50, 100, 150, 200 and 300 µM for **54**, at a final DMSO concentration of 2%. Hot agar (55 °C) was added to each falcon tube to a final volume of 30 mL and the solution was gently mixed through rotation. Each falcon tube was poured into separate Singer plates (Singer instruments, Somerset UK) and allowed to cool and set. Plates were not stacked to allow fast cooling and to avoid any potential breakdown of compound **23** and **54**, as reported by Davies.² Once cooled, a single plate from the *pdr1Δpdr3ΔxxxΔ* library was pinned using the singer RoToR HAD (Alphatech systems Ltd) robot onto all the plates in a 1536 format resulting in quadruplicate colonies. The plates were incubated for 24 hours and then photographed. Colony size and circularity was calculated using the colony-based screening program Gitter.¹²⁹ Results were compared to control, from which the residual growth could be calculated.

3.8 Chemical genetic screening

All chemical genetic screens were conducted using the *pdr1Δpdr3ΔxxxΔ* library.¹²⁵ Excluded from the *pdr1Δpdr3ΔxxxΔ* DMA are essential genes and 441 known slow growing deletion strains. The non-essential *pdr1Δpdr3ΔxxxΔ* yeast DMA is composed of 14 plates, arranged in a 386 format, which include a *pdr1Δpdr3Δhis3Δ* border to account for the effects of nutrition access on colony size. All plates were expanded to a 1536 format, allowing for quadruplicates of each colony.

Based on the results of the solid phase dose response assay (see section 3.17.2), 14 plates containing 40 mL of SC agar with no antibiotics were prepared with compound **23** at a concentration of 50 μ M (15 μ L from a 100 mM stock solution in 285 μ L DMSO) to a final DMSO concentration of 1%. This was repeated with **54** at a concentration of 160 μ M (48 μ L from a 100 mM stock solution in 252 μ L DMSO). The solution containing the corresponding drug and DMSO was not added to the agar until the agar had cooled to approximately 55 °C. For each drug treatment, corresponding control plates were also poured using SC agar, which contained 300 μ L of DMSO. Plates were then allowed to set over 1 hour before drying in the laminar flow (Holten Lamin Air). Using the singer RoToR HDA the *pdr1Δpdr3ΔxxxΔ* library was pinned from a previously prepared working copy onto the treated and controlled plates. A separate working copy was used for both **23** and **54** and their corresponding control plates. Plates were incubated at 33 °C and photographed at 18, 22 and 36 hours. Images were processed by the colony-based screening program Gitter,¹²⁹ where average circularity and size of each quadruplicate strain was calculated and compared against control. Results of the Gitter processing were run through a Screen Mill¹³⁰ search resulting in a *p*-value and z-score for all quadruplicate colonies compared to control.

Resistant strains were defined as having a *p*-value less than 0.05 and a z-value (for the ratio of growth control/growth treatment) of -1.95 or less. Sensitive strains are defined as a *p*-value of 0.05 or less and a z-score (for the ratio of growth control/growth treatment) of greater than 1.95.

3.9 Validation of chemical genetic screens

Validations of both resistant and sensitive *pdr1Δpdr3ΔxxxΔ* strains were conducted in liquid media. Results from the chemical genetic assay (section 3.18) indicated a large number of the resistant and sensitive deletion strains were the same for both compounds **23** and **54** suggesting both compounds have the same target. Therefore, resistant and sensitive strains were combined for the validation. A total of 169 resistant strains and 155 sensitive strains were inoculated into individual wells of four flat bottom 96 well plates (Corning Inc), containing 100 μL of SD-URA liquid media. A single row containing only *pdr1Δpdr3Δhis3Δ* was added on each plate as a control. Plates were placed on vortex for 30 sec at 1000 rpm and incubated at 33 °C for 48 hours until all strains were at saturation. Plates were then briefly vortexed for 30 seconds at 1000 rpm and pinned using the singer RoToR HDA to four working copy of flat bottom 96 well plates containing 100 μL of SD-URA. The new working copies were again placed upon the vortex for 30 seconds at 1000 rpm and incubated for 48 hours at 33 °C.

For compound **23**, four flat bottom 96 well plates were prepared consisting of 99 μL of SD-URA liquid media and 2 μL of **23** in DMSO in each well, at a final concentration of 40 μM. Resistant and sensitive strains were then pinned from the previously prepared working copy using the singer RoToR HDA. Plates were placed upon the vortex for 30 seconds at 1000 rpm and incubated at 33 °C. Optical density measurements were then acquired at 24, 30, 36 and 48 hour time periods. The above process was repeated with compound **54** at a final concentration of 160 μM. For both compounds each resistant and sensitive plate was run with an untreated control plate with final DMSO concentration of 2%. Resistant strains were defined as having residual growth 4 standard deviations above the residual growth *pdr1Δpdr3Δhis3Δ* strain. Sensitive strains were defined in a similar manner, in which residual growth was 2 standard deviations below the *pdr1Δpdr3Δhis3Δ* strains. Slow growing control strains were identified as having 40% reduction in growth compared to the mean control colony size and were subsequently excluded from the data.

Gene ontology analysis was conducted on all validated strains utilising both yeastmine^{131,132} and g:Profiler.^{133,134}

3.10 Mammalian cell experiments

PhD candidate Richard Little, in the School of Biological Sciences, Victoria University of Wellington, conducted the following mammalian MTT cell proliferation experiments.

Under normal cell culture conditions, human HL-60 promyelocytic leukemia cells at a concentration of 1×10^4 cells/well were seeded into a 96 well plate and incubated with **23** for 48 hours. Cell were then treated with 20 μ L of MTT and left to incubate for a further 2 hours at 37 °C. Addition of 100 μ L of a solubilising agent containing 45% DMF and 10% SDS (pH 4.5, adjusted with acetic acid addition) allowed for the solubilisation of the blue crystals overnight at 37 °C. Absorbance readings at 570 nm indicated MTT reduction. IC₅₀ values were calculated from a concentration-response curve using prism (Prism Graphpad Software Inc).

3.11 Affinity matrix synthesis

Epoxy-activated agarose (600 mg, 12 μ M) was added to 2 mL of dH₂O in a 15 mL falcon tube and allowed to swell for 2 hours. Sodium azide (6 mg, 90 μ M) was then added to the solution and stirred lightly via vortex, followed by the addition of cerium (III) chloride heptahydrate (1.4 mg, 5.4 μ M). The solution was rotated for 16 hours at 33 °C. The solution was then centrifuged at 2000 rpm for 1 min followed by the removal of the solvent. Purification was achieved by the successive addition of ethanol and diethyl ether, where ethanol (6 mL) was added and the solution and lightly shaken via vortex for 1 min before being centrifuged at 1000 rpm for 1 min. The solvent was removed and replaced with diethyl ether (6 mL). This was again shaken lightly via vortex and centrifuged at 1000 rpm for 1 min before being decanted off. The whole process was then repeated to ensure full conversions of epoxide to azide. The final white precipitate was then dried under vacuum.

3.12 Cell lysate preparation

Glass beads (0.5 mm diameter, dnature Ltd) were soaked in a solution of 1 M HCl overnight followed by extensive rinsing with water to ensure the pH was neutral. The glass beads were dried under vacuum at 80 °C overnight and chilled in ice before use. A *pdr1Δpdr3Δhis3Δ* yeast colony was inoculated into a 15 mL solution of SD-URA and allowed to shake overnight at 33 °C. The 15 mL yeast culture was centrifuged for 2 min at 20,000 rpm and the supernatant was decanted off. The *pdr1Δpdr3Δhis3Δ* culture was resuspended in 2 mL of lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% DMSO and 10 µL of protease inhibitor cocktail). Chilled glass beads were added to the solution at a ratio of 3 g per gram of yeast cell (wet weight). The solution was then shaken at full speed via vortex 5 times for 3 min, keeping the cells on ice for 2 min between vortex sessions. The glass beads were removed via pipette and the cellular debris was removed through centrifugation at 4 °C for 30 min at 16,000 rpm.

3.13 Copper-assisted azide-alkyne cycloaddition

From a prepared stock solution of **61** (100 mM) in DMSO, 40 µL was added to a 1 mL solution of prepared yeast lysate (section 3.12). The solution was allowed to shake for 6 hours at 33 °C before the addition of 25 mg of azide-activated agarose matrix (section 3.11), 0.1 mL of saturated copper sulfate and 5 mg of ascorbic acid neutralised in aqueous sodium hydroxide. The reaction mixture was left to shake at 33 °C for 12 hours before undergoing centrifugation at 7,000 rpm for 5 min. The supernatant was decanted providing the derivatised matrix, which was washed three times with a buffer solution (1 M Tris-HCl buffer pH 8), centrifuging at 7,000 rpm for 5 min and decanting the supernatant between each wash. The above method was repeated with the exclusion of the saturated copper sulfate and ascorbic acid solution as a control.

3.14 Isolation of binding proteins

A 500 μ L low salt wash solution (0.1% triton X-100 in 0.5 M Tris-HCl pH 7.6) was added to the lysate-matrix complex for both the treatments and negative control (section 3.12) and resuspended. The solution was then centrifuged for 5 min at 7,000 rpm and the supernatant decanted, followed by the addition of a 500 μ L high salt wash solution (1M NaCl, 0.1% triton X-100 in 0.5 M Tris-HCl pH 7.7). The solution was shaken via vortex, centrifuged at 7,000 rpm for 5 min and the supernatant was removed. An LDS wash was performed with the addition of 100 μ L of NuPAGE LDS sample buffer (4X) loading buffer. The solution was again shaken via vortex and centrifuged at 7,000 rpm for 2 min and the supernatant was collected. The lysate-matrix complex was then washed twice with dH₂O and twice with a buffer solution (100 mM aqueous ammonium bicarbonate) with the last buffer wash lasting 10 min. The washes were centrifuged at 7,000 rpm for 5 min and supernatant decanted. The lysate-matrix complex was then reduced with a 100 μ L solution of 10 mM DTT in 100 mM ammonium bicarbonate at 30 °C for 30 min then allowed to cool to room temperature before the addition of 1 μ L of a 1 M solution iodoacetamide to make a final concentration of 10 mM. The solution was incubated for 30 min at 35 °C with no exposure to light, followed by centrifugation (7,000 rpm, 5 min) and removal of supernatant before the lysate-matrix complex was washed with 50% acetonitrile in 100 mM ammonium bicarbonate buffer. The resulting solution was centrifuged (7,000 rpm, 5 min) and the supernatant decanted. The matrix was then rehydrated in 20 μ L of 50 mM ammonium bicarbonate, followed by the addition of 1 μ g of trypsin and incubated overnight at 33 °C. The solution was centrifuged (7,000 rpm, 5 min) and the supernatant was collected and concentrated via speed vac to afford the peptides.

3.15 ZipTip purification

The concentrated peptides were resuspended in 10 μ L of a 0.2% formic acid solution. For the preparation of the ZipTip (Millipore Corp.), 10 μ L of a 0.2% formic acid solution was aspirated twice. Care was taken to not pass any air through the ZipTip. The sample was then aspirated 10X followed by 2X aspiration with 10 μ L of a 0.2%

formic acid solution then 10X aspiration with a 1:1 formic acid:acetonitrile solution. Utilising the same ZipTip, this procedure was repeated ensuring binding of all the peptides onto the ZipTip followed by the final aspiration in 70% acetonitrile solution. The 1:1 formic acid:acetonitrile solution was then added to the 70% acetonitrile solution and concentrated via speed vac to yield the purified isolated peptides. The procedure was then repeated with the control sample.

3.16 Protein identification

Following protein purification and isolation, identification was undertaken utilising either linear ion trap (LTQ) spectroscopy (LTQ XL™ Linear Ion Trap Mass Spectrometer, Thermofischer), or MALDI (AB SCIEX TOF/TOF™ 5800).

3.16.1 LTQ tandem mass spectroscopy

Samples were prepared by resuspending the purified peptides in 50 µL of formic acid buffer (0.1% conc. formic acid in HPLC water). Ionisation of the peptide solution was conducted by electrospray with a silica tip emitter (360 µm OD X 20 µm ID X 10 µm tip opening, New objectives, USA), with a set voltage potential of 1.8 kV, a heat capillary set to 275 °C, and a tube lens at 120 V allowed the positive ions to enter. Ions with a mass to range of 200-1850 m/z were analysed in a 6 hour acquisition time utilising Fourier transform mass spectroscopy (FTMS) in the orbitrap. At a resolution of 30,000, a full scan in the FTMS identified the 6 top intensity ions were selected for collision-induced dissociation (CID) and detection in the LTQ. The following dynamic exclusion criteria were as stated: repeat duration 30 s, repeat counts 2, exclusion duration 90 s, exclusion list size 500.

3.16.2 MALDI spectroscopy

Preparation of the MALDI matrix was undertaken via the addition of 500 µL of acetonitrile, 500 µL of MilliQ water and 1 µL of concentrated trifluoroacetic acid (TFA). The solution was mixed via vortex before the addition of 10 mg of alpha-

cyano-4-hydroxycinnamic acid (CHCA). The solution was shaken at full speed via vortex followed by a centrifugation at 16,000 rpm for 5 min. The supernatant was decanted and used as matrix. A calibration mixture was also prepared consisting of 1 μ L of CalMix2 (Applied biosystems) in 100 μ L of the matrix solution.

The purified isolated peptides from treatment and control samples (see section 3.13) were resuspended in 10 μ L of MilliQ water. A series of dilutions were made to a total volume of 6 μ L consisting of a 1:1, 5:1 and 10:1 matrix:sample solution. The dilutions and their corresponding controls were each added as individual spots on the MALDI detection plate at a volume of 1 μ L. The corresponding calibration spot was also added. The prepared plate was allowed to dry.

Calibration was internally conducted utilising CalMix2 monoisotopic peaks of adrenocorticotrophic hormone (ACTH) clip 7-38 (m/z 3657.9294), ACTH clip 18-39 (m/z 2465.1990), ACTH clip 1-17 (m/z 2093.0870), angiotension 1 (m/z 1296.6770) and des-Arg1-brodykinin (m/z 904.4680). Criteria of calibration included a minimum of three matched peaks, mass tolerance of ± 0.1 m/z , signal to noise ratio ≥ 5 and a outlier error of 1 m/z . The spectrum was acquired through a positive reflector mode. Laser intensity was fixed at 4600 and a pulse range of 400 Hz. A total of 500 laser shots were taken and peptide fragments were collected in a mass range of 700 to 4000 Da. MS/MS sequence was imported into the ProteinPilot™ (Sciex) software. The generated peak list was sequence was analysed with the MASCOT algorithm (Matrix science) generating a peak list, which was run against the NCBI database for *S. cerevisiae* generating a list of isolated proteins.

Results

3.17 Dose-response assay in *Saccharomyces cerevisiae*

3.17.1 Liquid phase dose-response assay

Previously established results by Davies² indicated no bioactivity of compound **23** and other 1,2-cyclopropyl carbohydrates in the haploid wild-type yeast strain BY4741. However bioactivity of the 1,2-cyclopropyl carbohydrates synthesis by Davies was reported in the *pdr1Δpdr3ΔxxxΔ* strain due to the deletion in the *pdr1p* and *pdr3p* efflux pumps. Therefore all dose-response assays conducted during this study were undertaken using the *pdr1Δpdr3Δhis3Δ* strain as a surrogate for a wild type in the *pdr1Δpdr3ΔxxxΔ* library. A liquid growth assay (section 3.7) was used in order to establish an inhibitory concentration of compounds **23**, **54**, **55**, **61** and **62**. As shown in figure 3.2, inhibition of growth over a 24 hour period for compound **23** was recorded with an IC₅₀ value of 20.4 μM. The recorded IC₅₀ value varies slightly from that reported by Davies (44 μM).² IC₅₀ values were calculated from triplicate data and determined through fitting a sigmoidal curve from the dose response data. A maximal cell death was reported at 60.14 μM as shown in figure 3.2.

Figure 3.2: Compound **23** dose-response assay. IC₅₀ values of sensitised *pdr1Δpdr3Δhis3Δ* strain yeast to compound **23** were measured over a 24 hour period. The triplicate data was fitted by a sigmoidal curve and presented through a dose- response curve. Error bars represent standard error of the mean.

Of the 1,2-cyclopropyl carbohydrate library tested by Davies,² inhibitory effects were only noted in compounds containing the α -cyclopropyl ring. However, this was not the case in this study as inhibitory effects were observed in both the α - and β -stereoisomers of the 1,2-cyclopropyl carbohydrates (**54** and **55**) as well as (**61** and **62**), although, greater inhibition was observed in the α stereoisomers (**54** and **61**) compared to the β isomer. As shown in figure 3.3, both compounds **54** and **61** still inhibit in the low μM range, though a noticeable increase in IC_{50} is observed compared to compound **23**, with the calculated IC_{50} for **54** and **61** being 148 μM and 110 μM , respectively. IC_{50} were again recorded from triplicate data and calculated through the fitting of a sigmoidal curve (figure 3.3). All results were recorded at a 24 hour time point. Comparison of inhibition of compounds **54** and **55**, and **61** and **62** are demonstrated in figure 3.4A and 3.4B, respectively. It is worth noting that the increase in the residual growth demonstrated in figure 3.4 at higher concentrations is due to the precipitation of the compounds **54**, **55**, **61** and **62** in the liquid agar. Formation of the cloudy precipitate is noticeable immediately upon addition of the inhibitor.

Figure 3.3: Dose-response assay for both compound **54** (A) and **61** (B). IC_{50} values of sensitised *pdr1 Δ pdr3 Δ his3 Δ* strain yeast to compound **54** and **61** were measured over a 24 hour period. The triplicate data was fitted by a sigmoidal curve and presented through a dose- response curve. Error bars represent standard error of the mean.

Figure 3.4: A) Comparison of compounds **54** and **55**, indicating a prominent increase in the IC_{50} of the β product (**55**) compared to the α (**54**). B) Comparison of compounds **61** and **62**, indicating a prominent increase in the IC_{50} of the β product (**62**) compared to the α (**61**). Results are consistent with similar trends observed by Davies. Error bars show standard error of the mean.

3.17.2 Solid phase dose-response

A solid phase dose-response was conducted in order to establish the necessary dose required in the chemical genetic screen. A solid phase dose-response was only conducted on compounds **23** and **54** as these compounds displayed a low IC_{50} in the liquid dose-response. Due to limitations in the amount of compound, **61** did not undergo a solid dose-response and a chemical genetic analysis. Results shown in figure 3.5 are consistent with that of the liquid dose-response showing inhibition by both compounds. The treated plates were photographed at 24 hours and the images were processed to determine average circularity and colony size. Average circularity and colony sizes were then compared to the negative control and residual growth was calculated to determine the respective IC_{50} values. Solid phase inhibition at 24 hours of compounds **23** and **54** resulted in an IC_{50} values of 52.2 μ M and 158.5 μ M, respectively (figure 3.5). This data was not repeated in triplicates due to low compound amounts. Again, a small increase in the residual growth of compound **23** is noticeable at 200 μ M and 300 μ M, which is due to the precipitation of the compound within the agar when the plates are poured.

Figure 3.5: Solid phase dose-response of compound **23** and **54** indication IC_{50} values of 52.2 μ M and 158.5 μ M respectively. Data was not repeated in triplicates therefore standard error of the mean was not calculated. IC_{50} was calculated by fitting a sigmoidal curve.

3.18 Chemical genetic screens

Using the *pdr1Δpdr3ΔxxxΔ* deletion mutant library of *S. cerevisiae*, a high-throughput synthetic genetic array was conducted with compounds **23** and **54**. This would allow for the identification of any chemical genetic interactions and ideally identification of a mechanism of action of both compounds. From the solid phase dose response (section 3.17.2), an inhibitory concentration of both compounds **23** and **54** was selected that would give approximately a 50% reduction in growth. This would allow for the identification of both sensitive and resistant strains. The *pdr1Δpdr3ΔxxxΔ* library was expanded from 384 colonies to the 1536 colony format to produce quadruplicates of each strain, with each plate containing a *pdr1Δpdr3Δhis3Δ* border, which acts as a proxy wild-type strain. The *his3* deletion is neutral in the growth media and the *pdr1Δpdr3Δhis3Δ* strain has a common genetic background with the rest of the library bearing a full set of deletion markers. The deletion library was photographed at 24, 36 and 48 hours and the images were processed allowing for the determination of both colony size and circularity.¹²⁹ As shown in figure 3.6, resistant

strains were defined by an increase in growth with a p -value of >0.05 and a z -score of <-1.95 (for the ratio of growth control/growth treatment) against the negative control. Of the 4800 homozygous deletion strains tested, 132 were resistant against compound **23** and 83 strains were found to be resistant against compound **54**. In addition, sensitive strains were defined as having a decreased growth with a p -value of >0.05 and a z -score of >1.95 (for the ratio of growth control/growth treatment) against the control. This identified 67 strains sensitive to compound **23** and 106 strains sensitive to compound **54** (Figure 3.6). Identification of both resistant and sensitive strains allowed for early speculation of potential mechanisms of actions of both compounds. An early gene ontology analysis indicated enrichment among the resistant strain for cytoplasmic translation with a p -value of 2.17×10^{-6} and 0.018 for both compounds **23** and **54**, respectively. Ontology was found among the sensitive strains with corresponding p -values of 0.001 and 0.01 respectively for compounds **23** and **54**, which indicated a degree of involvement in protein folding in the endoplasmic reticulum (ER). Due to both compounds identifying similar resistant and sensitive strains as well as having matching ontology results, they were assumed to have the same target. However identified strains were validated before definitive conclusions can be made, as high-throughput techniques are susceptible to false positive and negative results.

Figure 3.6: Example of a single plate (plate 8) from the homozygous *pdr1Δpdr3ΔxxxΔ* deletion mutant library, screened against **23**, indicating both a resistant and sensitive strain compared to control (right plate). Each plate contains 306 deletion strains in quadruplicate colonies with a *pdr1Δpdr3Δhis3Δ* border around the edge of the plate acting as proxy wild-type strain.

3.19 Validation of chemical genetic hits

A number of factors in the high-throughput screens can lead to false positive or negative results. Variation in the amount of yeast collected during pinning, competition for nutrients with neighboring colonies and inherently fast or slow growing strains are all factors that can lead to errors in chemical genetic arrays. It was therefore decided to conduct the validations in liquid SD-URA media in order to eliminate some of these potential error-producing factors and to ensure sensitivity and resistance was robust across different growth media. Due to the similarities in the identified sensitive and resistant strains of both compounds **23** and **54** in the genomic screens, and the hypothesis that both compounds are hitting the same target, all identified hits were combined for validation. Therefore, a total of 168 resistant and 151 sensitive strains were chosen for validation against both compounds.

For these validations, resistant strains were defined as having a residual growth 4 standard deviations greater than the average Δ his control. Slow growing strains were identified as having a 40% in the control stain compared to the mean control OD. This identified 61 resistant strains for compound **23** and 12 resistant strains for compound **54** (Appendix, table 4.0). Gene ontology analysis showed significant interactions of both **23** and **54**. Utilising a Holm-Bonferroni test correction, enrichment of the cytosolic ribosome to a significant p -value of 0.019 for compound **23** was noted (table 3.0). However, with compound **54**, ontology enrichment was shown both in the SNARE complex and SNAP receptor activity. Both enrichments were statistically significant, indicated by a p -value less than 0.05 utilising the Holm-Bonferroni test correction (table 3.1).

Compound 23 Resistant			
Ontology	Enrichment	P-value	# of genes
Biological process	-	-	-
Cellular component	Cytosolic ribosome	1.37×10^{-7}	11
Molecular function	-	-	-

Table 3.0: Gene ontology enrichments of resistant validated hits for compound **23**.

Compound 54 Resistant			
Ontology	Enrichment	P-value	# of genes
Biological process	-	-	-
Cellular component	SNARE complex	3.14×10^{-3}	3
Molecular function	SNAP receptor activity	1.44×10^{-2}	3

Table 3.1: Gene ontology enrichments of resistant validated hits for compound **54**.

Additionally, sensitive strains were defined as having a residual growth 2 standard deviations below than the average Δ his control and again slow growing strains were identified as having a 40% in the control stain compared to the mean control OD. This allowed for the successful identification of 112 sensitive strains to compound **23** and 53 strains sensitive to compound **54** (appendix, table 4.1). Gene ontology analysis was again undertaken on the identified sensitive strains for both compounds **23** and **54**. Analysis using a Holm-Bonferroni test correction of the sensitive strains identified by compound **23** showed enrichment in protein folding in the endoplasmic reticulum, along with a large assortment of enrichments in the cellular process of the ER (table 3.2). Despite slightly varied results in compound **54** when compared to **23**, there were also noticeable similarities. Enrichment was found in intra-golgi vesicle-mediated transport utilising a Holm-Bonferroni test correction for both compounds. Interestingly, the same assortment of enrichments relating to the ER was found for **54** as they were for **23** (table 3.3). It is worth noting that gene ontology terms are “nested”, containing parental and daughter terms. Thus the list of enrichments shown in table 3.0, 3.1, 3.2, 3.3 and 3.4 include such nested terms and are found because of overlapping sets of sensitive deletion strains.

Ontology	Enrichment	P-value	# of genes
Biological process	Protein folding in endoplasmic reticulum	1.15×10^{-2}	5
Cellular component	Endoplasmic reticulum part	3.60×10^{-7}	26
	Endomembrane system	3.96×10^{-7}	40
	Endoplasmic reticulum	5.02×10^{-7}	29
	Endoplasmic reticulum membrane protein complex	5.65×10^{-6}	5
	Endoplasmic reticulum membrane	4.93×10^{-5}	5
	Nuclear outer membrane-endoplasmic reticulum membrane network	9.20×10^{-5}	22
	Integral component of endoplasmic reticulum membrane	6.15×10^{-3}	8
	Intrinsic component of endoplasmic reticulum membrane	6.15×10^{-3}	8
	Bounding membrane of organelle	1.71×10^{-2}	30

Table 3.2: Gene ontology interaction of sensitive validated hits for compound **23**.

Ontology	Enrichment	P-value	# of genes
Biological process	Intra-Golgi vesicle-mediated transport	2.45×10^{-2}	5
Cellular component	Endoplasmic reticulum part	7.19×10^{-6}	17
	Endomembrane system	9.40×10^{-7}	26
	Endoplasmic reticulum	3.55×10^{-5}	18
	Endoplasmic reticulum membrane protein complex	9.89×10^{-3}	3
	Endoplasmic reticulum membrane	1.21×10^{-4}	15
	Nuclear outer membrane-endoplasmic reticulum membrane network	1.92×10^{-4}	7
	Integral component of endoplasmic reticulum membrane	6.21×10^{-4}	7
	Intrinsic component of endoplasmic reticulum membrane	6.21×10^{-4}	7
	Bounding membrane of organelle	8.89×10^{-6}	24
	Organelle membrane	1.51×10^{-3}	25

Table 3.3: Gene ontology interaction of sensitive validated hits for compound **54**.

Following the hypothesis that compounds **23** and **54** are targeting the same receptor, resistant and sensitive strains were compared between the compounds and common validated strains were better evaluated, as they were believed to represent the the most robust chemical genetic interactions. The common hits between **23** and **54** were subjected to a gene ontology analysis and the results are indicated in table 3.4. Unfortunately, no enrichment was found for the common resistant strains.

Ontology	Enrichment	P-value	# of hits
Biological process	intra-Golgi vesicle-mediated transport	8.08×10^{-3}	5
	Macromolecule localisation	1.11×10^{-2}	18
	Cellular protein localisation	1.53×10^{-2}	14
	Cellular macromolecule localisation	2.52×10^{-2}	14
	Protein localisation	2.60×10^{-2}	16
	Intracellular protein transport	2.84×10^{-2}	12
	Cellular localisation	3.54×10^{-2}	18
	Protein transport	4.85×10^{-2}	14
Cellular component	Endomembrane system	9.17×10^{-8}	15
	Bounding membrane of organelle	1.132×10^{-5}	24
	Endoplasmic reticulum part	1.31×10^{-5}	15
	Endoplasmic reticulum membrane	3.87×10^{-5}	3
	Endoplasmic reticulum	3.96×10^{-5}	16
	Nuclear outer membrane-endoplasmic reticulum membrane network	6.00×10^{-3}	14
	Organelle membrane	9.01×10^{-4}	6
	Integral component of endoplasmic reticulum membrane	2.82×10^{-3}	6
	Intrinsic component of endoplasmic reticulum membrane	2.82×10^{-3}	21
	Endoplasmic reticulum membrane protein complex	5.13×10^{-3}	22
	Golgi transport complex	1.42×10^{-2}	3
	Membrane	2.17×10^{-2}	29

Table 3.4: Gene ontology for the common sensitive hits between **23** and **54**.

A manual analysis of the validated resistant and sensitive hits showed a high number of gene involved in specific protein complexes, as shown in table 3.5. For example all but one EMC gene of the EMC complex was defined as sensitive after the validations indicating a strong interaction with the complex. Similar results were obtained for other complexes (table 3.5). Where possible members of the complexes shown in table 3.5 that were not tested were all subjected to hypothesis testing in order to assess their sensitivity to compounds **23** and **54**. This allows for the testing of potential false negatives from the high-throughput genetic screening in method 3.8.

3.20 Hypothesis testing of non-validated strains

Where several members of a protein complex had been identified through the initial screen and validated, it was hypothesised that these complexes were important in the mode of action of the compounds. This hypothesis was tested through assessment of further members of each complex. Hypothesis testing was undertaken utilising the same methodology as the validation assay in order to reduce any potential variation in data. The hypothesis testing was run coherently with randomly selected strains from the *pdr1Δpdr3ΔxxxΔ* deletion mutant library, that were previously defined as not resistant or sensitive in order remove any potential biases in the results. By utilising the same parameters as the validation experiments, several of the genes previously untested in the complexes in table 3.5 were validated and defined as sensitive. This added further weight for the importance of the complexes in the mechanism of action of compounds **23** and **54**. Randomly selected strains that were included as a negative control showed no sign so sensitivity.

Complex	Member	Validation	Hypothesis testing
EMC	EMC1	23	
	EMC2	23 and 54	
	EMC3	23 and 54	
	EMC4	23	
	EMC5	Not tested	23 and 54
	EMC6	23 and 54	
COG	COG1	Not tested	Not in deletion library
	COG2	Not tested	Null mutant inviable
	COG3	Not tested	Not in deletion library
	COG4	Not tested	Null mutant inviable
	COG5	23 and 54	
	COG6	Not tested	23 and 54
	COG7	23 and 54	
	COG8	23 and 54	
GARP	VPS51	23 and 54	
	VPS52	Not tested	Not in deletion library
	VPS53	23 and 54	
	VPS54	Not tested	Not in deletion library
Sec63 Complex	SEC62	Not tested	Null mutant inviable
	SEC66	23 and 54	
	SEC72	Not tested	23
SSH1 complex	SSH1	23 and 54	
	SBH2	23	23 and 54
	SSS1	Not tested	Null mutant inviable

Table 3.5: Potential complexes involved in the mechanism of action of both **23** and **54** showing validated hits and hypothesis tested hits to respective protein complex.

3.21 Mammalian cell experiments

Compound **23** was subjected to MMT assay by PhD candidate, Richard Little, who utilised the HL-60 human promyelocytic leukemia cell line to conduct initial dose-response assays. As shown in figure 3.7, inhibition of growth was observed over a 48 hour period with a maximal inhibition dose of 76 μ M. Utilising triplicate data and

the best fitted sigmoidal curve, the IC_{50} value was calculated to be 24 μ M. Although mammalian cells tend to be more sensitive to small molecules due to their lack of *PDR* efflux pumps, these results are consistent with the IC_{50} value reported in the yeast liquid dose-response. It is important to note that the perceived increase in survival at a higher concentration of approximately 400 μ M is again due to the precipitation of compound **23** and not an increase in cell viability.

Figure 3.7: Dose-response assay of HL-60 mammalian cell line to compound **23** measured over a 48 hour period. The triplicate data was fitted by a sigmoidal curve a. Error bars represent standard error of the mean.

3.22 Protein isolation and identification via affinity purification

As the dose-response results (section 3.17.1) demonstrated bioactivity of the propargyl derivative **61**, a chemical proteomic analysis was undertaken in order to attempt to elucidate the potential target for the bioactive compounds synthesised in chapter 2. Both linear ion trap (LTQ) and MALDI-TOF mass spectroscopic methods were used in this study.

Description	Score	Coverage	Proteins	Unique peptides	Peptides	#AA
Fructose-bisphosphate aldolase	31.1	8.36%	1	2	2	359
Glyceraldehyde-3-phosphate dehydrogenase	15.55	9.34%	3	1	3	332
Enolase	14.17	11.90%	2	2	6	437
60S ribosomal protein	10.43	5.39%	1	1	2	297
Phosphoglycerate kinase	10.08	8.17%	1	1	3	416
Heat shock protein SSA2	6.31	9.23%	2	2	3	639
Phosphoglycerate mutase	5.27	7.29%	1	1	2	247
Pyruvate kinase	5.26	6.40%	1	2	3	500
40S ribosomal protein	3.97	11.76%	2	1	1	136
Elongation factor	2.69	2.40%	1	1	1	458

Table 3.6: Results from the LTQ mass spectrometer. Although results were recorded the low score and low protein coverage does not make these results significant, therefore no observations were made.

Analysis of the results from the LTQ mass spectroscopy was undertaken using Thermo fisher Proteome Discoverer 1.4.1.14, which yielded several identified proteins as shown in table 3.6. However all hits had scores below those considered meaningful. These low quality assignments were based off low percentage coverage of the protein as identifications were made from a very small number of peptides. For example the top result as determined by Thermo fisher Proteome Discoverer was Fructose-bisphosphate aldolase, which had the highest score of 31.1 although identification was made from two peptides with covered 8.36% of the peptide. This is not sufficient to identify the protein as a target, and most likely relates to a small amount of contamination in the sample.

Analysis of the MALDI results by protein pilot™ program and MASCOT algorithm yielded one potential match, the YAL028W-like protein, however this was also associated with a low score of 26, with only 4/108 peptide fragment matches. Therefore this data is also not significant and no observations were made.

Discussion

The decision to undergo biological analysis utilising the *pdr1Δpdr3Δ* DMA library was made based on the previous findings reported by Davies,² which indicated 1,2-cyclopropyl carbohydrates were potentially active substrates for ATP-binding cassette (ABC) efflux transporters. The general amphiphilicity of the 1,2-cyclopropyl carbohydrates synthesised by Davies was consistent with that of efflux substrates. To this end, Davies screened a small library of 1,2-cyclopropyl carbohydrates against the yeast wild-type strain BY4741, which resulted in no bioactivity, however, identical test runs against the *pdr1Δpdr3Δ* deletion library yielded toxicity in the mid to low micromolar concentration range, which was promising.

3.23 Dose response in relation to chemical structure

As stated in section 1.4.1, it was proposed that due to the high ring and torsional strain of the cyclopropane moiety and its sp^2 -like characteristics, 1,2-cyclopropyl carbohydrates would undergo either a ring opening or expansion reaction if exposed to an enzymatic nucleophile, allowing for a mechanism-based inhibition of a putative target enzyme. Through a number of SAR assays with a small library of 1,2-cyclopropyl carbohydrates, Davies² was able to postulate a potential mechanism of inhibition. For example, positioning good leaving groups at either the C-7 or C-3 position resulted in very little to no bioactivity, deeming ring expansion reactions shown in scheme 3.1 to be unlikely.

Scheme 3.1: Unlikely ring expansion mechanisms.

In contrast, synthesised compounds with the ability to stabilise a C-7 carbanion consistently showed bioactivity, suggesting ring opening reactions as a mechanism of inhibition as shown in scheme 3.2. This led to the proposed design of the new lead compound **54**, where the formation of an enolate anion (**81b**) was envisaged to occur leading to an increase stabilisation through resonance with **81a** and favoring the ring opening reaction. As expected, the synthesised target compounds in chapter 2 exhibited inhibition of *pdr1Δpdr3Δ* yeast strain in the low micromolar concentration range, adding further weight that the compounds undergo a ring opening reaction and formation of a carbanion. Disappointingly, however, the IC₅₀ values of the newly synthesised compounds were higher than the original compound **23**. This was envisaged to be due to unfavourable non-covalent interactions within the binding pocket. Moreover, the substitution of the electronegative chlorine for an alkyl chain could result in unfavourable hydrophobic interactions within the binding pocket, resulting in the increased IC₅₀ value. Nonetheless, observed bioactivity of all synthesised compounds was consistent with ring opening and carbanion stabilisation. The proposed mechanism shown in scheme 3.2 is thought to be the most likely mechanism of inhibition.

Scheme 3.2: Proposed mechanism of inhibition of compound **54**.

As demonstrated by Davies², the stereochemistry of the cyclopropane ring also has an important effect on the biological activity of the compounds, with bioactivity being reported only for the compounds with the cyclopropyl ring on the α -face on the pyranose ring. Although in this study bioactivity was reported for both the α - and β cyclopropyl compounds synthesised in chapter 2, a substantial reduction in bioactivity was observed in the β diastereoisomers compared to the α diastereoisomers. This suggested that either a steric interaction reduces the ability of the β diastereoisomers to access the active site of the target leading to a lower bioactivity, or that the stereochemistry of the β diastereoisomers interferes with either the enzymatic nucleophilic attack or the ability for the enzyme to aid in the stabilisation of the C-7 carbanion. As shown in figure 3.8, if an enzymatic nucleophile attacks the anomeric carbon from the β -face on the pyranose ring, the steric bulk of the cyclopropane and the attached substituents in the β isomer may hinder the nucleophilic attack from occurring, leading to a reduced affinity of the compound. Much like the positioning of the enzymatic nucleophile preference may also be imposed through the positioning of a catalytic residue within the binding site, which aids in the stabilisation of the C-7 carbanion. Irrespective of which of these effects underlies the facial preference, it is clear that a diastereoselective preference is consistent with a target based activation of

the compound. An example demonstrating a similar situation was reported by Todoroki and colleagues,¹³⁵ where the correct stereochemistry cyclopropane ring on the plant hormone abscisic acid was essential in eliciting bioactivity. Through the synthesis of several derivatives of abscisic acid and extensive biological testing, they conclude that the cyclopropane must be in the ‘down’ orientation due to the tight sterics of the binding site.¹³⁵

Figure 3.8: Enzymatic nucleophilic attack from the β face, as a proposed reason for decreased bioactivity of the β diastereoisomers.

As expected, the dose response of compound **56** gave no bioactivity, which was consistent with the results reported by Davies.² This negative result adds further weight that the C-6 OH is an essential pharmacophore element for the bioactivity of the synthesised compounds. This suggests that non-covalent interactions such as a hydrogen bond acceptor may be present within the binding pocket in close proximity to the C-6 OH. However, the lack of bioactivity may also be due to the larger steric bulk of the formate ester, preventing the compound from accessing the enzyme's active site.

It is important to note that although the results are consistent with the possibility that the synthesised 1,2-cyclopropyl carbohydrates are substrates for an enzymatic nucleophilic attack, it remains conceivable that the inhibition could be occurring through strictly non-covalent interactions or that nucleophilic attack is occurring via an activated water molecule.¹³⁶

3.24 Chemical genetic analysis

Investigation into the potential mechanism of compounds **23** and **54** through the use of chemical genetic profiling resulted in some fascinating outcomes, which allowed for the speculation of potential pathways and even targets involved in the activity of both compounds. The initial chemical genetic screen using the *pdr1Δpdr3ΔxxxΔ* DMA library¹²⁵ yielded a total of 132 resistant strain and 67 sensitive strains for compound **23** and 87 resistant and 106 sensitive strain for compound **54**. However due to the potential errors that can arise from high-throughput screening all identified strain were subjected to validation in order to identify potential false positive results. As stated in section 3.19, it was assumed that both compounds had a common target due the similarities in the resulting chemical genetic profiles. Therefore all genes identified as a 'hit' in the chemical genetic screens were combined in the validation and run against compounds **23** and **54**. The validation screens included randomly selected strains, which were not identified as a 'hit' in the high-throughput screens as negative controls.

Identification of the sensitive and resistant strains allowed for the analysis of biological and cellular enrichment through gene ontology. Although no enrichment was found for the common resistant genes, several processes were found to be significant for the sensitive strains with the general theme for protein localisation and trafficking associated with the endoplasmic reticulum and golgi (for the full ontology enrichments see, table 3.4). The common validated results also underwent further analysis with the Biological Networks Gene Ontology (BiNGO) tool which is an open-source Java application that determines which gene ontologies are significantly overexpressed.¹³⁷ This allowed for further analysis of the data indicating over representation of genes in specific pathways along with the graphical representation of the data (figure 3.9). As shown in figure 3.9, although significant enrichment is associated with such processes as protein folding in the endoplasmic reticulum and cellular calcium ion homeostasis, the overwhelming majority of enrichment is in protein localisation and intracellular transport, verifying the results from the initial ontological screens.

Figure 3.9: BiNGO analysis of the common validated sensitive strains indicated an over representation of genes involved in protein localisation and intracellular transport.

However although both gene ontology and BiNGO analysis indicated the potential mechanism of action of compounds **23** and **54** being involved in protein localisation and intracellular transport, further work needed to be done to narrow the parameters in which inhibition may be occurring. Therefore a manual analysis of the common validated hits was undertaken in order to identify the potential involvement of specific protein complexes in the mechanism of compounds **23** and **54**. As shown in table 3.5, five protein complexes were found to display sensitivity to both compounds.

Analysis of the complexes shown in table 3.5 leads to a speculative hypothesis of a potential mechanism of action of compounds **23** and **54**. The initial aim was to attempt to identify a set of essential genes that are not in the deletion library with a

paralog complex that would be identified as sensitive. With chemical genetic interactions, partial inhibition of the essential complex at a low concentration, i.e IC_{50} , level would lead to reduced growth of all colonies however the strain with the gene deletion in the paralog complex or associated pathways would exhibit hypersensitivity to the compound indicating the essential complex as a potential target.

Of the complexes shown in table 3.5 this was the case of the Sec61 complex. The Sec61 complex is essential for yeast viability, hence it is not in the deletion library, however, Sec61 is known to have a physical interaction with the Sec63 complex and it also has a paralog, the Ssh1 complex, both of which were found to be hypersensitive. The Sec61 complex is essential in protein translocation into the endoplasmic reticulum (ER) for both co- and post-translational translocation of protein. During co-translational translocation a signal recognising particle receptor (SRPR) recognises a specific signal recognising particle (SRP) at the amino terminal of the peptide within the translating ribosome. The SRPR transfers the ribosome to the Sec61 complex allowing the two complexes to directly bind in turn allowing translocation of the polypeptide through the Sec61 complex concurrently with the peptide synthesis (figure 3.10A).^{138,139} In contrast to co-translational translocation, post-translational translocation is the translocation of previously synthesised peptide from the cytosol to the ER. This requires the recruitment of the additional complex Sec63 and the Hsp70 chaperone protein BiP,¹⁴⁰ to aid in the unidirectional movement of the proteins into the ER from the cytosol. Upon Sec61 recognition of the SRP on the polypeptide, Sec63 recruits the BiP chaperone, which essentially pulls the polypeptide through the Sec61 complex and into the ER (Figure 3.10B).¹³⁹

Figure 3.10: A) Co-translational translocation: SRPR binds to SRP on the ribosome. Binding of the ribosome directly to Sec61 releases the SRP-induced break on translation and allows translocation to occur concurrently with translation, B) posttranslational translocation: Translated polypeptide binds to Sec61 which recruits the Sec52/63 complex. The polypeptide is pulled through via Hsp70 chaperone protein BiP. Figure adopted from Park *et al* with permission from the publisher.¹³⁹

In light of these results and findings Sec61 was identified as a potential target for compounds **23** and **54**. The results from the chemical genetic screens and the validations also seem to match that reported by Junne and colleagues,¹⁴¹ who reported the isolation and discovery of a new natural product that directly inhibited the translocation complex Sec61. Due to only a small amount of their chemical genetic profile being published, the authors were contacted directly to see if similarities were present between both their data set and that presented in this thesis. However after examination of their data and correspondence with the authors few similarities were found reducing the probability of Sec61 as a potential target for **23** and **54**. Consistent with this, analysis of the strains resistant to compound **23** shown enrichment in the cytosolic ribosome, suggesting that the reduction of translation into the ER causes resistance, most likely through reducing the build up of protein in the ER. This contradicts the suggestion of Sec61 complex as a potential target, as inhibition Sec61 would also slow translation and reduce the build up of protein in the ER giving similar results.

Analysis of the literature identified the use of both the GARP and the COG complexes in the process of vesicle tethering. Vesicle fusion to the plasma membrane is known to be mediated through the attachment of proteins known as target *N*-

ethylmaleimide sensitive factor attachment protein receptors (t-SNARE) located on the target plasma membrane and vesicle *N*-ethylmaleimide sensitive factor attachment protein receptor (v-SNAREs) located on the vesicle plasma membrane.¹⁴² However, although it is well known that attachment of both t-SNAREs and v-SNAREs is essential for the fusion of the vesicles to the plasma membrane, the fidelity of a specific vesicle to the desired target has been debated.¹⁴³ The wide distribution of SNAREs on vesicle and target plasma membranes is not enough to account for the specific localised distribution of vesicles to their target. An example of this was demonstrated by Brennwald and colleagues¹⁴⁴ in which they found that the yeast SNAREs Sso1p and Sso2p were widely distributed over the plasma membrane of both vesicles and target, however vesicles still arrived at specific destinations. This specificity is now thought to be attributed to the tether complexes such as the conserved oligomeric golgi (COG) and the golgi-associated retrograde protein (GARP), which have been demonstrated to form the first initial link between the vesicle and the plasma membrane aiding in the recognition of the vesicle and facilitating v-SNARE and t-SNARE binding.^{142,143,145}

The GOG tethering complex is apart of a class of tethering factors along with GARP and the exocyst complex known as the CATCHR (complexes associated with tethering containing helical rods). Although these tethering complexes belong to the same family they share few sequence similarities with the exception of a conserved helical bundle.¹⁴² As shown in figure 3.11A the COG complex consists of 8 subunits, which are organised in two to distinct lobes that have few structural and functional similarities.¹⁴⁶ The essential COG4 is proposed to bind directly to the v-SNARE, Syll and Vps45 through N-terminal helical coiled-coil mediating and stabilising the interaction with the vesicle. COG domains 6 and 7 also bind directly to the SNAREs via the same mechanism further aiding in stabilisation.¹⁴² The COG tethering complex has been stated to regulate the intra-golgi and endosome-to-golgi trafficking.^{143,147,146,142} All four non essential subunits were among the genes that defined as sensitive to both compounds **23** and **54** (see table 3.5).

Although the GARP complex is less extensively characterised than the remanding two members of the CATCHR family, the COG and exocyst complex, it is still known to facilitate retrograde trafficking of vesicles from the late endosome to the golgi.^{148,148}

An example of this was demonstrated by Fröhlich and colleagues,¹⁴⁹ who were investigating the importance of retrograde trafficking from the endosome to the golgi for sphingolipid homeostasis. Upon a quantitative genome-wide synthetic lethality assay in yeast they found one of the strongest hypersensitivity was towards genes of the GARP complex. This later allowed them to propose that the GARP complex was essential for retrograde trafficking of sphingolipid from the endosome to the golgi. As shown in figure 3.11B the GARP complex consists of 4 subunits of which Vps51 has been shown to be essential in the tethering process. It has been shown that in the absence of Vps51 the remaining GARP complex can still form however no binding to Stx6 occurs. However lack of one of the remaining subunits in the presence of Vps51 only slightly reduces binding.¹⁴⁸ As shown in figure 3.11, Vps52 and 53 also bind directly with the yeast SNARE stx6. With the exception of Vps52 and Vps53, which are not in the *pdr1Δpdr3ΔxxxΔ* deletion library, deletion of the other members of the GARP complex led to sensitivity.

Figure 3.11: Multi subunit tethering complexes COG and GARP interacting with membrane bound SNAREs, allowing for the stabilisation of vesicles. A) Indicating the 8 subunits of COG with the interactions of COG4, COG7 and COG6 directly with the SNAREs. B) The GARP complex, Vps51 is essential in the function of the GARP complex as lack of Vps51 has been shown to inhibit GARP tethering. Figure adopted from Hong and colleagues with rights from the publisher.¹⁴²

The final member of the tethering CATCHR family, the excoyst complex is an essential gene in the viability of yeast. Much like the COG and the GARP complex

the exocyst complex aids in vesicle tethering indirectly facilitating SNARE docking.^{142,145} As shown in figure 3.12 the exocyst complex consists of 8 subunits, of which Sec3 and Sec70 have been shown to interact strongly with the plasma membrane via electronic interactions and Sec4 interacts with the vesicle. Establishment of these interactions facilitates the interaction of the v-SNARE and the t-SNARE.^{142, 145} As stated above, partial inhibition of an essential gene with a low drug concentration would lead to a reduced growth in all viable strains however strains with coinciding deletions in related processes would lead to hypersensitivity. As hypersensitivity was seen in the strains which contained deletions in either the COG or GARP complex, which are closely related to the exocyst system, this allowed for the speculation of potential inhibition of a component of the exocyst complex or a protein closely associated with the exocyst complex. Interestingly the exocyst complex has also been shown to interact both physically and functionally with the Sec61 complex.^{150,151} Toikkanen and colleagues¹⁵⁰ showed that through overexpression of the Sec61 β gene they were able to suppress the growth defects of all exocyst mutants. Therefore, by expansion of this, Sec61 mutants should cause hypersensitivity and reduced growth in yeast when the exocyst complex is been inhibited. This is exactly what is observed in the chemical genetic screens further adding weight to potential inhibition of an aspect of the exocyst system or associated proteins.

Figure 3.12: The exocyst multi subunit tethering complex. indirectly facilitating the docking of t-SNAREs and v-SNAREs. Figure adopted from Hong and colleagues with rights from the publisher.¹⁴²

The final protein complex that was identified in the chemical screens as being sensitive to compounds **23** and **54** is the ER membrane protein complex (EMC). The EMC was initially proposed to be associated with the unfolded protein response (UPR),^{152,153} which would explain the gene ontology enrichment in protein folding (table 3.2, figure 3.9), however, new studies suggest that the complex is more likely involved in the process of retrograde and anterograde transport¹⁵⁴ with more recent publications proposing that the EMC complex directly aids in ER to mitochondrial vesicle tethering.¹⁵⁵ This adds further weight to the hypothesis that compound **23** and **54** are involved in the inhibition of protein tethering.

Although no enrichment was found in the common resistant strains, analysis of the strains resistant to compound **54** showed enrichment in SNARE complex and SNAP receptor activity. SNAP is one of the four essential α -helices that make up the SNARE complex. Although the exact interaction and involvement of **23** and **54** remains unknown resistance in both the SNAREs and the SNAPs reinforces the idea that the compounds are closely associated with vesicle tethering and the exocyst system.

3.25 Affinity purification of target protein

As the target of the synthesised 1,2-cyclopropyl carbohydrates remains unknown, attempts to isolate the target were undertaken through the use of an affinity purification resin. Although Davies² was unsuccessful in his attempts of isolating the target protein he did report potential involvement of the compounds in transcription and translation. It was therefore assumed that, like the majority of drugs, the target of the 1,2-cyclopropyl carbohydrates was a protein.

The unsuccessful attempts at target isolation by Davies² was found to be due the inactivity of his probe modified compounds which contained *p*-azidobenzyl substituents at carbons C-3 and C-4 position. This indicated a potential essential interaction with the target at these positions, therefore we hypothesised that attachment of a propargyl probe at the C-7 position might extend out of the binding pocket and allow of a copper-assisted azide-alkyne cycloaddition reaction to occur

with an azide modified resin. In order to test this hypothesis compound **61** was successfully synthesised and an initial biological analysis was conducted on the **61** which indicated bioactivity in the low micromolar concentration similar to that of **54**. However due to the addition of only 5 atoms onto the C-7 carbon speculation still remained whether the propargyl probe would be long enough to extend out of the binding pocket and interact with the azide modified resin.

Whole cell lysate from *S. cerevisiae* was treated with **61** followed by the addition of the azide modified agarose resin. Following the removal of the supernatant the resin was washed with a low salt solution removing any unbound proteins followed by a high salt concentration wash. Utilisation of both the low and the high salt wash had been shown to remove any non-specific bound protein.¹⁵⁶ A final wash using LDS-page was undertaken to remove any specific but non-covalently associated protein as it was assumed that the compound was covalently binding to the target. Finally the resin was then subjected to a trypsin digest followed by mass spec analysis.

As reported (section 3.22) no significant results were obtained from either the LTQ mass spectroscopy or the MALDI mass spectroscopy analysis. After analysis of the chemical genetic screens indicated a potential mechanism of action in protein trafficking, it was assumed that the target protein may be membrane bound therefore it would not be possible to isolate the target through an affinity purification as the insoluble membrane is removed in the first stage of preparing the cell lysate.

Summary

The initial dose-responses conducted on the compounds synthesised in chapter 2 showed pleasing results with bioactivity being recorded in the mid to low micromolar concentrations range. An SAR analysis confirmed that the mechanism of inhibition was most likely occurring through a cyclopropane ring opening reaction and formation of a carbanion. Variation in bioactivity between the α and β diastereoisomers suggest that either a potential steric interactions of the binding pocket or the position of the catalytic residue or nucleophile favors α diastereoisomers (figure 3.8). The chemical genetic analysis performed indicated involvement of the

compound in protein trafficking and translocation, with further analysis of specific complexes, suggesting involvement in vesicle tethering. Consistent with this, no cytosolic target could be isolated and identified by mass spectroscopy; however, lack of target identification could also be due to other factors such as steric bulk of the target protein inhibition the interaction of the azide and alkyne. Although no target was isolated through an affinity purification process, the exocyst complex was found to be an essential complex central to the identified sensitive strains, therefore future work would revolve around the exocyst system to better understand its involvement in the mechanism of action of the synthesised 1,2-cyclopropyl carbohydrates.

Chapter 4

Conclusion

4.1 Future directions

Although much work has been done throughout this thesis towards the elucidation of the mechanism of action of 1,2-cyclopropyl carbohydrates, many areas of investigation still remain. While the target 1,2-cyclopropyl carbohydrates **23**, **54** and **61** were successfully synthesised, it would be valuable to further increase the library of 1,2-cyclopropyl carbohydrate derivatives in order to better understand the structure-activity relationship needed for bioactivity and in turn increasing the overall biological activity. Further work to identify the target through affinity purification and/or better understand the mechanism of action would also be needed as the next essential step in the development the 1,2-cyclopropyl carbohydrates derivatives towards their use as pharmaceutical compounds.

4.1.1 Structure-activity relationship

As previous SAR assays conducted by Davies² identified several essential pharmacophore elements around the pyranose ring, such as benzyl groups at the C-3 and C-4 position along with a hydroxyl group at the C-6 position, the limitations of the binding pocket around the ethoxy- and propargyloxycarbonyl substituent of **54** and **61** remain largely unknown. Extension of the alkyl chain would allow investigation as to whether the chain is orientated such that it protrudes out of the binding pocket and in turn potentially eluding to the depth of the binding pocket. This would be indicated by either a constant bioactivity in the mid to low micro molar concentration, or, if the alkane chain does not protrude out of the binding pocket a decrease in bioactivity will be observed with an increasing chain length. Extending the alkane chain will also increase the LogP (The logarithm of the partition coefficient between water and 1-octanol). Increasing lipophilicity of the compound might allow

for easier access into the cell, alternatively use of a more polar substituent at this position may reduce the sensitivity of the compound to the action of efflux pumps. If extension of the alkane chain is successful and bioactivity is retained this may address previous concerns that the propargyl group on compound **61** was not protruding out of the binding pocket limiting its ability to interact with the derivatised matrix. Therefore addition of an alkyne group onto an extended alkane chain would overcome this issue increasing the ability for the copper-assisted azide-alkyne cycloaddition.

The use of alternative stereochemistry around the pyranose ring should also be investigated though a SAR assay. Use of galactose, mannose and allose would provide alternative stereochemistry around the pyranose ring, which may affect the bioactivity of the 1,2-cyclopropyl carbohydrate derivatives. Use of these alternatives substrates will also give vital information about the 1,2-cyclopropyl carbohydrates binding site.

4.1.2 Elucidation of the mechanism of action and target identification

Despite great progress being made in the identification of the mechanism of action, the exact mechanism and target of the 1,2-cyclopropyl carbohydrates still remains unknown. Use of yeast library tagged with green fluorescent protein (GFP) would allow for the visualisation of any changes in protein localisation in response to the 1,2-cyclopropyl carbohydrates. As the 1,2-cyclopropyl carbohydrate derivatives have indicated to be involved in post-translational protein trafficking this would indicate where protein trafficking is been stopped.¹⁵⁷ Alternatively attachment of a GFP to a propargyloxycarbonyl substituted 1,2-cyclopropyl carbohydrate like **61** and **62**, could allow for the visualisation of where the compounds are going within the cell.

Isolation and identification of the biological target may prove to be the most important next stage in understanding the potential pharmaceutical benefit of these 1,2-cyclopropyl carbohydrates. Unsuccessful target isolation and identification in this thesis was thought to be due to either the propargyl probe on compound **61** not extending out of the binding pocket or that the target was a membrane bound protein. To overcome these issues future work should initially focus on a membrane affinity

purification, which would isolate the target if it is membrane bound protein. If no target is isolated then the second attempt should focus on the derivatisation of the propargyloxycarbonyl in attempt to extend the alkane chain. This would potentially allow for the extension of the propargyl out of the binding pocket allowing it to interact with the modified matrix.

4.2 Conclusion

The initial aim of this Masters project was to synthesise a small library of 1,2-cyclopropyl carbohydrates in order to test whether these compound were undergoing mechanism-based covalent binding through a cyclopropane ring opening reaction. This hypothesis was tested through the preparation of compounds **23** and the carboxyalky 1,2-cyclopropyl carbohydrate derivatives **54** and **61**, which if subjected to an enzymatic nucleophilic attack at C-1 leading to a cyclopropane ring opening would be able to stabilise the carbanion formation through the electronegative chlorines of **23** and enolate stabilisation of **54** and **61**. Successful synthesis of **23**, **54** and **61** was achieved in yields of 32%, 22% and 9% respectively over 7 steps. Chemical genetic profiling of compounds **23**, **54** and **61** against a *pdr1Δpdr3Δ* yeast strain resulted in bioactivity in the low macromolar concentration indicating cyclopropane ring opening and carbanion formation as the likely mechanism of inhibition.

As these compounds were found to be bioactive identification of the mechanism of action was undertaken with compound **23** and **54** through a chemical genetic analysis utilising a PDR-deficient yeast deletion strain, *pdr1Δpdr3ΔxxxΔ* library. A high-throughput assay identified a total of 132 resistant and 67 sensitive strains for compound **23** with 83 resistant and 106 sensitive strains for compound **54**, which were all subjected further validation. Validated genes were subjected to a gene ontology analysis and enrichment was found in protein trafficking and localisation with notable involvement in vesicle tethering, revealed through chemical genetic interactions with the GARP, GOG, Sec63, Ssh1 and EMC complexes. Although no target protein was isolated through affinity purification the impressive specificity of chemical genetic screens strongly indicate the mechanism of action of the 1,2-

cyclopropyl carbohydrates in protein localisation and vesicle tethering. A potential role for the exocyst complex in the mechanism of action of these compounds is proposed for future evaluation.

Although much work still needs to be done through the chemical synthesis of more 1,2-cyclopropyl carbohydrates derivatives and further biological analysis to better understand their mechanism of action, the use of 1,2-cyclopropyl carbohydrates as potential pharmaceuticals or probes of protein trafficking functions shows much promise.

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Spectra

¹H NMR of the cyclopropane substrate **40** (500 MHz, CDCl₃)

^{13}C NMR of the cyclopropane substrate **40** (500 MHz, CDCl₃)

^1H NMR of the C-6 protected 1,2-cyclopropyl carbohydrate **41** (500 MHz, CDCl_3)

¹³C NMR of the C-6 protected 1,2-cyclopropyl carbohydrate **41** (500 MHz, CDCl₃)

¹³C NMR of the C-6 protected 1,2-cyclopropyl carbohydrate **42** (500 MHz, CDCl₃)

^1H NMR of the 1,2-cyclopropyl carbohydrate **23** (500 MHz, CDCl_3)

^{13}C NMR of the 1,2-cyclopropyl carbohydrate **23** (500 MHz, CDCl_3)

^1H NMR of the 1,2-cyclopropyl carbohydrate **43** (500 MHz, CDCl_3)

^{13}C NMR of the 1,2-cyclopropyl carbohydrate **43** (500 MHz, CDCl_3)

^1H NMR of the 1,2-cyclopropyl carbohydrate **54** (500 MHz, CDCl_3)

54

^{13}C NMR of the 1,2-cyclopropyl carbohydrate **54** (500 MHz, CDCl_3)

^1H NMR of the 1,2-cyclopropyl carbohydrate **55** (500 MHz, CDCl_3)

^{13}C NMR of the 1,2-cyclopropyl carbohydrate **55** (500 MHz, CDCl_3)

^1H NMR of the 1,2-cyclopropyl carbohydrate **56** (500 MHz, CDCl_3)

^{13}C NMR of the 1,2-cyclopropyl carbohydrate **56** (500 MHz, CDCl_3)

61

^1H NMR of the 1,2-cyclopropyl carbohydrate **61** (500 MHz, CDCl_3)

^{13}C NMR of the 1,2-cyclopropyl carbohydrate **61** (500 MHz, CDCl_3)

62

^1H NMR of the 1,2-cyclopropyl carbohydrate **62** (500 MHz, CDCl_3)

^{13}C NMR of the 1,2-cyclopropyl carbohydrate **62** (500 MHz, CDCl_3)

Chemical genetic hits

Resistant Validations

Compound 23		Compound 54	
ORF	Gene	ORF	Gene
YBL027W	RPL19B	YGL212W	VAM7
YBR089C-A	NHP6B	YHL033C	RPL8A
YBR161W	CSH1	YML058W	SML1
YBR181C	RPS6B	YMR017W	SPO20
YBR266C	SLM6	YMR085W	YMR085W
YBR267W	REI1	YNL147W	LSM7
YDL082W	RPL13A	YOR106W	VAM3
YDL121C	YDL121C	YPL086C	ELP3
YDL128W	VCX1	YPL120W	VPS30
YDR005C	MAF1	YPL178W	CBC2
YDR250C	YDR250C	YPR043W	RPL43A
YDR375C	BCS1	YGR055W	MUP1
YDR435C	PPM1		
YEL013W	VAC8		
YFL023W	BUD27		
YFL036W	RPO41		
YFR048W	RMD8		
YGL024W	YGL024W		
YGL174W	BUD13		
YGL175C	SAE2		
YGR037C	ACB1		
YGR206W	MVB12		
YHL033C	RPL8A		
YHR081W	LRP1		
YHR203C	RPS4B		
YIL138C	TPM2		
YIR005W	IST3		
YJR032W	CPR7		
YJR050W	ISY1		

YLL045C	RPL8B		
YLR185W	RPL37A		
YLR234W	TOP3		
YLR345W	YLR345W		
YLR346C	YLR346C		
YLR371W	ROM2		
YML010C-B	YML010C-B		
YML035C	AMD1		
YML058W	SML1		
YML107C	PML39		
YML129C	COX14		
YMR017W	SPO20		
YMR256C	COX7		
YMR269W	TMA23		
YMR269W	YMR269W		
YMR282C	AEP2		
YNL147W	LSM7		
YNL169C	PSD1		
YNL171C	YNL171C		
YNL242W	ATG2		
YNL246W	VPS75		
YNL316C	PHA2		
YOL089C	HAL9		
YOL160W	YOL160W		
YOR085W	OST3		
YPL080C	YPL080C		
YPL090C	RPS6A		
YPL157W	TGS1		
YPL178W	CBC2		
YPL222W	FMP40		
YPR043W	RPL43A		
YPR191W	QCR2		

Table 4.0: Resistant validated strain 1

Sensitive Validations

Sesistant strains			
Compound 23		Compound 54	
ORF	Gene	ORF	Gene
YAL027W	SAW1	YAL010C	MDM10
YAL028W	FRT2	YAL027W	SAW1
YBL028C	YBL028C	YBL048W	RRT1
YBL047C	EDE1	YBR022W	POA1
YBL048W	RRT1	YBR036C	CSG2
YBR022W	POA1	YBR089C-A	NHP6B
YBR023C	CHS3	YBR171W	SEC66
YBR036C	CSG2	YBR283C	SSH1
YBR042C	CST26	YDL006W	PTC1
YBR043C	QDR3	YDL020C	RPN4
YBR150C	TBS1	YDR058C	TGL2
YBR171W	SEC66	YDR074W	TPS2
YBR175W	SWD3	YDR127W	ARO1
YBR194W	AIM4	YDR207C	UME6
YBR283C	SSH1	YEL031W	SPF1
YCL045C	EMC1	YER090W	TRP2
YCL046W	YCL046W	YFL032W	YFL032W
YCR020C-A	MAK31	YGL005C	COG7
YDL006W	PTC1	YGL023C	PIB2
YDL020C	RPN4	YGL133W	ITC1
YDL199C	YDL199C	YGR028W	MSP1
YDR056C	YDR056C	YGR080W	TWF1
YDR057W	YOS9	YGR157W	CHO2
YDR058C	TGL2	YGR188C	BUB1
YDR072C	IPT1	YIL030C	SSM4
YDR074W	TPS2	YIL036W	CST6
YDR123C	INO2	YJL029C	VPS53
YDR127W	ARO1	YJL192C	SOP4

YDR183W	PLP1	YJR040W	GEF1
YDR199W	YDR199W	YJR059W	PTK2
YDR294C	DPL1	YJR087W	YJR087W
YDR297W	SUR2	YJR088C	EMC2
YDR304C	CPR5	YKL081W	TEF4
YDR305C	HNT2	YKL207W	EMC3
YDR326C	YSP2	YKR020W	VPS51
YEL031W	SPF1	YKR072C	SIS2
YER002W	NOP16	YKR082W	NUP133
YER019C-A	SBH2	YLL014W	EMC6
YFL032W	YFL032W	YLR262C	YPT6
YFR024C-A	LSB3	YLR338W	OPI9
YFR039C	OSW7	YML013W	UBX2
YGL005C	COG7	YML071C	COG8
YGL051W	MST27	YML097C	VPS9
YGL085W	LCL3	YMR123W	PKR1
YGL132W	YGL132W	YNL051W	COG5
YGL133W	ITC1	YNL231C	PDR16
YGL230C	YGL230C	YNL257C	SIP3
YGL231C	EMC4	YNR019W	ARE2
YGL261C	PAU11	YNR021W	YNR021W
YGR017W	YGR017W	YOR026W	BUB3
YGR028W	MSP1	YOR216C	RUD3
YGR079W	YGR079W	YPR060C	ARO7
YGR080W	TWF1	YPR170C	YPR170C
YGR093W	DRN1		
YGR100W	MDR1		
YGR111W	YGR111W		
YGR123C	PPT1		
YGR157W	CHO2		
YGR188C	BUB1		
YIR007W	EGH1		
YJL029C	VPS53		

YJL095W	BCK1		
YJL134W	LCB3		
YJL135W	YJL135W		
YJL150W	YJL150W		
YJL192C	SOP4		
YJR040W	GEF1		
YJR073C	OPI3		
YJR087W	YJR087W		
YJR088C	EMC2		
YJR108W	ABM1		
YJR110W	YMR1		
YJR145C	RPS4A		
YKL063C	YKL063C		
YKL081W	TEF4		
YKL207W	EMC3		
YKR020W	VPS51		
YKR029C	SET3		
YKR082W	NUP133		
YLL014W	EMC6		
YLR058C	SHM2		
YLR262C	YPT6		
YLR337C	VRP1		
YLR338W	OPI9		
YLR374C	YLR374C		
YLR415C	YLR415C		
YLR454W	FMP27		
YML013W	UBX2		
YML052W	SUR7		
YML071C	COG8		
YML097C	VPS9		
YML106W	URA5		
YMR102C	YMR102C		
YMR123W	PKR1		

YMR304W	UBP15		
YNL051W	COG5		
YNL079C	TPM1		
YNL162W	RPL42A		
YNL231C	PDR16		
YNL238W	KEX2		
YNL257C	SIP3		
YNR019W	ARE2		
YNR021W	YNR021W		
YNR031C	SSK2		
YOL087C	DUF1		
YOR006C	TSR3		
YOR026W	BUB3		
YOR216C	RUD3		
YOR265W	RBL2		
YPL108W	YPL108W		
YPL171C	OYE3		
YPR170C	YPR170C		

Table 4.1: Sensitive validated strain 1