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entitled

Synthesis of Carbohydrate-based Inhibitors of Antigen 85

By

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Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Chemistry

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An Abstract of

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The hydrophobic nature of the Mycobacterium tuberculosis (M.tb) cell wall contributes significantly to the organism’s drug resistance and virulence. Antigen 85 (Ag85), a class of related mycolyltransferase enzymes (Ag85A, Ag85B and Ag85C), plays a major role in the mycobacterial cell wall synthesis through the generation of trehalose dimycolate (TDM) and mycoly arabinogalactan (mAG), major hydrophobic components of the M.tb cell wall. We have extended the search for new inhibitors of mycobacterial cell wall synthesis by making a number of α-D-trehalose and β-D-arabinose-derived thioester, sulfonamide and 1,2-dicarbonyl compounds as synthetic probes or inhibitors of Ag85.

Our effort to develop new Ag85 synthetic probes started with synthesis of three thioester derivatives potentially useful for development of new mycolyltransferase assays.
Starting with commercially available materials, we have modified α-D-trehalose and α-D-methylglucoside at the C-6 position to make thioacetate and benzothioate analogs in fairly good yields (77% to 90%).

Continuing our search for potent inhibitors of Ag85, we have also synthesized three classes of D-trehalose and β-D-arabinose-derived esters, α-ketoesters and α-ketoamides, as transition state inhibitors of Ag85C. Incorporation of an ester, α-ketoester or α-ketoamide at the C-6 position was accomplished using Swern conditions to oxidize the primary hydroxyl group, followed by a Horner-Wadsworth-Emmons condensation to obtain the methyl ester. Treatment of the ester with LiOH resulted in a carboxylic acid derivative which was then coupled with a cyanophosphorane in the presence of EDCI to obtain cyananoketo-phosphorane derivative. Oxidation of the phosphorane with DMDO, followed by amidation of the resulting α-ketonitrile with relevant alkyl amines yielded α-ketoamide analogs. Inhibitory study using a newly developed colorimetric assay revealed that an arabinose methyl ester showed weak inhibition of Ag85C at 25 mM concentration, and was also co-crystallized with Ag85C. All dicarbonyl compounds made did not show inhibition against the growth of *M. smegmatis* ATCC 14468 using a Kirby-Bauer disk assay.

To develop a new lead series, trehalose-based sulfonamide and sulfone ester derivatives were synthesized as potential transition state inhibitors of Ag85 complex. Sulfonamides are known serine protease inhibitors. Synthesis of sulfone esters was accomplished by incorporation of ester moiety on the C-6 of trehalose substrate via a Horner-Wadsworth-Emmons condensation reaction with triethyl-α-phosphorylmethanesulfonate. Sulfonamides were installed through a similar process.
followed by base-promoted coupling of appropriate amine with the sulfonyl chloride intermediate. The sulfonamide analogs were successfully made in fairly good yields (42% - 91%).
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List of Abbreviations

Ac₂O …………………..Acetic anhydride
AcOH …………………..Acetic acid
Ag85…………………..Antigen 85
ARDS………………..Acute respiratory disease syndrom
Bn …………………..Benzyl
BnBr ………………….Benzyl bromide
Bu₄NI ………………….Tetrabutylammonium iodide
DCM …………………..Dichloromethane
CH₂Cl₂………………..Dichloromethane
DIPEA ……………….N,N-diisopropylethylamine
DIAD………………….Diisopropyl azodicarboxylate
DMAP ……………….4-(N,N-dimethylamino)pyridine
DMDO ……………….Dimethylidioxirane
DME ………………..Dimethoxyethane = dimethylglycol
DMF …………………..N,N-dimethylformamide
DMSO ………………Dimethylsulfoxide
DOTS…………………Direct observation therapy, short course
EDCI ………………..1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
Et ……………………..Ethyl
EtOAc ..................Ethyl acetate
Et3N ..................Triethyl amine
HIV........................Human immunodeficiency virus
H2O........................Water
iPr .........................Isopropyl
mCPBA ..................m-Chloroperoxybenzoic acid
LAM.....................Lipoarabinomannans
ManLAM.............. Mannosylated lipoarabinomannans
Me .....................Methyl
MeOH ................. Methanol
MDR.....................Multidrug resistance
MDR-TB ............. Multidrug resistant tuberculosis
MS......................Mass
\textit{M.\textit{tb}}} ............ \textit{Mycobacterium tuberculosis}
NBS .....................\textit{N}-bromosuccinimide
NaH ................. Sodium hydride
NMR ......................Nuclear Magnetic Resonance
PPh3 ....................Triphenylphosphine
Ph ......................Phenyl
py .....................Pyridine
r.t.....................Room temperature
\textit{iBu} ..................\textit{tert}-Butyl
TBAI ....................Tetrabutylammonium iodide
TB…………………Tuberculosis
TDM………………Trehalose dimycolate
TEA ………………Triethyl amine
TES ………………Triethylsilane
TFA ………………Trifluoroacetic acid
Tf₂O ………………Triflic anhydride
THF ………………Tetrahydrofuran
TIPS ……………Triisopropylsilane
TBDPS ……………tert-Butyldiphenylsilyl
TLC ………………Thin layer chromatography
TMP ………………2,4,6-Trimethylpyridine
TMM………………Trehalose monomycolate
TMSOTf …………Trimethylsilyl trifluoromethanesulfonate = trimethylsilyl triflate
Tn ………………..2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-galactopyranosyl
Ts (tosyl) ………….p-Toluenesulfonyl
TsCl……………...p-Toluenesulfonyl chloride
TrCl ………………Triphenylmethyl chloride
UV ……………….Ultraviolet
WHO……………..World health organization
XDR………………Extensively drug resistant
XDR-TB……………Extensively drug resistant tuberculosis
CHAPTER 1

Antigen 85 Complex as a Potential Target for Development of New Tuberculosis Drugs

1.1 Introduction

Tuberculosis (TB), is the leading cause of death in the world today from a single bacterial infectious disease.\textsuperscript{1-2} An estimated 1.8 billion people worldwide, roughly one third of the world population, are infected with TB – meaning they harbor latent TB.\textsuperscript{2} Approximately 8 – 10 million new cases of active TB are reported annually, with about 2 million people dying from the disease each year.\textsuperscript{2} There are also a growing number of TB cases worldwide associated with human immunodeficiency virus (HIV) infection.\textsuperscript{1-2} The resurgence of TB-HIV co-infection is posing significant challenges to the clinical management of tuberculosis, and frequently results in high mortality rates.

The prevalence of tuberculosis is further complicated by the emergence of new strains of tuberculosis which are resistant to a number of currently available drugs.\textsuperscript{1,2,3} The first antibiotic which was shown to be effective for the treatment of TB was streptomycin, which was quickly followed by the identification of streptomycin-resistant strains of \textit{Mycobacterium tuberculosis (M.t.b)}.\textsuperscript{4} Similarly, all single-drug TB treatments
developed since the identification of streptomycin resistant *M. tb* strains have led to the emergence of certain drug resistant strains. A response by physicians to single-drug resistant strains is to employ another first-line drug that inevitably selects for multidrug resistance (MDR) strains. There are two kinds of multi-drug resistant strains being observed: 1) Multidrug-resistant tuberculosis (MDR-TB). This is TB that is resistant to at least two first-line drugs used in treating all cases of TB, namely, isoniazid, ethambutol, pyrazinamide, and rifampicin (Figure 1.1). Treatment for this strain requires the use of second-line TB drugs, such as amikacin, kanamycin, capreomycin, ethionamide, cycloserine, ciprofloxacin (a fluoroquinolone) or *p*-aminosalicylic acid (Figure 2). It is reported that about 50 million people are currently infected with MDR-TB.\(^2,3\) Treatment of MDR-TB necessitates the use of less effective (and more expensive) second-line drugs. Treatment can last for up to two years. Complications resulting from this prolonged and costly treatment sometimes lead to patient non-compliance resulting in further drug resistance.

![Figure 1.1: First-line anti-tuberculosis drugs and the years they were introduced on the market.](image)

The resulting extensively drug resistant strains (XDR) initially seemed unmanageable.\(^5\) The first account of XDR-TB described an outbreak in South Africa in 2006 in which 52 out of 53 infected patients died within an average period of 16 days
from the time of diagnosis. However, a more recent study describing XDR-TB in Russia presented a more optimistic result with 48% of the patients reaching a favorable outcome after treatment. Extensively drug resistant TB (XDR-TB) is resistant to isoniazid and rifampicin, and is also resistant to any fluoroquinolone such as ciprofloxacin and at least one of three injectable second-line drugs - amikacin, kanamycin, or capreomycin (Figure 1.2).

Figure 1.2: Examples of second-line anti-tuberculosis drugs

While there is a possible danger of MDR-TB and XDR-TB developing in all TB patients who do not take their medicines regularly or as prescribed, the incidence of XDR-TB is highly associated with HIV-TB coinfection. For sure, the emergence of MDR and XDR TB threatens global management of the disease, and poseses a major risk
to both developing and developed countries. The World Health Organization (WHO) recommends the DOTS (Direct Observation Therapy, Short course) strategy\(^1,2\) in treating TB in order to both achieve high level of disease eradication as well as reduce the incidence of drug resistance in patients. The DOTS consists of an initial treatment with four drugs, namely, isoniazid, rifampicin, pyrazinamide and ethambutol; taken daily for two months. This is then followed immediately by treatment with isoniazid and rifampicin for another four months.

The DOTS strategy recommended by WHO is undoubtedly the best approach, but the process is lengthy, complex and involves an unpleasant combination of different drugs. It is easy to see how the DOTS strategy, though well intentioned for optimal result, may lead to poor patient compliance, lower cure rates and even increase in emergence of new cases of drug resistance. There is therefore, an urgent need to develop new therapeutics that will tackle the resistance shown by \(M.tb\), and make the prescription regimen simpler. There is an added urgency when it is realized that since the introduction of rifampicin in 1965, no truly new drug for TB has been developed.\(^3\)

### 1.2 Role of Cell Wall in Bacterial Virulence

\(M.tb\) is the causative agent of tuberculosis, and humans are the only known reservoirs. \(M.tb\) is a rod-shaped bacterium.\(^3\) It is an obligate aerobe, which is why in cases of active infection, \(M.tb\) complexes are found in the well-aerated upper lobes of the lungs. It is common practice not to classify \(M.tb\) as either Gram-positive or Gram-negative, since it does not have the chemical properties of either, although it contains
peptidoglycan in its cell wall. It should be noted, however, that if a Gram stain is performed on *M. tb*, it stains very weakly Gram-positive or not at all. 3, 8

Other pathogens belonging to the Mycobacterium genus include1, 2, 3: 1) *Mycobacterium bovis*. This is the causative agent of TB in cows. Although both cows and humans are both reservoirs, the bacterium is rarely found to cause TB in humans. 2) *Mycobacterium avium*. This bacterium causes TB-like disease which is mostly common in patients with HIV-infection. Mycobacterium avium is not restricted to the lungs, but rather is disseminated. 3 Due to complication with HIV-coinfection, clinical management of *Mycobacterium avium* is very difficult, and often resistant to first-line anti-tuberculosis drugs. 3) *Mycobacterium leprae*, which is the causative agent of leprosy.

The thick hydrophobic nature of *M. tb* cell wall is an important feature which contributes to its virulence. Indeed, the thick, multi-layered, extremely hydrophobic cell envelope, which results in very low cellular permeability, acts as a barrier against many classes of hydrophilic antibacterial drugs. 3, 8, 9 Therefore, the mycobacterial cell wall is very important for the long-term survival of of *M. tb* in the hostile environment of the macrophage of the host, as well as for the progression of tuberculosis. 8, 9 The general structure of the mycobacterial cell envelope is now well understood. The basic model, proposed by Minnikin, 10 identified a thick asymmetric lipid bilayer which is located beyond the plasma membrane, the peptidoglycan, and mycolyarabinogalactan (mAG) layer (Figure 1.3).
Broadly speaking, the *M. tb* cell wall is made up of four important components\textsuperscript{8, 10, 11, 12} as depicted in Figure 1.3, namely, the plasma membrane (PM), peptidoglycan (PG), arabinogalactan (AG), and an outer capsule-like layer. The PG, which is embedded in the plasma membrane, is covalently linked to the AG via a diglycosyl phosphoryl bridge; resulting in an important structural element of the *M. tb* cell wall known as the mycolyl-arabinogalactan-peptidoglycan (mAGP) complex (Figure 1.3).

Figure 1.3: Schematic representation of general structure of *M. tb* cell wall. Adapted from Umesiri et al.\textsuperscript{13}
Figure 1.4: Primary structure of the mAG
The arabino motif of the cell wall contains a 1,3-branched arabinofuranoside-based mycolated hexasaccharides, via ester linkages at each of the four primary hydroxyl groups to form the mycolyl-arabinan moiety (Figure 1.4). About 60% of the *M.tb* cell is composed of lipids such as trehalose monomycolate (TMM), trehalose dimycolate (TDM) and mycolic acids.

Chemical analysis of the cell envelope composition has also revealed the presence of diverse noncovalently-bound lipids such as phosphatidyl-*myo*-inositol mannosides (PIMs), and lipopolysaccharides such as lipoarabinomannans (LAM) and mannosylated lipoarabinomannans (ManLAM, Figure 1.5), depending on the *Mycobacterium* species. In slow-growing mycobacterial species, such as *M.tb*, proteins and polysaccharides are present in the outermost stratum known as the capsule.

Figure 1.5: Current model of *M.tb* ManLAM with structurally related components. Adopted from Umesiri *et al.*
The exact location of LAM in the cell envelope remains elusive although it is known that the phosphatidyl myo-inositol serves as a cell wall anchor in the bacterial plasma membrane,\textsuperscript{14} however LM and LAM have been shown to be exposed at the cell surface (Figures 1.3 and 1.5).\textsuperscript{15} LAM and mAG are the main lipopolysaccharides of the mycobacterial cell wall. These key components insulate the bacteria from its environment and are essential for mycobacterial survival.\textsuperscript{13} In addition, they play diverse roles in the bacteria-host interactions.\textsuperscript{15} Structurally, both LAM and mAG share an arabinan domain, but possess significantly different final superstructures (Figures 1.4 and 1.5). LAM is composed of a phosphatidylinositol group linked to a branched arabinomannan core (Figure 1.5),\textsuperscript{16} while mAG is a branched arabinogalactan polymer esterified with mycolic acids (Figure 4).\textsuperscript{15}

1.3 The Cord Factor

Whereas the inner layer of the cell wall is made up of mycolic acids covalently attached to the terminal portion of the AG moiety, the outer layer is composed mainly of glycolipids containing mycolic acids, such as TDM (the cord factor) and its biosynthetic precursor, TMM (Figure 1.6).\textsuperscript{17} These extractable glycolipids make up to 60% of the \textit{M.tb} cell.\textsuperscript{1}
In order to fully discuss the role of TDM in tuberculosis, a definition of primary, secondary and cavity tuberculosis is in order at this juncture. Primary tuberculosis occurs as result of initial exposure to infection. Usually, it starts in the lungs, spreads rapidly to the lymph nodes and then to the rest of the body. But in most cases, it heals spontaneously such that it rarely progresses to disseminated disease stage. Disseminated TB refers to the spread of the disease within the body as infected macrophages move through the blood and the lymph transports the bacteria to other sites. Once infected, symptoms of disseminated TB correspond to the locations infected. The infection can become reactivated if the mycobacteria are able to rupture the tubercle and spread through the lungs. This refers to secondary tuberculosis. Essentially, secondary tuberculosis, also known as post-primary tuberculosis, develops in persons who have developed sufficient immunity as a result of primary infection to limit disease to just the upper lobes of the lungs. Often, secondary TB leads to cavities in the lung which produce massive numbers of *M.tb* that are coughed up to infect new persons (Figure
This kind of tuberculosis is described as cavitary TB. It is generally accepted that *M. tb* survives because of its ability to produce cavities that mediate its escape to infect new people.\(^\text{18}\)

![Figure 1.7: Secondary and cavitary tuberculosis. Photograph of human lungs showing secondary tuberculosis with cavities in the right upper lobe. Foci of caseation necrosis (white areas) are also present in areas of tuberculous pneumonia in both upper lobes. (Reproduced by permission—Rosen, Y: Atlas of Granulomatous Diseases; http://www.granuloma.homestead.com).](image)

The cord factor is believed to contribute significantly to the virulence and persistence of mycobacteria.\(^\text{17-19}\) TDM is the most abundant lipid released by virulent *M. tb* which has long exhibited a puzzling behavior because it can change from non-toxic to highly toxic form when injected in an oily medium.\(^\text{17}\) In a micellar conformation (bound to bacterial cell wall for example), TDM is non-toxic and protects organism from host defenses.\(^\text{17-18}\) On the other hand, secreted TDM (monolayer conformation)
accumulates with host lipids in alveoli, which quickly leads to caseous necrosis resulting in cavities.\textsuperscript{17-18} Virulent \textit{M.tb} releases large amounts of TDM during growth within cavities, a process that is believed to perpetuate the cavity.\textsuperscript{18} This way, TDM plays a huge role in primary tuberculosis.\textsuperscript{17-19} Hunter \textit{et al.} observed that mice injected with TDM produced caseating granulomas (a sign of primary TB).\textsuperscript{18} In addition, Indrigo and co-workers\textsuperscript{20} on their part reported that the removal of lipids from the surface of \textit{M.tb} resulted in 99\% of them being killed by macrophages within three days in culture. Their study also showed that addition of purified TDM back to the culture medium restored almost completely the ability of \textit{M.tb} to survive in such culture media.\textsuperscript{20}

Hunter \textit{et al.} also observed that secondary TB typically begins as a lipid pneumonia (a kind of lung inflammation which develops when lipids such as TDM enter the bronchial track), rather than as a granulomatous disease (as in the case of primary TB).\textsuperscript{20} This pneumonia abruptly undergoes necrosis to produce cavities which initiate the final phase of tubercular infection. Since cavities produce large numbers of organisms that synthesize and release huge amounts of TDM, it is likely that TDM is a participant in their maintenance.\textsuperscript{20} Taken together, all these studies suggest that TDM is an important determinant for successful infection, as well as survival of \textit{M.tb} within macrophages.\textsuperscript{17-20} This is due to its contribution to the hydrophobicity of the cell wall, as well as it being a powerful antigen.\textsuperscript{17-22} As a result, TDM, TMM and their synthetic analogs are potential targets for reducing the pathogenicity of \textit{M.tb}.  

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1.4 Role of Mycolic Acid in Bacterial Cell Wall

Mycolic acids are long alpha-branched lipids found in cell walls of *M.tb* (Figure 1.8), and make up about 50% of the dry weight of the mycobacterium cell envelope.\textsuperscript{1,3,13} Mycolic acids are strong hydrophobic molecules that form a lipid shell around the organism and are known to significantly restrict permeability properties at the cell surface. Mycolic acids, along with other components of the cell wall, form a hydrophobic barrier that is thought to contribute significantly to the survival of the bacterium. The following are some of the ways by which the hydrophobic cell wall contributes to the virulence, and resistance of MTB\textsuperscript{1,8,11,12}: 1) impermeability to stains and dyes, 2) resistance to many antibiotics, 3) resistance to killing by acidic and alkaline compounds, 4) resistance to osmotic lysis via complement deposition, and 5) resistance to lethal oxidations and survival inside of macrophages.

![Chemical structures](image)

Figure 1.8: α-Alkyl-β-hydroxyl fatty acids with long alkyl side chain.\textsuperscript{12}
An important component of the lipid layer of bacterial cell wall is TDM, otherwise known as the cord factor. Trehalose monomycolates and trehalose dimycolates are mycolyl-carriers, and important substrates in the synthesis of the bacterial cell wall.

1.5 Enzymes Involved in Biosynthesis of mAG Portion of Cell Wall

Enzymes involved in the biosynthesis of mycobacterial LAM and mAG have frequently been targets of anti-tubercular drugs. For example, the antitubercular drug isoniazid works by inhibiting the biosynthesis of the mycolic acid component of the mAG. Specifically, isoniazid is activated by mycobacterial enzyme KatG. KatG is a catalase-peroxidase enzyme which reacts with isoniazid to generate reactive species: isonicotinic hydrazyl radical and the isonicotinoyl radical (Figure 1.9). These reactive species then form adducts with NAD+ and NADP+ that are potent inhibitors of inhA, an enoyl acyl-carrier enzyme involved in biosynthesis of mycolic acid.

![Figure 1.9: Structures of isoniazid, the isonicotinic lhydrazyl radical and the isonicotinoyl radical (also termed isonicotinic acyl radical).](image)

Similarly, ethambutol inhibits formation of the arabinan component of arabinogalactan by inhibiting the arabinosyl transferase enzyme; thereby inhibiting the formation of mycolyl-arabinogalactan-peptidoglycan complex in the cell wall (Figures 1.3 & 1.4).
A list of some of the enzymes already implicated in the biosynthetic pathway of mAG is given in Table 1.

As has been shown by the development of resistance to many single-drugs, the strategy of targeting a single step in a biosynthetic pathway may not be the most effective approach in the management of multi-drug resistant strains of mycobacteria. To minimize the probability of a single mutation leading to resistance, we have argued elsewhere \textsuperscript{13} that it may be beneficial to target multiple enzymes in the same or related biosynthetic pathway and to target enzymes representing metabolic “hubs” that are essential for multiple biosynthetic pathways. For example, EmbA, EmbB and EmbC enzymes are involved in various stages of the synthesis of both LAM and mAG components of mycobacterial cell wall. While EmbA and EmbB are involved in mAG biosynthesis, EmbC is involved the biosynthesis of LAM. Targeting all three enzymes simultaneously may be a potentially more effective approach in developing new anti-tubercular therapies.

Another important illustration is antigen 85 (Ag85), an enzymatic complex comprised of three related acyl transferase enzymes: Ag85A, Ag85B and Ag85C. This enzymatic complex catalyses transfer of mycolyl groups to terminal ends of AG of bacterial cell wall to form mAG, as well as the synthesis of TDM from TMM. It seems plausible then that attempting to simultaneously inhibit this enzymatic complex may help reduce incidence of resistance, since the possibility of all three enzymes developing mutations at the same time is limited.\textsuperscript{11,13}
<table>
<thead>
<tr>
<th>Enzyme, Rv #</th>
<th>Species found</th>
<th>Function</th>
<th>Essential?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv3806, UbiA</td>
<td>M. tuberculosis</td>
<td>Phosphoribosyl transferase</td>
<td>Yes</td>
</tr>
<tr>
<td>Rv3790, DprE1</td>
<td>M. tuberculosis</td>
<td>Decaprenylphosphoryl-D-ribose oxidase</td>
<td>Yes</td>
</tr>
<tr>
<td>Rv3791, DprE2</td>
<td>M. tuberculosis</td>
<td>Decaprenylphosphoryl-D-2'-keto erythro pentose reductase</td>
<td>Yes</td>
</tr>
<tr>
<td>Rv1086</td>
<td>M. tuberculosis</td>
<td>Prenyl diphosphate synthase, for synthesis of polyrenyl phosphate molecules (Dec-P)</td>
<td>Yes</td>
</tr>
<tr>
<td>Rv2361c</td>
<td>M. tuberculosis</td>
<td>Decaprenyl diphosphate synthase</td>
<td>Yes</td>
</tr>
<tr>
<td>Rv1302, ortholog to E. coli WecA</td>
<td>E. coli</td>
<td>UDP-GlcNAc transferase, loads GlcNAc-P to Dec-P</td>
<td>Yes</td>
</tr>
<tr>
<td>Rv3265c</td>
<td>M. smegmatis</td>
<td>Rhamnosyltransferase for formation of disaccharide linker</td>
<td>Yes</td>
</tr>
<tr>
<td>WbbL</td>
<td>M. smegmatis</td>
<td>UDP-Galp mutase, converts UDP-Galp to corresponding furanose</td>
<td>Yes</td>
</tr>
<tr>
<td>Rv3809c UGM</td>
<td>M. smegmatis</td>
<td>A galactosyltransferase (GlfT); transfers first galactofuranoside</td>
<td>Yes</td>
</tr>
<tr>
<td>GlfT2</td>
<td>M. tuberculosis</td>
<td>A GlfT; transfers remaining Galf sp.</td>
<td>Yes</td>
</tr>
<tr>
<td>Rv3808c</td>
<td>M. tuberculosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GlfT</td>
<td>M. tuberculosis</td>
<td>AftA; catalyses transfer of first arabinofuranosyl moiety to the galactan</td>
<td>Yes</td>
</tr>
<tr>
<td>Rv3792</td>
<td>AftA (emb locus)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rv3805c</td>
<td>C. glutamicum</td>
<td>Plays major role in formation of β(1→2)-linkages in arabinan biosynthesis</td>
<td>Yes</td>
</tr>
<tr>
<td>AftB</td>
<td>M. smegmatis</td>
<td>Required for formation of α(1→3)-linkages in arabinan biosynthesis</td>
<td>Yes</td>
</tr>
<tr>
<td>Rv2673, AftC</td>
<td>C. glutamicum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSMEG2785</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ncgl1822</td>
<td>M. tuberculosis</td>
<td>Transfers two Araf residues, completes Ara6 motif</td>
<td>Yes</td>
</tr>
<tr>
<td>EmbA</td>
<td>M. tuberculosis</td>
<td>Arabinosyltransferase</td>
<td>Yes?</td>
</tr>
<tr>
<td>Rv3794, EmbB, Rv3795</td>
<td>M. tuberculosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antigen85</td>
<td>M. tuberculosis</td>
<td>A cyltransferase enzymatic complex</td>
<td>Yes</td>
</tr>
</tbody>
</table>
For this reason, the study of the mAG biosynthetic pathways is also of interest for drug development by providing additional targets for the disruption of mycobacterial cell wall. Specifically, new strategies aimed at inhibiting the enzymes essential for mAG and TDM synthesis are likely to be important for the identification of new anti-tubercular therapies. Many of the enzymes involved in the biosynthetic pathway for the mAG are represented in Table 1, and a detailed review of recent attempts to inhibit individual enzymatic pathways have been covered. This research is interested in the last step of the biosynthesis of mAG, which involves the mycolation of the arabinogalacton moiety. This step is catalyzed by antigen 85 complex – the target of the inhibitory studies involved in this thesis.

1.6 Role of Antigen 85 Complex

The Ag85 complex is an important class of enzymes known to be involved in maintaining the hydrophobic nature of the mycobacterium cell wall. The enzymes were first isolated as the major secreted component of \textit{M.tbc} culture filtrates in 1965. These three proteins are homologous, with an amino acid sequence identity of 68-79\% in their mature secreted forms.

The Ag85 complex has been identified as the major immunodominant antigens of \textit{M.tbc} and confers immunoprotection over \textit{M.tbc} within host macrophages. The genes that encode Ag85A, Ag85B and Ag85C are the fibronectin-binding protein A (\textit{fbpA}), fibronectin-binding protein B (\textit{fbpB}) and fibronectin-binding protein C2 (\textit{fbpC2}) genes, respectively. Several past studies suggest that the differential expression of Ag85 genes may be a mechanism adopted by mycobacteria to confuse and evade host immune
For example, it seems that environmental factors regulate the levels of different antigen 85 proteins expressed per time. Mycobacteria grown in Souton media were shown to produce mRNA from only the \( fbpB \) and \( fbpC2 \) genes, while mycobacteria grown in resting macrophages produced mRNA from only \( fbpA \) gene.\(^{30}\) Similarly, mycobacteria grown in activated macrophages showed transcription of mRNA of \( fbpC2 \) gene.\(^{30}\) It is due to this immunosuppressant behavior that these 30/32-kDa proteins have become strong candidates in developing effective vaccines against tuberculosis.\(^{28}\) One study, for instance, has shown that vaccination of guinea pigs with a recombinant \textit{Mycobacterium bovis} bacillus Calmette-Guerin vaccine expressing and secreting the \textit{M. tb} 30-kDa protein (rBCG30) successfully induced stronger protective immunity against aerosol challenged with \textit{M. tb} than conventional \textit{M. bovis} bacillus Calmette-Guerin vaccine.\(^{28,32}\)

Clearly, Ag85 complex is critical for the survival of \textit{M. tb} in host macrophage, not only due to its immunogenicity, but also due to its role in maintenance of hydrophobic cell wall through mycolation activity.\(^{29-30,33}\) It has been shown to be an acyltransferase enzyme which helps to maintain integrity of the cell wall by:\(^{33}\) a) synthesis of TDM from TMM (Scheme 1.1) and b) catalyzing transfer of mycolic acids to terminal ends of arabinogalactan moiety of mycobacterial cell wall (Scheme 1.2). Our laboratory, through the work of Sanki \textit{et al}.\(^{34}\) has also demonstrated that derivatives of D-arabinose are capable of acting as substrates for Ag85C-mediated acyl transfer, and that acyl donor, \( p\)-nitrophenyl 6-O-octanoyl-\( \beta \)-D-glucopyranoside, can be used in the place of TMM to study Ag85C-catalysed acyl-transfer.
Scheme 1.1: Ag85 Catalyzed Synthesis of TDM from TMM.

Scheme 1.2: Ag85 Catalyzed Synthesis of Mycolyl-arabinan Portion of the mAGP from TMM.
1.7 Catalytic Mechanism of Mycolyl Transfer by Ag85

The catalytic triad of serine proteases such as chymotrypsin contain a Ser-His-Asp charge-relay system similar to that of Ser-His-Glu system found in Ag85 complex. Following several studies involving sequence analysis and x-ray crystallography resulted in placing Ag85 complex within the α/β hydrolase superfamily, which confirms serine as the initiating nucleophile in a hydrolytic attack on enzyme substrate. The implication of these studies is that a transfer mechanism, instead of hydrolytic mechanism in which water would be the nucleophile, is the mode of catalytic action utilized by Ag85 complex. A rather detailed mechanism for mycolyl transfer to the terminal ends of the arabinogalactan moiety by Ag85, based on the work of Ronning et al. has been proposed. An important feature of the mechanism is the attack on the carbonyl carbon of TMM by Ser124, resulting in the elimination of trehalose from the binding site while the mycoly group is retained as part of Ag85 complex (Scheme 1.3).

Consequently, a second molecule of TMM (or the terminal end of AGP is believed to enter the carbohydrate binding pocket (Figure 1.10). The mycolyl moiety attached to Ser124 of Ag85 is then transferred, forming either a TDM or a mAGP. This is the ping-pong mechanism. There is also an adjacent hydrophobic tunnel which is believed to accommodate the α-chain of the mycolate ester.
Scheme 1.3: Proposed Mechanism of Mycolyl Transfer to TMM or AGP to form TDM and mAGP, respectively. Adapted from Sanki et al.\textsuperscript{12}
1.8 Rationale for Targeting Ag85

Several studies have established the mycolytransferase role of Ag85, and hence, the importance of targeting Ag85 for the inhibition of mycobacterial cell wall synthesis. For example, Belisle et. al. targeted inhibition of Ag85 proteins in vivo in Mycobacterium aurum using 6-azido-6-deoxy-α-D-trehalose (ADT). The result showed a 87% decrease in the level of production of TDM and 44% reduction in the synthesis of TMM. The study also reported 60% inhibition of mycolytransferase activity of all three
Ag85s, with a minimum inhibitory concentration (MIC) of 200 µg/mL. Similarly, Jackson et al. demonstrated a 40% reduction in the amount of cell wall linked mycolic acids produced in Ag85C knocked out of M.tb, but did not result in any change in the quantity of non-covalently bound lipids such as TDM. On the other hand, inactivation of the genes encoding for Ag85A and Ag85B did not seem to have any effect in the amount of cell wall bound mycolates as well as noncovalently bound lipids such as TDM. Harth et al showed that disruption of fbpA resulted in poor growth of M.tb in a macrophage-like cell, although disruption of Ag85B did not appear to have any effect on mycobacterial growth. However, when the three fbp genes encoding for all three enzymes were simultaneously targeted by modified oligodeoxyribonucleotides to inhibit translation, 90% reduction in expression of Ag85 genes was observed, with 90% inhibition of M.tb growth. In fact, there was an 8-fold increase in bactericidal effect noticed when this antisense technology was used in combination with INH (isoniazid).

There are four inferences that could be drawn from these studies, namely, 1.) that whereas Ag85A or Ag85B could not compensate for the inactivation of fbpC2, these three enzymes are not fully redundant, 2.) that M.tb utilizes differential expression of these three enzymes in order to survive and evade host’s macrophages, 3.) that concurrent targeting of all three Ag85 enzymes is a more likely approach to succeed in overcoming mycobacterial resistance than single enzyme targets; and 4), the study demonstrates the synergistic effect that a drug targeting Ag85 could have in tandem with other mycobacterial drugs – thereby greatly improving prospects for combination therapy.
There is also another rationale for targeting Ag85 for the development of novel antitubercular therapeutics. That is tied to the fact that Ag85 complex is extracellularly located, and as a result, the usual bacterial attack against antibiotics, such as drug-modifying enzymes which act intracellularly and efflux pumps within the bacterial membrane, would have little or no effect on drugs targeting Ag85. Finally, since Ag85 complex is not found in humans, the xenobiotic nature of any specific inhibitor containing trehalose or arabinose (natural substrates) may present minimal side effects. Ag85 is also an attractive target for development of new therapeutics against tuberculosis due to the hypothesis that all three enzymes use TMM a common mycolyl donor. This hypothesis is based on genetic data, biochemical data, and thorough characterization and inspection of the six available crystal structures. Consequently, the possibility of all three Ag85 enzymes using TMM as a common mycolyl-donor simplifies inhibitor development and allows for simultaneous targeting of all three Ag85 enzymes with the same compound.

1.9 Inhibition of Antigen 85: An Emerging Strategy for Development of Anti-tuberculosis Drugs.

Perhaps as a mark of its importance in development of new therapeutics for tuberculosis, Ag85 complex has become the target of different classes of synthetic inhibitors in recent years. After the report by Belisle et al. that ADT inhibited Ag85 activity in their assay, other inhibitors have been designed and synthesized. However, relatively few libraries of inhibitors targeting Ag85 have been reported. There are possibly two reasons why this is so: first, the crystal structure of antigen 85 was only
recently solved\textsuperscript{29-30} and second, development of a robust high-throughput assay to quickly and easily test new synthetic inhibitors have been rather slow and difficult.\textsuperscript{11,17} In spite of this challenge, however, various libraries based upon phosphonate compounds 1-6 (Figure 1.11),\textsuperscript{40-41} sulfonate compounds 7 (Figure 1.11)\textsuperscript{42} and two types of trehalose analogs 8-14 (Figure 1.12)\textsuperscript{43-44} have been reported.

\begin{align*}
1 & \ n = 3; \text{IC}_{50} = 3.56 \mu M \\
2 & \ n = 4; \text{IC}_{50} = 1.06 \mu M \\
4 & \ n = 4, \ x = 1, \ R = \text{Et}; \text{IC}_{50} = 1.31 \mu M \\
5 & \ n = 3, \ x = 2, \ R = \text{H}; \text{IC}_{50} = 4.39 \mu M \\
6 & \ n = 4, \ x = 2, \ R = \text{H}; \text{IC}_{50} = 1.47 \mu M \\
7 & \text{IC}_{50} = 4.3 \mu M
\end{align*}

Figure 1.11. Current synthetic inhibitors of Ag85 complex: phosphonate compounds (1-6),\textsuperscript{40-41} sulfonate compound (7).\textsuperscript{42}

As the Ag85 enzymes catalyze mycolyl transfer using a common ping-pong mechanism that proceeds through a covalent tetrahedral transition state, similar to the mechanism of serine proteases,\textsuperscript{29-30} strategies used to inhibit serine proteases can potentially be used to inhibit members of the Ag85 complex. Based on the proposed mechanism, phosphonate and sulfonate compounds (1-7) were designed to act as transition state analogs.\textsuperscript{40-43} Once synthesized, the compounds were assayed using the
radiometric assay described by Belisle et al.\textsuperscript{33} The first trehalose-based compounds 8 and 9 were designed before the crystal structure of Ag85C was reported.\textsuperscript{43} The second trehalose-based library 10-14 was derived from mechanistic studies and included substrate analogs.\textsuperscript{44} The two libraries of compounds were assayed using a disk diffusion assay against \textit{M. smegmatis} (ATCC 14468) and their inhibitory activities are presented in Figure 1.12. Only the most potent compounds are shown.

\textbf{Figure 1.12:} Current synthetic inhibitors of Ag85 complex: trehalose-based analogs (8-14).\textsuperscript{43-44}

Boucau \textit{et al.} also reported a spectrophotometric coupled assay measuring \textit{M.\textit{tb}} Ag85C activity.\textsuperscript{11} This assay was used in two different studies. First, it was used on a moderate scale for the characterization of formed products.\textsuperscript{11, 34} Belisle \textit{et al.} had previously shown the production of TDM using Ag85C.\textsuperscript{33} Therefore, Sanki \textit{et al} synthesized a number of arabinofuranosides which were tested as acyl acceptors in the Ag85C catalyzed transfer reaction.\textsuperscript{34} By employing the use of both NMR and ESI-MS,
this study clearly demonstrated that Ag85C readily catalyzes acylation at the 5-OH position of arabinofuranose-based compounds.\textsuperscript{13,34} While both $\alpha$- and $\beta$ arabinofuranoside were modified at the 2 and 5 positions, an arabinosylarabinoside more closely mimicking the non-reducing termini of the AG was modified only at the 5 position, even though a 2-OH was available for acylation. This assay by Boucau et al was also tested for feasibility in high-throughput screening applications using the NIH clinical collection (NCC). The screening test showed that two known mycobacterial drugs, ebselen and clofazimine, both showed inhibition of Ag85C activity (unpublished data), thereby indicating that the Ag85 complex may be one of the targets inhibited by these compounds. The assay was then used to test the possible inhibitory activity of methyl $S$-alkyl-$S$-thio-D-arabinofuranosides (\textsuperscript{15-20}, Figure 1.13) synthesized by Sanki et al.\textsuperscript{12,13} While compounds containing a 5-$S$-octyl side chain showed activity in a growth inhibition assay against \textit{M. smegmatis} ATCC 14468, the compounds were inactive in the spectrophotometric coupled assay. Most recently, Elamin et al have also reported a novel colorimetric assay using the natural substrate, TMM.\textsuperscript{17} The assay is based on the quantification of glucose from degradation of trehalose, which is the product from the catalytic activity of Ag85A. This assay is reported to be quite robust, with a low coefficient of variance of 0.04 in 96-well plates, and shows a \textit{Z'} factor of 0.67-0.73.
1.10 Conclusion

The emergence of drug resistant strains of *M.tb* and co-infection with HIV has made the need for new anti-tuberculosis drugs even more urgent. It is generally accepted that the hydrophobic nature of *M.tb* cell wall serves as barrier to drugs and also contributes to its ability to survive. The important role of *M.tb* cell wall is further highlighted by the fact that such well known anti-tuberculosis drugs such as isoniazid and ethambutol work by inhibiting the biosynthesis of major components of the *M.tb* cell wall.

Currently, one approach to developing new drugs has been to focus on new pathways or enzymatic targets not specifically targeted by existing drugs. The idea is to identify new targets to which *M.tb* has not yet developed resistance. One of such new enzymatic targets currently generating interest is Ag85. This report has cited several studies underscoring the viability of Ag85 as potential target for developing new anti-tuberculosis drugs due to its ability to catalyse the biosynthesis of major components of the *M.tb* cell wall, namely, TDM and mAG. The case for targeting Ag85 seems strong enough. Studies have shown that drugs targeting this enzymatic complex can work
synergistically with current tuberculosis drugs such as isoniazid. Research also suggests that targeting all three Ag85 enzymes is not only a more viable approach than targeting individual members, it may also help in reducing the incidence of drug resistance especially in combination therapy with other drugs. It has also been noted that since Ag85 is extracellularly located, it is possible to reduce the effect of bacterial active efflux and drug-modifying enzymes that act intracellularly. Finally, the fact that Ag85 is not found in humans presents an opportunity to develop drugs that select for Ag85 with possibility of reduced side effects.

We have also reviewed current small molecule inhibitors of Ag85. From ADT studied by Belisle and coworkers, sulfonates, phosphonates, trehalose-based compounds, to arabinose based thiols developed by Sanki et al, we have shown that some of these compounds not only inhibited Ag85 mycolyltransferase activity but also exhibited antibacterial activity in many cases. This underscores the fact that Ag85 is an emerging target of interest in developing new tuberculosis drugs.
CHAPTER 2

Synthesis of Carbohydrate-based Thioester Derivatives for Development of Antigen 85 Mycolytransferase Assay

2.1 Introduction

There has been significant amount of knowledge gained over the past several years concerning the crystal structure and protein-substrate interactions of Ag85 complex.28-30 Yet, the design of potent inhibitors of Ag85 mycolytransferase activity has been rather slow in coming. The reason for this, among others, is due to the fact that the development of rapid and easy assays suitable for high-throughput screening (HTS) has been even slower. To date, three mycolytransferase assays, two of which are suitable for HTS, have been reported.11,17,33,45-46

The first reported mycolytransferase assay was in 1987.45-46 In order to determine the enzymes and genes responsible for mycolic acid deposition, Belisle et al.33 developed a mycolyltransferase assay in which non-radioactive mycolic acids from lipid-soluble TMM were transesterified to radioactive water-soluble \([^{14}\text{C}]{\alpha}-\text{D-trehalose},\) resulting in the formation of lipid-soluble \([^{14}\text{C}]\) TMM and \([^{14}\text{C}]\) TDM. Hence, this mycolyltransferase assay involves the isolation of TMM from \(M.\text{tb}\), the enzymatic transfer of mycolic acids from a lipid-soluble TMM molecule to a radioactive water-soluble trehalose molecule,
the manipulation of the radioactive products in a two-phase reaction, and a subsequent aqueous workup with extraction and thin layer chromatography which then allows for visualization of the products. The transferase activity was assessed in terms of product formation. Typically, this mycolyltransferase assay was carried out by suspending 250 µg of TMM in 450 µL of 0.1 M potassium phosphate (pH 7.5), 0.1 mM dithiothreitol (DTT). To this was added 10 to 50 µg of enzyme, and the mixture was sonicated for 30 s. [\textsuperscript{14}C] Trehalose (20 nmol, 30.4 mCi/mmol) was then added, and the mixture was incubated at 37 °C for 30 min. The reaction was stopped by adding 5 mL of CHCl\textsubscript{3}:CH\textsubscript{3}OH (2:1) and 1.4 mL of water.\textsuperscript{33,45}

Figure 2.1: A synthetic substrate, 6-azido-6-deoxy-\(\alpha\)-D-trehalose (ADT) used in a radiometric assay used by Belisle et al.\textsuperscript{33} ADT showed inhibition of the mycolyltransferase activity of all Ag85s by 60%; inhibition of synthesis of TDM (87%), and TMM (44%); and completely suppressed the growth of Mycobacterium aurum with MIC of 200 µg/mL concentration.\textsuperscript{33}

Belisle and coworkers used this assay successfully to assess Ag85 mycolyltransferase activity.\textsuperscript{33} In fact, the use of 6-azido-6-deoxy-\(\alpha\)-D-trehalose (ADT) as antagonist established that this complex is not only essential for activity but also a potential target for development of new anti-tubercular drugs. For example, their study
showed that treating Ag85 complex with ADT (Figure 2.1) resulted in decreased production of TMM by 44% and TDM by 87%, and inhibited over-all antigen 85 activity by up to 60% at 100 µg/mL concentration.33

Although this mycolyltransferase assay was successful in assessing antigen 85 activity, its complexity and the use of radioactive compounds makes it unsuitable for HTS. Consequently, our laboratory in conjunction with the Ronning laboratory developed another mycololytransferase assay suitable for HTS.11 The design of the assay was premised on choosing a substrate that adequately resembles the natural substrate of the enzyme in order to ensure enzyme specificity, as well as ensure that the reaction velocity observed accurately reflects the activity of the antigen 85, and not some other coupled enzyme. Since the mycolic acid in TMM is linked at the O6 position of the glucose moieties in trehalose, Boucau and coworkers reasoned that an octanoyl moiety linked at O6 of glucose would be a reasonable structural analog.11 Therefore, a glucose derivative with an acyl chain should bear enough structural similarities with the natural substrate to be recognized by the enzyme. Additionally, it has been shown that the active site of the Ag85 enzymes readily accommodates a variety of carbohydrates and glycoconjugates. For instance, both disaccharide molecules such as trehalose and glycoconjugates containing alkyl moieties such as octylthioglucoside have been shown to bind within the active sites of Ag85B and Ag85C.28-30 Consequently, a glucose derivative esterified at the 6-O position with an eight-carbon acyl chain and a p-nitrophenyl moiety attached by a β-linkage onto O1 of glucose was chosen as the substrate for the assay (compound 1 in Scheme 2.1). As shown by Figure 2.2, this assay worked really well in predicting antigen 85C activity.
Scheme 2.1: Coupled Colorimetric Assay for Ag85C Activity Developed by Boucau et al. Works in Two Steps. First, a nucleophilic attack on the ester linkage of the octanoyl chain of \( \text{1} \) by Ag85C releases \( p \)-nitrophenyl- \( \beta \)-D-glucoside \( \text{2} \), which, in the second step, is then hydrolyzed by \( \beta \)-glucosidase into glucose \( \text{3} \) and \( p \)-nitrophenolate \( \text{4} \). The rate of \( p \)-nitrophenolate release is then observed by direct measurement of the absorbance over time. By measuring the absorbance at 405 nm, one can directly monitor the change in \( p \)-nitrophenolate concentration, which is an indication of the enzyme activity. An excess of \( \beta \)-glucose is also added to the reaction to function as an acyl acceptor and to promote turnover of the enzyme.\(^{11}\)

This assay was not only successful in assessing inhibitory activity of synthetic substrates,\(^{12,34,47}\) it was also very useful for HTS purposes.\(^{11,13}\) However, attempt to use this assay to screen newer libraries of synthetic inhibitors proved rather difficult. It was noticed that background noise coming from the \( \beta \)-glucosidase was variable and sometimes too high. Hence, our laboratory in conjunction with the Ronning laboratory
again embarked on the design and synthesis of new natural analogs suitable for developing HTS mycolyltransferase assays.

Figure 2.2: Positive and negative control reactions for colorimetric assay developed by Boucau et al. Reactions were performed with no enzyme (\(\square\)), glucosidase only (+), Ag85C and glucosidase (X). The concentration of the substrate in each reaction was 175 µM.


At about the time we were working on the substrates discussed in this chapter, however, another successful mycolyltransferase assay suitable for HTS was reported by Elamin et al.¹⁷ This assay was different from the one published by Boucau and
coworkers\textsuperscript{4} in that it utilized the natural substrate (TMM) of Ag85 instead of a synthetic substrate. TMM was extracted from \textit{M. smegmatis} (mc\textsuperscript{2}155) grown in Middlebrook 7H9 medium supplemented with ADC enrichment (BD Diagnostic Systems, Sparks, MD).\textsuperscript{5}

Scheme 2.2: Reaction Scheme for Novel Mycolyltransferase Activity Assay Developed by Elamin \textit{et al.}\textsuperscript{17} Ag85A converts two molecules of TMM to TDM and trehalose. Trehalose is converted by trehalase to two molecules of glucose, which is then oxidized to gluconic acid and hydrogen peroxidase by glucose oxidase. Hydrogen peroxide reacts with o-dianisidine in the presence of peroxidase to produce a colored product (oxidized o-dianisidine), which will be converted to a stable colored product by sulfuric acid. The colored product is measured at 540 nm.\textsuperscript{17}

Since it was based on TMM, the assay was reported to not only give an indication of the mycolyltransferase activity of Ag85, but also provided a means to quantify, albeit indirectly, the amount of TMM present in cell wall. This quantification of TMM in cell
wall also provided an indication of the fitness of the mycobacterial cell wall within the macrophage.

The series of steps involved in this assay are illustrated in Scheme 2.2. First Ag85A converts two molecules of TMM to TDM and one molecule of trehalose. Then, trehalose is converted to two molecules of glucose by trehalase. The amount of glucose is then assayed by the glucose oxidase assay. Control experiments (Figure 2.3) show that this assay is an accurate measurement of antigen 85A activity.

Figure 2.3: Time course and control of the assay developed by Elamin et al. Without trehalase and antigen 85A (■), with trehalase (○), and with antigen 85A and trehalase (Δ). Reactions were performed with 150 mM TMM and 175 nM antigen 85A. The experiment was carried out in a final volume of 300 µL in a 96-well microplate at 37 °C. “Reprinted from J. Microbio. Methods/ 79 / Elamin et al, The mycolyltransferase 85A, a putative drug target of Mycobacterium tuberculosis: Development of a novel assay and quantification of glycolipid-status of the mycobacterial cell wall / 358-363, Copyright 2009, with permission from Elsevier.”
2.2 Design of Thioester Derivatives as Substrates for Assay Development

In designing substrates for a new assay, we took note of the fact that previous studies have shown that the active site of antigen 85 shows tolerance for a range of carbohydrate-based substrates.\textsuperscript{11,28,30,33} Figure 2.4 shows four synthetic substrates that this project was set out to explore. It is important that the substrate chosen should be as close to the natural substrate, TMM, as to reflect the native activity of the enzyme. This is why we have chosen trehalose as a substrate, and have designed mono-thioacid and thiophosphoryl derivatives to closely mimic the mono-acylated trehalose monomycolate. We have also chosen α-methylglucose as another substrate to study since a previous study\textsuperscript{11} has shown that Ag85C recognizes glucose substrate.

![Figure 2.4: Synthetic targets for development of coupled spectrophotometric assay for Ag85 activity.](image)

2.3 Choice of Thioester Derivatives

Sulfur is a known isostere of oxygen. In fact, replacement of oxyester with thioester functions provides alternate lipolytic enzyme substrates that are considered close structural mimics of physiological lipid substrates.\textsuperscript{49} Since hydrolysis of thiolipid
substrates can be easily followed in coupled spectroscopic assays, they are believed to have analytical advantages over traditional radiochemical assays.49-51

In certain cases, carbohydrate-based drugs have shown limited stability in vivo as a result of the ability of glycosidase enzymes to act on them.49 However, a number of studies have reported that S-linked synthetic analogs in which the interglycosidic oxygen has been replaced by sulfur have sometimes shown greater hydrolytic stability.52-54 The relative stability of a colorimetric product is very important as it allows enough time for colorimetric measurements.11,17 In this case, the expected fluorescent product (12, Scheme 2.3) is a viable target. S-linked carbohydrate drug candidates have also been shown to participate in hydrogen bonding, as well as accurately mimic natural carbohydrates.55-57 By incorporating a hydrophobic phenyl group in 6 and 7 (Figure 2.4), it is expected that they will each provide better binding interaction with the active site, since the hydrophobic group is expected to fit in nicely in the hydrophobic binding pocket of the active site. The proposed TMM binding site (Figure 1.10) within Ag85 has hydrophobic pockets where the α-alky chain and the meromycolate branch of a mycolic acid are proposed to fit in. We therefore propose that a phenyl group will more likely provide a better hydrophobic interaction with the hydrophobic binding pocket, than will a methyl group as in the case of compound 5.

It is with this background that our laboratory designed simple trehalose-derived thioesters 6 and 7, as well as glucose-based thiobenzoate 7 (Figure 2.4) as substrate for Ag85C. Since Ag85 belongs to the α/β hydrolase superfamily, it was reasoned that it could have the ability to transfer the acyl group, leaving a trehalose thiol which could
then react with bromobimane to give a fluorescent tag that could be used to track the activity of Ag85 (Scheme 2.3).

Scheme 2.3: A Proposed Coupled Spectrophotometric Assay for Ag85 Activity, Showing the Two Steps Involved in the Assay. First step is the hydrolysis of carbohydrate-based ester to release a thiol. Bromobimane is then introduced in the second step to react with released thiol to form a colorimetric compound. Measurement of fluorescence at 478 nm gives a direct indication of the concentration of the trehalose-based thiobimane compound, which accurately reflects the enzyme activity.
2.4 Expected Mechanism

Antigen 85 works by a nucleophilic attack on the carbonyl carbon of the acyl chain, thereby transferring an acyl group (in this case, a mycolyl group) to another molecule of trehalose or to the terminal end of arabinogalactan moiety through the primary hydroxyl oxygen. In this way, a trehalose or glucose-based thioacid derivative closely resembles TMM. Since thioacid derivatives are reactive intermediates which are easily susceptible to nucleophilic attack to give a thiol and an esterified product, it is expected that the attack of serine OH of antigen 85 on the carbonyl carbon of the thioester group in 9 would release a thiol 10 and an esterified antigen 85. This would be the first step in a two-step mechanism (Scheme 2.3). In the second step, the released carbohydrate-based thiol 10 then reacts with bromobimane 11 via a nucleophilic attack to give a fluorescent compound 12. Bromobimane is a heterocyclic compound which is often used to alkylate thiol groups, replacing the H with a fluorescent tag ($\lambda_{\text{emission}} = 478$ nm). As a result, bromobimane is often used as a fluorescent tag to monitor reaction kinetics.\textsuperscript{57-58} Hence, measuring the fluorescence at 478 nm allows direct monitoring of the change in trehalose-thiobimane 12 concentration, which is an indication of the enzyme activity. An excess of $\beta$-glucose is also added to the reaction to act as an acyl acceptor, thereby serving to promote turnover of the enzyme.

2.5 Synthesis of thioacid derivatives

The goal of this part of the project was the successful synthesis of compounds 5-8 (Figure 2.4). Scheme 2.4 shows a five-step synthesis designed to give compound 5, the first in the series. Attempt to directly tosylate trehalose regioselectively at only one
primary hydroxyl failed even after repeated attempts. It was this need to achieve regioselectivity that informed the design of this multi-step synthetic pathway. Consequently, the first target 5 was assessed in five steps beginning with trehalose, 13.

Scheme 2.4: Multi-step Synthesis of Thioacetate analog (5). Reagents and conditions: a) NaH, BnBr, Bu₄NI, DMF, 0 °C to r.t., 4 hr; b) 1.Ac₂O, TMSOTf, CH₂Cl₂, -78 °C to r.t. 5hr, 2. MeONa/MeOH, Amberlite IR 120 (H⁺ form), 0.5hr ; c) TsCl, Pyridine, DMAP (catalytic), 50 °C, 8 hrs; d) MeOH/EtOAc (1:1), Pd(OH)₂/C (20% Degussa type), H₂, 24 hrs, r.t.; e) AcSK, DMF, 50 °C, 4 hr.

The synthesis of carbohydrate-based thioester 5 began with the synthesis of per-benzylated trehalose 14. This pathway was chosen due to our desire to find a relatively easy way to access a fully protected trehalose with only one free primary hydroxyl. The
synthesis of 14 was rather straightforward, and followed the protocol adopted by Gilbertson et al.\textsuperscript{59} Briefly, this protocol required dissolving anhydrous trehalose in DMF under nitrogen gas, and catalytic amount of tetrabutylammonium iodide (Bu$_4$NI) was added. The reaction mixture was then transferred to an ice-water bath maintained at 0 °C, before adding enough NaH (60%) to deprotonate the hydroxyl groups (two equivalents of NaH per OH). To reduce effervescence, NaH was added in small portions, each time taking care to minimize exposure of reaction mixture to air and moisture. Reaction mixture was then stirred for about 1 hour at 0 °C, after which 1.5 equivalents of benzyl bromide per OH was added dropwise. Reaction mixture was then stirred, allowing temperature to warm up to room temperature. Monitored by TLC (20% ethyl acetate in hexane), the reaction was complete within 5 hours, however, it would usually be left for about 10-12 hours. Once reaction is done, it was then quenched by addition of anhydrous methanol and stirred for 30 minutes more. Aqueous work-up followed with flash column chromatography often results in very high yield of about 99%.

Selective deprotection of one primary hydroxyl group in 14 to give a mono-ol 15 was achieved by an adaptation of similar protocol reported by Wang et al.\textsuperscript{43} This protocol involves TMSOTf-assisted acetolysis of the more reactive primary OBn. This reaction was performed in anhydrous dichloromethane at -78 °C. The reaction mixture was often stirred for 9 hours, allowing the temperature to warm up to room temperature over that time. The resulting acetyl intermediate was then de-acetylated with NaOMe in MeOH within 45 minutes. Amberlite IR 120 (H$^+$) was often added at this stage to slightly acidify the medium, resulting in mono-deprotected alcohol 15 in 43% yield. Depending on the
amount of starting materials, at about 4-5 hours, the mono-ol has fully formed with the rest being the starting material.

In order to make the free primary OH a good leaving group, and hence effect nucleophilic substitution at C-6, the OH was protected with a tosyl group starting with TsCl in pyridine.$^{55,59}$ Although this is a relatively easy reaction that could be run at low temperature of $0 – 4$ °C,$^{55}$ the reaction did not proceed quickly when first attempted. Perhaps the extensive benzyl groups created a stearic environment that limited the ease with which the reaction could proceed. To kick-start the reaction, catalytic amount of DMAP was added and the reaction medium was maintained at 50 °C for about 8 hours or more. Under these conditions, the reaction proceeded well to yield the product 16 in good yield of about 82%. Compound 16 was subjected to global deprotection via hydrogenolysis$^{43}$ to give 17 before proceeding to the thioesterification step.$^{52,60-63}$ The final step proceeded well to give 5 in fairly good yield of 77% after reverse phase purification with C-18.$^{60-63}$

2.6 Synthesis of benzothioate derivatives (6 and 7)

Although compound 5 was successfully isolated as the ESI-MS shows, it proved rather unstable. In addition, the purification process was challenging. Based on these problems, we modified our design to come up with compounds 6 and 7 (Figure 2.4). Compounds 6 and 7 will have two distinct advantages over compound 5: First, from the perspective of active site interaction, compounds 6 and 7 are expected to bind better due to the increased hydrophobicity introduced by the phenyl group. Second, from a synthetic
approach, either 6 or 7 can be readily assessed through a one-step synthesis via a Mitsunobu type reaction\textsuperscript{64-65} using thiobenzoic acid (Schemes 2.5 and 2.6).

With the improved design, the synthesis of 6 was embarked upon, but only compound 18 (the di-substituted analog) was successfully isolated in 72\% yield (Scheme 2.5). Although only 1.5 mole equivalent of thiobenzoic acid was used, the reaction was so fast that the two primary hydroxyl groups were readily esterified even at low temperature of 0 °C. Compound 18 was characterized by both $^1$H and $^{13}$C NMR. For example, the $^{13}$C NMR showed the characteristic thio-carbonyl peak at 190.96 ppm. Since the compound is symmetric, only thirteen distinct carbon peaks were expected as confirmed by the $^{13}$C NMR. Although a few impurities showed up in the $^1$H NMR, the remainder of the peaks accurately reflects compound 18. Also, high resolution mass spectrometry (HR-MS) identified M + 23 peak at $m/z = 605.11$ confirming the presence of compound 18.

![Scheme 2.5: Synthesis of α-D-Trehalose-derived Benzothioate Analog (18). Reagents and conditions: a. PPh$_3$, DIAD, anhydrous THF, 0 °C to r.t, 8 hr.](image_url)
In an attempt to retain the advantages of 6 (similarity to natural substrate, TMM, and greater binding interaction due to the phenyl group), compound 7 was designed (Scheme 2.6). It makes sense to suppose that since there is only one primary hydroxyl group in α-methyl-α-D-glucose that this reaction would precede without difficulty. And in fact, the synthesis of 7 in one-step using Mitsunobu conditions (Scheme 2.6)\textsuperscript{30-31} proceeded well in about 90% yield. Purification by flash column chromatography was also possible due to the presence of only three hydroxyls. The presence of 7 was confirmed by both $^1$H and $^{13}$C NMR. Although there remained traces of impurity, overall, NMR data confirmed the presence of 7. For instance, in the $^{13}$C NMR, the thio-carbonyl peak was clearly present at 206.39 ppm. There was also a second carbonyl peak noticed at 191.73 ppm. This second peak seems to suggest the presence of a partial thioacylation of a second OH group, which is consistent with the observations of Knight \textit{et al} \textsuperscript{20-21} on a glucose substrate. High resolution mass spectrometry (HS-MS) also recorded a (M + Na) $m/z$ = 337.0, which is a good match for the calculated (M + Na) $m/z$ = 337.08.

\begin{center}
\includegraphics[width=0.7\textwidth]{image.png}
\end{center}

\textit{Scheme 2.6: Synthesis of α-D-Methylglucoside-derived Benzothioate Analog (7).}

\textit{Reagents and conditions:} f. PPh$_3$, DIAD, anhydrous THF, 0 °C to r.t., 8 hours.
2.7 Conclusion

The fact that there are currently two mycolyltransferase assays suitable for HTS screening, motivated the design and synthesis of carbohydrate-based thioesters described in this chapter. We chose α-D-trehalose and α-methyl-α-D-glucose as substrates because trehalose is a natural substrate and glucose has been shown in a previous study to be well tolerated by Ag85C. Compounds 5, 6 and 7 were designed to resemble TMM which is mono-substituted.

The synthesis of compounds 5 and 7 was successfully accomplished. Starting with commercially available materials, we have modified α-D-trehalose at the C-6 position through a multi-step synthesis to make thioacetate 5 in 77% yield. Although compound 5 was successfully isolated and characterized, purification proved challenging. Partial purification was achieved by using silica gel column chromatography, as well as C-18 reverse phase column chromatography.

Similarly, α-methyl-α-D-glucose was successfully modified at the C-6 position using Mitsunobu conditions in one step to give thiobenzoate 7 in 90% yield. Attempt to synthesize mono-substituted trehalose-based thiobenzoate 6 using a similar protocol proved challenging. Instead, the di-substituted analog 18 was successfully isolated in 72% yield. The reaction was so fast that the two primary OH groups were modified at the same time. Efforts were made to control regioselectivity by reducing the mole equivalent of thiobenzoic acid used and by lowering the temperature of the reaction. However, these changes failed to yield the mono-substituted analog in any significant amount to allow for isolation and characterization.
Attempt to use 5 as an acyl donor in the mycolytransferase assay was not successful. There was a very high background noise possibly coming from residual thiol in compound 5. This is not surprising since we have already noted the difficulty encountered in purifying compound 5. In addition, we note that this possible interference from residual thiol is a major draw back in using bromobimane as a fluorescent tag. As a result of this difficulty, the Ronning laboratory in conjunction with our laboratory, decided to abandon this assay in favor of another assay that will not use bromobimane. This assay is currently being developed as a separate project. Hence, by the time synthesis of compounds 7 and 18 was completed, a decision was made not to continue their use as probes for Ag85 at this time.

To be able to use this assay in the future, it is important to ensure total purity of these compounds. Perhaps one way to do this is to investigate whether the use of ion-exchange chromatography may be a better way to purify a polar compound such as 5. Notwithstanding the difficulty encountered at the assay stage, the successful synthesis, isolation and characterization of 5, 7 and 18 is an important synthetic contribution, and provides a basis for further development of similar synthetic probes for Ag85.
2.8 Experimental Section

α-D-Trehalose and other fine chemicals were purchased from commercial suppliers and were used without further purification. All solvents used for reactions were dried following the standard procedures.66 Triethylamine was dried using MS 4Å. Thin-layer chromatography (TLC, silica gel 60, f254) were performed in distilled solvents as specified and visualized under UV light or by charring in the presence of 5% H2SO4/MeOH. Flash column chromatography was performed on silica gel (230-400 mesh) column using solvents as received. 1H NMR were recorded either on Varian VXR-400 (400 MHz) or INOVA-600 (600 MHz) spectrometer in CDCl3, CD3OD or DMSO-d6 using residual CHCl3, CH3OH or DMSO as internal references, respectively. 13C NMR were recorded either on Varian VXR-400 (100 MHz) or INOVA-600 (150 MHz) spectrometer in CDCl3, CD3OD or DMSO-d6 using the triplet centered at δ77.3 for CDCl3, septet centered at δ49.0 for CD3OD, or septet centered at δ39.5 for DMSO-d6 as internal reference, respectively. The 1H NMR data are presented as follows: chemical shift, multiplicity (s = singlet, d = doublet, dd = doublet of doublet, t = triplet, q = quartet, m = multiplet), coupling constants in Hertz, integration, and assignments. Low resolution mass spectra were taken on Esquire-LC electrospray ionization (ESI) mass spectrometer operated in the positive ion mode. High resolution mass spectrometry (HRMS) was performed on a mass spectrometer located at the Mass Spectrometry and Proteomics Facility, The Ohio State University.
2.8.1 Synthesis of 2,2',3,3',4,4',6,6'-Octa-O-benzyl-α-D-trehalose (14)\textsuperscript{59}

Anhydrous α-D-trehalose 13 (8.71 mmol, 3.00 g) was dried for about 30 minutes under high vacuum, and then dissolved in 100mL of anhydrous DMF under nitrogen atmosphere. To this solution was added 0.3 g (0.87 mmol) of tetrabutylammonium iodide, and cooled to 0 °C. NaH (139.2 mmol, 3.3 g) was added to the reaction mixture in portions over 30 minutes, and the reaction mixture stirred for 1 hour at 0 °C. Benzyl bromide (104 mmol, 12.4 mL) was added drop-wise to the reaction mixture at 0 °C. The reaction mixture was stirred under nitrogen, allowing the temperature to warm up to room temperature. Reaction progress was monitored by TLC using hexane and ethyl acetate (8:2). The reaction was completed after 4 hours, and was quenched by adding 30 mL of anhydrous MeOH, and stirred for another 1 hour at room temperature. The organic layer was diluted with CH₂Cl₂, washed twice with 30 mL of saturated NaHCO₃, then washed with 50 mL water twice, and dried over anhydrous Na₂SO₄. The organic layers were combined and concentrated \textit{in vacuo} to give crude product. The crude product was purified by silica gel flash column (230-400 mesh 15 × 6.5 cm). Elution with 8:2 hexane: ethylacetate afforded 9.28 g of colorless gummy product 14 in 99% yield; \(R_f = 0.67\) (3:7 EtOAc/hexanes). \(^1\)H NMR (400 MHz, CDCl₃) \(\delta\) 7.17-3.36 (m, 40H), 5.24 (d, \(J = 3.0\) Hz, 1H, H-1), 5.23 (d, \(J = 3.3\) Hz, 1H, H-1’), 5.01 (d, \(J = 10.9\) Hz, 2H), 4.68-4.98 (m, 8H, PhCH₂ O), 4.57 (d, \(J = 11.2\) Hz, 1H), 4.54 (d, \(J = 12.2\) Hz, 1H), 4.46 (d, \(J = 10.9\) Hz, 1H), 4.44 (d, \(J = 11.9\) Hz, 1H), 4.01-4.36 (m, 3H), 3.69 (dd, \(J = 9.9\) Hz, 9.2 Hz, 1H), 3.51-3.61 (m, 7H), 3.36 (d, \(J = 10.6\) Hz, 1H); \(^{13}\)C NMR (100 MHz, CDCl₃) \(\delta\) 140.46, 139.90, 139.75, 139.38, 129.93, 129.60, 129.48, 129.28, 129.11, 129.08, 128.98, 96.08, 88.40, 80.95, 79.26, 79.02, 78.69, 78.38, 77.23, 76.71, 75.09, 74.27, 72.20, 69.68; ESI-
MS Calculated for C\textsubscript{68}H\textsubscript{70}O\textsubscript{11}Na \( m/z = 1085.49([\text{M+Na}^+] \); observed \( m/z = 1085.95 ([\text{M+Na}^+] \). Data was consistent with what was reported in literature.\textsuperscript{59}

### 2.8.2 Synthesis of 2,2',3,3',4,4',6-Hepta-O-benzyl-\(\alpha\)-D-trehalose (15)\textsuperscript{43}

A solution of 14 (1.2 mmol, 1.3 g) in anhydrous CH\textsubscript{2}Cl\textsubscript{2} (10 mL) and acetic anhydride, Ac\textsubscript{2}O (3.6 mmol, 0.45 mL) was prepared and maintained at -78 °C. To this solution was added TMSOTf (1.6 mmol, 0.3 mL) drop-wise under \( \text{N}_2 \) atmosphere while stirring. After addition of TMSOTf, reaction mixture was allowed warm up to room temperature. The reaction progress was monitored by TLC (hexane: EtOAc, 7:3). The reaction was complete in 5 hours, after which a solution of NaOMe (2 M in MeOH, 3 mL) was added and stirred for another 30 minutes. The solution was neutralized with Amberlite IR 120 (H\textsuperscript{+} form), filtered and concentrated. Crude material was purified by silica gel flash column (230-400 mesh, 10 x 6.5 cm). Elution with 8:2 and 6:4 hexane:EtOAc resulted in 0.51 g of clear gum-like oil 15 in 42% yield; \( R_f = 0.47 \) (3:7 EtOAc/hexanes); with 0.42 g of starting material recovered. \( ^1\text{H} \) NMR (600 MHz, CDCl\textsubscript{3}) \( \delta \) 7.1–7.4 (m, 35H), 5.21 (d, \( J = 3.0 \) Hz, 1H), 5.20 (d, \( J = 3.3 \) Hz, 1H, H-1'), 5.01 (d, \( J = 10.9 \) Hz, 2H, PhCH\textsubscript{2} O), 4.7–4.9 (m, 8H), 4.66 (d, \( J = 11.2 \) Hz, 1H), 4.56 (d, \( J = 12.2 \) Hz, 1H), 4.48 (d, \( J = 10.9 \) Hz, 1H), 4.40 (d, \( J = 11.9 \) Hz, 1H), 4.0–4.2 (m, 3H), 3.69 (dd, \( J = 9.9 \) Hz, \( J = 9.2 \) Hz, 1H), 3.5–3.6 (m, 7H), 3.39 (d, \( J = 10.6 \) Hz, 1H), 2.0 (s, OH); \( ^{13}\text{C} \) NMR (150 MHz, CDCl\textsubscript{3}) \( \delta \) 138.96, 138.90, 138.44, 138.35, 138.25, 138.21, 137.9, 128.56, 128.47, 128.44, 128.2, 128.08, 128.06, 127.98, 127.78, 127.72, 127.62, 127.5, 94.4, 94.2, 81.89, 81.68, 79.64, 79.45, 77.8, 77.45, 75.70 (2 carbons), 75.16 (2 carbons), 73.61, 73.0, 72.92, 71.3, 70.8, 68.3, 61.6; ESI-MS Calculated for C\textsubscript{61} H\textsubscript{64} O\textsubscript{11}Na
m/z = 995.44 ([M+Na]⁺); observed m/z = 995.43 ([M+Na]⁺). Data was consistent with that reported in the literature.43

2.8.3 Synthesis of 2,2’,3,3’,4,4’,6-Hepta-O-benzyl-6’-O-tosyl-α-D-trehalose (16)⁵⁵,⁵⁹

To a solution of 3 (0.31 mmol, 0.30 g) dissolved in 3 mL of pyridine was added TsCl (3.15 mmol, 0.60 g; in 2 mL of pyridine) dropwise at room temperature under N₂; as well as catalytic amount of 4-dimethylaminopyridine (DMAP). Reaction mixture was stirred at 50 °C for 8 hours, monitored by TLC (hexane:EtOAc, 7:3). Then the reaction mixture was quenched with water, extracted with CH₂Cl₂, and washed with NaHCO₃. The organic layers combined, dried over anhydrous NaSO₄ and concentrated. Crude material was purified by silica gel flash column (230-400 mesh, 10 X 4.5 cm). Elution with 7:3 hexane: EtOAc resulted in resulted in 0.29 g of oily gum 16 in 82% yield; Rf = 0.57 (3:7 EtOAc/hexanes). ¹H NMR (400 MHz, CDCl₃) δ 7.12-7.71 (m, 39H), 5.12 (d, J = 3.0 Hz, 1H), 5.00 (d, J = 3.3 Hz, 1H, H-1’), 4.98 (d, J = 10.9 Hz, 2H), 4.7–4.9 (m, 8H), 4.66 (d, J = 11.2 Hz, 1H), 4.56 (d, J = 12.2 Hz, 1H), 4.48 (d, J = 10.9 Hz, 1H), 4.40 (d, J = 11.9 Hz, 1H), 4.0–4.2 (m, 3H), 3.69 (dd, J = 9.9 Hz, J = 9.2 Hz, 1H), 3.5–3.6 (m, 7H), 3.39 (d, J = 10.6 Hz, 1H), 2.38 (s, 3H). ¹³C NMR (100 MHz, CDCl₃) δ 144.96, 138.88, 138.77, 138.20, 138.11, 137.97, 137.86, 132.79, 129.93, 129.85, 128.54, 128.49, 128.15, 128.09, 127.87, 94.67, 94.29, 81.87, 79.21, 73.05, 72.71, 70.81, 68.88, 68.14, 21.75. ESI-MS Calculated for C₆₈H₇₀O₁₃SNa m/z = 1149.45 ([M+Na]⁺); observed m/z = 1149.60 ([M+Na]⁺).
2.8.4 Synthesis of 6-O-Tosyl-α-D-trehalose (17)\(^{43}\)

0.24 g (0.48 mmol) of 16 was dissolved in 5 mL of MeOH:EtOAc 1:1 and degassed under high vacuum. Then 0.30 g of Pd(OH)\(_2\)/C (20% Degussa type) was added to the solution, and degassed again. The reaction mixture was stirred at room temperature under atmospheric H\(_2\) pressure. The reaction was completed in 12 hours, monitored by TLC (EtOAc: MeOH 9:2). Reaction mixture was filtered through Celite, washed with MeOH, and concentrated to give 0.10 g of 17 as a dirty white solid in 91% yield; \(R_f = 0.30\) (3:7 EtOAc/hexanes). \(^1\)H NMR (600 MHz, CDOD\(_3\)) \(\delta\) ppm 7.6 (d, \(J = 3.7\) Hz, 2 H), 7.2 (d, \(J = 3.4\) Hz, 2 H), 4.8 (s, 2 H), 4.0 (d, \(J = 4.6\) Hz, 2 H), 3.8 (d, \(J = 4.9\) Hz, 1 H), 3.6 (m, 3 H), 3.5 (d, \(J = 11.5\) Hz, 1 H), 3.2 (d, \(J = 9.3\) Hz, 1 H), 3.2 (m, 1 H), 3.1 (m, 3 H), 3.1 (m, 1 H), 2.2 (d, \(J = 3.4\) Hz, 3 H). \(^{13}\)C NMR (150 MHz, CDOD\(_3\)) \(\delta\) ppm 146.43, 134.19, 131.03, 130.96, 95.09, 74.27, 73.75, 72.98, 71.73, 71.10, 70.68, 62.51, 49.00, 48.86, 48.77, 48.72, 48.63, 48.58, 21.70. ESI-MS Calculated for C\(_{19}\)H\(_{28}\)O\(_{13}\)Na m/z = 519.13 ([M+Na]\(^{+}\)); observed m/z = 519.00 ([M+Na]\(^{+}\)).

2.8.5 Synthesis of α-D-trehalose-derived thioacetate (5)\(^{55}\)

To a solution of 17 (0.20 mmol, 0.10 g) in 1 mL of DMF was added potassium thioacetate, AcSK (0.23mmol, 0.025 g, dried for 10 minutes under high vacuum before use) under N\(_2\) atmosphere. Reaction mixture was stirred at room temperature, and monitored by TLC (EtOAc:MeOH, 8:2). After completion in 4 hours, reaction mixture was concentrated and purified by reverse phase column chromatography using C-18 (PrepSep C-18, 8 X 1.5 cm) resulting in 0.060 g of compound 5 in 77% yield; \(R_f = 0.23\).
(2:8 methanol/dichloromethane). $^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 5.08 (d, IH), 5.05 (d, IH), 4.51 (s, IH), 3.90 (s, 2H), 3.79 (t, 2H), 3.76-3.43 (m, 5H), 3.33-3.12 (m, 4H), 2.36 (s, 3H), 2.31 (s, 5H); $^{13}$C NMR (100 MHz, D$_2$OD) $\delta$ 197.28, 95.13, 94.87, 75.11, 74.76, 74.36, 74.02, 73.37, 73.33, 72.13, 72.03, 62.72, 32.11, 30.56; HR-MS calculated for C$_{14}$H$_{24}$O$_{11}$SNa $m/z$ = 423.10 ([M+Na]$^+$); observed $m/z$ = 423.40 ([M+Na]$^+$).

### 2.8.6 Synthesis of α-D-trehalose-derived benzothioate (18)$^{61}$

To a solution of triphenylphosphine, PPh$_3$ (2.2 mmol, 0.58 g) in 10 mL anhydrous THF at 0 °C under N$_2$, was added diisopropyl azodicarboxylate, DIAD (2.2 mmol, 0.44 mL) dropwise. The reaction solution was stirred for about 30 – 60 mins. Then trehalose 13 (0.11 mmol, 0.32 g) and thiobenzoic acid (2.2 mmol, 0.26 ml) in 20 mL anhydrous THF was added slowly over 20 minutes to the flask containing solution of PP$_3$ and DIAD, and stirred at 0 ºC for 1 hour. Reaction mixture was stirred at room temperature for about 8 hours, monitored by TLC (Chloroform:acetone:methanol, 6:2:2). After completion of reaction, mixture was concentrated in vacuo. The crude material thus obtained was purified by silica gel flash column (230-400 mesh, 10 X 3.1 cm). Elution with chloroform: methanol (7:3) afforded 0.40 g of 18 as an off-white solid in 72% yield; $R_f$ = 0.43 (2:8 methanol:dichloromethane). Attempt to selectively form the mono analog by varying reagent amounts and reaction conditions failed even after repeated attempts. $^1$H NMR (600 MHz, d-DMSO) $\delta$ 7.9 (d, $J = 8.1$ Hz, 3 H), 7.7 (m, 1 H), 7.5 (t, $J = 7.1$ Hz, 2 H), 4.8 (s, 2 H), 4.1 (s, 1 H), 3.9 (s, 1 H), 3.6 (d, $J = 12.2$ Hz, 1 H), 3.5 (s, 1 H), 3.4 (s, 11 H), 3.2 (s, 3 H), 2.5 (d, $J = 1.2$ Hz, 3 H), 1.0 (td, $J = 7.0$, 1.2 Hz, 1 H). $^{13}$C NMR (150 MHz, DMSO) $\delta$ 191.50, 137.61, 134.10, 129.21, 127.40, 95.21, 73.62, 72.10, 71.50,
70.01, 48.30, 32.10, 18.10; ESI-MS calculated for \( \text{C}_{26}\text{H}_{30}\text{O}_{11}\text{S}_{2} \ \ ([\text{M+Na}]^+) \) \( m/z = 605.1127 \); observed \( m/z = 605.1136 \ ([\text{M+Na}]^+) \).

### 2.8.7 Synthesis of \( \alpha\)-\( \beta\)-methylglucoside-derived benzothioate (7) \(^{61} \)

To a solution of triphenylphosphine (2.2 mmol, 0.58 g) in 10 mL anhydrous THF at 0 °C under \( \text{N}_2 \), was added diisopropyl azodicarboxylate, DIAD (2.22 mmol, 0.44 ml) dropwise. The reaction mixture was stirred for about 1 hour. Then \( \alpha\)-\( \beta\)-methylglucoside \( 19 \) (0.2 g, 1.1 mmol) and thiobenzoic acid (2.2 mmol, 0.26 mL) in 20 mL THF was added slowly over 20 minutes to the mixture, and stirred at 0 °C for 1 hour. Reaction mixture was then stirred at room temperature for another 8 hours, monitored by TLC (CH\(_2\)Cl\(_2\): methanol; 8:2). After completion of reaction, 10 mL of 10% NaHCO\(_3\) solution was added to reaction flask and extracted with EtOAc. The organic layers were dried over anhydrous NaSO\(_4\) and concentrated in \( \text{vacuo} \). Crude material was purified by silica gel flash column (230-400 mesh; 10 x 3.1 cm). Elution with dichloromethane:methanol, 9:1 resulted in 0.28 g of 7 as a colorless solid in 78% yield; \( R_f = 0.52 \) (2:8 methanol/dichloromethane).

\(^1\)H NMR (400 MHz, (CD\(_3\))\(_2\)CO) \( \delta \) 8.0 (d, \( J = 8.1 \) Hz, 4H), 7.7 (dd, \( J = 7.7, 1.5 \) Hz, 2H), 7.6 (m, 4H), 4.6 (m, 2H), 4.6 (d, \( J = 4.0 \) Hz, 1H), 4.4 (s, 1H), 3.9 (d, \( J = 7.3 \) Hz, 1H), 3.8 (d, \( J = 2.6 \) Hz, 1H), 3.7 (s, 2H), 3.6 (s, 2H), 3.4 (s, 2 H), 3.3 (d, \( J = 1.8 \) Hz, 6H), 3.3 (s, 2H), 3.1 (dd, \( J = 4.9, 1.6 \) Hz, 1H), 2.1 (m, 2H). \(^{13}\)C NMR (100 MHz, (CD\(_3\))\(_2\)CO) \( \delta \) 206.39, 191.73, 137.97, 134.45, 129.76, 127.83, 100.92, 74.93, 74.78, 71.53, 55.32, 31.87, 29.92. HR-MS calculated for \( \text{C}_{14}\text{H}_{18}\text{O}_{6}\text{SNa} \ \ m/z = 337.0722 \ ([\text{M+Na}]^+) \); observed \( m/z = 337.0706 \ ([\text{M+Na}]^+) \).
CHAPTER 3

Synthesis of Sugar-derived Esters, α-Ketoesters and α-Ketoamides as Inhibitors for *Mycobacterium Tuberculosis* Antigen 85C

3.1 Introduction: Rationale for Research Design

Continuing our search to identify potential inhibitors of Ag85s, our laboratory hypothesized that 1,2-dicarbonyl compounds such as α-ketoamides and α-ketoesters, known transition state inhibitors of related serine proteases\(^ {67-68}\) may have inhibitory activity against Ag85s. Therefore, using the available X-ray structure of Ag85 (A, B, and C)\(^ {29-30}\) and existing catalytic models,\(^ {29-30,12,69}\) a number of sugar-based analogs have been designed that could bind the active site of Ag85 and form a tetrahedral intermediate capable of inhibiting acyltransferase activity of antigen 85. Catalytic triad of both serine protease (Ser-His-Asp)\(^ {70-71}\) and Ag85 (Ser-His-Glu)\(^ {29-30}\) have similar charge relay system, and share Ser124 in common. Based on this, we proposed that Ser124 of Ag85 could be trapped by similar mechanism, serving as a nucleophile to form either a serine-Ag85 adduct or a tetrahedral intermediate.\(^ {29-30,35}\) Hence, this chapter is focused on describing our work in the design and synthesis of sugar-based esters, α-ketoesters, and α-ketoamides as potential inhibitors of antigen 85 complex enzymatic activity. Past research efforts have shown that interfering with the biosynthesis of key components of
the cell wall is important in hindering the virulence and resistance of the *M.tbc*[^10][^33][^35]. For example, Belisle *et al.* demonstrated that ADT inhibits both the mycolyltransferase activity of all Ag85s, and the growth of *Mycobacterium aurum*[^33]. The details of their report have been discussed in chapters one and two. However, the conclusion drawn from their work is that trehalose dimycolate is critical for the maintenance of the mycobacterial cell wall, and supports our running hypothesis that disruption of the mycobacterial cell wall could be a viable target for development of new tuberculosis drugs.

To further support our hypothesis, we note that some of the well known anti-tubercular drugs work by interfering with the synthesis and processing of mycobacterial cell wall components such as the mAG. As noted in chapter 1.5, for example, isoniazid (Figure 1.1) inhibits biosynthesis of mycolic acids by reacting with KatG enzyme to generate reactive species, isonicotinic hydrazyl radical and the isonicotinoyl radical, that ultimately inhibit enzymes involved in mycolic acid synthesis.[^29][^30][^72] On the other hand, ethambutol (Figure 1.1) inhibits formation of the arabinan component of arabinogalactan by inhibiting arabinosyl transferase enzyme.[^69] There is an added benefit in the use of trehalose and arabinose moieties as substrate analogs. Since trehalose and arabinose are natural substrates found in the active site of antigen 85 complex, it is hoped that trehalose and arabinose-based substrates will selectively interact with Ag85 since mycolyltransferases are not found in humans.[^11][^30]
3.2 α-Ketoamides as Serine Protease Inhibitors

There is good reason to choose α-ketoamides and α-ketoesters as targets for development of new TB drugs: 1,2-dicarbonyl compounds such as α-ketoamides, α-ketoesters and α-diketones are known serine traps and act as inhibitors of related serine proteases.\textsuperscript{67-68} In particular, α-ketoamides have proven to be valuable electrophilic functional groups for the inhibition of serine proteases since the α-keto group is up to ten times more electrophilic than an adjacent amide in a peptide.\textsuperscript{73} The model generally accepted for interaction of α-ketoamides with serine protease active site is that α-ketoamides bind competitively and reversibly with the serine hydroxyl nucleophile of serine proteases to form a covalent hemiketal adduct (Scheme 3.1).\textsuperscript{73}

Scheme 3.1: A Model for the Interaction of α-Ketoamide with Serine Protease in Active Site. P\textsubscript{1} Represents a Peptide Fragment.\textsuperscript{73}

In their search for new therapeutics against hepatitis which is caused by hepatitis C virus (HCV), Han et al. from DuPont Pharmaceutical Company showed that not only were α-ketoamides and α-ketoesters potent inhibitors of the HCV NS3 serine protease, they found that an α-ketoamide analog (A) was 7-fold more potent than the corresponding carboxylic acid analog (B, Figure 3.1).\textsuperscript{67} Perhaps the more electrophilic
nature of α-ketoamides and other dicarbonyls facilitates ready attack of the serine hydroxyl on the carbonyl, forming a covalent adduct.

Figure 3.1: Peptide-based serine protease inhibitors of NCV NS3 protease, showing: A. α-ketoamides analog with IC\textsubscript{50} of 0.34 µM; B. Carboxylic acid analog with IC\textsubscript{50} of 2.5 µM.\textsuperscript{67}

In another example, Sheha \textit{et al.}\textsuperscript{74} synthesized a series of tri-peptide analogs containing α-ketoamides as new core structures for the inhibition of human immunodeficiency virus-1 protease (HIV-1 PR). They compared the activity of α-ketoamides (B, Figure 3.2) to the activity of the tripeptide A isosteres (Figure 3.2), a highly selective and potent HIV-1 protease inhibitor in advanced clinical studies.\textsuperscript{16} As Figure 3 shows, the α-ketoamide isosteres exhibited enhanced inhibitory activity relative to their parent analogs. Inhibitory activity was determined as percentage of enzyme inhibition at 50 nM. This further underscores the importance of α-ketoamides as serine protease inhibitors.
Figure 3.2: α-Ketoamide isosteres (B) of a potent HIV-1 protease inhibitor showed enhanced inhibitory activity compared to their parent analogs (A).\textsuperscript{74}

3.3 Design of Synthetic Targets

In designing new inhibitors of Ag85s, we took note of results from a structure activity relationship study performed by Wang \textit{et al.} using trehalose amide analogs as potential inhibitors of Ag85s.\textsuperscript{43} Specifically, their study showed that trehalose compounds modified with two hydrocarbon chains at the 6- and 6'- positions have similar or better activities against Ag85 than compounds with one hydrocarbon chain. Another important conclusion from their study was that the optimal chain length for maximal activity is around C\textsubscript{8} or C\textsubscript{9}.\textsuperscript{43}
Figure 3.3: Methyl esters (1 and 4), α-ketoesters (2 and 5), and α-ketoamides (3 and 6) designed as potential inhibitors of Ag85.

With the foregoing design features in mind, we designed six different classes of carbonyl compounds consisting of methyl esters (1 and 4), α-ketoesters (2 and 5), and α-ketoamides (3 and 6) derived from β-D-arabinose and D-trehalose, respectively (Figure 3.3). D-Arabinose and D-trehalose were chosen as templates for the inhibitor design since both are natural substrates for Ag85. The target compounds were conceived to be accessed starting from an arabinose methyl glycoside or trehalose, followed by modification at the hydroxymethyl moiety with either a methyl ester, α-ketoester, or an α-ketoamide. Our design rationale involves the expectation that the methyl esters would act as potential suicide substrates (Figure 3.4B) capable of forming a covalent adduct.
with serine at the Ser124 of Ag85C, while α-ketoesters and α-ketoamides would act as transition state inhibitors (Figure 3.4, A).\textsuperscript{34,47,76}

Figure 3.4: Model of tetrahedral intermediate in the case of ketoamides (A), and substrate suicide intermediate in the case methyl esters (B).\textsuperscript{47}

**3.4 Synthesis of Arabinose-based Methyl Ester, α-Ketoester and α-Ketoamide**

To demonstrate that our design could lead to competitive inhibitors of Ag85, our laboratory embarked on the synthesis of an ester, α-ketoester and an α-ketoamide starting from methyl β-D-arabinofuranoside 7 (Schemes 3.2 and 3.3). The synthesis of these arabinose-based methyl ester, α-ketoester and α-ketoamide was primarily accomplished by Aditya Sanki\textsuperscript{21} and are presented here in order to provide a complete report of the project.
3.4.1 Synthesis of Arabinose Methyl Ester (1)

The synthesis of arabinose methyl ester 1 was carried out through a multi-step synthesis beginning with 7 (Scheme 3.2).\textsuperscript{30,77} First, trityl protecting group was incorporated at C-5 position of 7 with trityl chloride in the presence of DMAP in pyridine to generate 8 in good yield.\textsuperscript{59} Compound 8 was benzylation in 81\% yield with benzyl bromide and sodium hydride, and the product was detritylated with 80\% acetic acid to generate alcohol 9\textsuperscript{77} in 89\% yield. Compound 9 was oxidized by Swern oxidation using oxalyl chloride and DMSO in CH₂Cl₂ at -60 °C in high yield, to give 10.\textsuperscript{78}

![Scheme 3.2: Synthesis of Arabinose Methyl Ester (1). Reagents and conditions:](image)

The aldehyde 10 was subjected to a Horner-Wadsworth-Emmons olefination\textsuperscript{43} in the presence of methyl diethylphosphonoacetate and sodium hydride in THF to produce α,β-
unsaturated ester 12 in 61% yield. Compound 12 when reduced with Pd (OH) 2-C/H2 afforded ester 1 in 54% yield (Scheme 3.2).43

3.4.2 Synthesis of α-Ketoester (2) and α-Ketoamide (3)

The double bond of 12 was selectively reduced with Wilkinson’s catalyst under H2 pressure (50 psi)78 in anhydrous THF to get 13 in 83% yield (Scheme 3.3). On treatment with LiOH in the presence of THF-H2O, 13 was converted to carboxylic acid derivative 14 in 73% yield.43

Scheme 3.3: Synthesis of α-Ketoester (2) and α-Ketoamide (3). Reagents and conditions:
g. (PPh3)3RhCl, THF, H2 (50 psi), r.t., 24 hr; h. LiOH.H2O, THF-H2O (3:1), r.t., 24 hr; i. Ph3PCHCN, EDCI, DMAP, CH2Cl2, r.t., 4 hr; j. (i) DMDO (2 equiv.), MeOH, r.t., 0.5 hr, 16 = 85% yield; (ii) DMDO (equiv.), CH2Cl2, -65 ºC, 20 min.; and n-butylamine, -65 ºC, 1 hr, 17 = 24% yield; k. (i) Pd-C/H2, THF, r.t., 12-24 hr, 2 = 56% yield, and 3 = 73% yield.
Compound 14 was coupled with cyanophosphorane in the presence of EDCI and DMAP in CH2Cl2 to furnish α-keto- cyanophosphorane derivative 15 in 87% yield.α-Ketoester 16 was obtained by oxidation with DMDO in MeOH in 85% yield. Similarly, α-ketoamide 17 was synthesized in 24% yield by oxidation with DMDO in CH2Cl2, followed by amidation of the α-ketonitrile intermediate with butylamine. Finally, α-ketoester 16 and α-ketoamide 17 were debenzylated with Pd-C/H2 to obtain unprotected methyl α-ketoester 2 and its congener α-ketoamide 3 in 56% and 73% yields, respectively (Scheme 3.3).

3.5 Synthesis of trehalose-based methyl ester (4), α-ketoester (5) and α-ketoamide (6)

In order to synthesize trehalose-based analogs, 4-6, it is important to obtain the starting material 20 in fairly good yields. To achieve that goal, an attempt was first made to adapt the protocol used by Gilbertson et al.59 First, the primary hydroxyl groups of 18 were protected with trityl group in the presence of DMAP in pyridine to generate 19.23 Crude compound 19 was dissolved in DMF and benzylated using benzyl bromide and sodium hydride; and the product 20 was detritylated with TsOH in MeOH-CH2Cl2 (1:1) to generate the diol 21 in 57% yield at milligram scale. However, attempt to scale up the synthesis of 20 to gram scale resulted in very low yield of 18% pure diol. Hence, there was the need to slightly modify the synthetic protocol. This was achieved by protecting the primary hydroxyls with TBDPS group instead of trityl group (Scheme 3.4). The result of the modification was the successful synthesis of 21 in multi-gram scale in fairly high yields.
Scheme 3.4: Synthesis of Di-ol Starting Material (21). Reagents and conditions: a. TrCl, DMAP, Pyridine., r.t., 24 hr; b. BnBr, NaH, DMF, 7 h; c. TsOH, MeOH/CH$_2$Cl$_2$ (1:1), Et$_3$N, 12 hr.

3.5.1 Synthesis of Trehalose Methyl Ester (4)

The synthesis of α-D-trehalose ester 4 was achieved starting from D-trehalose (18). The first step of the synthesis was a regioselective silylation of C-6 and C-6′ positions with tert-butyldiphenylsilyl chloride (TBDPSCI) in the presence of imidazole in DMF using standard conditions (Scheme 3.5). When the reaction was carried out at room temperature, both mono- and di-silylated products 22 and 23 were obtained, which were isolated as their benzylated derivatives 25 (24% yield) and 26 (31% yield) in the next step (Scheme 3.5). It was also observed that during benzylation of the mixture (22 and 23) with benzyl bromide and sodium hydride in DMF in the presence of tetrabutylammonium iodide, a small amount of octa-O-benzyl-D-trehalose 24 (8%
yield) was isolated as a side product. But when silylation of \( \textbf{18} \) was performed at 35 °C under similar reaction conditions, compound \( \textbf{23} \) was isolated in 84% yield. Further, benzylolation of the pure \( \textbf{23} \) produced desired product \( \textbf{26} \) in 45% yield. Finally, to obtain \( \textbf{21} \), the silyl protective groups of \( \textbf{26} \) were removed with tetrabutylammonium fluoride trihydrate (TBAF.3H2O) in THF to produce \( \textbf{21} \) in 84% yield.

![Diagram of molecular structures]

**Scheme 3.5: Synthesis of Trehalose-derived Methyl Ester (4).** *Reagents and conditions:* a. TBDPSCl, imidazole, DMF, r.t.-35 °C, 16 hr, 84%; b. BnBr, NaH, TBAI, DMF, r.t., 24 hr: \( \textbf{25} \) (24%), \( \textbf{24} \) (8%) and \( \textbf{26} \)(45%); c. TBAF.3H2O, THF, r.t., 24 hr, 84%; d. 1). (COCl)\(_2\), DMSO, TEA, CH\(_2\)Cl\(_2\), -78 to -75 °C, 1.5 hr; 2). MeOC(O)CH\(_2\)P(O)(OEt)\(_2\), NaH, THF, 0 °C, 12 hr; e. Pd(OH)\(_2\)/C, H\(_2\), THF, r.t., 16 hr, 79%.
Compound 21 was then converted to 27 in high yield by oxidation\textsuperscript{17} followed by Horners-Wadsworth-Emmons condensation using the previously described conditions.\textsuperscript{17} Methyl ester 4 was finally obtained in 79% yield by catalytic reduction\textsuperscript{43} with Pearlman’s catalyst (Pd(OH)\textsubscript{2}/C) in anhydrous THF under H\textsubscript{2} atmosphere (Scheme 3.5).

### 3.5.2 Synthesis of Trehalose-based \(\alpha\)-Ketoester (5) and \(\alpha\)-Ketoamide (6)

Synthesis of 5 and 6 started with regioselective reduction of \(\alpha,\beta\)-unsaturated double bonds in 27 using Wilkinson’s catalyst, (PPh\textsubscript{3})\textsubscript{3}RhCl, under 50 psi of H\textsubscript{2} to afford 28 in 67% yield (Scheme 3.6).\textsuperscript{78} Compound 28 was treated with LiOH in THF-H\textsubscript{2}O to obtain the carboxylic acid derivative 29 in 95% yield.\textsuperscript{73} Compound 29 was then coupled with cyanophosphorane in the presence of EDCI and DMAP in CH\textsubscript{2}Cl\textsubscript{2} to furnish \(\alpha\)-keto-cyanophosphorane derivative 30 in 10% yield.\textsuperscript{79} Trehalose \(\alpha\)-ketoester 31 was obtained by oxidation of 30 with DMDO in MeOH. Similarly, \(\alpha\)-ketoamide 32 was synthesized by oxidation of \(\alpha\)-keto-cyanophosphorane derivative 30 with DMDO in CH\textsubscript{2}Cl\textsubscript{2}, followed by amidation of the \(\alpha\)-ketonitrile intermediate \textit{in situ} with corresponding alkyl amine, from C4-C8 (Scheme 3.6).\textsuperscript{43,47}
Scheme 3.6: Synthesis of Bicyclo-ester (33) and Bicyclo-amide (34). Reagents: f. 
(PPh₃)₃RhCl, H₂ (50 psi), r.t., 48 hr, 84%; g. LiOH·H₂O, THF/H₂O (3:1), r.t., 12 hr, 95%; 
h. PPh₃CHCN, EDCI·HCl, DMAP, CH₂Cl₂, r.t., 2 hr, 78%; i. 1). for 31; DMDO, MeOH, 
r.t., 0.5 hr, 64%; 2). for 32; DMDO, CH₂Cl₂, -78 °C, 15 min.; then BuNH₂, CH₂Cl₂, -78 
°C, 0.5 hr, 46%; j. 1). for 33; Pd/C (10%), H₂, THF, r.t., 24 hr, 62%; 2). for 34; Pd/C 
(10%), H₂, THF, r.t., 24 hr, 65%.

Although we employed the similar reaction conditions for accessing trehalose-
derived α-ketoester 5 and its amide congener 6 from their precursors 31 and 32,
respectively, bicyclo-ester 33 and its amide congener 34 formed readily during the palladium-charcoal reduction at the final step (Scheme 3.6). This could be accounted for by the proximity of hydroxyl groups at C-4/C-4′. As expected, no α-keto carbonyl was found around 200 ppm in the 13C NMR spectra of bicyclo-products of 33 or 34. The carbonyl peak for α-keto-functionality of compound 33 was absent (Figure 3.5), rather an additional characteristic carbon peak at δ = 95.4 ppm for C-8/C-8′ appeared in the vicinity of the anomeric carbon, δ = 95.9 (C-1/C-1′). Similarly, a set of two adjacent carbon peaks at δ = 94.8 and 94.9 ppm was found in 13C NMR of 34. A literature search shows that the configuration at C-8/C-8′ resembles the C-2 carbon of N-acetyl neuraminic acid, which appears at δ = 96.4 ppm value. By comparing the δ-value of C-8/C-8′ with that for anomeric carbon of the acid, we concluded C-8/C-8′ as having the similar configuration in both 33 and 34.
3.6 Enzymatic Studies

Having synthesized compounds 1-6, their in vitro activity was evaluated using a recently developed Ag85C acyltransferase assay. It should be noted that the enzymatic studies was done with our collaborator in Dr. Ronning’s laboratory, Dr. Boucau Julie. Trehalose-derived analogues (4 and 33-34) did not produce any inhibitory activity (Figure 3.6A). Among the arabinose-derived substrates, only methyl ester 1 showed inhibition of the enzymatic activity of Ag85C at millimolar concentration (Figure 3.6A). It was further observed that when the enzyme was preincubated with 1 prior to the experiment, 50% inhibition of enzymatic activity was observed at 25 mM concentration of 1 (Figure 3.6B). By comparison, 20% inhibition of the enzymatic activity was observed without incubation at the same inhibitor concentration (Figure 3.6B). Clearly, then, it seems that the time dependent nature of the results suggests weak binding
between the Ag85C and 1 leading to the formation of a covalent bond between the enzyme and the inhibitor.47

Because the low inhibitory activity of 1 required the use of relatively high concentrations to observe any inhibition, insufficient amounts were available for performing a thorough kinetic analysis. Consequently, from the current data, it is not possible to ascertain the type of inhibition imparted by 1 on Ag85C. Considering that 1 is structurally analogous to the true substrate, it is our continuing hypothesis that 1 is a competitive inhibitor.

Attempt to confirm this hypothesis using crystallographic studies of Ag85C (Figure 3.7) was helpful but inconclusive. For example, although strong electron density is observed in the carbohydrate-binding pocket of the enzyme, the identity of the compound or compounds producing this density was not fully known.

Since two different compounds were added to the protein crystals, it is conceivable that each of three different possibilities could account for the observed electron density. First, compound 1 could be binding within the active site and forming a covalent intermediate with the nucleophile Ser124. The density between Ser124 and the density within the carbohydrate-binding pocket are bordering each other indicating that a bond has been formed. However, the positions of the catalytic triad residues do not deviate between this structure and the native form. This is surprising because if a bond is being formed, this result seemingly contradicts earlier contention by Ronning et al.29-30 that the conformational change seen in the Ag85C-DEP (diethylphosphate) structure is induced upon formation of the covalent bond between the Ser124 nucleophile and the substrate/inhibitor. However, it is still possible that within the context of the protein
crystal, the tendency of the enzyme to undergo a conformational change is not sufficient to overcome the lattice energy of the crystal.

Figure 3.6: A. Michaelis-Menten fit of initial velocity versus substrate concentration curves obtained for different concentrations of inhibitors (1 and 4). B. Percent initial velocity (reaction with inhibitor compared to control without inhibitor) versus concentration of 1.47

The second scenario is that the electron density is glycerol. In the previous structure of Ag85C, no electron density resembling glycerol was observed in the carbohydrate-binding pocket. However, in those previous studies, the crystal was
exposed to glycerol for less than 10 seconds before flash cooling to 100 K. In preparing the sample for this diffraction study, the crystal was incubated in a solution containing both glycerol at a final concentration of 20% v/v and 1 for an extended period of time. It is likely that the significantly higher concentration of glycerol in the cryoprotectant solution promoted the displacement of 1 from the active site, leaving the glycerol bound within that same site. Of course this hypothesis presumes that no covalent bond is formed between the inhibitor and the enzyme. This scenario is strengthened by the fact that the current structure exhibits no change in the active site.47

Figure 3.7: Molecular surfaces representing the carbohydrate-binding pocket of Ag85C. A) Two glycerol molecules are shown fit in the Fo-Fc map of the current structure. The map is contoured at 3σ. B) Methyl β-D-arabinofuranoside (1) modeled within the same density and forming a covalent bond with Ser124.47

The third explanation is that the electron density represents a combination of both previously mentioned states, where glycerol and 1 are bound at less than 100%
occupancy. Considering all of the available information, multiple glycerol molecules were placed within the enzyme active site (Figure 3.7A).\textsuperscript{47}

Although the crystallographic studies of Ag85C and 1 remain inconclusive, the information garnered from that structure still offers important insights to how Ag85C can interact with various carbohydrates. To better understand how 1 may inhibit Ag85C activity, the available electron density from the current crystal structure was used as a basis for modeling 1 within the carbohydrate-binding pocket (Fig 3.7B). The most reasonable explanation is that the binding energy between protein molecules required for crystal packing cannot be overcome by formation of the covalent bond in the active site. This lack of an observed conformational change offers the advantage of understanding the interactions between the carbohydrate binding site of Ag85C and potential substrates and inhibitors.\textsuperscript{47}

3.7 Conclusion

1,2-Dicarbonyl compounds such as α-ketoamides and α-ketoesters are known transition state inhibitors of related serine proteases. While Ag85 complex is not a serine protease, its catalytic triad (Ser-His-Glu) has a similar charge relay system as the catalytic traid of serine protease (Ser-His-Asp). Therefore, we have designed and synthesized three classes of D-trehalose and β-D-arabinose-derived esters, α-ketoesters and α-ketoamides, as transition state inhibitors of Ag85C.

Synthesis of methyl esters 1 and 4 was accomplished by modifying α-D-trehalose and β-D-arabinose at the C-6 position. Using Swern conditions, the primary hydroxyl
group was oxidized to an aldehyde, followed by a Horner-Wadsworth-Emmons condensation and hydrogenolysis to obtain the methyl ester 1 and 4 in fairly good yields of 54% and 79% respectively.

In order to obtain α-D-trehalose and β-D-arabinose-derived α-ketoesters 2 and 5 or α-ketoamides 3 and 6, the appropriate protected methyl ester was treated with LiOH resulting in a carboxylic acid derivative which was then coupled with a cyanophosphorane in the presence of EDCI to obtain cyanoketo-phosphorane derivative. Oxidation of the phosphorane with DMDO in MeOH resulted in successful isolation of arabinose-derived α-ketoester 2 in 56% yield. Amidation of the resulting α-ketonitrile with relevant alkyl amine also yielded arabinose-derived α-ketoamide 3 in 73% yield.

However, attempt to synthesize α-D-trehalose-derived α-ketoesters 5 and α-ketoamide 6 was only partially successful. Although we employed similar reaction conditions for accessing trehalose-derived α-ketoester 5 and α-ketoamide 6 from their precursors 31 and 32, respectively, we observed instead the formation of bicyclo-ester 33 and its amide congener 34 during the palladium-charcoal reduction at the final step. This was not totally unexpected. The observation was accounted for by the proximity of hydroxyl groups at C-4/C-4′, which could react with α-keto carbonyl to form a stable six-membered ring. As expected, no α-keto carbonyl was found around 200 ppm in the 13C NMR spectra of bicyclo-products of 33 or 34.

Inhibitory study using a newly developed colorimetric assay described in Scheme 2.1 revealed that arabinose methyl ester 1 showed weak inhibition of Ag85C at 25 mM concentration. Although crystallographic study involving methyl ester 1 and Ag85C is
not conclusive, the electron density observed within the active site suggests a covalent interaction between Ag85C and arabinose methyl ester 1.

Finally, compounds 1-4, 33 and 34 were tested for activity against *M. smegmatis* ATCC 14468 using a Kirby-Bauer disk assay. Unfortunately, compounds 1-4, 33 and 34 did not show inhibition against the growth of *M. smegmatis* ATCC 14468 in a Kirby-Bauer disk assay.

### 3.8 Experimental Section

**General Method.** Starting materials such as D-arabinose and D-trehalose, and all fine chemicals were purchased from commercial suppliers and were used without further purification. All solvents were obtained from Fisher and used as received. Silica (230-400 mesh) for flash column chromatography was obtained from Sorbent Technologies; thin-layer chromatography (TLC) precoated plates were from EMD. TLCs (silica gel 60, f_{254}) were visualized under UV light or by charring (5% H$_2$SO$_4$-MeOH). Flash column chromatography was performed on silica gel (230-400 mesh) using solvents as received. $^1$H NMR were recorded either on a Varian VXRS 400 MHz or an INOVA 600. MHz spectrometer in CDCl$_3$, CD$_3$OD or DMSO-$d_6$ using residual CHCl$_3$, CH$_3$OH and DMSO as internal references, respectively. $^{13}$C NMR were recorded on a Varian VXRS 100 MHz or Varian INOVA 150 MHz in CDCl$_3$ using the triplet centered at $\delta$ 77.3, in CD$_3$OD using the septet centered at $\delta$ 49.0 or in DMSO-$d_6$ using septet as internal
references, respectively. High resolution mass spectrometry (HRMS) was performed on a Micromass Q-TOF2 instrument.

3.8.1 Methyl 2,3-di-O-benzyl-5-O-trityl-β-D-arabinofuranoside (9)

To a well-stirred suspension of sodium hydride (142 mg, 5.93 mmol; obtained by washing 237 mg of 60% w/w dispersion in mineral oil with dry n-hexane) in anhydrous DMF (5 mL) was added methyl 5-O-trityl-β-D-arabinofuranoside 8 (0.86 g, 2.11 mmol) in DMF (5 mL) dropwise under N₂ atmosphere. Benzyl bromide (0.71 mL, 5.93 mmol) was added dropwise and the resulting solution was stirred at ambient temperature. The reaction was monitored by TLC and appeared complete after 7 h. Two-three ice-cubes were added into the reaction mixture and solution was stirred for 15 minutes to hydrolyze the excess reagents. The reaction mixture was diluted with 50 mL water and aqueous layer was extracted with ether (3 x 40 mL). Combined organic layer was dried (anhydrous Na₂SO₄) and filtered. The filtrate was concentrated under reduced pressure to get a crude material, which was purified by silica gel flash column chromatography (230-400 mesh; 10 x 5.5 cm) using 1:9 (250 mL) and then 1:4 (750 mL) EtOAc/hexanes to produce 1.01 g of 9 as a yellow gum in 81% yield; \( R_f = 0.46 \) (1:4 EtOAc/hexanes). \(^1\)H NMR (600 MHz, CDCl₃): \( \delta \) 3.18-3.24 (m, 2H), 3.22 (s, 3H), 4.03 (dd, 1H, \( J = 4.2, 6.6 \) Hz), 4.07 (dd, 1H, \( J = 6.0, 10.8 \) Hz), 4.12 (t, 1 H, \( J = 7.2 \) Hz), 4.55 (d, 1 H, \( J = 11.4 \) Hz), 4.61 (m, 3 H), 4.69 (d, 1 H, \( J = 4.2 \) Hz), 7.21 (m, 5 H), 7.27 (m, 10 H), 7.34 (m, 4 H), 7.46 (m, 6 H); \(^1\)C NMR (150 MHz, CDCl₃): \( \delta \) 55.1, 66.0, 72.6, 72.7, 80.6, 82.8, 84.2, 101.5, 127.2, 127.7, 127.8, 128.0, 128.1, 128.3, 128.5, 128.6, 128.9, 137.8, 138.24,
144.1; ESI-MS calculated for C_{20}H_{24}O_{5} ([M+Na]^+) \( m/z = 367.14 \); observed \( m/z = 367.15 \) ([M+Na]^+).

3.8.2 Methyl 2,3-di-\(O\)-benzyl-\(\beta\)-D-arabinofuranoside (10)\(^{81}\)

Compound 8 (1.04 g, 1.77 mmol) was dissolved in 80\% AcOH-H\_2O and the resulting mixture was stirred at 70 °C following a literature procedure.\(^{81}\) TLC showed no starting material after 1.5 hr. The reaction mixture was diluted with 100 mL water and aqueous layer was extracted with ether (4 x 40 mL). The organic phase was washed with saturated aqueous NaHCO\_3 until pH = neutral (checked by pH paper) followed by brine. The ether layer was dried (anhydrous Na\_2SO\_4) and filtered. The filtrate was concentrated under reduced pressure to get a crude material. Purification of the crude material by silica gel flash column chromatography (230-400 mesh; 8 x 3.1 cm) using 1:4 (250 mL) and then 2:3 (250 mL) EtOAc/hexanes generated 0.51 g of 10 as a colorless gum in 83\% yield; \( R_f = 0.11 \) (3:7 EtOAc/hexanes).

3.8.3 Methyl (5E)-2,3-di-\(O\)-benzyl-5,6-dideoxy-\(\beta\)-D-arabino-hept-5-enofuranosiduronic acid methyl ester (12)

To a cooled solution of oxalyl chloride (0.57 mL, 6.67 mmol) in CH\_2Cl\_2 (5 mL) at -78 °C was added DMSO (0.95 mL, 13.4 mmol) in CH\_2Cl\_2 (3 mL) dropwise under N\_2 atmosphere over a period of 5 minutes following a literature method.\(^{73}\) After stirring the reaction for 10 minutes at -78 °C, reaction mixture was allowed to attain -60 °C and compound 9 (1.53 g, 4.45 mmol) in CH\_2Cl\_2 (25 mL) was added dropwise for 30 minutes. The resulting reaction mixture was stirred for 45 minutes and then temperature was again
raised to -45 °C and anhydrous triethylamine (3.75 mL, 26.7 mmol) was added dropwise. After the reaction was stirred for 0.5 hr, temperature was raised to -10 °C. Cold CH₂Cl₂ (80 mL) was added at 0 °C followed by cold-water (100 mL). Organic layer was separated by separatory funnel and the aqueous phase was extracted with CH₂Cl₂ (2 x 50 mL). Combined organic phase was dried (anhydrous Na₂SO₄) and filtered. The filtrate was concentrated under reduced pressure to get the crude aldehyde 11.

Methyl diethylphosphonoacetate (1.4 mL, 8.01 mmol) was dissolved in THF (10 mL) and NaH (213 mg, 8.90 mmol; obtained by washing 356 mg of 60% w/w dispersion in mineral oil with dry n-hexane) in THF (5 mL) was added to the flask dropwise at -20 °C. Resulting solution was stirred for 0.5 hr and then crude aldehyde 11 without further purification was dissolved in anhydrous THF (10 mL) and added dropwise under N₂ atmosphere. The reaction was stirred for 3 hr at -20 °C. After completion of the reaction (monitored by TLC), ether (60 mL) was added and organic phase was washed with saturated aqueous NH₄Cl and brine, and dried (anhydrous Na₂SO₄). The filtrate was concentrated under reduced pressure to get a crude material, which was purified by silica gel flash column chromatography (230-400 mesh; 10 x 5.5 cm) using 1:9 (1 L) and then 3:17 (1 L) EtOAc/hexanes to produce 1.08 g of compound 12 as a colorless oil in 61% yield; Rₐ = 0.32 (1:4 EtOAc/hexanes). ¹H NMR (600 MHz, CDCl₃): δ 3.41 (s, 3H), 3.75 (s, 3H), 4.06 (dd, 1H, J = 4.2, 7.8 Hz), 4.17 (t, 1H, J = 6.6 Hz), 4.42 (ddd, 1H, J = 1.4, 6.3, 6.3 Hz), 4.60 (d, 1H, J = 12.0 Hz), 4.65 (s, 2 H), 4.73 (m, 2H), 5.99 (dd, 1H, J = 1.2, 15.6 Hz), 6.90 (dd, 1H, J = 6.0, 15.6 Hz), 7.29-7.39 (m, J = 10 Hz); ¹³C NMR (150 MHz, CDCl₃): δ 51.6, 55.4, 72.7, 72.9, 79.9, 83.6, 85.0, 101.8, 127.9, 127.93, 128.1, 128.2,
128.5, 137.6, 137.8, 146.9, 166.5; HR-MS calculated for C_{23}H_{26}O_{6}Na \ m/z = 421.1603 ([M+Na]^+); observed m/z = 421.1627 ([M+Na]^+).

### 3.8.4 Methyl β-D-arabino-heptafuranosiduronic acid methyl ester (1).

Compound 12 (0.34 g, 0.86 mmol) was dissolved in anhydrous MeOH (10 mL) and to the flask was added Pearlman catalyst, Pd(OH)\_2/C (85 mg).\textsuperscript{43} The suspension was degassed carefully using low vacuum and a balloon of H\_2 was connected to the flask via a needle. The reaction mixture was stirred at room temperature and monitored by TLC. After completion of the reaction (4.5 hr), catalyst was filtered through filter paper and the filtrate was concentrated under reduced pressure to provide a crude material. Purification by silica gel flash chromatography (230-400 mesh; 8 x 4.5 cm) with 3:2 EtOAc/hexanes (1 L) afforded 0.103 g of compound 1 as a colorless gum in 54% yield; R\_f = 0.19 (4:1 EtOAc/hexanes); \textsuperscript{1}H NMR (600 MHz, CDCl\_3): \(\delta\) 1.87 (m, 1H), 2.0 (m, 1H), 2.42 (ddd, 1H, \(J = 6.6, 8.4, 16.2\) Hz), 2.48 (ddd, 1H, \(J = 6.0, 9.0, 15.0\) Hz,), 3.36 (1H), 3.39 (s, 3H), 3.66 (s, 3H), 3.75 (m, 1H), 3.90 (t, 1H, \(J = 7.2\) Hz), 4.03 (m, 2H), 4.74 (d, 1H, \(J = 4.8\) Hz); \textsuperscript{13}C NMR (150 MHz, CDCl\_3): \(\delta\) 30.2, 30.4, 52.0, 55.3, 78.2, 79.4, 80.8, 102.0, 174.4; HS-MS calculated for C\_9H\_16O\_6Na \ m/z = 243.0845 ([M+Na]^+); observed \(m/z = 243.0844 ([M+Na]^+).\

### 3.8.5 Methyl 2,3-di-O-benzyl-β-D-arabino-heptafuranosiduronic acid methyl ester (13).

Compound 12 (0.40 g, 1.00 mmol) was dissolved in distilled THF (4 mL) and Wilkinson’s catalyst (0.28 g, 0.30 mmol) was added to the flask. H\_2 gas was flashed two
times finally the reaction was stirred at 50 psi pressure of H$_2$. The reaction was monitored by TLC and appeared complete after 24 hr. Excess solvent was removed under reduced pressure and the crude material thus obtained was purified by silica gel column (230-400 mesh; 10 x 4.5 cm) using 3:17 EtOAc/hexanes (1 L) to yield 0.33 g of 13 as a yellow oil in 83% yield; $R_f = 0.22$ (1:4 EtOAc/hexanes). $^1$H NMR (600 MHz, CDCl$_3$): $\delta$ 1.6-2.0 (m, 2H), 2.38 (ddd, 1H, $J = 7.2, 9.0, 16.2$ Hz), 2.47 (ddd, 1H, $J = 6.0, 9.0, 15.0$ Hz), 3.36 (s, 3H), 3.67 (s, 3H), 3.88 (m, 1H), 4.03 (m, 2H), 4.62 (dd, 2H, $J = 12.0, 21.6$ Hz), 4.67 (dd, 2H, $J = 12.0, 60.6$ Hz), 4.68 (d, 1H, $J = 3.5$ Hz), 7.28-7.39 (m, 10H); $^{13}$C NMR (150 MHz, CDCl$_3$): $\delta$ 30.5, 31.2, 51.7, 55.1, 72.5, 72.6, 80.1, 84.4, 85.2, 101.6, 127.9, 128.1, 128.4, 128.5, 128.6, 137.7, 138.1, 173.8; HS-MS calculated for C$_{23}$H$_{28}$O$_6$Na $m/z = 423.1778$ ([M+Na]$^+$); observed $m/z = 423.1784$ ([M+Na]$^+$).

3.8.6 Methyl 2,3-di-O-benzyl-β-D-arabino-heptafuranosiduronic acid (14).

A solution of 13 (0.68 g, 1.69 mmol) and LiOH.H$_2$O (0.25 g, 5.94 mmol) in THF-H$_2$O (3:1, 11 mL) was stirred at room temperature. The reaction was monitored by TLC and appeared complete after 24 hr. Excess base was neutralized with Amberlite 120 H resin (requisite quantity) (checked by pH paper). Resin was filtered off through filter paper. The filtrate was concentrated under reduced pressure to obtain crude residue, which was purified by silica gel flash column (230-400 mesh; 5.5 x 4.5 cm) with 3:7 (500 mL) and then 2:3 (500 mL) EtOAc/hexanes to furnish 0.59 g of 14 as a colorless thick gum in 90% yield; $R_f = 0.22$ (2:3 EtOAc/hexanes); $^1$H NMR (600 MHz, CDCl$_3$): $\delta$ 1.87-1.99 (m,), 2.42 (ddd, 1 H, $J = 6.6, 8.4, 16.2$ Hz), 2.50 (ddd, 1 H, $J = 6.0, 9.0, 16.8$ Hz), 3.36 (s, 3H), 3.89 (m, 1H), 4.04 (m, 2 H), 4.62 (dd, 2H, $J = 12.0, 22.2$ Hz), 4.67 (dd, 2H,
J = 12.0, 64.2 Hz), 4.69 (d, 1 H, J = 3.6 Hz), 7.28-7.39 (m, 10 H); \textsuperscript{13}C NMR (150 MHz, CDCl\textsubscript{3}): \(\delta\) 30.5, 31.1, 55.2, 72.5, 72.7, 80.0, 84.4, 85.1, 101.7, 128.0, 128.0, 128.2, 128.4, 128.60, 137.7, 138.1, 179.4; HR-MS calculated for C\textsubscript{22}H\textsubscript{26}O\textsubscript{6}Na \(m/z = 409.1612 ([M+Na]^{+}); observed m/z = 409.1627 ([M+Na]^{+}).

3.8.7 Methyl 2,3-di-O-benzyl-7-keto-8-(triphenylphosphanylidene)-\(\beta\)-D-arabinono-nonafuranosiduronitrile (15).

To a well-stirred solution of 14 (0.59 g, 1.53 mmol), EDCI (0.44 g, 2.30 mmol), DMAP (0.018 g, 0.15 mmol) in CH\textsubscript{2}Cl\textsubscript{2} (15 mL) was added dropwise a solution of (cyanomethylene)triphenylphosphorane (0.57 g, 1.69 mmol) in CH\textsubscript{2}Cl\textsubscript{2} (10 mL) under N\textsubscript{2} atmosphere. The resulting solution was stirred at ambient temperature. The reaction was monitored by TLC and appeared complete after 4.5 hr. The reaction mixture was diluted with CH\textsubscript{2}Cl\textsubscript{2} (50 mL) and water (100 mL) was added. Organic phase was pooled and aqueous layer was extracted with CH\textsubscript{2}Cl\textsubscript{2} (2 x 40 mL). Combined organic phase was washed with saturated aqueous NaHCO\textsubscript{3} and brine, and then dried (anhydrous Na\textsubscript{2}SO\textsubscript{4}) and filtered. The filtrate was concentrated under reduced pressure to render the crude mass, which was purified by silica gel flash column (230-400 mesh; 6 x 4 cm). Elution with 2:3 (750 mL) and then 1:1 (750 mL) EtOAc/hexanes produced 0.96 g of compound 15 as a white fluffy hygroscopic material in 89% yield; \(R_f = 0.29\) (3:2 EtOAc/hexanes). \textsuperscript{1}H NMR (600 MHz, CDCl\textsubscript{3}): \(\delta\) 1.93-2.05 (m, 2H), 2.79 (m, 1H), 2.91 (m, 1H), 3.38 (s, 3H), 3.91 (m, 1H), 4.04 (dd, 1H, \(J = 4.2, 6.6\) Hz), 4.08 (t, 1H, \(J = 6.0\) Hz), 4.60-4.69 (m, 4H), 4.71 (d, 1H, \(J = 4.2\) Hz), 7.25 (t, 1H, \(J = 7.2\) Hz), 7.29-7.38 (m, 9H), 7.49 (m, 6H), 7.56-7.63 (m, 9H). \textsuperscript{13}C NMR (150 MHz, CDCl\textsubscript{3}): \(\delta\) 32.1, 35.9, 35.9, 48.1, 48.9, 55.2,
72.1, 72.5, 80.85, 84.6, 85.8, 101.5, 122.6, 122.7, 123.1, 123.7, 127.7, 128.0, 128.1, 128.4, 128.5, 128.6, 129.2, 129.3, 132.2, 132.25, 133.2, 133.2, 133.2, 133.7, 133.7, 137.8, 138.4, 196.3, 196.3; \( ^{31} \text{P NMR (80.95 MHz, CDCl}_3 \): \( \delta \) 20.88; HS-MS calculated for \( \text{C}_{42}\text{H}_{40}\text{NO}_5\text{PNa} \) \( m/z = 692.2545 \) ([M+Na]\(^+\)); observed \( m/z = 692.2542 \) ([M+Na]\(^+\)).

3.8.8 Methyl 2,3-di-\( \text{O}\text{-benzyl-7-keto-}\beta\text{-D-arabino-octafuranosiduronic acid methyl ester (16).}

Compound 15 (0.1 g, 0.153 mmol) was dissolved in anhydrous MeOH (5 mL) and freshly prepared DMDO in excess of 2 equivalents was added dropwise at room temperature under \( \text{N}_2 \) atmosphere. The resulting mixture was stirred for 10 minutes and monitored by TLC. After 1 hr, reaction was complete. Excess solvent was evaporated under reduced pressure and crude material thus obtained was purified by silica gel flash column (230-400 mesh; 6.5 x 4.2 cm). Elution with 1:3 EtOAc/hexanes (1 L) afforded 0.78 g of 16 as a colorless thick gum in 85% yield; \( R_f \) = 0.78 (3:2 EtOAc/hexanes). \( ^1\text{H NMR (600 MHz, CDCl}_3 \):} \( \delta \) 1.88-2.00 (m, 2H), 2.89 (ddd, 1H, \( J = 6.0, 7.8, 18.6 \) Hz), 3.01 (ddd, 1H, \( J = 6.0, 7.8, 18.0 \) Hz), 3.34 (s, 3H), 3.85 (s, 3H), 3.88 (m, 1H), 4.03 (m, 2H), 4.62 (dd, 2H, \( J = 12.0, 19.2 \) Hz), 4.66 (d, 1H, \( J = 3.6 \) Hz), 4.68 (dd, 2H, \( J = 12.0, 82.8 \) Hz), 7.28-7.38 (m, 10H). \( ^{13}\text{C NMR (150 MHz, CDCl}_3 \):} \( \delta \) 29.6, 35.9, 53.2, 55.3, 72.6, 72.8, 79.9, 84.3, 85.0, 101.7, 128.0, 128.1, 128.2, 128.4, 128.6, 128.7, 137.7, 138.1, 161.3, 193.7; HR-MS calculated for \( \text{C}_{24}\text{H}_{28}\text{O}_7\text{Na} \) \( m/z = 451.1715 \) ([M+Na]\(^+\)) \( \text{C}_{24}\text{H}_{28}\text{NaO}_7 \) requires 451.1733 ([M+Na]\(^+\)).
3.8.9 Methyl 7-keto-β-D-arabino-octafuranosiduronic acid methyl ester (2).

Compound 16 (0.072 g, 0.168 mmol) and Pd/C (35 mg; 10% Pd on activated carbon, anhydrous version) were taken together and placed in vacuum for 15 minutes before addition of anhydrous THF (4 mL). Resulting suspension was stirred at room temperature under a balloon pressure of H₂. The reaction was monitored by TLC and appeared complete after 24 hr. Suspended solid was filtered off through a pad of Celite®. The Celite® bed was washed with two bed volumes of anhydrous THF and the combined filtrate was concentrated under reduced pressure. The crude product thus obtained was purified by a silica gel flash column (230-400 mesh; 9.5 x 2 cm). Elution with 1:19 MeOH/CHCl₃ (0.5 L) generated 0.023 g of 2 as a yellow gum in 56% yield; R_f = 0.26 (1:9 MeOH/CHCl₃). ¹H NMR (600 MHz, CDCl₃): δ 1.92 (m, 1H), 2.03 (m, 1H), 2.15 (br.hump, 1H), 2.95 (ddd, 1H, J = 6.6, 7.8, 18.6 Hz), 2.98 (br.hump, 1H), 3.05 (ddd, 1H, J = 6.6, 7.8, 18.6 Hz), 3.40 (s, 3H), 3.79 (m, 1H), 3.87 (s, 3H), 3.90 (m, 1H), 4.01 (t, 1H, J = 6.6 Hz), 4.75 (d, 1H, J = 4.2 Hz). ¹³C NMR (150 MHz, CDCl₃): δ 28.7, 35.6, 53.3, 55.6, 78.5, 79.9, 80.6, 102.0, 161.5, 193.9; HR-MS calculated for C₁₀H₁₆O₇Na m/z = 271.0793 ([M+Na]⁺); observed m/z = 271.0794 ([M+Na]⁺).

3.8.10 Methyl N-butyl-2,3-di-O-benzyl-7-keto-β-D-arabino-octafuranosiduronamide (17).

To a well-stirred solution of 15 (0.18 g, 0.262 mmol) in CH₂Cl₂ (3 mL) was added anhydrous freshly prepared DMDO in excess of two equivalents at -78 °C. The reaction was monitored by TLC for 0.5 hr when maximum starting material converted to the product ketonitrile (based on TLC). Butylamine (30 µL, 0.288 mmol) in CH₂Cl₂ (1 mL)
was added dropwise to the flask and stirring was continued. TLC showed maximum conversion of the starting ketonitrile after 30 minutes. Excess solvent was removed under reduced pressure at low temperature and crude material was dried in high vacuum pump for 1 h before being loaded on the silica gel flash column (230-400 mesh; 10 x 3.1 cm). Elution with 1:3 (0.5 L) and then 1:1 (0.5 L) EtOAc/hexanes yielded 0.03 g of 17 as a yellow gum in 24% yield; \( R_f = 0.7 \) (1:1 EtOAc/hexanes). \(^1\)H NMR (600 MHz, CDCl\(_3\)): \( \delta \) 0.93 (t, 3H), 1.35 (m, 2H), 1.52 (m, 2H), 1.91 (m, 2H), 2.99 (ddd, 1H, \( J = 6.0, 7.8, 18.6 \) Hz), 3.12 (ddd, 1H, \( J = 6.0, 8.4, 19.2 \) Hz), 3.28 (dd, 2H, \( J = 7.2 \) Hz), 3.34 (s, 3H), 3.89 (m, 1H), 4.04 (m, 2H), 4.62 (m, 3H), 4.67 (d, 1H, \( J = 3.6 \) Hz), 4.73 (d, 1H, \( J = 12.0 \) Hz), 6.92 (br.s, 1H), 7.28-7.38 (m, 10H). \(^1\)C NMR (150.83 MHz, CDCl\(_3\)): \( \delta \) 13.9, 20.3, 30.0, 31.5, 33.5, 39.3, 55.3, 72.6, 72.8, 80.1, 84.5, 85.4, 101.7, 128.0, 128.1, 128.2, 128.4, 128.5, 128.6, 128.7, 128.7, 137.8, 138.3, 160.1, 199.0; HR-MS calculated for \( \text{C}_{27}\text{H}_{35}\text{O}_6\text{N}\text{Na} \) \( m/z = 492.2247 \) ([M+Na]\(^+\)); observed \( m/z = 492.2362 \) ([M+Na]\(^+\)).

3.8.11 Methyl \( N\)-butyl-7-keto-\( \beta \)-D-arabino-octafuranosiduronamide (3).

Compound 17 (0.033 g, 0.071 mmol) and Pd/C (50 mg; 10% Pd on activated carbon, anhydrous version) were taken together and placed in vacuum for 15 minutes before addition of anhydrous THF (3 mL). Resulting suspension was stirred at room temperature under a balloon pressure of H\(_2\). The reaction was monitored by TLC and appeared complete after 24 hr. Suspended solid was filtered off through a pad of Celite\(^\circ\). The Celite\(^\circ\) bed was washed with two bed volumes of anhydrous THF and the combined filtrate was concentrated under reduced pressure. The crude product thus obtained was purified by a silica gel flash column (230-400 mesh; 9 x 1.5 cm). Elution with 1:19
MeOH/CHCl₃ (0.3 L) generated 0.015 g of 3 as a yellow glassy hygroscopic solid in 73% yield; 
\( R_f = 0.42 \) (1:9 MeOH/CHCl₃). \(^1\)H NMR (600 MHz, CDCl₃): \( \delta \) 0.93 (t, 1H), 1.35 (m, 2H), 1.53 (m, 2H), 189-2.02 (m, 2H), 3.02 (dt, 1H, 
\( J = 7.2, 7.2, 18.6 \) Hz), 3.11 (dt, 1H, 
\( J = 7.2, 7.2, 18.6 \) Hz), 3.29 (dd, 2H, 
\( J = 6.6 \) Hz), 3.41 (s, 3H), 3.81 (dd, 1H, 
\( J = 6.6, 13.2 \) Hz), 3.93 (t, 1H, 
\( J = 7.2 \) Hz), 4.03 (t, 1H, 
\( J = 6.6 \) Hz), 4.76 (d, 1H, 
\( J = 4.2 \) Hz), 7.03 (br.s, 1H). \(^{13}\)C NMR (150.83 MHz, CDCl₃): \( \delta \) 13.9, 20.2, 28.9, 32.5, 33.0, 39.4, 55.5, 78.6, 79.9, 80.8, 102.0, 160.3, 199.0; HR-MS calculated for C₁₃H₂₃O₅NNaO₅ 
\( m/z = 312.1375 \) (\([\text{M+Na}^+]\)); observed 
\( m/z = 312.3185 \) (\([\text{M+Na}^+]\)).

### 3.8.12 6, 6′-Di-O-tert-butyldiphenylsilyl-α-D-trehalose (23).

To a well-stirred solution of trehalose 18 (2.0 g, 5.84 mmol) and imidazole (0.99 g, 14.6 mmol) in anhydrous DMF (15 mL) was added TBDPSCl (3.8 mL, 14.6 mmol) dropwise at room temperature under N₂ atmosphere. The resulting solution was stirred at 35 °C for 16 h. After completion of the reaction (TLC), 2 mL MeOH was added to the reaction flask and the mixture was stirred for 0.5 hr. Excess solvent was removed under reduced pressure to get a crude material. Purification of crude material by silica gel flash chromatography (230-400 mesh; 10 x 6.1 cm) using 3:17 MeOH/EtOAc (2 L) afforded 4.05 g 23 as white amorphous solid in 84% yield; 
\( R_f = 0.6 \) (1:3 MeOH/EtOAc). \(^1\)H NMR (600 MHz, DMSO-\( d_6 \)): \( \delta \) 0.99 (s, 18H), 3.24 (m, 2H), 3.27 (m, 2H), 3.65 (m, 2H), 3.78 (m, 2H), 3.83 (m, 4H), 7.38-7.44 (m, 12H), 7.68 (m, 8H). \(^{13}\)C NMR (150 MHz, DMSO-\( d_6 \): \( \delta \) 19.0, 26.6, 63.4, 69.9, 71.7, 72.3, 73.2, 92.8, 127.7, 129.6, 129.7, 133.3, 133.5, 135.2, 135.3; HR-MS calculated for C₄₄H₅₈O₁₁Si₂Na 
\( m/z = 841.3419 \) (\([\text{M+Na}^+]\)); observed
When the reaction was carried out at room temperature, both 22 and 23 were formed and the mixture was separated after benzylation in the next step.

3.8.13 2,2′,3,3′,4,4′,6′-Hepta-O-benzyl-6,6′-di-O-tert-butyldiphenyl-silyl-α-D-
trehalose (25), and 2,2′,3,3′,4,4′-Hexa-O-benzyl-6,6′-di-O-tert-butyldiphenylsilyl-α-D-
trehalose (26).

To the suspension of NaH (3.93 g, 98.4 mmol-60% label washed thoroughly with dry hexanes; three times) in anhydrous DMF (30 mL) was added 23 (4.03 g, 4.92 mmol) in DMF dropwise. The resulting solution was stirred under N₂ atmosphere for 0.5 h and then benzyl bromide (11.77 mL, 98.41 mmol) and tetrabutylammonium iodide (TBAI; 0.54 g, 1.47 mmol) were added. The reaction mixture was stirred at room temperature. The reaction was monitored by TLC and complete to a major extent within 3 h but stirring was continued for 24 hr. MeOH (2 mL) was added to the reaction flask and the mixture was stirred for 0.5 hr. Water (60 mL) was added to the flask and the aqueous phase was extracted with ether (4 x 60 mL). Combined ether layers were dried (anhydrous Na₂SO₄) and filtered. The filtrate was concentrated to dryness under reduced pressure and crude material thus obtained was purified by flash chromatography (15 x 6.5) using hexanes (1.5 L) and EtOAc/hexanes-1:19 (2 L), 1:9 (1 L) and 1:4 (1.5) to generate 24₁⁵ (8% yield), 25 and 26; 1.44 g of 25 in 24% yield; \( R_f = 0.49 \) (1:4 EtOAc/hexanes). \(^1\)H NMR (600 MHz, CDCl₃): \( \delta \) 1.09 (s, 9H), 3.42 (dd, 1H, \( J = 3.0, 10.8 \) Hz), 3.55 (dd, 1H, \( J = 3.0, 12.0 \) Hz), 3.60 (dd, 1H, \( J = 3.6, 9.6 \) Hz), 3.67 (m, 2H), 3.71 (t, 1H, \( J = 9.6 \) Hz), 3.82 (dd, 1H, \( J = 2.4, 11.4 \) Hz), 3.92 (t, 1H, \( J = 9.6 \) Hz), 4.06 (t, 1H, \( J = 9.6 \) Hz), 4.09 (m, 1H), 4.12 (t, 1H, \( J = 9.6 \) Hz), 4.21 (m, 1H), 4.44 (d, 1H, \( J = 12.0 \) Hz),
4.49 (d, 1H, \( J = 10.8 \) Hz), 4.54 (d, 1H, \( J = 12.0 \) Hz), 4.61 (dd, 2H, \( J = 4.2, 12.0 \) Hz), 4.77-5.05 (m, 9H), 5.23 (d, 1H, \( J = 3.6 \) Hz), 5.32 (d, 1H, \( J = 4.2 \) Hz), 7.01 (t, 2H, \( J = 7.8 \) Hz), 7.10 (m, 3H), 7.16 (m, 2H), 7.28-7.44 (m, 32H), 7.69 (d, 2H, \( J = 6.6 \) Hz), 7.75 (d, 2H, \( J = 6.6 \) Hz). ¹³C NMR (150 MHz, CDCl₃): \( \delta \) 19.5, 27.0, 62.3, 68.3, 70.7, 71.8, 72.8, 73.0, 73.7, 75.3, 75.5, 75.8, 76.1, 77.8, 77.84, 79.6, 80.2, 82.0, 82.1, 94.3, 127.4, 127.5, 127.55, 127.6, 127.7, 127.73, 127.85, 127.87, 127.88, 127.9, 128.1, 128.16, 128.2, 128.4, 128.5, 128.6, 128.65, 129.78, 129.8, 133.4, 133.8, 135.8, 136.1, 137.9, 138.1, 138.5, 138.6, 138.7, 139.0, 139.1. HR-MS calculated for C₇₇H₈₂O₁₁SiNa \( m/z = 1233.552734 \) ([M+Na]⁺; observed \( m/z = 1233.5524 \) 34 ([M+Na]⁺; for compound 26: yield 3.02 g (45%); \( R_f = 0.49 \) (1:4 EtOAc/hexanes); ¹H NMR (600 MHz, CDCl₃): \( \delta \) 1.05 (s, 18H), 3.56 (dd, 2H, \( J = 3.6, 9.6 \) Hz), 3.58 (m, 2H), 3.72 (dd, 2H, \( J = 1.8, 11.4 \) Hz), 3.84 (t, 2H, \( J = 9.6 \) Hz), 4.02 (m, 2H), 4.03 (t, 2H, \( J = 9.6 \) Hz), 4.55 (d, 2H, \( J = 11.4 \) Hz), 4.63 (d, 2H, \( J = 12.0 \) Hz), 4.70 (d, 2H, \( J = 10.8 \) Hz), 4.86 (d, 2H, \( J = 10.8 \) Hz), 4.91 (d, 2H, \( J = 10.2 \) Hz), 4.97 (d, 2H, \( J = 10.8 \) Hz), 5.19 (d, 2H, \( J = 3.6 \) Hz), 6.99 (t, 4H, \( J = 7.8 \) Hz), 7.07 (t, 5H, \( J = 6.6 \) Hz), 7.28-7.40 (m, 25H), 7.45 (m, 4H), 7.65 (d, 4H, \( J = 7.2 \) Hz), 7.72 (d, 4H, \( J = 6.6 \) Hz); ¹³C NMR (150 MHz, CDCl₃): \( \delta \) 19.6, 27.1, 62.4, 71.7, 73.0, 75.4, 76.1, 77.8, 80.3, 82.1, 94.4, 127.3, 127.5, 127.7, 127.86, 127.87, 127.9, 128.1, 128.4, 128.6, 128.7, 129.8, 129.84, 133.6, 133.8, 135.9, 136.2, 138.3, 138.7, 139.0; HR-MS calculated for C₈₆H₉₄O₁₁Si₂Na ([M+Na] m/z = 1381.6218 (M+Na)⁺; observed \( m/z = 1381.6232 \) ([M+Na]⁺).
3.8.14 2,2′,3,3′,4,4′-Hexa-β-benzyl-α-D-trehalose (21) 43

Compound 26 (2.98 g, 2.2 mmol) and TBAF.3H2O (4.15 g, 13.15 mmol) were taken together and dried in high vacuum pump for 15 minutes before addition of anhydrous THF (30 mL). The resulting yellow solution was stirred at ambient temperature. The reaction was monitored by TLC and disappearance of starting material was complete after 36 h. Excess solvent was removed and crude material thus obtained was purified by silica gel flash column (230-400 mesh; 10 x 6.5 cm) with 3:7 (1 L), 2:3 (1 L) and 3:2 (2.5 L) EtOAc/hexanes to produce 1.63 g of 21 as a colorless foamy glassy mass in 84% yield; \( R_f = 0.1 \) (1:1 EtOAc/hexanes).

3.8.15 Bis(methyl-α-D-glucopyranosyluronate) ether (4)

A suspension of 27 (0.21 g, 0.21 mmol) and Pd(OH)2/C (100 mg) in a 2.5:1 mixture of anhydrous MeOH/EtOAc was degassed under vacuum and a balloon of \( \text{H}_2 \) was connected to the reaction vessel. The resulting suspension was stirred at ambient temperature under \( \text{H}_2 \) atmosphere. The reaction was monitored by TLC and appeared complete after 16 hr. The suspension was filtered through a pad of Celite® and Celite® bed was washed with MeOH and combined filtrate was concentrated under reduced pressure to obtain a crude residue, which was precipitated from 1:1 MeOH/EtOAc to get 0.076 g of compound 4 as a white amorphous solid in 79% yield; \( R_f = 0.21 \) (1:3 MeOH/EtOAc). 1H NMR (600 MHz, CD3OD): \( \delta \) 1.62 (m, 2H), 2.13 (m, 2H), 2.36 (m, 2H), 2.43 (m, 2H), 3.01 (t, 2H, \( J = 9.0 \) Hz), 3.40 (dd, 2H, \( J = 4.2, 10.2 \) Hz), 3.61 (s, 6H), 3.66 (t, 2H, \( J = 9.6 \) Hz), 3.71 (ddd, 2H, \( J = 2.4, 9.6 \) Hz), 4.92 (d, 2H, \( J = 3.6 \) Hz). 13C NMR (100 MHz, CD3OD): \( \delta \) 28.4, 31.5, 52.3, 72.1, 73.4, 74.6, 75.9, 95.2, 176.2; HR-MS
calculated for C_{18}H_{30}O_{13}Na \quad m/z = 477.1590 ([M+Na])^+; \text{ observed } m/z = 477.1584 ([M+Na])^+.

3.8.16 Bis(methyl-2,3,4-tri-\textit{O}-benzyl-\textit{\textalpha-}-\textit{D}-\textit{gluco}-octopyranosyluronate) ether (28)

To a solution of 27 (0.56 g, 0.57 mmol) in THF (7 mL) was added Wilkinson’s catalyst (0.47 mg, 513.4 mmol) and the solution was purged with H\textsubscript{2} thrice before a constant pressure of 50 psi of H\textsubscript{2} is maintained. The reaction was stirred at ambient temperature and monitored by TLC. After completion of the reaction (36-48 hr), excess solvent was removed under reduced pressure to get the crude material. The crude material was purified by silica gel flash chromatography (230-400 mesh; 8 x 4.5 cm). Elution with 1:9 EtOAc/hexanes generated 28 as a colorless amorphous solid: yield 0.38 g (67%); \textit{Rf} = 0.37 (3:7 EtOAc/hexanes). \textsuperscript{1}H NMR (600 MHz, CDCl\textsubscript{3}): \textit{\delta} 1.74 (m, 2H), 2.01 (m, 2H), 2.1 (m, 2H), 2.26 (m, 2H), 3.25 (t, 2H, \textit{J} = 9.0 Hz), 3.57 (dd, 2H, \textit{J} = 3.6, 9.6 Hz), 3.62 (s, 6H), 4.01 (ddd, 2H, \textit{J} = 3.0, 9.6, 9.6 Hz), 4.10 (t, 2H, \textit{J} = 9.6 Hz), 4.65 (d, 2H, \textit{J} = 11.4 Hz), 4.75 (d, 2H, \textit{J} = 12.6 Hz), 4.81 (d, 2H, \textit{J} = 12.6 Hz), 4.88 (d, 2H, \textit{J} = 10.8 Hz), 4.93 (d, 2H, \textit{J} = 10.8 Hz), 4.98 (d, 2H, \textit{J} = 10.8 Hz), 5.09 (d, 2H, \textit{J} = 3.6 Hz), 7.24-7.38 (m, 30H). \textsuperscript{13}C NMR (150 MHz, CDCl\textsubscript{3}): \textit{\delta} 27.0, 30.1, 51.6, 70.2, 73.3, 75.3, 75.7, 79.6, 81.8, 81.9, 92.7, 127.5, 127.7, 127.8, 127.9, 128.07, 128.1, 128.5; HR-MS calculated for C\textsubscript{60}H\textsubscript{56}O\textsubscript{13}Na \quad m/z = 1017.4395 ([M+Na])^+; \text{ observed } m/z = 1017.4401.

3.8.17 Bis(methyl-2,3,4-tri-\textit{O}-benzyl-\textit{\textalpha-}-\textit{D}-\textit{gluco}-octopyranosyluronic acid) ether (25)

To a well-stirred solution of 28 (0.77 g, 0.78 mmol) in 3:1 THF/H\textsubscript{2}O (10 mL) was added LiOH.H\textsubscript{2}O (0.21 g, 5.06 mmol) at room temperature. The resulting suspension was
stirred at room temperature for 12 hr and monitored by TLC. The reaction appeared complete after 12 hr. Reaction mixture was neutralized by Amberlite 120 H⁺ resin (checked by pH paper). Resin was filtered off through a cotton plug and excess solvent was concentrated under reduced pressure. The crude material thus obtained was purified by silica gel flash column (230-400 mesh; 10 x 3.1 cm). Elution with 1:19 and then 1:9 MeOH/CHCl₃ afforded 0.71 g of 29 as a yellow fluffy compound in 95% yield; Rᵢ = 0.5 (1:9 MeOH/CHCl₃). ¹H NMR (600 MHz, CDCl₃): δ 1.75 (m, 2H), 1.95 (m, 2H), 2.12 (t, 4H, J = 7.2 Hz), 3.23 (t, 2H, J = 9.0 Hz), 3.57 (dd, 2H, J = 3.6, 9.6 Hz), 3.91 (ddd, 2H, J = 2.4, 9.6, 9.6 Hz), 4.09 (t, 2H, J = 9.0 Hz), 4.61 (d, 2H, J = 11.4 Hz), 4.74 (d, 2H, J = 12.0 Hz), 4.81 (d, 2H, J = 12.0 Hz), 4.86 (d, 2H, J = 10.8 Hz), 4.91 (d, 2H, J = 10.8 Hz), 4.96 (d, 2H, J = 10.8 Hz), 5.11 (d, 2H, J = 3.0 Hz), 7.23 (m, 6H), 7.28-7.37 (m, 24H). ¹³C NMR (150 MHz, CDCl₃): δ 26.7, 29.9, 70.4, 73.5, 75.3, 75.7, 79.7, 81.8, 81.9, 92.5, 127.5, 127.7, 127.8, 128.0, 128.1, 128.15, 128.6, 128.62, 128.63, 138.3, 138.4, 138.8, 180.2; HR-MS calculated for C₅₈H₆₂O₁₃Na m/z = 989.4086 ([M+Na])⁺; observed m/z = 989.4088 ([M+Na])⁺.

3.8.18 Bis(2,3,4-tri-O-benzyl-9-cyano-8-oxo-9-(triphenylphosphanylidene)-α-D-glucopyranosylurononitrile) ether (30)

Compound 29 (0.71 g, 0.74 mmol), EDCI (0.42 g, 2.22 mmol) and DMAP (9 mg, 0.07 mmol) were taken together and dried under vacuum for 15 minutes before CH₂Cl₂ (5 mL) was added. Cyanophosphorane derivative (0.28 g, 0.84 mmol) dissolved in CH₂Cl₂ (5 mL) was added dropwise to the flask under N₂ atmosphere at ambient temperature.
The reaction was monitored by TLC and appeared complete in 2 hr. The reaction mixture was diluted with CH₂Cl₂ (50 mL) and washed with water, saturated aqueous NaHCO₃, and brine. Aqueous phases were back extracted with CH₂Cl₂ (2 x 40 mL). Combined organic phases were dried (anhydrous Na₂SO₄), filtered and the filtrate was concentrated under reduced pressure to obtain the crude residue. The crude material was purified by silica gel flash chromatography (230-400 mesh; 10 x 3.5 cm). Elution with 3:2 EtOAc/hexanes yielded 0.89 g of 30 as a white fluffy mass in 78% yield; \( R_f = 0.96 \) (1:9 MeOH/CHCl₃). \(^1\)H NMR (600 MHz, CDCl₃): \( \delta \) 1.88 (m, 2H), 1.99 (m, 2H), 2.53 (m, 2H), 2.78 (m, 2H), 3.29 (t, 2H, \( J = 9.6 \) Hz), 3.63 (dd, 2H, \( J = 3.6, 9.6 \) Hz), 4.11 (m, 4H), 4.63 (d, 2H, \( J = 10.8 \) Hz), 4.76 (d, 2H, \( J = 12.0 \) Hz), 4.83 (d, 2H, \( J = 10.8 \) Hz), 4.87 (d, 2H, \( J = 10.8 \) Hz), 4.96 (d, 2H, \( J = 10.8 \) Hz), 5.32 (d, 2H, \( J = 3.0 \) Hz), 7.19 (m, 6H), 7.24-7.32 (m, 20H), 7.39 (m, 4H), 7.47 (m, 12H), 7.55-7.61 (m, 18H). \(^1^3\)C NMR (100 MHz, CDCl₃): \( \delta \) 26.6, 35.12, 35.2, 47.3, 48.5, 70.6, 72.9, 75.0, 75.5, 79.7, 81.8, 82.1, 92.0, 122.5, 122.6, 123.1, 124.0, 127.2, 127.4, 127.5, 127.7, 127.9, 128.0, 128.3, 128.36, 129.1, 129.2, 133.1, 133.2, 133.6, 133.7, 138.7, 138.8, 139.1, 196.5, 196.53; \(^3^1\)P (80.95 MHz, CDCl₃): \( \delta \) 20.8; HR-MS calculated for C₉₈H₉₀O₁₁N₂P₂Na \( m/z = 1555.5864 \) ([M+Na]⁺); observed \( m/z = 1555.5918 \) ([M+Na]⁺).

3.8.19 Bis(methyl-2,3,4-tri-O-benzyl-8-oxo-\( \alpha \)-D-gluco-nonapyanosyluronate) ether (31)

To a well-stirred solution of 30 (0.14 g, 0.092 mmol) in MeOH (1 mL) was added DMDO in acetone (in excess of 2 equivalents) and the resulting solution was stirred at ambient temperature. The reaction was monitored by TLC and appeared complete in 0.5
hr. Excess solvent and reagents were removed under reduced pressure to get a gummy material. Purification of the crude material by silica gel flash column (230-400 mesh; 9 x 3.1 cm) with 1:3 EtOAc/hexanes produced 0.062 g of 31 as a colorless thick gum in 64% yield; \(R_f = 0.6\) (1:1 EtOAc/hexanes). \(^1\)H NMR (600 MHz, CDCl₃): \(\delta\) 1.73 (m, 2H), 1.93 (m, 2H), 2.45 (m, 2H), 2.55 (m, 2H), 3.23 (t, 2H, \(J = 6.0\) Hz), 3.51 (dd, 2H, \(J = 3.6, 9.6\) Hz), 3.79 (s, 6H), 4.01 (m, 2H), 4.05 (t, 2H, \(J = 9.6\) Hz), 4.62 (d, 2H, \(J = 11.4\) Hz), 4.74 (d, 2H, \(J = 11.4\) Hz), 4.82 (d, 2H, \(J = 12.0\) Hz), 4.87 (d, 2H, \(J = 12.0\) Hz), 4.91 (d, 2H, \(J = 11.4\) Hz), 4.96 (d, 2H, \(J = 3.6\) Hz), 4.97 (d, 2H, \(J = 14.4\) Hz), 7.22 (m, 6H), 7.26-7.36 (m, 24H). \(^1^3\)C NMR (150 MHz, CDCl₃): \(\delta\) 25.9, 35.2, 53.0, 70.0, 73.4, 75.3, 75.8, 79.7, 81.5, 81.9, 93.3, 127.4, 127.7, 127.8, 128.1, 128.14, 128.2, 128.3, 128.6, 128.64, 128.7, 138.4, 138.41, 138.8, 161.4, 193.8; HR-MS calculated for C₆₂H₆₆O₁₅Na \(m/z = 1073.4303\) ([M+Na]+); observed \(m/z = 1073.4299\) ([M+Na]+).

3.8.20 Bis(N-butyl-2,3,4-tri-O-benzyl-8-oxo-\(\alpha\)-D-glucosyluronamide) ether (32)

Compound 30 (0.1 g, 0.065 mmol) was dissolved in CH₂Cl₂ (4 mL) and freshly prepared DMDO in acetone (in excess of 4 equiv.) at -78 °C was cannulated into the reaction mixture at -78 °C. The resulting solution was stirred for 15 min. under N₂ atmosphere when TLC showed disappearance of starting material. Excess DMDO was removed by applying vacuum for 15 minutes. Butylamine (16 µL, 0.16 mL) in CH₂Cl₂ (2 mL) was added dropwise at -78 °C and the reaction mixture was stirred for 0.5 hr. After disappearance of starting material (TLC), the reaction was allowed to attain room temperature. Excess solvent was removed under reduced pressure. The crude material
was purified by silica gel flash column (230-400 mesh; 4 x 3.5 cm) using 1:3 EtOAc/hexanes to afford 0.034 g of compound 32 as a colorless gum in 46% yield; \( R_f = 0.63 \) (1:1 EtOAc/hexanes). \(^1\)H NMR (600 MHz, CDCl\(_3\)): \( \delta \) 0.92 (t, 6H, \( J = 7.2 \) Hz), 1.35 (m, 4H), 1.51 (m, 4H), 1.78 (m, 2H), 1.96 (m, 2H), 2.62 (m, 2H), 2.73 (m, 2H), 3.27 (m, 6H), 3.54 (dd, 2H, \( J = 3.6, 9.6 \) Hz), 3.99 (m, 2H), 4.07 (t, 2H, \( J = 9.6 \) Hz), 4.63 (d, 2H, \( J = 10.8 \) Hz), 4.80 (dd, 4H, \( J = 12.0, 17.4 \) Hz), 4.86 (d, 2H, \( J = 9.6 \) Hz), 4.91 (d, 2H, \( J = 9.6 \) Hz), 4.96 (d, 2H, \( J = 11.4 \) Hz), 5.03 (d, 2H, \( J = 3.6 \) Hz), 6.96 (t, 2H, \( J = 5.4 \) Hz), 7.21 (m, 6H), 7.28-7.37 (m, 24H). \(^1\)C NMR (150 MHz, CDCl\(_3\)): \( \delta \) 13.9, 20.2, 26.3, 29.9, 31.5, 33.3, 39.2, 70.2, 73.3, 75.3, 75.8, 79.8, 81.8, 82.0, 92.6 (C-1), 127.5, 127.6, 127.8, 127.9, 128.2, 128.6, 128.61, 138.5, 138.6, 138.9, 160.5, 199.5; HR-MS calculated for C\(_{68}\)H\(_{80}\)O\(_{13}\)N\(_2\)Na \( m/z = 1155.5566 ([M+Na])^+ \); observed \( m/z = 1155.5558 ([M+Na])^+ \).

### 3.8.21 Synthesis of trehalose-derived bicyclo-methyl ester (33)

To a well-stirred solution of 31 (0.05 g, 0.0475 mmol) in THF (4 mL) was added Pd/C (70 mg) and a balloon of H\(_2\) was connected to the reaction flask. The resulting suspension was stirred at room temperature. The reaction was monitored by TLC and appeared complete after 24 hr. The catalyst was filtered through a pad of Celite\(^\circledR\) and excess solvent was removed under reduced pressure. The crude material thus obtained was purified by silica gel flash column (230-400 mesh; 6 x 3.5 cm) using 3:3:19 MeOH/acetone/CHCl\(_3\) to generate 0.015 g of 33 as a yellow glassy solid in 62% yield; \( R_f = 0.26 \) (1:3 MeOH/EtOAc). \(^1\)H NMR (600 MHz, CD\(_3\)OD): \( \delta \) 1.77 (m, 4H), 1.89 (m, 4H), 3.48 (dd, 2H, \( J = 4.2, 9.6 \) Hz), 3.51 (t, 2H, \( J = 9.6 \) Hz), 3.71 (s, 6H), 3.81 (m, 2H), 3.83 (t, 2H, \( J = 9.6 \) Hz), 4.98 (d, 2H, \( J = 4.2 \) Hz). \(^1\)C NMR (150MHz, CD\(_3\)OD): \( \delta \) 25.3, 32.3,
3.8.22 Synthesis of trehalose-derived bicyclo-N-butylamide (34)

To a well-stirred solution of 32 (0.05 g, 0.044 mmol) in THF (4 mL) was added Pd/C (54 mg) and a balloon of H₂ was connected to the reaction flask. The resulting suspension was stirred at room temperature. The reaction was monitored by TLC and appeared complete after 24 hr. The catalyst was filtered through a pad of Celite® and excess solvent was removed under reduced pressure. The crude material thus obtained was purified by silica gel flash column (230-400 mesh; 7.5 x 2.1 cm) using 3:3:19 MeOH/acetone/CHCl₃ to generate 0.017 g of 34 as a yellow glassy solid in 65% yield; \( R_f = 0.42 \) (1:1 MeOH/EtOAc). ¹H NMR (600 MHz, 1:1 CD₃OD/CDCl₃): \( \delta \) 1.08 (t, 6H, \( J = 7.2 \) Hz), 1.51 (m, 4H), 1.66 (m, 4H), 1.83 (m, 2H), 2.0 (m, 4H), 2.24 (m, 2H), 3.32 (m, 2H), 3.40 (m, 2H), 3.74 (m, 4H), 3.92 (m, 2H), 4.05 (t, 2H, \( J = 9.6 \) Hz), 5.23 (d, 2H, \( J = 3.6 \) Hz). ¹³C NMR (100 MHz, 1:1 CD₃OD/CDCl₃): \( \delta \) 14.1, 20.7, 24.7, 30.3, 32.1, 39.7, 67.4, 71.3, 73.1, 75.0, 94.8, 94.9, 173.1; HR-MS calculated for C₂₆H₄₄O₁₃N₂Na \( m/z = 615.2744 \) ([M + Na]⁺); observed \( m/z = 615.2741 \) ([M + Na]⁺).

3.8.23 Antibacterial Assay

Arabinofuranoside analogues 1-3 and trehalose congeners 4, 33 and 34 were screened for their ability to inhibit \( M. \) smegmatis ATCC 14468 using Kirby-Bauer disk diffusion assay.⁸² Isoniazid (INH) was used as a positive control and DMSO, the diluent, as a negative control. \( M. \) smegmatis ATCC 14468 was used as a fast growing non-pathogenic
surrogate for *M. tuberculosis*.\(^{83}\) The strain was previously used for screening antimycobacterial compounds.\(^{43,84}\) Unfortunately 1-4 and 33-34 showed no inhibitory activity against this organism.\(^{47}\)
CHAPTER 4

Trehalose-derived Sulfonamides as Potential Inhibitors of Antigen 85

4.1 Introduction: Sulfonamides in Drug Development.

Sulfonamides and related analogs such as sulfones and sulfonate esters are an important class of compounds found in many drugs. In fact, many pharmacological agents possessing sulfonamide moieties in their core structure have exhibited antitumor, antibacterial, antidiuratic, anticarbonic anhydrase, antiviral and anti-inflammatory activity. Searching through relevant literature to find alternate protease inhibitors, we found that sulfonamides have been shown to be potent protease inhibitors. For example, amprenavir, a potent aspartic HIV protease inhibitor is currently approved for treatment of AIDS and HIV infection (Figure 4.1). Amprenavir has the advantage that it is potent with $K_i = 0.6 \text{ nM}$, water-soluble, and a high oral bioavailability (>70%). With a half life ($t_{1/2}$) of 7-10 h, it means that amprenavir is administered less frequently thereby having the potential for less side effects with respect to other marketed HIV protease inhibitors.

A number of sulfonamide serine protease inhibitors have also shown promise as new drugs. In this group will be Silvistat (Figure 4.1), an inhibitor of human neutrophil
elastase (HNE) registered in Japan for the treatment of acute respiratory distress syndrome (ARDS). HNE is a serine protease which has been implicated in a number of inflammatory diseases such as ARDS, Cystic fibrosis, and ischemia reperfusion injury. HNE is a glycoprotein of 33 kDa weight which possesses the classical catalytic triad of Ser195-His57-Asp102. Silvistat was shown to be a competitive inhibitor with $K_i = 0.2 \, \mu\text{M}$ and $\text{IC}_{50} = 0.044 \, \mu\text{M}$. The relative success of Silvistat is an indication that sulfonamide and other sulfone derivatives are promising targets for the development of new drugs that work through inhibition of related serine proteases.

Figure 4.1: Amprenavir, a HIV drug and Silvistat, a drug for ARDS. Both are protease inhibitors with sulfonamide core.

In their search for next generation drugs against hepatitis C virus (HCV), Raboisson et al. have reported the synthesis of a dipeptide with sulfonamide moiety as an inhibitor of HCV non-structural protein 3 (NS3/4A) serine protease (Figure 4.2). This compound was shown to be a potent inhibitor of HCV NS3/4A serine protease with $K_i = 0.2 \, \text{nM}$, and $\text{EC}_{50} = 3.76 \, \text{nM}$. It was also metabolically more stable than other analogs without sulfonamide core, and it is currently undergoing preclinical trials.
Although the exact role of the sulfonamide or related moiety in drug targets that are already in advanced clinical stages is not yet well understood, it is clear that the sulfonyl motif provides greater bioavailability and more interaction with active site through hydrogen bonding. Since the catalytic triad of both serine protease (Ser-His-Asp) and Ag85 (Ser-His-Glu) has serine as a common nucleophile, we reasoned that the design of trehalose analogues with sulfonamide moiety could be a viable inhibitor of antigen 85. Therefore, this chapter discusses the design and synthesis of novel trehalose-based sulfonamides as potential inhibitors of Ag85 acyl-transferase activity.

4.2 Design of Trehalose Sulfonamides as Inhibitors of Ag85

We have chosen to make trehalose-derived sulfonamides because trehalose is a natural substrate in Ag85. In their work using trehalose sulfonamide analogs of
type A (Figure 4.3), Rose et al. showed that trehalose-derived sulfonamides are viable leads for the development of new anti-tuberculosis drugs. They made trehalose sulfonamides of the type A represented in Figure 3, and tested them for inhibition against the growth of *M.tb* strain H37Ra and *M. avium*. The minimum inhibitory concentration (MIC) found for trehalose-derived sulfonamides of type A was 16-32 µg/mL for *M.tb* H37Ra, and 32-64 µg/mL for all *M. avium* strains. As figure 4.3 shows, the optimal alkyl length for activity is C-8.

\[
\begin{align*}
R &= \text{CH}_3(\text{CH}_2)_3\text{SO}_2\text{NH}, \text{MIC} > 250 \mu\text{g/mL}.
R &= \text{CH}_3(\text{CH}_2)_7\text{SO}_2\text{NH}, \text{MIC} = 16-32 \mu\text{g/mL}.
R &= \text{CH}_3(\text{CH}_2)_{15}\text{SO}_2\text{NH}, \text{MIC} > 32 \mu\text{g/mL}.
R &= \text{C}_6\text{H}_5\text{SO}_2\text{NH}, \text{MIC} = \text{ND}.
\end{align*}
\]

**Type A Sulfonamides**

Figure 4.3: Trehalose-derived sulfonamides of type A synthesized by Rose et al.

Continuing our effort to find potent inhibitors of Ag85, we chose to design trehalose-derived sulfonamides of type B (Figure 4.4). Our design for type B sulfonamides took a different route from type A sulfonamides already made by Rose et al. First, we rationalized that a mono-substituted trehalose analog could be a better inhibitory target than the di-substituted analog used by Rose et al. The rationale for this is based on the fact that the main substrate for the synthesis of TDM or mAGP of the cell wall is the TMM, a mono-acylated trehalose. Studies have shown that the binding mode
of TMM and mycolyl transfer from TMM follows a ‘ping pong’ mechanism, meaning that one molecule of TMM binds the carbohydrate binding site and after its mycolyl group is successfully transferred to serine, it is released out of the active site as trehalose, making room for another molecule of TMM. This mechanism presents an opportunity to explore mono-substituted trehalose analogs as potent inhibitors of Ag85. We note that the work of Rose et al. was done before the crystal structure Ag85 was available. Figure 4.5 shows a proposed binding model for sulfonamides within the active site of Ag85.

![Binding Model for Sulfonamides](image)

**Type B Sulfonamides**

Figure 4.4: Trehalose-derived sulfonamides of type B. This type closely mimics TMM and allows for easy access to sulfonamide, sulfone and sulfone ester derivatives from one synthetic pathway.

Second, we have chosen the Type B analogs because the synthetic design allows us to develop sulfonamide and sulfone derivatives in one synthetic pathway. This provides a versatile method to access various derivatives of sulfones, sulfonate esters and sulfonamides as potential inhibitors of Ag85. Hence, it is easier to develop a library of
sulfone analogs using Type B approach. In addition to all the points noted above, the type B sulfonamides discussed in this chapter are terminal sulfonamides (Figure 4.4).

![Figure 4.5: Proposed model for the binding of sulfonamide analogs within the active site of Ag85.](image)

4.3 Synthesis of trehalose-based sulfonic ester (7)

The synthesis of sulfone derivatives is based on the adaptation of the method employed by Reddick et al.\textsuperscript{101} Trehalose 1 is benzylated,\textsuperscript{59} and one primary hydroxyl group is selectively de-benzylated\textsuperscript{43} as described in chapter two to give mono-hydroxyl analog 3. Compound 3 is converted to aldehyde 4 using Swern oxidation.\textsuperscript{43} Sulfone ester was then installed on the trehalose substrate via Horner-Wadsworth-Emmons condensation\textsuperscript{43,102} reaction with triethyl-\(\alpha\)-phosphorylmethanesulfonate (5) to give trehalose sulfonate ester 6 in 91% yield (Scheme 4.1). Global reduction of 6 via Pd-catalysed hydrogenolysis resulted in compound 7 in good yields (71%).

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Scheme 4.1: Synthesis of Sulfonate Ester (7). Reagents and conditions: a) NaH, BnBr, Bu₄NI, anhydrous DMF, 0 ºC to r.t., 4 hr; b) Ac₂O, TMSOTf, CH₂Cl₂, -78 ºC to r.t., Amberlite IR 120 (H⁺ form), 5 hr; c) (COCl)₂, anhydrous DMSO, anhydrous CH₂Cl₂, Et₃N, -78 ºC to -10 ºC; d) triethyl α-phosphorylmethanesulfonate (5), NaH, anhydrous THF, 0 ºC to r.t., 7 hr; e) MeOH/EtOAc (1:1), Pd/C (10% Degussa type), H₂, 24 hr, r.t.

4.4 Synthesis of trehalose-derived sulfonamides (13-16)

In order to facilitate nucleophilic substitution reaction with an appropriate amine, compound 6 was first converted to the sulfonyl chloride intermediate 8. This was achieved by reacting 6 with tetrbutylammonium iodide at 50 ºC, and then with sulfuryl chloride at room temperature. After removal of solvent in vacuo, compound 8 was used
for the next step without further purification. Sulfonamides 9, 10, 11 and phenyl sulfonate ester 12 were synthesized through base-promoted coupling of appropriate amine (or phenol) with sulfonyl chloride intermediate 8 in 79%, 96%, 55% and 53% yield respectively.\textsuperscript{12,25} When subjected to Pd/C/H\textsubscript{2} reduction reaction,\textsuperscript{43} compounds 9 and 10 were successfully reduced to compounds 13 and 14 in 76.2%, 77.3% yield respectively (Scheme 4.2). Attempts to reduce 11 and 12 to 15 and 16 respectively proved unsuccessful.

Scheme 4.2: Synthesis of Sulfonamides (13-15) and Phenyl Sulfonate Ester (16). 

Reagents and conditions: f 1). Bu\textsubscript{4}NI, acetone, 50 °C, 24 hr; 2). SO\textsubscript{2}Cl\textsubscript{2}, PPh\textsubscript{3}, anhydrous CH\textsubscript{2}Cl\textsubscript{2}, 0 °C, 2-3 hr; g) DMAP, anhydrous CH\textsubscript{2}Cl\textsubscript{2}, appropriate amine of phenol, 0 °C to r.t., 1 hr; to give 9 (79.0% yield), 10 (96.1% yield), 11 (55.1% yield) and 12 (53.2% yield); h) Pd/C (10% Degussa type), H\textsubscript{2}, 24 hr, r.t. to give 13 and 14 in 76.2%, 77.3% yield respectively.
4.5 Conclusion

Sulfonamides and related sulfone derivatives are known inhibitors of serine proteases. Since the catalytic triad (Ser-His-Glu) of Ag85 has a similar charge relay system as the catalytic triad of serine protease (Ser-His-Asp), it is expected that it will function by similar mechanism as related serine proteases. Therefore, we have designed and synthesized two classes of D-trehalose-derived sulfonate esters 7 and 16, and sulfonamides 13-15 as inhibitors of Ag85. Compounds 7, 13-16 were designed to mimic TMM.

The synthesis of sulfone ester 7 was accomplished by incorporation of ester moiety on the C-6 of α-D-trehalose substrate via a Horner-Wadsworth-Emmons condensation reaction with triethyl-α-phosphorylmethanesulfonate. Hydrogenolysis of the protected precursor 6 resulted in isolation of compound 7 in 71% yield.

Sulfonamides 13-15 were installed through a similar process, followed by base-promoted coupling of appropriate amine with the sulfonyl chloride intermediate. Trehalose-derived sulfonamides 13 and 14 were successfully isolated in 76% yield and 77% yield respectively. However, attempt to de-protect 11 and 12 to access sulfonamide 15 and sulfonate ester 16 was unsuccessful. In both cases, it is possible that delocalization between the lone pair of electrons on nitrogen (as in 11) or oxygen (as in 12) and the benzene ring prevented the desired product from forming.

The Ronning laboratory is currently developing an assay that could be used to screen these compounds and others from our laboratory. In the future, it will be important to develop a larger library of sulfonamides which could be tested for inhibitory activity against Ag85 and bacterial growth. Since the protocol for accessing both sulfonamides
and sulfone derivatives of type B via one synthetic pathway have been worked out in this project, developing a larger library in the future will not be difficult. This is an important contribution made by this study.

4.6 Experimentals

**General Methods.** α-D-Trehalose and other fine chemicals were purchased from commercial suppliers and were used without further purification. All solvents used for reactions were dried following the standard procedures. Triethylamine was dried using MS 4Å. Thin-layer chromatography (TLC, silica gel 60, f254) were performed in distilled solvents as specified and visualized under UV light or by charring in the presence of 5% H2SO4/MeOH. Flash column chromatography was performed on silica gel (230-400 mesh) column using solvents as received. 1H NMR were recorded either on Varian VXR-400 (400 MHz) or INOVA-600 (600 MHz) spectrometer in CDCl3, CD3OD or DMSO-d6 using residual CHCl3, CH3OH or DMSO as internal references, respectively. 13C NMR were recorded either on Varian VXR-400 (100 MHz) or INOVA-600 (150 MHz) spectrometer in CDCl3, CD3OD or DMSO-d6 using the triplet centered at δ 77.273 for CDCl3, septet centered at δ 49.0 for CD3OD, or septet centered at δ 39.5 for DMSO-d6 as internal reference, respectively. The 1H NMR data are presented as follows: chemical shift, multiplicity (s = singlet, d = doublet, dd = doublet of doublet, t = triplet, q = quartet, m = multiplet), coupling constants in Hertz, integration, and assignments. Low resolution mass spectra were taken on Esquire-LC electrospray ionization (ESI) mass spectrometer operated in the positive ion mode. High resolution mass spectrometry (HRMS) was
performed on a mass spectrometer located at the Mass Spectrometry and Proteomics Facility, The Ohio State University.

4.6.1 Synthesis of trehalose-derived vinyl ethylsulfonic ester (6)\textsuperscript{101,102}

To a solution of oxalyl chloride (0.49 mmol, 0.08 mL) in 1 mL of anhydrous CH\textsubscript{2}Cl\textsubscript{2} at -78 °C, was added anhydrous DMSO (0.99 mmol, 0.07 mL) and the resulting solution was stirred for 15 minutes. Reaction solution was stirred under nitrogen atmosphere, and was allowed to warm up to -65 °C in 30 minutes. A solution of mono-ol 3 (0.33 mmol, 0.32 g) in 1 mL of anhydrous CH\textsubscript{2}Cl\textsubscript{2} was added drop-wise over 20 minutes. The reaction mixture was stirred for another 45 minutes, allowing the temperature to warm up to -45°C. To this solution was added anhydrous triethylamine (1.30 mmol, 0.20 mL) dropwise. The reaction mixture was stirred and allowed to warm up to between -10°C to 0°C over 40 minutes. The reaction progress was monitored by TLC (hexane:EtOAc, 7.5:2.5). When the reaction was completed, 5 mL of 1 N HCl was added to quench the reaction. Reaction system was diluted with ethyl ether. Organic layers were combined and washed with cold water three times, brine, and then dried over anhydrous Na\textsubscript{2}SO\textsubscript{4}. Removal of solvent \textit{in vacuo} resulted in 3.01 g of crude mono aldehyde 4 as an oil in 92% yield; \(R_f = 0.40\) (3:7 EtOAc/hexanes). This product was dried and used immediately in its crude form for the next reaction.

To a 0 °C suspension of NaH (60% in mineral oil, 0.24 g, 10.0 mmol) in anhydrous THF (5 mL) was added a solution of triethyl α-phosphorylmethanesulfonate (5) (0.24 g, 0.91 mmol) in 2 mL of anhydrous THF drop-wise. The reaction mixture was...
stirred at 0 ºC for 30 minutes. The icebath was removed, and crude aldehyde \(4\) (0.45 g, 0.46 mmol) in 2 mL of anhydrous THF was added dropwise. Reaction mixture was stirred under nitrogen atmosphere for 6 hours. After completion of reaction (monitored by TLC, Hexane:EtOAc, 7:3), reaction was quenched with water and diluted with ethyl acetate. Organic layers were combined and concentrated \textit{in vacuo}. Crude material was purified by silica gel flash column (230-400 mesh, 10 x 4.5 cm). Elution with 7:3 hexane/ethyl acetate afforded 0.35 g light yellowish product \(6\) in 71.4% yield; \(R_f = 0.63\) (3:7 EtOAc/hexanes). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) ppm 1.2 (t, \(J = 6.8\) Hz, 2H), 3.5 (m, 2H), 3.6 (m, 1H), 3.9 (d, \(J = 3.7\) Hz, 1H), 4.0 (dt, \(J = 14.0, 7.1\) Hz, 2H), 4.5 (m, 3H), 4.7 (m, 2H), 4.8 (m, 1H), 4.9 (s, 1H), 6.50 (d, 1H), 6.83 (d, 1H), 7.1 (d, \(J = 2.6\) Hz, 3H), 7.2 (d, \(J = 13.9\) Hz, 10H), 7.3 (s, 4H). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) ppm 15.21, 67.28, 68.57, 71.23, 72.44, 72.55, 73.23, 73.93, 75.44, 75.99, 76.06, 77.28, 77.61, 77.77, 77.92, 79.12, 82.01, 93.83, 122.92, 127.80, 127.93, 127.99, 128.11, 128.23, 128.28, 128.34, 128.39, 128.44, 128.53, 128.61, 128.77, 128.84, 128.88, 129.01, 129.29, 130.09, 138.15, 138.19, 138.39, 138.47, 138.64, 139.14. HS-MS calculated for C\(_{64}\)H\(_{68}\)O\(_{13}\)SNa \(m/z = 1099.4278 ([M+Na]^+)\); observed \(m/z = 1099.4236 ([M+Na]^+)\).

4.6.2 General procedure for the synthesis of trehalose-derived vinyl sulfonamides (9, 10, 11 and 12)

Tetrabutylammonium iodide (0.15 g, 0.40 mmol) was added to a solution of trehalose sulfonate ester \(6\) (0.34 g, 0.33 mmol) in 2 mL of acetone, and then the reaction system was refluxed for 24 hours at 50 ºC under nitrogen. Reaction was cooled to room temperature and solvent removed \textit{in vacuo} to give crude tetrabutylammonium sulfonate
salt as yellow syrup. The crude syrup was dissolved in CH$_2$Cl$_2$ and washed with water and brine. Organic layers were combined and dried over anhydrous Na$_2$SO$_4$, filtered and concentrated to give 0.29 g of oily material which was then taken to the next step without further purification.

Sulfuryl chloride (0.83 mmol, 0.10 mL, 2.5 equivalent) was added to a solution of triphylphosphosphine (0.83 mmol, 0.22 g, 2.5 equivalent) in 3 mL of anhydrous CH$_2$Cl$_2$ at 0 ºC and allowed to stir for about 5 minutes under nitrogen. The icebath was removed and oil product from above (0.29 g dissolved in 2 mL of CH$_2$Cl$_2$) was added dropwise to the reaction solution. Reaction medium was stirred at room temperature, and monitored by TLC (Hexane:EtOAc, 8:2). After 2.5 hours, reaction was completed. Reaction mixture was concentrated in vacuo to obtain 0.31 g of 8 as a crude oily product. Compound 8 was dried and used for the next step without further purification.

To a 0 ºC solution of sulfonyl chloride 8 (0.60 mmol) in anhydrous CH$_2$Cl$_2$ (1 mL) was added a solution of appropriate amine (1.8 mmol) and dimethylamino pyridine, DMAP (1.8 mmol) in 1 mL of CH$_2$Cl$_2$. The reaction was stirred at 0 ºC, and monitored by TLC (Hexane:EtOAc, 7:3). After completion of reaction, usually within 2 hours, reaction was quenched with water, and diluted with CH$_2$Cl$_2$. Organic layers were combined, washed with 10 mL each of NaHCO$_3$ and brine. Organic layers were dried over anhydrous Na$_2$SO$_4$, and concentrated in vacuo. Crude product was purified by silica gel flash column (230-400 mesh, 10 X 4.5 cm). Elution with 9:1, 8:2 and 7:3 hexane/ethyl acetate resulted in 45 mg of phenylethylsulfonamide analog 9 in 79% yield, $R_f = 0.52$ (3:7 EtOAc/hexanes); 53 mg of butylsulfonamide analog 10 in 96% yield, $R_f = 0.56$ (3:7 EtOAc/hexanes); 22 mg of aniline sulfonamide analog 11 in 55% yield, $R_f = 0.59$ (3:7 EtOAc/hexanes).
EtOAc/hexanes); and 28 mg of phenylsulfonate ester 12 in 53% yield, $R_f = 0.70$ (3:7 EtOAc/hexanes).

**Phenylethylsulfonamide 9:** $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ ppm 1.6 (s, 1H), 2.7 (d, $J = 6.2$ Hz, 1H), 3.1 (d, $J = 6.2$ Hz, 1H), 3.5 (d, $J = 4.8$ Hz, 1H), 3.5 (s, 1H), 4.1 (s, 2H), 4.5 (m, 2H), 4.7 (d, $J = 11.4$ Hz, 1H), 4.8 (m, 1H), 5.0 (d, $J = 9.9$ Hz, 1H), 6.22 (d, 1H), 6.73 (d, 1H) 7.1 (m, 2H), 7.2 (s, 2H), 7.3 (m, 10H). $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ ppm 36.76, 44.51, 68.57, 69.51, 71.29, 73.30, 73.56, 74.02, 75.67, 75.96, 76.15, 76.26, 77.23, 77.54, 77.86, 78.12, 79.56, 81.89, 81.98, 82.32, 94.44, 95.11, 127.39, 127.82, 127.91, 128.10, 128.14, 128.20, 128.27, 128.38, 128.51, 128.87, 128.90, 128.95, 128.98, 129.10, 129.19, 129.31, 137.88, 138.19, 138.36, 138.41, 138.67, 138.97, 139.21, 141.65. HR-MS calculated for C$_{70}$H$_{73}$NO$_{12}$SNa $m/z = 1174.4751$ ([M+Na]$^+$); observed $m/z = 1174.4706$ [M+Na]$^+$. 

**Butylsulfonamide 10:** $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ ppm 0.9 (m, 2H) 1.2 (m, 2 H) 1.4 (m, 1 H) 1.6 (s, 1 H) 2.8 (d, $J = 6.6$ Hz, 1 H) 3.6 (m, 2 H) 4.1 (d, $J = 9.5$ Hz, 1 H) 4.6 (dd, $J = 15.7$, 12.1 Hz, 1 H) 4.7 (s, 1 H) 4.8 (m, 2 H) 5.0 (dd, $J = 10.4$, 7.9 Hz, 1 H) 7.1 (s, 1 H) 7.3 (s, 3 H) 7.3 (dd, $J = 17.0$, 7.5 Hz, 10 H). $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ ppm 13.58, 19.65, 29.70, 31.90, 42.69, 68.06, 69.04, 70.78, 72.81, 73.06, 73.51, 75.14, 75.50, 75.64, 75.76, 76.70, 79.03, 79.10, 81.37, 81.61, 81.80, 93.93, 94.60, 127.25, 127.39, 127.44, 127.58, 127.62, 127.72, 127.76, 127.86, 127.89, 128.00, 128.05, 128.38, 128.43, 128.59, 137.38, 137.67, 137.86, 137.90, 138.16, 138.45, 138.71, 141.00. HS-MS
calculated for C\textsubscript{66}H\textsubscript{73}NO\textsubscript{12}SNa \textit{m/z} = 1126.4751 ([M+Na])\textsuperscript{+}; observed \textit{m/z} = 1126.4714 ([M+Na])\textsuperscript{+}.

**Phenylsulfonamide 11:** \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) \(\delta\) ppm 3.5 (m, 1H), 4.0 (d, \(J = 9.2\) Hz, 1H), 4.1 (m, 1H), 4.4 (s, 1H), 4.5 (d, \(J = 19.0\) Hz, 1H), 4.7 (s, 2H), 4.8 (d, \(J = 9.9\) Hz, 1H), 4.9 (d, \(J = 8.8\) Hz, 1H), 7.0 (s, 2H), 7.2 (d, \(J = 5.5\) Hz, 3H), 7.3 (m, 14H). \textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}) \(\delta\) ppm 68.05, 68.87, 70.74, 72.76, 73.03, 73.49, 75.03, 75.13, 75.47, 75.83, 76.63, 78.81, 79.00, 81.33, 81.73, 93.81, 94.50, 121.05, 125.27, 127.08, 127.34, 127.55, 127.60, 127.69, 127.82, 127.99, 128.05, 128.28, 128.37, 128.43, 128.47, 128.59, 129.39, 136.24, 137.33, 137.63, 137.67, 137.89, 138.15, 138.36, 138.73, 143.36. ESI-MS calculated for C\textsubscript{68}H\textsubscript{69}NO\textsubscript{12}SNa \textit{m/z} = 1146.45 ([M+Na])\textsuperscript{+}; observed \textit{m/z} = 1145.60 [M+Na]\textsuperscript{+}.

**Phenylsulfonic ester 12:** \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) \(\delta\) ppm 1.26 (s, 1H) 1.58 (s, 1H), 3.5 (s, 2 H), 3.7 (s, 1H), 4.0 (d, \(J=15.4\) Hz, 1H), 4.5 (s, 1H), 4.7 (s, 2H), 4.8 (m, 2H), 5.0 (s, 1H), 7.1 (s, 2H), 7.3 (m, 14H). \textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}) \(\delta\) ppm 29.99, 68.34, 69.22, 71.11, 73.18, 73.57, 73.79, 75.35, 75.86, 75.90, 76.14, 76.97, 77.87, 78.00, 79.26, 81.53, 82.07, 94.03, 94.75, 122.58, 122.70, 123.94, 127.31, 127.44, 127.55, 127.64, 127.85, 127.91, 128.01, 128.08, 128.25, 128.41, 128.48, 128.65, 128.71, 128.78, 128.83, 130.03, 137.26, 137.75, 137.93, 138.12, 138.39, 138.57, 138.95, 147.31. ESI-MS calculated for C\textsubscript{68}H\textsubscript{68}NO\textsubscript{13}SNa \textit{m/z} = 1147.38 ([M+Na])\textsuperscript{+}; observed \textit{m/z} = 1147.20 ([M+Na])\textsuperscript{+}.
4.6.3 General procedure for hydrogenation reactions giving trehalose-derived sulfonamides 7, 13, 14\(^{43}\)

The relevant starting material (6, 9, 10, 11 or 12) was dissolved in 5 mL of degassed MeOH-EtOAc (1:1). Then catalytic amount of Pd/C (10\%) was added and further degassed. The reaction mixture was then stirred at room temperature under atmospheric H\(_2\) pressure for 24 hours. Reaction progress was monitored by TLC (methanol:chloroform, 7:3). After completion of reaction, reaction mixture was filtered through Celite, and the residue was washed with MeOH to give 5 mg of 7 in 50\% yield, \(R_f = 0.65\) (4:6 methanol/dichloromethane); 16 mg of 13 in 76\% yield, \(R_f = 0.70\) (3:7 methanol/dichloromethane); and 17 mg of 14 in 77.3\% yield, \(R_f = 0.61\) (3:7 methanol/dichloromethane).

**Ethylsulfonic ester 7**: \(^1\)H NMR (400 MHz, CD\(_3\)OD) \(\delta\) ppm 1.4 (t, \(J = 6.6\) Hz, 3H), 1.9 (s, 1H), 2.3 (s, 1H), 3.1 (s, 1H), 3.2 (s, 1H), 3.3 (m, 4H), 3.5 (d, \(J = 9.2\) Hz, 2H), 3.8 (m, 4H), 4.3 (q, \(J = 6.3\) Hz, 2H), 5.0 (m, 3H). \(^{13}\)C NMR (100 MHz, CD\(_3\)OD) \(\delta\) ppm 15.61, 27.30, 47.75, 62.61, 68.05, 71.08, 71.90, 73.27, 73.94, 74.41, 75.61, 95.47, 95.72. HS-MS calculated for C\(_{15}\)H\(_{28}\)O\(_{13}\)SNa \(m/z = 471.1148\) ([M+Na])\(^{+}\); observed \(m/z = 471.1135\) ([M+Na])\(^{+}\).

**Phenylethylsulfonamide 13**: \(^1\)H NMR (400 MHz, CD\(_3\)OD) \(\delta\) ppm 1.92 (s, 2H), 2.8 (s, 2H), 3.1 (s, 2H), 3.3 (m, 10H), 3.5 (d, \(J = 8.8\) Hz, 2H), 3.7 (s, 2H), 3.8 (s, 4H), 4.9 (s, 16H), 5.1 (s, 1H), 7.3 (d, \(J = 18.7\) Hz, 4H). \(^{13}\)C NMR (100 MHz, CD\(_3\)OD) \(\delta\) ppm 36.72, 44.39, 61.37, 69.89, 70.68, 72.07, 72.69, 73.23, 74.47, 94.03, 126.34, 128.37,
128.73, 138.90; HS-MS calculated for C_{21}H_{33}NO_{12}SNa \textit{m/z} = 546.1621 ([M+Na])^+; observed \textit{m/z} = 546.1609 ([M+Na])^+.

Butylsulfonamide 14: $^1$H NMR (400 MHz, CD$_3$OD) δ ppm 0.9 (s, 3H), 2.0 (s, 2H), 3.0 (m, 2H), 3.3 (m, 4H), 3.5 (s, 1H), 3.8 (s, 1H), 3.8 (d, \textit{J} = 11.4 Hz, 2H), 5.1 (d, \textit{J} = 18.7 Hz, 4H). $^{13}$C NMR (100 MHz, CD$_3$OD) δ ppm 12.84, 19.64, 26.12, 32.29, 42.48, 61.36, 69.93, 70.68, 72.00, 72.69, 73.25, 74.52, 94.07, 94.30; HS-MS calculated for C$_{17}$H$_{33}$NO$_{12}$SNa \textit{m/z} = 498.1621 ([M+Na])^+; observed \textit{m/z} = 498.1640 ([M+Na])^+. 
References


82. National Committee for Clinical Laboratory Standards. Approved Standard: M2-
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   Canioni, D.; Emile, J.F.; Fischer, A.; Blanche, S.; Gaillard, J.L.; Casanova, J.L. 


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![Chemical Structure](image)
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HS-MS Spectrum of Ethylsulfonic ester (7)

Internal Standard

Calculated M+Na 471.1148
Measured M+Na 471.1138
2.8ppm
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$^{13}$C NMR of Vinyl phenylethylsulfonamide (9)
HS-MS Spectrum of Vinyl phenylethylsulfonamide (9)
HS-MS Spectrum of Vinyl phenylethylsulfonamide (9)
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HS-MS Spectrum of Vinyl butylsulfonamide (10)
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