MECHANISM-BASED DESIGN OF BIOACTIVE CYCLOPROPANATED SUGARS

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Abstract

Carbohydrates are important feed stocks in synthesis of natural products and so attract the interest of many organic researchers throughout the world, most notably in the last 10 years. The work described within explores the manipulation of the glucose-derived glucal. The addition of a reactive substituted cyclopropane across the alkene has been employed synthetically for many years, the subsequent ring breaking/expansion has been identified in the lab as slow and needing the support of catalysts. We ask the question, "Will cyclopropanated carbohydrates undergo the slow ring breaking/expansion in the presence of proteins, and are we able to identify which of the two types of mechanisms the reaction is going through?" The cyclopropane will act as a warhead to bind to proteins through Ferrier like rearrangements, resulting in irreversible inhibition. To identify the potential of such compounds, a combination of techniques are used to identify potential pathways, protein targets and reactivity through structure activity relationships.

The key steps involved in finding out the potential of cyclopropanated carbohydrates are to determine biological activities through bio-assays, structure activity relationships, selective binding, chemical genetics and chemical proteomics. The bio-assays together with structure activity relationships provides evidence on which chemical mechanism is occurring when the biological target is interacting with the bioactive cyclopropanated carbohydrates. The most active compound, benzose **(7)**, was subjected to chemical genetic analysis to determine the pathways and processes that are involved with the mode of action. The chemical genetic analysis was complimented by chemical proteomics to identify the direct biological target. Analogues of benzose were synthesised by the addition of azide groups to undergo a Huisgen Cyclisation within a cell lysate to facilitate binding to an alkyne-substituted matrix.

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Abbreviation list

^1H NMR	Proton nuclear magnetic resonance
^{13}C NMR	Carbon nuclear magnetic resonance
δ	Chemical Shift (ppm)
ABC	ATP-binding cassette
Ac_2O	Acetic anhydride
Ally	$\text{CH}_2\text{CHCH}_2-$
AIBN	Azobisisobutyronitrile
Bn	Benzyl
Bz	Benzoyl
bs	Broad singlet
b.p.	Boiling point
CDCl_3	Deuterated chloroform
CHCA	alpha-Cyano-4-hydroxycinnamic acid
ClonNAT	Nourseothricin
Conc.	Concentration or concentrated
COSY	Correlation spectroscopy
d	Doublet
dd	Doublet of doublets
ddd	Doublet of doublets of doublets
ddH_2O	Double distilled water
DCM	Dichloromethane

DMF	<i>N,N</i> -dimethylformamide
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
G418	Geneticin
GFP	Green fluorescent protein
GO	Gene Ontology
eq.	Equivalents
Et ₃ N	Triethylamine
Et ₂ O	Diethylether
EtOAc	Ethyl acetate
HMBC	Heteronuclear multi bond correlation
IR	Infrared
IUPAC	International union of pure and applied chemistry
LDA	Lithium diisopropylamide
m.p.	Melting point
MDR	Multi-drug resistance
NBS	N-bromosuccinimide
NMR	Nuclear magnetic resonance
NOESY	Nuclear Overhauser effect spectroscopy
Nu	Nucleophile
OD	Optical density

PDR	Pleiotropic drug resistance
ppm	Parts per million
q	Quartet
r.t.	Room temperature
<i>R_f</i>	Retention factor
s	Singlet
sat.	Saturated
SC	Synthetic
SD	Synthetic dropout media
SGA	Synthetic genetic array
t	Triplet
TEBAC	Benzyltriethylammonium chloride
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin layer chromatography
Tr	Trityl
UPR	Unfolded protein response
UPRE	UPR element
wt	Wild-type
xxxΔ	Deletion from the DMA
YGDS	Yeast genome deletion set
YPD	Yeast extract peptone dextrose media

1.0 INTRODUCTION

We introduce here the background to cyclopropanes and carbohydrates and how these can be utilised in the field of drug development.

This introduction shows the importance of enzymes in drug development, and how this information can be used in designing drugs to prevent or alleviate health related diseases. A method in drug development is introduced that has not been used to the potential we believe it presents. Finally, the use of yeast as a model organism in identifying potential pathways and processes that bioactive compounds target is presented, along with the use of yeast in identifying potential lead drugs for further optimisation.

1.1 Enzymes in drug development

Enzymes are crucial for existence of all forms of life. Without the ability to catalyse reactions, life would not exist (Szostak et al. 2001). These biological catalysts have evolved to exhibit a high degree of specificity and catalyse reactions with highly conserved mechanisms. Enzymes have been a point of focus for drug development, due to their role in metabolic pathways and potential for manipulation in diseases that involve abnormal protein expression (Neurath & Walsh, 1976; Marik et al. 2011).

Drugs that inhibit over-expressed proteins are used to decrease, delay or prevent the progression of the related disease (Arnoldo, 2008). For example, a large area of cancer research is focused on designing novel anticancer drugs that are based on the normal substrate of enzymes. These enzymes are normally highly expressed in cancerous cells (Shah et al. 2004), and it has been shown that inhibiting these proteins can cause a decrease in cancerous growth (Marik et al. 2011). To be therapeutically

useful, an inhibitor must suppress the activity of a protein that is essential for disease progression, with limited adverse effects (Dombernowsky et al. 1998; Semenza, 2002). A well designed inhibitor typically has a K_d value in the nanomolar (nM) range (Friedman et al. 1993).

Common practise in drug development is to identify a protein target that is over-expressed in a related health problem. For example, up regulation of the HER-mediated signalling network has been implicated in breast cancer, resulting from the amplification of the HER-2 gene (Slamon et al. 1987). HER-2 has been found to have a specific activity of 11 nmol/min/mg and protein over-expression is present in approximately 20% of human breast cancers and is associated with decreased survival (Slamon et al. 1989). A successful strategy to decrease the effect of overexpressed HER-2 protein involved the development of inhibitors that decrease the HER-2 signalling pathways which play a role in the cancer cell proliferation. The extracellular binding domain F of HER-2 by trastuzumab, a humanized monoclonal antibody, has been shown to decrease cell proliferation (Vogel et al. 2002; Cameron et al. 2008). HER-2 is also targeted by the small-molecule inhibitor Lapatinib, the structure is shown in figure 1.1 (Cameron et al. 2008).

Enzymes can be targeted in not just humans. Bacterial related health

Figure 1.1. Lapatinib a bioactive small molecule inhibitor of HER-2.

problems are also being researched to develop new methods of inhibiting enzymes associated with antibiotic resistant infections.

Antibiotic-resistant infections have become a major challenge for healthcare professionals (Neidell et al. 2012). More die as a result from methicillin-resistant *Staphylococcus aureus* (MRSA) (approximately 19,000 a year) than complications associated AIDS in USA (Klevens et al. 2008). To overcome drug resistance, a new drug target or new drug classes are needed. Multiple approaches will then increase the rate of the discovery of new target proteins to combat antibiotic-resistant infections (Smith, 2005; Lizawa et al. 2004; Van Bambeke et al. 2008). One unexploited target in antibacterial drug design is present in the *de novo* purine biosynthetic pathway, 5-(carboxylamino)imidazole ribonucleotide synthase (CAIR-synthase). This pathway is a useful antibacterial target as it is different to that of humans (Firestine et al. 2009; Thoden et al. 2008). This gives an important potential target for an enzyme inhibiting drug and demonstrates the importance of designing single target inhibitors for drug discovery. The next step of finding bioactive compounds that can selectively inhibit CAIR-synthase can be achieved by synthesising a compound (based on the pre-existing knowledge of the CAIR-synthase active site) or testing a library of compounds against the protein. Firestine et al. (2009) tested 48 000 drug-like compounds against CAIR-synthase at inhibitor concentrations between 5- 27 μM and initially found 212 potential inhibitors. Of these 14 showed dose-response relationships. Thoden et al. (2008) were also the first research group to find selective, non-nucleotide inhibitors for CAIR-synthase.

In order to achieve advances in drug design and identification of bioactive compounds it is vital to adopt multiple approaches (Balani et al. 2005).

1.2 Approaches to drug design and discovery:

Drug design has been approached from many angles and each angle has its advantages and disadvantages (Patani & LaVoie, 1996; Wolber et al. 2008). The approaches to drug design can be broken down to three common methods.

1) Ligand-Based Drug Design

Designing a compound that interacts with a target, based on knowledge of existing ligands that interact with the target site. Typically this approach uses quantitative structure activity relationship (QSAR) analysis and requires activity profiles of many ligands (Yang & Huang, 2006). It is especially valuable when high quality target site information is sparse, as can often be the case with membrane-associated target proteins.

2) High Throughput Screening

Discovery of a lead compound, typically from natural products or libraries of combinatorially synthesised compounds. For example the Maybridge, ChemDic, Chembridge and MS Spectrum Libraries consisting of over 49,000+ drug like compounds, are commercially available libraries that can be used for high throughput screens. Depending on the screening methodology adopted, this approach does not require any prior knowledge of either target or drug-target interactions. The library screens can then be spilt up into three types:

- 1) Structure based screens;
- 2) Activity based screens; and
- 3) High throughput libraries screens.

Once a lead compound is discovered, it will then be optimised through medicinal chemistry (Costantino & Barlocco, 2006; Kolb & Sharpless, 2003) and/or QSAR approaches as described above.

3) Target-Based Drug Design

Designing a compound that interacts with a target, based on existing knowledge of the target's active site (Ricci et al. 2005; Skulnick et al. 1995). Typically this approach is used for the design of mechanism-based drugs.

The three methods of drug discovery are different, however, all drug development approaches follow a similar path:

1. Synthesis, extraction or compound library;
2. screening against a cell line/target to find activity;
3. identification of targets or interactions if unknown; and
4. optimisation.

A fourth method will be presented and discussed in this thesis. This method is based around well-known organic mechanisms and how they could be catalysed by enzymes. In most cases the approach to drug design is completed against a biological target, ie a protein of interest. However, when the target is unknown, it is essential to identify what the bioactive compound is interacting with.

1.3 Methods of target identification

One of the most important steps in drug development is the identification of the protein target of the bioactive molecule (Phillip, 2002). Knowledge of the interaction between the protein target and the bioactive molecule is essential for understanding the mechanism of the bioactive molecule within a cell (Cho & Kwon, 2012).

A classical method of target identifying is genetic based, involving mutations introduced into living cells (James et al. 1996). The introduced mutations can include knockout genes or over-expression of certain proteins. When this is done on a large scale with multiple permutations it is referred to as a genomic library (James et al. 1996). A genomic library screened against a drug-like compound will identify resistant or sensitive strains, where particular genes that are knocked out or up-regulated may play a pivotal role in the mechanism of action of the drug-like compound. For example, a deletion library in *Saccharomyces cerevisiae* treated with rapamycin was able to identify resistant and sensitive strains (Xie et al. 2005). This information was then used to identify that the TOR (target of rapamycin) pathways were being affected. Further studies identified that rapamycin inhibits downstream signalling from the target TOR proteins inhibiting protein synthesis. Chemical genomics together with other approaches of identification can be used to generate strong evidence towards identifying a bioactive compound's target and function (Huang et al. 2004).

A direct approach for drug target identification involves proteomic profiling, which identifies proteins by peptides. Identification of a target can be completed in forward or reverse chemical proteomic methods, both of which can utilise affinity based interactions (Kramer & Cohen, 2004). Forward chemical proteomics involves the identification of the target protein of a newly-discovered bioactive molecule (Lokey, 2003). The compound of interest is attached to a resin, then a cell lysate or protein mixture is washed past the resin (affinity pull-down) as shown in figure 1.2. The proteins are then removed by washing or tryptic digest (Bantscheff et al. 2012). The interacting targets can then be identified by mass spectrometry (von Rechenberg et al. 2005; Cho & Kwon, 2012).

Figure 1.2. Classic affinity purification for drug target discovery, involving direct immobilization of the bioactive compound onto a solid resin. (Figure adapted from Sleno & Emili, 2008)

If the bioactive compound of interest does not contain a suitable functional group (most commonly; an amine, carboxyl, or hydroxyl group) to attach to the resin, chemical synthesis of an appropriate functionalised analogue of the compound is required. The resulting analogue will then need to be evaluated as to whether it retains the required target affinity or biological activity (Bantscheff, 2012).

With a method that is successfully able to identify a biologically active compound's target, it is then possible to identify what type of interactions are occurring. Using methods like mechanism-based design, it is possible to design a compound to interact with a biological target in a certain way, giving you an idea of the interactions that could possibly occur.

1.4 Mechanism-based inhibitors

Mechanism-based inhibition is a lesser-used method in drug discovery, but has become an essential method in identifying irreversible inhibitors of target enzymes (Walsh, 1982; Silverman, 1988).

The mechanism-based design approach is split into two types, one is based on having understanding of how a compound undergoes a reaction in the chemistry lab. Applying that idea in context with cells and asking the question, "Is it possible for this mechanism to be catalysed by an enzyme within a cellular environment"? This type will be explored within this thesis.

The second type, which is the reverse of above was used by Borthwick et al. (1998) to identify novel inhibitors of Human Herpes Virus Retinitis Pneumonitis (HHVRP), where Borthwick designed an inhibitor based on the active site of HHVRP. The information generated from the structure and interactions of HHVRP with its substrate, gave Borthwick the required information to identify what chemical mechanism would achieve a covalent mechanism-based attachment to the HHVRP. Borthwick was then able to design a compound with all the requirements shown to be needed to inhibit HHVRP.

Cyclopropanes have been employed frequently in this field due to their high ring strain and their propensity to ring open when exposed to nucleophiles. Nucleophiles are able to attack, breaking the cyclopropane to irreversibly form covalent bonds (Hanzlik et al. 1979; Wiseman & Abeles, 1979; Paech et al. 1980; Silverman & Hoffman, 1980).

Mechanism-based design of inhibitors has many challenges to overcome to become a more commonly used method. Understandably, a major challenge is the fear of unexpected side effects through build-up of

covalently bound non-targets (Overall & Kleifeld, 2006). This has led to the majority of drug development focusing on non-covalent inhibitors. Interestingly, 35% of the currently licensed enzyme inhibitors for human use in the USA covalently modify the target or enzyme-substrate complex (Robertson, 2005). These drugs have proven efficacy by targeting binding but more importantly the catalytic mechanism. Since the active moiety of the bioactive molecule is only active to the target enzyme, it proves to be highly specific (Overall & Kleifeld, 2006).

The interactions between enzymes and substrates or inhibitors are usually non-covalent, for example through hydrogen bonding, electrostatic and hydrophobic interactions, but irreversible covalent modifications can also occur. Suicide inhibitors are a class of inhibitors that utilise an enzyme's catalytic mechanism, to leave the inhibitor covalently attached to the enzyme. These inhibitors are most often used when cell death is the desired outcome, as in the potential treatment of cancers (Ricci et al. 2005). Suicide inhibitors are also used to trigger cell death in the case of antibiotics. The β -lactam-based antibiotics are a good example of this. Scheme 1.1 shows a ring opening of the penicillin β -lactams (Labischinski, 1992). The attack at the carbonyl group of the β -lactam ring, typically by an active site serine of a bacterial transpeptidase, results in the nitrogen-carbon bond breaking, and the binding of penicillin through an ester to the enzyme.

Scheme 1.1. Ring opening of penicillin by transpeptidase

The work proposed in this thesis will be looking at a class of potential suicide inhibitors that utilise the reactivity of strained functionalised cyclopropanes.

1.4.1 Cyclopropanated compounds

Freund reported the first synthesis of cyclopropane (C_3H_6) in 1881 (Freund, 1881). The compound was discovered to be highly flammable and reactive to nucleophilic attack (Blomberg et al. 1987). Cyclopropanes have also been found to have an anaesthetic effect (Waters & Schmidt, 1934) as well as some other interesting properties. For example, a cyclopropane has a higher degree of general similarity to an alkene than a cyclobutane (Meijere, 1979; Meijere, 2003). An alkene is usually more reactive than a cyclopropane, but there are no fundamental differences in behaviour (Baeyer, 1885; Meijere, 2003).

Three years after Freund's synthesis of cyclopropane, the first synthesis of a cyclopropane derivative was reported (Perkin, 1884). Current research on the chemical and biological uses of cyclopropanes continues to provide advances in understanding their utility (Meijere, 2003). The cyclopropane ring has biological relevance as it has high torsional strain and is susceptible to nucleophilic attack, for example by proteins (Wong et al. 1989).

Thousands of cyclopropane containing compounds have been characterised (Brandt & Wessjohann, 2003), with many being biologically active. Cyclopropane moieties appear to be involved in some interesting enzyme catalysed reactions, including the inhibition of methanol dehydrogenase (Frank et al. 1989). These biologically active cyclopropanated structures found naturally makes them useful tools for enzyme inhibitor design. Cyclopropanes are found in several natural

product classes, including hormones, amino acids, fatty acids, alkaloids, and terpenoids.

The importance of cyclopropanes relates in part to the properties that they engender on their parent molecule. An increase in overall rigidity, activity through constraint in flexibility and also potential energy (created by torsional and ring strain) of cyclopropane-containing molecules, can be factors that help drive further reactions towards opening the cyclopropane (Chu-Moyer & Danushefsky, 1993). An enzymatic nucleophile can attack the cyclopropane and form a covalent bond, as occurs with dihydrofolate reductase with cyclopropane substituted pteridine and pyrimidine in schemes 1.3 & 1.4 (Haddow et al. 1987; Suckling, 1988). The resulting enzymatic attack on a cyclopropane causes inhibition by leading to the ring opening of the cyclopropane and irreversible inhibition as the enzyme is covalently attached. (Haddow et al. 1987; Suckling, 1988 Chu-Moyer & Danushefsky; 1993).

Harnessing cyclopropanes that undergo ring opening or breaking reactions that lead to irreversible inhibition is the ideal outcome in the research proposed here. The active compounds described in this chapter contain a cyclopropane with a carbohydrate backbone, together are known to undergo catalytic ring opening and ring breaking reactions.

Scheme 1.2. Cyclopropanated pteridine as a mechanism-based inhibitor of dihydrofolate reductase

Scheme 1.3. Cyclopropanated pyrimidine as a mechanism-based inhibitor of dihydrofolate reductase

1.4.2 Carbohydrate-cyclopropanes

The incorporation of cyclopropanes into carbohydrates provides interesting chemistry through the combination of the strained cyclopropyl ring with the chirality inherent to the parent carbohydrate. The literature in this field has been based mainly on such compounds as intermediates in the synthesis of bioactive compounds and new synthetic methods, focusing on the pyranoside- and furanoside-based carbohydrates (Meldgaard & Wengel, 2000; Hewitt & Harvey, 2010). Examples include the synthesis of seven-membered rings and their application in the synthesis of natural products and analogues (Brandt & Wessjohann, 2003).

1,2-Cyclopropanated carbohydrates can be synthesised by the addition of a carbene to a glycal. Many (protected) glycals are commercially available, while they are also simply synthesised from the corresponding sugar. 1,2-Cyclopropanated carbohydrates are very interesting as they can undergo a range of ring expansion and ring breaking reactions. The ring expansion reactions provide oxepines and ring breaking leads to 2-C-branched pyranosides (Hewitt & Harvey, 2010; Moore, 2012).

Schemes 1.4 and 1.5 depict a reaction of a cyclopropanated carbohydrate with a nucleophile which attacks the C-1 of the sugar; the product is a 7-membered ring formed through ring expansion (Salaün, 2000). The ring expansion can go by either of two pathways. The first (scheme 1.4) results in the loss of the C-3 substituent, which is promoted by a Lewis acid or electrophile, resulting in an unsaturated product. The second pathway results in the loss of a leaving group from the C-7 position (X=halogen, hydrogen), as shown in scheme 1.5. Several research groups have studied cyclopropane ring expansions of functionalised *gem*-dichloro-1,2-cyclopropanated carbohydrates (Hewitt & Harvey, 2010), for example, leading to oxepines following ring expansion in refluxing toluene in the presence of a nucleophile under basic conditions (Jayaraman & Ganesh 2008; Jayaraman & Ganesh 2007).

Another method of cyclopropane ring opening results in the C-2-branched pyranoside (Hewitt, 2008). Scheme 1.6 shows a generalised mechanism for this transformation as proposed by Hewitt. There are numerous potential rearrangement reactions that can then follow such reactions of the cyclopropanated sugar.

The mechanisms described above have the potential of being able to be catalysed by an enzyme. A potential enzyme will need a nucleophile, electrophile a binding site that will accommodate the cyclopropanated sugar. If an enzyme has the right characteristics, these compounds may covalently bind to that enzyme (scheme 1.6). In the absence of an external nucleophile a cyclopropane sugar can react through an intermolecular cyclisation with the C-6, resulting in the generation of a bicyclic compound (Hoberg & Cousins, 2000). These reactions illustrate the general mechanisms associated with breaking of the cyclopropane ring. (Hoberg & Bozell, 1995).

In summary, cyclopropanated sugar derivatives can undergo reactions with nucleophiles and electrophiles that lead to either:

- i) Ring-opening of the cyclopropane, giving carbohydrate-like pyrans with variant C-2 substitution or
- ii) Ring expansion of the pyran, giving oxepines.

In both cases, either a catalyst, promoter or harsh reaction conditions are required and the nucleophile is covalently bonded to the C-1 position.

Figure 1.3. Structure of cyclopropanated carbohydrate, showing numbers of carbons.

Scheme 1.5. Nucleophile-mediated ring expansion with the loss of C-3 substituent

Scheme 1.4. Nucleophile-mediated ring expansion with the loss of C-7 substituent

Scheme 1.6. Nucleophile-mediated ring breaking with the loss of C-7 substituent through a stabilised carbanion intermediate.

Designing bioactive compounds based on carbohydrates offers many benefits including: cost effectiveness, pre-existing stereochemistry, and well known chemistry. Once a compound of this class has been identified to have bioactivity, it will then be optimised to increase the associated toxicity.

1.5 Yeast as a model organism

Saccharomyces cerevisiae (baker's yeast) is a valuable tool for the search of novel therapeutic agents. *S. cerevisiae* screens are inexpensive and often quicker and simpler to run compared to mammalian screens. The unicellular *S. cerevisiae* are small in size and are able to double in 90 min under optimum conditions.

The yeast genome consists of 16 chromosomes; ~6400 open reading frames (ORFs) of which ~1000 genes are classed as "essential". This term is given to genes that once deleted leaves the yeast non-viable. Over 4800 genes are non-essential and have been replaced singly by integration of an antibiotic resistance marker and synthetic barcode sequence to create a comprehensive non-essential deletion mutant array (DMA). This has given rise to a yeast genome deletion set (YGDS) which comprises of ~4800 strains that each have a single non-essential gene deleted. The deleted gene is replaced by a gene cassette (KanMX4) conferring resistance to kanamycin. The cassette is accompanied by a unique molecular bar code surrounding every gene deletion. The YGDS is a tool that can be used to determine pathways in which compounds may act, by identifying strains that are either hyper-sensitive or resistant compared to the wild type of yeast as described by Tong et al. (2004). High throughput drug assays make it possible to categorise compounds that can act on particular pathways or processes.

The *S. cerevisiae* genome has been completely sequenced and has a relatively low number of genes and associated proteins compared to mammalian cells, making it a simple medium to use (Geoffeau et al. 1996). *S. cerevisiae* also has other important factors that make it a model organism for the study of genetic disorders in humans. Almost 1,000 yeast genes have orthologs that are implicated in human disease. *S. cerevisiae*

can also mimic multiple human disease, through genetic manipulation (Botstein et al. 1997; Kataoka et al. 1985). This fact has been a driving force in the importance of using yeast as the first stepping stone in the discovery of new drugs that can treat or prevent the progression of a human disease.

A study by Andrade and colleagues identified 285 yeast proteins that are similar to 155 proteins associated to human disease (Andrade et al. 1998). If one is to inhibit a protein in yeast, then most likely, a similar protein in humans will also be inhibited (Bork et al. 2004). This gives a direct method for testing inhibitory drugs in a robust, easily cultured, simplified and well-characterised system. One such example is the immunosuppressant compound FK506 that has an inhibitory concentration (IC_{50}) of 9 nM and was found to inhibit calcineurin function in yeast (Breuder et al. 1994). As well as human diseases, *S. cerevisiae* also represents an entry point for identifying antifungal agents (Joyce & Palsson, 2006).

What has been presented here in this introduction gives a pathway to drug development; from synthesising a new compound, to identifying its potential in the future of drug design. All together this leads to a hypothesis that will be explored as part of my Masters in Biomedical Science based on carbohydrate-based cyclopropanes and their potential use in drug development.

1.6 Research hypothesis and objectives

Cyclopropanated sugars that can undergo ring opening or ring expansion reactions have ideal characteristics for suicide inhibitors. The research hypothesis will be tested to determine whether these reactions (scheme 1.4, 1.5 and/or 1.6) can be utilised in drug development. These reactions do not occur at a significant rate naturally without the use of strong nucleophiles, activators or high temperatures which make them ideal substrates for enzymes. Addition of a protein may provide nucleophilic and catalytic functionalities either ring opening or ring expansion, providing the products at ambient temperatures. If the nucleophile was provided by the protein, this would leave the binding site alkylated by the bioactive substance. It is proposed that there will be a limited number of proteins that will provide the essential functionality and capacity to both bind the artificial substrate and complete the reaction, resulting in good target selectivity.

The main objectives of this research are to

- 1) Prepare a range of cyclopropanated glucal-derived compounds;
- 2) Conduct a structure activity relationship (SAR) analysis to determine the most active compound against *S. cerevisiae*;
- 3) Identify potential pathways of interactions by chemical genomics; and
- 4) Determine protein targets by mass spectrometry

1) Prepare a range of cyclopropanated compounds

Starting with glucal, a range of cyclopropanated compounds will be synthesised to create a small library. The derivatives are designed to provide information on which proposed ring opening/ ring expansion mechanism is taking place within the cell.

2) SAR analysis to determine the most active compound against *S. cerevisiae*

Each compound that is made will be subjected to an IC₅₀ analysis in *S. cerevisiae*. In addition to identifying a lead compound, structural features that promote or suppress activity will be determined and a likely mechanism for any covalent modification be proposed.

3) Identify potential pathways of interactions by chemical genomics

The lead compound from 2 (above) will be subjected to a chemical genomic screen to identify any pathways that are being affected in yeast. The lead compound will also be subjected to an assay to determine whether it is cytotoxic or cytostatic.

4) Determine protein targets

A parallel study to 3 (above) will be to modify the lead compound to allow for an attachment point of a matrix or molecular probe. Ideally this will be achieved without a decrease in activity. The lead compound-probe will be then treated with *S. cerevisiae* cell lysate. Target proteins will be isolated by use of sepharose resin and identified by tryptic digest and MS/MS to determine the protein target.

This thesis is split into two general threads, chapter two focuses on the chemistry aspect, and chapter three has the emphasis on the biological testing of the compounds synthesised in chapter two.

2.0 CHEMISTRY

2.1 Aim and Objectives

The aim of this chemistry was to design and synthesis a range of cyclopropanated carbohydrates in order to identify a lead compound. The lead compound would then be carried through to the biological testing which is discussed in chapter 3. The objectives outlined in section 1.6 that will be addressed here include;

- 1) To prepare a range of cyclopropanated compounds
- 4) Determine protein targets

The structure of each compound presented with this thesis is numbered as depicted in figure 2.1. This is in accordance to IUPAC recommendations.

2.2 Introduction

2.2.1 Cyclopropanes

In 1959 Simmons and Smith discovered a procedure that produced cyclopropanes from alkenes with carbenes without side products usually present in other methods current at the time (Simmons & Smith, 1959). Their method involves treatment of an alkene with CH_2I_2 and a zinc-copper couple, and is thought to go through an organozinc intermediate. A high degree of stereoselectivity can be achieved, for example when directed by a hydroxyl group. The zinc coordinates to the oxygen species which directs the reaction to occur on the same face as the oxygen functionality. This is shown in scheme 2.1, where the cyclic allylic alcohol (cyclopent-2-en-1-ol) is cyclopropanated forming the bicyclic alcohol (bicyclo[3.1.0]hexan-2-ol).

Scheme 2.1. Synthesis of bicyclo[3.1.0]hexan-1ol by Simmons and Smith methodology.

gem-Dihalocyclopropyl groups are important functional groups in organic chemistry. They provide routes to functionalised and unfunctionalised cyclopropanes, including those found in natural products. *gem*-Dichloro/*gem*-dibromocyclopropanes are synthesised by the addition of a carbene, through the treatment of an alkene with chloroform or bromoform. The Makosza dichlorocarbene addition is directed by steric bulk, such that

addition occurs on the opposite face to the C-3 substituent, due to the C-3 hydroxyl protecting group blocking the top face.

Nagarajan demonstrated the preparation of a range of 1,2-cyclopropanated sugars through the Simmons-Smith and Makosza methodologies as exemplified by the reaction of tri-O-benzyl-D-glucal shown in scheme 2.2 (Nagarajan et al. 1997). Firstly, the cyclopropane was synthesised using a zinc-copper couple, CH_2I_2 and acetyl chloride in refluxing diethyl ether, producing the “up facing” cyclopropane in 89% yield. Makosza cyclopropanation with chloroform provided the “down facing” dichlorocyclopropane in 85% yield. A modified Makosza cyclopropanation was used to synthesise the “down facing” dibromocyclopropane with an 84% yield, scheme 2.2.

Scheme 2.2. Simmon-Smith cyclopropanation, Makosza dichlorocyclopropanation and modified Makosza dibromocyclopropanation cyclopropanation (Nagarajan et al. 1997).

The above methodology was extended here to the formation of a library of cyclopropanated sugars, varying the oxygen substituents and cyclopropane stereoisomers. The library was created to identify a lead compound and give further evidence towards the likely mechanism introduced in section 1.4.2. The library was extended once the lead compound was identified to further optimise the inhibitory effect on *S. cerevisiae*.

2.2.2 Optimising inhibitors

Once a lead inhibitor is found, it can then be altered to enhance any desirable effects. A study by Marik and colleagues (2011) shows a good example in optimisation of genistein, a known bioactive compound that inhibits breast cancer cell lines (Shah et al. 2004; Marik et al. 2011). To enhance the activity of the anticancer properties, seven derivatives of genistein were synthesised. Marik identified three derivatives that showed a 10-15 increase in toxicity (IC_{50} genistein 14 μ M, MA-6 0.8 μ M) against ER positive breast cancer cell lines.

Designing bioactive compounds for biologically known and unknown targets is important to enhance the field of drug discovery. Cyclopropanes have already been shown to be important in mechanism-based inhibition of proteins. However, with a carbohydrate backbone increased protein specificity may occur due to the associated stereochemistry, which is essential towards decreasing negative side effects. Identifying biological targets of these cyclopropane-containing compounds will determine the associated selectivity and, together with chemical genetics, would present a pathway to developing new commercial drugs. To fully utilise chemical genetics it is beneficial to compliment it with chemical proteomics. For this, it is crucial to have a molecular probe that is able to bind to the lead compound and then trap the protein target. Ideally, selective binding will occur.

2.2.3 Molecular probes

The identification of a potential protein target of an active compound can be achieved by the use of a molecular probe (Jeffery & Bogyo, 2003). Resin-based probes consist of a water-insoluble matrix as well as a linker and a point of attachment, for example an epoxide. (Blanks & McLaughlin, 1988). Used together with mass spectrometry it is possible to identify proteins that are interacting with the resin; additionally, it is possible to attach the active compound of interest to the linker of the resin and to use that compound as “bait” for the proteins.

Traditionally, a lead compound is developed through SAR, then the identification of the biological target is undertaken if unknown. However, achieving the attachment of an affinity resin without the lead compound losing its activity is a challenge, and in some circumstances impossible. In many situations the compound of interest has to be chemically modified to allow for the attachment of the resin (Leslie & Hergenrother, 2008). The modification ideally has to have no effect on binding to a target or there is a risk the protein pull-down maybe different to that of the target of the non-resin bound compound.

The incorporation of an azide into the compound of interest allows a well-known method of attachment to a probe, though the Huisgen Cyclisation chemistry (Huisgen et al. 1984; Moses & Moorhouse, 2007). Azides react with alkynes in the presence of a copper species, and ascorbic acid (only if Cu^{II}) through Huisgen-type cycloadditions. Typically, the copper-assisted Huisgen Cyclisation reaction is the ideal method to react azides with the corresponding alkyne, and is valuable as both azides and alkynes are inert within the cell. The identification of proteins using Huisgen-Cyclisation is shown in scheme 2.3.

Scheme 2.3. A proposed attachment of a glycoside by a Huisgen Cyclisation reaction to determine protein targets by mass spectrometry

2.4 Results and discussion

2.4.1 Synthesis of cyclopropanated carbohydrates derivatives **4** to **10** and **13** to **18**

The synthesis of cyclopropanes can be achieved in multiple ways, commonly by the addition of carbenes to alkenes (Simmons & Smith, 1959). The use of 1,2-unsaturated sugars (glucals) as substrates extends the range of products to highly functionalised stereo-chemically pure systems. This application has been pioneered, based on the research by Simmons and Smith, Makosza (Makosza & Wawrsyniewicz, 1969), and Nagarajan (Nagarajan et al. 1997). For this project two main glucal substrates were used, 3,4-di-O-benzyl-6-O-trityl-*D*-glucal (**4**) and 3,4,6-tri-O-benzyl-*D*-glucal (**28**), both of which have a high degree of literature precedence for their cyclopropanation, and are easily made from *D*-glucose (Gammon et al. 2007; Nagarajan et al. 1997). A general scheme is presented in scheme 2.4.

Scheme 2.4. A general scheme of a cyclopropanation from *D*-glucose. R = Bn, Tr; R'R'' = Bn

The pioneering work of Emil Fischer and Karl Zach (Fischer & Zach, 1912) was employed in the synthesis of tri-O-acetyl-*D*-glucal from *D*-glucose in three steps beginning with acetic anhydride and a catalytic amount of perchloric acid (scheme 2.5). Addition of HBr in acetic acid provided the α -bromoglycoside as a white solid. This was dissolved in diethyl ether and reacted with zinc in the presence of aqueous acetic acid to provide crude 3,4,6-tri-O-acetyl-*D*-glucal in 93% over the three steps.

Scheme 2.5. Synthesis of 3,4,6-Tri-O-acetyl-D-glucal **1**

The acetyl groups were removed using catalytic sodium methoxide in methanol followed by purification as described by Risseeuw (Risseeuw et al. 2009): The crude product was dissolved in methanol and treated with portions of acetone and then diethyl ether which resulted in the impurities precipitating out; the resulting pure *D*-glucal was concentrated, providing a pleasing 99% yield. Selective re-protection of the C-6 hydroxyl with trityl chloride provided **3** in a good yield of 80% after purification by silica column chromatography. However, this yield was typically only achieved when a pink colour was seen on the addition of the trityl chloride to the pyridine, indicating the presence of the trityl cation. If this was not observed the yield was reduced to ca. 60% most likely due to wet pyridine. In all cases, the yield based on recovered starting material was typically 97%, indicating incomplete conversion with wet pyridine. Protection of C-3 and C-4 hydroxyl groups was then achieved with benzyl

Scheme 2.6. Synthesis of 3,4-di-O-benzyl-6-O-trityl-D-glucal (**4**)

bromide in DMF and NaH which provided the desired product **4** as a white solid in a good yield of 93% (scheme 2.6).

The next step in the synthesis was cyclopropanation, forming non-halogenated or halogenated (bromide and chloride) cyclopropane derivatives. The Simmons-Smith reaction afforded the unsubstituted cyclopropane, while the halogenated derivatives were generated by Makosza cyclopropanation with bromoform or chloroform. The Simmons-Smith method applied CH_2I_2 , zinc dust and $\text{Cu}(\text{OAc})_2$, whereby the zinc carbenoid intermediate reacts with the alkene **4** with high stereoselectivity due to coordination to the C-3 oxygen substituent. Purification afforded **17** as a white solid with yields from 75 to 84% with characteristic H-1 and H-2 signals in the ^1H NMR spectrum and the two protons on the cyclopropane (H-7a, b) characteristic of the product.

Dibromocyclopropanation was performed as reported by Hewitt and Harvey (Hewitt & Harvey, 2009) using a suspension of potassium fluoride in a 33% (w/w) solution of sodium hydroxide which resulted in yields of between 60- 80% of the major product **13** and yields of the minor isomer **14** between 10-20%. The maximum yield of 80% was afforded after the reaction time was extended to 5 days. The characteristic H-1 and H-2 signals in the ^1H NMR spectrum were again as distinctive as in **17**. An alternative cyclopropanation method with 33% sodium hydroxide, in the absence of potassium fluoride allowed preparation of the dichlorocyclopropane. Higher yields were achieved with chloroform, with yields between 70 to 85%. Purification by silica column chromatography yielded an off-white powder and colourless oil for the major and minor products, respectively. The ^1H NMR spectrum of the major product **5** matched the data of Moore (2012) (scheme 2.7). The diastereomer at C-1, C-2 of **5** and **13** were also prepared in yields of 10 to 20%

Scheme 2.7. Synthesis of **5** and **6**, **13** and **14** and **17**

Removal of the trityl group from the C-6 position of **5**, **6**, **13**, **14** and **17** was done with formic acid in diethyl ether resulted in the corresponding alcohols **7**, **9**, **15**, **16**, **18** in a range of yields between 20 and 60%. These low to moderate yields were due to the formation of a formate ester, which was unable to be converted to 6-OH due to the propensity of cyclopropane ring opening under formate deprotection conditions. The amount of formate ester formed depended on time of the reaction, but it was found that the dibromocyclopropanes did not form any formate esters if the reaction was limited to two hours. Concurrent biological testing of compounds **8** and **10** showed that the compounds were inactive (discussed in section 3.4.3) therefore the synthesis of the formate ester dibromocyclopropane was not pursued.

The deprotection of compounds **5** and **6** (scheme 2.8) found that two hours provided a good balance of product to formate by-product, as shown in table 2.1. Purification of the mixture by silica column chromatography gave semi-pure dichloro- and dibromocyclopropanes products **7** and **13**, further purification by -14 °C hexane recrystallisation resulted in pure white powder. The deprotection of **5** and the

Compound number	Time hours	Deprotected OH	Formate ester	Starting material
7	2	45%	22%	20%
7	4	39%	34%	13%
7	6	21%	68%	10%
13	2	17%	0%	58%

Table 2.1. Rate of formate ester formation compared to recovered starting material and desired deprotection.

characterisation of **7** and **8** was consistent with that of Moore: for **7** the characteristic H-1 signal at 3.87 (doublet, $^3J_{H-H} = 8.3$ Hz) and H-2 at 1.84 ppm (doublet of doublets, $^3J_{H-H} = 8.6, 3.9$ Hz) peaks of the cyclopropane and the aromatic region (7.43 - 7.29 ppm) showing only 10 protons, consistent with two benzyl protecting groups (C-3 and C-4). The formate ester **8** displayed very similar peaks for H-1 and H-2 and the 10 aromatic protons. However, there was an H-8 formyl proton at 8.03 ppm (quartet, $^3J_{H-H} = 0.7$ Hz) which corresponded to the formate ester.

The same characterization technique was used to identify **9** and **10**, formed from the deprotection of dichlorocyclopropane **6**. The characterization of **9** and **10** were again consistent with that of Moore (2012): for **9** the characteristic H-1 at 3.97 (doublet, $^3J_{H-H} = 9.4$ Hz) and H-2 at 2.13 ppm (triplet, $^3J_{H-H} = 8.2$ Hz) peaks of the cyclopropane and the aromatic region (7.43-7.29 ppm) showing only 10 protons. The formate ester **10** displayed very similar peaks for H-1 and H-2 and the 10 aromatic protons. However, there was an H-8 proton at 8.00 ppm (singlet) which corresponded to the formate ester.

In the interest of generating a library of compounds all side products were kept for the biological studies to determine SARs.

Scheme 2.8. Synthesis of compounds **7** and **8**, **9** and **10**, **15** and **16**. Figure 2.1. Guide to numbering glucals (left), and carbohydrate-cyclopropanes (right).

2.4.2 Synthesis of cyclopropanated carbohydrate derivatives **29** to **33**

Compound **29** has been reported in the literature to be active against HeLa cell lines (Sanhueza et al. 2007), it was decided to synthesise this compound to compare whether the activity can also be found in yeast. The dibromocyclopropane **32** was also synthesised to determine if the activity of **29** was related to the possible mechanism of inhibition.

As described previously, the deprotection of the tri-*O*-acetyl-*D*-glucal was achieved by the use of catalytic sodium methoxide in methanol followed by purification. The resulting pure *D*-glucal **2** was obtained in a 98% yield. Subsequent re-protection with benzyl bromide provided the desired tri-*O*-benzyl-*D*-glucal **28** as a white solid in a good yield of 80% (scheme 2.9).

The tri-*O*-benzyl-*D*-glucal was then subjected to dichlorocyclopropanation which resulted in a 79% yield of the desired major product **29** with no minor **30** product being observed initially. However, on repeating the reaction the minor isomer was observed in 12% yield along with the major isomer in 82% yield. As previously mentioned the characteristic NMR signals of H-1 and H-2 were used to identify the products of cyclopropanation reactions.

The tri-*O*-benzyl-*D*-glucal was also subjected to dibromocyclopropanation providing a yield of 65% of the major isomer **32** and 14% of the minor isomer **33**. Complete deprotection of the benzyl groups within **29** was accomplished by hydrogenation with H₂ and catalytic Pd/C, in methanol with catalytic acetic acid, as described by Murali (Murali et al. 2007) albeit with alterations to the temperature. Heating of the reaction mixture was effective in dissolving the tribenzyl derivative more effectively. Purification through a silica plug afforded an 83% yield of the triol **31**. Compound **31** was used as a substrate in later reactions.

Scheme 2.9. Synthesis of **29** and **30**, **31** and **32** and **33**

2.4.3 Synthesis of cyclopropanated carbohydrate derivatives **25**, **26**, **27** and **31**

To increase the scope of the SAR study, a range of compounds were prepared with partial protection of the hydroxyl groups at C-3, C-4 and/or C-6. The conditions of the cyclopropanation required protecting groups on C-3, C-4 and C-6 to have partial protection this had to occur after the cyclopropanation steps.

Compound **5** was subjected to deprotection of both the trityl and benzyl substituents by hydrogenolysis with catalytic Pd/C, in methanol, as described by Murali, but using warm ethanol as solvent, which provided higher levels of solubility than methanol. The deprotection yielded the triol **31**. After 24 hours of stirring under a H₂ atmosphere, four spots developed on the TLC. These were separated by flash chromatography to yield fully and partially deprotected products **25**, **27** and **31** (scheme 2.10).

Scheme 2.10. Synthesis of **25**, **27** and **31**.

2.4.4 Synthesis of cyclopropanated carbohydrate derivative **12**

Further derivatisations of the C-6 hydroxyl material were carried out in an attempt to attach either a fluorescent probe or matrix to aid in the identification of any potential protein targets (see section 3.3.10).

A Swern oxidation, pioneered by Swern and Omura (1978) was attempted, with the aim of accessing the C-6 aldehyde. However, over-oxidation was observed, with the alcohol converting to the carboxylic acid **12** as shown in scheme 2.11. The expected aldehyde proton signal was not present in the ^1H NMR spectra for either the crude or purified product. This was not the original intention but, due to the nature of this project, the carboxylic acid was in itself useful as it could be explored in biological testing and SAR studies, to date compound **12** has yet to be tested for biological activity. Further oxidations at this stage have yet to be attempted.

Scheme 2.11. Synthesis of **12**

2.4.5 Synthesis of cyclopropanated carbohydrate derivative **11**

To further understand which of the proposed chemical mechanisms is occurring within the biological environment (see section 1.6), it was decided to remove one of the chlorides on the cyclopropane as this would decrease the level of carbanion stability. Therefore, the synthesis of **11** was attempted (scheme 2.12). It is known that when *gem*-dichlorocyclopropane carbohydrates are treated with excess LiAlH_4 , the des-halo product is formed. We attempted to synthesise the mono halide via use of limited equivalents of LiAlH_4 . Reduction of the chloride of compound **7** was decided. At this stage of the project **7** was the most biologically active compound. Reduction of the chloride was achieved using LiAlH_4 in THF under N_2 , for three days. Although three products were noted in the TLC of the reaction mixture, only the monochloro derivative **11** could be purified sufficiently for characterisation. The SAR analysis of this compound led to further indication of the mechanism that is occurring within the cell (section 3.4.3).

Scheme 2.12. Synthesis of **11**

2.4.6 Synthesis of compounds **36**, **38** and **37**; exploration of probe attachment

With the knowledge that bioactivity may be completely lost if a probe is attached to the C-6 hydroxyl in compound **7**, it was decided to attempt to selectively protect either the C-3 or C-4 hydroxyl to probe these sites as likely attachment points of a probe.

Compounds **36** and **37** were synthesised in one step from **3** as described by Mereyala & Kulkarni (1989), shown in scheme 2.13. For **37** 0.8 equivalents of benzoyl chloride was added to a solution of **3** in DMF and pyridine. Selective protection of the C-3 hydroxyl was observed with a yield of 80% **37**, 8% **36** and 12% recovered starting material. This method was repeated with 2.5 equivalents of benzoyl chloride with a yield of 95% **36** was achieved in four hours with no mono protected glucal being observed. Compounds **36** and **37** were unable to be cyclopropanated as the conditions of the cyclopropanation (strong aqueous base) are ideal conditions to remove the ester functionalities. Research by Hewitt (2008) showed that under these conditions, the cyclopropanation of the triacetyl glucal **1** was unable to form the corresponding cyclopropane. It was then decided that **37** could be used to generate selectively a benzyl protected C-4 hydroxyl, this would then allow C-3 position to be deprotected and reprotected with a protecting group that would stay intact in under the cyclopropanation reaction conditions. However prior to this, **3** was reacted with one equivalent of benzyl chloride and one equivalent of sodium hydride to determine if there was any selectivity of between C-4

Scheme 2.13. Synthesis of **36** and **37**

and C-3 hydroxyl groups in the benzylation. This might occur if the acidity of the proton is influencing the selectivity.

In several instances, different selectivity in mono protection of C-3 and C-4 was observed. The alkylation of 6-O-trityl-D-glucal with one equivalent of benzyl bromide primarily led to the addition of a single benzyl group on the C-4 position of **3** to give **38** (scheme 2.14). This is in accordance with the computational and experimental findings of Hamann et al. (1999), who showed that this selectivity arises as a result of greater acidity of the hydroxyl group at C-4 over other positions. Hamann et al. focus their argument on the stability of the oxyanion at the C4 position. This is opposite to the outcome of the mono benzylation of the C-3 and C-4 hydroxyl with benzoyl chloride. The C-3 hydroxyl is preferred due to the accessibility of the site of acylation, the allylic C-3 hydroxyl is also more nucleophilic. It was found that the benzyl protection with one equivalent of benzyl bromide was not as selective as the benzoyl in a parallel experiment, 60% and 80% respectively. Although both reactions resulting in **36** and **38** were more selective than that previously completed in literature (Mereyala & Kulkarni, 1989; Moore, 2012).

Scheme 2.14. Selective synthesis of **5** and **38**

2.4.7 Synthesis of cyclopropanated carbohydrate derivative **24**; the probe linker

Initial reactions were attempted to attach a fluorescein to the C-6 hydroxyl of **7** were unsuccessful. These results are not discussed within this thesis. Due to repeated failed attempts of attaching a probe to **7**, it was decided to attempt attachment of a probe to the C-3 and/or C-4 position of an analogue of **7**. To achieve this, a *p*-azidobenzyl protecting group was synthesised.

The *p*-azidobenzyl bromide protecting group was synthesised in two steps from *p*-toluidine, as described by Kloss. (Kloss et al. 2011). *p*-Toluidine reacted with sodium nitrate in water and 37% HCl to form a diazonium salt that, upon treatment with sodium azide, afforded *p*-azidotoluene **19** as a bright orange liquid in a yield of 70%. The radical bromination of *p*-azidotoluene was carried out as described by Mornet and colleagues (Mornet et al. 1984) utilising AIBN and NBS, and provided the desired product in 80% yield (scheme 2.15). Care was needed during purification by silica chromatography due to the nature of silica being nucleophilic and able to react with *p*-azidobenzyl bromide. As long as the column was completed within 10 min, pure *p*-azidobenzyl bromide **20** was obtained in a good yield of 79%. Personal care was taken due to the tendency of **20** to act as an irritant (greater than that of benzyl bromide).

Scheme 2.15 Synthesis of **20**

For selective *p*-azidobenzyl protection of the C-4 hydroxyl group of **3**, two equivalents of 6-*O*-trityl-*D*-glucal **3** was added to one equivalent of *p*-azidobenzyl bromide. Selective protection of C-4 hydroxyl group was observed and **22** was identified by proton NMR and HMBC. A modest 63% yield was achieved after purification by silica chromatography. *p*-Azidobenzyl protection at the C-3 hydroxyl group of the 6-*O*-trityl-*D*-glucal was not observed in TLC or crude NMR. This observation was expected as previously discussed. Further protection of the C-3 hydroxyl was completed with the addition of NaH and BnBr in DMF as described previously (section 3.4.6), this resulted in the fully protected glucal required for the dichlorocyclopropanation reaction. However the cyclopropanated sugar did not form under Makosza cyclopropanation conditions. This may have been due to an impurity noted in the starting material glucal, which appeared as an aldehyde-like peak that at first was thought to have been DMF in the ¹H NMR spectrum of the glucal **22**. To overcome this, the trityl glucal **3** was subjected to four equivalents of *p*-azidobenzyl bromide to form the di-*p*-azidobenzyl protected **21** in 65% yield (scheme 2.16).

Scheme 2.16. Synthesis of **21**, **23**, and **24**.

It is speculated that the *p*-azidobenzyl bromide was reacting with DMF to form the aldehyde species, this side compound could not be separated by means of column chromatography or recrystallization. Due to time constraints, at this stage of the project it was decided to ignore this by-product and carry on with a somewhat impure compound **21**. The cyclopropanation yielded only the major species, with the cyclopropane opposite the C-3 substituent with a yield of 15%, consistent with previous cyclopropanations. It is likely that if the reaction had been high yielding a minor isomer would have also been seen. Subsequent removal of the trityl protecting group on C6-OH of **23** yielded 46% of pure alcohol **24**. Compound **24** was then attached to an alkyne-substituted sepharose matrix through Huisgen Cyclisation chemistry in a cell lysate (section 3.3.10).

2.4.8 Synthesis of Alkyne-substituted matrix; the probe

To complement the synthesis of **24** an alkyne matrix was identified to allow the attachment of **24** via Huisgen Cyclisation. However, due to the cost of alkyne-substituted matrices, synthetic modifications of a standard epoxy-activated sepharose matrix was attempted. A small number of alkynes were added to the epoxide to determine which would be used for the Huisgen Cyclisation conditions. Propargyl alcohol could not be linked directly to the epoxide, probably due to the weakly nucleophilic hydroxyl group. It was decided that a stronger nucleophile was needed, a secondary amine would provide the greater nucleophilicity and would also not require the addition of a strong base. To achieve this, it was decided to couple an amine to propargyl bromide. Use of *N*-propylamine and isopropylamine was attempted, but these amines were too volatile to give an acceptable yield of monopropargylated amine. 3-Aminopropanol proved effective, resulting in 3-(prop-2-ynylamino)propanol **37** in a yield of 85% along with 12% of the bis product **38**. The addition of **37** to the epoxide-activated sepharose is covered in section 3.3.10.

Scheme 2.17. Synthesis of **37** and **38**

2.6 Experimental

Unless otherwise stated, the following conditions apply. All reactions were performed under an atmosphere of N₂ in oven-dried glassware using HPLC grade solvents.

Reaction progress was monitored using thin layer chromatography (TLC) plates pre-coated with silica DC-Fertigfolien Polygram® SIL G/UV₂₅₄ and visualised by either UV radiation (254 nm) or potassium permanganate dip. Purification by silica chromatography was conducted using a column filled with silica gel 60, with solvent systems indicated. ¹H NMR and ¹³C NMR spectra were recorded on either a Varian Unity Inova 300 (300 MHz for ¹H and 75 MHz for ¹³C), a 500 Varian Unity Inova 500 (500 MHz for ¹H and 125 MHz for ¹³C). All chemical shifts (δ) were referenced to solvent peaks (7.26 ppm ¹H- residual CHCl₃ and 77.2 ppm ¹³C- CDCl₃ or 4.80 ppm ¹H- residual D₂O. Infrared spectra were obtained on Bruker Tensor 27 FTIR spectrometer. High-resolution mass spectra were obtained on an Agilent Q-TOF LC/MS 6530 series. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter in DMSO unless otherwise stated.

Tri-O-acetyl-D-glucal (**1**)

Using a modification of Fischer & Zach's procedure (Fischer & Zach, 1912), acetic anhydride (200 mL) was added to a 500 mL flask, D-glucose (150 mg, 0.83 mmol) was dissolved under stirring, followed by drop-wise addition of perchloric acid. Further D-glucose (19 g, 0.105 mol) was added slowly over 1.5 h at a rate such that the temperature of the reaction stayed between 40-50 °C. Once the reaction was cooled to r.t., fuming hydrogen bromide in an acetic acid solution (78 mL) was added and left to stir for 1.5 h at r.t. The solution was diluted with dichloromethane (200 mL), the organic layer was washed with water (2 x 100 mL), then sat. sodium bicarbonate solution (6 x 80 mL). The organic layers were combined and dried with magnesium sulphate, filtered and concentrated to afford an off white powder. Recrystallization from ethyl acetate/hexanes, provided **(1)** (27.1 g, 93.1%) as a white solid. The spectral data matched those reported in the literature (Zhao et al. 2010)

1) R_f = 0.3 (3:1 hexanes/ethyl acetate); $[\alpha]_D^{23.4} -8.26 (C\ 0.97, DMSO)$; m.p. 50-51 °C (lit m.p. = 50-52 °C); 1H NMR: (500 MHz, $CDCl_3$) δ 6.46 (dd, J = 6.1, 1.2 Hz, 1H, H-1), 5.33 (m, 1H, H-3), 5.22 (dd, J = 7.5, 5.6 Hz, 1H, H-4), 4.84 (dd, J = 6.1, 3.2 Hz, 1H H-2), 4.39 (dd, J = 12.2, 5.9 Hz, 1H, H-6a), 4.25 (m, 1H, H-5), 4.19 (dd, J = 12.2, 3.2 Hz, 1H, H-6b), 2.09 (s, 3H, CH_3CO), 2.07 (s, 3H, CH_3CO), 2.04 (s, 3H, CH_3CO); ^{13}C NMR: (500 MHz, $CDCl_3$) δ 170.8 (CH_3CO), 170.6 (CH_3CO), 169.8 (CH_3CO), 145.8 (CH, C-1), 99.1 (CH, C-2), 74.1 (CH, C-5), 67.6 (CH, C-3), 67.3 (CH, C-4), 61.5 (CH_2 , C-6), 21.1 (CH_3 , CH_3CO), 20.94 (CH_3 , CH_3CO), 20.88 (CH_3 , CH_3CO); IR (Film from CH_2Cl_2): ν_{max} 2961, 1738, 1650, 1369, 1215, 1032, 912, 730 cm^{-1} .

D-Glucal (2)

Solid sodium (62 mg, 2.70 mmol) was reacted with methanol (50 mL), then treated with a solution of **1** (2.32 g, 8.52 mmol) in methanol (20 mL). The reaction was stirred at r.t. for 10 min, the solution was concentrated to provide crude D-glucal. The crude product was purified by successive addition of methanol (3 mL), acetone (10 mL) and diethyl ether (20 mL), which lead to precipitation of impurities. The solution was filtered and concentrated to afford **2** (1.037 g, 84%) as a yellow solid/oil. The spectral data matched literature values (Moore, 2012).

2) $[\alpha]_D^{26.5} -9.29$ (C 1.09, DMSO) ^1H NMR: (500 MHz, D_2O) δ 6.41 (dd, $J = 6.1, 1.6$ Hz, 1H, H-1), 4.82–4.81 (complex 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 (complex m, 3H, H-5, H-6a, H-6b), 3.67 (dd, $J = 9.0, 7.1$ Hz, 1H, H-4); ^{13}C NMR: (500 MHz, D_2O) δ 143.6 (CH, C-1), 102.7 (CH, C-2), 78.1 (CH, C-5), 68.7 (CH, C-4), 68.2 (CH, C-3), 60.0 (CH_2 , C-6); IR (KBr): ν_{max} 3372, 2887, 1653, 1416, 1385, 1232, 1137, 1098, 1073, 1045, 1017 cm^{-1}

1,5-Anhydro-2-deoxy-6-O-trityl-D -arabino-hex-1-enitol (3)

As reported by Esswein et al. (1990) **2** (1.81 g, 12.3 mmol) was added in a 1:1 mixture of dichloromethane and pyridine (25 mL) trityl chloride (4.50 g, 16.1 mmol) was then added over 3 min at room temperature. The solution was stirred for 18 h at r.t. before the solution was quenched with water, the resulting mixture was separated and the organic layer was washed with Sat. sodium bicarbonate solution (2 x 60 mL), dried over magnesium sulphate and concentrated to provide a crude yellow oil. The crude oil was purified by column chromatography (hexanes/ethyl acetate 1:1) to afford **3** as a white solid in 3.82 g (80%) yield. The spectral data matched to what had previously been reported (Moore, 2012).

3) $R_f = 0.53$ (2:1 Ethyl acetate/hexanes); $[\alpha]_D^{25} +27.2 (C\ 1.00, DMSO)$; m.p. 61.0-63°C (lit m.p. = 62-64 °C); 1H NMR (500 MHz, $CDCl_3$): δ 7.20 - 7.45 (m, 15H, Ph), 6.40 (dd, $J = 6.1, 1.6$ Hz, 1H, H-1), 4.76 (dd, $J = 6.1, 2.2$ Hz, 1H, H-2), 4.24 (m, 1H, H-3), 3.9 (ddd, $J = 9.5, 7.1, 3.5$ Hz, 1H, H-4), 3.84 (dt, $J = 9.5, 3.4$ Hz, 1H, H-5), 3.57 (dd, $J = 10.4, 3.5$ Hz, 1H, H-6a), 3.36 (dd, $J = 10.4, 3.5$ Hz, 1H, H-6b), 2.33 (d, $J = 3.5$ Hz, 1H, OH-4); ^{13}C NMR: ($CDCl_3$) δ 144.7 (CH, C-1), 143.6 (C, Ph), 128.7 (CH, Ph), 128.2 (CH, Ph), 127.4 (CH, Ph), 102.6 (CH, C-2), 87.1 (C, CPh_3), 76.7 (CH, C-5), 71.8 (CH, C-4), 69.6 (CH, C-3), 63.0 (CH_2 , C-6); IR (Film from CH_2Cl_2): ν_{max} 3382, 3061, 3033, 2920, 2882, 1647, 1491, 1449, 1229, 1099, 1056, 1032, 1002, 735, 702 cm^{-1}

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

In a slight modification of Esswein et al. (1990) procedure, **3** (0.440 g, 2.03 mmol) was dissolved in dimethylformamide (20 mL) and cooled to -10 °C in an ice/salt bath. Sodium hydride (0.671 g, 16.78 mmol) was added, portion-wise over 1 min and the solution stirred at -10 °C for 15 min followed by addition of benzyl bromide (0.712 g, 4.2 mmol). The solution was allowed to warm to room temperature and stirred for 16 h. The reaction was then quenched with water (20 mL) and extracted with chloroform (3 x 25 mL). The organic fractions were combined, dried over magnesium sulphate and concentrated to give the crude product as a pale yellow solid. The crude product was purified by column chromatography (hexanes/ethyl acetate 9:1) to afforded **4** as a white solid. The spectral data matched to what had previously been reported (Nagarajan et al. 1997 & Moore, 2012).

5) $R_f = 0.40$ (9:1 hexanes/ethyl acetate); 115-117 °C (lit m.p. = 116 °C); 1H NMR: (500 MHz, $CDCl_3$) δ 7.51-7.04 (complex m, 25H, Ph), 6.52 (dd, $J = 6.1, 1.5$ Hz, 1H, H-1), 4.90 (dd, $J = 6.1, 2.4$ Hz, 1H, H-2), 4.76 (d, $J = 11.0$ Hz, 1H,

PhCH₂), 4.65 (d, J = 11.6 Hz, 1H, PhCH₂), 4.58 (d, J = 11.6 Hz, 1H, PhCH₂), 4.53 (d, J = 11.0 Hz, 1H, PhCH₂), 4.20 (m, 1H, H-3), 4.04 (m, 1H, H-5), 4.01 (dd, J = 8.8, 5.9 Hz, 1H, H-4), 3.56 (dd, J = 10.2, 2.2 Hz, 1H, H-6a), 3.43 (dd, J = 10.2, 4.1 Hz, 1H, H-6b); ¹³C NMR: (CDCl₃) δ 145.0 (CH, C-1), 144.0 (C, Ph), 138.5 (C, Ph), 138.2 (C, Ph), 128.9 (CH, Ph), 128.6 (CH, Ph), 128.4 (CH, Ph), 128.1 (CH, Ph), 127.96 (CH, Ph), 127.94 (CH, Ph), 127.8 (CH, Ph), 127.7 (CH, Ph), 127.1 (CH, Ph), 99.9 (CH, C-2), 86.6 (C, CPh₃), 77.1 (CH, C-5), 76.2 (CH, C-3), 74.8 (CH, C-4), 74.0 (CH₂, PhCH₂), 71.0 (CH₂, PhCH₂), 62.3 (CH₂, C-6); IR (Film from CH₂Cl₂): ν_{max} 3061, 3031, 2927, 2878, 1647, 1491, 1449, 1095, 1066, 1026, 908, 732, 695 cm⁻¹.

1,5-Anhydro-4-O-benzyl-2-deoxy-6-O-trityl-D-arabino-hex-1-enitol (**36**)

In a slight modification of Schmidt et al. procedure, **3** (0.550 g, 4.1 mmol) was dissolved in dimethylformamide (20 mL) and cooled in an ice/salt bath to -10 °C. Sodium hydride (0.169 g, 4.25 mmol) was added slowly, portion-wise over 1 min and the solution stirred at -10 °C for 15 min followed by addition of benzyl bromide (0.712 g, 4.21 mmol). The solution was allowed to warm to room temperature and stirred for 16 h, followed by quenching with water (20 mL) and extraction with chloroform (3 x 25 mL). The organic fractions were combined, dried over magnesium sulphate and concentrated to give the crude product as a pale yellow solid. The crude product was purified by column chromatography (hexanes/ethyl acetate gradient elution 9:1 to 0:1) afforded **38** as a yellow oil (64%). The spectral data matched to what had previously been reported (Moore, 2012).

38) R_f = 0.37 (3:1 hexanes/ethyl acetate); ¹H NMR: (500 MHz, CDCl₃) δ 7.51–7.07 (complex m, 20H, Ph), 6.50 (dd, J = 6.1, 1.4 Hz, 1H, H-1), 4.76 (dd, J = 6.1, 2.4 Hz, 1H, H-2), 4.62 (d, J = 11.5 Hz, 1H, PhCH₂), 4.48 (d, J = 11.5 Hz, 1H, PhCH₂), 4.31 (m, 1H, H-3), 3.95 (apparent dt, J = 9.3, 2.9 Hz, 1H, H-5), 4.82 (dd, J = 9.3, 6.6 Hz, 1H, H-4), 3.65 (dd, J =

10.3, 2.4 Hz, 1H, H-6a), 3.29 (dd, $J = 10.3, 3.4$ Hz, 1H, H-6b), 1.96 (bs, 1H, OH-3); ^{13}C NMR: (CDCl_3) δ 144.9 (CH, C-1), 143.9 (C, Ph), 138.3 (C, Ph), 128.9 (CH, Ph), 128.6 (CH, Ph), 128.1 (CH, Ph), 127.99 (CH, Ph), 127.97 (CH, Ph), 127.2 (CH, Ph), 102.5 (CH, C-2), 86.6 (C, CPh_3), 77.5 (CH, C-4), 77.0 (CH, C-5), 74.0 (CH_2 , PhCH_2), 69.4 (CH, C-3), 62.3 (CH_2 , C-6); IR (Film from CH_2Cl_2): ν_{max} 3372, 3061, 3030, 2927, 2879, 1650, 1492, 1449, 1091, 1029, 1001, 901, 744, 696 cm^{-1} .

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

As reported by Nagarajan et al. (1997), a solution of **4** (800 mg, 1.93 mmol) and benzyltriethylammonium chloride (10 mg, 0.044 mmol) in chloroform (5 mL) was treated drop-wise with 33% sodium hydroxide (10 mL) with rapid stirring. The biphasic mixture was stirred at r.t for 10 hrs. The biphasic mixture was then diluted with water (10 mL) and extracted with dichloromethane (3 x 20 mL). The organic fractions were combined and dried over magnesium sulphate and concentrated to provide a crude mixture of cyclopropanes **5** and **6**. The crude product was purified by column chromatography (14:1 hexanes: ethyl acetate) liberated the major cyclopropane **5** as a white solid in a yield of 626 mg (62%) and the minor cyclopropane **6** as an oil in a yield of 151 mg (15%) The spectral data matched those previously reported (Nagarajan et al. 1997 & Moore, 2012).

5) $R_f = 0.40$ (9:1 hexanes/ethyl acetate); m.p. 50.0–51.0 $^{\circ}\text{C}$; ^1H NMR: (500 MHz, CDCl_3) δ 7.47–7.22 (complex m, 25H, Ph), 4.81 (d, $J = 11.6$ Hz, 1H, PhCH_2), 4.80 (d, $J = 10.7$ Hz, 1H, PhCH_2), 4.70 (d, $J = 11.6$ Hz, 1H, PhCH_2), 4.25 (d, $J = 10.7$ Hz, 1H, PhCH_2), 4.06 (d, $J = 8.0$ Hz, 1H, H-1), 3.88–3.84 (complex m, 2H H-4, H-5),

3.78 (dd, $J = 8.8, 4.9$ Hz, 1H, H-3), 3.48 (dd, $J = 10.2, 3.0$ Hz, 1H, H-6a), 3.17 (dd, $J = 10.2, 3.0$ Hz, 1H, H-6b), 1.87 (dd, $J = 8.0, 4.9$ Hz, 1H, H-2); ^{13}C NMR: (CDCl_3) δ 143.8 (C, Ph), 138.1 (C, Ph), 137.7 (C, Ph), 128.8 (CH, Ph), 128.7 (CH, Ph), 128.4 (CH, Ph), 128.11 (CH, Ph), 128.10 (CH, Ph), 128.08 (CH, Ph), 128.06 (CH, Ph), 127.8 (CH, Ph), 127.3 (CH, Ph), 86.9 (C, CPh_3), 80.5 (CH, C-5), 77.6 (CH, C-3), 75.9 (CH, C-4), 75.0 (CH_2 , PhCH_2), 72.2 (CH_2 , PhCH_2), 63.7 (CH_2 , C-6), 61.8 (C, C-7), 59.3 (CH, C-1), 34.7 (CH, C-2); IR (Film from CH_2Cl_2): ν_{max} 3087, 3061, 3031, 2919, 2869, 1492, 1449, 1158, 1093, 1029, 736, 698 cm^{-1} .

6b $R_f = 0.30$ (9:1 hexanes/ethyl acetate); m.p. 50–51 $^{\circ}\text{C}$; ^1H NMR: (500 MHz, CDCl_3) δ 7.51–7.17 (complex m, 23H, Ph), 6.90–6.88 (complex m, 2H, Ph), 4.88 (d, $J = 11.9$ Hz, 1H, PhCH_2), 4.75 (d, $J = 11.9$ Hz, 1H, PhCH_2), 4.70 (d, $J = 10.6$ Hz, 1H, PhCH_2), 4.34 (d, $J = 10.6$ Hz, 1H, PhCH_2), 4.14–4.08 (complex m, 2H, H-3, H-4), 4.05 (d, $J = 8.0$ Hz, 1H, H-1), 3.56 (dd, $J = 10.3, 2.0$ Hz, 1H, H-6a), 3.41 (ddd, $J = 9.7, 3.9, 2.0$ Hz, 1H, H-5), 3.19 (dd, $J = 10.3, 3.9$ Hz, 1H, H-6b), 2.12 (apparent t, $J = 7.7$ Hz, 1H, H-2); ^{13}C NMR: (CDCl_3) δ 144.0 (C, Ph), 138.3 (C, Ph), 138.0 (C, Ph), 129.0 (CH, Ph), 128.7 (CH, Ph), 128.3 (CH, Ph), 128.2 (CH, Ph), 128.09 (CH, Ph), 128.08 (CH, Ph), 128.0 (CH, Ph), 127.9 (CH, Ph), 127.0 (CH, Ph), 86.7 (C, CPh_3), 78.4 (CH, C-5), 78.2 (CH, C-4), 76.6 (CH, C-3), 74.9 (CH_2 , PhCH_2), 71.6 (CH_2 , PhCH_2), 62.33 (CH_2 , C-6), 62.25 (CH, C-1), 62.1 (C, C-7), 33.1 (CH, C-2); IR (Film from CH_2Cl_2): ν_{max} 3061, 3031, 2930, 2875, 1492, 1449, 1182, 1090, 1023, 910, 736, 698 cm^{-1} .

1,5-Anhydro-3,4-di-O-benzyl-2-deoxy-1,2-C-(dichloromethylene)-D-glycero-D-gulo-hexitol (7), and 1,5-anhydro-3,4-di-O-benzyl-6-O-(formyl)-2-deoxy-1,2-C-(dichloromethylene)-D-glycero-D-gulo-hexitol (8)

A solution of **5** (50 mg, 0.09 mmol) in diethyl ether (0.3 mL) was treated with formic acid (0.4 mL) and stirred at room temperature for 1 h. The

reaction was diluted with diethyl ether (4 mL) and washed with sat. sodium bicarbonate solution (4 x 10 mL). The organic layer was dried over magnesium sulphate and concentrated. The crude product was purified by column chromatography (hexanes/ethyl acetate gradient 14:1 to 5:1) to give **7** in a yield of 16.56 mg (45%) as a white solid and **8** in a yield of 8.65 mg (22%) as an oil. The spectral data matched reported (Moore 20012).

7) R_f = 0.45 (3:1 hexanes/ethyl acetate); $[\alpha]_D^{25} +63.2$ (C 1.00, DMSO); m.p. 80.0–81 °C; ^1H NMR: (500 MHz, CDCl_3) δ 7.43–7.29 (complex m, 10H, Ph), 4.93 (d, J = 11.2 Hz, 1H, PhCH_2), 4.81 (d, J = 11.7 Hz, 1H, PhCH_2), 4.72 (d, J = 11.7 Hz, 1H, PhCH_2), 4.67 (d, J = 11.2 Hz, 1H, PhCH_2), 3.87 (d, J = 8.3 Hz, 1H, H-1), 3.81 (dd, J = 9.5, 3.9 Hz, 1H, H-3), 3.87 (dt, J = 8.6, 3.9 Hz, 1H, H-5), 3.68 (d, J = 3.9, 2H, H-6a,b), 3.60 (dd, J = 9.5, 8.6 Hz, 1H, H-4), 1.84 (dd, J = 8.6, 3.9 Hz, 1H, H-2), 1.68 (s, 1H, 6-OH); ^{13}C NMR: (CDCl_3) δ 138.1 (C, Ph), 137.6 (C, Ph), 128.7 (CH, Ph), 128.6 (CH, Ph), 128.3 (CH, Ph), 128.15 (CH, Ph), 128.12 (CH, Ph), 79.6 (CH, C-5), 77.2 (CH, C-3), 75.0 (CH_2 , PhCH_2), 74.8 (CH, C-4), 72.2 (CH_2 , PhCH_2), 62.8 (CH_2 , C-6), 60.9 (C, C-7), 58.9 (CH, C-1), 33.9 (CH, C-2); IR (Film from CH_2Cl_2): ν_{max} 3375, 3032, 2976, 2864, 1496, 1454, 1365, 1095, 1026, 733, 696 cm^{-1} .

8) R_f = 0.30 (9:1 hexanes/ethyl acetate); ^1H NMR: (500 MHz, CDCl_3) δ 8.03 (q, J = 0.7 Hz, 1H, HC(O)O), 7.43–7.27 (complex m, 10H, Ph), 4.95 (d, J = 11.2 Hz, 1H, PhCH_2), 4.81 (d, J = 11.3 Hz, 1H, PhCH_2), 4.70 (d, J = 11.3 Hz, 1H, PhCH_2), 4.63 (d, J = 11.2 Hz, 1H, PhCH_2), 4.30 ddd, J = 12.0, 5.1, 0.7, 1H, H-6a), 4.15 (ddd, J = 12.0, 2.7, 0.7, 1H, H-6b), 3.87 (dddd, J = 8.3, 5.1, 2.7, 0.7 Hz, 1H, H-5), 3.86 (d, J = 8.1 Hz, 1H, H-1), 3.80 (dd, J = 9.5, 4.4 Hz, 1H, H-3), 3.57 (dd, J = 9.5, 8.3 Hz, 1H, H-4), 1.82 (dd, J = 8.1, 4.4 Hz, 1H, H-2); ^{13}C NMR: (CDCl_3) δ 160.6 (C, CHO), 137.7 (C, Ph), 137.5 (C, Ph), 105 128.73 (CH, Ph),

128.66 (CH, Ph), 128.5 (CH, Ph), 128.2 (CH, Ph), 128.13 (CH, Ph), 128.08 (CH, Ph), 77.9 (CH, C-5), 77.2 (CH, C-3), 74.9 (CH₂, PhCH₂), 74.5 (CH, C-4), 72.1 (CH₂, PhCH₂), 63.3 (CH₂, C-6), 61.0 (C, C-7), 58.6 (CH, C-1), 34.1 (CH, C-2); IR (Film from CH₂Cl₂): $\nu_{\max}$ 3062, 3031, 2919, 2869, 1726, 1496, 1453, 1152, 1128, 1089, 1027, 735, 697 cm⁻¹.

1,5-Anhydro-3,4-di-O-benzyl-2-deoxy-1,2-C-(dichloromethylene)-D-glycero-D-gulo-hexitol (9), and 1,5-anhydro-3,4-di-O-benzyl-6-O-(formyl)-2-deoxy-1,2-C-(dichloromethylene)-D-glycero-D-talo-hexitol (10)

A solution of **6** (50 mg, 0.09 mmol) in diethyl ether (0.3 mL) was treated with formic acid (0.4 mL) and stirred at r.t. for 10 h. The mixture was diluted with diethyl ether (4 mL) and washed with saturated sodium bicarbonate solution (4 x 10 mL). The organic layer was dried over magnesium sulphate and concentrated. The crude product was purified by column chromatography (hexanes/ethyl acetate gradient 14:1 to 5:1) to give **19** in a yield of 17 mg (30%) of **9** a colourless oil and 5 mg (9%) of **10** as a yellow oil. The spectral data matched to what had previously been reported (Moore 2012).

9) R_f = 0.38 (3:1 hexanes/ethyl acetate); ¹H NMR: (500 MHz, CDCl₃) δ 7.43–7.23 (complex m, 10H, Ph), 4.81 (d, J = 11.2 Hz, 1H, PhCH₂), 4.78 (d, J = 11.7 Hz, 1H, PhCH₂), 4.72 (d, J = 11.7 Hz, 1H, PhCH₂), 4.67 (d, J = 11.2 Hz, 1H, PhCH₂), 4.05 (d, J = 8.3 Hz, 1H, H-3), 3.80 (t, J = 9.4 Hz, 1H, H-1), 3.59 (dt, J = 8.6, 3.9 Hz, 1H, H-5), 3.78 (d, J = 3.9, 2H, H-6a,b), 3.58 (dd, J = 9.5, 8.6 Hz, 1H, H-4), 2.11 (t, J = 8.2 Hz, 1H, H-2); ¹³C NMR: (CDCl₃) δ 140.1 (C, Ph), 137.6 (C, Ph), 128.9 (CH, Ph), 128.6 (CH, Ph), 128.3 (CH, Ph), 128.2 (CH, Ph), 128.2 (CH, Ph), 79.7 (CH, C-5), 77.4 (CH, C-3), 75.0 (CH₂, PhCH₂), 75.1 (CH, C-4), 72.2 (CH₂, PhCH₂), 62.9 (CH₂, C-6), 60.9 (C, C-7), 60.0 (CH, C-1), 33.1 (CH, C-2). IR (Film from CH₂Cl₂): $\nu_{\max}$ 3378, 3033, 2979, 2862, 1494, 1454, 1365, 1098, 1030, 736, 697 cm⁻¹.

10) R_f = 0.25 (9:1 hexanes/ethyl acetate); ^1H NMR: (500 MHz, CDCl_3) δ 8.0 (s, 1H, HC(O)O), 7.42-7.23 (complex m, 10H, Ph), 4.88 (d, J = 11.8 Hz, 1H, PhCH_2), 4.85 (d, J = 11.0 Hz, 1H, PhCH_2), 4.70 (d, J = 11.8 Hz, 1H, PhCH_2), 4.57 (d, J = 11.0 Hz, 1H, PhCH_2), 4.47 (dd, J = 12.0, 1.2 Hz, 1H, H-6a), 4.22 (dd, J = 12.0, 5.1 Hz, 1H, H-6b), 4.19 (apparent t, J = 7.8 Hz, 1H, H-3), 3.96 (d, J = 8.1 Hz, 1H, H-1), 3.80 (dd, J = 10.4, 7.6 Hz, 1H, H-4), 3.50 (ddd, J = 10.4, 5.1, 1.7 Hz, 1H, H-5), 2.12 (t, J = 8.1 Hz, 1H, H-2); ^{13}C NMR: (CDCl_3) δ 160.7 (C, COH), 137.8 (C, Ph), 137.7 (C, Ph), 128.8 (CH, Ph), 128.6 (CH, Ph), 128.24 (CH, Ph), 128.19 (CH, Ph), 128.18 (CH, Ph), 128.1 (CH, Ph), 77.9 (CH, C-3), 76.2 (CH, C-5), 75.9 (CH, C-4), 74.7 (CH_2 , PhCH_2), 71.3 (CH_2 , PhCH_2), 62.5 (CH_2 , C-6), 62.2 (CH, C-1), 61.4 (C, C-7), 32.8 (C, C-2); IR (Film from CH_2Cl_2): ν_{max} 3065, 3031, 2918, 2870, 1724, 1497, 1454, 1170, 1088, 1027, 734, 698 cm^{-1} .

1,5-Anhydro-3,4-di-O-benzyl-2-deoxy-1,2-C-(chloromethylene)-D-glycero-D-gulo-hexitol (11),

To a solution of **7** (40 mg, 0.10 mmol) in tetrahydrofuran (1 mL) was added lithium aluminium hydride (11.2 mg, 0.29 mmol) and stirred at r.t. under nitrogen for 3 days. The mixture was quenched with water (2 mL), extracted with ethyl acetate (3 x 5 mL). The combined organic layers were dried over magnesium sulphate and concentrated. The crude product was purified by column chromatography (hexanes/ethyl acetate 4:1) to give **11** in a yield of 19 mg (47.4 %) as an oil.

11) R_f = 0.40 (3:1 hexanes/ethyl acetate) ^1H NMR: (500 MHz, CDCl_3) δ 7.42–7.30 (complex m, 10H, Ph), 4.79 (d, J = 11.4 Hz, 1H, PhCH_2), 4.79 (d, J = 11.7 Hz, 1H, PhCH_2), 4.68 (d, J = 11.5 Hz, 2H, PhCH_2), 3.92 (dd, J = 9.6, 4.4 Hz, 1H, H-1), 3.81 (m, 1H, H-3), 3.72 (m, 1H, H-5), 3.69–3.60 (m, 3H, H-4, H-6a,b), 3.15 (dd, J = 5.1, 3.2 Hz, 1H, H-2), 1.83 (t, J = 6.3 Hz, 1H, OH), 1.37–1.33 (m, 1H, H-8); ^{13}C NMR:

(CDCl₃) δ (C, Ph), (C, Ph), (CH, Ph), (CH, Ph), (CH, Ph), (CH, Ph), (CH, Ph), (CH, C-5), (CH, C-3), (CH₂, PhCH₂), (CH, C-4), (CH₂, PhCH₂), (CH₂, C-6), (C, C-7), (CH, C-1), (CH, C-2) IR (Film from CH₂Cl₃): $\nu_{\max}$ 3370, 3042, 2972, 2864, 1499, 1456, 1369, 1092, 1027, 732, 696 cm⁻¹; C₂₁H₂₃ClO₄Na⁺ [M+Na]⁺ calculated 374.1285, found 374.1353.

**1,5-anhydro-3,4-di-O-benzyl-6-O-(carboxyl)-2-deoxy-1,2-C
(dichloromethylene)- D-glycero-D-gulo-hexitol (12)**

Dimethyl sulfoxide (86.17 mg, 1.103 mmol) and dichloromethane (3 mL), was cooled to – 78 °C. Oxalyl chloride (69.9 mg, 0.551 mmol) was then added and stirred for 40 min before **7** (150 mg, 0.362 mmol) was added and stirred for another 30 min before triethylamine (22.34 mg, 2.208 mmol) was added, stirred for another 30 min, quenched with Sat. sodium chloride (1 mL) and warmed to r.t. The organic layer was then dried over magnesium sulphate and concentrated to afford a crude material 170 mg. The acid product was separated by a base wash to give **12** in a yield of 47 mg (30.5%). No further purification performed.

12) R_f = 0.1 (3:1 hexanes/ethyl acetate) ¹H NMR: (500 MHz, CDCl₃) δ 11.87 (bs, 1H, COOH), 7.41–7.20 (complex m, 10H, Ph), 4.92 (d, J = 11.3 Hz, 1H, PhCH₂), 4.80 (d, J = 11.4 Hz, 1H, PhCH₂), 4.65 (t, J = 11.3 Hz, 2H, PhCH₂), 3.98 (d, J = 9.6 Hz, 1H, H-1), 3.84 (m, 1H, H-3), 3.76 (m, 2H, H-4,5), 3.60 (d, J = 8.6 Hz 1H, H-6a), 3.34 (d, J = 8.6 Hz 1H, H-6b) 1.78 (dd, J = 8.6, 4.4 Hz, 1H, H-2); further analysis is required.

1,5-Anhydro-3,4-di-O-benzyl-6-O-(triphenylcarbinyl)-2-deoxy-1,2-C-(dibromomethylene)-D-glycero-D-gulo-hexitol (13) and 1,5-anhydro-3,4-di-O-benzyl-6-O-(triphenylcarbinyl)-2-deoxy-1,2-C-(dibromomethylene)-D-glycero-D-talo-hexitol (14)

A solution of **4** (508 mg, 0.87 mmol) and benzyltriethylammonium chloride (23 mg, 0.101 mmol) in bromoform (1.5 mL) was treated with a drop-wise solution of 33% aqueous sodium hydroxide (3 mL) and potassium fluoride (2.0 g, 34.42 mmol) in water (2.0 mL). The biphasic mixture was stirred for five days at r.t. The mixture was diluted with water (6 mL), extracted with diethyl ether (3 x 15 mL). The combined organic layer was washed with brine (10 mL), dried over magnesium sulphate and concentrated. The crude product was purified by column chromatography (hexanes/ethyl acetate 9:1) to give the major stereoisomer **13** in a yield of 300 mg (49.8%) as an oil, in addition to the minor stereoisomer **14** in a yield of 83 mg (12.8%) as a yellow oil with a combined yield of 63%.

13) R_f = 0.35 (9:1 hexane/ethyl acetate) ^1H NMR: (500 MHz, CDCl_3) δ 7.43–7.21 (complex m, 23H, Ph), 7.02 (dd, 2H, Ph), 4.83 (t, J = 11.6 Hz, 2H, PhCH_2), 4.73 (d, J = 11.8 Hz, 1H, PhCH_2), 4.25 (d, J = 10.7 Hz, 1H, PhCH_2), 4.14 (d, J = 7.8 Hz, 1H, H-1), 3.90–3.94 (m, 2H, H-3, H-4), 3.72 (dd, J = 8.8, 4.9 Hz, H-5) 3.52 (d, J = 1.5 Hz, 1H, H-6a), 3.2 (d, J = 3.3 Hz, 1H, H-6b), 2.01 (dd, J = 7.8, 4.9 Hz, 1H, H-2), ^{13}C NMR: (CDCl_3) δ 143.8 (C, Ph), 138.1 (C, Ph), 137.9 (C, Ph), 128.8 (CH, Ph), 128.6 (CH, Ph), 128.3 (CH, Ph), 128.1 (CH, Ph), 128.09 (CH, Ph), 128.08 (CH, Ph), 128.06 (CH, Ph), 127.8 (CH, Ph), 127.3 (CH, Ph), 86.9 (C, CPh_3), 80.8 (CH, C-5), 80.1 (CH, C-4), 75.7 (CH, C-3), 75.0 (CH_2 , PhCH_2), 72.1 (CH_2 , PhCH_2), 63.7 (CH_2 , C-6), 59.7 (CH, C-1), 35.7 (C, C-2), 34.3 (CBr_2 , C-7), IR (Film from CHCl_3): ν_{max} 3434, 3081, 3012, 2918, 1710, 1449, 1364, 1084, 745, 697, 632 cm^{-1} ; $\text{C}_{40}\text{H}_{23}\text{BrO}_4\text{Na}^+$ $[\text{M}+\text{Na}]^+$ calculated 738.0980, found 738.1185.

14) $R_f = 0.28$ (9:1 hexane/ethyl acetate) ^1H NMR: (500 MHz, CDCl_3) δ 7.53–7.31 (complex m, 23H, Ph), 6.91 (dd, $J = 7.6, 1.8$ Hz, 2H, Ph), 4.92 (d, $J = 2$ Hz, 1H, PhCH_2), 4.75 (d, $J = 1.9$ Hz, 1H, PhCH_2), 4.72 (d, $J = 0.7$ Hz, 1H, PhCH_2), 4.37 (d, $J = 10.8$ Hz, 1H, PhCH_2), 4.22 (dd, $J = 7.55, 10.25$ Hz, 1H, H-1), 4.11–4.15 (m, 1H, H-3), 3.57 (dd, $J = 10.3, 1.7$ Hz, H-4), 3.42–3.46 (m, 1H, H-5), 3.31 (dd, $J = 10.3, 1.7$ Hz, 1H, H-6a), 3.20 (dd, $J = 10.3, 1.7$ Hz, 1H, H-6b), 2.15 (t, $J = 8.1$ Hz, 1H, H-2), ^{13}C NMR: (CDCl_3) δ 144.1 (C, Ph), 138.8 (C, Ph), 138.1 (C, Ph), 128.9 (CH, Ph), 128.8 (CH, Ph), 128.7 (CH, Ph), 128.1 (CH, Ph), 128.1 (CH, Ph), 128.1 (CH, Ph), 128.1 (CH, Ph), 127.9 (CH, Ph), 87.4 (C, CPh_3), 81.1 (CH, C-5), 80.5 (CH, C-4), 75.5 (CH, C-3), 75.1 (CH_2 , PhCH_2), 72.1 (CH_2 , PhCH_2), 63.9 (CH_2 , C-6), 60.7 (CH, C-1), 37.5 (C, C-2), 36.3 (CBr_2 , C-7), IR (Film from CH_2Cl_2): ν_{max} 3434, 3082, 3032, 2918, 1632, 1448, 1088, 1020, 913, 737, 697 cm^{-1} ; $\text{C}_{40}\text{H}_{23}\text{BrO}_4\text{Na}^+ [\text{M}+\text{Na}]^+$ calculated 738.0980, found 738.1000

1,5-Anhydro-3,4-di-O-benzyl-2-deoxy-1,2-C-(dichloromethylene)-D-glycero-D-gulo-hexitol (15)

A solution of **13** (50 mg, 0.08 mmol) in diethyl ether (0.3 mL) was treated with formic acid (0.4 mL) and stirred at r.t. for 1 h. The mixture was diluted with diethyl ether (4 mL) and washed with saturated sodium bicarbonate solution (4 x 10 mL). The organic layer was dried over magnesium sulphate and concentrated. The crude product was purified by column chromatography (hexanes/ ethyl acetate gradient 14:1 to 5:1) to give **15** in a yield of 12 mg (34%) as an oil.

15) $R_f = 0.27$ (3: 1 hexanes/ ethyl acetate) ^1H NMR: (500 MHz, CDCl_3) δ 7.43–4.25 (m, 10H, Ph), 4.94 (d, $J = 11.4$ Hz, 1H, Ph-CH_2), 4.80 (d, $J = 11.7$ Hz, 1H, Ph-CH_2), 4.71 (d, $J = 11.4$ Hz, 1H, Ph-CH_2), 4.60 (d, $J = 11.3$ Hz, 1H, Ph-CH_2), 3.90 (d, $J = 8.4$ Hz, 1H, H-1), 3.89 (m, 1H, H-4), 3.71 (dd, $J = 9.0, 4.1$ Hz, 1H, H-3), 3.60 (d, $J = 3.9$ Hz, 2H, H-

6a, H-6b), 3.64-3.60 (m, 1H, H-5), 1.99 (dd, $J = 8.4, 4.5$ Hz, 1H, H-2); ^{13}C NMR: (CDCl_3) δ (C, COH), (C, Ph), (C, Ph), (CH, Ph), (CH, Ph), (CH, Ph), (CH, Ph), (CH, Ph), (CH, Ph), (CH, C-3), (CH, C-5), (CH, C-4), (CH_2 , PhCH_2), (CH_2 , PhCH_2), (CH_2 , C-6), (CH, C-1), (C, C-7), (C, C-2);

1,5-Anhydro-3,4-di-O-benzyl-2-deoxy-1,2-C-(dichloromethylene)-D-glycero-D-talo-hexitol (16)

A solution of **14** (50 mg, 0.08 mmol) in diethyl ether (0.3 mL) was treated with formic acid (0.4 mL) and stirred at r.t. for one h. The mixture was diluted with diethyl ether (4 mL) and washed with saturated sodium bicarbonate solution (4 x 10 mL). The organic layer was dried over magnesium sulphate concentrated. The crude product was purified by flash chromatography (hexanes/ethyl acetate gradient 14:1 to 5:1) to give **19** in a yeild of 6 mg (17%) as an oil.

16) $R_f = 0.15$ (3:1 hexanes/ethyl acetate) ^1H NMR: (500 MHz, CDCl_3) δ 7.43-4.25 (m, 10H, Ph), 4.94 (d, $J = 11.4$ Hz, 1H, Ph- CH_2), 4.83 (d, $J = 11.7$ Hz, 1H, Ph- CH_2), 4.71 (d, $J = 11.4$ Hz, 1H, Ph- CH_2), 4.61 (d, $J = 11.3$ Hz, 1H, Ph- CH_2), 4.00 (d, $J = 8.4$ Hz, 1H, H-1), 3.90 (m, 1H, H-4), 3.77 (dd, $J = 9.0, 4.1$ Hz, 1H, H-3), 3.61 (d, $J = 3.9$ Hz, 2H, H-6a, H-6b), 3.69-3.65 (m, 1H, H-5), 2.09 (t, $J = 8.4$ Hz, 1H, H-2); ^{13}C NMR: (CDCl_3) δ (C, COH), (C, Ph), (C, Ph), (CH, Ph), (CH, Ph), (CH, Ph), (CH, Ph), (CH, Ph), (CH, Ph), (CH, C-3), (CH, C-5), (CH, C-4), (CH_2 , PhCH_2), (CH_2 , PhCH_2), (CH_2 , C-6), (CH, C-1), (C, C-7), (C, C-2)

1,5-Anhydro-3,4-di-O-benzyl-6-O-(triphenylcarbinyl)-2-deoxy-1,2-C-(methylene)-D-glycero-D-gulo-hexitol (17)

To a solution of zinc (0.314 g, 4.8 mmol), copper iodide (0.091 g, 0.48 mmol) and methyl iodide (6.25 mL) in ether (3 mL) under nitrogen was treated with drop-wise with acetyl chloride (0.16 mL). The solution was heated in an oil bath for five min at 45 °C. A solution of **4** (300 mg, 0.53 mmol) in ether (5 mL) was added drop-wise to the heated mixture over 30 min, additional methyl iodide (6.25 mL) was added then the combined mixture was refluxed under nitrogen for 1 h. The reaction mixture was cooled in an ice bath then quenched drop-wise with saturated ammonium chloride then filtered. The filtrate was washed with hexanes (3 x 5 mL). The combined organic fractions were washed with 10% sodium hydroxide (3 x 5 mL), combined organic fractions were dried over magnesium sulphate and concentrated. The crude product was purified by column chromatography (hexanes/ethyl acetate 5:1) to give **17** with a yield of 282 mg (89%) as a whit solid. The spectral data matched to what had previously been reported (Nagarajan et al. 1997).

17) R_f = 0.25 (5:1 hexane/ethyl acetate) ^1H NMR (500 MHz, CDCl_3): δ 0.82 (app. dt, J = 9.8, 5.8 Hz, 1H, H-8), 0.91 (dt, J = 6.8, 2.4 Hz, 1H, H-9), 1.33 (app. dq, J = 9.7, 7.0 Hz, 1H, H-2), 3.12 (dd, J = 10.1, 4.3 Hz, 1H, H-3), 3.33-3.43 (m, 3H, H-4, H-6, H-7), 3.81 (ddd, J = 7.0, 5.8, 2.8 Hz 1H, H-1), 4.06 (t, J = 6.9 Hz, 1H, H-5), 4.38 (d, J = 10.8 Hz, 1H, CH_2Ph), 4.61 (d, J = 11.7 Hz, 1H, CH_2Ph), 4.70 (d, J = 10.7 Hz, 1H, CH_2Ph), 4.83 (d, J = 11.7 Hz, 1H, CH_2Ph), 6.93 (dd, J = 7.5, 1.8 Hz, 2H, Ph), 7.18-7.49 (m, 23H, Ph), ^{13}C NMR: (CDCl_3) δ 138.50 (C, Ph), 138.42, (CH, Ph), 138.43 (CH, Ph) 128.43 (CH, Ph), 128.03 (CH, Ph), 127.87 (CH, Ph), 127.74 (CH, Ph), 127.57 (CH, Ph), 78.74 (CH, C-3), 78.55 (CH, C-5), 77.92 (CH, C-4), 74.21 (CH_2 , PhCH_2), 70.0 (CH_2 , PhCH_2), 62.52 (CH_2 , C-6), 55.10 (CH, C-1), 15.69 (C, C-7), 11.98 (C, C-2).

1,5-Anhydro-3,4-di-O-benzyl-2-deoxy-1,2-C-(methylene)-D-glycero-D-gulo-hexitol (**18**)

A solution of **21** (50 mg, 0.08 mmol) in diethyl ether (0.3 mL) was treated with formic acid (0.4 mL) and stirred at r.t. for 1 h. The mixture was diluted with diethyl ether (4 mL) and washed with saturated sodium bicarbonate solution (4 x 10 mL). The organic layer was dried over magnesium sulphate and concentrated. The crude product was purified by column chromatography (hexanes/ethyl acetate gradient 14:1 to 5:1) to give **22** with a yield of 18 mg (65%) as an oil. The spectral data matched to what had previously been reported (Nagarajan et al. 1997).

18) R_f = 0.12 (3:1 hexane/ethyl acetate). ^1H NMR (500 MHz, CDCl_3): δ 7.35-7.29 (m, 10H, Ph), 4.89 (t, 2H, CH_2Ph), 4.74 (t, 2H, CH_2Ph), 3.87 (m, 1H, C-1), 3.40 (m, 3H, C-3, C-4, C-5), 2.11 (dd, 2H, C-6, C-6'), 1.08-0.93 (m, 1H, C-2), 0.81-0.69 (m, 2H, C-7, CPH_3); ^{13}C NMR: (CDCl_3) δ 138.49 (C, COH), 138.35, (CH, Ph), 128.51 (CH, Ph), 128.0 (CH, Ph), 127.79 (CH, Ph), 126.98 (CH, Ph), 79.61 (CH, C-3), 76.91 (CH, C-5), 76.53 (CH, C-4), 73.61 (CH_2 , PhCH_2), 71.25 (CH_2 , PhCH_2), 62.58 (CH_2 , C-6), 49.98 (CH, C-1), 14.43 (C, C-7), 10.29 (C, C-2).

p-Azidotoluene (**19**)

p-Toluidine (1 g, 9.2 mmol) in water (12 mL) and hydrochloric acid (37%, 12 mL) were reacted with sodium nitrite (0.756 g, 10.8 mmol) for 2 h of 0°C. The diazonium salt was then treated with sat. sodium acetate (15.1 g, 368 mmol) and sodium azide (1.2 g, 18.4 mmol). The resulted mixture was extracted with portions of 2:1 ethyl acetate/hexane (20 mL) and the combined organic extracts were treated with brine (20 mL) and dried with magnesium sulphate. Resulting in p-azidotoluene as a yellow/orange oil with a distinct smell **23** (95% yield). The spectral data matched to what had previously been reported (Smith et al. 2011)

19) ^1H NMR (300 MHz; CDCl_3): δ = 6.91-7.18 (m, 4H, CH), 2.34 (s, 3H, CH_3) ppm. ^{13}C NMR (500 MHz; CDCl_3): δ = 20.8 (1C, $\text{N}_3\text{-C-CH}$), 118.8 (2C, $\text{N}_3\text{-C-CH}$), 130.3 (2C, $\text{CH}_3\text{-C-CH}$), 134.6 (1C, $\text{CH}_3\text{-C}$),

p-Azidobenzyl bromide (20)

A solution of **19** (0.665 g, 5 mmol), *N*-bromosuccinimide (0.98 g, 5.5 mmol) and azobisisobutyronitrile (0.08 g) was heated in the dark under nitrogen in refluxing dry benzene for 5 h. The mixture was poured into water and diethyl ether. The organic layer was dried over magnesium sulphate and concentrated. The residue was dissolved in a small volume of light petroleum and purified by column chromatography (hexanes) resulting in *p*-azidobenzyl bromide with a yield of 65% as an orange liquid. The spectral data matched to what had been previously reported (Gubbens et al. 2009). Please note; *p*-azidobenzyl bromide is a strong irritant to the eyes that causes a high degree of discomfort and should be kept in a closed system and prior to cleaning be quenched with water within the fumehood.

20) ^1H NMR (500 MHz, CDCl_3): δ 7.38 (d, J = 8.5 Hz, 2H, Ph), 7.00 (d, J 8.5 Hz, 2H, Ph), 4.48 (s, 2H, CH_2); ^{13}C NMR (75.5 MHz, CDCl_3) δ 45.6 (1C, C-1), 119.1, 130.1, 134.1, 140.1.

1,5-Anhydro-3,4-di-O-(p-azidophenyl)methoxy-2-deoxy-6-O-trityl-D-arabino-hex-1-enitol (21)

Trityl-D-glucal (400 mg, 0.810 mmol) was dissolved in dimethylformamide (10 mL) and cooled in an ice bath to -10°C . sodium hydride (48 mg, 2.20 mmol) was then added portion-wise over 1 min and the solution was

stirred for 10 min, which was then followed by the addition of *p*-azidobenzyl bromide (**20**) (400 mg, 1.89 mmol). The solution was warmed to r.t. and stirred for 16 h, followed by quenching with water (20 mL) and extraction with chloroform (3 x 10 mL). The organic layers were combined and reduced to 10 mL and washed with aqueous sat. copper sulphate (4 x 10 mL). The organic layer was then dried with magnesium sulphate and concentrated, to provide a crude mixture that was purified by column chromatography (hexanes/ethyl acetate 9:1) resulting in **21** in a yield of 250 mg (47.6%) as an oil.

21) R_f = 0.7 (5:1 hexanes/ethyl acetate) ¹H NMR: (500 MHz, CDCl₃) δ 7.42- 7.30 (complex m, 7H, Ph), 7.30- 7.05 (complex m, 12H, Ph), 6.88 (d, J = 8.4 Hz, 2H, PhN₃), 6.79 (d, J = 8.4 Hz, 2H, PhN₃), 6.43 (d, J = 6.3 Hz, 1H, H-2), 4.79 (dd, J = 6.3, 3.1 Hz, 1H, H-1), 4.69 (d, J = 11.3 Hz, 1H, PhCH₂), 4.65 (d, J = 11.4 Hz, 1H, PhCH₂), 4.45 (d, J = 11.3 Hz, 1H, PhCH₂), 4.35 (d, J = 11.3 Hz, 1H, PhCH₂), 4.15-

4.00 (complex m, 2H, H-3,4), 3.90 (d, J = 6.3 Hz, 1H, H-5), 3.48 (dd, J = 10.2, 2.4 Hz, 1H, H-6a), 3.25 (d, J = 10.1, 2.4 Hz, 1H, H-6b); ¹³C NMR (75.5 MHz, CDCl₃) δ 144.9 (CH, C-1), 143.8 (C, Ph), 134.9 (C, Ph), 129.5 (C, Ph), 128.7 (CH, Ph), 127.8 (CH, Ph), 127.0 (CH, Ph), 118.8 (C, CN₃), 99.7 (CH, C-2), 76.9 (C, CPh₃), 76.3 (CH, C-5), 74.6 (CH, C-4), 73.2 (CH₂, PhCH₂), 70.8 (CH₂, PhCH₂), 62.0 (CH₂, C-6); IR (Film from CH₂Cl₃) ν_{max}

1,5-Anhydro-4-O-(*p*-azidophenyl)-2-deoxy-6-O-trityl-D-arabino-hex-1-enitol (**22**)

Trityl-D-glucal **3** (1.8 g, 3.7 mmol) was dissolved in dimethylformamide (20 mL) and cooled in an ice/calcium chloride bath to -10 °C. Sodium hydride (48 mg, 2.20 mmol) was then added portion-wise over 1 min and the solution was stirred for 10 min, which was then followed by the addition of *p*-azidobenzyl bromide (**20**) (400 mg, 1.89 mmol). The solution was warmed

to r.t and stirred for 16 h, followed by quenching with water (40 mL) and extraction with chloroform (3 x 20 mL) the organic layers were combined and reduced to 10 mL which was then washed by aqueous sat. copper sulphate (6 x 20 mL). The organic layer was then dried with magnesium sulphate and concentrated, to provide a crude mixture that was purified by column chromatography (hexanes/ethyl acetate 9:1) resulting in **21** in a yield of 439 mg (36.4%) as an oil.

22) R_f = 0.28 (5:1 hexanes/ ethyl acetate) ^1H NMR: (500 MHz, CDCl_3) δ 7.51- 7.45 (m, 7H, Ph), 7.33-7.23 (m, 8H, Ph), 7.04 (d, J = 8.3 Hz, 2H, PhN_3), 6.90 (d, J = 8.5 Hz, 2H, PhN_3), 6.51 (d, J = 6.1 Hz, 1H, H-2), 4.77 (dd, J = 6.1, 2.4 Hz, 1H, H-1), 4.62 (d, J = 11.2 Hz, 1H, PhCH_2), 4.45 (d, J = 11.2 Hz, 1H, PhCH_2), 4.32 (t, J = 6.1 Hz, 1H, H-3),

3.93 (dd, J = 9.3, 3.0 Hz, 1H, H-4), 3.82 (dd, J = 9.2, 6.7 Hz, 1H, H-5), 3.65 (dd, J = 10.3, 2.2 Hz, 1H, H-6a), 3.27 (dd, J = 10.3, 3.4 Hz, 1H, H-6b), 1.91 (apparent t, 1H, OH);

1,5-Anhydro-3,4-di-O-(p-azidophenyl)methoxyl-6-O-(triphenyl-c-arynyl)-2-deoxy-1,2-C-(dichloromethylene)-D-glycero-D-gulo-hexitol

Following the methodology of Nagarajan et al. (1997) a solution of **21** (200 mg, 0.3 mmol) and benzyltriethylammonium chloride (10 mg, 0.04 mmol) in chloroform (5 mL) was treated drop-wise with 33% aqueous sodium hydroxide (10 mL) with rapid stirring. The biphasic mixture was stirred at r.t. for 10 h. The Biphasic mixture was then diluted with water (10 mL) and extracted with dichloromethane (3 x 20 mL). The organic fractions were combined and dried by magnesium sulphate and concentrated to provide a crude mixture. The crude product was purified by column chromatography (hexanes/ethyl acetate 14:1) giving the major cyclopropane **23** in a yield of 20 mg (9%) as a colourless oil.

23) $R_f = 0.3$ (9:1 hexanes /ethyl acetate); ^1H NMR: (500 MHz, CDCl_3) δ 7.47 (d, $J = 8.3$ Hz, 2H, PhN_3), 7.22 (d, $J = 8.3$ Hz, 2H, PhN_3), 7.05 (d, $J = 8.3$ Hz, 2H, PhN_3), 7.95 (d, $J = 8.0$ Hz, 2H, PhN_3), 4.81 (d, $J = 11.6$ Hz, 1H, PhCH_2), 4.78 (d, $J = 10.7$

Hz, 1H, PhCH_2), 4.70 (d, $J = 11.6$ Hz, 1H, PhCH_2), 4.30 (d, $J = 10.7$ Hz, 1H, PhCH_2), 4.10 (d, $J = 8.0$ Hz, 1H, H-1), 3.88–3.80 (complex m, 2H H-4,5), 3.75 (dd, $J = 8.4, 4.2$ Hz, 1H, H-3), 3.49 (dd, $J = 10.2, 3.0$ Hz, 1H, H-6a), 3.23 (dd, $J = 10.2, 3.0$ Hz, 1H, H-6b), 1.90 (dd, $J = 8.0, 4.9$ Hz, 1H, H-2); ^{13}C NMR: (CDCl_3) δ (C, Ph), (C, Ph), (CH, Ph), (CH, Ph), (CH, Ph), (CH, Ph), (CH, Ph), (CH, C-5), (CH, C-3), (CH_2 , PhCH_2), (CH, C-4), (CH_2 , PhCH_2), (CH_2 , C-6), (C, C-7), (CH, C-1), (CH, C-2); IR (Film from CH_2Cl_2) ν_{max} 3016, 2970, 2113, 1444, 1365, 1092, 1025, 900, 760, 701 cm^{-1}

1,5-Anhydro-3,4-di-O-(p-azidophenyl)methoxy-2-deoxy-1,2-C-(dichloromethylene)-D-glycero-D-gulo-hexitol

A solution of **23** (20 mg, 0.027 mmol) in diethyl ether (0.3 mL) was treated with formic acid (0.4 mL) and stirred at r.t. for one h. The mixture was diluted with diethyl ether (4 mL) and washed with saturated sodium bicarbonate solution (4 x 10 mL). The organic layer was dried over magnesium sulphate and concentrated. The crude product was purified by column chromatography (hexanes/ethyl acetate gradient 14:1 to 5:1) to give **24** in a yield of 6.5 mg (48%) as an oil, and **25** (1 mg 7%).

24) $R_f = 0.30$ (3:1 hexanes/ethyl acetate) ^1H NMR: (500 MHz, CDCl_3) δ 7.45 (d, $J = 8.3$ Hz, 2H, PhN_3), 7.36 (d, $J = 8.3$ Hz, 2H, PhN_3), 7.14 (d, $J = 8.3$ Hz, 2H, PhN_3), 7.10 (d, $J = 8.0$ Hz, 2H, PhN_3), 4.91 (d, $J = 11.3$ Hz, 1H, PhCH_2), 4.79 (d, $J = 12$

Hz, 1H, PhCH_2), 4.68 (t, $J = 11.1$ Hz, 1H, PhCH_2), 3.89 (d, $J = 9.5$ Hz, 1H, H-1), 3.79 (dd, $J = 9.3, 3.9$ Hz, 1H, H-4), 3.75 (m, 1H, H-3), 3.71 (m, 2H, H-6a,b), 3.62 (t, $J = 9.2$, Hz, 1H, H-5), 1.82 (dd, $J = 8.3, 3.9$ Hz, 1H, H-2); ^{13}C NMR: (CDCl_3) δ

135.8 (C, Ph), 135.8 (C, Ph), 135.1 (CH, Ph), 134.5 (CH, Ph), 129.1 (CH, Ph), 129.1 (CH, Ph), 118.8 (CN₃, Ph), 118.7 (CN₃, Ph), 79.5 (CH, C-5), 76.1 (CH₂, PhCH₂), 74.9 (CH, C-4), 74.2 (CH₂, PhCH₂), 71.4 (CH, C-3), 62.6 (CH₂, C-6), 60.8 (C, C-7), 58.8 (CH, C-1), 33.7 (CH, C-2); IR (Film from CH₂Cl₃) $\nu_{\max}$ 3415, 2980, 2358, 2346, 2340, 1611, 1512, 1359, 1095, 841, 670 cm⁻¹.

1,5-Anhydro-6-O-(triphenylcarbinyl)-2-deoxy-1,2-(dichloromethylene)-D-glycero-D-gulo-hexitol (25) 1,5-Anhydro-4-O-benzyl-2-deoxy-1,2-C-(dichloromethylene)-D-glycero-D-gulo-hexitol (27)

Compound **5** (920 g, 1.45 mmol) was taken up in warm ethanol (30 mL) under nitrogen flow and 10% Pd/C (~80 mg) and three drops of acetic acid were added to the mixture. The reaction vessel was purged with hydrogen gas and the mixture was vigorously stirred for 24 h. The reaction mixture was filtered through celite and concentrated. The crude product was purified by column chromatography (hexanes/ethyl acetate gradient 9:1 to 1:1) resulting in four colourless oils **7** in a yield of 180 mg (30%), **25** in 30 mg (5%), **26** in 103 mg (31%) and **27** in 103 mg (31%).

25) R_f = 0.8 (1:1 hexanes/ethyl acetate) ¹H NMR: (500 MHz, CDCl₃) δ 7.51- 7.23 (m, 15H, Ph), 3.86 (d, J = 8.6 Hz, 1H, H-1), 3.80- 3.65 (m, 2H, H-3, 4), 3.62- 3.58 (m, 1H, H-5), 3.41 (dd, J = 6.8, 1.5 Hz, 1H, H-6a), 3.31 (dd, J = 1.4, 6.9 Hz, 1H, H-6b), 3.12 (bs, 2H, OH), 1.89 (dd, J = 8.6, 3.6 Hz, 1H, H-2), ¹³C NMR: (CDCl₃) 146.9 (C, Ph), 144.4 (C, Ph), 143.5 (C, Ph), 128.6 (CH, Ph), 128.6 (CH, Ph), 128.0 (CH, Ph), 127.7 (CH, Ph), 127.3 (CH, Ph), 126.8 (CH, Ph), 82.0 (C, C-Ph₃), 78.2 (CH, C-5), 69.4 (CH, C-4), 68.9 (CH, C-3), 68.5 (CCl₂, C-7), 62.5 (CH₂, C-6), 58.9 (CH, C-1), 34.1 (CH, C-2); IR (Film from CH₂Cl₃) $\nu_{\max}$ 3460, 3058, 3021, 1591, 1492, 1444, 1328, 1156, 1010, 758, 706, 638 cm⁻¹; C₂₆H₂₄Cl₂O₄Na⁺ [M+Na]⁺ calculated 470.1052, found 470.1046

27) $R_f = 0.2$ (1:1 hexanes/ethyl acetate) ^1H NMR: (500 MHz, CDCl_3) δ 7.40- 7.30 (m, 5H, Ph), 4.79 (d, $J = 11.7$ Hz, 1H, PhCH_2), 4.76 (d, $J =$, 1H, PhCH_2), 3.89 (d, $J = 11.5$ Hz, 1H, H-1), 3.78 (s, 1H, H-3), 3.69 (m, 2H, H-4, 5), 3.60 (dd, $J = 8.6, 3.8$ Hz, 2H, H-6a, 6b) 2.62 (bs, 1H, OH), 2.0 (bs, 1H, OH), 1.79 (dd, $J = 8.5, 3.6$ Hz, 1H, H-2). ^{13}C NMR: (CDCl_3) 136.9 (C, Ph), 128.7 (CH, Ph), 128.4 (CH, Ph), 128.3 (CH, Ph), 79.1 (CH, C-5), 76.1 (CH_2 , PhCH_2), 72.1 (CH, C-4), 68.2 (CH, C-3), 63.1 (CH_2 , C-6), 61.1 (CCl_2 , C-7), 58.8 (CH, C-1), 29.7 (CH, C-2); IR (Film from CH_2Cl_3) ν_{max} 3375, 2016, 2928, 1635, 1384, 1161, 1110, 1053, 987 cm^{-1}

1,5-Anhydro-2-deoxy-1,2-C-(dichloromethylene)-D-glycero-D-gulo-hexitol (31)

Compound **30** (626 mg, 1.2 mmol) was taken up in warm methanol (30 mL) under nitrogen flow and 10% Pd/C (80 mg) and three drops of acetic acid were added to the mixture. The reaction vessel was purged with hydrogen gas and the mixture was vigorously stirred for 36 h. The reaction mixture was filtered through celite and concentrated. The crude product was purified by column chromatography (hexanes/ethyl acetate 1:1) resulting in a colourless oil **26** in a yield of 241.2 mg (87.5%).

26) $R_f = 0.05$ (1:1 hexanes/ ethyl acetate) ^1H NMR: (500 MHz, CDCl_3) δ 3.88 (d, $J = 8.5$ Hz, 1H, H-1), 3.85 (dd, $J = 9.0, 3.3$ Hz, 1H, H-4), 3.80 (d, $J = 3.2$ Hz, 2H, H-6a, H-6b), 3.69- 3.61 (complex m, 2H, H-3, H-5), 1.92 (dd, $J = 8.6, 3.3$ Hz, 1H, H-2); ^{13}C NMR: (CDCl_3) 80.9 (CH, C-5), 70.0 (CH, C-4), 68.9 (CH, C-3), 62.9 (CH_2 , C-6), 62.0 (CCl_2 , C-7), 58.8 (CH, C-1), 34.0 (CH, C-2); IR (Film from CH_2Cl_3) ν_{max} 3376, 3016, 2928, 1635, 1061, 1010, 953, 785 cm^{-1}

Tri-O-benzyl-D-glucal (**28**)

A solution of **2** (1.54 g, 9.72 mmol) in dimethylformamide (20 mL) was cooled to 0 °C and then treated with sodium hydride (1.5 g, 62.5 mmol). The solution was stirred at 0 °C for 25 min, then treated with benzyl bromide (3 g, 37.5 mmol) and allowed to warm to room temperature. After 17 h, the solution was quenched with water (5 mL) and then extracted with diethyl ether (3 x 40 mL). The organic fractions were combined, dried, filtered and concentrated to give the crude product **28** as a yellow oil. The crude product was purified by column chromatography (hexanes/ethyl acetate 9:1) resulting in **28** in a yield of 3.9 g (91%) as a white crystalline solid. The spectral data matched those reported previously (Nagarajan et al. 1997).

28) R_f = 0.35 (9:1 hexanes/ ethyl acetate); m.p. 53.5–55.0 °C (lit.107 m.p. 53– 55 °C); $[\alpha]^{23.4}_D$ –4.90 (c 1.02, CH₂Cl₂); ¹H NMR: (500 MHz, CDCl₃) δ 7.33–7.23 (complex m, 15H, Ph) 6.43 (dd, J = 6.1, 1.3 Hz, 1H, H-1), 4.88 (dd, J = 6.4, 2.7 Hz, 1H, H-2), 4.83 (d, J = 11.3 Hz, 1H, PhCH₂), 4.640 (d, J = 11.3 Hz, 1H, PhCH₂), 4.637 (d, J = 11.8 Hz, 1H, PhCH₂), 4.60 (d, J = 12.0 Hz, 1H, PhCH₂), 4.564 (d, J = 12.0 Hz, 1H, PhCH₂), 4.562 (d, J = 11.8 Hz, 1H, PhCH₂), 4.61–4.53 (complex m, 3H, PhCH₂), 4.21 (ddd, J = 6.4, 2.7, 1.3 Hz, 1H, H-3), 4.06 (ddd, J = 8.8, 5.1, 3.0 Hz, 1H, H-5), 3.86 (dd, J = 8.8, 6.1 Hz, 1H, H-4), 3.80 (dd, J = 10.5, 5.1 Hz, 1H, H-6a), 3.75 (dd, J = 10.5, 3.0 Hz, 1H, H-6b); ¹³C NMR: (CDCl₃) δ 144.9 (CH, C-1), 138.5 (C, Ph), 138.3 (C, Ph), 138.1 (C, Ph), 128.6 (CH, Ph), 128.4 (CH, Ph), 128.1 (CH, Ph), 127.94 (CH, Ph), 127.89 (CH, Ph), 127.80 (CH, Ph), 127.79 (CH, Ph), 100.1 (CH, C-2), 76.8 (CH, C-5), 75.9 (CH, C-3), 74.5 (CH, C-4), 73.9 (CH₂, PhCH₂), 73.7 (CH₂, PhCH₂), 70.6 (CH₂, PhCH₂), 68.7 (CH₂, C-6); IR (Film from CH₂Cl₂): ν_{max} 3087, 3064, 3030, 2865, 1648, 1496, 1454, 1239, 1100, 1070, 1027, 735, 697 cm⁻¹.

1,5-Anhydro-3,4,6-tri-O-benzyl-2-deoxy-1,2-C-(dichloromethylene)-D-glycero-D-gulo-hexitol (29) and 1,5-anhydro-3,4,6-tri-O-benzyl-2-deoxy-1,2-C-(dichloromethylene)-D-glycero-D-talo-hexitol (30)

As reported by Nagarajan et al. (1997) a solution of **28** (800 mg, 1.93 mmol) and benzyltriethylammonium chloride (10 mg, 0.04 mmol) in chloroform (5 mL) was treated drop-wise with a 33 % aqueous solution of sodium hydroxide (10 mL) with rapid stirring. The biphasic mixture was stirred at room temperature for 10.5 h, diluted with water (10 mL) and extracted with dichloromethane (3 x 20 mL). The organic fractions were combined, dried over magnesium sulphate and concentrated to provide a crude mixture. Which was purified by column chromatography (hexanes/ethyl acetate 14:1) giving **29** as a white solid in 626 mg (62%) and **30** as a yellow oil in 61.9 mg (6%) in a combined yield of 68%. Spectral data matched those which have been previously reported (Nagarajan et al. 1997).

29 R_f = 0.40 (9:1 hexanes/ ethyl acetate (CH_2Cl_2); ^1H NMR: (500 MHz, CDCl_3) δ 7.43–7.22 (complex m, 15H, Ph), 4.90 (d, J = 11.2 Hz, 1H, PhCH_2), 4.80 (d, J = 11.7 Hz, 1H, PhCH_2), 4.69 (d, J = 11.7 Hz, 1H, PhCH_2), 4.55 (d, J = 11.2 Hz, 1H, PhCH_2), 4.53 (d, J = 12.2 Hz, 1H, PhCH_2), 4.44 (d, J = 12.2 Hz, 1H, PhCH_2), 3.89 (d, J = 8.0 Hz, 1H, H-1), 3.84 (m, 1H, H-5), 3.78–3.76 (complex m, 2H, H-3,4), 3.56 (dd, J = 10.6, 2.8 Hz, 1H, H-6a), 3.52 (dd, J = 10.6, 3.7 Hz, 1H, H-6b), 1.79 (m, 1H, H-2); ^{13}C NMR: (CDCl_3) δ 138.3 (C, Ph), 138.1 (C, Ph), 137.8 (C, Ph), 128.65 (CH, Ph), 128.58 (CH, Ph), 128.5 (CH, Ph), 128.3 (CH, Ph), 128.04 (CH, Ph), 128.02 (CH, Ph), 127.93 (CH, Ph), 127.89 (CH, Ph), 127.8 (CH, Ph), 80.0 (CH, C-5), 77.6 and 75.3 (CH, C-3, and CH, C-4), 74.7 (CH_2 , PhCH_2), 73.5 (CH_2 , PhCH_2), 72.0 (CH_2 , PhCH_2), 70.3 (CH_2 , C-6), 61.6 (C, C-7), 59.1 (CH, C-1), 34.4 (CH, C-2); IR (Film from CH_2Cl_2) ν_{max} 3088, 3064, 3031, 2862, 1496, 1454, 1363, 1251, 1093, 1026, 733, 696 cm^{-1} .

30) $R_f = 0.25$ (9:1 hexanes/ ethyl acetate); ^1H NMR: (500 MHz, CDCl_3) δ 7.46–7.17 (complex m, 15H, Ph), 4.89 (d, $J = 11.7$ Hz, 1H, PhCH_2), 4.83 (d, $J = 11.0$ Hz, 1H, PhCH_2), 4.72 (d, $J = 11.7$ Hz, 1H, PhCH_2), 4.64 (d, $J = 12.0$ Hz, 1H, PhCH_2), 4.56 (d, $J = 12.0$ Hz, 1H, PhCH_2), 4.55 (d, $J = 11.0$ Hz, 1H, PhCH_2), 4.18 (apparent t, $J = 7.8$ Hz, 1H, H-3), 4.00 (d, $J = 8.1$ Hz, 1H, H-1), 3.86 (dd, $J = 10.3, 7.6$ Hz, 1H, H-4), 3.77 (dd, $J = 11.0, 1.9$ Hz, 1H, H-6a), 3.67 (dd, $J = 11.0, 5.3$ Hz, 1H, H-6b), 3.48 (ddd, $J = 10.3, 5.3, 1.9$ Hz, 1H, H-5), 2.13 (apparent t, $J = 8.1$ Hz, 1H, H-2); ^{13}C NMR: (CDCl_3) δ 138.3 (C, Ph), 138.2 (C, Ph), 137.9 (C, Ph), 128.7 (CH, Ph), 128.6 (CH, Ph), 128.53 (CH, Ph), 128.49 (CH, Ph), 128.2 (CH, Ph), 128.1 (CH, Ph), 128.0 (CH, Ph), 127.9 (CH, Ph), 127.7 (CH, Ph), 78.6 (CH, C-5), 78.0 (CH, C-3), 76.8 (CH, C-4), 74.9 (CH_2 , PhCH_2), 73.7 (CH_2 , PhCH_2), 71.4 (CH_2 , PhCH_2), 69.1 (CH_2 , C-6), 62.4 (CH, C-1), 61.8 (C, C-7), 32.9 (CH, C-2); IR (Film from CH_2Cl_2) ν_{max} 3088, 3064, 3031, 2917, 2862, 1496, 1454, 1364, 1091, 1026, 731, 696 cm^{-1} .

1,5-Anhydro-3,4,6-tri-O-benzyl-2-deoxy-1,2-C-(dibromomethylene)-D-glycero-D-gulo-hexitol (32) and 1,5-anhydro-3,4,6-tri-O-benzyl-2-deoxy-1,2-C-(dibromomethylene)-D-glycero-D-talo-hexitol (33)

As reported by Nagarajan et al. (1997()) a solution of **28** (800 mg, 1.93 mmol) and TEBAC (10 mg, 0.04 mmol) in bromoform (5 mL) was treated drop-wise with a solution of 33% aqueous sodium hydroxide (5 mL), and potassium fluoride (10 mg, 0.17 mmol) in water (1 mL). The biphasic mixture was stirred at r.t. for three days. The solution was diluted with water (5 mL) and extracted with diethyl ether (3 x 20 mL), then the organic fractions were combined and washed with brine (30 mL). The solution was dried, over magnesium sulphate and concentrated to provide a crude mixture in bromoform and purified by column chromatography (hexanes/ethyl acetate 14:1) gave **32** as a white solid in 497 mg (65%) and **33** as a yellow

oil in 159 mg (14%) in a combined yield of 79%. Spectral data matched those which have been previously reported (Moore 2012).

32: *R_f* = 0.40 (9:1 hexanes/ethyl acetate); m.p.

60.5–62.0 °C ¹H NMR: (500 MHz, CDCl₃) δ 7.47–7.22 (complex m, 15H, Ph), 4.90 (d, *J* = 11.2 Hz, 1H, PhCH₂), 4.81 (d, *J* = 12.0 Hz, 1H, PhCH₂), 4.70 (d, *J* = 12.0 Hz, 1H, PhCH₂), 4.534 (d, *J* = 11.2 Hz,

1H, PhCH₂), 5.527 (d, *J* = 12.2 Hz, 1H, PhCH₂), 4.44 (d, *J* = 12.2 Hz, 1H, PhCH₂), 3.95 (d, *J* = 7.8 Hz, 1H, H-1), 3.87 (ddd, *J* = 7.6, 3.7, 2.7 Hz, 1H, H-5), 3.79 (dd, *J* = 9.5, 7.6 Hz, 1H, H-4), 3.68 (dd, *J* = 9.5, 4.9 Hz, 1H, H-3), 3.55 (dd, *J* = 10.5, 2.7 Hz, 1H, H-6a), 3.51 (dd, *J* = 10.5, 3.7 Hz, 1H, H-6b), 1.87 (dd, *J* = 7.8, 4.9 Hz, 1H, H-2); ¹³C NMR: δ (CDCl₃) C 138.3 (C, Ph), 138.1 (C, Ph), 137.9 (C, Ph), 128.7 (CH, Ph), 128.6 (CH, Ph), 128.51 (CH, Ph), 128.3 (CH, Ph), 128.03 (CH, Ph), 128.01 (CH, Ph), 127.94 (CH, Ph), 127.88 (CH, Ph), 127.81 (CH, Ph), 80.4 (CH, C-5), 80.1 (CH, C-3), 75.2 (CH, C-4), 74.8 (CH₂, PhCH₂), 73.5 (CH₂, PhCH₂), 71.9 (CH₂, PhCH₂), 70.3 (CH₂, C-6), 59.4 (CH, C-1), 35.4 (CH, C-2), 34.1 (C, C-7); IR (Film from CH₂Cl₂): ν_{max} 3063, 3030, 2860, 1496, 1454, 1092, 1027, 734, 696 cm⁻¹.

33) *R_f* = 0.25 (9:1 hexanes/ethyl acetate); ¹H

NMR: (500 MHz, CDCl₃) δ 7.50–7.18 (complex m, 15H, Ph), 4.92 (d, *J* = 11.7 Hz, 1H, PhCH₂), 4.83 (d, *J* = 11.1 Hz, 1H, PhCH₂), 4.72 (d, *J* = 11.7 Hz, 1H, PhCH₂), 4.67 (d, *J* = 12.1 Hz, 1H, PhCH₂), 4.57 (d, *J*

= 12.1 Hz, 1H, PhCH₂), 4.56 (d, *J* = 11.1 Hz, 1H, PhCH₂), 4.18 (t, *J* = 7.8 Hz, 1H, H-3), 4.08 (d, *J* = 7.8 Hz, 1H, H-1), 3.96 (dd, *J* = 10.3, 7.4 Hz, 1H, H-4), 3.78 (dd, *J* = 11.0, 1.7 Hz, 1H, 1H, H-6a), 3.68 (dd, *J* = 11.0, 5.4 Hz, 1H, H-6b), 3.52 (ddd, *J* = 10.3, 5.4, 1.7 Hz, 1H, H-5), 2.29 (apparent t, *J* = 7.9 Hz, 1H, H-2); ¹³C NMR: (CDCl₃) δ 138.3 (C, Ph), 138.2 (C, Ph), 137.9 (C, Ph), 128.7 (CH, Ph), 128.4 (CH, Ph), 128.2 (CH, Ph), 128.1 (CH, Ph), 128.0 (CH, Ph), 127.83 (CH, Ph), 127.79 (CH, Ph), 127.7 (CH, Ph), 78.6 (CH, C-3), 78.5 (CH, C-5), 76.6 (CH, C-4), 74.8 (CH₂, PhCH₂), 73.7 (CH₂, PhCH₂), 71.1 (CH₂, PhCH₂), 69.1 (CH₂, C-6),

62.8 (CH, C-1), 33.8 (CH, C-2), 30.9 (C, C-7); IR (Film from CH₂Cl₂): ν_{max} 3063, 3030, 2861, 1496, 1454, 1091, 1027, 734, 696 cm⁻¹.

3-(prop-2-ynylamino)propan-1-ol (**34**)

A solution of 3-aminopropanol (1 g, 0.013 mol) and K₂CO₃ (1.8 g, 0.013 mol) in acetonitrile (45 mL) was stirred at r.t. under N₂ for 5 min. A solution of propargyl bromide (0.158 g, 0.0013 mol) dissolved in acetonitrile (6 mL) was added drop-wise with stirring. After stirring for 24 h the solvent was evaporated. The residue was dissolved in ethyl acetate (60 mL), washed with 10% sodium hydroxide in (3x 25 mL) and dried, over magnesium sulphate and concentrated. The crude product was purified by column chromatography (ethyl acetate to dichloromethane/methanol 4:1) to give **34** (146 mg (98.6%).

34) *R*_f = 0.20 (4:1 CH₂Cl₂/MeOH); ¹H NMR: (300 MHz, CDCl₃) δ 3.80 (t, *J* = 5.4 Hz, 2H, H-1), 3.45 (d, *J* = 2.5 Hz, 2H, H-4), 2.95 (t, *J* = 5.8 Hz, 2H, H-3), 2.45 (bs, 1H, OH), 2.23 (t, *J* = 1.5 Hz, 1H, H-5), 1.73 (p, *J* = 5.9, 11.2 Hz, 2H, H-2); ¹³C NMR:

3-(prop-2-ynylamino)propan-1-ol derivatised sepharose (**35**)

To a solution of N-propargyl-3-aminopropanol (126 mg, 1.1 mmol) in tetrahydrofuran (3 mL) and dH₂O (4 mL) was added epoxy-activated sepharose matrix (600 mg). The mixture was shaken at 33 °C for 16 h before the mixture was centrifuged down at 2 000 rpm for 1 min. The solvent was decanted off, and ethanol (6 mL) was added. The ethanol mixture was vortex for 1 min then centrifuged at 2 000 rpm for 1 min, the solvent was decanted and replaced with diethyl ether (6 mL). The solution was vortex for 1 min then centrifuged at 2 000 rpm for 1 min before the

solvent being decanted, this last step was repeated. The resulting matrix was further dried under vacuum.

35) The derivatised resin was not amenable to spectroscopic analysis

1,5-Anhydro-3,4-di-O-benzoyl-2-deoxy-6-O-trityl-D-arabino-hex-1-enitol (36) and 1,5-Anhydro-4-O-benzoyl-2-deoxy-6-O-trityl-D-arabino-hex-1-enitol (37)

To a solution of **2** (4.0 g, 10.3 mmol) in dimethylformamide (32 mL) and pyridine (4 mL) at 0 °C was slowly added benzoyl chloride (1.14 g, 8.11 mmol). The solution was then stirred at r.t. for 4 h, diluted with water (5 mL), and extracted with dichloromethane, dried over magnesium sulphate and concentrated. The crude product was purified by column chromatography (hexanes/ethyl acetate 3:1) to

afford **36** in 3.57 g (80%) and **37** in 0.40 g (8%). The spectral data matched those reported in the literature (Mariela & Kulkarni, 1988).

To a solution of **2** (1 g, 2.57 mmol) in dimethylformamide (8 mL) and pyridine (1 mL) at 0 °C was slowly added benzoyl chloride (0.9 g, 6.43 mmol). The solution was then stirred at r.t. for 4 h, diluted water, and extracted with dichloromethane, dried over magnesium sulphate and concentrated. The crude product was purified by column chromatography (hexanes/ethyl acetate 3:1) to afford **37** in 3.66 g (95%).

36) ^1H NMR: (500 MHz, CD_3OD) δ 8.34– 7.06 (m, 25H, Ph), 6.6 (app dd, 1H, H-1), 5.73 (t, 1H, J = 6.1 Hz, H-4), 5.62 (ddd, 1H, J = 6.1, 1.8 Hz, H-3), 5.04 (dd, 1H, J = 6.1, 3.2 Hz, H-2), 4.54– 4.27 (m, 1H, H-5), 3.51– 3.57 (m, 2H, H-6a & H-6b); ^{13}C NMR: (CDCl_3) δ 168.2 (C, OCOPh), 162.6 (OCOPh), 142.8 (CH, Ph), 134.6 (CH, Ph), 133.1 (CH, Ph), 130.6 (CH, Ph), 128.8 (CH, Ph), 127.8 (CH, Ph), 127.3 (CH, Ph), 127.0 (CH, Ph), 98.7 (CH, C-2), 87.1 (C, CPh_3), 76.1 (CH, C-5), 68.4 (CH, C-4), 68.2 (CH, C-3), 62.1 (CH_2 , C-6).

37) ^1H NMR: (500 MHz, CD_3OD) δ 7.80– 7.10 (m, 20H, Ph), 6.55 (dd, 1H, H-1), 5.55 (ddd, 1H, 4.8, 1.8 Hz, H-3), 4.90 (dd, 1H, J = 6.0, 3.4 Hz, H-2), 3.81–4.0 (m, 2H, H-4, H-5), 3.33 (dd 1H, H-6a), 3.64 (dd, 1H, H-6b), 2.42 (bs, OH); ^{13}C NMR: (CDCl_3) δ 168.2 (OCOPh), 146.8 (CH, Ph), 144.1 (CH, Ph), 133.6 (CH, Ph), 130.8 (CH, Ph), 129.1 (CH, Ph), 128.3 (CH, Ph), 128.2 (CH, Ph), 127.4 (CH, Ph), 99.7 (CH, C-2), 87.4 (C, CPh_3), 78.1 (CH, C-5), 73.4 (CH, C-4), 68.2 (CH, C-3), 63.1 (CH_2 , C-6).

3.0 BIOLOGY

3.1 Aims and objectives

The overall aim of the biology study is to identify the most active cyclopropane-containing glycoside and its cellular protein target. To achieve this, objectives listed will be completed.

- Conduct a structure activity relationship (SAR) analysis to determine the most active compound against *S. cerevisiae* (2);
- Identify potential pathways of interactions by chemical genomics (3); and
- Determine protein targets by mass spectrometry (4).

This chapter describes the biological testing of the compounds synthesised in chapter 2. The library of compounds will be subjected to initial dose response assays *in vivo*, (*S. cerevisiae*) to determine the lead compound, which will also give evidence of the mechanism of action. The lead compound will be then used in the chemical genomic screen, to determine pathways or interactions within the cell. Finally, modifications will be made to the lead compound to isolate the biological target.

Note. Compound **7** will now be called benzose from here on.

3.2 Introduction

3.2.1 Structure Activity Relationship

The purpose and importance of a Structure Activity Relationship (SAR) is to predict the properties of new compounds from similar known pre-existing molecules.

In the context of bioactive compounds, SARs are relationships between the molecular structure and biological activity of chemicals. Similarity can be assessed by structure or quantitatively by determining the mathematical relationships between molecular descriptors and activity of the compound. Studies of the latter are called quantitative structure activity relationships (QSAR). This thesis will focus on SAR not QSAR. The basic idea of SAR studies originated in the 19th century, when a study by Crum-Brown and Fraser (1868) indirectly discovered that it was the quaternary ammonium group of curare and other quaternary alkaloids that was responsible for their activity of muscular paralysis.

The first direct attempt to identify the relationship between pharmacological effect and physicochemical properties was described by Richet in 1893. Richet's study identified the effect of narcotic action on tadpoles by a class of organic compounds was related to the solubility of these compounds. Since these early studies, the ideas of SAR have stayed the same, but the uses have continued to be developed. SAR-based studies have been used to great effect in finding new drug candidates (Baumert et al. 2012). SAR can also be used to suggest a likely mechanism of action; by no means can it definitively determine the mechanism of action but it can give supporting evidence. The rationale behind determining the mechanism of reaction comes from an in-depth knowledge of the chemistry of the substituents. For example, some reactions are able to proceed via a chlorine-containing compound whereas the reaction is less favourable with bromine at the same position.

The rate of the reaction is determined by the stability of the anion intermediate; the compound that has the most stabilised intermediate can facilitate the reaction most effectively. With an understanding of the chemistry it is possible to take a range of similar compounds and determine a proposed mechanism; this is further demonstrated in section 3.5.2.

3.2.2 Affinity isolation

The early applications of affinity matrices were related to the use of "immunoabsorbents" for the purification of antibodies. These early applications have also been used for the purification of nucleotides, complementary strands of nucleic acids, certain species of transfer RNA and enzymes (Silman & Katchalski, 1966; Sander et al. 1966; Cuatrecasas, 1970). It was not until later that enzyme isolation started to have a huge impact in this field.

Affinity chromatography matrices attached to bioactive compounds are a major tool for finding direct targets. The identification of target proteins for bioactive compounds is an essential process of modern pharmaceutical drug development because it is a prelude to identification of novel drugs with greater selectivity through mechanism-based and structure-based design. Traditionally for this method, compounds that are bound to the matrix interact with proteins by non-covalent interactions. However, if a matrix-bound bioactive compound covalently binds to a target protein, it is believed this protein can be removed by trypsin digestion off the matrix-bound protein. The peptide fragments can then be identified using MALDI-TOF analysis; an ionization technique used in mass spectrometry, allowing the analysis of molecules.

The bioactive compound can be either attached to the matrix before or after it has interacted with proteins. If attached after, the chemical reaction of attaching the molecule and matrix must not affect any potential protein targets. Figure 3.1 by Speers & Cravatt (2004) demonstrates the binding of a matrix to a compound to identify the molecule's target. The compound, with either an azide or alkyne attached can be either added to cells or it can be added to a cell lysate. The matrix is then attached in the lysate under the Huisgen Cyclisation conditions (copper sulphate, neutralised ascorbic acid).

Figure 3.1. Cartoon representation of an azide-containing molecule binding to an enzyme then the attachment to an alkyne-substituted matrix (Speers & Cravatt 2004). The biologically active compound is first treated with a protein lysate, selectively binding a protein. The bound protein is then removed from the lysate by attaching it to a resin through a Huisgen Cyclisation with the alkyne. This resulting in the bioactive compound being irreversibly attached to the resin.

3.2.3 Yeast gene deletion sets (*Saccharomyces cerevisiae*)

The yeast gene deletion set (YDS) provides one of the most successful methods used in the identification of pathways affected by small drug-like compounds (Parsons et al. 2004). For *S. cerevisiae*, deletion mutations have been made for all ~6300 known or suspected genes, identifying ~1000 essential yeast genes and ~4800 viable haploid gene-deletion mutants (Tong et al. 2004). These findings show yeast has the ability to withstand deletions of a substantial number of individual genes and remain viable in standard laboratory conditions (Suter et al. 2006). While

many single gene deletions cause no change in growth, combinations of two such deletions can lead to reduction in, or loss of, viability (Boone et al. 2007).

Synthetic non-viable relationships may occur for genes acting in a single process, for example, two pathways with the same end point (Boone et al. 2007). To enable high-throughput synthetic lethal analysis of a bioactive compound, a library with a single deletion can be used, while the bioactive compound acts equivalently to a second deletion. The deletion strains that die or have decreased growth will identify potential pathways that are involved in the bioactivity of the compound (Boone et al. 2007). Figure 3.2 demonstrates the viability of the deletion strains when exposed to the bioactive compound. In some cases an introduced bioactive compound shows only low levels of activity due to the cell's abilities for detoxification, for example via efflux pumps (Prasad & Goffeau, 2012). To overcome this it is possible to remove the activity of efflux pumps, by deletion of the genes that encode for them or their transcription factors (Wendler et al. 1997). This provides another example of gene deletions that have no effect until treated with a bioactive compound. Removing the activity of efflux pumps is not the only method for increasing sensitivity of bioactive compounds, for example co-treating bioactive compounds with efflux pump inhibitors.

Figure 3.2. A chemical genomic approach to identify drug pathways and interactions. While the cell is viable when either treated with a biologically active compound or related gene is disrupted, it is unable to survive the double insult. By indentifying the pattern of phenotype related to treatment with the biologically active compound in the chemical genomic screen, target pathways and interactions can be identified. (Figure adapted from Parsons et al. 2004)

Double and triple mutants are also possible. The $\Delta pdr1\Delta pdr3\Delta xxx$ (where xxx donates a single knockout) has been used in our laboratory for drug discovery and pathway identification (Coorey, 2012). The *PDR1* and *PDR3* genes encode transcription factors for ABC transporters including the *pdr5p* efflux pump, which is able to export a vast range of compounds (Talibi & Raymond, 1999; Prasad & Goffeau, 2012). Due to the nature of drug-like compounds, the majority of them are substrates for efflux pumps because of their hydrophobicity (Prasad & Goffeau, 2012). By removing or reducing efflux pump activity, it is possible to identify lead-drug candidates that would normally be missed in a drug screen.

The gene *SYS1* codes for an integral membrane protein in the golgi and is required for targeting of the ARf-like GTPase *Ar13p* to the golgi. The deletion of *SYS1* causes increased sensitivities to many different drug-like compounds (Setty et al. 2004). The *SYS1* gene knock out has no effect on cell growth until the cell is put under this type of stress. Gene deletions of this sort that sensitise to multiple drugs or conditions are referred to as multi drug sensitising, or colloquially “frequent fliers” (Hillenmeyer et al. 2008). A

paper presented by Hillenmeyer et al. (2008) lists the currently known frequent flier genes whose deletion strains are sensitive to many bioactive compounds and therefore are generally ignored in chemical genomic screens.

In this study the triple deletion mutant library ($\Delta pdr1 \Delta pdr3 \Delta xxx$) was used as the compounds being tested are exported by ABC transporters in *S. cerevisiae*.

3.3 Materials and methods

3.3.1 Chemicals and compounds

The range of cyclopropane-containing glucals were either synthesised by myself (chapter 2.0) or donated by Peter Moore of the Organic Chemistry Group, School of Chemical and Physical Sciences, Victoria University of Wellington, and were purified to >95% pure as assessed by NMR spectroscopy. In each case, each study was completed using a single batch apart from the lead compound which was identified by batch number to keep experiments consistent. All samples were stored at -20 °C in DMSO at a concentration of either 10 mM or 50 mM. All other reagents were purchased from Sigma-Aldrich unless otherwise stated.

3.3.1.2 Yeast strains and cultures

Yeast strains were either obtained from the $\Delta pdr1\Delta pdr3\Delta xxx$ knockout library, originally prepared by Namal Coorey (within the Chemical Genetic Group, School of Biological Sciences, Victoria University of Wellington) or from a copy of the yeast gene deletion library, a gift from Charles Boone (University of Toronto, Canada).

Strain	Genotype
BY4741	<i>MATa his3Δ0/his3Δ0 leu2Δ0/leu2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0</i>
$\Delta pdr1\Delta pdr3$	<i>MATa pdr1Δ::natR pdr3Δ::URA3 can1Δ::STE2pr-Sp_his5; lyp1Δ; his3Δ1; leu2Δ0; ura3Δ0; met15Δ0 LYS2</i>
$\Delta pdr1\Delta pdr3\Delta xxx$	<i>pdr1Δ::NATpdr3Δ::URA3:xxxΔ::kanR can1Δ::STE2pr-Sp_his5 Lyp1Δ; his3Δ1; leu2Δ0; ura3Δ0; met15Δ0 LYS2</i>
4xUPRE-GFP	<i>MATa met15Δ::4xUPRE-GFP-URA3 can1Δ::STE2pr-Sp_his5; lyp1Δ; his3Δ1; leu2Δ0; ura3Δ0; met15Δ0 LYS2</i>

3.3.2 Yeast growth conditions

All *S. cerevisiae* strains were cultured in one of the following media supplemented with appropriate antibiotics when required; ClonNat (WERNER BioAgents) final concentration of 100 mg/L and geneticin (G418) final concentration 200 mg/L. All media were autoclaved at 121 °C for 22 min before use.

Yeast peptone dextrose (YPD) Broth: Was prepared from 8 g yeast extract (Becton, Dickinson and Company), 16 g Bacto-Peptone (Becton, Dickinson and Company), and 0.096 g adenine (Sigma-Aldrich) mixed into 760 mL of distilled water. The resulting mixture was sterilised by autoclave; once cooled, 40 mL of 40% glucose (Sigma-Aldrich) solution was added along with appropriate antibiotics when required.

Solid YPD media was prepared as above but with the addition of 16 g agar (Invitrogen).

Synthetic complete (SC) broth: Was prepared from 1.7 g of yeast nitrogen base without amino acids or ammonium sulphate (Becton, Dickinson and Company), 1 g of monosodium glutamate (Sigma-Aldrich), and 2 g amino acid mixture (see below) was added to 760 mL of distilled water and sterilized by autoclave. Once cooled, 40 mL of 40% glucose (Sigma-Aldrich) solution was added along with appropriate antibiotics when required.

Solid SC media was prepared as above but with the addition of 16 g agar (Sigma-Aldrich).

Synthetic Drop out (SD-ura) broth: as above (SC) media with the removal of uracil in the amino acid mixture.

Amino acid mixture for SC media: 3 g adenine, 2 g uracil, 2 inositol, 0.2 g p-aminobenzoic acid, 2 g alanine, 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 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 and 2 g valine.

3.3.3 Initial IC₅₀ assay in *Saccharomyces cerevisiae*

Liquid phase assay

The yeast strains BY4741 wild type and $\Delta pdr1\Delta pdr3$ were used to determine the initial IC₅₀ of benzose and compounds **1- 18**.

With the initial concentration of 1 mM, serial dilutions in $-\log(\sqrt{10} \approx 3.162$ -fold) decrements were made for each of the glucal derived compounds by dilution in DMSO (dimethyl sulfoxide). Serial dilutions were transferred to wells in a 96 well V-bottom plate (KJ511-3, Interlab) along with DMSO alone for the negative control to determine the residual growth for each yeast strain in the absence of growth inhibitory compound, the residual growth is determined from the treated cell growth divided by the control cell growth.

A single colony of yeast strain of interest (BY4741 or $\Delta pdr1\Delta pdr3$) was inoculated in 4 mL of each of the three broths (YPD, SC, SD) with the appropriate antibiotics. The cultures were incubated on a rotating drum (40 rpm) at 31 °C for 18 hours. Cell titre was determined using a spectrometer (UNICAM 8625 UV/VIS) and 5×10^5 cells/mL were inoculated into 10 mL of the respective broth for each yeast strain and vortexed. From each broth plus cells, 99 μ L were delivered into the wells of a 96 well flat-bottomed plate (TCP-000-096, JET BIOFIL) using a multichannel pipette, for each strain in each drug condition in triplicate. Subsequently, 2 μ L of half

log drug dilutions and DMSO were added to the yeast cells (final DMSO concentration 2%). Individual rows of SC, SD and YPD were included for each plate to determine the optical density (OD) of the growth media. The contents of the wells were mixed by vortexing the plates at 1000 rpm for 30 seconds using the Eppendorf Mix Mate (Krackeler Scientific Inc. Albany, New York) and were incubated at 30 °C for 18 hours.

The cultures were resuspended by vortexing at 1000 rpm for 30 seconds and the OD in each well was measured at 590 nm using Envision™ Xcite™ 2102 Multilabel Reader (Perkin-Elmer, Turku, Finland) with HTS microplate reader software version 1.12. The OD in each well was subtracted from the average OD for media alone control. The residual growth ($OD_{590Exp}/OD_{590DMSO}$ control x100) was determined and the average residual growth plotted using Microsoft Excel (2013).

Solid phase assay

Hot SC-agar (50 °C) was poured into 33 Singer plates (Singer Instruments, Somerset UK), with benzose at a range of concentrations from 0 to 200 µM, in increments of 20 µM. In triplicate, a single plate from the $\Delta pdr1\Delta pdr3\Delta xxx$ library (384 array) was chosen and plated onto each of the 30 compound-containing agar plates and along with three DMSO alone plates for the negative context by the use of Singer RoToR HDA robot (Singer Instruments, Somerset, UK). The plates were imaged photographically after 24 hours incubation at 30 °C. Visual inspection and use of Colony HT (2006, version 110) were used to identify a concentration that led to ~40% reduction in colony size compared to control, allowing identification of both resistant and sensitive yeast strains in further assays.

3.3.4 Serial dilution assay

The serial spot dilution assay to determine cytotoxic or cytostatic behaviours was followed directly from the SC liquid phase IC₅₀ experiment of benzose against BY4741 and $\Delta pdr1 \Delta pdr3$ (Methods 3.7.4). One hundred μL of culture from the benzose concentrations: 1000, 314, 100, 31, 10, 3.14, 1.0 and 0.31 μM as well as the 2% DMSO control were transferred into a 96 well plate from which five sets of 1:4 serial dilutions in dH₂O were made. The 96 well plates were then transferred onto SC agar plates by pipetting 1.5 μL of each dilution with a multi-channel pipette. This resulted in four lines of five serial dilutions on each half of the SC agar plate as shown below. The agar plates were incubated at 30 °C for 36 hours. Photographs were recorded at 18, 24 and 36 hours.

3.3.5 Mammalian cell experiments

All mammalian cell based experiments were completed by Arun Kanakkantharan in the School of Biological Science, Victoria University of Wellington.

MTT Cell proliferation assay

1A9 human ovarian carcinoma cells and HL-60 human promyelocytic leukemia cells were seeded in 96-well plates at 1×10^4 cells/well and incubated with benzose for 48 hours (for HL-60 cells) and 72 hours (for 1A9 cells) under normal cell culture conditions. Twenty microlitres of MTT was added to each well and incubated for two hours at 37°C. The blue crystals were then solubilized at 37°C overnight in 100 μL of solubilizing solution containing 10% SDS, 45% dimethylformamide (pH 4.5, adjusted with acetic acid), and MTT reduction was measured the following day by absorbance at a wavelength of 570 nm in a multiwell-plate reader (Versamax, Molecular Devices, Sunnyvale, CA). The half maximal inhibitory

concentration (IC_{50}) of the compound was determined from a concentration-response curve using Sigma Plot software version 8 (Systat Software Inc., Point Richmond, CA, USA).

3.3.6 Cell lysate preparation

Glass beads (425-600 μ m diameter, Sigma Aldrich) were soaked in 1 M HCl for 16 hours then thoroughly rinsed in dH₂O, followed by drying for 16 hours. The prepared glass beads were chilled in ice before use.

Fifteen mL of yeast culture was centrifuged at 20 000 rpm (Eppendorf centrifuge 5810) and the media was decanted off. The cells were re-suspended in 2 mL of lysis buffer (50 mM Tris.HCl, pH 7.6, 1 mM EDTA and 10 μ L protease inhibitor mixture). To the buffered culture was added 6 g of the prepared glass beads, this was then vortexed at full speed 5 times for 8 min, between each cycle the cells were kept on ice for 2 min. After the 5 cycles, the glass beads were removed by pipetting out the media and the cell debris was removed by ultra-centrifugation at 4 °C for 30 min at 45 000 rpm (OptimaTML-100 XP Ultracentrifuge).

3.3.7 Bradford protein quantification

Ten μ L of the cell lysate was diluted 10-fold with 50 mM Tris.HCl pH 7.5 buffer, of which 1-5 μ L aliquots were taken and made up to 10 μ L with the appropriate volume of 50 mM Tris.HCl pH 7.5 buffer in a 96 well plate. Two hundred μ L of Bradford reagent was added. A BSA standard with a defined concentration (200-1500 μ g/ml) was set up on the same 96 well plate. The absorbance was read by (plate reader) at 340 nm and the results were graphed with the linear regression of the standards.

3.3.8 Affinity matrix preparation

To a solution of 1.26 mg of *N*-propargyl-3-aminopropanol in 3 mL of THF and 4 mL of dH₂O was added 600 mg of epoxy-activated sepharose matrix (Sigma-Aldrich). The mixture was shaken at 33 °C for 16 hours before the mixture was centrifuged down at 2 000 rpm for 1 min. The solvent was decanted off, and 6 mL ethanol was added. The ethanol mixture was vortexed for 1 min then centrifuged at 2 000 rpm for 1 min, the solvent was decanted and replaced with 6 mL of diethyl ether. The solution was vortexed for 1 min then centrifuged in an enclosed falcon tube at 2 000 rpm for 1 min before the solvent being decanted, this last step was repeated. The resulting matrix was further dried in a vacuum.

3.3.9 Affinity matrix derivatisation

To a solution of 20 mg of 6-[2-(4-azidophenyl)ethyl]azulene in 1 mL of THF and 1 mL of cell lysate (*S. cerevisiae*) was added 5 mg of ascorbic acid and sodium hydroxide to neutralise the solution, then 0.1 mL sat. aqueous copper sulphate was added. The solution was stirred for 5 min before 300 mg of the alkyne-substituted sepharose matrix was added. A second sample was made as above but with the unmodified epoxy-activated sepharose matrix. The mixtures were shaken at 33 °C overnight. Both solutions were then centrifuged at 2 000 rpm for 2 min and the solvents were then decanted off. The following wash steps were then carried out on both samples: 2x 6 mL water, 2x 6 mL ethanol, 2x 6 mL diethyl ether. For each wash, the sample was centrifuged in an enclosed falcon tube at 2 000 rpm for 1 min then solvent decanted. A solution of 2 M Ammonia was then used to dissolve any remaining copper, this was followed by the removal of the ammonia by centrifugation at 2 000 rpm the matrix was then washed multiple times with dH₂O.

3.3.10 Huisgen Cyclisation of **24** to the alkyne-substituted sepharose matrix

To a prepared yeast lysate (section 3.3.6), compound **24** was added and shaken at 33 °C for 6 h before the alkyne-substituted sepharose matrix was added, along with 0.1 mL saturated copper sulphate, and 5 mg of ascorbic acid neutralised by aqueous sodium hydroxide. The mixture was shaken at 33 °C for 12 h. Centrifugation at 4 000 rpm for 5 min and decanting off the supernatant provided the derivatised matrix, which was then washed with 1 M Tris.HCl buffer pH 7.5, again centrifuged at 4 000 rpm for 5 min and the buffer decanted off. The above was repeated with the alkyne-substituted sepharose matrix without the copper species (Discussion 3.5.4) as a control.

3.3.11 Peptide preparation

One hundred µL of 100 mM Tris.HCl pH 8.8 with 0.5 M urea was added to the lysate-matrix prepared in section 3.3.10. This was then vortexed. To the suspension, 1 µL of NuPage reducing agent (Life Technologies) was added, this was then incubated at 55 °C for 35 min. After this time, the sample was cooled to room temperature. One µL of freshly made 0.5 M iodoacetamide (0.023 g in 250 µL MilliQ water) was added and left for 30 min in the dark. To the sample, 0.5 µL of 0.5 M calcium chloride and 1 µg of trypsin in 20 µL of 100 mM Tris pH 8.8 with 0.5 M urea was added and incubated over night at 35 °C with rotation.

The matrix was removed by centrifugation at 2 000 rpm for 3 min, the buffer was evaporated by centrivap concentrator. The tryptic peptides were re-suspended in 20 µL of 0.1% TFA.

3.3.12 ZipTip purification

A PerfectPure C-18 Tip (Eppendorf) was attached to a pipette and washed before peptides were attached. The wash consisted of 3x 10 μ L acetonitrile, 3x 10 μ L 50:50 acetonitrile/ 0.1% TFA and then 5x 10 μ L 0.1% aqueous TFA. Following the wash, the samples of peptides in 20 μ L 0.1% aqueous TFA prepared in section 3.3.11 were aspirated and expelled 20x to attach the peptides to the tip. The tips were then washed with 3x 10 μ L with 0.1% aqueous TFA. The peptides were then eluted off the tip with 1.5 μ L acetonitrile/ 0.1% aqueous TFA (80:20). The sample was allowed to dry in positive air flow. The above was repeated with the alkyne-substituted sepharose control sample.

3.3.13 Protein identification

Proteins were identified by peptide mass fingerprinting. Proteins were cleaved from the alkyne-substituted matrix as described in 3.7.11 and 3.7.12. The resulting peptide fragments were then subjected to MALDI analysis on an AB SCIEX TOF-TOF™ 5800 spectrometer. The sequences acquired from this process were then searched against the yeast protein database using the MASCOT algorithm.

3.3.13.1 *Preparation of protein spots for MALDI analysis*

The dried peptides from section 3.3.12 were resuspended in 1.5 μ L of CHCA (alpha-Cyano-4-hydroxycinnamic acid) Matrix (8 mg/mL CHCA, 1% aqueous TFA and 50% acetonitrile 1:1). Resuspended samples were transferred onto a MALDI plate and left to dry.

3.3.13.2 *MALDI-TOF MS/MS analysis*

MALDI TOF-TOF MS/MS analysis of samples was carried out using an AB SCIEX TOF-TOF™ 5800 spectrometer (Applied Biosystems, Foster City, CA)

equipped with a diode pulse laser (355 nm, 1 kHz). The spectrometer was controlled by TOF-TOF™ Series Explorer™ software.

3.3.13.3 Acquisition of spectra for MS

Spectra for MS analysis were acquired in positive reflector mode with CID off. Peptide fragments were collected in the mass range 700- 4000 Da. The laser intensity was fixed at 5500 with a pulse rate of 400 Hz. The laser was fired in a uniform random pattern. A total of 400 laser shots were collected per spectrum.

3.3.13.4 Calibration of spectra for MS

MS spectra were internally calibrated using CalMix2 (Applied Biosystems) monoisotopic peaks of des-Arg1-Brodykinin (904.4680), angiotensin 1 (m/z 1296.6770), adrenocorticotrophic hormone (ACTH) clip 1-17 (m/z 2093.0870), ACTH clip 18-39 (m/z 2465.1990) and ACTH clip 7-38 (3657.9294). Peak matching criteria for calibration included signal to noise ratio greater than or equal to five, mass tolerance of ± 0.1 m/z, a minimum of three matched peaks, and maximum outlier error of 1 m/z. A list of reference masses is shown in appendix 8.0.

3.3.13.5 Precursor ion selection for MS/MS

Selection criteria for precursors to undergo MS/MS analysis included a signal to noise ratio greater than or equal to 20, mass within the range 800-4000 Da, and exclusion of precursor ions within a resolution of 200 of the selected precursor ion. Known adduct, calibration and common contaminant peaks, such as trypsin were excluded. A full list of exclusion masses is shown in appendix 8.0. A maximum of 60 precursor ions were selected per spot. MS/MS data were acquired from strongest precursor ion first.

3.3.13.6 Acquisition of spectra for MS/MS

Spectra for MS/MS analysis were acquired in 1 kV positive reflector mode, with CID and metastable suppressor on. The laser intensity was fixed at 5800 with a pulse rate of 1000 Hz. The laser was fired in a random uniform

pattern. Acquisition of spectra was stopped after four sub-spectra were collected. A total of 1000 laser shots were collected per full spectrum.

3.3.13.7 *Calibration of spectra for MS/MS*

The monoisotopic angiotensin 1 peak (1296.4680) was selected as a precursor ion for calibration. MS/MS spectra were then calibrated using known fragment ion masses for this precursor ion (appendix 8.0). Peak matching criteria for calibration included a signal to noise ratio greater than or equal to 10, mass tolerance ± 0.03 m/z, a minimum of five matched peaks, and maximum outlier error of 100 ppm

3.3.13.8 *Comparison of peptide sequences to S. cerevisiae data base*

Peptide fingerprints and MS/MS sequences were imported into Protein Pilot™ 3.0 software. The MASCOT algorithm was used to search the generated peaks list against the NCBI non-redundant data base (2008/09/02, 21331 sequences) for *S. cerevisiae* (905409 sequences) for proteins obtained from *S. cerevisiae* samples.

3.3.15 Validation of benzose breakdown in agar

Hot SC-agar (50 °C) containing 60 μ M benzose was poured in to five Singer plates. At time intervals of four, 10, 14 and 18 h after pouring, the plates were pinned by the use of Singer RoToR HDA with plate 10 of the $\Delta pdr1 \Delta pdr3 \Delta xxx$ library. After 24 h of incubation at 33 °C each plate was photographed and the growth of each spot was determined by colony HT analysis.

3.3.14 Genomic screen

The genomic screen used in these studies was modified by Namal Coorey within the Chemical Genetic Group, School of Biological Sciences, Victoria University of Wellington. The screen was adapted from Tong & Boone 2006 to create a library containing the background with null mutations in the PDR1 and PDR3 transcription factors. The library was maintained as haploid MATa mutants in 1536 colonies per plate format on 14 plates. Each plate contained 384 strains in quadruplicate with a *his3Δ::kanR* control stain border comprising 144 colonies along the side of the plate. Since the genomic screen identifies sensitive and resistant strains by colony size, the *his3Δ::kanR* border acts as a buffer to prevent strains from being susceptible to edge effects such as, more access to media and less competition. The *his3Δ::kanR* was used as the border because it does not show any genetic interaction with any query mutations since histidine prototrophy is achieved via *can1Δ:STE2pr-sp-HIS5* in the recombinant MATa strains.

The $\Delta pdr1\Delta pdr3\Delta xxx$ gene deletion library was copied on to SC agar plates in a 1536 arrangement which consisted of four replicates arranged in a box formation, as well as a $\Delta his3$ border that consisted of the two outer rows and columns of the plates. Fifty six 40 mL SC agar plates were poured after autoclaving, with 14 having no drug and 800 μ L DMSO as the control and the remaining 42 having 60 μ M benzose (48 μ L of 50 mM stock in DMSO) and 752 μ L DMSO, both having a DMSO concentration of 2%. The DMSO benzose solution was not added until the agar had cooled to 50 °C. Plating of the 1536 array yeast on to the drug and control plates was done using the Singer RoTor HDA. The treated and control plates were incubated at 30 °C. At 18, 24 and 36 h, photographs were taken of each plate, these were then subjected to Colony HT analysis to determine the size of each yeast colony.

Resistant strains were defined as those where the residual growth of benzose-treated strains was found to be above two standard deviations

from the total average residual growth in at least one of the technical replicates in 2 of 3 biological replicates. Residual growth is defined as the average of the quadruplet of benzose treated in each strain divided by the average of the corresponding quadruplet of DMSO control. If one of the four colonies of the quadruplet was outside of two standard deviation points from the average colony was rejected.

Sensitive strains were defined as those where the residual growth of benzose treated strains was found to be below two standard deviations from the total residual growth average in at least one of the technical replicates in 2 of 3 biological replicates.

3.3.16 Validation of the genomic screen

Each yeast strain that was shown to be resistant in the genomic screen was treated to 30, 50 and 70 μM benzose, following the methods described for IC_{50} analysis liquid phase assay (section 3.3.4).

For each strain, an aliquot of 98 μL of 5×10^6 cells/ mL was removed from the mid log culture and placed into 96 well round bottom plate. To each aliquoted yeast strain was added 2 μL the appropriate concentration of benzose in DMSO. The samples were sealed by a plastic film and left in an incubator for 36 hours. After this time the samples were shaken before being read by the plate reader (OD_{590}). To be validated as a resistant strain, the strain had to have a residual growth greater than 0.4 at 50 μM of benzose, 10 standard deviations above Δhis3 residual growth average (0.362 ± 0.004).

This method was also used to validate the sensitive strains but with lower benzose concentrations of 10, 30 and 50 μM . To be validated, strains had to have residual growth below 0.555 at 30 μM of benzose, two standard deviations below Δhis3 residual growth average (0.685 ± 0.065).

3.3.17 Unfolded protein response element (UPRE) assay

The UPRE assay was carried out by James. H Matthews (within the Chemical Genetic Group, School of Biological Sciences, Victoria University of Wellington) with the 4×UPRE-GFP (Unfolded Protein Response Element-Green Fluorescent Protein) strain. A single colony of YCG266 was inoculated into 3 mL SC and grown overnight at 30 °C on a rotating drum. Cell titre was diluted to 5×10^6 cells/mL with SC and resuspended. Fifty μ L aliquots of this cell suspension were dispensed into a PerkinElmer 384-well CellCarrier confocal microscopy plate, with a concentration range of 200, 100, 50, 25, 12.5, 6.75, 3.85 μ M dilutions of benzose and DTT at 0.025 mM. ddH₂O and 2% DMSO were aliquoted into separate wells as controls. The treated cultures were incubated at 30 °C for 4 h, before being read with an Opera High Content Screening System confocal microscope (PerkinElmer). Excitation and emission wavelengths were 488 and 520 nm (GFP), and 561 and 600 nm (RFP) respectively. Data from this assay was analysed using Acapella software (PerkinElmer), with $\text{Ln}(\text{GFP}/\text{mCherry})$ being calculated to determine activation of the unfolded protein response.

3.4 Results

The aim of this study was to determine the potential target of a lead compound and to investigate which cellular pathways it affects. To identify the lead compound, a structure activity relationship (SAR) assay was completed. The SAR assay was also designed to help determine the likely binding mechanism that is occurring when interacting with the protein of interest (assuming covalent interactions). Identification of proteins that interact directly with the lead compound was attempted using affinity chromatography and mass spectrometry. A yeast-based genetic deletion approach was undertaken using a drug sensitised $\Delta pdr1 \Delta pdr3 \Delta xxx$ genetic mutant array.

3.4.1 Benzose is active in $\Delta pdr1 \Delta pdr3$ dual knockout *S. cerevisiae*

The first synthesised compound (benzose) was subjected to a general inhibitory assays against *S. cerevisiae* BY4741 wild type and $\Delta pdr1 \Delta pdr3$ dual knock out strains and two stains of human cell lines, HL-60 (human promyelocytic leukaemia) and 1A9 (human ovarian cancer) cells. Each assay was completed in triplicate. The BY4741 wild type yeast was not sensitive towards benzose at any of the concentrations tested, with a growth rate within 10% of the non-treated control at each concentration. The $\Delta pdr1 \Delta pdr3$ strain had an IC_{50} of 44 μM ; maximal cell death was observed at 100 μM (figure 3.3). At the higher end of the concentration range the cell survival apparently increased. This was due to benzose precipitating in the aqueous solution at these higher concentrations. When the DMSO carrier was increased from 2% to 4%, the increase in survival was no longer observed.

Figure 3.3. *S. cerevisiae* $\Delta pdr1 \Delta pdr3$ dual knock out and BY4741 treated with benzose. Benzose showed activity against $\Delta pdr1 \Delta pdr3$ with an IC_{50} of 44 μM , however, no activity was found against BY4741 cells. This strongly suggests that benzose is a substrate for the membrane ATP-binding cassette (ABC) transporter, which is regulated by the *PDR1* and *PDR3* transcription factors. Results were measured at OD_{590} , in triplicates. Values shown are the average of triplicate experiments, with the error bars representing the standard deviation.

3.4.2 Serial dilution assay

The serial spot dilution assay was undertaken as described in section 3.3.5, to determine whether benzose acts as a cytotoxic or cytostatic compound. The two strains of yeast were grown in liquid culture at the concentrations shown in figure 3.4; cells were serially diluted then transferred onto drug-free agar. It was found that at high concentrations (314 μ M above) benzose is cytotoxic in the initial treatment, leading to no growth on the drug-free agar. At lower concentrations (314 μ M and below) benzose acts as cytostatic compound, and growth resumed unimpeded following transfer. Figure 3.4 confirms that BY4741 is not effected by benzose. However, $\Delta pdr1\Delta pdr3$ at high concentrations is showing no growth after the 2nd serial dilution at 314 μ M and the 3rd dilution at 101 μ M. At lower concentrations the $\Delta pdr1\Delta pdr3$ are able to

Figure 3.4. Benzose is cytostatic and cytotoxic. Shown here, a double serial dilution, the vertical dilution is a decreasing concentration of benzose, and the horizontal is a 1:4 dilution of cells. As the concentration is increased, cellular growth is decreased showing both cytostatic and cytotoxic characteristics. The serial dilution assay was repeated in triplicate, shown here is representative of the three replicates. Note, at high concentrations benzose precipitates out of solution resulting in increased cell growth, BY4741 at 1000 μ M its due to plating error.

fully recover at all serial dilutions. It has to be noted that 1000 μM a pipetting error occurred due to a defective channel (hence no growth for the BY4741 strain) and benzose precipitates out of solution resulting in decreased toxicity.

3.4.3 Identification of the lead compound through SAR analysis

The structure activity relationship study was undertaken as described in section 3.3.4. The study was designed to identify the most active compound in *S. cerevisiae*, the study was also designed to identify the potential mechanism by which the compounds act on their biological target. Structural variations were limited to the groups on oxygen at C-3, C-6 and varying the substituent on the cyclopropanes. As discussed in section 1.4.2, should these cyclopropanated glycals bind covalently to their target protein, they are likely to do so through one of three possible mechanisms.

The compound library consisted of 27 glycals (table 3.1), of which 20 contain a cyclopropane; 12 of these are chlorine containing and six contain bromides; the remaining two cyclopropanes are unsubstituted. All glycals were tested under the same conditions as benzose (section 3.3.4) against two strains of yeast, the BY4741 wild type and $\Delta pdr1\Delta pdr3$. Only four compounds (**7**, **11**, **15**, **25** and **27**) were sufficiently active in $\Delta pdr1\Delta pdr3$ to permit determination of an IC_{50} . Others were inactive or only partially active below their solubility limit in 2% DMSO; only compound **27** showed activity in the BY4741 wild type yeast.

The most active compound was then tested against the human cell lines HL-60 and 1A9, benzose was inactive in both human cell lines tested.

Compound number	R ¹	R ²	R ³	R ⁴	R ⁵	Cyclo- propane	$\Delta pdr1$	
							BY4741	$\Delta pdr3$
							μM	μM
1	CH ₂ OAc	Ac	Ac	-	-	-	-	-
3	CH ₂ OTr	H	H	-	-	-	-	-
4	CH ₂ OTr	Bn	Bn	-	-	-	-	-
5	CH ₂ OTr	Bn	Bn	Cl	Cl	Down	-	-
6	CH ₂ OTr	Bn	Bn	Cl	Cl	Up	-	-
7	CH ₂ OH	Bn	Bn	Cl	Cl	Down	-	40 ± 1.2
8	CH ₂ OCHO	Bn	Bn	Cl	Cl	Down	-	-
9	CH ₂ OH	Bn	Bn	Cl	Cl	Up	-	-
10	CH ₂ OCHO	Bn	Bn	Cl	Cl	Up	-	-
11	CH ₂ OH	Bn	Bn	Cl	H	Down	-	300 ± 15
12	COOH	Bn	Bn	Cl	Cl	Down	-	-
13	CH ₂ OTr	Bn	Bn	Br	Br	Down	-	-
14	CH ₂ OTr	Bn	Bn	Br	Br	Up	-	-
15	CH ₂ OH	Bn	Bn	Br	Br	Down	-	150 ± 19
16	CH ₂ OH	Bn	Bn	Br	Br	Up	-	-
17	CH ₂ OTr	Bn	Bn	H	H	Up	-	-
18	CH ₂ OH	Bn	Bn	H	H	Up	-	-

24	CH ₂ OH	p-N ₃ Bn	p-N ₃ Bn	H	H	Down	-	-
25	CH ₂ OTr	Bn	H	Cl	Cl	Down	-	200 ± 22
26	CH ₂ OH	H	Bn	Cl	Cl	Down	-	-
27	CH ₂ OH	Bn	H	Cl	Cl	Down	500 ± 9	400 ± 24
28	CH ₂ OBn	Bn	Bn	-	-	-	-	-
30	CH ₂ OBn	Bn	Bn	Cl	Cl	Down	-	-
30	CH ₂ OBn	Bn	Bn	Cl	Cl	Up	-	-
31	CH ₂ OH	H	H	Cl	Cl	Down	-	-
32	CH ₂ OBn	Bn	Bn	Br	Br	Down	-	-
33	CH ₂ OBn	Bn	Bn	Br	Br	Up	-	-

Table 3.1. IC₅₀ analysis of the library synthesised in chapter 2 against yeast strains BY4741 and $\Delta pdr1\Delta pdr3$. All compounds were tested. Those with no measurable activity are shown with a dash (-). Five compounds were identified as active against $\Delta pdr1\Delta pdr3$, with one showing activity against BY4741. Each compound was measured against the two strains of *S. cerevisiae* at 1, 0.314, 0.100, 0.031, 0.010, 0.003 and 0.001 mM in three technical and two biological replicates.

3.4.4 Matrix derivatisation

The matrix derivatisation was undertaken as described in section 3.3.9 of the methods. The study was carried out to synthesise a matrix that would have an alkyne that would act as an attachment point to **24**, which contains an azide moiety which is able to undergo a Huisgen cyclization reaction within a cell lysate. This was carried out in two steps, 1) The attachment of the alkyne-substituted amine to the epoxide and 2) subsequent Huisgen cyclization. An experiment was carried out to confirm that the resin can capture the azide derivative, as indicated by a colour change since traditional identification techniques (NMR, IR and MS) are not suitable for resin-supported chemistry. An azulene-azide derivative (6-[2-(4-azidophenyl)ethyl]azulene) that is coloured bright blue and contains a p-azidobenzyl group was chosen as a substrate to show that cyclization is possible. The bright blue colour could not be removed from the matrix when washed with multiple solvents (water, ethanol and ether), showing the cyclization worked and the 6-[2-(4-azidophenyl)ethyl]azulene was attached via the copper-activated Huisgen Cyclization.

Figure 3.5. Proposed structure of the azulene-azide derivative undergoing a Huisgen Cyclisation, resulting in “staining” of the matrix bright blue.

3.4.5 Chemical proteomic analysis

A chemical proteomic analysis study was undertaken as described in section 3.3.13. The study was employed to determine the potential biological target(s) of benzose.

An MS/MS analysis was used to determine the identity the peptides that were digested off the lysate treated **24**-matrix. The control resin (alkyne-substituted matrix without **24** attached) was shown to have interacted with three proteins with a Z-score greater than 56 which was determined by Protein Pilot™ 3.0 to be the cut off for statistically relevant results. All proteins found were part of the glyceraldehyde-3-phosphate dehydrogenase family, presumably retained due to residual epoxide groups that had not fully reacted with 3-aminopropan-1-ol (McCaul & Byers, 1976). The study with the **24**-matrix presented four proteins with three matching those isolated by the control resin; the glyceraldehyde-3-phosphate dehydrogenase family. The remaining protein was with a Z-score of 73, Tef2p, *TEF1* encodes the Translational elongation factor EF-1 alpha, however this was also then identified in the control resin upon validations. Subsequent bioactivity analysis of **24** indicates that the compound is inactive, and this is likely to explain the inability of the **24**-derivatised matrix to retain proteins (see section 3.4.3). Currently no biological targets have been identified.

3.4.6 Benzose breaks done in agar

The IC_{50} of benzose in solid agar had to be determined before undertaking the chemical genomic screen. The initial experiment unexpectedly showed no activity of benzose against any strain of plate 10 of the $\Delta pdr1\Delta pdr3\Delta xxx$ library, up to 100 μM . Subsequently a second experiment was carried out at concentrations up to 200 μM , in this case an IC_{50} was recorded at 70 μM , which was inconsistent to the previous study. It was proposed that the benzose might be breaking down in agar. To test this, an experiment was carried out where plates were left for 4, 10, 14 and 18 hours from pouring. Plates older than 14 hours showed a marked decrease in activity of benzose; the change in IC_{50} with time is shown in figure 3.6. On the basis of these results, 4 hours after the plates were poured was determined to be the best for the subsequent chemical genomic screen, permitting the agar to cool and set, but retain activity. Temperature was also determined to be a factor in the breaking down of benzose, it was decided to add benzose to the agar directly before each plate was poured to limit the contact with heated agar.

Figure 3.6. Breakdown of benzose in agar. It was found that benzose breaks down to unknown products over time, decreasing the activity against $\Delta pdr1\Delta pdr3$. The data points represent average concentration of benzose required to reach the IC_{50} at each time point. The error bars equate to the standard deviation between the three replicates.

3.4.7 Chemical genomic Assay

The *PDR1* and *PDR3* genes encode transcription factors for elements of the pleiotropic drug resistance network including the major efflux pump *pdr5*. The library of $\Delta pdr1\Delta pdr3\Delta xxx$ modified yeast strains is thus sensitised toward drug-like compounds, while also harbouring a different gene deletion in each strain of the library. This was tested against benzose to identify strains that had elevated sensitivity or resistance to the compound. To be classified as resistant, the yeast strain had to show residual growth above 0.40 at 50 μ M, 10 standard deviations above $\Delta pdr1\Delta pdr3\Delta his$ (0.36 ± 0.004), which was used as a proxy for wild type. For a strain to be classed as sensitive it had to show residual growth below 0.36 at 30 μ M, two standard deviations below $\Delta pdr1\Delta pdr3\Delta his$ (0.68 ± 0.065).

The ~4800 strains were tested in a 1536 strain array format (394x four replicates per plate) as shown in figure 3.7. The strains that have a square box around them have been classified as showing enhanced resistance. The screen was performed with three biological replicates as well as two technical replicates with four colonies of each strain.

Figure 3.7. Example of a single resistant gene from plate 8, left DMSO control, right benzose (70 μ M) treated. The 1536 pinned plate consists of 384x four replicates, 309 different single deletion mutants, the border consists of *his3* (56x four replicates).

Of the 4800 strains, 56 were identified as having enhanced resistance. The 56 strains are presented in table 3.3. Of these, 13 were annotated as dubious open reading frames. Notably 25 strains were found to be involved in transcription or translation.

A total of 18 $\Delta pdr1\Delta pdr3\Delta xxx$ sensitive strains were identified (table 3.2). Of the 18, (*APL5*, *ATP11*, *CHO2*, *CIT1*, *IRE1*, *NUP133*, *OPI3*, *PDR16*, *PHO80*, *RPN4*, *SAP190*, *SIP3*, *SSD1*, *TGL2*, *TPM1*, *YJR087w*, *YKL207w*, and *YNR021w*) three (*YJR087w*, *YKL207w*, and *YNR021w*) were annotated as dubious open reading frames or uncharacterised; and seven (*APL5*, *CIT1*, *OPI3*, *PDR16*, *PHO80*, *SIP3*, and *TGL2*) were annotated as multi drug sensitising (Hillmeyer 1997) deletion strains (frequently flyers as described in section 3.2.3). Of the remaining eight, five (*ATP11*, *IRE1*, *NUP133*, *RPN4* and *SSD1*) were found to be involved in transcription or translation.

Systematic name	Alias	Description of biological process
YKL207W	AIM27	required for efficient folding of proteins in the ER
YPL195W	APL5	vesicle-mediated transport; protein binding AP-3 adaptor complex
YNL315C	ATP11	protein complex assembly; unfolded protein binding
YGR157W	CHO2	telomere maintenance; phosphatidylethanolamine N-methyltransferase activity
YNR001C	CIT1	tricarboxylic acid cycle; citrate (Si)-synthase activity; mitochondrion
YHR079C	IRE1	protein amino acid phosphorylation; protein serine/threonine kinase activity.
YKR082W	NUP133	mRNA export from nucleus; structural molecule activity; nuclear pore
YJR073C	OPI3	phosphatidylcholine biosynthetic process
YNL231C	PDR16	response to drug; phosphatidylinositol transporter activity; cytoplasm
YOL001W	PHO80	telomere maintenance; cyclin-dependent protein kinase regulator activity
YDL020C	RPN4	telomere maintenance; transcription factor activity

YKR028W	SAP190	G1/S transition of mitotic cell cycle; protein serine/threonine phosphatase activity
YNL257C	SIP3	transcription initiation from RNA polymerase II promoter
YDR293C	SSD1	intracellular mRNA localization; negative regulation of translation; regulation of fungal-type cell wall organization
YDR058C	TGL2	lipid metabolic process; triacylglycerol lipase activity; cellular component
YNL079C	TPM1	pseudohyphal growth; actin lateral binding; bud neck contractile ring
YJR087W		Dubious open reading frame that overlaps EMC2 which is required for efficient protein folding
YNR021W		biological_process unknown. Localises to the endoplasmic reticulum

Table 3.2. Gene deletion strains identified as sensitive in the chemical genomic screen treated with Benzose (70 μ M). The strains identified as frequent flyers are identified by a grey background.

Systematic name	Alias	Description of biological process
YDR524C	AGE1	ADP-ribosylation factor; GTPase activating protein effector involved in secretory and endocytic pathways
YDR525W	API2	dubious open reading frame
YDR376W	ARH1	oxidoreductase of the mitochondrial inner membrane, involved in cytoplasmic and mitochondrial iron homeostasis
YMR116C	ASC1	G-protein beta subunit and guanine nucleotide dissociation inhibitor for Gpa2p
YLR393W	ATP10	mitochondrial inner membrane protein required for assembly of the F0 sector of mitochondrial F1F0 ATP synthase
YFL023W	BUD27	protein involved in bud-site selection
YPL178W	CBC2	possibly to package mRNA for export from the nucleus; contains RNA-binding motif
YER061C	CEM1	beta-keto-acyl synthase with possible role in fatty acid synthesis
YGL019W	CKB1	beta regulatory subunit of casein kinase 2 has many substrates including transcription

		factors and all RNA polymerases
YKL139W	CTK1	catalytic subunit of C-terminal domain of kinase I, Phosphorylates both RNA pol II to affect transcription and pre-mRNA 3'end processing and ribosomal protein
YBR026C	ETR1	a probable role in fatty acid synthesis
YFL237C	HAP2	subunit of the heme-activated, glucose-repressed HAP2p/3p/4p/5p CCAAT-binding complex, a transcriptional activator and global regulator of respiratory gene expression
Control	HIS3	imidazoleglycerol-phosphate dehydratase, catalyzes the sixth step in histidine biosynthesis; mutations cause histidine auxotrophy and sensitivity to Cu, Co, and Ni salts; transcription is regulated by general amino acid control via GCN4p
YDR528W	HLR1	regulation of cell wall composition and integrity and response to osmotic stress
YHR187W	IPK1	Inositol 1,3,4,5,6-pentakisphosphate 2-kinase, nuclear protein required for synthesis of 1,2,3,4,5,6-hexakisphosphate (phytate), which is integral to cell function; has 2 motifs conserved in other fungi
YGL086W	MAD1	coiled-coil protein involved in the spindle-assembly checkpoint
YDR005C	MAF1	highly conserved negative regulator of RNA polymerase III
YRP070W	MED2	subunit of the RNA polymerase mediator complex; essential for transcriptional regulation
YFR034C	PHO4	basic helix-loop-helix transcription factor of the myc-family; activates transcription cooperatively with PHO2p
YDR276C	PMP3	small plasma membrane, deletion causes hyperpolarization of the plasma membrane
YPL157W	QCR2	subunit A21:D40nit 2 of the ubiquinol cytochrome-c reductase complex involved in electron transport chain
YER083C	RMD5	component of GID Complex that confers ubiquitin ligase (U3) activity; necessary for polyubiquitination and degradation of the gluconeogenic enzyme fructose-1,6-bisphosphatase
YFR048W	RMD7	involved in insertion of proteins into the ER membrane; required for the retrieval of HDEL proteins from the Golgi and ER

YBL027w	RPL19b	ribosomal 60s subunit protein
YPR043W	RPL43A	ribosomal 60s subunit protein
YLL045C	RPL8B	ribosomal 60S subunit protein L8B
YJL191W	RPS14b	protein component of the small (40S) ribosomal subunit; required for ribosome assembly and 20S pre-rRNA.
YNL302C	RPS1a	ribosomal protein 10 (RP10) of the small (40S) subunit
YPL090C	RPS6A	ribosomal 40s subunit protein
YBL025W	RRN10	protein involved in promoting high level transcription of rDNA, subunit of UAF for RNA polymerase I
YNL254C	RTC4	protein of unknown function; null mutation suppresses CDC13-1 temperature sensitivity;
YDL225W	SHS1	component of the septin ring that is required for cytokinesis; septins are GTP-binding proteins that assemble into rod-like hetero-oligomers that can associate with other rods to form filaments
YBR289W	SNF5	subunit of the SWI/SNF chromatin remodeling complex; involved in transcription regulation
YHR008C	SOD2	protects cells against oxygen toxicity
YGL127C	SOH1	subunit of the RNA polymerase II mediator complex; associates with core polymerase subunits to form the RNA polymerase II holoenzyme; involved in telomere maintenance
YMR179W	SPT2	protein involved in negative regulation of transcription
YER161C	SPT21	protein with a role in transcriptional silencing; required for normal transcription at several loci including HTA2-HTB2 and HHF2-HHT2, but not required at the other histone loci
YOR247W	SRL1	mannoprotein that exhibits a tight association with the cell wall, required for cell wall stability in the absence of GPI-anchored mannoproteins
YGL024W	SST3	dubious open reading frame unlikely to encode a protein that overlaps with <i>PGD1</i> , a RNA polymerase II mediator, and STE18 involved in mating signalling.
YPR191W	TGS1	trimethyl guanosine synthase; required for nucleolar assembly and splicing of meiotic pre-mRNAs
YDR354W	TRP4	anthranilate phosphoriboyl transferase of the tryptophan biosynthetic pathway.

YPR066W	UBA3	protein that acts together with ULA1p to activate RUB1p before its conjugation to proteins which may play a role in protein degradation
YDR092W	UBC13	ubiquitin-conjugating enzyme involved in the error-free DNA postreplication repair pathway; interacts with MMS2p to assemble ubiquitin chains at the Ub Lys-63 residue; DNA damage triggers redistribution from the cytoplasm to the nucleus
YER151C	UBP3	ubiquitin-specific protease involved in transport and osmotic response; interacts with Bre5p to co-regulate anterograde and retrograde transport between the ER and Golgi; involved in transcription elongation in response to osmotic stress through phosphorylation at Ser695 by HOG1p
YNL197C	WHI3	RNA binding protein that sequesters CLN3 mRNA in cytoplasmic foci; Tpk1 (PKA) mediated phosphorylation of WHI3 affects the G1/S phase transition by modulating CLN3 transcription
YDR250C		dubious open reading frame unlikely to encode a protein
YGL036W		dubious open reading frame unlikely to encode a protein
YLR365W		dubious open reading frame unlikely to encode a protein
YLR366W		dubious open reading frame unlikely to encode a protein which overlaps RPS22B
YNL170W		dubious open reading frame unlikely to encode a protein. Overlaps with PSD1 (Phosphatidylserine decarboxylase)
YPL257W		putative protein of unknown function; homozygous diploid deletion strain exhibits low budding index

Table 3.3. Genes deletion strains identified as resistant in the chemical genomic screen treated with Benzose (70 μ M)

3.4.8 Validation of high throughput results

High throughput assays are prone to errors. False positives, where sensitivity or resistance is erroneously indicated, can be identified by careful validation of the identified deletion strains. Such false positives can arise from a number of sources. Competition can cause a decrease in growth, when normal growing yeast is situated next to fast growing strains or an apparent increase when neighbouring a slower growing stain. Also mispinnings lead to colonies where fewer cells were collected during pinning, leading to apparent reduced growth across a quadruplet for a strain. Finally it is unclear if the breakdown product(s) observed in section 3.4.6, may have an effect on some strains of yeast. For these reasons it was decided to validate the resistant and sensitive strains in liquid SC culture.

3.4.8.1 *Validation of Resistant strains*

The validation of the resistant strains were undertaken as described in section 3.3.16 of the methods. Of the 56 validated strains assessed, 34 strains showed enhanced resistance compared to the $\Delta his3$ control to 50 μM benzose in SC culture, shown in graph 3.9 and an example of the resistance against HIS control is shown in figure 3.8. No further studies were completed to determine the precise IC_{50} of any resistant strain.

Δ TGS1					Δ HIS3				
		1	2	3		1	2	3	
DMSO control	A	0.447	0.467	0.481	A	0.433	0.407	0.420	
30 μ M Benzose	B	0.441	0.420	0.445	B	0.357	0.229	0.277	
50 μ M Benzose	C	0.419	0.450	0.431	C	0.148	0.152	0.156	
70 μ M Benzose	D	0.490	0.429	0.425	D	0.161	0.140	0.148	

Figure 3.8. Sample of resistant validations from the chemical genetic screen. Each strain was subjected to three concentrations of benzose (30, 50, 70 μ M). Strains that showed residual growth greater than 0.400 at 50 μ M treatment of benzose (10 stdev of $\Delta HIS3$ control average) were classed as resistant. $\Delta TGS1$ shows resistance against benzose at all concentrations of benzose, at 50 μ M $\Delta TGS1$ has an average (three replicates) residual growth of 0.932 ± 0.016 at least 0.5 greater than $\Delta HIS3$.

3.4.8.2 Validation of sensitive strains

The validation of the sensitive strains was carried out as described in section 3.3.16 of the methods. All 18 strains that were identified as sensitive in the high throughput screen validated as sensitive in SC media (*OPI3*, *CIT1*, *APL5*, *YKL207w*, *TGL2*, *PHO80*, *SIP3*, *PDR16*, *SAP190*, *YNR021w*, *YJR087w*, *CHO2*, *IRE1*, *ATP11*, *TPM1*, *RPN4*, *SSD1* and *NUP133*). The validation of these sensitive strains was undertaken with lower benzose concentrations; 50– 10 μ M. The results showed that four of these strains were sensitive below 10 μ M.

Figure 3.9. Residual growth of all strains identified as resistant to benzene in the chemical genomic screen. The resistant genes were subjected to validations against 50 μ M of benzene in SC media. Genes that were validated as resistant are dark blue those that were identified as not equal are represented as light blue. The bars represent the average residual growth

3.4.9 Benzose has no effect on the unfolded protein response

In order to determine if the unfolded protein response (UPR) was activated by benzose treatment, YCG266-4xUPRE-GFP yeast cells were treated with the compound and examined for UPR activation via confocal microscopy. Benzose treated cells were compared to cells treated with a known UPR inducer, dithiotheritol (DTT). No evidence was seen for induction of the UPR by treatment with benzose.

Figure 3.10. UPR assay to determine protein misfolding, benzose showed no increase in protein folding. DTT was used as a control, as it is a known protein misfolding inducer. Whole cell GFP intensity, measured at 520 nm, increases with concentration in DTT treated cells, indicating UPR activation. GFP intensity remains relatively constant in benzose treated cells, indicating a non-existent response.

3.4.10 Antibiotic assay

Current experiments have shown no activity in bacteria (*E. coli* DH5a and Psa-V 18800).

3.5 DISCUSSION

This project identified a range of biologically active compounds. The library of compounds synthesised consisted mainly of cyclopropane-carbohydrates with a range of glucal precursors and by-products. This library was screened for inhibitory biological activity for mechanism and mode of action studies in *S. cerevisiae*. Twenty-seven compounds were synthesised and screened for inhibitory activity, producing five active compounds.

Of the synthetic compounds identified as active, the most potent was found to be benzose, therefore, was selected for further study.

3.5.1 Dose response assays

A series of dose response assays were carried out in SC broth against *S. cerevisiae* BY4741 and $\Delta pdr1\Delta pdr3$ strains to determine the characteristics of the studied compounds with regards to general toxicity.

A liquid phase response assay showed that benzose, and compounds **11**, **15**, **20** and **27** inhibited yeast growth in the low to mid micromolar range. Benzose inhibited all cell growth with an IC_{50} concentration of 40 μ M in the $\Delta pdr1\Delta pdr3$ strain, but was inactive against BY4741, identifying efflux pumps as having an effect on toxicity. Benzose was shown to have no activity against human cell lines HL-60 and 1A-9. Furthermore benzose was also found to have no activity as an antibacterial agent.

The $\Delta pdr1\Delta pdr3$ strain has a reduced ability to efflux xenobiotics through ATP-binding cassette (ABC) transporters (Taibi & Taymound, 1999). The major ABC transporters are able to export a wide range of substrates (Jungwirth & Kuchler, 2006) and the structure and hydrophobicity of benzose fits the character of an ABC transporter substrate. For many

drugs, including benzose, the *PDR1* + *PDR3* combined deletions are sufficient to increase the sensitivity of the cell (Nourani et al. 1997; Rogers et al. 2001). *PDR1* and *PDR3* are genes that produce transcription factors that are considered to be master regulators of the pleiotropic drug resistance systems, which includes the major ABC efflux pumps (Onda et al. 2004). These multideleted knock-outs are therefore sensitive to a wide array of compounds and can be used for screens to identify weakly-active lead structures which can often be missed by classic drug screening (Rogers et al. 2001). The yeast $\Delta pdr1\Delta pdr3\Delta xxx$ library was used as it is a valuable tool for the genomic screening, coupling its increased sensitivity with the utility of a deletion library for revealing chemical genetic profiling to probe benzose's mode of action (Ferrari et al. 2010; Coorey, 2012). The $\Delta pdr1\Delta pdr3$ and BY4741 dose response was further utilised to identify whether benzose acts in a cytostatic or cytotoxic manner.

3.5.1.1 Cytostatic and cytotoxic

In the initial dose response assays with benzose, only partial inhibition of growth was observed for the PDR-deficient strain $\Delta pdr1\Delta pdr3$ between 0.1 μM to 1000 μM (section 3.4.2). Growth was only observed at 0.1 μM to 100 μM concentrations while 314 μM showed no signs of growth beyond the second cell dilution. This may be due to irreversible inhibition of growth at high concentrations but a reversible inhibitory effect at lower concentrations. This has been previously reported in literature (Van Goietsenoven et al. 2012; Dumont et al. 2007). For example, narciclasine, a potent eEF1 α inhibitor, induces cytotoxic mitochondrial apoptosis at ≥ 200 nM whereas cytostatic impairment of actin cytoskeleton organisation is induced at 50 nM (Van Goietsenoven et al. 2012; Dumont et al. 2007). The results above suggest that, like narciclasine, benzose is exhibiting both cytostatic and cytotoxic effects.

The remaining compounds did not show activity or have any effect at low enough concentration to be considered as further lead compounds. However, the dose response assay was able to give insight into the biological mechanism through structure activity relationships.

3.5.2 Structure activity relationship of cyclopropanated carbohydrates

The structures and properties of each of the five active compounds (benzose, **11**, **15**, **20** and **27**) against $\Delta pdr1\Delta pdr3$ were analysed to assess why they have different levels of biological activity. Five of the 27 compounds synthesised were active against $\Delta pdr1\Delta pdr3$, with benzose being the most active (40 μ M). However only one, **17** (0.4 mM) was active against BY4741 (*S. cerevisiae*), as described in section 3.4.1. To understand the activity the compounds will be discussed in turn based on the three proposed mechanisms discussed (section 1.4.2).

The following assumptions are also made in this analysis;

- I) All compounds share the same target; and
- II) the compounds function through covalent interactions with their target or, must be covalently modified prior to interaction with their target.

Given a proteins' ability to catalyse reactions, the inherent activity of an individual substrate may be masked. Thus activity will arise from a combination of a compound's ability to bind to the target and its ability to undergo the appropriate transformation.

3.5.2.1 Mechanism 1

As introduced in sections 1.4.1 and 2.2.1, the cyclopropane is a strained three-membered ring that is susceptible to nucleophilic attack. Of the active compounds (benzose, **11**, **15**, **20**, **27**), all have a cyclopropane and all can undergo the proposed ring breaking mechanism (scheme 3.1). For a compound to go via this mechanism it must be able to stabilise a carbanion, and have a good leaving group on the cyclopropane (Hewitt & Harvey, 2008). All five active compounds are able to fulfil these attributes. Compound **15** is the only one of the five active compounds that has a dibromocyclopropane. Bromines are large halogens that act as very good leaving groups (Bartoli & Todesco, 1977), however their ability to

stabilise a carbanion, is not to the same degree as chlorine (Bickelhaupt et al. 2005). It has been shown computationally by Bickelhaupt et al. (2005) that α -stabilization of the carbanion by CH_2X^- is strongest for fluorine and become less so along the series $\text{X} = \text{F}, \text{Cl}, \text{Br}$ and I . This is shown to follow the observed decrease in basicity of CH_2X^- along this series, the reduced basicity corresponds to a decrease in proton affinity of CH_2X^- . When applying this computational model to the anion stabilisation proposed in mechanism 1, it would be expected that the dibromine will be less active than the dichlorocyclopropane-carbohydrate. This is seen in the difference in activity between benzose and **15**. The monochlorocyclopropane **11** is eight-fold less active than benzose. This change in activity can be attributed to the removal of one of the two chlorides from benzose. The loss of a chloride would reduce the effect of carbanion stabilisation (Brinkman et al. 1994), resulting in lower activity. Furthermore, the activity of **11** is less than **15**, which adheres to the stabilisation of the carbanion effect, two bromines have a greater stabilisation effect over a single chlorine.

Scheme 3.1. Mechanism 1

3.5.2.3 Mechanism 2

The two proposed ring opening mechanisms may be operating, however the evidence is presently against this. Scheme 3.2 shows the loss of a substituent of the cyclopropane in a Ferrier-like rearrangement, for this to occur the substituents on the cyclopropane must be good leaving groups, this is the case. However a bromine is a much better leaving group than chlorine (Alonso & Rossi, 1980) so it would be expected **15** would be more reactive than benzose, this is not the case as shown in section 3.4.3. Therefore, it is doubtful that this is the mechanism of action.

Scheme 3.2. Mechanism 2

3.5.2.2 Mechanism 3

The mechanism shown in scheme 3.3 is similar to the above, however in this case the leaving group is on the C-3 position. Compounds **11**, **15** and benzose all have an OBn, which can act as a leaving group when activated by an electrophile (Rooseboom et al. 2004). However two of the five compounds have a hydroxyl group, which is a very poor leaving group (Qin et al. 2007). Although catalytic activation by the protein target may permit this transformation, it remains less likely.

Scheme 3.3. Mechanism 3

At this point the evidence discussed here is pointing towards the carbanion intermediate mechanism, as depicted in scheme 3.1.

The orientation of the cyclopropane proved to be important for activity as all five active compounds had the cyclopropane in the “down” position (ie on the same face as the C-6 substituent of the pyran), whereas the stereoisomers in the “up” position showed no activity. This effect could be caused by multiple factors. Sterics play an important part of all drug design. If the structure does not fit with the target it will not be active. For example a study by Carotti et al. (2002) demonstrated that removing the methyl group in the 3' position of geiparvarin increased its level of inhibition of monoamine oxidase. This was due to reduction in the steric hindrance. Todoroki et al. (1996) showed that the “down” unfunctionalised cyclopropane of abscisic acid analogues was inactive against the

stomatal opening of the epidermal strips of spiderwort, lettuce seed germination, α -amylase induction by gibberellin A-3 in barley half-seeds and elongation of the second leaf sheath of rice seedlings, whereas the “up” cyclopropane was active, the difference in activity was contributed to the stereochemistry of the cyclopropane in a tight binding pocket.

3.5.2.4 C-6 hydroxyl is needed for activity

Benzose has a C-6 hydroxyl, it was found that when this was protected by either a trityl **5**, formate ester **8** or benzyl **29** the activity is completely lost (excluding **25** for reasons later discussed). This suggests either an unfavourable steric interaction or that H-bonding is required for activity. Interestingly, **25** which retains OTr but lacks a C-3 OBn is somewhat active, albeit at a five-fold higher concentration than benzose. This may be because of hydrogen bonding within the pocket as both the C-3 hydroxyl and C-6 hydroxyl may be able to interact with the same hydrogen bond acceptor shown in figure 3.11. The enzyme acts as a hydrogen bond donor and the compound the acceptor because compound **9** with the formate esters would be able to accept a hydrogen bond, however it is

Figure 3.11. Proposed hydrogen bonding in the active site with C-3 and C-6 hydroxyl. A: represents the hydrogen acceptor in the hydrogen bond formation. The blue hydrogen bond represents compounds; **9, 11, 15, 16, 18, 24, 27, 26** and benzose, the orange hydrogen bond represents compounds; **25, 27** and **26**.

inactive.

Surprisingly, **24** was found to be inactive due to the addition of an azide in the *para*-position of the benzyl group. This suggests that the benzyl protecting groups are situated inside the active site pocket.

In all cases of activity the cyclopropane was in the “down” position and the “up” was always found to be inactive. It is proposed that benzose is in a tight-binding enzymatic pocket, and the enzymatic nucleophile is attacking the anomeric centre (C-1), and is positioned so it can undergo nucleophilic attack at the required angle as shown in figure 3.12. However, in the proposed position with compound **8** the nucleophile would be unable to attack the “up” cyclopropane, resulting in inactivity.

Figure 3.12. Proposed nucleophilic attack of the aromatic centre of compound **9** and benzose. Benzose is active, whereas **9** is inactive suggesting steric hindrance as a factor, or the nucleophile is situated at an angle that can attack the “down” cyclopropane but not the “up” cyclopropane. A: represents the proton acceptor in the hydrogen bond formation (Nitrogen or Oxygen), Nu: represents the nucleophile attacking the aromatic centre.

3.5.3 The chemistry of benzose

It is possible, as we have seen in the structure activity relationships of the cyclopropanated-carbohydrates and their precursors, to predict their chemistry within a cell. There are several determinations that can be made about benzose in this manner. Benzose (1,5-Anhydro-3,4-di-O-benzyl-2-deoxy-1,2-C-(dichloromethylene)-D-glycero-D-gulo-hexitol) is a non-polar, cyclopropanated carbohydrate. The apparent affinity of the compounds for ABC transporters suggests that the toxicity of benzose is limited by the detoxification pathways of the cell. This was expected as benzose had been designed to be highly selective with its target. The proposed mechanism of benzose (scheme 3.4) shows that ring breaking will not occur unless the structural requirements are met. This is further backed up by the SAR analysis that shows the addition of either an azide, C-6 hydroxyl protecting group, or “up” cyclopropane results in full loss of activity.

The mechanism proposed in scheme 3.4 would see the nucleophile within the targets active site being irreversibly bound to benzose. The nucleophile attacks at C-1, results in ring breaking and the formation of a stabilised anion, which is stabilised by the two chlorines. Through acid-base catalysis, the anion attacks a proton, a base attacks the C-2 proton with results in the formation of an alkene and elimination of chlorine. However, this mechanism can stop once C-7 has been protonated.

This reaction could have a range of deleterious effects including covalent modification of the proteins structure, blocking the active site, or interfering with protein-protein interactions (Way, 2000; Johnson, 2004; Stadtman, 1990). Covalent modification may not be occurring if the protein is attacking the cyclopropane via an activated water molecule (Liu et al. 1995). This mechanism will still occur although benzose will not irreversibly bind to the protein.

Benzose is non-polar with a polar alcohol region, which is likely to form hydrogen bonds within the active site of the enzyme. Non-polar drugs are able to diffuse through the phospholipid bilayer membrane (Schwendener & Schott, 2010), meaning they are able to be incorporated into the cell without the need of active transport. Hydrophobic compounds are more likely to be “drug-like” as they have a high ligand-lipophilic efficiency score which is associated with drug-likeness (Leeson & Springthorpe, 2007). Ligand-lipophilic efficiency is a parameter used to link potency and lipophilic nature associated with a compound to estimate its potency as a

drug.

The proposed mechanism of benzose suggests a likely pathway of how the compound acts to inhibit yeast survival. The primary biological effect of toxicity suggests that inhibition of cellular growth is a result of selective inhibition of an essential protein.

Scheme 3.4. The proposed enzymatic step of benzose within the active site of an unknown protein, where a nucleophile is present that is able to attack the C-1 (anomeric) position. The addition of the nucleophile causes the cyclopropane to break resulting in the rearrangement of bonds forming a stabilised anion, which is then able to attack a proton then elimination of H-2 by a base and elimination of Cl.

3.5.4 Alkyne-substituted sepharose matrix

This study was undertaken to identify possible methods of attaching benzose to a resin in order to create a tool for identification of potential benzose-interacting proteins. The investigation yielded a possible method of attachment and also a method to identify whether the chemistry was viable, as described in results section 3.4.4.

Through testing a range of cyclopropane-containing glycosides and glucals for activity against $\Delta pdr1 \Delta pdr3$, benzose was found to be the most promising candidate for pull-down studies due to the low micromolar activity. However there were no suitable points of attachment on benzose for the matrix attachment, as any modification to the C-6 hydroxyl would most likely cause loss of activity as shown by **6**, **9** and **30** in the SAR as previously mentioned in 3.5.2.

Therefore a strategy was proposed to add the attachment point to the C-3 or C-4 hydroxyl. A viable option was the use of p-azidobenzyl as it is already similar to the benzyl on the C-3 and C-4 hydroxyl and has been previously used to attach a photoaffinity probe (Young, 1983). Additionally the p-azidobenzyl could undergo a Huisgen Cyclisation reaction, within a cell lysate, to a corresponding alkyne resin leading to identification of the biological target (Wang *et al.* 2009). By doing so it would overcome the problem of binding benzose to a matrix. Azides are small and have no biological effect and was anticipated to have little effect on the binding of benzose. Compound **24** was therefore synthesised as a tool to identify the likely target(s) of benzose.

It was decided to modify an epoxy-activated sepharose 6B resin to an alkyne-substituted sepharose resin. The epoxy-activated sepharose resin has an advantageous 13-atom spacer which is linked to an epoxide through which an alkyne can be attached. It has also been reported that alkyne-modified probes display suitable stability for profiling enzyme

activities in cells (Speers & Cravatt, 2004), as they can undergo the Huisgen Cyclisation with azide-containing compounds such as **24**.

The epoxide matrix was treated with three different alkyne derivatives to determine which would lead to a desired alkyne-substituted sepharose matrix. The short optimised synthesis of the alkyne- substituted sepharose 6B resulted from the propargylation of 3-aminopropan-1-ol with propargyl bromide. Two other amines were also tried but they did not present a viable option. The amines were too volatile, which resulted in their loss under the reaction conditions required with the propargyl bromide. The 3-(prop-2-ynylamino)propan-1-ol was subsequently reacted in a vast excess with the epoxy-activated sepharose 6B matrix to give an unknown yield (scheme 3.5). Traditional chemical identification techniques are not amenable to quantifying the outcome of matrix derivatisations of this sort, therefore a proof of principle using colour was used to identify the product.

Scheme 3.5. Synthesis of the alkyne-sepharose matrix.

A fellow chemist in the School of Chemical and Physical Sciences (Victoria University of Wellington) has been working on azulene derived compounds that are densely coloured even at nanomolar concentrations (Bevan, 2010). Given its colour and availability, 6-methylazulene was reacted with p-azidobenzyl bromide. The product 6-[2-(4-azidophenyl)ethyl]azulene was then reacted under Huisgen Cyclisation conditions (Speers & Cravatt, 2004) in a cell lysate with the alkyne-substituted sepharose 6B matrix. No yield was recordable. However, when compared to a control reaction with the click conditions and underivatized epoxide activated resin, it was clear the p-azidobenzyl azulene substituted the alkyne-substituted matrix, giving a persistent blue colour. As figure 3.13 shows, there is retention of colour on the azulene substituted matrix. Ammonia was used to remove any copper species, by forming tetraamminecopper(II) sulphate, ensuring the only remaining blue colour could come from immobilised azulene. The Huisgen Cyclisation conditions lead to a blue sepharose matrix and without the click conditions a white sepharose matrix was present. While these results were only an indication that the matrix was undergoing a Huisgen Cyclisation reaction via the alkyne of the modified matrix, it was believed to be strong enough evidence to proceed. The azulene capture

Figure 3.13. Demonstrates visual results for azulene-sepharose matrix compared to the alkyne-sepharose matrix; for proof of principle purposes. Two samples of alkyne-sepharose matrix was subjected to azulene-azide; one with the Huisgen Cyclisation condition one without, to determine if azulene will “click” to the alkyne matrix. Both the alkyne-sepharose matrices were multiple solvent washes, then to an ammonia wash followed by multiple water washes. The sample treated under the cyclisation conditions lead to a blue matrix, while the other lead to white matrix, indication the reaction was successful.

from the cell lysate proved the principle, and the approach may be valuable in other cases (ie attempts to identify targets of other bioactive compounds).

Unfortunately, **24** has been shown to have no activity against $\Delta pdr1 \Delta pdr3$ (*S. cerevisiae*). However, pull-down from lysate was attempted as weak binding, metabolic degradation or reduced cell entry could have been the cause of the lack of activity. It could also indicate whether **24** is binding to proteins that do not lead to cell death. Unfortunately, and in line with the lack of activity, no proteins were selectively isolated using the affinity matrix, which indicates substantial constraints operating with this part of the target's binding pocket. Future work may identify a compound that can utilise the alkyne-modified matrix.

3.5.5 Chemical genomic screen gives insight to the biological role of benzose

During this study it was identified that benzose breaks down in agar (section 3.4.6), but through careful preparation of benzose-containing agar plates, the $\Delta pdr1 \Delta pdr3 \Delta xxx$ deletion library was screened identifying 53 resistant and 18 sensitive strains. All sensitive strains were verified in liquid culture, along with 34 of the 53 resistant strains.

Benzose belongs to a class of cyclopropanated carbohydrates which have been shown to have activity against cancer cell lines (Sanhueza et al. 2007). Benzose may bind and inhibit a protein synthesis related protein via one of the proposed mechanisms (section 1.4.2) as indicated by the genomic screen results, and so inhibit growth in $\Delta pdr1 \Delta pdr3$ cells. However, no inhibition was observed in the HL-60 and 1A9 mammalian cell lines or the bacterial strains Psa-V 18800 and *E.coli* DH5a, suggesting that the role of benzose in inhibition is selective to *S. cerevisiae*. Compound **29** showed no activity in $\Delta pdr1 \Delta pdr3$ cells however, literature showed **29** as

having an IC₅₀ in the low micromolar range against HeLa (Sanhueza et al. 2007).

Figure 3.14. Compound **29** and benzose (**7**)

A number of benzose-resistant deletion mutants were identified in the chemical genetic screen, but failed to show functional enrichment by Gene Ontology (GO) analysis. However, a very high proportion of genes whose deletion lead to resistance encode for proteins involved in ribosomal transcription or translation (*CKB1*, *CTK1*, *MED1*, *RMD7*, *RPL19b*, *PLR43a*, *RPL43a*, *RPS14b*, *RPS1a*, *RPS6a*, *RRN10*, *SNF5*, *SPT2*, *SPT21*, *UBP3*, *SIP3*, *IRE1*, *ATP11*, and *RPN4*). The deletion of these genes could cause impairment in protein synthesis. Limiting protein synthesis has been identified as a method of increasing cell growth under pressures of toxicity such as an increase in misfolded proteins caused by bioactive compounds (Ding 2007; Steffen et al. 2012).

A previously reported chemical genetic screen using tunicamycin, a known N-linked glycosylation inhibitor in the ER, is often used to prompt the unfolded protein response. Yeast strains with ribosomal protein knockouts treated with tunicamycin have been shown to result in resistance, the associated resistance varied dramatically depending on the corresponding ribosomal protein knock out. Tunicamycin resistance was found to be partially *Hac1* independent. *Hac-1* independent resistance to ER stress in yeast is thought to activate transcriptional response to ER stress. The stress is reliant on the contact between RNA Pol II and the core promoter of ER chaperone genes (Schroder et al. 2003). Interestingly, a majority of the hits found in the genetic screen identify ribosomal protein

knock outs, resulting in resistance. Also, RNA Pol II related knockouts were identified as sensitive and resistant, with suppressors as sensitive and up regulators as being resistant.

Wild-type yeast was co-treated with tunicamycin and cycloheximide to determine whether reduced translation generally resulted in tunicamycin resistance, this was found to be true (Steffen et al. 2012). If this holds true as a comparison to benzose, a strain that has a decreased rate of protein synthesis will show resistance. Reducing the rate of protein synthesis will allow the cell to “keep up” with the increased misfolding. To identify whether decreased protein synthesis is causing resistance it would be advisable to co-treat with benzose and a known protein synthesis inhibitor. Recently, benzose was subjected to a protein misfolding assay.

These results suggest that benzose like Tunicamycin may induce the unfolded protein response, to determine this, the UPR assay was conducted.

3.5.6.1 The effect of benzose on the unfolded protein response UPRE

Benzose may have one or more roles in protein synthesis. For example, it may lead to production of misfolded proteins. Misfolded proteins are degraded and recycled to prevent toxic effects (Das et al. 2013; Goldberg, 2003). If benzose is causing an increase in misfolding of proteins, the cell's protein recycling pathways will be unable to keep up with the increased workload. Amongst the sensitive hits were $\Delta ire1$, $\Delta YJR087w$ (overlaps *emc2*), and $\Delta aim27$, all of which are associated with protein misfolding, indicating this as a potential function of benzose which will likely trigger the unfolded protein response (UPR) (Rao et al. 2004; Lin et al. 2007; Jonikas et al. 2009). The primary causes of UPR in a cellular context are elevated synthesis of secretory proteins, expression of mutant proteins prone to misfolding, and altered glycosylation (Kaufman, 2002). However, inhibition of proteins essential to protein folding will also lead to upregulation of the UPR system.

An assay was conducted to assess the ability of benzose to perturb protein folding. The UPR assay uses a strain of yeast in which the expression of a red fluorescent reporter protein (RFP) is controlled by the unfolded protein responsive element (UPRE). An increase in RFP levels acts as an indicator of UPR activation (Jonikas *et al.* 2009; Bernales *et al.* 2006; Okamura *et al.* 2000). Unfortunately, this UPR assay strain does not have the $\Delta pdr1\Delta pdr3$ background, and so it was recognised that the efficient efflux of benzose that ablates growth inhibition in the presence of a functioning PDR system could also lead to a negative response in this UPR assay system. Whilst a positive result in the assay would have confirmed an effect on protein folding, unfortunately the negative result obtained in this assay is not informative. Work is ongoing to place the UPR reporter construct into the $\Delta pdr1\Delta pdr3$ background.

The primary causes of UPR in a cellular context are elevated synthesis of secretory proteins, expression of mutant proteins prone to misfolding, and altered glycosylation (Kaufman, 2002). In this experiment, benzose did not produce a response from the UPR GFP reporter expression.

3.5.6.2 Deletion mutants sensitive to benzose

The 18 benzose hypersensitive strains were subjected to the same analysis as the resistant strains. However, eight of these strains have been previously identified as multidrug sensitising (Hillemeyer *et al.* 2008). The term multidrug sensitising is given to a gene deletion that leads to sensitivity to multiple drugs of diverse structure and activity. For example, a gene that encodes for a protein involved in cell wall synthesis, when removed may allow for a higher concentration of drug to diffuse into the cell, making the cell more susceptible to cell death (Hillemeyer *et al.* 2008; Parsons *et al.* 2004; Parsons *et al.* 2006). Of the 10 genes that are not multidrug sensitising, three were found to be involved in RNA localisation, one in the negative regulation of transcription from RNA polymerase II promoter in response to stress and three were found to be involved in the

negative regulation of cellular protein biosynthesis. Therefore, as observed with the resistant genes, the majority of sensitive genes are found to be involved in transcription and/or translation.

There were no statistically significant enriched processes relating to these sensitive strains, although removing multiple hypothesis test corrections (such as the Bonferroni correction), results in the phospholipid biosynthesis pathway being noted as enriched, albeit on the basis of only two strains (CHO2 & OPI3). Interestingly, IRE1, CHO2 and OPI3 genes result in a Yeastmine (www.yeastmine.org) publication enrichment for an article "The membrane stress response buffers lethal effects in disequilibrium by reprogramming the protein homeostasis" (Thibault *et al.* 2012).

3.9.6.3 Mutants effected by benzose

Fifteen of the gene deletion strains identified as either benzose sensitive or resistant encode ribosomal subunits or proteins involved in the regulation of protein synthesis, five of which were directly associated with RNA-polymerase I and II. The yeast ribosome consists of 78 ribosomal proteins, encoded by 137 genes, two per ribosomal protein. Twenty of the 30 most abundant mRNAs encode ribosome proteins. The mRNAs from these genes have significantly shorter half-lives compared to other mRNAs, and an estimated 50% of the RNA-polymerase-II-mediated transcription events involve ribosomal proteins (Warner, 1999). *S. cerevisiae* utilises transcription as its primary means of regulating ribosomal synthesis, whereas eubacteria utilise translation, albeit in very different ways.

To date benzose has only been found to be active in *S. cerevisiae*. It can therefore be suggested that benzose may have an effect on a step in translation or subsequent processing of, for example, a ribosomal subunit; however the exact biological mechanism through which benzose acts remains open for further investigation.

4.0 Overall discussion

Over the course of this study benzose has been developed as the most potent glycal inhibitor of growth in the *S. cerevisiae* strain $\Delta pdr1\Delta pdr3$, from the library synthesised. The genomic assay suggests a role within transcription and/or translation. It is currently believed benzose has a direct or indirect role on ribosomal proteins. Selective covalent binding may have occurred, this remains to be investigated, and there are many questions still to be answered.

4.1 Assessment of experimental strategies and protocols

The overall strategies and protocols described in this thesis were designed to identify a lead compound and its biological mode of action in a rational manner. The SAR analysis allowed the bioactive compounds to be identified and a lead found. However, a larger library of glucosides needs to be tested to add more scope to the study.

The chemical genomic screen using benzose was able to identify some likely pathways and processes that are being affected. The major problem found here was the breakdown of benzose and the associated activity reduction of benzose in agar. This could be repeated in liquid culture to determine if the lack of reactivity limited the genetic screen. The 1536-format agar-based high throughput is efficient from the perspective of compound use, practical simplicity and internal replication. The amount of benzose required for a liquid-based high throughput screen would not act as a deterrent, as benzose is synthesised in four steps from D-glucal.

A limitation in the chemical proteomics was found to be the lack of activity of **24**. Attaching a probe to benzose on the C-6 hydroxyl of benzose would most likely lead to loss of activity, as evident in the SAR analysis which showed that the formate ester exterminated all activity against the $\Delta pdr1\Delta pdr3$ strain. Further investigations are required to

identify a method of attaching a biological probe to benzose in order to identify the target, without the loss of activity.

Within the chemistry section of this thesis it was determined that the synthesis of each compound was achieved in the fewest steps currently known. Methods of dihalocyclopropanation without the need for a fully protected glucal would lead to a much easier synthesis of further compounds destined to join the SAR. A method of dichlorocyclopropanation of an alkene via a dichlorocarbene, magnesium and ultrasonication, had been previously presented by Lin *et al.* (2003). Scheme 3.6 demonstrates this reaction on 3,4-dihydro-2H-pyran resulting in the 7,7-dichloro-5-oxabicyclo[4.1.0]heptane, in an impressive yield of 95%. This high yield would reflect the lack of competing functionality in this system, no compounds with C-3 functionality have been described at present in the literature. However, it would be worth investigating this cyclopropanation method on D-glucal. This method may offer a way of synthesising novel dichlorocyclopropanated carbohydrates.

Scheme 3.6. Synthesis of 7,7-dichloro-5-oxabicyclo[4.1.0] heptane via a dichlorocarbene, magnesium and ultra-sonication with a yield of 95%.

4.2 Assessment of the aims and objectives

The overall aim of this project was to determine whether cyclopropanated sugars that can undergo ring opening or ring expansion reactions have ideal characteristics for suicide inhibitors. In this respect, it was partially successful. Of the four objectives set out in this thesis, three were achieved.

Objective one was to design and synthesise a range of compounds that would allow identification of bioactivity, and to give an idea of the mechanism of action. A range of compounds were synthesised consisting of cyclopropanated carbohydrates and their glucal precursors.

With a range of compounds synthesised, objective 2 was to identify a lead compound, which was benzose. The library allowed identification of the most likely chemical mechanism through which the compound might covalently modify a target protein. If covalent modification occurs, it is proposed to go via a stabilised carbanion. Again the objective was met, the lead compound was then subjected to a chemical genetic screen.

Objective three consisted of trying to identify the likely mode of action of the selected lead compound within the biological environment. This objective was completed with benzose, which was found to be both cytostatic and cytotoxic depending on concentration. It appears to interact specifically with translation and transcription pathways.

The final objective was to identify the likely protein target(s) of the lead compound. A modified matrix and a benzose-like compound **24** were synthesised to achieve this target. However, compound **24** was found to be inactive, which raises the questions of the ability to use a chemical proteomic approach for these compounds. With the lack of time, objective four has yet to be accomplished.

5.0 Future directions

A considerable amount of progress has been made towards the application of mechanism-based inhibitors, consisting of a cyclopropane on a carbohydrate backbone. While many structures have been synthesised, many more could be made for further investigations. Identification of the biological target would be a major step forward in presenting the "big picture" in which benzose has its effect.

5.1 SAR where to now?

The development of a range of cyclopropanated derivatives has yielded a varying degree of activity, however there is a large area of stereochemistry yet to be investigated. Allose and galactose offer differing stereochemical arrangements compared to glucose, and would offer a good starting point to extend the structure activity relationship. The substituents on the C-3 and C-4 hydroxyls also offer an area that warrant more investigation. As discussed in section 3.5.2, the most likely mechanism of action is through the carbanion intermediate, although this remains to

Figure 5.1. A cyclopropanated carbohydrate showing all possible stereo isomers, showing the use of multiple 6-carbon sugars.

$R_1, R_2 = \text{Cl, Br, I, H, NHR, CONH}_2, \text{CONHR,}$

$R_3 = \text{OH, OBn, OBz, Me, Ac, alkyl chain, NHR,}$

$R_4 = \text{OH, OBn, OBz, OMe, OAc, alkyl chain, NHR,}$

$R_5 = \text{OH, OBn, OBz, OMe, OAc, alkyl chain, NHR,}$

be proven. The research into benzose has proposed two methods of moving forward with the SAR studies. Firstly, ignore the protein target and make more cyclopropanated carbohydrates (figure 5.1) to try and increase the toxicity on the whole cell, or secondly focus on identifying the biological target and design potent, benzose-like compounds for this target.

Benzose its self may never offer a biologically relevant compound, however the ring breaking mechanism offers a novel mechanism-based inhibition that warrants more research. The use of the cyclopropane with the carbohydrate backbone has been shown to be active, most likely through the ring breaking mechanism, which answers the question "Will cyclopropanated carbohydrates undergo the slow ring breaking/expansion in the presence of proteins, and are we able to identify what of three types of mechanisms the reaction is going through?" The question can now be changed to further identify key aspects of activity, "will modifications to the structure provide selective binding to a biologically relevant target?"

6.0 Conclusion

We set out to identify the potential in drug discovery of known and novel cyclopropanated-carbohydrates. With the background knowledge of known chemistry that is well preceded in the literature, we proposed that this known chemistry could be catalysed by a selective protein.

Over the course of this project, a library of cyclopropanated carbohydrate based compounds has been synthesised, with regio- and stereoselective chemistry in mind. Five compounds (all cyclopropane-containing) were found to display cytotoxic activity against *S. cerevisiae* ($\Delta pdr1\Delta pdr3$), with the most active being benzose. A derivative of benzose was then synthesised to act as a molecular probe to give a tool to identify the protein target. However, this compound lacked activity and failed to pull down any proteins for identification. The current mechanism through which benzose acts in *S. cerevisiae* is unknown, but it is believed to be involved in transcription or translation, potentially via a ribosomal target, further investigations are needed.

7.0 References

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8.0 Appendix

Exclusion masses for MALDI TOF-TOF MS/MS		
m/z monoisotopic	NAME	TOLERANCE (m/z)
659.384	Trypsin	0.03
805.417	Trypsin	0.03
861.060	CHCA	0.10
877.000	Polyethylene glycol	0.10
906.505	Trypsin	0.03
1020.503	Trypsin	0.03
1153.574	Trypsin	0.03
1175.523	Trypsin	0.03
1296.680	Angiotensin 1	0.03
1433.721	Trypsin	0.03
1493.599	Trypsin	0.03
1676.777	Trypsin	0.03
1774.851	Trypsin	0.03
2093.080	ACTH (clip 1-17)	0.03
2163.057	Trypsin	0.03
2193.003	Trypsin	0.03
2193.995	Trypsin	0.03
2273.160	Trypsin	0.03
2289.155	Trypsin	0.03
2305.150	Trypsin	0.03
2465.190	ACTH clip (18-39)	0.03
2514.339	Trypsin	0.03
2550.233	Trypsin	0.03
2612.181	Trypsin	0.03
2613.350	Trypsin	0.03
3211.475	Trypsin	0.03
3227.400	Trypsin	0.03

m/z monoisotopic	NAME	TYPE	CHARGE
904.468	des-Arg1-bradykinin	r	+1
1296.685	Angiotensin	r	+1
1570.677	Glu1-fibrinopeptide	r	+1
2093.087	ACTH (1-17)	r	+1
2465.199	ACTH (18-39)	r	+1
3657.929	ACTH (7-38)	r	+1

m/z monoisotopic	NAME	TYPE	CHARGE
255.1090	B2-17(-NH3) fragment ion	r	+1
354.1780	B3-17(-NH3) fragment ion	r	+1
784.4110	B6 fragment ion	r	+1
1181.6580	Angiotensin Y9 fragment ion	r	+1
1183.6010	Angiotensin fragment ion	r	+1

Sample Name: benzocel
Data Collected on: vnmr500.sops.vuw.ac.nz-inova500
Archive directory: /home/ralph/dylan
Sample directory: benzocel
Fidfile: PROTON_20130630_cdc13_500_02
Pulse Sequence: PROTON (szpul)
Solvent: cdcl3
Data collected on: Jun 30 2013

Sample #44, Operator: Dylan
Relax. delay 0.010 sec
Pulse 60.0 degrees
Acq. time 3.454 sec
Width 4743.8 Hz
64 repetitions
OBSERVE H1, 499.8139667 MHz
DATA PROCESSING
Ft size 32768
Total time 3 min 42 sec

Sample Name:

benzoesl

Data Collected on:

vnmr500.sops.vnu.ac.nz-inova500

Archive directory:

/home/walrup/Dylan

Sample directory:

benzoesl

Fidfile: goosy_20130630_cdcl3_500_01

Pulse Sequence: goosy

Solvent: cdcl3

Data collected on: Jun 30 2013

Sample #44, Operator: Dylan

Relax. delay 2.000 sec

Acq. time 0.150 sec

Width 4743.8 Hz

2D Width 4743.8 Hz

Single scan

512 increments

OBSERVE H1, 499.7739667 MHz

DATA PROCESSING

Sq. sine bell 0.075 sec

F1 DATA PROCESSING

Sq. sine bell 0.064 sec

FT size 2048 x 2048

Total time 19 min

Sample Name: benzose1
 Data Collected on: vnmr500,seps,vuv.ac.nz-lnova500
 Archive directory: /home/walshup/Dylan
 Sample directory: benzose1
 F1 file: ghmbcad_20130630_0d013_500_50-
 Pulse Sequence: ghmbcad
 Solvent: dcl3
 Data collected on: Jun 30 2013
 Sample #44, Operator: Dylan
 Relax, delay 1.500 sec
 Acq. time 0.150 sec
 Width 4743.8 Hz
 2D Width 27643.4 Hz
 8 repetitions
 2 x 512 increments
 OBSERVE H1, 499.7739667 MHz
 DATA PROCESSING
 Sg, sine bell 0.075 sec
 F1 DATA PROCESSING
 Gauss apodization 0.017 sec
 FT size 4096 x 4096
 Total time 3 hr, 59 min

Sample Name: Benzocel
 Data Collected on: yunm500.sops.yun.ac.nz-incra500
 Archive directory: /home/yalkup/Dylan
 Sample directory: Benzocel
 FIDFile: HSGCAD_20130630_00013_500-90
 Pulse Sequence: HSGCAD
 Solvent: cdcl3
 Data collected on: Jun 30 2013
 Sample #44, Operator: Dylan
 Relax. delay 1.400 sec
 Acq. time 0.150 sec
 Width 4743.8 Hz
 2D Width 20105.6 Hz
 4 repetitions
 2 x 512 increments
 OBSERVE H1, 499.7739667 MHz
 DECOUPLE C13, 125.6783173 MHz
 Power 36 db
 on during acquisition
 off during delay
 H40_autocx modulated
 DATA PROCESSING
 Gauss apodization 0.069 sec
 F1 DATA PROCESSING
 Gauss apodization 0.024 sec
 FT size 4096 x 4096
 Total time 1 hr, 50 min

Sample Name:
608-1_441803101
Data Collected on:
vnmr500.scp.vnmr-400-1000500
Archive directory:
/home/vnmr/Dylan
Sample directory:
608-1_441803101
FID file: PROTON_20121119_06013_569_01

Pulse Sequence: PROTON (zgpg3)
Solvent: cdcl3
Data collected on: Nov 19 2012

Sample #19, Operator: Dylan

Pulse 60.0 degrees
Acq. time 4.096 sec
Width 8000.0 Hz
64 repetitions
OBSERVE HL 499.776924 MHz
DATA PROCESSING
F2 alias 65516
Total time 4 min 23 sec

Sample Name:
 608-1_461801050001
 Data Collected on:
 vnmx500.srps.vnm.nr-608500
 Archive directory:
 /home/vnmr/Dylan
 Sample directory:
 608-1_461801050001
 File(s): 608000_20121119_00011_500_01
 Pulse sequence: CARGO (42901)
 Solvent: cdcl3
 Data collected on: Nov 29 2012

Sample #39, Operator: Dylan
 Pulse 45.0 degrees
 Acq. time 2.084 sec
 Width 31446.5 Hz
 1736 repetitions
 OBSERVE CH, 125.668911 MHz
 DECOUPLE H1, 499.778713 MHz
 Power 45 dB
 continuously on
 WALTZ-16 modulated
 DATA PROCESSING
 Line broadening 0.5 Hz
 F2 alias 11072
 Total time 1 hr

Sample Name:
2,6-dichloro-4,5-epoxy-3,4-dihydro-2H-pyran-2-ol
Data Collected on:
vnmr500-emp.vw.ac.nz-lmvr500
Acquire directory:
/home/vmlhup/Dy2an
Sample directory:
3,6-dichloro-4,5-epoxy-3,4-dihydro-2H-pyran-2-ol
FIDFile: PROTON_2012109_00013_500_01
Pulse Sequence: PROTON (zgpg3)
Solvent: cdcl3
Data collected on: Dec 9 2012
Sample #21, Operator: Dy2an
Pulse 59.0 degrees
Acq. time 4.096 sec
MkDch 8000.0 Hz
64 repetitions
OBSERVE HI, 499.7765734 MHz
PR also 65536
total time 4 min 23 sec

Sample Name:
3,6-dichloro-4-hydroxy-1,5-dioxane-2,5-diol
Data Collected on:
Vnmr500.spc, vnmr.ac-nu-lmvr500
Archive directory:
/home/vnmr500/dylen
Sample directory:
3,6-dichloro-4-hydroxy-1,5-dioxane-2,5-diol
File: CARBON_2012109_0601_500_01

Pulse Sequence: zgpg30 (zgpg30)
Solvent: cdcl3
Data collected on: Dec 9 2012

Sample #13, Operator: Dylen
Pulse 45.0 degrees
Acq. time 2.084 sec
Width 3144.5 Hz
1736 repetitions
OBSERVE C13, 125.668391 MHz
DECOUPLE H1, 439.7750713 MHz
Power 45 dB
continuously on
NUC1-16 isolated
DATA PROCESSING
Line broadening 0.5 Hz
F2 size 131072
Total time 1 hr

Sample Name: 2,4,3,6-tetra-O-acetyl-beta-D-glucopyranose
 Data Collected on: vnmr500.scs, vnmr-40, NE-100v500
 Archive directory: /home/walshp/Dy1an
 Sample directory: 2,4,3,6-tetra-O-acetyl-beta-D-glucopyranose
 Filename: PROTON_10120401_0001_500_01
 Pulse sequence: PROTON (*2pul)
 Solvent: d6o13
 Data collected on: Apr 2 2012
 Sample #35, Operator: Dy1an
 Pulse 50.0 degrees
 Acq. time 4.096 sec
 Width 8000.0 Hz
 64 repetitions
 Observed: 31, 459.776724 MHz
 DATA PROCESSING
 FT also 65536
 Total time 4 min 23 sec

Sample Name: 1,3,2012p02
 Data Collected on: VNMZ500-600s-vw.ac.ac-14094566
 Archive directory: /home/walrus/Dylan
 Sample directory: 1,3,2012p02
 File(s): PROTON_20120911_cdc11_506_01
 Pulse Sequence: PROTON (zgpg3)
 Solvent: cdcl3
 Data collected on: Mar 1 2012
 Sample #28, Operator: Dylan
 Pulse 60.0 degrees
 Acq. time 4.094 sec
 Width 8000.0 Hz
 64 repetitions
 OBSERVE M1, 439.765770 MHz
 DATA PROCESSING
 PC also 65516
 Total time 4 min 23 sec

Sample Name:
 triol_
 Data Collected on:
 vnmrj500.spep.vnmr.nc.na-luova500
 Archive directory:
 /home/vnlug/Dylan
 Sample directory:
 triol_
 P1/Pr1: PROTON_20130625_cdc13_500.01

Pulse Sequence: PROTON (zgpg3)
 Solvent: cdcl3
 Data collected on: Jun 25 2013

Sample #43, Operator: Dylan

Solax. delay 0.010 sec
 Pulse pr. 0 degrees
 Acq. time 4.095 sec
 Width 8000.0 Hz
 64 repetitions
 OBSERVE EL 439.773667 MHz
 DATA PROCESSING
 PC also 65516
 Total time 4 min 23 sec

Extra bit

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