SYNTHESES AND ANTIGENIC ANALYSES OF OLIGOSACCHARIDES DERIVED FROM *BACILLUS ANTHRACIS*, *BURKHOLDERIA PSEUDOMALLEI* AND *BURKHOLDERIA MALLEI*

by

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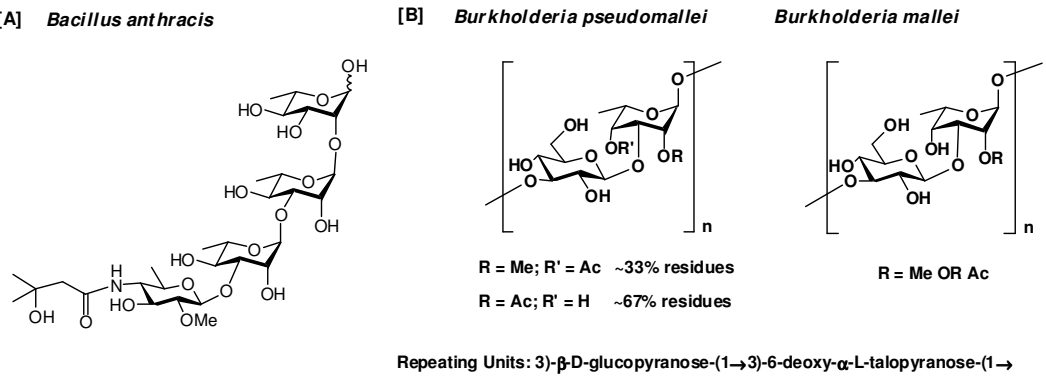
(Under the Direction of GEERT-JAN BOONS)

ABSTRACT

Bacillus anthracis, *Burkholderia pseudomallei*, and *Burkholderia mallei* are the etiological agents of anthrax, melioidosis, and glanders, respectively. These pathogens have gained notoriety due to their potential for use in biological warfare and bioterrorism, and have heightened concerns and awareness among researchers worldwide, emphasizing the need to develop efficient tools and therapies for rapid detection and identification of these pathogens and for the prevention of deadly infectious diseases caused by them. Recently, the structures of cell-wall oligosaccharides of *B. anthracis, B. pseudomallei and B. mallei* have been elucidated. These species-specific carbohydrate epitopes are interesting targets for development of vaccines and diagnostics for infectious diseases. In order to evaluate their antigenicity, these oligosaccharides were chemically synthesized in their pure forms.

The BclA glycoprotein, an important constituent of the exosporium of *B. anthracis* spores, is substituted with an oligosaccharide that contains a unique monosaccharide, trivially named as anthrose. In order to study the antigenicity of anthrose, efficient

syntheses of an anthrose-containing trisaccharide and structurally related analogs were developed. Protein conjugates of these oligosaccharides were prepared and used in immunological studies. Ongoing studies will determine whether these antigens could be used as vaccine components and/or diagnostic markers for anthrax.



***B. anthracis*, *B. pseudomallei* and *B. mallei* cell-wall carbohydrate structures**

A convergent synthesis of tetrasaccharide containing two disaccharide repeating units, having the 4-*O*-acetyl and the 2-*O*-methyl substitution in the 6-deoxy-talose unit, was developed. Immunological studies using protein-conjugate of this tetrasaccharide will determine its potential for use as vaccine candidate or diagnostic marker for melioidosis. A glycosylation at the sterically challenging C-3 hydroxyl of 6-d-Tal was achieved.

INDEX WORDS: Anthrax, melioidosis, glanders, glycoconjugates, oligosaccharides, vaccines

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DEDICATION

“Pain is temporary, it may last a minute, or an hour, or a day, or a year, but eventually it will subside and something else will take its place. If I quit, however, it lasts forever.”

Lance Armstrong

To

My Grandmothers

…who embarked on a journey to heaven in 2007 and I could not wish them bon voyage.

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ABBREVIATIONS

Å	Angstrom
Ac	Acetyl
AcOH	Acetic acid
BF_3•Et_2O	Borontrifluoride diethyletherate
Bn	Benzyl
Bz	Benzoyl
Cbz (Z)	Benzyloxycarbonyl
DCC	*N,N'*-Dicyclohexylcarbodiimide
DCM	Dichloromethane
DDQ	2,3-Dicyano-5,6-dichloro quinone
DIPEA	*N,N*-Diisopropylethylamine
DMAP	4-(Dimethylamino)pyridine
DMF	*N,N*-Dimethylformamide
DMSO	Dimethyl sulfoxide
EDC	3-(3-Dimethylaminopropyl)-1-ethylcarbodiimide
Et_3SiH	Triethyl silane
EtOAc	Ethyl acetate
EtOH	Ethyl alcohol
h	Hour
HATU	*O*-(7-Azabenzotriazol)- 1-yl-*N,N,N',N'*-tetramethyluronium hexafluorophosphate
HOAt	1-Hydroxy-7-azabenzotriazole
Hz	Hertz
m	Multiplet
m/z	Mass to charge ratio
MeOH	Methanol

mM	Millimolar
mmol	Millimole
MS	Molecular sieves
NBS	*N*-Bromosuccinimide
NIS	*N*-Iodosuccinimide
NMR	Nuclear Magnetic Resonance
q	Quartet
Rf	Retention factor
rt	Room temperature
s	Singlet
t	Triplet
TBAI	Tetrabutyl ammoniumiodide
t-BuOH	*tert*-Butyl alcohol
TEA	Triethylamine
TfOH	Trifluoromethanesulfonic acid
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TMSOTf	Trimethylsilyl trifluromethanesulfonate

Chapter I

Introduction and Literature Review

1.1 General Introduction

For decades carbohydrates were regarded primarily as energy sources and structural components in living systems. This preconception has been dispersed by the realization that carbohydrates also play important roles in a wide range of biological processes. They are involved in a number of processes such as cell-cell recognition, fertilization, embryogenesis, neuronal development, hormone activities, the proliferation of cells and their organization into specific tissues, viral and bacterial infection, and tumor cell metastasis. The carbohydrate family comprises monosaccharides, oligosaccharides, and polysaccharides; with monosaccharides being the simplest and the smallest ones that cannot be hydrolyzed further to smaller constituent units. Carbohydrates, when covalently linked to lipids and proteins, provide an important class of biopolymers named glycolipids and glycoproteins, respectively.

1.2 Oligosaccharides

Oligosaccharides (OSs) are a unique class of biopolymer distinct from proteins and nucleic acids. Unlike proteins and nucleic acids, in which monomers are connected by a single type of linkages such as amide bonds and phosphotriester linkages,

respectively; oligosaccharides have monomers connected by more than one type of glycosidic linkages. Anomeric linkages in oligosaccharides can adopt an axial or equatorial orientation. The lengths of oligosaccharides are described by prefix naming, i.e. disaccharides and trisaccharides are composed of two- and three monosaccharide units, respectively. Oligosaccharides can be linear as well as branched. No borderline can be drawn strictly between oligo- and polysaccharides; however, the term “oligosaccharide” is more commonly used to refer to well-defined structures as opposed to a polymer of unspecified length and composition. Strictly speaking, the term “oligosaccharide” used to denote saccharides containing up to and including ten monosaccharide units. Recently, this term has been used freely to denote any synthetic saccharide and even saccharides obtained by partial degradation of natural polysaccharides.

It is also believed that a single oligosaccharide can play numerous roles in an organism, depending on the carrier to which it is attached as well as on the site of attachment. An enormous number of potential isomers of even small oligosaccharides can be obtained by varying the sugar monomers, the site of glycosylation, anomeric configuration, and branching and functional group substitution. Glycoproteins and glycolipids are thus among the most functionally and structurally diverse naturally occurring molecules. Advances in chemical and enzymatic synthesis have made it possible to construct well-defined oligosaccharides for use in elucidating biological functions and structure-activity relationship. A variety of synthetic strategies can be exploited in order to achieve a desired oligosaccharide. Regio- and stereoselectivity in glycosylations are two major factors to be considered while designing such a synthesis.

Proper protecting group manipulations of the glycosyl acceptor can help achieve the desired regioselectivity. The stereoselectivity can be controlled by carefully selection of reaction conditions and by the presence or absence of participating functionality on the donor molecule. The reactivity of a glycosyl donor or acceptor can be modulated by variations in protecting groups.

1.3 Synthetic Oligosaccharide-Based Bacterial Vaccine

Oligo- and polysaccharides (PSs) are major cell wall components of human pathogens and these structures confer mechanical stability to the surface of microorganisms. The polysaccharide capsule is also believed to be implicated in interfering with the elimination process of invading encapsulated bacteria from the circulation.[1] Capsular polysaccharides have an ability to impair phagocytosis, complement activation and humoral response in the early stages of acute infections, and thereby enhancing the virulence of bacteria. Thus, polysaccharide capsule is often referred to as a virulence factor and can serve as an important protective antigen. Most bacterial cell-surface saccharides have strain-specific carbohydrate sequence with a few exception in which bacteria express closely similar carbohydrate structures that are specific to mammalian tissues. This molecular mimicry may play a vital role in their escaping recognition by the host.[2]

The development of carbohydrate-based vaccine has a long history. As early as 1923, Heidelberger and Avery[3] described pneumococci-specific soluble substances that consist most likely of PSs and being typical for the serotype.[4] Later in 1930, Francis and Tillet[5] noted that intradermal administration of purified, type specific capsular

polysaccharides (CPSs) of pneumococci induced the development of PS-specific serum antibodies in healthy adults.[2, 4, 5] Heidelberger et al.[6] established that pneumococcal CPS-specific antibodies offer long lasting, type specific immunity and could be used as vaccines. This pioneering research led to the developments of purified CPS-based human vaccines. However, the development of chemotherapeutics and antibiotics led to a loss in interest in further development of CPS-based vaccines. Renewed interest for preventive vaccination was triggered once again to combat a threat posed by the emerging antibiotic resistant bacterial strains. A few examples include a 23-valent pneumococcal vaccine, Pneumovax^{TM} 23, consists of the type specific CPSs of the 23 most virulent pneumococci; the Vi-polysaccharide vaccine against typhoid; and the *Neisseria meningitidis* groups A, C, Y, and W-135 four component vaccine.[2, 4] The Centers for Disease Control and Prevention (CDC) has recommended that the pneumococcal vaccine be administered to all adults 65 years of age or older.[2, 7]

1.4 Vaccine at a Glance

The origin of vaccine trails back to 1796 and it has an interesting history. Edward Jenner performed first successful vaccination in a teenage boy, James Phipps, in an attempt to protect him from possibly deadly effect of smallpox. Jenner observed that a number of milkmaids who had previously contracted cowpox were resistant to smallpox, a similar virus. Phipps was then “vaccinated” (from the Latin term *vacca*, for cow) with pustular material from the hand of Sarah Nelmes, who contracted the infection from a cow named Blossom, whose hide hangs today at St. George’s Hospital in London. Jenner challenged Phipps with virulent smallpox, six weeks after vaccination, and

fortunately Phipps did not contract the disease. Louis Pasteur first used the terms “immune” and “immunity” in the scientific sense almost 75 years later; however, he retained the word “vaccination” to describe his own accomplishments in the prevention of rabies and anthrax to acknowledge Jenner’s pioneering research. Since then, the field of vaccine development has gotten momentum and vaccines have been developed to manage and reverse infections caused by bacteria, viruses and parasites, notably those for diphtheria (von Behring) and polio (Salk) among many others.[8-10]

In the past decades, an increasing trend in the bacterial resistance to antibiotics has been observed, leading to a serious threat for successful treatment for bacterial infections. Vaccination is the most cost-efficient and powerful medical intervention in the control, prevention and eradication of many diseases that affect human population. In vaccination, the first step includes isolation of a pathogen or toxin followed by preparation of a specific antigenic and innocuous immunogen, which is then formulated into a vaccine that maintains and ideally augments immunogenicity.[1]

Recent advancement in understanding of the immune system, molecular architecture and biology of pathogens has lead to a tremendous improvement in vaccine efficacy and has created opportunity to educate and stimulate the immune system.[1] Construction of vaccines that contain highly purified antigenic molecules or haptens derived from or modeled on the most immunogenic domains of a pathogen has become a common practice.

1.5 Immunization

The objective of immunization (vaccination) is to utilize infectious agents prophylactically to develop resistance to infection within a population. A various types of immunization procedures are summarized in Fig. 1.1.

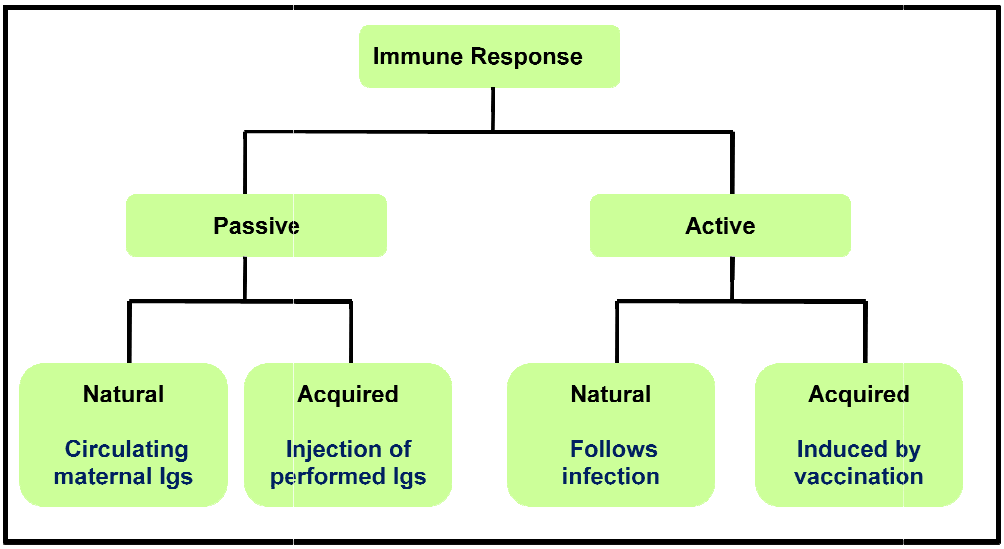

Figure 1.1. Basics of immune response.

Passive immunization can be achieved by using immunoglobulins (Ig or antibodies) derived from human or humanized serum. This approach is generally used when: a) active vaccine is unavailable; b) immunocompromised person is at risk or susceptible to endemic infection; and c) time is the limiting factor once exposure occurs and no active immunization is possible. This approach provides a temporary protection, up to 3 months, after which, the Ig levels drops below a minimum level required for protection. On the other hand, it requires several months for the immune system to provide adequate rapid protection against pathogens after active immunization

However, immunity achieved in this fashion is long lasting, persisting for many years or even a lifetime. A quick and vigorous antibody response, known as immunological memory, is obtained upon any recurrence of infection by the same pathogen.

1.6 Immune System

The prime function of the immune system is to provide protection against invading foreign agent and the immune responses must begin as soon as the host recognizes that a pathogenic microorganism an outsider. The immune system is composed of natural (innate) immunity and acquired (adaptive) immunity. A non-specific protection against infectious agents can be achieved through mechanical, chemical and biological barriers in case of innate immunity, whereas adaptive immunity involves immunological memory, offers rapid and specific protection against a definite antigen and heighten immune responses upon re-exposure to that antigen.

1.7 Immune Responses to T-Cell Independent and T-Cell Dependent Antigens

PSs have been classified as T-cell independent type 2 (TI-2) antigens based on their ability to stimulate antigen-specific B-cells in the absence of T-cells and they may be recognized by resting B-lymphocytes through their membrane-bound antibody receptors.[2, 11] The multivalency and large size of these antigens triggers cross linking and clustering of B-cell receptors and induce antibody secretion.[12] Pure PS cause the maximum immune response with one exposure, but fails to result in a booster effect upon repeated exposure to the same antigen as they do not induce immunologic memory.[2] The booster injections regain antibody levels that have fallen after original

immunization, but fail to produce massive response.[1, 13] In contrast to PSs, most proteins are univalent and require cooperation of B-cells with T-cells to induce antibody synthesis and thus, are T-cell dependent (TD) antigens.[14] Thus, proteins mount heightened antibody response on booster injection and, unlike TI-2 response, promote antibody class switching from IgM to IgG class, due to active participation of T-helper cells. Moreover, in a TD response the B-cells distinguish into a subset of B-memory cells, imparting an immunological memory of the specific antigen involved.[1]

Antigens, which are small molecules, often fail to provoke an active immune response. These very small antigens, known as haptens, when chemically conjugated to larger carrier molecules become immunogenic.[15, 16] Large antigens or hapten-carrier conjugates capable of eliciting an active immune response are referred to as immunogens. Low molecular PSs and OSs behave as haptens. These carbohydrate haptens are rendered immunogenic by covalently linking them to carrier proteins resulting in the preparation of glycoconjugates, thereby, converting TI carbohydrate antigens into TD antigens for use in vaccines. This observation was first made by Avery and Goebel, who demonstrated that the type 3 pneumococcal PS may be rendered immunogenic by its coupling to horse serum globulin.[17] The CPSs of *Haemophilus influenza* type b (Hib), TI-2 immunogens, become TD immunogens when conjugated to proteins.[18-20]

Generally, infants possess immature B-cells and express only surface bound IgM, at a time when the diminishing maternal antibodies render them most susceptible to infection. Pure PSs do not induce immune response for protective antibody production in children less than 2 years of age. The immune system in elderly and

immunocompromised people also responds poorly to PSs. Hence, it is desirable to convert TI-2 type polysaccharide antigens to TD antigens for use in vaccines in order to boost their immunity.

1.8 PSs and OSs as Vaccine Components

In the late 1980s, general use of first PS-protein conjugate vaccine against Hib in the developed countries led to an almost eradication of meningitis of neonates, infants and children and, eventually the whole population.[21] This success was reflected in the development of a meningococcal Group C conjugate vaccine[22], subsequently followed by, a fourvalent meningococcal conjugate vaccine, Menactra™, containing the CPSs of N. meningitides A, B, W135, and Y. The CDC recommended the combination vaccine for routine use in children of 11-12 years of age.[23] In 2000, Prevnar™, a heptavalent pneumococcal conjugate vaccine against serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F, were developed and it is included in routine pediatric practice in the United States.[24, 25] The further developments of 9-valent[26] and 11-valent[27] pneumococcal vaccines are underway. The possibility of using protein conjugates of relatively small OSs as vaccines was raised after overwhelming success of native[20] and degraded[28] PS-based conjugate vaccines against Hib. Early observations made by Goebel that a protein-conjugate of the cellobiuronic acid disaccharide, a repeating unit of the CPS of Streptococcus pneumonia type 3, was capable of eliciting CPS-specific antisera in rabbits.[2, 29]

Advances made in the last two decades in chemical synthesis of OSs, including new protecting group strategies and glycosylation methodologies[30] make the assembly

of unprotected OSs up to the 25-mer range[31, 32] possible. Additionally, the arrival of efficient methods for conjugation of OSs to proteins and peptides adds to the development of novel, chemically synthesized glycoconjugate vaccines.[33, 34] The chemically synthesized constructs presents numerous advantages over those produced microbiologically, including product homogeneity, well-characterized and reproducible conjugation, absence of microbial contamination, and possible analysis. Chemical synthesis facilitates mapping of the structural parameters that affect carbohydrate-specific immunogenicity, such as the length and the chemical composition of OSs, e.g., presence or absence of non-carbohydrate appendages.[2] The choice of OS-based vaccines instead of whole PSs offers the construction of safe vaccines that contains "only the necessary antigenic subunits", and thereby, eliminates "undesirable side effects."[35] The earlier developments and recent trends in synthetic carbohydrate-based bacterial vaccines have been summarized in a few interesting reviews.[2, 36-39]

1.9 Synthetic Oligosaccharide-Based Vaccine in Clinical Practice

Haemophilus influenzae Type B (Hib)

The success of immunization with the purified CPS conjugate vaccines of Hib to eradicate childhood meningitis spurred an interest in exploring alternative approaches those utilize chemical synthesis to obtain well-characterized epitopes of the native polymer, which can be coupled to immunogenic protein carriers. Following the path paved by van Boom[40] and Just [41], Verez-Bencomo and Roy prepared protein conjugates of fully synthetic fragments of the CPS of Hib.[42-44] In their attempts, they prepared the poly(ribosyl-ribitol-phosphate) conjugates (Fig. 1.2), and demonstrated

that the antigenicity of this synthetic conjugate matched that of the native CPS linked to the same protein carrier. The spacer linked polymer containing 6-9 repeating units of the CPS of Hib was conjugated to the outer membrane protein complex (OMP) of *Neisseria meningitidis* and tetanus toxoid (TT).

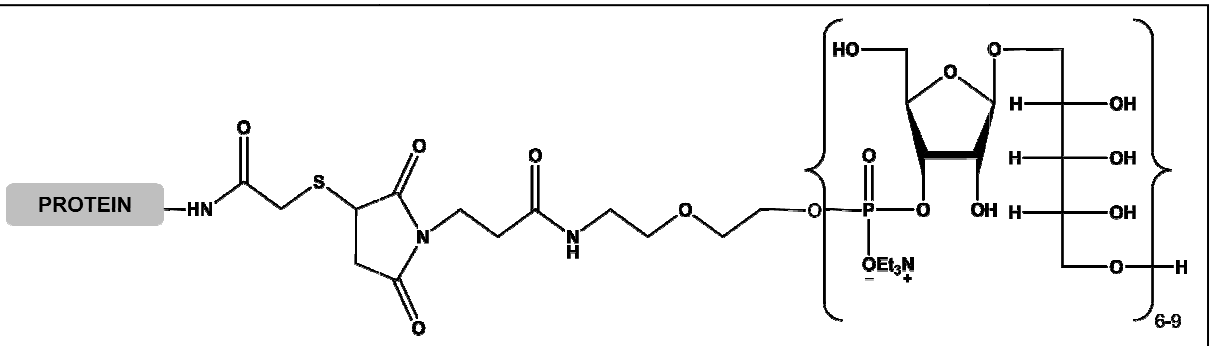

Figure 1.2. The synthetic Hib conjugate.

Several successful clinical trials with adults, four-year olds, and newborns, with a few rare cases of minor adverse effects, proved the safety and immunogenicity of the synthetic Hib-TT conjugate vaccine.[2] In 2003, the synthetic Hib-conjugate vaccine, Quimi-Hib™, was licensed and commercially available in Cuba, and is now in routine use for prophylactic immunization of infants and children[2, 45] More than 1.2 million doses were administered until early 2006[2, 45] and "a very reduced number of slight adverse effects" was observed [2, 46]

1.10 Anthrax (*Bacillus anthracis*)

Anthrax is one of the acute infectious diseases caused by bacterium *Bacillus anthracis. B. anthracis* was so called after the Greek word for black coal ('anthracis'), due to the black necrotic ulcers characteristic of cutaneous anthrax infection. *B. anthracis* has the ability to form spores that can live in the soil for many years. Anthrax is most commonly seen in wild and domestic lower vertebrates (cattle, sheep, goats, camels, antelopes, and other herbivores), but humans can also contract the disease upon exposure to infected animals or tissue from infected animals or by inhaling anthrax spores from contaminated animal products. Anthrax can also be spread by eating undercooked meat from infected animals. Anthrax is diagnosed by isolating *B. anthracis* from the blood, skin lesions, or respiratory secretions or by measuring specific antibodies in the blood of persons with suspected cases.[18]

To date there are three main forms of anthrax infections known namely cutaneous (skin), inhalation, and gastrointestinal. In case of cutaneous anthrax, the infections occur when the bacterium enters a cut or abrasion on the skin. Skin infection begins as a raised itchy bump that develops into a vesicle and then a painless ulcer with a black necrotic area in the center. The case fatality rate reported for the patients with cutaneous anthrax, not treated with antibiotics, is 20%. Initial symptoms of inhalation anthrax may resemble that of the common cold. After a few days, severe breathing problem and shock may arise. Inhalation anthrax is believed to be fatal, and even with all possible supportive care including antibiotics, the case fatality rate is very high, approximately 75%. The consumption of contaminated meat may be followed by the intestinal anthrax infection and is characterized by an acute inflammation of the

gastrointestinal tract. The case fatality rate for the gastrointestinal anthrax is estimated to be 25%-60%.[18]

B. anthracis, the etiological agent of anthrax, is a gram-positive, nonmotile, aerobic, facultative anaerobic, spore-forming, rod-shaped bacterium.[47] In 1877, Robert Koch grew the organism in pure culture, demonstrated its ability to form endospores, and produced experimental anthrax by injecting it into animals.[48]

B. anthracis, like other bacillus species, forms endospores (or spores) when vegetative cells are deprived of an essential nutrient. Dormant spores are highly resistant to adverse environmental and chemical conditions. These characteristics allow the spore to survive for a long period in the soil until it encounters a suitable environment to reestablish vegetative growth. Humans and animals can typically contract anthrax by contact with spores.

B. anthracis spores cannot be detected and identified easily as they do not possess a characteristic appearance (e.g., color), smell, or taste. And thus, the anthrax spores can be used as biological weapons. It has been an unfortunate reality in the United States in 2001 when anthrax was deliberately spread through the postal system by sending letters with powder containing *B. anthracis* spores. Because the possibility of a terrorist attack using bioweapons is especially difficult to predict, detect, or prevent, it is among the most feared terrorism scenarios.[47] The anthrax attacks of 2001 have increased concern about the feasibility of large-scale aerosol bioweapons attacks by terrorist groups.

In order to combat this threat, problems such as rapid diagnosis of anthrax infection, vaccination, therapy, postexposure prophylaxis, and decontamination of the environment need to be addressed.

1.11 Anthrax Toxins

In 1955, Smith and coworkers first demonstrated the presence of toxins in filter-sterilized serum of guinea pigs.[49] To date three major proteins acting in binary combinations,[50] now known as protective antigen (PA); lethal factor (LF), and edema factor (EF) mainly compose the anthrax toxins. PA has an ability to elicit a protective immune response against anthrax.[51] The toxicity studies of the binary combinations of these proteins demonstrated that intravenous injection of PA+LF (LeTx, lethal toxin) provokes death of experimental animals,[52] whereas edema in the skin was produced upon intradermal injection of PA+EF (EdTx, edema toxin).[50] None of these proteins is toxic when administered individually[47, 50, 52] and they represent a unique combination of the A-B toxins.[47, 53] PA acts as the common cell-binding domain (B) and cell damage occurs when it interacts with two different enzyme domains (A), EF and LF.

Protective Antigen

Protective antigen (PA) is the principal antigen responsible for the generation of toxin-neutralizing antibodies and, immunity to PA afforded primary protection against anthrax.[54, 55] The mature protein, PA_{83}, is 735 amino acids that is folded into four functional domains each of which is required for a particular step in the intoxication process.[47, 56] However, the antigenic epitopes on PA are yet to be fully defined and it is

evident from recent studies that PA has several neutralizing epitopes, in particular, on domail-4 and -1.[57, 58] Domain 1 (residues 1-249) contains the proteolytic activation site and cleavage of the PA monomer releases the subdomains 1a and 1b, which are the *N*-terminal fragment of 20 kDa (PA_{20}) and the 63 kDa (PA_{63}), respectively.[47, 56] The later can oligomerize to form ring-shaped heptamer.[59] A β-barrel core containing a large flexible loop that has been involved in pore formation represents domain 2 (residues 250-487).[56] At low pH, a rearrangement of the seven loops of the heptamer takes place, which results into conformational changes in the heptamer and converts from prepore to pore.[47] A chymotrypsin cleavage site is present in this loop (Phe^{313}-Phe^{314}), which is required for toxicity.[60] The heptamer forms cation-selective channels in artificial and cell membranes.[47, 61, 62] The smallest of the four domains containing the hydrophobic patch that is believed to be involved in protein-protein interactions is domain 3 (residues 488-594).[56] The relatively isolated domain that is required for binding to the cell receptor is domain 4 (residues 595-735). A small modification in this domain including short deletion of the *C*-terminal end or of part of the solvent-exposed loop (704-722), or amino acid substitutions, diminish or eliminate the PA-receptor interaction.[47] The receptor binding can also be blocked by monoclonal antibodies directed to this region.[63] PA has become an excellent target for the prevention and therapy of anthrax because of its pivotal role in the pathophysiology of anthrax. Additionally, it has been demonstrated recently that purified lethal factor (LF) complexed with PA_{63} heptamer exhibits similar biological and functional activities to that of the free LF, suggesting consideration of both the individual toxin components and the active complex as critical targets for anthrax therapeutic design.[64]

Lethal Factor

Lethal Factor is a mature protein containing 776 residues, in which the central part of the molecule is composed of a series of four imperfect repeats. The protein becomes unstable and inactive when this region is subjected to any deletions.[65] Leppla and coworkers demonstrated that the catalytic domain in LF resides in the C-terminal part and identified residues 686-690 as the zinc metalloprotease consensus sequence.[66] Mutations of these residues prevent interaction of Zn^{2+} and LF and destroy lethal toxin activity, indicating that LF is a zinc protease and the zinc metalloprotease consensus sequence is required for lethal toxin activity.[66] Recently, it has been reported that LF inactivate mitogen-activated protein kinases (MAPKKs), and thereby, disrupt the transcriptional machinery that modulates gene expression.[67, 68] This may have a role in anthrax pathogenesis, however, the cascade of events following the cleavage remains unclear.[47]

Edema Factor

Edema factor is a mature protein containing 767 residues and it was the first of the two anthrax toxins to be characterized. It resembles the eukaryotic protein calmodulin by acting as an adenylate cyclase that increases the concentration of cyclic AMP in eukaryotic cells.[69] Edema toxin has a 24-amino acid stretch containing the ATP-binding sequence present in the eukaryotic adenylate cyclase[47, 70]; and this sequence is involved in catalysis.[71, 72] An interaction between EF and lipid is pH independent.[73] Unlike LF, EF show tendency to associate to the vesicle membrane after translocation

to the cytosol, suggesting that, EF may resemble the membrane-associated eukaryotic adenylate cyclase.[74]

1.12 Mode of Action of Anthrax Toxins

A general model for anthrax toxin mechanism and toxin entry into host cells has evolved as a result of the combination of structural and cellular analysis (Fig. 1.3).

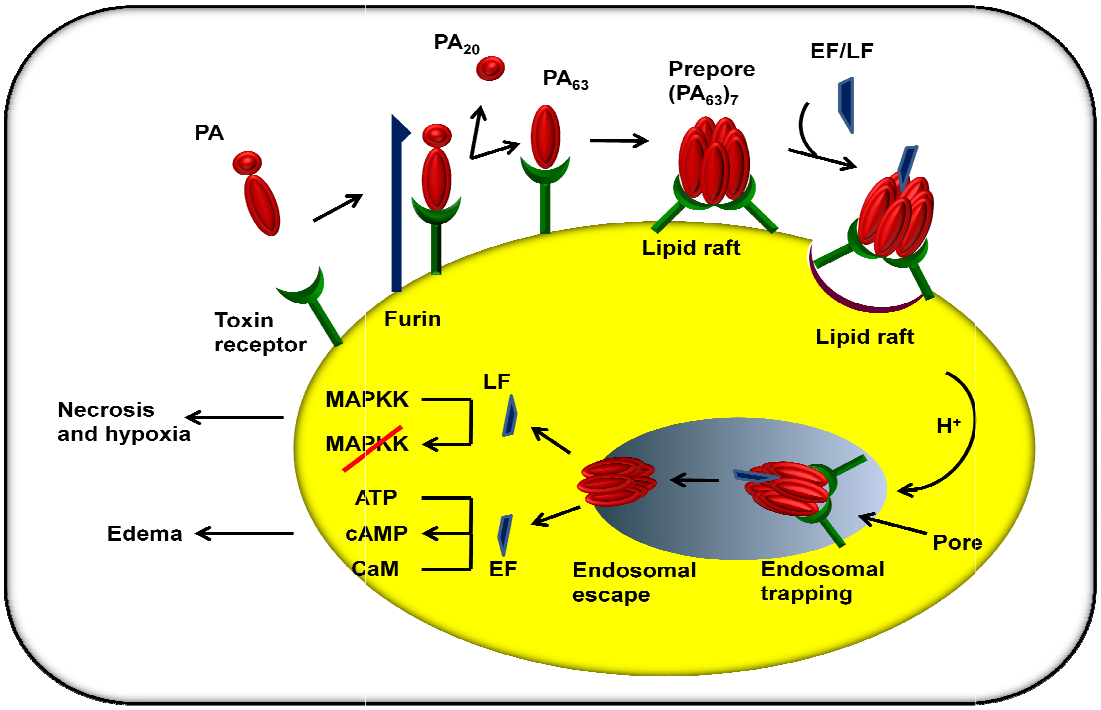

Figure 1.3. Model illustrating mode of action of anthrax toxin. CaM: Calmodulin; cAMP: Cyclic adenosine monophosphate; EF: Edema factor; LF: Lethal factor; PA: Protective antigen. Modified from references 76 and 82.

The exact sequence of events causing cellular death and death of the infected host remains unclear even after the partial elucidation of the molecular details of toxin entry.[75] An enzymatic activity of anthrax toxin is now linked to the loss of host

immunity.[76] *Bacillus anthracis* spores turn into vegetative anthrax bacilli after invading the host system and then vegetative bacilli utilize a three-component toxin system that induces cell death. PA, the main component, helps transport two exotoxins, LF and EF, into the host cells. This process begins with the highly specific binding of PA to the cell surface receptor, which is followed by an activation of PA upon cleavage by furin or a furin-like protease into two fragments.[53] After proteolytical cleavage, the small 20 kDa fragment (PA20) diffuses away and the remaining 63 kDa receptor-bound fragment (PA_{63}) oligomerize into ring-shaped self heptamer, known as prepore $(PA_{63})_7$[59, 77], exposing the binding sites for LF and EF. Oligomerization of PA_{63} triggers receptor-meidated endocytosis.[78] Initially, clustering of the toxin receptors takes place, which is followed by association of the complex with lipid rafts and exposure of binding domains to the LF or EF, and finally, internalization via the classic clathrin-dependent endocytic pathway.[79]

A conformational change in complex occurs at low pH in the endosomal compartment[80] and the prepore is converted into a pore. This transition occurs by insertion of a flexible loop of each PA molecule into the lipid bilayer membrane; followed by insertion of the pore into the membrane, and this pore serve as a transmembrane channel.[79] This channel facilitates the translocation of the enzymatic toxin proteins, LF/EF, across the cell membrane, releasing them into the host cell cytoplasm.[81-83] EF increases intracellular cAMP levels causing massive edema,[69] whereas LF cleaves MAPKKs and disrupts MAP kinase signaling pathway.[67, 68] The toxin seems to trigger an apoptotic pathway in infected cells, demonstrating additional downstream targets of the toxin, resulting in tissue hypoxia and cell lysis usually within 1-2 h.[79, 84] Both LeTx and

EdTx are expressed within 3 h of germination and corrupt a key antibacterial killing mechanism of macrophage [79] During progression of disease, overproduction of cytokines caused by the increasing level of lethal toxin results in shock-like death.[85]

1.13 Existing and Future Anthrax Prophylaxes – PA-Based Vaccines

In the famous field trial of Pouilly Le Fort in 1881, the attenuated *B. anthracis* strain was the first live whole-cell vaccine to be used successfully. Since then anthrax vaccines have progressed through pXO2-negative spores in the 1930s, to culture filtrates adsorbed to aluminium hydroxide in 1970 and probably to recombinant PA in near future. Stern-strain derivatives producing genetically detoxified LF and EF presents safer, nontoxic, live vaccine candidates.[86, 87] The live vaccine is still used for veterinary purposes.[88] Two first-generation acellular vaccine formulations prepared from filtered culture supernatants of an attenuated (nonencapsulated, toxin-producing) *B. anthracis* strain, by adsorbing the supernatant onto aluminum hydroxide and by precipitation of the supernatant with alum, have been licensed in USA (anthrax vaccine adsorbed [AVA], now called BioThrax®) and one in the UK, respectively.[75] Both anthrax vaccines have been reported to cause local adverse effects.[89, 90] The currently licensed AVA in the USA and the UK has been evaluated in human volunteers in order to determine antibody response.[91-93] The efficiency of immunization in humans could be evaluated by using surrogate markers provided by toxin neutralization.

A second-generation anthrax vaccine development effort is mainly directed to subunit vaccines that contain nontoxic, purified mutants of PA.[58, 94, 95] Unlike their predecessors, these vaccines' composition is fully defined, they are free from any

adverse effects and are producible on large scale and they can be stored at room temperature. Additionally, numerous trials conducted show promising efficacy of the second-generation vaccines.[58, 94-100]

Numerous attempts have been made to generate multigram quantities of highly pure rPA protein from a variety of recombinant organisms[96, 101, 102] in order to make sufficient quantities of PA available for mass protection of the human population. Currently, at least two rPA-based vaccines are being developed and US NIH has sponsored human safety and immunogenicity trials. A vaccine based on Stern strain is produced by VaxGen Inc. in the USA, whereas the other vaccine is produced from E. coli and is manufactured by Avecia in the UK. A recombinant multicopy plasmid-based expression system that expresses higher levels of PA is developed by VaxGen.[103] The Avecia vaccine comprises a nucleotide-codon-optimized version of the PA gene expressed from *E. coli*.[100] Recently, VaxGen disclosed encouraging preliminary results of immunogenicity and tolerance from its clinical trials of its new vaccine, rPA102.[104] However, the US federal health officials cancelled the contract with VaxGen on December 20, 2006.

1.14 Glanders (*Burkholderia mallei)*

Burkholderia mallei is a non-motile, encapsulated, Gram-negative, rod-shaped bacterium that causes a severe systemic disease known as glanders. Glanders is primarily a disease affecting solipeds (horses, mules, donkeys), however, humans and other animals can also contract this disease. The disease has not been seen in the United States since the 1940s. However, the organism has been considered as a

potential biowarfare agent and is classified as category B bioterrorism agent by the CDC because of their potential to cause illnesses with significant morbidity and mortality. It is commonly seen among domestic animals in Africa, Asia, the Middle East, and Central and South America. The organism is associated with infections in laboratory workers as very few organisms are required to spread disease. Humans contract glanders if they come in contact with infected animals. The cases of human-to-human transmission have also been reported.[105] The transmission of organisms to humans occurs through abraded skin, mucous membranes or inhalation into the lungs. Route of infection determines the symptoms of glanders. In humans, disease can occur in four basic forms: acute localized infection, septicemic illness, acute pulmonary infection, or chronic cutaneous infections.[105] The common symptoms of glanders include, fever, muscle aches, chest pain, muscle tightness, and headache. The disease is diagnosed by isolating *B. mallei* from blood, sputum, urine or skin lesions. Currently, there is no vaccine available for glanders. As the disease is uncommon in humans, information regarding antibiotic treatment of the organism is limited.

1.15 Melioidosis *(Burkholderia pseudomallei)*

Melioidosis, also known as Whitmore's disease, is caused by bacterium *Burkholderia pseudomallei*. Clinically and pathologically, the organism is closely related to *B. mallei*. The disease is endemic to Southeast Asia. However, the organism has been considered as a potential biowarfare agent and is classified as category B bioterrorism agent by the CDC. The organisms are found in contaminated water and soil and, transmission occurs by direct contact with the contaminate source. Human-to-

human transmissions have been reported.[106] Melioidosis can occur in four basic forms: acute localized infection, pulmonary infection, bloodstream infection or chronic infection.[106] Melioidosis is diagnosed by isolating *B. pseudomallei* from the blood, urine, sputum or skin lesions. Currently, there is no vaccine for the disease. Most cases of the disease can be treated with appropriate antibiotics.

1.16 Research Objectives

Recently, structure of cell-wall oligosaccharide (Fig. 1.4, [A], tetrasaccharide) of *B. anthracis* has been elucidated.[107] Although a vaccine and diagnostic tools for *B. anthracis* are available, there is still a need for improved alternative vaccines that target pre-germination spores and replicating cells, while not focusing exclusively on the protective antigen (PA) component of the anthrax toxin complex. There is a need for rapid diagnostic tests, which are specific and sensitive enough to detect *B. anthracis* at the earliest possible stage of infection. The long term objective of the research presented in this dissertation is to utilize the structure of oligosaccharide derived from the *B. anthracis* exosporium to prepare and test the chemically synthesized improved carbohydrate-protein conjugate vaccines as well as to develop efficient and sensitive immunological detection methods. The short term goals were to chemically construct the smallest possible fragments, trisaccharides and analogs (Fig. 1.4, **1-4**), of the native tetrasaccharide; evaluate their antigenicity and potential to be used as diagnostic markers/vaccine components; and identify the minimal structural components required for immunological responses.

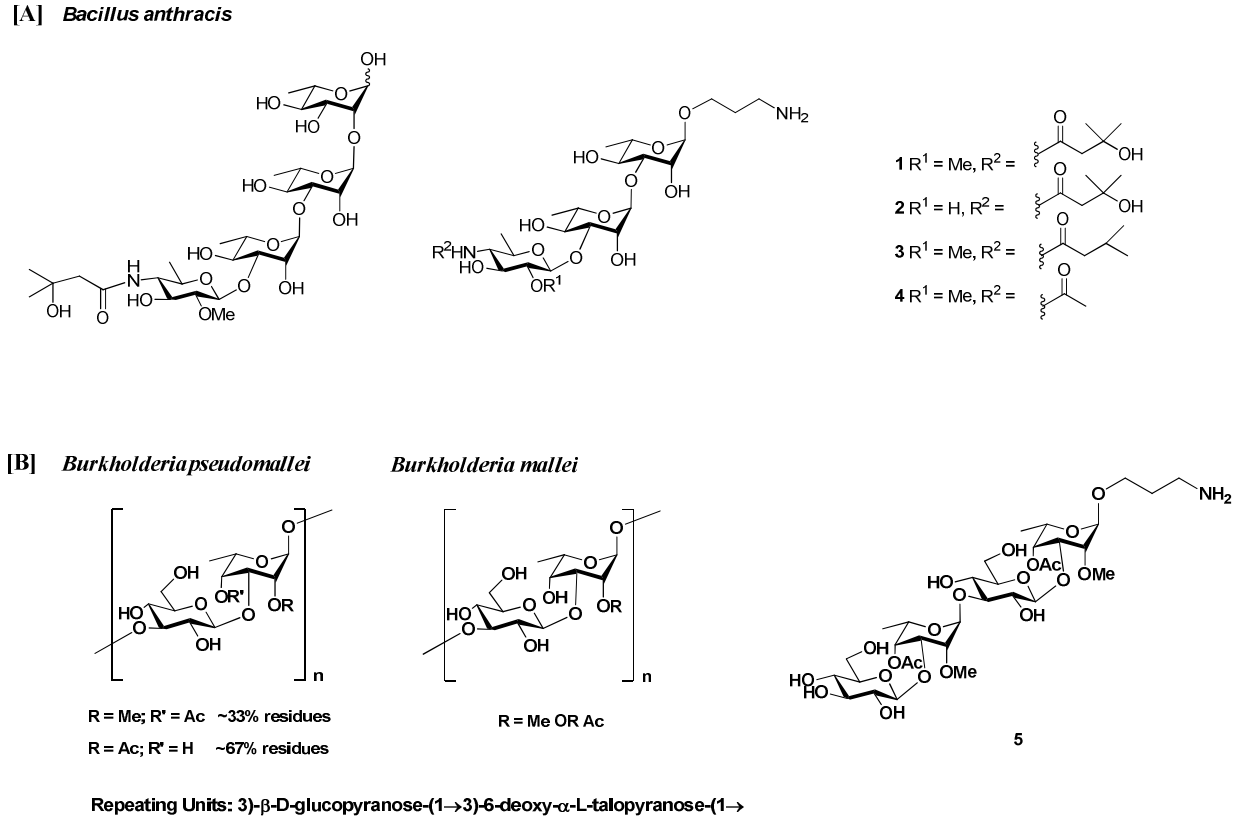

Figure 1.4. Oligosaccharides of the Glycoprotein BclA [A] and Structures of *B. pseudomallei* and *B. mallei* O-PS [B].

Recently, complex carbohydrate fragments derived from the lipopolysaccharide *O*-antigens, O-PS and O-PS II for *B. mallei* and *B. pseudomallei*, respectively, have been identified (Fig. 1.4, [B]). The O-PS II moiety produced by *B. pseudomallei* is an unbranched heteropolymer consisting of disaccharide repeating units having the structure *3)-β-D-glucopyranose-(1→3)-6-deoxy-α-L-talopyranose-(1→* in which ~33% of the 6-deoxy-*α*-L-talopyranose (6dTal*p*) residues have 2-*O*-methyl and 4-*O*-acetyl substitution while ~67% of residues possess only 2-*O*-acetyl substitution.[108-110] The O-antigen (O-PS) expressed by *B. mallei* is virtually identical to O-PS II except that it lacks

acetyl substitutions at the C-4 OH of the 6dTal*p* residues.[108] To evaluate the antigenicity of these carbohydrates and their possible uses as diagnostic markers and/or vaccine components, the attention will be paid to develop highly convergent syntheses of spacer-linked oligosaccharide fragments derived from the *O*-side chain of *B. pseudomallei* (Fig. 1.4, **5**). The long term goal is to conjugate tetrasaccharide **5** to carrier proteins and investigate its potential as vaccine component/diagnostic marker for *B. pseudomallei*.

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Chapter II

Synthesis and Antigenic Analysis of the BclA Glycoprotein Oligosaccharide from the *Bacillus anthracis* Exosporium*

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2.1 Abstract

The glycoprotein BclA is an important constituent of the exosporium of *Bacillus anthracis* spores. This glycoprotein is substituted with an oligosaccharide composed of a β-L-rhamnoside substituted with the previously unknown terminal saccharide, 2-*O*-methyl-4-(3-hydroxy-3-methylbutanamido)-4,6-dideoxy-D-glucopyranose, also referred to as anthrose. Anthrose has not been found in spores of *B. Cereus* and *B. thuringiensis*, making it a potential species-specific marker for *B. anthracis*. In order to study the antigenicity of anthrose, efficient syntheses of an anthrose-containing trisaccharide and a series of structurally related analogues were developed. The analogues lacked either the methyl ether at C-2 or contaminated modified C-4 amino functionalities of anthrose. The synthetic compounds were equipped with an aminopropyl spacer to facilitate conjugation to the carrier proteins mariculture Keyhole Limpet Hemocyanin (mcKLH) and bovine serum albumin (BSA). Serum antibodies of rabbits immunized with live or irradiated spores of *B. anthracis* Sterne 34F_2 were able to recognize the synthetic trisaccharide-mcKLH conjugate. The specificity of the interaction was confirmed by competitive inhibition with the free and BSA-conjugated trisaccharides. Inhibition using the trisaccharide analogues demonstrated that the isovaleric acid moiety of anthrose is an important structural motif for antibody recognition. These data demonstrate that 1) anthrose is a specific antigenic determinant of the *B. antrhacis* Sterne spore; 2) this antigen is presented to the immune system of rabbits receiving the anthrax live-spore vaccine; 3) synthetic analogues of the oligosaccharide retain the antigenic structure; and 4) the antigenic region is localized to specific terminal groups of the oligosaccharide. Collectively these data provide an

important proof-of-concept step in the synthesis and development of spore-specific reagents for detection and targeting of non-protein structures in *B. anthracis.*

Keywords: *anthrax. glycoconjugates.oligosaccharides. vaccines*

2.2 Introduction

Bacillus anthracis is a gram-positive, spore-forming bacterium that causes anthrax in humans and other mammals.[1, 2] Because of the high resilience of *Bacillus anthracis* spores to extremes of their environment they can persist for many years until encountering a signal to germinate.[3, 4] When spores of *B. anthracis* are inhaled or ingested they may germinate and establish populations of vegetative cells which release anthrax toxins, often resulting in the death of the host.[5] The relative ease by which *B. anthracis* may be weaponized and the difficulty in early recognition of inhalation anthrax due to the non-specific nature of its symptoms were demonstrated by the deaths of five people who inhaled spores from contaminated mail.[6-8] Consequently, considerable efforts are being directed towards the development of early disease diagnostics and there is a renewed interest in anthrax vaccines. Sterile, cell-free vaccines containing the protective antigen (PA) component of anthrax toxin have proven safe and effective.[9, 10] The anthrax vaccine that provides the most comprehensive protection is, however, the *B. anthracis* Sterne $34F_2$ live-spore vaccine.[11, 12] Although not licensed for human use in the US or EU, the live-spore vaccine has proven highly efficacious as a veterinary vaccine and similar live-spore preparations have been used extensively in humans and animals in eastern Europe and Asia.[13] Although these live-spore vaccines may elicit lower antitoxin antibodies than the licensed cell-free anthrax

vaccines, their documented efficacy is attributed to additional adjuvant properties and as yet undefined protective epitopes contributed by the spores or outgrowing vegetative cells.[14] It is feasible, but as yet unexplored, that specific carbohydrate antigens may contribute to the enhanced efficacy of the live spore vaccines.

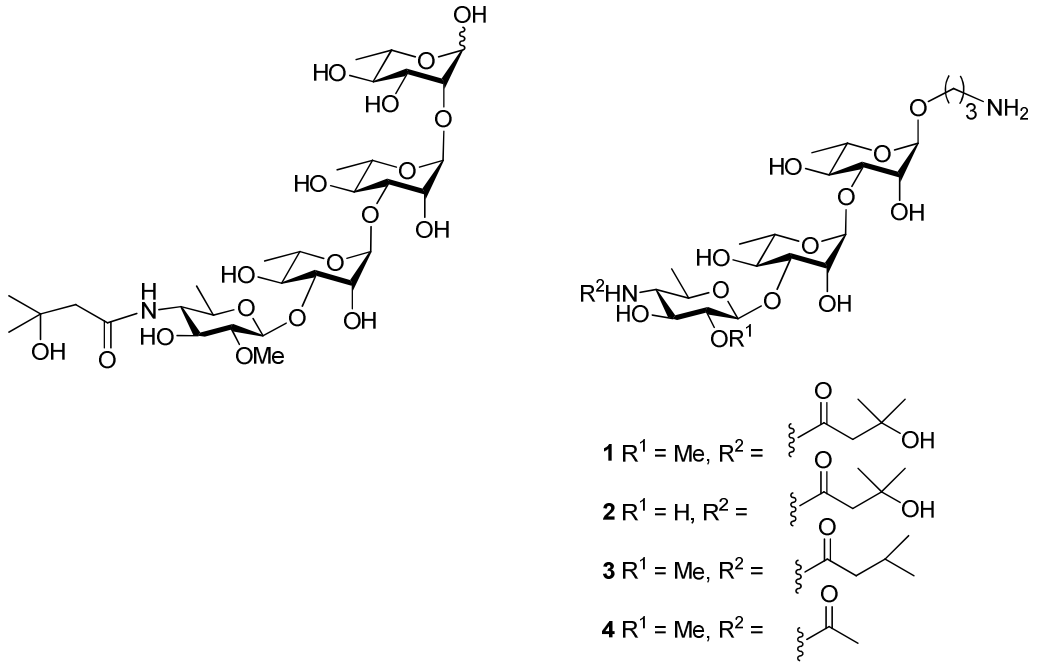


Figure 2.1. Oligosaccharide of the glycoprotein BclA and synthetic targets.

Spores of *B. anthracis* are enclosed by a prominent loose fitting layer called the exosporium, which consists of a paracrystalline basal layer composed of a number of different proteins and an external hair-like nap.[15-19] The filaments of the nap are formed by the highly immunogenic glycoprotein BclA, which has a long, central collagen-like region containing multiple X-X-Gly repeats where X can be any amino acid.[20] Almost all of the repeating units contain a threonine (Thr) residue, which provides sites for potential glycosylation.[21, 22] Recently, it was shown that the BclA glycoprotein contains

an *O*-linked saccharide, the structure of which was determined by a combination of NMR spectroscopy and mass spectrometry.[23] The oligosaccharide is probably attached to the protein through a GalNAc moiety, which was lost during the hydrazine-mediated release from the BclA glycoprotein.[23] The structure of the tetrasaccharide is depicted in Fig. 2.1.

The previously unknown non-reducing terminal saccharide, 2-O-methyl-4-(3-hydroxy-3-methylbutanmido)-4, 6-dideoxy-d-glucopyranose, was named anthrose and has not been found in spores of *B. cereus* and *B. thuringiensis*, making it a potential species-specific marker for *B. anthracis*. It may also be a new target for therapeutic intervention or vaccine development.[23]

In this dissertation, we report the synthesis of an anthrose-containing trisaccharide and a series of structurally related analogs. We demonstrate that 1) serum of rabbits immunized by live or irradiated spores of B. *anthracis* Sterne $34F_2$ recognize the trisaccharide **1,** which is derived from the glycoprotein BclA; 2) the antigenic nature of the trisaccharide can be altered by modification of specific side groups in the terminal glycosyl structure; and 3) a 3-methyl butyryl substituent is essential for recognition by anti-spore antiserum.

2.3 Results and Discussion

Synthesis

To study the immunological properties of the oligosaccharide of BclA, we examined whether antisera from rabbits immunized with live or irradiated spores of *B. anthracis* Sterne $34F_2$ were able to recognize the synthetic anthrose-containing BclA

oligosaccharide[24-27] and selected analogues. Although challenging, chemical synthesis offers an opportunity to obtain almost every oligosaccharide target in sufficient quantity and purity for these biological studies. Furthermore, chemical synthesis has the advantage that a target compound can be equipped with an artificial spacer for convenient conjugation to a carrier protein, and offers opportunities for obtaining analogues for structure-activity relationship studies.

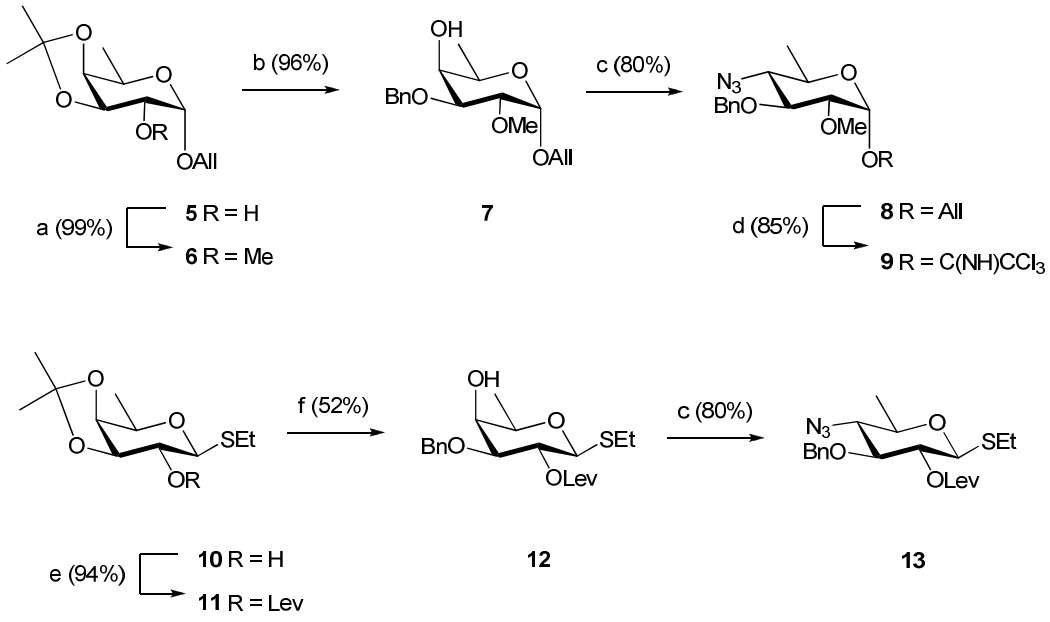
Compounds **1**-**4** (Fig. 2.1) were selected as targets for chemical synthesis. Compound **1** is derived from the oligosaccharide of BclA and contains an intact anthrose moiety. Compound **2** lacks the methyl ether at C-2 and derivatives **3** and **4** contain modified C-4 amino functionalities of anthrose. We anticipated that compound **1** conjugated to bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH) would be attractive material for determining whether live or irradiated spores of *B. anthracis* Sterne $34F_2$ can induce an anti-carbohydrate antibody response, and derivatives **2**-**4** valuable to examine which chemical moieties of anthrose are critical for binding with antibodies.

Compounds **1**-**4** were synthesized from monosaccharide precursors **14**, **15** and **9** or **13** (Schemes 2.1 and 2.2). Thus, glycosyl donor **14**[28] can be coupled with a benzyloxycarbonyl protected amino propyl spacer to give compound **16**, which immediately can be used in a subsequent glycosylation with rhamnoside **15** to give disaccharide **17**. After removal of the levulinoyl (Lev) ester of **17**, the resulting glycosyl acceptor can be coupled with an appropriately protected anthrose donor. The benzoyl ester at C-2 of **15** will ensure that only α-glycosides will be obtained during glycosylation due to neighboring group participation.

The anthrose moieties of target compounds **1**-**4** are linked through a β-glycoside to the C-3 hydroxyl of the rhamnoside. Thus, an obvious strategy to introduce this moiety would be the use of a glycosyl donor which carries a selectively removable ester at C-2. At a late stage of the synthesis, this protecting group can be removed to reveal an alcohol, which can then be methylated. However, this strategy is complicated by the fact that the methylation has to be performed under neutral or mildly acidic conditions due to the presence of a number of base sensitive ester protecting groups. In general, such procedures provide relatively low yields of product, especially when applied to a complex compound. Alternatively, the methyl ether can be introduced at the monosaccharide stage by using strongly basic conditions; however, this approach may suffer from the formation of anomeric mixtures during the introduction of the anthrose glycoside. In order to examine both strategies, glycosyl donors **9** and **13** were prepared and coupled with glycosyl acceptor **18**. Compounds **9** and **13** contain an azido moiety at C-4, which at a late stage of the synthesis can be reduced to an amine and then acylated with different reagents to provide compounds **1**-**4**.

Glycosyl donor **9** was synthesized from selectively protected allyl 6-deoxygalactoside **5** (Scheme 2.1).[29] Thus, methylation of the C-2 hydroxyl of **5** could easily be accomplished by treatment of **5** with methyl iodide in the presence of sodium hydride to give compound **6** in a yield of 99%. The 3,4-*O*-isopropylidene acetal of **6** could easily be removed by using aqueous acetic acid to give a diol, which was selectively benzylated at C-3 to give compound **7**, by first stannylene acetal formation by reaction with dibutyltin oxide in refluxing methanol followed by treatment with benzyl bromide and CsF in DMF.[30, 31] Next, an azido group was introduced at C-4 with

inversion of configuration to give compound **8** by conversion of the hydroxyl of **7** into a triflate by reaction with triflic anhydride and pyridine followed by displacement with sodium azide in DMF.[32] Fully protected **8** was converted into trichloroacetimidate **9** by removal of the anomeric allyl ether by treatment with $PdCl_2$ and NaOAc followed by reaction of the resulting lactol with trichloroacetonitrile in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU).[33, 34]



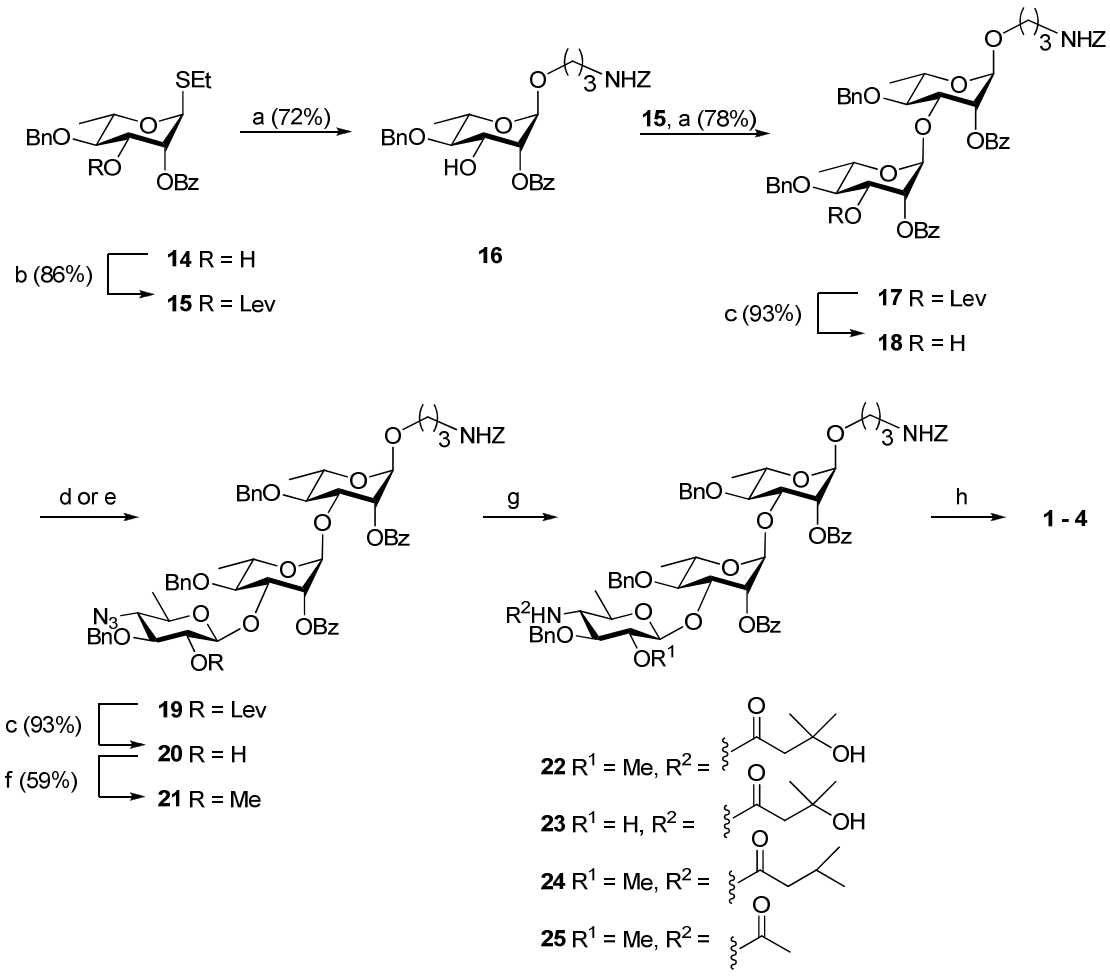
Scheme 2.1. Reagents and conditions: a) MeI, NaH, DMF, rt; b) i) 60% HOAc in H_2O, 90 °C, ii) Bu_2SnO, MeOH, reflux, iii) CsF, BnBr, DMF, rt; c) i) Tf_2O, py, CH_2Cl_2, 0 °C, ii) NaN_3, DMF, 40 °C; d) i) $PdCl_2$, NaOAc, 90% HOAc/H_2O, rt, ii) CCl_3CN, DBU, CH_2Cl_2, rt; e) levulinic acid, DCC, DMAP, CH_2Cl_2, rt; f) i) 60% aq HOAc, 90 °C, ii) Bu_2SnO, toluene, reflux, iii) Bu_4NBr, BnBr, toluene, reflux.

Glycosyl donor **13** was synthesized from known thioglycoside **10**.[35] Thus, a levulinoyl (Lev) ester at C-2 of compound **10** was installed by treatment with levulinic acid, 1,3-dicyclohexylcarbodiimide (DCC), and 4-(dimethylamino)pyridine (DMAP) in CH_2Cl_2 to give compound **11** in excellent yield.[36] Next, the isopropylidene acetal of **11**

was removed by treatment with aqueous acetic acid to give the corresponding diol. Attempts to selectively benzylate the C-3 hydroxyl of this compound by intermediate stannylene acetal formation, using conditions described for the preparation of **7**, gave **12** in a low yield due to cleavage of the Lev ester. However, a moderate yield of **12** was obtained when the stannene acetal formation was performed by refluxing the diol and dibutyltin oxide in toluene followed by treatment with benzyl bromide and tetrabutylammonium bromide (Bu_4NBr). Finally, triflation of **12** followed by nucleophilic displacement with sodium azide gave the required thioglycosyl donor **13**.

Next, attention was focused on the preparation of rhamnosyl acceptor **18** and installment of the anthrose moiety. Thus, an *N*-iodosuccinimide/trifluoromethanesulfonic acid (NIS/TfOH) mediated glycosylation[37] of thioglycosyl donor **14** with benzyloxycarbonyl protected aminopropanol gave spacer modified **16** as only the α-anomer. No self-condensation of **14** was observed due to a much higher glycosyl acceptor reactivity of *N*-benzyloxycarbonylaminopropanol. Compound **16** was immediately used in a second glycosylation with glycosyl donor **15**, using NIS/TfOH as the activator to give disaccharide **17** in a good yield. Next, the levulinoyl ester of **17** was selectively removed by treatment with hydrazine acetate,[36] to afford glycosyl acceptor **18** in a yield of 93%. Coupling of trichloroacetimidate **9** with **18** in the presence of BF_3-Et_2O in acetonitrile at -40 °C gave trisaccharide **21** in a good yield (86%) as a 1:4 mixture of α/β-anomers. In this case, the modest β-selectivity was achieved by the formation of an intermediate α-nitrilium ion.[38, 39]

Anomerically pure **22** was obtained after reduction of the azido group of **21** to give an amine, which was acylated with 3-hydroxy-3-methyl-butyric acid using *O*-(7-azabenzotriazol-1-yl-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate/1-hydroxy-7-Azabenzotriazole/diisopropylethylamine (HATU/HOAt/DIPEA) as the activating reagent.



Scheme 2.2. Reagents and conditions: a) $HO(CH_2)_3NHZ$, NIS, TfOH, CH_2Cl_2, 0 °C; b) levulinic acid, DCC, DMAP, CH_2Cl_2, rt; c) NH_2NH_2, HOAc, MeOH, CH_2Cl_2, rt; d) **13**, NIS, TfOH, CH_2Cl_2, 0 °C, 76%; e) **9**, BF_3•Et_2O, MeCN, -40 °C, 86%, α/β 1:4; f) MeI, Ag_2O, Me_2S, THF, rt; g) i) 1,3-propanedithiol, TEA, pyridine, H_2O, ii) for **22**, **23**, **24**, HOAt, HATU, DIPEA, rt, 61-78%, for **25**, Ac_2O, pyridine, rt, **22:** 63%, **23:** 78%, **24:** 61%, **25:** 66%; h) i) NaOMe, MeOH, rt, ii) Pd/C, H_2 (g), *t*BuOH/H_2O/AcOH 40:1:1, rt, **1:** 98%, **2:** 96%, **3:** 94%, **4:** 92%.

As expected, an NIS/TfOH mediated coupling of thioglycosyl donor **13** with acceptor **18** gave trisaccharide **19** as only the β-anomer due to the neighboring group participating Lev ester at C-2. The Lev group of **19** was selectively removed by treatment with hydrazine acetate[36] and the hydroxyl of the resulting trisaccharide **20** was methylated by treatment with methyl iodide and freshly prepared Ag_2O in the presence of dimethyl sulfide. Despite a prolonged reaction time, the product was obtained in a modest yield of 51%. Thus, the advantage of using glycosyl donor **13** in trisaccharide formation was off-set by a low yielding methylation reaction.

Reduction of the C-4'' azido moiety of **21** followed by the coupling with 3-hydroxy-3-methyl-butyric acid gave compound **22**. Deprotection of **22** could easily be accomplished by a two-step procedure entailing removal of the benzoyl esters using sodium methoxide in methanol, followed by cleavage of the benzyl ethers and benzyloxycarbamate by hydrogenation over Pd/C in a mixture of *tert*-butanol/water/acetic acid.

Analogue **2**, lacking a methyl ether at C-2 of anthrose, was prepared by reduction of the azido group of **20** followed by introduction of the 3-hydroxy-3-methyl-butyric acid moiety and deprotection using standard procedures. Compounds **3** and **4** were obtained by reduction of the azido moiety of **21** followed by acylation of the resulting amine using appropriate reagents to give compounds **24** and **25**, which were deprotected using standard procedures.

Preparation of Carbohydrate-Protein Conjugates

Trisaccharide **1** was linked to the carrier protein mariculture Keyhole Limpet Hemocyanin (mcKLH; Pierce Biotechnology, Rockford, IL) for immunological evaluation.

To this end, the amino functionality of trisaccharide **1** was derivatized with an acetyl thioacetic acid moiety by reaction with *S*-acetylthioglycolic acid pentafluorophenyl ester to afford the corresponding thioacetate derivative, which after purification by size-exclusion chromatography, was directly de-*S*-acetylated using 7% ammonia (g) in DMF just prior to conjugation. The de-*S*-acylation was performed under a strict argon atmosphere to prevent formation of the corresponding disulfide. KLH was activated with succinimidyl 3-(bromoacetamido) propionate (SBAP) in a sodium phosphate buffer (pH 7.2) containing 0.15 M sodium chloride and then purified by a centrifugal filter device with a nominal molecular-weight limit of 30 kDa. The bromoacetyl activated KLH (KLH-BrAc) was subsequently incubated with the thiolated trisaccharide in a 0.1 mM sodium phosphate buffer (pH 8.0) containing 5 mM ethylene diamine tetraacetate (EDTA). The afforded glycoconjugate (KLH-BrAc-**1**) carried 1042 copies of trisaccharide **1** per KLH molecule as determined by Lowry's protein concentration test and quantitative carbohydrate analysis by HPAEC-PAD. For the purpose of evaluating the binding specificity of antibodies raised against the *B. anthracis* spores, the thiol derivative of trisaccharide **1** was conjugated to maleimide activated BSA (BSA-MI, Pierce Endogen, Inc.) in a phosphate buffer (pH 7.2) containing sodium azide and EDTA. After a reaction time of 2 h, the glycoprotein was purified using a centrifugal filter device with a nominal molecular weight cut-off of 10 kDa. The average number of trisaccharide copies per BSA molecule was determined to be 18:1. The same conjugation method and thiolated derivatives of trisaccharides **2**, **3**, and **4** were used to give the corresponding BSA-MI-**2**, BSA-MI-**3**, and BSA-MI-**4** glycoconjugates with a sacchride/protein ratio of 10:1, 9:1, and 4:1, respectively.

Antibody Binding Analyses

To explore the immunogenicity of the saccharide moieties of BclA, rabbits were immunized four times at biweekly intervals with live or irradiated spores of *B. anthracis* Sterne 34F_2. First, it was investigated whether the post-immune sera have the ability to recognize the synthetic anthrose-containing trisaccharide **1**. For this purpose, an ELISA was performed whereby microtiter plates were coated with the KLH-BrAc-**1** conjugate and serial dilutions of sera added. An anti-rabbit IgG antibody labeled with horseradish peroxidase was employed as a secondary antibody for colorimetric detection (OD, optical density). Binding was observed between the antisera and KLH-trisaccharide conjugate whereas no interaction was detected for native KLH, indicating that the saccharide epitopes of BclA are antigenic (Figure 2.2a). Rabbits immunized with irradiated Sterne34F_2 spores elicited lower but detectable titers of anti-saccharide antibodies. The fact that the irradiated spores elicited IgG antibodies indicates that the saccharide epitopes were not damaged during the irradiation process.

Next, the specificity of the interaction of the antisera with the KLH-BrAc-**1** conjugate was further investigated using a competitive inhibition ELISA. Thus, microtiter plates were coated with the KLH-BrAc-**1** conjugate, and serial dilutions of antisera mixed with free trisaccharide **1** were added. As depicted in Figure 2.2b, a six-fold excess of trisaccharide **1** (as compared to a concentration of trisaccharide used for coating microtiter wells), resulted in a significant drop in OD at all serum dilutions tested. Also, increasing the excess of the competing trisaccharide **1** resulted in a further reduction in OD. It is evident that the inhibition is dose dependent, thus demonstrating that the interaction of the elicited antibodies with **1** is specific. The interaction of antisera

from rabbits immunized with irradiated spores with **1** could also be inhibited in a dose response manner (Figure 2c).

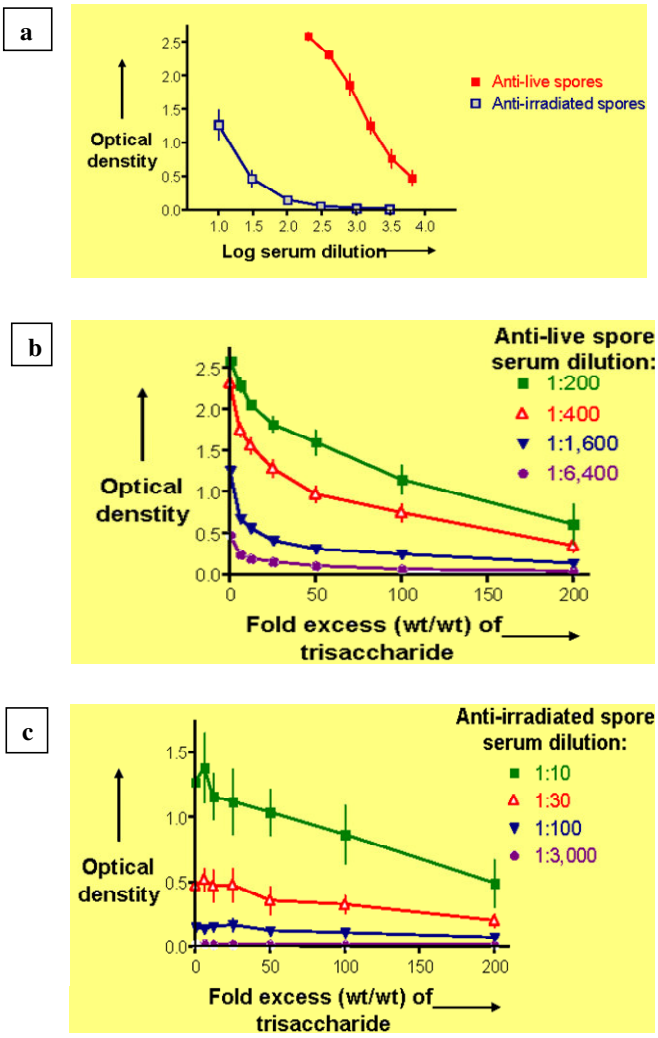


Figure 2.2. ELISA and competitive inhibition of anti-live and anti-irradiated spore antiserum. Microtiter plates were coated with KLH-BrAc-**2** conjugate (0.5 µg per mL conjugate, corresponding to 0.03 µg per mL trisaccharide). Rabbit anti-live (1:200→1:6400 diluted) or anti-irradiated (1:10→1:3000 diluted) spore *B. anthracis* Sterne $34F_2$ antisera were applied to coated microtiter plates (**a**). For the inhibition assay the serum was first mixed with free trisaccharide **2** (0–200-fold excess, wt/wt) (**b** and c). Unspecific binding was tested with uncoated wells with 200-fold "excess" trisaccharide or 200-fold "excess" KLH (data not shown). The data are reported as the means ±SD of triplicate measurements. Modified from reference 42.

Having established that Sterne $34F_2$ spores are able to induce an anti-carbohydrate antibody response, we sought to further evaluate which structural motifs of the anthrose moiety are critical for antibody recognition. To this end, the ability of BSA-MI-**1** and BSA-MI-conjugates of the three structural analogues **2**, **3**, and **4** to inhibit the interaction of the antisera with KLH-BrAc-**1** was determined (Figure 3). For these experiments, BSA conjugates were employed in an effort to conserve synthetic material. Microtiter plates were again coated with the KLH-BrAc-**1** conjugate and treated with an antisera dilution of 1:1600. The importance of the 2''-*O*-methyl ether of anthrose was established using the BSA-MI-**2** conjugate. This conjugate carries trisaccharide analogue **2**, which lacks the 2''-*O*-methyl ether but has an intact *N*-(3-hydroxy-3-methyl-butyryl) moiety at C-4''. As shown in Figure 2.3, this conjugate is a potent inhibitor of antibody binding with as low as a 2-fold weight excess eliciting >95% reduction in reporter signal, compared to the BSA-MI-conjugate carrying the native trisaccharide **1**, for which no significant difference in inhibition was observed in the concentration range investigated. These data indicate that the methyl ether is not critical for anti-spore antibody binding. To elucidate the importance of the 3-hydroxy-3-methyl-butyryl moiety of anthrose, conjugates BSA-MI-**3** and BSA-MI-**4** were prepared. Trisaccharide **3** carries a 3-methyl-butyryl moiety at the C-4'', thus only lacking the hydroxyl group of the native C-4-moiety of the anthrose monosaccharide, whereas trisaccharide **4** is *N*-acetylated at the C-4'', thus lacking most of the 3-hydroxy-3-methyl-butyryl moiety. Interestingly, a two-fold excess of trisaccharide **3** reduced OD by 85% compared to the control. In contrast, a similar concentration of analogue **4** resulted in reduction in OD of only 17%. Very high concentrations of BSA-MI-**4** were required to achieve considerable

inhibition (a 500-fold excess of BSA-MI-**4** resulted in a 50% drop in OD, data not shown). These results indicate that the 4''-(3-methylbutyryl)-moiety is an important structural motif of the authentic saccharide epitope on the surface of *B. anthracis* Sterne spores.

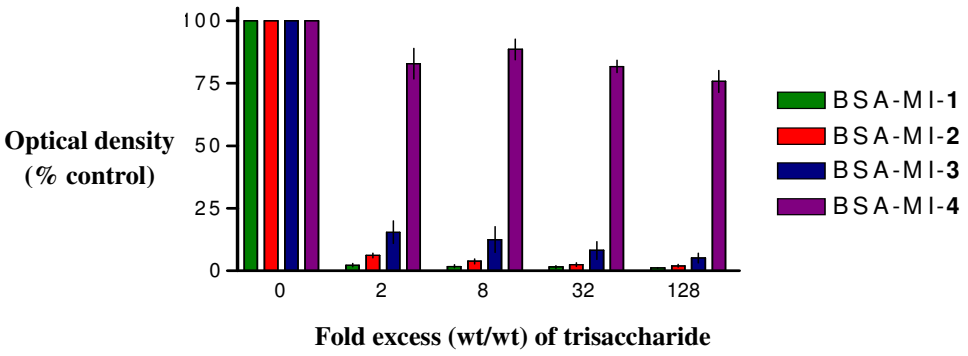


Figure 2.3. Competitive inhibition of anti-live spore antiserum binding to synthetic anthrose-containing trisaccharide by synthetic analogue conjugates. Microtiter plates were coated with KLH-BrAc-**2** conjugate (0.5 µg per mL conjugate corresponding to 0.03 µg per mL trisaccharide). Rabbit anti-live spore *B. anthracis* Sterne $34F_2$ antiserum (1:1600 dilute) was first mixed with BSA–trisaccharide conjugates (0-128-fold excess, wt/wt based on carbohydrate concentration) and then applied to the coated microtiter plate. Unconjugated BSA mixed with antiserum did not have any effect (data not shown). OD values were normalized for the OD values obtained without BSA-trisaccharide conjugate (0-fold "excess", 100%). Non-specific binding was tested with uncoated wells containing antiserum and buffer (data not shown). The data are reported as the means ±SD of triplicate measurements. Modified from reference 42.

2.4 Conclusion

The significance of the observations described in this paper is two-fold. First, we have demonstrated that, by using antilive spore antisera and anti-irradiated spore

antisera, the anthrose-containing trisaccharide of BclA is antigenic and exposed on the surface of *B. anthracis* Sterne 34F2 spores when presented in rabbits. Second, we have located an important antigenic component of this reactivity in the terminal 3-methyl-butyryl structures of the saccharide and confirmed its specificity using synthetic saccharide analogues. These data provide an important proof-of-concept step in the development of spore-specific reagents for detection and targeting of non-protein structures in *B. anthracis*. These structures may in turn provide a foundation for directing immune responses to spore structures during the early stages of the *B. anthracis* infection process. During the preparation of this manuscript Seeberger and co-workers reported that the anthrax oligosaccharide conjugated to KLH could elicit antibodies that recognize *B. anthracis* spores.[40] Our data are complementary to these findings in that *B. anthracis* spores elicit anti-carbohydrate antibodies, which may be harnessed for diagnosis. Ongoing studies will demonstrate whether these and additional saccharide structures are present and accessible on the spores from other *B. anthracis* isolates, including the highly virulent *B. anthracis* Ames and other *B. anthracis* cured of virulence plasmids pXO1 and pXO2.

2.5 Experimental Section

General

^{1}H NMR spectra were recorded in $CDCl_3$ or D_2O on Varian Merc-300 or Varian Inova-500 spectrometers equipped with Sun workstations at 300 K. TMS (δ_H 0.00) or D_2O (δ_H 4.67) was used as the internal reference. ^{13}C NMR spectra were recorded in $CDCl_3$ or D_2O at 75 MHz on a Varian Merc-300 spectrometer, respectively, using the

central resonance of $CDCl_3$ (δ_C 77.0) as the internal reference. COSY, HSQC, HMBC and TOCSY experiments were used to assist signal assignment of the spectra. The different monosaccharide units are referred to as a, b, and c, respectively, with a denoting the reducing end monosaccharide. Mass spectra were obtained on Applied Biosystems Voyager DE-Pro MALDI-TOF (no calibration) and Bruker Daltonics 9.4T (FTICR, external calibration with BSA). Optical rotatory power was obtained on Jasco P-1020 polarimeter at 300 K.

Chemicals were purchased from Aldrich or Fluka and used without further purification. CH_2Cl_2, acetonitrile and toluene were distilled from calcium hydride; THF from sodium; and MeOH from magnesium and iodine. Mariculture keyhole limpet hemocyanin (mcKLH), maleimide activated bovine serum albumin (BSA-MI), and succinimidyl 3-(bromoacetamido)propionate (SBAP) were purchased from Pierce Endogen, Rockford, IL. Aqueous solutions are saturated unless otherwise specified. Molecular sieves were activated at 350 ℃ for 3 h in vacuo. All reactions were performed under anhydrous conditions under argon and monitored by TLC on Kieselgel 60 F254 (Merck). Detection was by examination under UV light (254 nm) and by charring with 10% sulfuric acid in methanol. Silica gel (Merck, 70-230 mesh) was used for chromatographies. Iatrobeads 6RS-8060 was purchased from Bioscan.

General procedure for levulination

A solution of DCC (6 equiv) and DMAP (0.015 equiv) in CH_2Cl_2 was added under argon to a solution of **10** or **14** (1 equiv) and levulinic acid (10 equiv) in CH_2Cl_2 (at a concentration of 0.06 mol saccharide per L). The reaction mixture was stirred at room temperature for 2 h, and then filtered through Celite. The filtrate was washed twice with

water. The organic layer was dried ($MgSO_4$), filtered, and concentrated to dryness. Purification of the crude product by column chromatography on silica gel afforded the desired product **11** or **15**.

General procedure for isopropylidene removal

A solution of **6** or **11** (1 equiv) in acetic acid/water (3:2, at a concentration of 0.5 mol saccharide per L) was heated under reflux at 90 °C for 15 min, and then concentrated to dryness. The residue was co-distilled with toluene twice. Purification of the crude product by column chromatography on silica gel afforded the desired diol product.

General procedure for introduction of the C-4 azide group

Trifluoromethanesulfonic anhydride (1.5 equiv) was added slowly at 0 °C to a solution of **7** or **12** (1 equiv) in pyridine (10 equiv) and dry CH_2Cl_2 (at a concentration of 0.2 mol saccharide per L). The reaction mixture was stirred at 0 °C for 1 h, and then diluted with CH_2Cl_2. The solution was washed with H_2O and saturated $NaHCO_3$. The organic layer was dried ($MgSO_4$), filtered, and concentrated to dryness. To a solution of this residue in DMF (at a concentration of 0.08 mol saccharide per L) was added sodium azide (5 equiv). The reaction mixture was stirred at 40 °C overnight, and then concentrated to dryness. The residue was dissolved in ethyl acetate, and the solution was washed with saturated $NaHCO_3$ and brine. The organic layer was dried ($MgSO_4$), filtered, and concentrated to dryness. Purification of the crude product by column chromatography on silica gel afforded the desired product **8** or **13**.

General procedure for cleavage of the levulinoyl ester

A solution of hydrazine acetate (1 equiv) in dry MeOH (0.4 mol L^{-1}) was added under argon to a solution of **17** or **19** (1 equiv) in dry CH_2Cl_2 (at a concentration of 0.04 mol saccharide per L). The reaction mixture was stirred at room temperature for 4 h, and then concentrated to dryness. The residue was dissolved in CH_2Cl_2, and then washed with water. The organic layer was dried ($MgSO_4$), filtered, and concentrated to dryness. Purification of the crude product by column chromatography on silica gel afforded the desired product **18** or **20**.

General procedure for azide reduction and introduction of C-4'' moiety

TEA (15 equiv) was added to a solution of **20** or **21** (1 equiv) and 1,3-propanedithiol (20 equiv) in pyridine (at a concentration of 0.014 mol saccharide per L) and H_2O (at a concentration of 0.1 mol saccharide per L). The reaction mixture was stirred at room temperature overnight, and then concentrated to dryness. The residue was co-evaporated with toluene twice and ethanol twice. Purification of the crude product by column chromatography on silica gel (CH_2Cl_2/MeOH/TEA 100:5:1) afforded the free amine compounds. β-Hydroxyisovaleric acid or isovaleric acid (2 equiv) was activated by HOAt (4 equiv) and HATU (4 equiv) in DMF (at a concentration of 0.01 mol saccharide per L) for 1 h, and then DIPEA (8 equiv) was added. The resulting yellow solution was added dropwise to the free amine compound (1 equiv) in DMF (at a concentration of 0.02 mol saccharide per L). The reaction mixture was stirred at room temperature for 4 h, and then concentrated to dryness. Purification of the crude product by column chromatography on silica gel afforded the desired product **22**, **23** or **24**. Alternatively, a solution of free amine (1 equiv) in Ac_2O (2 equiv), pyridine (2 equiv) and

DMAP (0.1 equiv) was stirred at room temperature overnight, and then concentrated to dryness. The residue was co-evaporated with toluene twice. Purification of the crude product by column chromatography on silica gel afforded the desired product **25**.

General precedure for global deprotection

NaOMe (pH 8-10) was added to a solution of **22**, **23**, **24**, or **25** in dry MeOH (at a concentration of 0.06 mol saccharide per L). The reaction mixture was stirred at room temperature overnight, and then neutralized by the addition of Dowex 650 H^+. The suspension was filtered through Celite, and washed with MeOH/CH_2Cl_2 1:1. The combined filtrates were concentrated to dryness. Purification of the crude product by column chromatography on silica gel afforded the desired deacetylated product. To a solution of the partially deprotected compound in *tert*-butanol/H_2O/AcOH (40:1:1, 0.01 mol L^{-1}) was added Pd/C (cat.) under an atmosphere of hydrogen. The reaction mixture was stirred at room temperature overnight, and then filtered through Celite. The filtrate was concentrated to dryness. Purification of the crude product by Iatro beads afforded the desired product **1**-**4**.

Allyl 3,4-*O*-isopropylidene-2-*O*-methyl-α-D-fucopyranoside (6)

NaH (3.25 g, 67.63 mmol, 50% in mineral oil) was added to a solution of **5** (8.26 g, 33.81 mmol) in DMF (90 mL). The reaction mixture was stirred at 0 °C for 1 h, and then methyl iodide (4.21 mL, 67.62 mmol) was added dropwise. The reaction mixture was stirred at room temperature for 6 h, and then poured into ice water. The solution was extracted with CH_2Cl_2 (100 mL) and washed with water (100 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated to dryness. Purification of the crude product by column chromatography on silica gel (hexane/EtOAc 4:1) afforded the

desired product **6** as colorless oil (8.66 g, 99%). R_f = 0.74 (hexane/EtOAc 2:1); $[\alpha]_D^{27}$ = +67.7 (c = 3.6 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.26 (d, 3H, $J_{5,6}$ = 6.3 Hz, H-6), [1.29, 1.47, 2 × s (CH_3CCH_3)], 3.30 (dd, 1H, $J_{1,2}$ = 3.6, $J_{2,3}$ = 8.1 Hz, H-2), 3.44 (s, 3H, OCH_3), 3.94-4.10 (m, 3H, H-4, H-5, OCH_2CHCH_2), 4.14 (dd, 1H, J = 5.4, 12.9 Hz, OCH'_2CHCH_2), 4.18 (dd, 1H, $J_{2,3}$ = 8.1, $J_{3,4}$ = 5.7 Hz, H-3), 4.88 (d, 1H, $J_{1,2}$ = 3.6 Hz, H-1), 5.16 (dd, 1H, J = 1.2, 10.2 Hz, OCH_2CHCH_2), 5.28 (dd, 1H, J = 1.5, 17.1 Hz, $OCH_2CHCH'_2$), 5.87 (m, 1H, OCH_2CHCH_2); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.2 (C-6), [26.3, 28.3 (CH_3CCH_3)], 58.5 (OCH_3), 63.1 (C-5), 68.2 (OCH_2CHCH_2), 75.7 (C-4), 76.0 (C-3), 79.1 (C-2), 95.3 (C-1), 108.7 (CH_3CCH_3), 117.9 (OCH_2CHCH_2), 133.6 (OCH_2CHCH_2); MALDI-TOF/MS: m/z: calcd for $C_{13}H_{22}O_5Na$: 281.1365; found: 281.7 $[M+Na]^+$.

Allyl 3-*O*-benzyl-2-*O*-methyl-α-D-fucopyranoside (7)

Treatment of **6** (8.66 g, 33.53 mmol) in acetic acid/water (40.2 mL/26.8 mL) as described in the general procedures gave the diol as a white solid (7.39 g, 33.86 mmol, quantitative). R_f = 0.30 (CH_2Cl_2/MeOH 19:1); $[\alpha]_D^{27}$ = +4.9 (c = 2.5 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.21 (d, 3H, $J_{5,6}$ = 6.6 Hz, H-6), 2.56 (s, 1H, OH), 3.40 (s, 3H, OCH_3), 3.47 (dd, 1H, $J_{1,2}$ = 3.0, $J_{2,3}$ = 9.6 Hz, H-2), 3.75 (s, 1H, H-4), 3.89-3.97 (m, 2H, H-3, H-5), 4.00 (dd, 1H, J = 6.3, 12.6 Hz, OCH_2CHCH_2), 4.14 (dd, 1H, J = 3.6, 12.9 Hz, OCH'_2CHCH_2), 4.99 (d, 1H, $J_{1,2}$ = 3.0 Hz, H-1), 5.16 (d, 1H, J = 10.5 Hz, OCH_2CHCH_2), 5.28 (d, 1H, J = 17.1 Hz, $OCH_2CHCH'_2$), 5.87 (m, 1H, OCH_2CHCH_2); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.1 (C-6), 57.7 (OCH_3), 65.6 (C-5), 68.2 (OCH_2CHCH_2), 69.4 (C-3), 71.5 (C-4), 77.9 (C-2), 94.5 (C-1), 117.9 (OCH_2CHCH_2), 133.8 (OCH_2CHCH_2); MALDI-TOF/MS: m/z: calcd for $C_{10}H_{18}O_5Na$: 241.1052; found: 241.7 $[M+Na]^+$.

Dibutyltin oxide (8.43 g, 33.86 mmol) was added to a solution of the diol (7.39 g, 33.8 mmol) in dry MeOH (300 mL). The reaction mixture was heated under reflux until the solution became clear. After cooling to room temperature, the reaction mixture was concentrated to dryness. Benzyl bromide (4.0 mL, 33.86 mmol) and CsF (5.15 g, 33.86 mmol) were added to a solution of the residue in DMF (130 mL). The reaction mixture was stirred at room temperature overnight, and then concentrated to dryness. The residue was dissolved in CH_2Cl_2 (100 mL), and the solution was washed with H_2O (100 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated to dryness. Purification of the crude product by column chromatography on silica gel (hexane/EtOAc 3:1) afforded the desired product **7** as colorless oil (10.03 g, 32.53 mmol, 96%). R_f = 0.34 (hexane/EtOAc 2:1); $[\alpha]_D^{27}$ = +86.6 (c = 2.0 in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.21 (d, 3H, $J_{5,6}$ = 6.5 Hz, H-6), 3.45 (s, 3H, OCH_3), 3.55 (dd, 1H, $J_{1,2}$ = 3.5, $J_{2,3}$ = 9.5 Hz, H-2), 3.59-3.78 (m, 2H, H-3, H-5), 3.86 (dd, 1H, $J_{2,3}$ = 7.0, $J_{3,4}$ = 7.0 Hz, H-4), 3.99 (dd, 1H, J = 7.0, 13.0 Hz, OCH_2CHCH$_2$), 4.12 (dd, 1H, J = 5.5, 13.0 Hz, OCH'_2CHCH$_2$), 4.61 (d, 1H, J = 12.0 Hz, PhCH_2), 4.72 (d, 1H, J = 12.0 Hz, PhCH'_2), 4.94 (d, 1H, $J_{1,2}$ = 3.5 Hz, H-1), 5.15 (d, 1H, J = 10.5 Hz, OCH$_2$CHCH_2), 5.26 (d, 1H, J = 17.0 Hz, OCH$_2$CHCH'_2), 5.89 (m, 1H, OCH$_2$CHCH$_2$), 7.19-7.29 (m, 5H, H_{arom}); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.1 (C-6), 58.9 (OCH_3), 65.3 (C-4), 68.2 (OCH$_2$CHCH$_2$), 70.2 (C-3), 72.7 (PhCH$_2$), 77.5 (C-2), 77.9 (C-5), 95.5 (C-1), 117.9 (OCH$_2$CHCH$_2$), [127.7, 127.8, 128.4, 133.9 (C_{arom})], 138.3 (OCH$_2$$CHCH_2$); MALDI-TOF/MS: m/z: calcd for $C_{17}H_{24}O_5Na$: 331.1521; found: 331.2 [M+Na]$^+$.

Allyl 4-azido-3-*O*-benzyl-4,6-dideoxy-2-*O*-methyl-α-D-glucopyranoside (8)

Treatment of **7** (10.03 g, 32.53 mmol) in pyridine (28.6 mL, 0.33 mol) and CH_2Cl_2 (160 mL) with trifluoromethanesulfonic anhydride (8.2 mL, 48.66 mmol) followed by treatment of triflate residue in DMF (400 mL) with sodium azide (10.40 g, 0.16 mol) was performed according to the general procedure to give compound **8** as colorless oil (8.67 g, 80%). R_f = 0.41 (hexane/EtOAc 5:1); $[\alpha]_D^{27}$ = +130.5 (*c* = 2.4 in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.30 (d, 3H, $J_{5,6}$ = 6.5 Hz, H-6), 3.02 (t, 1H, $J_{3,4}$ = 9.0, $J_{4,5}$ = 10.0 Hz, H-4), 3.27 (dd, 1H, $J_{1,2}$ = 3.5, $J_{2,3}$ = 9.5 Hz, H-2), 3.44 (s, 3H, OCH_3), 3.52 (m, 1H, H-5), 3.72 (t, 1H, $J_{2,3}$ = 9.5, $J_{3,4}$ = 9.0 Hz, H-3), 3.98 (dd, 1H, *J* = 7.0, 13.0 Hz, OCH_2CHCH$_2$), 4.12 (dd, 1H, *J* = 5.0, 13.0 Hz, OCH'_2CHCH$_2$), 4.72 (d, 1H, *J* = 10.5 Hz, PhCH_2), 4.84 (d, 1H, *J* = 10.5 Hz, PhCH'_2), 4.89 (d, 1H, $J_{1,2}$ = 3.5 Hz, H-1), 5.18 (d, 1H, *J* = 10.5 Hz, OCH$_2$CHCH_2), 5.28 (d, 1H, *J* = 17.5 Hz, OCH$_2$CHCH'_2), 5.87 (m, 1H, OCH$_2$CHCH$_2$), 7.19-7.35 (m, 5H, H_{arom}); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 18.4 (C-6), 58.7 (OCH_3), 66.1 (C-5), 67.9 (C-4), 68.2 (OCH$_2$CHCH$_2$), 75.5 (PhCH$_2$), 79.8 (C-3), 82.3 (C-2), 94.8 (C-1), 118.3 (OCH$_2$CHCH$_2$), [127.8, 128.2, 128.4, 133.6 (C_{arom})], 138.2 (OCH$_2$$CHCH_2$); MALDI-TOF MS: *m*/*z*: calcd for $C_{17}H_{23}N_3O_4Na$: 356.16; found: 356.7 [*M*+Na]$^+$.

Ethyl 3,4-*O*-isopropylidene-2-*O*-levulinoyl-1-thio-β-D-fucopyranoside (11)

Treatment of **10** (1.34 g, 5.40 mmol) and levulinic acid (5.53 mL, 54.00 mmol) in CH_2Cl_2 (90 mL) with DCC (6.69 g, 32.42 mmol) and DMAP (9.90 mg, 0.081 mmol) in CH_2Cl_2 (9 mL) according to the general procedure gave compound **11** as colorless oil (1.76 g, 94%). R_f = 0.71(hexane/EtOAc 1:1); $[\alpha]_D^{27}$ = +1.3 (*c* = 0.7 in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.19 (t, 3H, *J* = 7.5 Hz, SCH$_2$CH_3), 1.28 (s, 3H, CH_3), 1.35 (d, 3H, $J_{5,6}$ = 7.0 Hz, H-6), 1.49 (s, 3H, CH'$_3$), 2.12 (s, 3H, CH_3C(O)CH$_2$CH$_2$C(O)O), 2.53-

2.78 (m, 6H, $CH_3C(O)CH_2CH_2C(O)O$, SCH_2CH_3, $CH_3C(O)CH_2CH_2C(O)O$), 3.78-3.82 (m, 1H, H-5), 3.98 (dd, $J_{3,4}$ = 5.5, $J_{4,5}$ = 2.5 Hz, H-4), 4.06 (dd, 1H, $J_{2,3}$ =7.5, $J_{3,4}$ = 5.5 Hz, H-3), 4.25 (d, 1H, $J_{1,2}$ = 10.0 Hz, H-1), 4.92 (dd, 1H, $J_{1,2}$ = 10.0, $J_{2,3}$ = 7.5 Hz, H-2); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 14.7 (SCH_2CH_3), 16.8 (C-6), [23.8, 26.4 (CH_3)], [27.8, 28.1 ($CH_3C(O)CH_2CH_2C(O)O$, SCH_2CH_3)], 29.8 ($CH_3C(O)CH_2CH_2C(O)O$), 38.0 ($CH_3C(O)CH_2CH_2C(O)O$), 71.8 (C-2), 72.7 (C-5), 76.4 (C-4), 77.2 (C-3), 82.2 (C-1), 171.7 ($CH_3C(O)CH_2CH_2C(O)O$), 206.3 ($CH_3C(O)CH_2CH_2C(O)O$); MALDI-TOF/MS: *m*/*z*: calcd for $C_{16}H_{26}O_6SNa$: 369.13; found: 369.5 $[M+Na]^+$.

Ethyl 3-*O*-benzyl-2-*O*-levulinoyl-1-thio-β-D-fucopyranoside (12)

Treatment of **11** (1.75 g, 5.05 mmol) in acetic acid/water (6.0 mL/4.0 mL) according to the general procedure for isopropylidene removal gave the diol as a white solid (1.55 g, quantitative). R_f = 0.38(CH_2Cl_2/MeOH 19:1); $[\alpha]_D^{27}$ = -3.5 (*c* = 1.2 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.19 (t, 3H, *J* = 7.5 Hz, SCH_2CH_3), 1.28 (d, 3H, $J_{5,6}$ = 6.0 Hz, H-6), 2.13 (s, 3H, $CH_3C(O)CH_2CH_2C(O)O$), 2.51-2.86 (m, 6H, $CH_3C(O)CH_2CH_2C(O)O$, SCH_2CH_3, $CH_3C(O)CH_2CH_2C(O)O$), 3.57-3.68 (m, 2H, H-3, H-5), 3.75 (d, *J* = 2.7 Hz, H-4), 4.32 (d, 1H, $J_{1,2}$ = 9.9 Hz, H-1), 4.98 (t, 1H, $J_{1,2}$ = 9.9, $J_{2,3}$ = 9.3 Hz, H-2); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 14.8 (SCH_2CH_3), 16.6 (C-6), 23.7 (SCH_2CH_3), 28.2 ($CH_3C(O)CH_2CH_2C(O)O$), 29.8 ($CH_3C(O)CH_2CH_2C(O)O$), 38.4 ($CH_3C(O)CH_2CH_2C(O)O$), [71.5, 71.8 (C-2, C-4)], 73.8 (C-3), 74.7 (C-5), 82.7 (C-1), 172.7 ($CH_3C(O)CH_2CH_2C(O)O$), 206.3 ($CH_3C(O)CH_2CH_2C(O)O$); MALDI-TOF/MS: *m*/*z*: calcd for $C_{13}H_{22}O_6SNa$: 329.10; found: 330.2 $[M+Na]^+$.

Dibutyl tin oxide (1.26 g, 5.06 mmol) was added to a solution of the diol (1.55 g, 5.06 mmol) in dry toluene (50 mL). The reaction mixture was heated under reflux with a

Dean-Stark apparatus for 3 h, and then cooled to 60 °C. Benzyl bromide (0.60 mL, 5.06 mmol) and tetrabutylammonium iodide (1.68 g, 5.06 mmol) were added and the resulting reaction mixture was heated under reflux for 3 h. After cooling to room temperature, the reaction mixture was concentrated to dryness. The residue was dissolved in EtOAc (50 mL), and the resulting solution was washed with H_2O (50 mL). The organic layer was dried ($MgSO_4$) filtered, and concentrated to dryness. Purification of the crude product by column chromatography on silica gel (hexane/EtOAc 2:1) afforded the desired product **12** as colorless oil (1.04 g, 52%). R_f = 0.43 (hexane/EtOAc 1:1); $[\alpha]_D^{27}$ = -4.0 (*c* = 0.8 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.16 (t, 3H, *J* = 7.5 Hz, SCH_2CH_3), 1.28 (d, 3H, $J_{5,6}$ = 6.3 Hz, H-6), 2.12 (s, 3H, $CH_3C(O)CH_2CH_2C(O)O$), 2.48-2.76 (m, 6H, $CH_3C(O)CH_2CH_2C(O)O$, SCH_2CH_3, $CH_3C(O)CH_2CH_2C(O)O$), 3.43-3.54 (m, 2H, H-3, H-5), 3.75 (d, *J* = 3.0 Hz, H-4), 4.23 (d, 1H, $J_{1,2}$ = 9.9 Hz, H-1), 4.57 (d, 1H, *J* = 12.0 Hz, $PhCH_2$), 4.61 (d, 1H, *J* = 11.1 Hz, $PhCH'_2$), 5.14 (t, 1H, $J_{1,2}$ = 9.6, $J_{2,3}$ = 9.6 Hz, H-2), 7.19-7.31 (m, 5H, H_{arom}); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 14.7 (SCH_2CH_3), 16.6 (C-6), 23.4 (SCH_2CH_3), 28.1 ($CH_3C(O)CH_2CH_2C(O)O$), 29.9 ($CH_3C(O)CH_2CH_2C(O)O$), 37.9 ($CH_3C(O)CH_2CH_2C(O)O$), [69.1, 69.2 (C-2, C-4)], 71.7 ($PhCH_2$), 74.5 (C-3), 79.7 (C-5), 82.9 (C-1), [127.9, 128.1, 128.5, 137.5 (C_{arom})], 171.7 ($CH_3C(O)CH_2CH_2C(O)O$), 206.3 ($CH_3C(O)CH_2CH_2C(O)O$); MALDI-TOF/MS: *m*/*z*: calcd for $C_{20}H_{28}O_6SNa$: 419.15; found: 419.5 $[M+Na]^+$.

Ethyl 4-azido-3-*O*-benzyl-4,6-dideoxy-2-*O*-levulinoyl-1-thio-β-D-glucopyranoside (13)

Treatment of **12** (0.50 g, 1.26 mmol) in pyridine (1.0 mL, 12.61 mmol) and CH_2Cl_2 (6.5 mL) with trifluoromethanesulfonic anhydride (0.32 mL, 1.90 mmol) followed by

treatment of triflate residue in DMF (16 mL) with sodium azide (0.41 g, 6.31 mmol) according to the general procedure for introduction of the C-4 azide group gave compound **13** as colorless oil (0.42 g, 79%). R_f = 0.32 (hexane/EtOAc 4:1); $[\alpha]_D^{27}$ = +13.1 (*c* = 0.5 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.67 (t, 3H, *J* = 7.5 Hz, SCH_2CH_3), 1.30 (d, 3H, $J_{5,6}$ = 5.7 Hz, H-6), 2.10 (s, 3H, $CH_3C(O)CH_2CH_2C(O)O$), 2.44-2.49 (m, 2H, $CH_3C(O)CH_2CH_2C(O)O$), 2.57-2.67 (m, 4H, SCH_2CH_3, $CH_3C(O)CH_2CH_2C(O)O$), 3.11-3.24 (m, 2H, H-4, H-5), 3.48 (t, $J_{3,4}$ = 9.0, $J_{2,3}$ = 9.0 Hz, H-3), 4.27 (d, 1H, $J_{1,2}$ = 9.9 Hz, H-1), 4.68 (d, 1H, *J* = 11.1 Hz, $PhCH_2$), 4.71 (d, 1H, *J* = 11.1 Hz, $PhCH'_2$), 4.94 (dd, 1H, $J_{1,2}$ = 9.9, $J_{2,3}$ = 9.0 Hz, H-2), 7.22-7.29 (m, 5H, H_{arom}); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 14.8 (SCH_2CH_3), 18.7 (C-6), 23.9 (SCH_2CH_3), 28.0 ($CH_3C(O)CH_2CH_2C(O)O$), 29.8 ($CH_3C(O)CH_2CH_2C(O)O$), 37.8($CH_3C(O)CH_2CH_2C(O)O$), 67.7 (C-4), 72.2 (C-2), 74.9 ($PhCH_2$), 75.1 (C-5), 82.3 (C-3), 83.2 (C-1), [127.9, 128.0, 128.2, 128.4, 137.5 (C_{arom})], 171.5 ($CH_3C(O)CH_2CH_2C(O)O$), 206.1 ($CH_3C(O)CH_2CH_2C(O)O$); MALDI-TOF/MS: *m*/*z*: calcd for $C_{20}H_{27}N_3O_5SNa$: 444.15; found: 444.1 $[M+Na]^+$.

Ethyl 2-*O*-benzoyl-4-*O*-benzyl-3-*O*-levulinoyl-1-thio-α-L-rhamnopyranoside (15)

Treatment of **14** (4.93 g, 12.25 mmol) and levulinic acid (12.5 mL, 122.50 mmol) in CH_2Cl_2 (180 mL) with DCC (15.18 g, 73.57 mmol) and DMAP (22.45 mg, 0.18 mmol) in CH_2Cl_2 (18 mL) according to the general procedure for levulination gave compound **15** as colorless oil (5.29 g, 86%). R_f = 0.34 (hexane/EtOAc 3:1); $[\alpha]_D^{27}$ = -18.9 (*c* = 2.6 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.23 (t, 3H, *J* = 7.2 Hz, SCH_2CH_3), 1.33 (d, 3H, $J_{5,6}$ = 6.0 Hz, H-6), 2.02 (s, 3H, $CH_3C(O)CH_2CH_2C(O)O$), 2.35-2.39 (td, 2H, *J* = 6.9, 9.6 Hz, $CH_3C(O)CH_2CH_2C(O)O$), 2.46-2.72 (m, 4H, SCH_2CH_3, $CH_3C(O)CH_2CH_2C(O)O$), 3.58 (t, $J_{4,5}$ = 9.3, $J_{3,4}$ = 9.6 Hz, H-4), 4.18 (m, 1H, H-5), 4.59 (d, 1H, *J* =11.1 Hz,

PhCH_2), 4.66 (d, 1H, J = 11.1 Hz, PhCH'_2), 5.22 (s, 1H, H-1), 5.28 (dd, 1H, $J_{2,3}$ = 3.3, $J_{3,4}$ = 9.6 Hz, H-3), 5.51 (dd, 1H, $J_{1,2}$ = 1.5, $J_{2,3}$ = 3.3 Hz, H-2), 7.19-8.00 (m, 10H, H_{arom}); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 14.9 (SCH_2CH_3), 18.0 (C-6), 25.4 (SCH_2CH_3), 27.9 ($CH_3C(O)CH_2CH_2C(O)O$), 29.7 ($CH_3C(O)CH_2CH_2C(O)O$), 37.8($CH_3C(O)CH_2CH_2C(O)O$), 68.3 (C-5), 72.5 (C-2), 72.6 (C-3), 74.9 (PhCH_2), 78.9 (C-4), 81.9 (C-1), [127.8, 127.9, 128.4, 128.5, 129.7, 129.8, 133.4, 137.9 (C_{arom})], 165.5 (PhC(O)O), 171.7 ($CH_3C(O)CH_2CH_2C(O)O$), 206.2 ($CH_3C(O)CH_2CH_2C(O)O$); MALDI-TOF/MS: *m*/*z*: found: 524.1 [*M*+Na]$^+$; MALDI-FTICR/MS: *m*/*z*: calcd for $C_{27}H_{32}O_7SNa$: 523.1766; found: 523.1761 [*M*+Na]$^+$.

3-[(*N*-Benzyloxycarbonyl)amino]propyl 2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranoside (16)

Glycosyl donor **14** (3.79 g, 9.42 mmol), 3-(*N*-benzyloxycarbonyl)aminopropanol (3.94 g, 18.83 mmol) and 4 Å powdered molecular sieves (7.73 g) in CH_2Cl_2 (150 mL) in the presence of NIS (2.33 g, 10.36 mmol) and TfOH (0.166 mL, 1.88 mmol) were reacted according to the general procedure for NIS glycosylation to give compound **16** as white solid (3.73 g, 72%). R_f = 0.26 (hexane/EtOAc 2:1); $[\alpha]_D^{27}$ = +11.3 (*c* = 1.8 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.32 (d, 3H, $J_{5,6}$ = 6.0 Hz, H-6), 1.73 (m, 2H, $OCH_2CH_2CH_2NHZ$), 2.11 (d, 1H, J = 4.5 Hz, O*H*), 3.24 (dd, 2H, J = 6.3, 12.6 Hz, $OCH_2CH_2CH_2NHZ$), 3.36-3.45 (m, 2H, $OCH_2CH_2CH_2NHZ$, H-4), 3.65-3.75 (m, 2H, $OCH'_2CH_2CH_2NHZ$, H-5), 4.12 (dd, 1H, $J_{2,3}$ = 3.3, $J_{3,4}$ = 8.4 Hz, H-3), 4.69 (d, 1H, J = 11.1 Hz, PhCH_2), 4.76 (s, 1H, H-1), 4.79 (d, 1H, J = 11.1 Hz, PhCH'_2), 4.85 (broad, 1H, N*H*), 5.02 (s, 2H, PhCH_2OC(O)), 5.25 (dd, 1H, $J_{1,2}$ = 1.5, $J_{2,3}$ = 3.3 Hz, H-2), 7.18-7.99 (m, 15H, H_{arom}); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 18.2 (C-6), 29.6 ($OCH_2CH_2CH_2NHZ$),

38.6 ($OCH_2CH_2\mathit{C}H_2NHZ$), 65.6 ($O\mathit{C}H_2CH_2CH_2NHZ$), 66.6 (C-5), 67.6 ($Ph\mathit{C}H_2OC(O)$), 70.5 (C-3), 73.2 (C-2), 75.2 ($Ph\mathit{C}H_2$), 81.6 (C-4), 97.5 (C-1), [127.9, 128.1, 128.4, 129.7, 129.9, 130.4, 133.3, 136.6, 138.1 (C_{arom})], 156.3 ($PhCH_2O\mathit{C}(O)$), 166.3($Ph\mathit{C}(O)O$); MALDI-TOF/MS: *m*/*z*: found: 572.9 $[M+Na]^+$; MALDI-FTICR/MS: *m*/*z*: calcd for $C_{31}H_{35}NO_8Na$: 572.2260; found: 572.2259 $[M+Na]^+$.

3-[(*N*-Benzyloxycarbonyl)amino]propyl *O*-(2-*O*-benzoyl-4-*O*-benzyl-3-*O*-levulinoyl-α-L-rhamnopyranosyl)-(1→3)-2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranoside (17)

Glycosyl donor **15** (3.04 g, 6.07 mmol), glycosyl acceptor **16** (3.03 g, 5.51 mmol) and 4 Å powdered molecular sieves (6.07 g) in CH_2Cl_2 (100 mL) in the presence of NIS (1.51 g, 6.71 mmol) and TfOH (0.11 mL, 1.22 mmol) was treated according to the general procedure for the linker glycosylation to give compound **17** as colorless oil (4.26 g, 78%). R_f = 0.34 (hexane/EtOAc 2:1); $[\alpha]_D^{27}$ = +23.6 (c = 1.8 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.11 (d, 3H, $J_{5,6}$ = 6.0 Hz, H-6b), 1.28 (d, 3H, $J_{5,6}$ = 6.0 Hz, H-6a), 1.72 (m, 2H, $OCH_2C\mathit{H_2}CH_2NHZ$), 1.99 (s, 3H, $C\mathit{H_3}C(O)CH_2CH_2C(O)O$), 2.32-2.39 (m, 2H, $CH_3C(O)CH_2C\mathit{H_2}C(O)O$), 2.50-2.67 (m, 2H, $CH_3C(O)C\mathit{H_2}CH_2C(O)O$), 3.22 (dd, 2H, *J* = 6.0, 12.3 Hz, $OCH_2CH_2C\mathit{H_2}NHZ$), 3.39-3.49 (m, 2H, $J_{3,4}$ = 9.6, $J_{4,5}$ = 9.6 Hz, $OC\mathit{H_2}CH_2CH_2NHZ$, H-4b), 3.56 (t, $J_{3,4}$ = 9.3, $J_{4,5}$ = 9.3 Hz, H-4a), 3.62-3.72 (m, 2H, $OC\mathit{H'_2}CH_2CH_2NHZ$, H-5a), 3.84 (m, 1H, H-5b), 4.17 (dd, 1H, $J_{2,3}$ = 3.0, $J_{3,4}$ = 9.0 Hz, H-3a), 4.45 (d, 1H, *J* = 11.4 Hz, $PhC\mathit{H_2}$), 4.50 (d, 1H, *J* = 11.4 Hz, $PhC\mathit{H'_2}$), 4.66 (d, 1H, *J* = 10.8 Hz, $PhC\mathit{H''_2}$), 4.80 (broad, 2H, H-1a, N*H*), 4.95 (d, 1H, *J* = 10.8 Hz, $PhC\mathit{H'''_2}$), 4.99 (s, 2H, $PhC\mathit{H_2}OC(O)$), 5.06 (s, 1H, H-1b), 5.29 (d, 1H, $J_{2,3}$ = 3.3 Hz, H-2a), 5.32 (dd, 1H, $J_{2,3}$ = 3.0, $J_{3,4}$ = 9.6 Hz, H-3b), 5.52 (dd, 1H, $J_{1,2}$ = 1.8, $J_{2,3}$ = 3.0 Hz, H-2b), 7.05-8.00 (m, 25H, H_{arom}); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 17.8 (C-6b), 18.2 (C-6a), 28.0

($CH_3C(O)CH_2CH_2C(O)O$), 29.7 ($OCH_2CH_2CH_2NHZ$, $CH_3C(O)CH_2CH_2C(O)O$)), 37.8 ($CH_3C(O)CH_2CH_2C(O)O$), 38.5 ($OCH_2CH_2CH_2NHZ$), 65.6 ($OCH_2CH_2CH_2NHZ$), 66.5 ($PhCH_2OC(O)$), 67.9 (C-5a), 68.6 (C-5b), 70.7 (C-2b), 71.8 (C-3b), 72.7 (C-2a), [73.9, 75.8 ($PhCH_2$)], 78.2 (C-4b), 79.3 (C-3a), 79.8 (C-4a), 97.0 (C-1a), 99.7 (C-1b), [127.5, 127.7, 127.9, 128.2, 128.3, 128.4, 128.5, 129.5, 129.6, 129.7, 129.8, 133.4, 136.6, 137.9, 138.0 (C_{arom})], 156.3 ($PhCH_2OC(O)$), [165.3, 166.1 ($PhC(O)O$)], 171.7 ($CH_3C(O)CH_2CH_2C(O)O$), 206.2 ($CH_3C(O)CH_2CH_2C(O)O$); MALDI-TOF/MS: *m*/*z*: found: 1011.6 $[M+Na]^+$; MALDI-FTICR/MS: *m*/*z*: calcd for $C_{56}H_{61}NO_{15}Na$: 1010.3939; found: 1010.3932 $[M+Na]^+$.

3-[(*N*-Benzyloxycarbonyl)amino]propyl *O*-(2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranosyl)-(1→3)-2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranoside (18)

Treatment of **17** (4.26 g, 4.31 mmol) in CH_2Cl_2 (100 mL) with hydrazine acetate (397 mg, 4.31 mmol) in MeOH (10 mL) according to the general procedure for cleavage of the levulinoyl ester gave compound **18** as white solid (3.56 g, 93%). R_f = 0.42 (hexane/EtOAc 2:1); $[\alpha]_D^{27}$ = +21.9 (*c* = 2.2 in $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$): δ = 1.17 (d, 3H, $J_{5,6}$ = 6.0 Hz, H-6b), 1.26 (d, 3H, $J_{5,6}$ = 6.0 Hz, H-6a), 1.72 (m, 2H, $OCH_2CH_2CH_2NHZ$), 3.22 (d, 2H, *J* = 6.0 Hz, $OCH_2CH_2CH_2NHZ$), 3.30-3.41 (m, 2H, $J_{3,4}$ = 9.6, $J_{4,5}$ = 9.3 Hz, $OCH_2CH_2CH_2NHZ$, H-4b), 3.54 (t, 1H, $J_{3,4}$ = 9.3, $J_{4,5}$ = 9.3 Hz, H-4a), 3.62-3.71 (m, 2H, $OCH'_2CH_2CH_2NHZ$, H-5a), 3.78 (dd, 1H, $J_{4,5}$ = 9.3 Hz, $J_{5,6}$ = 6.0 Hz, H-5b), 4.04 (dd, 1H, $J_{2,3}$ = 2.1, $J_{3,4}$ = 9.6 Hz, H-3b), 4.18 (dd, 1H, $J_{2,3}$ = 3.0, $J_{3,4}$ = 9.0 Hz, H-3a), 4.57-4.63 (m, 3H, $PhCH_2$), 4.77 (s, 1H, H-1a), 4.86 (d, 1H, *J* = 10.8 Hz, $PhCH'_2$), 4.99 (s, 2H, $PhCH_2OC(O)$), 5.11 (s, 1H, H-1b), 5.28 (d, 1H, $J_{2,3}$ = 3.0 Hz, H-2a), 5.33 (dd, 1H, $J_{2,3}$ = 2.1 Hz, H-2b), 7.12-8.01 (m, 25H, H_{arom}); ^{13}C NMR (75 MHz, $CDCl_3$): δ =

17.9 (C-6b), 18.1 (C-6a), 29.5 ($OCH_2CH_2CH_2NHZ$), 38.5 ($OCH_2CH_2CH_2NHZ$), 65.6 ($OCH_2CH_2CH_2NHZ$), 66.5 ($PhCH_2OC(O)$), 67.9 (C-5a), 68.3 (C-5b), 69.8 (C-3b), 72.8 (C-2b), 73.1 (C-2a), [74.0, 75.6 ($PhCH_2$)], 77.6 (C-3a), 80.3 (C-4a), 81.1 (C-4b), 97.1 (C-1a), 99.5 (C-1b), [127.7, 127.8, 127.9, 128.2, 128.3, 128.4, 128.5, 129.6, 129.7, 129.8, 133.2, 133.3, 137.8, 138.1 (C_{arom})], 156.3 ($PhCH_2OC(O)$), [165.8, 165.9 ($PhC(O)O$)]; MALDI-TOF/MS: *m*/*z*: found: 913.5 $[M+Na]^+$; MALDI-FTICR/MS: *m*/*z*: calcd for $C_{51}H_{55}NO_{13}Na$: 912.3571; found: 912.3559 $[M+Na]^+$.

3-[(*N*-Benzyloxycarbonyl)amino]propyl *O*-(4-azido-3-*O*-benzyl-4,6-dideoxy-2-*O*-levulinoyl-β-D-glucopyranosyl)-(1→3)-*O*-(2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranosyl)-(1→3)-2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranoside (19)

Glycosyl donor **13** (80 mg, 0.19 mmol), glycosyl acceptor **18** (151 mg, 0.17 mmol) and 4 Å powdered molecular sieves (0.23 g) in CH_2Cl_2 (3 mL) in the presence of NIS (47 mg, 0.21 mmol) and TfOH (3 μL, 0.034 mmol) was treated according to the general procedure for the linker glycosylation to give compound **19** as colorless oil (161 mg, 76%). R_f = 0.30 (hexane/EtOAc 2:1); $[\alpha]_D^{27}$ = +15.3 (*c* = 0.8 in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.89 (d, 3H, $J_{5,6}$ = 6.5 Hz, H-6c), 1.06 (d, 3H, $J_{5,6}$ = 6.5 Hz, H-6b), 1.27 (d, 3H, $J_{5,6}$ = 6.5 Hz, H-6a), 1.73 (m, 2H, $OCH_2CH_2CH_2NHZ$), 1.88 (s, 3H, $CH_3C(O)CH_2CH_2C(O)O$), 1.99-2.10 (m, 2H, $CH_3C(O)CH_2CH_2C(O)O$), 2.12-2.22 (m, 2H, $CH_3C(O)CH_2CH_2C(O)O$), 2.76 (m, 1H, H-5c), 2.91 (t, 1H, $J_{3,4}$ = 9.5, $J_{4,5}$ = 10.0 Hz, H-4c), 3.17-3.23 (m, 3H, $OCH_2CH_2CH_2NHZ$, H-3c), 3.41-3.44 (m, 2H, $OCH_2CH_2CH_2NHZ$, H-4a), 3.56 (t, 1H, $J_{3,4}$ = 9.5, $J_{4,5}$ = 9.5 Hz, H-4b), 3.66-3.74 (m, 3H, $OCH'_2CH_2CH_2NHZ$, H-5a, H-5b), 3.97 (dd, 1H, $J_{2,3}$ = 3.0, $J_{3,4}$ = 9.5 Hz, H-3a), 4.20 (m, 2H, H-1c, H-3b), 4.45 (d, 1H, *J* = 12.0 Hz, $PhCH_2$), 4.53 (d, 1H, *J* = 11.5 Hz, $PhCH'_2$), 4.61 (d, 1H, *J* = 12.0

Hz, PhC*H''*$_2$), 4.63 (d, 1H, *J* = 11.5 Hz, PhC*H'''*$_2$), 4.71 (d, 1H, *J* = 11.5 Hz, PhC*H''''*$_2$), 4.77 (s, 1H, H-1a), 4.84 (m, 2H, N*H*, PhC*H'''''*$_2$), 4.95 (t, 1H, $J_{1,2}$ = 8.0, $J_{2,3}$ = 10.5 Hz, H-2c), 5.00 (s, 2H, PhCH_2OC(O)), 5.14 (s, 1H, H-1b), 5.30 (d, 1H, $J_{2,3}$ = 3.0 Hz, H-2a), 5.32 (d, 1H, $J_{2,3}$ = 3.0 Hz, H-2b), 7.13-8.05 (m, 30H, H_{arom}); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 17.7 (C-6c), 17.9 (C-6b), 18.1 (C-6a), 27.6 ($CH_3C(O)CH_2CH_2C(O)O$), 29.6 ($CH_3C(O)CH_2CH_2C(O)O$), 31.6 ($OCH_2CH_2CH_2NHZ$), 37.3 ($CH_3C(O)CH_2CH_2C(O)O$), 38.5 ($OCH_2CH_2CH_2NHZ$), 65.6 ($OCH_2CH_2CH_2NHZ$), 66.6 (PhCH_2OC(O)), 67.2 (C-4c), 67.8 (C-5a), 68.6 (C-5b), 70.6 (C-5c), 71.9 (C-2a), 72.7 (C-2b), [73.4, 74.3, 74.4 (PhCH_2)], 75.4 (C-2c), 77.2 (C-3b), 78.0 (C-3a), 79.6 (C-4a), 80.2 (C-4b), 80.9 (C-3c), 97.2 (C-1a), 98.8 (C-1b), 100.3 (C-1c), [127.0, 127.3, 127.8, 128.0, 128.1, 128.2, 128.3, 128.4, 128.5, 129.8, 129.9, 130.1, 133.0, 133.3, 133.4, 136.6, 137.5, 137.9, 138.6 (C_{arom})], 156.3 ($PhCH_2OC(O)$), [165.7, 165.8 (Ph*C*(O)O)], 171.1 ($CH_3C(O)CH_2CH_2C(O)O$), 206.1 ($CH_3C(O)CH_2CH_2C(O)O$); MALDI-TOF/MS: *m*/*z*: found: 1271.7 [*M*+Na]$^+$; MALDI-FTICR/MS: *m*/*z*: calcd for $C_{69}H_{76}N_4O_{18}Na$: 1271.5052; found: 1271.4893 [*M*+Na]$^+$.

3-[(*N*-Benzyloxycarbonyl)amino]propyl *O*-(4-azido-3-*O*-benzyl-4,6-dideoxy-β-D-glucopyranosyl)-(1→3)-*O*-(2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranosyl)-(1→3)-2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranoside (20)

Treatment of **19** (116 mg, 0.093 mmol) in CH_2Cl_2 (2.3 mL) with hydrazine acetate (8.6 mg, 0.093 mmol) in MeOH (0.23 mL) according to the general procedure for cleavage of the levulinoyl ester gave compound **20** as white solid (100 mg, 93%). R_f = 0.36 (hexane/EtOAc 2:1); $[\alpha]_D^{27}$ = +11.0 (*c* = 0.06 in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.88 (d, 3H, $J_{5,6}$ = 6.0 Hz, H-6c), 1.13 (d, 3H, $J_{5,6}$ = 6.0 Hz, H-6b), 1.26 (d, 3H, $J_{5,6}$ =

5.5 Hz, H-6a), 1.73 (m, 2H, $OCH_2CH_2CH_2NHZ$), 2.74 (m, 1H, H-5c), 2.83 (t, 1H, $J_{3,4}$ = 9.5, $J_{4,5}$ = 10.0 Hz, H-4c), 3.12 (t, 1H, $J_{2,3}$ = 9.0, $J_{3,4}$ = 9.5 Hz, H-3c), 3.22 (m, 2H, $OCH_2CH_2CH_2NHZ$), 3.30 (t, 1H, $J_{1,2}$ = 8.0, $J_{2,3}$ = 9.0 Hz, H-2c), 3.40 (m, 1H, $OCH_2CH_2CH_2NHZ$), 3.49 (t, 1H, $J_{3,4}$ = 9.0, $J_{4,5}$ = 10.0 Hz, H-4b), 3.54 (t, 1H, $J_{3,4}$ = 9.0, $J_{4,5}$ = 10.0 Hz, H-4a), 3.64-3.71 (m, 2H, $OCH'_2CH_2CH_2NHZ$, H-5a), 3.78 (m, 1H, H-5b), 4.05 (dd, 1H, $J_{2,3}$ = 3.0, $J_{3,4}$ = 9.5 Hz, H-3b), 4.08 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1c), 4.21 (dd, 1H, $J_{2,3}$ = 3.0, $J_{3,4}$ = 9.0 Hz, H-3a), 4.56 (d, 1H, J = 10.5 Hz, $PhCH_2$), 4.60 (d, 1H, J = 10.5 Hz, $PhCH'_2$), 4.66 (d, 1H, J = 11.0 Hz, $PhCH''_2$), 4.71 (d, 1H, J = 12.0 Hz, $PhCH'''_2$), 4.75 (d, 1H, J = 12.0 Hz, $PhCH''''_2$), 4.76 (s, 1H, H-1a), 4.83 (broad, 1H, NH), 4.92 (d, 1H, J = 11.0 Hz, $PhCH'''''_2$), 5.00 (s, 2H, $PhCH_2OC(O)$), 5.12 (s, 1H, H-1b), 5.32 (d, 1H, $J_{2,3}$ = 3.0 Hz, H-2a), 5.36 (d, 1H, $J_{2,3}$ = 3.0 Hz, H-2b), 7.19-8.01 (m, 30H, H_{arom}); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 17.9 (C-6c), 18.1 (C-6a, C-6b), 29.6 ($OCH_2CH_2CH_2NHZ$), 38.5 ($OCH_2CH_2CH_2NHZ$), 65.6 ($OCH_2CH_2CH_2NHZ$), 66.6 ($PhCH_2OC(O)$), 66.9 (C-5c), 67.9 (C-5a), 68.7 (C-5b), 70.6 (C-4c), 72.5 (C-2a, C-2b), 74.6 (C-2c), [74.7, 75.0, 75.3 ($PhCH_2$)], 75.4 (C-3b), 77.6 (C-3a), 80.1 (C-4a, C-4b), 82.2 (C-3c), 97.1 (C-1a), 99.0 (C-1b), 103.0 (C-1c), [127.8, 127.9, 128.0, 128.1, 128.2, 128.3, 128.4, 128.5, 128.6, 129.8, 130.0, 133.3, 133.4, 136.6, 137.8, 138.0 (C_{arom})], 156.4 ($PhCH_2OC(O)$), [165.6, 165.8 ($PhC(O)O$)]; MALDI-TOF/MS: m/z: found: 1172.7 $[M+Na]^+$; MALDI-FTICR/MS: m/z: calcd for $C_{64}H_{70}N_4O_{16}Na$: 1173.4685; found: 1173.4588 $[M+Na]^+$.

3-[(*N*-Benzyloxycarbonyl)amino]propyl *O*-(4-azido-3-*O*-benzyl-4,6-dideoxy-2-*O*-methyl-β-D-glucopyranosyl)-(1→3)-*O*-(2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranosyl)-(1→3)-2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranoside (21)

Method A: Sodium acetate (0.63 g, 7.68 mmol) and $PdCl_2$ (0.38 g, 2.14 mmol) was added to a solution of **8** (0.594 g, 1.78 mmol) in AcOH/H_2O 9:1 (60 mL). The reaction mixture was stirred at room temperature overnight, and then filtered through Celite. The filtrate was concentrated to dryness and the residue was co-evaporated with toluene (2×60 mL). Purification of the crude product by column chromatography on silica gel (hexane/EtOAc 2:1) afforded the hemiacetal compound. To a solution of this compound in CH_2Cl_2 (30 mL) was added trichloroacetonitrile (1.79 mL, 17.85 mmol) and DBU (0.11 mL, 0.74 mmol). The reaction mixture was stirred at room temperature for 5 h, and then concentrated to dryness. Purification of the crude product by column chromatography on silica gel (hexane/EtOAc 2:1+0.5%TEA) afforded imidate donor **9** as an α/β mixture 9:1 (0.622 g, 85%). A mixture of acceptor **18** (1.22 g, 1.37 mmol), donor **9** (0.622 g, 1.51 mmol) and 4 Å powdered molecular sieves (1.85 g) in dry acetonitrile (23 mL) was stirred at 0 °C for 1 h, and then cooled to -40 °C. A solution of $BF_3 \cdot Et_2O$ (0.28 mL, 2.27 mmol) was added slowly. The mixture was stirred at -40 °C for 1 h, and then neutralized with triethylamine. The solution was filtered through Celite, washed with MeOH/CH_2Cl_2 5:95 (20 mL), and the combined filtrates were concentrated to dryness. Purification of the crude product by column chromatography (hexane/EtOAc 3:1) on silica gel afforded the desired product **21** as α/β 1:4 mixture (1.38 g, 86%).

Method B: Methyl iodide (0.20 mL, 3.24 mmol) and silver(I) oxide (0.37 g, 1.60 mmol) were added to a solution of **20** (93 mg, 0.08 mmol) in THF (2 mL). Dimethyl

sulfide (1 µL, 0.014 mmol) was added as catalyst. The flask was wrapped by aluminium foil to exclude light. The reaction mixture was stirred at room temperature overnight, and then filtered through Celite. The filtrate was concentrated to dryness. Purification of the crude product by column chromatography (hexane/EtOAc 3:1) on silica gel afforded the desired product **21** as colorless oil (48 mg, 51%). R_f = 0.56 (hexane/EtOAc 2:1); $[\alpha]_D^{27}$ = +82.0 (*c* = 0.2 in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.83 (d, 3H, $J_{5,6}$ = 6.0 Hz, H-6c), 1.12 (d, 3H, $J_{5,6}$ = 6.0 Hz, H-6b), 1.24 (d, 3H, $J_{5,6}$ = 5.5 Hz, H-6a), 1.73 (m, 2H, $OCH_2CH_2CH_2NHZ$), 2.74 (m, 1H, H-5c), 2.84 (t, 1H, $J_{3,4}$ = 10.5, $J_{4,5}$ = 10.0 Hz, H-4c), 2.91 (t, 1H, $J_{1,2}$ = 8.0, $J_{2,3}$ = 9.0 Hz, H-2c), 3.11 (t, 1H, $J_{2,3}$ = 9.0, $J_{3,4}$ = 9.5 Hz, H-3c), 3.22 (m, 2H, $OCH_2CH_2CH_2NHZ$), 3.36 (s, 3H, OCH_3), 3.39 (m, 1H, $OCH_2CH_2CH_2NHZ$), 3.50 (t, 1H, $J_{3,4}$ = 9.5, $J_{4,5}$ = 10.0 Hz, H-4b), 3.54 (t, 1H, $J_{3,4}$ = 9.5, $J_{4,5}$ = 10.0 Hz, H-4a), 3.67 (m, 2H, $OCH'_2CH_2CH_2NHZ$, H-5a), 3.76 (m, 1H, H-5b), 4.10 (dd, 1H, $J_{2,3}$ = 3.0, $J_{3,4}$ = 9.5 Hz, H-3b), 4.20 (dd, 1H, $J_{2,3}$ = 3.0, $J_{3,4}$ = 9.5 Hz, H-3a), 4.31 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1c), 4.52 (d, 1H, *J* = 11.0 Hz, $PhCH_2$), 4.59 (d, 1H, *J* = 10.5 Hz, $PhCH'_2$), 4.65 (d, 1H, *J* = 11.0 Hz, $PhCH''_2$), 4.72 (d, 1H, *J* = 11.5 Hz, $PhCH'''_2$), 4.75 (d, 1H, *J* = 12.0 Hz, $PhCH''''_2$), 4.76 (s, 1H, H-1a), 4.80 (d, 1H, *J* = 10.5 Hz, $PhCH'''''_2$), 4.82 (broad, 1H, NH), 5.00 (s, 2H, $PhCH_2OC(O)$), 5.14 (s, 1H, H-1b), 5.30 (d, 1H, $J_{2,3}$ = 3.0 Hz, H-2a), 5.38 (d, 1H, $J_{2,3}$ = 3.0 Hz, H-2b), 7.19-8.02 (m, 30H, H_{arom}); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 17.8 (C-6c), 18.0 (C-6b), 18.1 (C-6a), 29.6 ($OCH_2CH_2CH_2NHZ$), 38.5 ($OCH_2CH_2CH_2NHZ$), 60.4 (OCH_3), 65.6 ($OCH_2CH_2CH_2NHZ$), 66.6 ($PhCH_2OC(O)$), 67.3 (C-5c), 67.9 (C-5a), 68.6 (C-5b), 70.2 (C-4c), 72.7 (C-2a), 73.2 (C-2b), [74.2, 75.2, 75.5 ($PhCH_2$)], 75.9 (C-3b), 78.0 (C-3a), 80.0 (C-4a), 80.5 (C-4b), 82.5 (C-3c), 84.3 (C-2c), 97.1 (C-1a), 99.2 (C-1b), 102.9 (C-1c), [127.6, 127.8, 127.9, 128.0, 128.2, 128.3, 128.4,

128.5, 129.7, 129.8, 129.9, 130.1, 133.0, 133.3, 137.8, 137.9, 138.2 (C_{arom})], 156.3 ($PhCH_2OC(O)$), [165.5, 165.8 ($PhC(O)O$)]; MALDI-TOF/MS: *m*/*z*: found: 1187.8 $[M+Na]^+$; MALDI-FTICR/MS: *m*/*z*: calcd for $C_{65}H_{72}N_4O_{16}Na$: 1187.4841; found: 1187.4715 $[M+Na]^+$.

3-[(*N*-Benzyloxycarbonyl)amino]propyl *O*-(4-(3-hydroxy-3-methylbutanamido)-3-*O*-benzyl-4,6-dideoxy-2-*O*-methyl-β-D-glucopyranosyl)-(1→3)-*O*-(2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranosyl)-(1→3)-2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranoside (22)

Treatment of **21** (0.71 g, 0.61 mmol), 1,3-propanedithiol (1.26 mL, 12.55 mmol) in pyridine (43 mL) and H_2O (6.1 mL) with TEA (1.28 mL, 9.15 mmol) according to the general procedure for azide reduction and introduction of C-4'' moitety gave free amine(0.69 g, 99%). Treatment of the free amine (0.47 g, 0.41 mmol) in DMF (20 mL) with β-hydroxyisovaleric acid (88 µL, 0.82 mmol) which was activated with HOAt (0.23 g, 1.64 mmol) and HATU (0.62 g, 1.64 mmol) in DMF (10 mL) for 1 h, and then added DIPEA (0.57 mL, 3.28 mmol) gave compound **22** as colorless oil (0.32 g, 63%) and its α-isomer (76 mg, 15%). R_f = 0.26 (hexane/EtOAc 1:1); $[\alpha]_D^{27}$ = +9.4 (*c* = 0.3 in $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$): δ = 0.73 (d, 3H, $J_{5,6}$ = 5.5 Hz, H-6c), 1.09 (s, 3H, $(CH_3)_2C(OH)CH_2C(O)NH$), 1.12 (d, 3H, $J_{5,6}$ = 6.0 Hz, H-6b), 1.18 (s, 3H, $(CH'_3)_2C(OH)CH_2C(O)NH$), 1.24 (d, 3H, $J_{5,6}$ = 5.5 Hz, H-6a), 1.74 (m, 2H, $OCH_2CH_2CH_2NHZ$), 1.99 (d, 1H, *J* = 15.0 Hz, $(CH_3)_2C(OH)CH_2C(O)NH$), 2.09 (d, 1H, *J* = 15.0 Hz, $(CH_3)_2C(OH)CH'_2C(O)NH$), 2.91 (m, 1H, H-5c), 2.98 (t, 1H, $J_{1,2}$ = 8.0, $J_{2,3}$ = 8.5 Hz, H-2c), 3.15 (t, 1H, $J_{2,3}$ = 8.5, $J_{3,4}$ = 9.0 Hz, H-3c), 3.22 (m, 2H, $OCH_2CH_2CH_2NHZ$), 3.38 (s, 3H, OCH_3), 3.39 (m, 2H, $OCH_2CH_2CH_2NHZ$, H-4c), 3.52 (t,

1H, $J_{3,4}$ = 9.0, $J_{4,5}$ = 9.5 Hz, H-4b), 3.54 (t, 1H, $J_{3,4}$ = 9.0, $J_{4,5}$ = 9.5 Hz, H-4a), 3.67 (m, 2H, OC*H'*$_2$CH$_2$CH$_2$NHZ, H-5a), 3.76 (m, 1H, H-5b), 4.12 (dd, 1H, $J_{2,3}$ = 3.5, $J_{3,4}$ = 9.0 Hz, H-3b), 4.21 (dd, 1H, $J_{2,3}$ = 3.0, $J_{3,4}$ = 9.0 Hz, H-3a), 4.34 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1c), 4.48 (d, 1H, J = 11.0 Hz, PhC*H*$_2$), 4.54 (d, 1H, J = 11.0 Hz, PhC*H'*$_2$), 4.60 (d, 1H, J = 10.5 Hz, PhC*H''*$_2$), 4.71 (d, 1H, J = 12.5 Hz, PhC*H'''*$_2$), 4.77 (s, 1H, H-1a), 4.83 (d, 1H, J = 11.5 Hz, PhC*H''''*$_2$), 4.85 (broad, 1H, N*H*), 4.95 (d, 1H, J = 11.0 Hz, PhC*H'''''*$_2$), 5.00 (s, 2H, PhC*H*$_2$OC(O)), 5.15 (s, 1H, H-1b), 5.30 (d, 1H, $J_{2,3}$ = 3.0 Hz, H-2a), 5.39 (d, 1H, $J_{2,3}$ = 3.5 Hz, H-2b), 7.14-8.00 (m, 30H, H_{arom}); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 17.7 (C-6c), 17.8 (C-6b), 18.1 (C-6a), 29.2 (OCH$_2$*C*H$_2$CH$_2$NHZ), [29.3, 29.7 ((*C*H$_3$)$_2$C(OH)CH$_2$C(O)NH)], 38.5 (OCH$_2$CH$_2$*C*H$_2$NHZ), 47.7 ((CH$_3$)$_2$C(OH)*C*H$_2$C(O)NH), 55.7 (C-4c), 60.3 (OCH_3), 65.6 (O*C*H$_2$CH$_2$CH$_2$NHZ), 66.6 (Ph*C*H$_2$OC(O)), 67.9 (C-5a), 68.6 (C-5b), 69.4 ((CH$_3$)$_2$*C*(OH)CH$_2$C(O)NH), 70.6 (C-5c), 72.8 (C-2a), 73.1 (C-2b), [73.5, 74.1, 75.5 (Ph*C*H$_2$)], 76.1 (C-3b), 78.1 (C-3a), 79.8 (C-3c), 80.0 (C-4a), 80.5 (C-4b), 84.4 (C-2c), 97.1 (C-1a), 99.2 (C-1b), 103.0 (C-1c), [127.5, 127.7, 127.8, 127.9, 128.0, 128.1, 128.2, 128.3, 128.4, 128.5, 129.7, 129.8, 129.9, 130.2, 133.0, 133.3, 137.9, 138.3, 138.5 (C_{arom})], 156.4 (PhCH$_2$O*C*(O)), [165.6, 165.9 (Ph*C*(O)O)], 172.2 ((CH$_3$)$_2$C(OH)CH$_2$*C*(O)NH); MALDI-TOF/MS: *m*/*z*: found: 1261.4; MALDI-FTICR/MS: *m*/*z*: calcd for $C_{70}H_{82}N_2O_{18}Na$: 1261.5460; found: 1261.5427 [*M*+Na]$^+$.

3-[(*N*-Benzyloxycarbonyl)amino]propyl *O*-(4-(3-hydroxy-3-methylbutanamido)-3-*O*-benzyl-4,6-dideoxy-β-D-glucopyranosyl)-(1→3)-*O*-(2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranosyl)-(1→3)-2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranoside (23)

Treatment of **20** (21 mg, 0.018 mmol), 1,3-propanedithiol (0.04 mL, 0.40 mmol) in pyridine (1.28 mL) and H_2O (0.92 mL) with TEA (0.03 mL, 0.27 mmol) according to the

general procedure for azide reduction and introduction of C-4'' moitety gave free amine (20 mg, 98%). Treatment the free amine (20 mg, 0.018 mmol) in DMF (2 mL) with β-hydroxyisovaleric acid (4 µL, 0.037 mmol) which was activated with HOAt (10 mg, 0.074 mmol) and HATU (28 mg, 0.074 mmol) in DMF (1 mL) for 1 h, and then added DIPEA (26 µL, 0.15 mmol) gave compound **23** as colorless oil (17 mg, 78%). R_f = 0.61 (hexane/EtOAc 1:2); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.79 (d, 3H, $J_{5,6}$ = 6.5 Hz, H-6c), 1.11 (s, 3H, $(CH_3)_2C(OH)CH_2C(O)NH$), 1.12 (d, 3H, $J_{5,6}$ = 6.5 Hz, H-6b), 1.14 (s, 3H, $(CH'_3)_2C(OH)CH_2C(O)NH$), 1.25 (d, 3H, $J_{5,6}$ = 5.5 Hz, H-6a), 1.74 (m, 2H, $OCH_2CH_2CH_2NHZ$), 2.06 (d, 1H, J = 15.0 Hz, $(CH_3)_2C(OH)CH_2C(O)NH$), 2.14 (d, 1H, J = 15.0 Hz, $(CH_3)_2C(OH)CH'_2C(O)NH$), 2.98 (m, 1H, H-5c), 3.21(m, 3H, $OCH_2CH_2CH_2NHZ$, H-3c), 3.36-3.42 (m, 3H, $OCH_2CH_2CH_2NHZ$, H-2c, H-4c), 3.52 (t, 1H, $J_{3,4}$ = 9.0, $J_{4,5}$ = 9.5 Hz, H-4b), 3.54 (t, 1H, $J_{3,4}$ = 9.0, $J_{4,5}$ = 10.0 Hz, H-4a), 3.68 (m, 2H, $OCH'_2CH_2CH_2NHZ$, H-5a), 3.77 (m, 1H, H-5b), 4.08 (dd, 1H, $J_{2,3}$ = 3.0, $J_{3,4}$ = 9.0 Hz, H-3b), 4.14 (d, 1H, $J_{1,2}$ = 7.5 Hz, H-1c), 4.21 (dd, 1H, $J_{2,3}$ = 3.0, $J_{3,4}$ = 9.0 Hz, H-3a), 4.49 (d, 1H, J = 11.0 Hz, $PhCH_2$), 4.57 (d, 1H, J = 11.0 Hz, $PhCH'_2$), 4.60 (d, 1H, J = 10.5 Hz, $PhCH''_2$), 4.67 (d, 1H, J = 11.0 Hz, $PhCH'''_2$), 4.73 (d, 1H, J = 11.0 Hz, $PhCH''''_2$), 4.77 (s, 1H, H-1a), 4.86 (broad, 1H, NH), 4.94 (d, 1H, J = 10.5 Hz, $PhCH'''''_2$), 5.00 (s, 2H, $PhCH_2OC(O)$), 5.13 (s, 1H, H-1b), 5.32 (d, 1H, $J_{2,3}$ = 3.0 Hz, H-2a), 5.38 (d, 1H, $J_{2,3}$ = 3.5 Hz, H-2b), 5.43 (d, 1H, J = 9.0 Hz, $(CH_3)_2C(OH)CH_2C(O)NH$), 7.19-8.02 (m, 30H, H_{arom}); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 17.7 (C-6c), 17.9 (C-6b), 18.1 (C-6a), [29.3, 29.4 ($(CH_3)_2C(OH)CH_2C(O)NH$)], 29.7 ($OCH_2CH_2CH_2NHZ$), 38.5 ($OCH_2CH_2CH_2NHZ$), 47.8 ($(CH_3)_2C(OH)CH_2C(O)NH$), 55.3 (C-4c), 65.6 ($OCH_2CH_2CH_2NHZ$), 66.6 ($PhCH_2OC(O)$), 67.9 (C-5a), 68.7 (C-5b), 69.5 ($(CH_3)_2C(OH)CH_2C(O)NH$), 70.9 (C-5c), 72.7 (C-2a), 72.8

(C-2b), [72.5, 74.6, 75.4 (Ph*C*H_2)], 74.9 (C-2c), 77.2 (C-3b), 78.0 (C-3a), 79.6 (C-3c), 80.0 (C-4a), 80.1 (C-4b), 97.1 (C-1a), 99.1 (C-1b), 103.1 (C-1c), [127.8, 127.9, 128.0, 128.1, 128.2, 128.3, 128.4, 128.6, 129.7, 129.8, 130.0, 133.1, 133.3, 137.9, 138.0, 138.4 (C_{arom})], 151.7 (PhCH_2O*C*(O)), [165.7, 165.9 (Ph*C*(O)O)], 172.3 ((CH_3)$_2$C(OH)CH_2*C*(O)NH); MALDI-TOF/MS: *m*/*z*: calcd for $C_{69}H_{80}N_2O_{18}Na$: 1247.5304; found: 1249.7 [*M*+Na]$^+$.

3-[(*N*-Benzyloxycarbonyl)amino]propyl *O*-(4-(3-methylbutanamido)-3-*O*-benzyl-4,6-dideoxy-2-*O*-methyl-α-D-glucopyranosyl)-(1→3)-*O*-(2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranosyl)-(1→3)-2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranoside (24)

The azide of compound **20** was reduced as described in the general procedures. Treatment of the free amine (0.12 g, 0.11 mmol) in DMF (5 mL) with DIPEA (0.15 mL, 0.86 mmol) and isovaleric acid (24 µL, 0.22 mmol) that was pre-activated with HOAt (57 mg, 0.42 mmol) and HATU (0.16 g, 0.42 mmol) in DMF (2.6 mL) for 1 h, gave compound **24** as colorless oil (78 mg, 0.064 mmol, 61%) and its α-isomer (19 mg, 0.016 mmol, 15%). R_f = 0.39 (hexane/EtOAc 1:1); $[\alpha]_D^{27}$ = +18.3 (*c* = 0.6 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.73 (d, 3H, $J_{5,6}$ = 6.0 Hz, H-6c), 0.78 (s, 3H, (C*H*$_3$)$_2$CHCH$_2$C(O)NH), 0.82 (s, 3H, (C*H'*$_3$)$_2$CHCH$_2$C(O)NH), 1.12 (d, 3H, $J_{5,6}$ = 6.0 Hz, H-6b), 1.24 (d, 3H, $J_{5,6}$ = 5.5 Hz, H-6a), 1.71 (m, 2H, OCH$_2$C*H*$_2$CH$_2$NHZ), 1.80-1.98 (m, 3H, (CH$_3$)$_2$CHC*H*$_2$C(O)NH, (CH$_3$)$_2$C*H*CH$_2$C(O)NH), 2.92 (m, 1H, H-5c), 2.97 (t, 1H, $J_{1,2}$ = 7.8, $J_{2,3}$ = 9.0 Hz, H-2c), 3.15-3.23 (m, 3H, OCH$_2$CH$_2$C*H*$_2$NHZ, H-3c), 3.37 (s, 3H, OCH$_3$), 3.39 (m, 2H, OC*H*$_2$CH$_2$CH$_2$NHZ, H-4c), 3.51 (t, 1H, $J_{3,4}$ = 9.0, $J_{4,5}$ = 9.0 Hz, H-4b), 3.56 (t, 1H, $J_{3,4}$ = 9.0, $J_{4,5}$ = 9.0 Hz, H-4a), 3.66 (m, 2H, OC*H'*$_2$CH$_2$CH$_2$NHZ, H-5a), 3.76 (m, 1H, H-5b), 4.13 (dd, 1H, $J_{2,3}$ = 3.0, $J_{3,4}$ = 9.0 Hz, H-3b), 4.21 (dd, 1H, $J_{2,3}$ = 3.0,

$J_{3,4}$ = 9.0 Hz, H-3a), 4.34 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1c), 4.48 (d, 1H, J = 12.0 Hz, PhCH_2), 4.53 (d, 1H, J = 10.8 Hz, PhCH'_2), 4.60 (d, 1H, J = 10.8 Hz, PhCH''_2), 4.69 (d, 1H, J = 12.0 Hz, PhCH'''_2), 4.77 (s, 1H, H-1a), 4.83 (d, 1H, J = 10.8 Hz, PhCH''''_2), 4.85 (broad, 1H, NH), 4.96 (d, 1H, J = 10.8 Hz, PhCH'''''_2), 5.00 (s, 2H, PhCH_2OC(O)), 5.15 (d, 1H, $J_{1,2}$ = 1.2 Hz, H-1b), 5.30 (dd, 1H, $J_{1,2}$ = 1.2, $J_{2,3}$ = 3.0 Hz, H-2a), 5.39 (dd, 1H, $J_{1,2}$ = 1.8, $J_{2,3}$ = 3.0 Hz, H-2b), 7.18-8.06 (m, 30H, H_{arom}); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 17.6 (C-6c), 17.8 (C-6b), 18.1 (C-6a), [22.4, 22.5 ($(CH_3)_2$CHCH$_2$C(O)NH)], 25.9 ($(CH_3)_2$CHCH$_2$C(O)NH), 29.5 (OCH$_2$CH$_2$CH$_2$NHZ), 38.4 (OCH$_2$CH$_2$CH$_2$NHZ), 46.2 ($(CH_3)_2$CHCH$_2$C(O)NH), 55.7 (C-4c), 60.3 (OCH_3), 65.6 (OCH$_2$CH$_2$CH$_2$NHZ), 66.5 (PhCH$_2$OC(O)), 67.8 (C-5a), 68.5 (C-5b), 70.7 (C-5c), 72.7 (C-2a), 73.1 (C-2b), [73.3, 74.1, 75.5 (PhCH$_2$)], 76.0 (C-3b), 78.2 (C-3a), 79.7 (C-3c), 79.8 (C-4a), 80.4 (C-4b), 84.3 (C-2c), 97.0 (C-1a), 99.2 (C-1b), 103.0 (C-1c), [127.5, 127.6, 127.7, 127.9, 128.0, 128.1, 128.2, 128.3, 128.4, 128.5, 129.7, 129.8, 129.9, 130.1, 133.0, 133.3, 136.6, 137.9, 138.3, 138.4 (C_{arom})], 156.3 (PhCH$_2$OC(O)), [165.6, 165.9 (PhC(O)O)], 172.2 ($(CH_3)_2$CHCH$_2$C(O)NH); MALDI-TOF/MS: *m*/*z*: found: 1245.4; MALDI-FTICR/MS: *m*/*z*: calcd for $C_{70}H_{82}N_2O_{17}Na$: 1245.5511; found: 1245.5510 $[M+Na]^+$.

3-[(*N*-Benzyloxycarbonyl)amino]propyl *O*-(4-acetamido-3-*O*-benzyl-4,6-dideoxy-2-*O*-methyl-β-D-glucopyranosyl)-(1→3)-*O*-(2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranosyl)-(1→3)-2-*O*-benzoyl-4-*O*-benzyl-α-L-rhamnopyranoside (25)

The azide of compound **20** was reduced as described in the general procedures. Treatment the free amine (94 mg, 0.083 mmol) with acetic anhydride (0.016 mL, 0.17 mmol) in pyridine (0.014 mL, 0.17 mmol) and DMAP (1 mg, 0.008 mmol) gave compound **25** as colorless oil (64 mg, 66%) and its α-isomer (17 mg, 17%). R_f = 0.25

(hexane/EtOAc 2:3); $[\alpha]_D^{27}$ = +7.2 (*c* = 0.4 in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.72 (d, 3H, $J_{5,6}$ = 6.0 Hz, H-6c), 1.12 (d, 3H, $J_{5,6}$ = 6.0 Hz, H-6b), 1.23 (d, 3H, $J_{5,6}$ = 6.5 Hz, H-6a), 1.70 (s, 3H, C*H*$_3$C(O)NH), 1.73 (m, 2H, OCH$_2$C*H*$_2$CH$_2$NHZ), 2.90 (m, 1H, H-5c), 2.97 (t, 1H, $J_{1,2}$ = 8.0, $J_{2,3}$ = 8.5 Hz, H-2c), 3.12 (t, 1H, $J_{2,3}$ = 8.5, $J_{3,4}$ = 9.5 Hz, H-3c), 3.22 (m, 2H, OCH$_2$CH$_2$C*H*$_2$NHZ), 3.33 (t, 1H, $J_{3,4}$ = 9.5, $J_{4,5}$ = 10.0 Hz, H-4c), 3.38 (s, 3H, OCH$_3$), 3.39 (m, 1H, OC*H*$_2$CH$_2$CH$_2$NHZ), 3.51 (t, 1H, $J_{3,4}$ = 9.0, $J_{4,5}$ = 10.0 Hz, H-4b), 3.54 (t, 1H, $J_{3,4}$ = 9.5, $J_{4,5}$ = 8.5 Hz, H-4a), 3.67 (m, 2H, OC*H'*$_2$CH$_2$CH$_2$NHZ, H-5a), 3.75 (m, 1H, H-5b), 4.13 (dd, 1H, $J_{2,3}$ = 3.0, $J_{3,4}$ = 9.0 Hz, H-3b), 4.21 (dd, 1H, $J_{2,3}$ = 2.5, $J_{3,4}$ = 9.5 Hz, H-3a), 4.34 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1c), 4.48 (d, 1H, *J* = 11.5 Hz, PhC*H*$_2$), 4.53 (d, 1H, *J* = 11.0 Hz, PhC*H'*$_2$), 4.59 (d, 1H, *J* = 10.5 Hz, PhC*H''*$_2$), 4.70 (d, 1H, *J* = 12.0 Hz, PhC*H'''*$_2$), 4.77 (s, 1H, H-1a), 4.81 (d, 1H, *J* = 11.0 Hz, PhC*H''''*$_2$), 4.83 (broad, 1H, N*H*), 4.95 (d, 1H, *J* = 10.5 Hz, PhC*H'''''*$_2$), 5.00 (s, 2H, PhC*H*$_2$OC(O)), 5.15 (s, 1H, H-1b), 5.30 (d, 1H, $J_{2,3}$ = 2.5 Hz, H-2a), 5.40 (d, 1H, $J_{2,3}$ = 3.0 Hz, H-2b), 7.19-8.00 (m, 30H, H_{arom}); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 17.5 (C-6c), 17.8 (C-6b), 18.1 (C-6a), 23.5 (*C*H$_3$C(O)NH), 29.7 (OCH$_2$*C*H$_2$CH$_2$NHZ), 38.5 (OCH$_2$CH$_2$*C*H$_2$NHZ), 55.9 (C-4c), 60.3 (OCH$_3$), 65.6 (O*C*H$_2$CH$_2$CH$_2$NHZ), 66.6 (Ph*C*H$_2$OC(O)), 67.8 (C-5a), 68.5 (C-5b), 70.7 (C-5c), 72.8 (C-2a), 73.1 (C-2b), [73.5, 74.2, 75.5 (Ph*C*H$_2$)], 76.0 (C-3b), 78.3 (C-3a), 79.6 (C-3c), 79.8 (C-4a), 80.5 (C-4b), 84.5 (C-2c), 97.0 (C-1a), 99.3 (C-1b), 103.0 (C-1c), [127.6, 127.8, 127.9, 128.0, 128.2, 128.3, 128.4, 128.5, 129.7, 129.8, 129.9, 130.2, 133.0, 133.3, 136.6, 137.9, 138.3, 138.5 (C_{arom})], 156.3 (PhCH$_2$O*C*(O)), [165.6, 165.9 (Ph*C*(O)O)], 169.8 (CH$_3$*C*(O)NH); MALDI-TOF/MS: *m*/*z*: found: 1204.3; MALDI-FTICR/MS: *m*/*z*: calcd for $C_{67}H_{76}N_2O_{17}Na$: 1203.5042; found: 1203.5040 [*M*+Na]$^+$.

3-Aminopropyl *O*-(4-(3-hydroxy-3-methylbutanamido)-4,6-dideoxy-2-*O*-methyl-β-D-glucopyranosyl)-(1→3)-*O*-(α-L-rhamnopyranosyl)-(1→3)-α-L-rhamnopyranoside (1)

Treatment of **22** (138.0 mg, 111.3 µmol) in MeOH/CH_2Cl_2 (2 mL/2 mL) with NaOMe (pH 8-10) according to the general procedure for global deprotection gave deacetylated product (110.1 mg, 96%). Treatment of the partially deprotected compound (110.1 mg, 106.7 µmol) in *tert*-butanol/H_2O/AcOH (10 mL/0.25 mL/0.25 mL) with a catalytic amount of Pd/C under an atmosphere of hydrogen gave compound **1** as white solid (65.5 mg, 98%). R_f = 0.50 (CH_3CN/H_2O/AcOH 40:20:1); ^{1}H NMR (500 MHz, D_2O): δ = 1.13 (d, 3H, $J_{5,6}$ = 6.0 Hz, H-6c), 1.21 (broad, 12H, (CH_3)$_2$C(OH)CH_2C(O)NH, H-6a, H-6b), 1.92 (m, 2H, $OCH_2CH_2CH_2NH_2$), 2.36 (s, 2H, $(CH_3)_2C(OH)CH_2C(O)NH$), 3.00-3.15 (m, 4H, $OCH_2CH_2CH_2NH_2$, H-2c, H-4c), 3.42-3.52 (m, 5H, $OCH_2CH_2CH_2NH_2$, H-4a, H-4b, H-3c, H-5c), 3.53 (s, 3H, OCH_3), 3.61 (m, 1H, H-5a), 3.69-3.76 (m, 3H, H-3a, H-5b, $OCH'_2CH_2CH_2NH_2$), 3.90 (d, 1H, $J_{3,4}$ = 10.0 Hz, H-3b), 3.93 (s, 1H, H-2a), 4.17 (s, 1H, H-2b), 4.63 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1c), 4.65 (s, 1H, H-1a), 4.93 (s, 1H, H-1b); ^{13}C NMR (75 MHz, D_2O): δ = [16.7, 16.8 (C-6a, C-6b)], 17.2 (C-6c), 26.8 ($OCH_2CH_2CH_2NH_2$), [28.2, 28.4 ((CH_3)$_2$C(OH)CH_2C(O)NH)], 37.6 ($OCH_2CH_2CH_2NH_2$), 49.0 ($(CH_3)_2C(OH)CH_2C(O)NH$), 56.7 (C-4c), 60.2 (OCH_3), 65.0 ($OCH_2CH_2CH_2NH_2$), 68.9 (C-5a), 69.4 (C-5b), 69.9 ($(CH_3)_2C(OH)CH_2C(O)NH$), 70.0 (C-2a), 70.3 (C-2b), [70.9, 71.2, 71.4, 72.9 (C-4a, C-4b, C-3c, C-5c)], 78.4 (C-3a), 79.7 (C-3b), 83.4 (C-2c), 99.8 (C-1a), 102.3 (C-1b), 103.8 (C-1c), 174.2 ($(CH_3)_2C(OH)CH_2C(O)NH$); MALDI-TOF/MS: *m*/*z*: found: 649.6; MALDI-FTICR/MS: *m*/*z*: calcd for $C_{27}H_{50}N_2O_{14}Na$: 649.3160; found: 649.3156 $[M+Na]^+$.

3-Aminopropyl *O*-(4,6-dideoxy-4-(3-hydroxy-3-methylbutanamido)-β-D-glucopyranosyl)-(1→3)-*O*-(α-L-rhamnopyranosyl)-(1→3)-α-L-rhamnopyranoside (2)

Treatment of **23** (17.0 mg, 13.9 µmol) in MeOH/CH_2Cl_2 (0.5 mL/0.5 mL) with NaOMe (pH 8-10) according to the general procedure for global deprotection gave the deacetylated product (14.0 mg, 99%). Treatment of the partially deprotected compound (14.0 mg, 13.8 µmol) in *tert*-butanol/H_2O/AcOH (2 mL/0.05 mL/0.05 mL) with a catalytic amount of Pd/C under an atmosphere of hydrogen gave compound **2** as white solid (8.1 mg, 96%). R_f = 0.30 (CH_3CN/H_2O/AcOH 40:20:1); ^{1}H NMR (300 MHz, D_2O): δ = 1.11 (d, 3H, $J_{5,6}$ = 6.0 Hz, H-6c), 1.17 (broad, 12H, $(CH_3)_2C(OH)CH_2C(O)NH$, H-6a, H-6b), 1.84 (m, 2H, $OCH_2CH_2CH_2NH_2$), 2.33 (s, 2H, $(CH_3)_2C(OH)CH_2C(O)NH$), 2.96 (m, 2H, $OCH_2CH_2CH_2NH_2$), 3.11 (t, 1H, $J_{3,4}$ = 7.2, $J_{4,5}$ = 7.2 Hz, H-4c), 3.27 (t, 1H, $J_{1,2}$ = 7.8, $J_{2,3}$ = 8.4 Hz, H-2c), 3.36-3.60 (m, 6H, $OCH_2CH_2CH_2NH_2$, H-4a, H-5a, H-4b, H-3c, H-5c), 3.64-3.75 (m, 3H, H-3a, H-5b, $OCH'_2CH_2CH_2NH_2$), 3.86 (m, 2H, H-2a, H-3b), 4.14 (s, 1H, H-2b), 4.58 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1c), 4.63 (s, 1H, H-1a), 4.88 (s, 1H, H-1b); ^{13}C NMR (75 MHz, D_2O): δ = [16.7, 17.2 (C-6a, C-6b, C-6c)], 27.1 ($OCH_2CH_2CH_2NH_2$), [28.2, 28.4 ($(CH_3)_2C(OH)CH_2C(O)NH$)], 37.6 ($OCH_2CH_2CH_2NH_2$), 49.0 ($(CH_3)_2C(OH)CH_2C(O)NH$), 56.7 (C-4c), 65.1 ($OCH_2CH_2CH_2NH_2$), 68.9 (C-5a), 69.1 (C-5b), 69.9 ($(CH_3)_2C(OH)CH_2C(O)NH$), 70.0 (C-2a), 70.3 (C-2b), [71.1, 71.3, 71.4, 73.5 (C-4a, C-4b, C-3c, C-5c)], 74.2 (C-2c), 78.4 (C-3a), 79.7 (C-3b), 99.8 (C-1a), 102.3 (C-1b), 103.6 (C-1c), 174.2 ($(CH_3)_2C(OH)CH_2C(O)NH$); MALDI-TOF/MS: *m*/*z*: found: 635.3; MALDI-FTICR/MS: *m*/*z*: calcd for $C_{26}H_{48}N_2O_{14}Na$: 635.3003; found: 635.3000 $[M+Na]^+$.

3-Aminopropyl *O*-(4,6-dideoxy-4-(3-methylbutanamido)-2-*O*-methyl-β-D-glucopyranosyl)-(1→3)-*O*-(α-L-rhamnopyranosyl)-(1→3)-α-L-rhamnopyranoside (3)

Treatment of **24** (47.0 mg, 38.4 µmol) in MeOH/CH_2Cl_2 (0.5 mL/0.5 mL) with NaOMe (pH 8-10) according to the general procedure for global deprotection gave the deacetylated product (39.0 mg, 100%). Treatment of the partially deprotected compound (39.0 mg, 38.4 µmol) in *tert*-butanol/H_2O/AcOH (4 mL/0.1 mL/0.1 mL) with a catalytic amount of Pd/C under an atmosphere of hydrogen gave compound **3** as white solid (22.1 mg, 94%). R_f = 0.40 (CH_3CN/H_2O/AcOH 60:20:1); ^{1}H NMR (500 MHz, D_2O): δ = 0.77 (m, 6H, $(CH_3)_2CHCH_2C(O)NH$), 1.06 (d, 3H, $J_{5,6}$ = 6.0 Hz, H-6c), 1.14 (m, 6H, H-6a, H-6b), 1.84 (broad, 3H, $OCH_2CH_2CH_2NH_2$, $(CH_3)_2CHCH_2C(O)NH$), 1.99 (m, 2H, $(CH_3)_2CHCH_2C(O)NH$), 2.94-2.99 (m, 4H, $OCH_2CH_2CH_2NH_2$, H-2c, H-4c), 3.33-3.46 (m, 5H, $OCH_2CH_2CH_2NH_2$, H-4a, H-4b, H-3c, H-5c), 3.47 (s, 3H, OCH_3), 3.55 (m, 1H, H-5a), 3.63 (dd, 1H, $J_{2,3}$ = 3.5, $J_{3,4}$ = 9.5 Hz, H-3a), 3.69 (m, 2H, H-5b, $OCH'_2CH_2CH_2NHZ$), 3.83 (dd, 1H, $J_{2,3}$ = 3.0, $J_{3,4}$ = 10.0 Hz, H-3b), 3.87 (s, 1H, H-2a), 4.12 (s, 1H, H-2b), 4.57 (d, 1H, $J_{1,2}$ = 8.5 Hz, H-1c), 4.61 (s, 1H, H-1a), 4.86 (s, 1H, H-1b); ^{13}C NMR (75 MHz, D_2O): δ = [16.7, 16.8 (C-6a, C-6b)], 17.2 (C-6c), [21.7, 21.8 ($(CH_3)_2CHCH_2C(O)NH$)], 22.3 (CH_3COOH), 26.2 ($(CH_3)_2CHCH_2C(O)NH$), 26.8 ($OCH_2CH_2CH_2NH_2$), 37.6 ($OCH_2CH_2CH_2NH_2$), 45.5 ($(CH_3)_2CHCH_2C(O)NH$), 56.7 (C-4c), 60.2 (OCH_3), 65.1 ($OCH_2CH_2CH_2NH_2$), 69.0 (C-5a), 69.4 (C-5b), 70.0 (C-2a), 70.1 (C-2b), [71.0, 71.3, 71.5, 73.0 (C-4a, C-4b, C-3c, C-5c)], 78.4 (C-3a), 79.8 (C-3b), 83.5 (C-2c), 99.9 (C-1a), 102.3 (C-1b), 103.8 (C-1c), 177.2 (CH_3COOH), 179.7 ($(CH_3)_2CHCH_2C(O)NH$); MALDI-TOF/MS: *m*/*z*: found: 633.2; MALDI-FTICR/MS: *m*/*z*: calcd for $C_{27}H_{50}N_2O_{13}Na$: 633.3211; found: 633.3207 $[M+Na]^+$.

3-Aminopropyl *O*-(4-acetamido-4,6-dideoxy-2-*O*-methyl-β-D-glucopyranosyl)-(1→3)-*O*-(α-L-rhamnopyranosyl)-(1→3)-α-L-rhamnopyranoside (4)

Treatment of **25** (27.2 mg, 23.0 µmol) in MeOH/CH_2Cl_2 (0.5 mL/0.5 mL) with NaOMe (pH 8-10) according to the general procedure for global deprotection gave the deacetylated product (22.9 mg, quantitative). Treatment of the partially deprotected compound (22.9 mg, 23.5 µmol) in *tert*-butanol/H_2O/AcOH (4 mL/0.1 mL/0.1 mL) with a catalytic amount of Pd/C under an atmosphere of hydrogen gave compound **4** as white solid (12.1 mg, 92%). R_f = 0.45 (CH_3CN/H_2O/AcOH 40:20:1); ^{1}H NMR (500 MHz, D_2O): δ = 1.04 (d, 3H, $J_{5,6}$ = 5.5 Hz, H-6c), 1.13 (d, 3H, $J_{5,6}$ = 6.5 Hz, H-6b), 1.16 (d, 3H, $J_{5,6}$ = 6.5 Hz, H-6a), 1.87 (s, 3H, C*H*$_3$C(O)NH), 1.89 (m, 2H, $OCH_2CH_2CH_2NH_2$), 2.93-3.08 (m, 4H, $OCH_2CH_2CH_2NH_2$, H-2c, H-4c), 3.34-3.46 (m, 5H, $OCH_2CH_2CH_2NH_2$, H-4a, H-4b, H-3c, H-5c), 3.47 (s, 3H, OCH_3), 3.55 (m, 1H, H-5a), 3.64-3.71 (m, 3H, H-3a, H-5b, $OCH'_2CH_2CH_2NHZ$), 3.83 (d, 1H, $J_{3,4}$ = 10.0 Hz, H-3b), 3.87 (s, 1H, H-2a), 4.12 (s, 1H, H-2b), 4.59 (d, 1H, $J_{1,2}$ = 8.0 Hz, H-1c), 4.61 (s, 1H, H-1a), 4.87 (s, 1H, H-1b); ^{13}C NMR (75 MHz, D_2O): δ = [16.8, 17.0 (C-6a, C-6b, C-6c)], 22.3 (CH_3COOH), 26.3 ($CH_3C(O)NH$), 26.8 ($OCH_2CH_2CH_2NH_2$), 37.6 ($OCH_2CH_2CH_2NH_2$), 56.9 (C-4c), 60.2 (OCH_3), 65.1 ($OCH_2CH_2CH_2NH_2$), 68.2 (C-5a), 69.0 (C-5b), 69.4 (C-2a), 70.0 (C-2b), [71.0, 71.3, 71.5, 73.0 (C-4a, C-4b, C-3c, C-5c)], 78.4 (C-3a), 79.7 (C-3b), 83.3 (C-2c), 99.9 (C-1a), 102.3 (C-1b), 103.8 (C-1c), 174.8 ($CH_3C(O)NH$), 178.4 (CH_3COOH); MALDI-TOF/MS: *m*/*z*: found: 591.2; MALDI-FTICR/MS: *m*/*z*: calcd for $C_{24}H_{44}N_2O_{13}Na$: 591.2741; found: 591.2737 $[M+Na]^+$.

General procedure for *S*-acetylthioglycolylamido derivatization of the aminopropyl spacer

The oligosaccharide **1** (10 mg, 0.016 mmol) was slurried in dry DMF (500 µL) and SAMA-OPfp (7.2 mg, 0.024 mmol) was added followed by dropwise addition of DIPEA (5.6 µL, 0.032 mmol). After stirring at room temperature for 2 h, the mixture was concentrated, co-evaporated twice with toluene and the residue purified by size-exclusion chromatography (Biogel P2 column, eluated with H_2O containing 1% *n*-butanol) to give, after lyophilization, the corresponding thioacetate (10.6 mg, 0.0144 mmol, 90%) as a white powder. In this manner, the thioacetamido derivatives of compounds **1**-**4** were prepared in yields of 85-95%.

General procedure for *S*-deacetylation

7% NH_3 (g) in DMF solution (200 µL) was added to a solution of the thioacetate derivative corresponding to trisaccharide **1** (2.6 mg, 3.5 µmol) in ddH_2O (40 µL) and the mixture was stirred under argon atmosphere. The reaction was monitored by MALDI-TOF showing the product peak of $[M+Na]^+$. After 1 h the solvent was evaporated under high-vacuum. The thiol derivatized trisaccharide was further dried under high vacuum for 30 min and then used immediately in conjugation without further purification.

General procedure for the conjugation of thiol derivatized trisaccharides to BSA-MI

The conjugations were performed as instructed by Pierce Endogen Inc. In short, the thiol derivative (2.5 equiv excess to available MI-groups on the protein), deprotected just prior to conjugation as described above, was dissolved in ddH_2O (100 µL) and added to a solution of the maleimide activated protein (2 mg) in conjugation buffer

sodium phosphate pH 7.2 containing EDTA and sodium azide (200 µL). The mixture was incubated for 2 h at room temperature and then purified by Millipore Centriplus centrifugal filter devices with a 10 kDa molecular cut-off. All centrifugations were performed at 8 ℃ for 25 min, spinning at 13×g. The reaction mixture was centrifuged and the filter washed with 10 mM Hepes buffer pH 6.5 (3×200 µL). The conjugate was retrieved and taken up in sodium phosphate buffer pH 7.4, 0.15 M sodium chloride (1 mL). This gave glycoconjugates with a carbohydrate/BSA ratio of 18:1 for trisaccharide **1**, 10:1 for 2''-OH-trisaccharide **2**, 9:1 for 4''-isovaleric acid trisaccharide **3** and 4:1 for trisaccharide 4''-HNAc-trisaccharide **4** as determined by Dubois' phenol-sulfuric acid total carbohydrate assay, quantitative monosaccharide analysis by HPAEC/PAD and Lowry protein concentration test.

Conjugation of thiol derivatized trisaccharide to KLH-BrAc

A solution of KLH (15 mg) in 0.1 M sodium phosphate buffer pH 7.2 containing 0.15 M NaCl (1.5 mL) was added to a solution of SBAP (6 mg) in DMSO (180 µL). The mixture was incubated for 2 h at room temperature and then purified using Millipore Centriplus centrifugal filter devices with a molecular cut-off of 30 kDa. All centrifugations were performed at 8 ℃ for 25 min. spinning at 3000 rpm. The reaction mixture was centrifuged off and the filter washed with conjugation buffer (2×750 µL). The activated protein was retrieved by spinning at 3000 rpm for 15 min at 8 ℃ and taken up in 0.1 mM sodium phosphate buffer pH 8.0 containing 5 mM EDTA (2 mL). The activated protein was added to a vial containing de-S-acetylated trisaccharide (2.6 mg) and the mixture was incubated at room temperature for 18 h. Purification was achieved using centrifugal filters as described above for the BSA-MI-trisaccharide conjugates. This

gave a glycoconjugate with 1042 trisaccharide residues/KLH molecule as determined by phenol/sulfuric acid total carbohydrate assay, quantitative monosaccharide analysis by HPAEC/PAD and Lowry protein concentration test.

Preparation of *Bacillus anthracis* Sterne $34F_2$ spores

Bacillus anthracis Sterne $34F_2$ was obtained from the CDC culture collection. Spores of *B. anthracis* Sterne $34F_2$ were prepared from liquid cultures of PA medium[41] grown at 37 °C, 200 rpm for six days. Spores were washed two times by centrifugation at 10000×g in cold (4 °C) sterile deionized water, purified in a 50% Reno-60 (Bracco Diagnostics Inc., Princeton, NJ) gradient (10000×g, 30 min, 4 °C) and washed a further four times in cold sterile deionized water. After suspension in sterile deionized water, spores were quantified with surface spread viable cell counts on brain heart infusion (BHI) agar plates (BD BBL, Sparks, MD). Spore suspensions were stored in water at -80 °C.

For the preparation of killed spores, 500-µL aliquots of spore suspensions in water, prepared as described above and containing approximately 3×10^8 CFU, were irradiated in 2.0 mL Sarstedt freezer tubes (Sarstedt, Newton, NC) in a gammacell irradiator with an absorbed dose of 2 million rads. Sterility after irradiation was monitored by spread-plating 10-µL aliquots of irradiated spore suspension on BHI agar plates. The plates were incubated for 72 h at 37 °C and monitored for colony growth. Absence of growth was taken as an indicator of sterility.

Preparation of antispore antiserum

All antisera were prepared in female New Zealand White rabbits (2.0-3.5 kg) purchased from Myrtle's Rabbitry (Thompson Station, TN). For antiserum production

each of two rabbits were inoculated intramuscularly at two sites in the dorsal hind quarters with 0.5 mL of washed live-spore or irradiated spore inoculum (3×10^6 total spores). Rabbits were vaccinated at 0, 14, 28, and 42 days. Serum was collected prior to the first immunization (pre-immune serum) and at 7 and 14 d after each injection of antigen. Terminal bleeds were collected 14 d after the last immunization. All animal protocols were approved by the CDC Animal Care and Use Committee (ACUC) and implemented under the direction of the CDC attending veterinarian.

Antibody-binding analyses

Binding of rabbit anti-live spore antiserum to synthetic oligosaccharide conjugates was done by enzyme-linked immunosorbent assay (ELISA). Briefly, Immulon II-HB flat bottom 96-well microtiter plates (Thermo Labsystems, Franklin, MA) were coated with 100 µL per well of the KLH-BrAc-**1** conjugate at a concentration of 0.03 µg per mL of carbohydrate content, corresponding to 0.5 µg per mL by protein content, or by the protein mcKLH by itself (0.5 µg per mL protein) in coating buffer (0.01 M PBS, pH 7.4). Plates were washed three times in wash buffer (0.01 M PBS, pH 7.4, 0.1% Tween-20) using an ELX405 microplate washer (BioTek Instruments Inc., Winooski, VT). Serial dilutions (100 µL per well) in blocking solution (0.01 M PBS, pH 7.4, 5% skim milk, 0.5% Tween-20) of either rabbit anti-spore antiserum from the day 49 bleed or pre-immune serum were then added and plates were incubated for 1 h 37 ℃. After incubation the plates were washed three times in wash buffer at which time a goat anti-rabbit IgG horseradish peroxidase conjugate (ICN Pharmaceuticals, Aurora, OH) was added (100 µL per well) and the incubation continued for 1 hour at 37 ℃. Plates were then washed three times in wash buffer and 100 µL per well of ABTS peroxidase

substrate was added (KPL, Gaithersburg, MD). Color development was stopped after 15 minutes at 37 °C by addition of 100 µL per well of ABTS peroxidase stop solution (KPL, Gaithersburg, MD). Optical density (OD) values were read at a wavelength of 410 nm (490 nm reference filter) with a MRX Revelation microtiter plate reader (Thermo Labsystems, Franklin, MA).

To test for competitive inhibition, the rabbit anti-live spore antiserum or the rabbit anti-irradiated spore antiserum was added together with unconjugated trisaccharide in blocking solution at a 6-, 12-, 25-, 50-, 100-, or 200-fold weight excess compared to weight of carbohydrate used for coating. The negative control consisted of uncoated wells incubated with the respective antiserum plus trisaccharide **1** at a concentration corresponding to "200-fold excess" of trisaccharide.

To explore competitive inhibition by synthetic saccharide analogues conjugated to bovine serum albumin (BSA; Pierce Biotechnology, Rockford, IL), rabbit anti-live spore antiserum was diluted 1:1600 in blocking solution. For each well 100 µL of the serum were mixed with either 100 µL blocking solution or 100 µL of BSA-MI-conjugate in blocking solution with a concentration corresponding to a 2-, 4-, 8-, 16-, 32-, 64-, or 128-fold weight excess of carbohydrate compared to carbohydrate used for coating. The four conjugates tested were: BSA-MI-**1**, BSA-MI-**2**, BSA-MI-**3**, and BSA-MI-**4**. First the serum and then the BSA-saccharide conjugate solutions were added to an uncoated microtiter plate and mixed by pipetting up and down before the well contents were transferred to a coated plate. The microtiter plates were incubated and developed as described above.

The data are reported as the means ±SD of triplicate measurements.[42]

2.6 References

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Chapter III

First Total Synthesis of a Tetrasaccharide Antigen Derived from *Burkholderia pseudomallei* and *Burkholderia mallei* – Creation of a Vaccine Candidate against Glanders and Meliodosis*

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3.1 Abstract

The *O*-polysaccharide (O-PS) antigens of *Burkholderia pseudomallei* and *Burkholderia mallei* lipopolysaccharides have become interesting targets for development of vaccines for melioidosis and glanders, respectively. The structures of the *O*-antigen polysaccharides of *B. pseudomallei* and *B. mallei* strains have recently been characterized and consist of the disaccharide repeating units, *3)-β-D-glucopyranose-(1→3)-6-deoxy-α-L-talopyranose-(1→*. In *B. pseudomallei*, ~33% of the 6-deoxy-α-L-talopyranose (6dTal*p*) residues have 2-*O*-methyl and 4-*O*-acetyl substitution while ~67% of residues possess only 2-*O*-acetyl substitution, whereas, *B. mallei* lacks 4-*O*-acetyl substitution and contains the 2-*O*-methyl or 2-*O*-acetyl substitution in the talose residue. In order to study the antigenicity of these carbohydrate epitopes, a convergent synthesis of tetrasaccharide containing two disaccharide repeating units, having the 4-*O*-acetyl and 2-*O*-methyl substitution in the 6dTal*p*, was developed. A glycosylation at the sterically hindered position (C-3 OH) of the 6dTal*p* residue was developed for the first time while constructing the *β-D-Glc-(1→3)-6-d-α-L-Tal* linkage. The oligosaccharide will be evaluated for its potential for use as vaccine component/diagnostic marker for melioidosis. Removal of the 4-*O*-acetyl substitution will provide the carbohydrate epitope, characteristic of *B. mallei* O-PS and further immunological studies will determine its importance in development of vaccine or diagnostic for glanders.

3.2 Introduction

Since its separation from the genus *Pseudomonas* in 1992, more than 20 species of *Burkholderia* have been identified. Although most species of *Burkholderia* are saprophytes or plant pathogens, two species, namely *Burkholderia pseudomallei* and *Burkholderia mallei* cause serious and potential fatal diseases in humans. *B. pseudomallei* and *B. mallei*, causative agents for infectious diseases such as melioidosis (Whitmore's disease) and glanders respectively,[1] have the potential for use as agents in biological warfare and terrorism. Both bacteria are listed as category B bioterrorism agents.[2] *Burkholderia* produce a number of cell surface carbohydrates including exopolysaccharides,[3-5] lipopolysaccharides with two different *O*-chain polysaccharides,[6, 7] and an unusual glycolipid.[8, 9] Recently, complex carbohydrate fragments derived from the lipopolysaccharide *O*-antigens (O-PS and O-PS II for *B. mallei* and *B. pseudomallei*, respectively) of these pathogens have been identified. The O-PS II moiety produced by *B. pseudomallei* is an unbranched heteropolymer consisting of disaccharide repeating units having the structure *3)-β-D-glucopyranose-(1→3)-6-deoxy-α-L-talopyranose-(1→* in which ~33% of the 6-deoxy-α-L-talopyranose (6dTal*p*) residues have 2-*O*-methyl and 4-*O*-acetyl substitution while ~67% of residues possess only 2-*O*-acetyl substitution.[6, 7, 10] The O-antigen (O-PS) expressed by *B. mallei* is virtually identical to O-PS II except that it lacks acetyl substitutions at the C-4 OH of the 6dTal*p* residues.[10] To evaluate the antigenicity of these carbohydrates and their possible uses as diagnostic markers and/or vaccine components, we aim to develop highly convergent syntheses of spacer-linked oligosaccharide fragments derived from

the *O*-side chain of *B. pseudomallei* and *B. mallei*. These compounds will be examined as vaccine and diagnostic candidates.

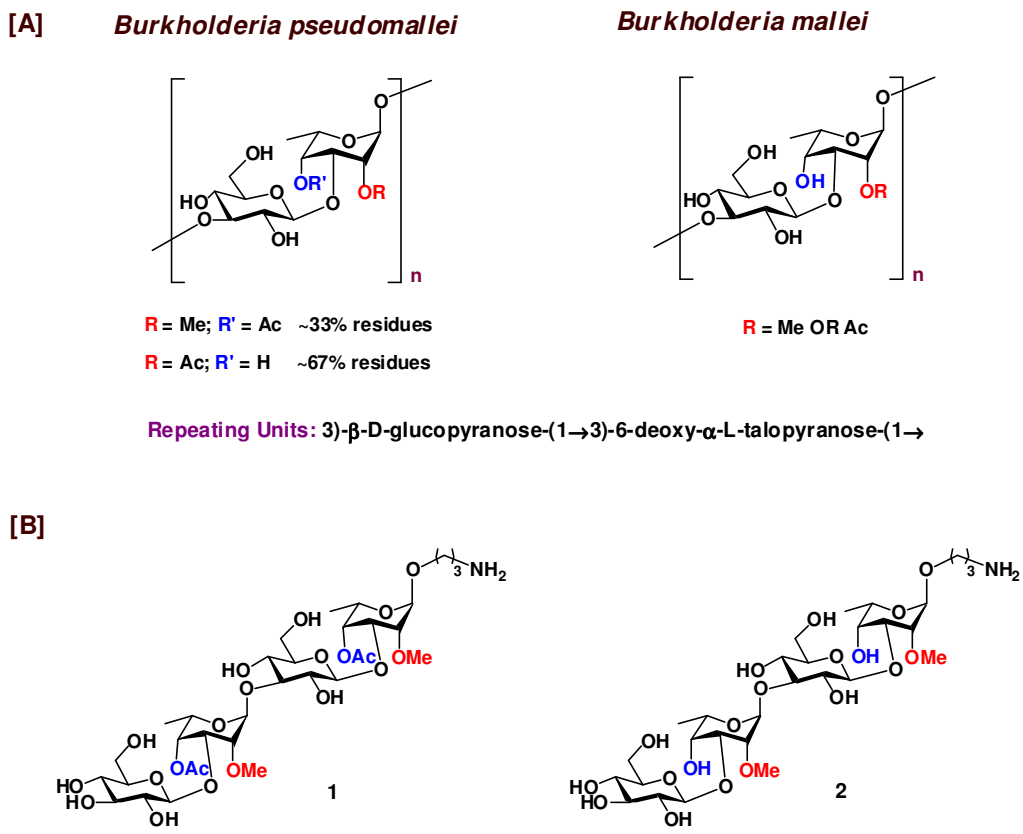


Figure 3.1. [A] Structures of *B. pseudomallei* and.*B. mallei* O-PSs; [B] Synthetic targets.

In this manuscript, we report the synthesis of tetrasaccharide (**1**). This is the first report that demonstrates a successful glycosylation at the sterically hindered C-3 hydroxyl of the 6-deoxy-talopyranoside. An antigenic evaluation of this carbohydrate epitope is underway.

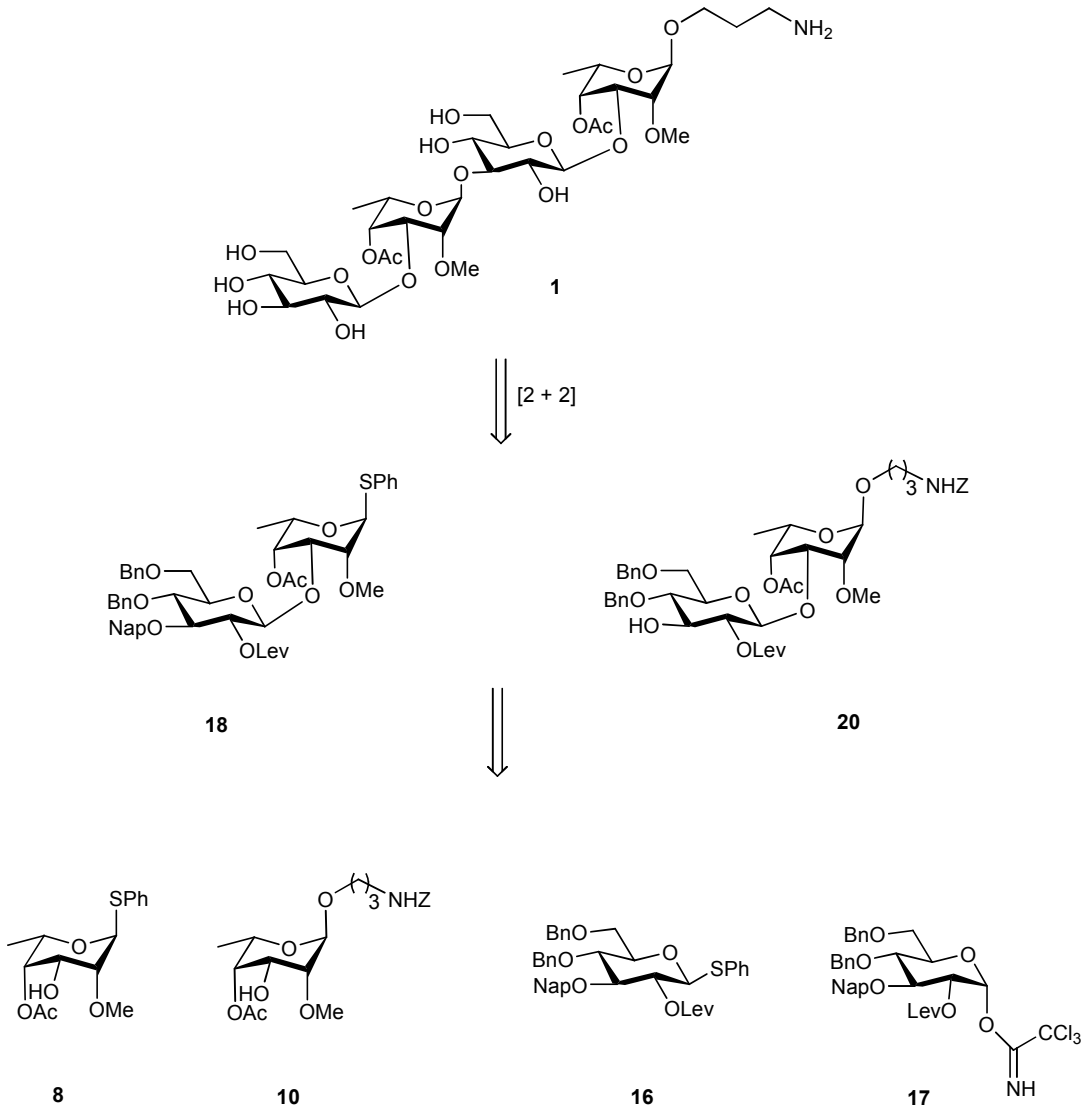
3.3 Results and Discussion

Synthesis

Initially, to study the immunological properties of the oligosaccharide fragments derived from *B. pseudomallei* and *B. mallei*, we designed tetrasaccharides **1** and **2** (Fig. 3.1 [B]) containing two disaccharide-repeating unit components of *B. pseudomallei* and *B. mallei* O-PSs, respectively, and are equipped with an artificial aminopropyl spacer that would facilitate conjugation to a carrier protein. Compound **1** was selected as a primary target for synthesis and immunological evaluation as it offers an option to selectively remove the C-4 acyl group by treatment with base to achieve compound **2** in future. Tetrasaccharide **1** contains the structure *3)-β-D-glucopyranose-(1→3)-6-deoxy-α-L-talopyranose-(1→* in which the 6dTal*p* residues have 2-*O*-methyl and 4-*O*-acetyl substitution. The *β-D-glucopyranose-(1→3)-6-deoxy-α-L-talopyranose* linkage has not been achieved previously by chemical synthesis.

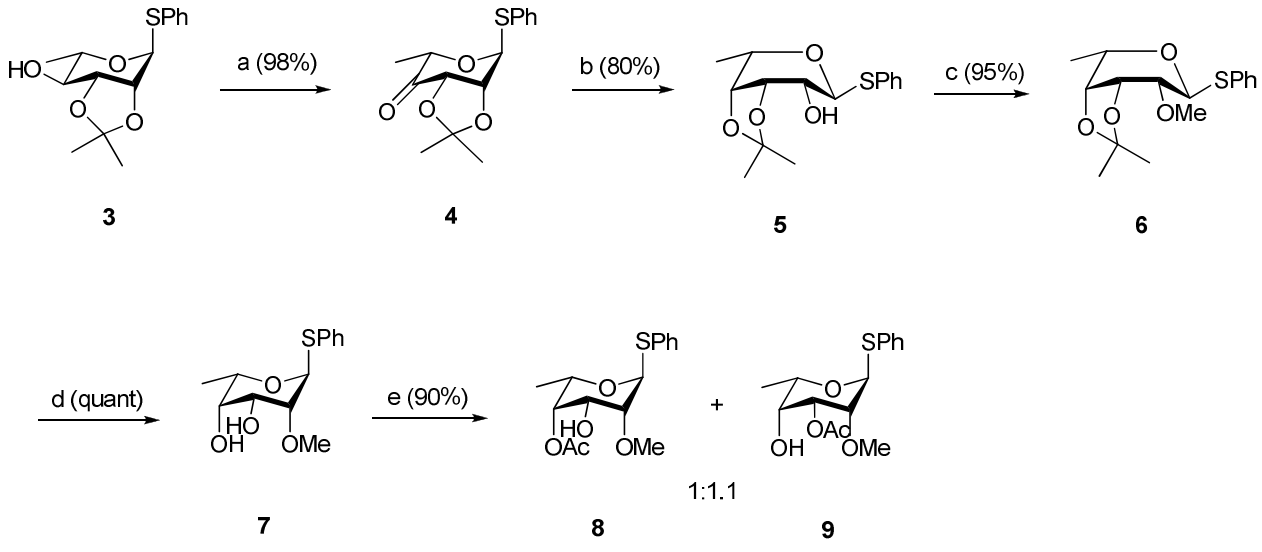
The synthetic strategy to construct **1** is depicted in scheme 3.1. Compound **1** was synthesized from monosaccharide precursors **8**, **10**, **16** and **17** (Schemes 3.1). Tetrasaccharide was prepared by assembling the disaccharide building blocks **18** and **20**. The C-4 and the C-2 hydroxyls of the 6dTal*p* units were masked as acetyl ester and methyl ether, respectively, as they were already present in the final compound. The C-4 and the C-6 hydroxyls of glucopyranoside were permanently protected as benzyl ethers. An orthogonal participating functionality at the C-2 hydroxyl of glucopyranoside was required to ensure formation of the β-linkage as well as to facilitate selective removal of the C-2 ester functionality of glucopyranoside in presence of the C-4 acetyl ester in the 6dTal*p* units at the end of synthesis. Hence, the C-2 hydroxyl of **16** was protected with

the levulinoyl (Lev) group. An orthogonal ether functionality was required at the C-3 hydroxyl of glucose that can selectively be removed in presence of benzyl ethers to give disaccharide acceptor (**20**). The naphthylmehtyl (Nap) group was chosen to serve this purpose.



Scheme 3.1. Synthetic strategies.

Synthesis of the 6dTal*p* glycosyl donor **8** commenced from the rhamnosyl acetal [11] (Scheme 3.2). A key feature in synthesis of the 6dTal*p* residue is an inversion of stereochemistry at the C-4 hydroxyl of rhamnoside building block by performing an oxidation-reduction sequence. An initial attempt of oxidation with tetrapropylammonium perruthenate in the presence of 4-methylmorpholine *N*-oxide[12] resulted in the oxidation of anomeric thiophenyl to form corresponding sulfoxide. Later on, we found out that Swern oxidation of **3** was a good method to achieve the 4-ulose **4**.[13] However, chromatographic purification of 4 could not be performed successfully maybe because it streaked on silica gel, presumably owing to equilibration between the ketone and its hydrate.[13] The crude ketone **4** was immediately reduced with sodium borohydride in a 1:1 mixture of 2-propanol-dichloromethane at 0 °C, affording compound **5** along with a mixture of other impurities[13]. Nevertheless, a careful chromatographic purification provided a good yield of compound **5**.



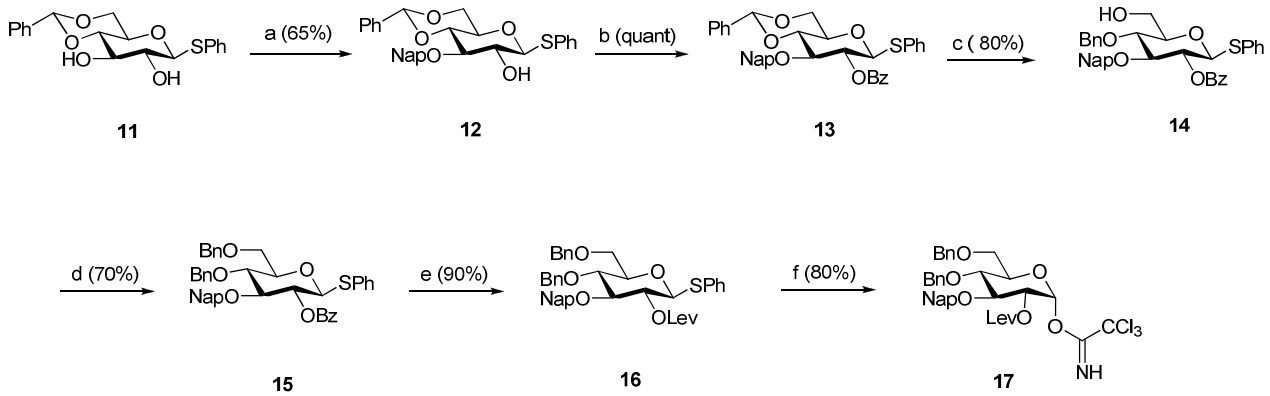
Scheme 3.2. Reagents: a) $(COCl)_2$, DMSO, CH_2Cl_2, Et_3N, -78 °C to rt; b) NaBH4, CH_2Cl_2:IPA (1:1), 0 - 20 °C; c) MeI, NaH, DMF, 0 °C - rt; d) 90% aq AcOH, 80 °C; e) i) $CH_3(OCH_3)_3$, DMF, 50 °C, ii) AcOH:H_2O (4:1), rt.

The isopropylidene acetal migration was observed in **5** in several repetitions of this step. The formation of 3,4-*O*-acetal was confirmed by acetylating **5**. A change in the chemical shift of H-2 to the downfield region compared to that in **5**, indicated the presence of the C-2 hydroxyl and the 3,4-*O*-isopropylidene acetal in compound **5**. An important conformational change of the taloside was also observed following the migration of the isopropylidene acetal to form 3,4-*O*-linked bicyclic acetal. The coupling constant ($J_{1,2}$ = 7.8 Hz) indicated an almost diaxial orientation of H-1 and H-2, consistent with a boat or twist-boat conformation of the ring instead of the more common chair conformation of the precursors ($J_{1,2}$ = 1.5 Hz). Heidelberg and Martin observed a similar conformational behavior during an acid catalyzed rearrangement of 4-pentenyl 6-deoxy-2,3-*O*-isopropylidene talopyranoside to form 4-pentenyl 6-deoxy-3,4-*O*-isopropylidene talopyranoside.[14] The migration of acetal, however, worked in our favor and, gave us an edge on selective protection of the C-2 hydroxyl as methyl ether (**6**). The methylation of **5** could easily be accomplished by treatment of **5** with methyl iodide in the presence of sodium hydride to afford compound **6** in 95% yield. The isopropylidene acetal of **6** could easily be removed by using aqueous acetic acid, reverting back to $^1C_4$ conformation.

The transformation of the diol **7** into orthoester and subsequent ring opening resulted into a 1:1.1 mixture of the C-4 axial acetate (**8**) and the C-3 equatorial acetate (**9**), surprisingly, **9** being the major compound. Both regio-isomers could be separated easily by column chromatography. This may be due to a change in conformation during in situ orthoester formation, giving rise to an intermediate that facilitates ring opening in both, axial and equatorial, fashion. An *N*-iodosuccinimide/trifluoromethanesulfonic acid (NIS/TfOH) mediated coupling[15] of thioglycosyl donor **8** with benzyloxycarbonyl

protected aminopropanol afforded spacer modified **10** as only the α-anomer. Self-condensation of compound **8** was not observed, probably, due to the much higher acceptor reactivity of *N*-Cbz protected spacer.

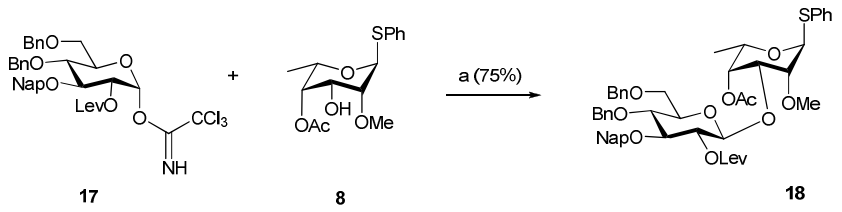
Glycosyl donor **17** was synthesized from the benzylidene acetal **11**[16] (Scheme 3.3). The diol **11** was selectively protected as 2-methylnaphthyl (NAP) ether[17, 18] **12**, by first stannylene acetal formation by reaction with dibutyltin oxide in refluxing methanol followed by treatment with 2-bromomethyl naphthalene and CsF in DMF.[19, 20] The remaining hydroxyl was temporarily protected as benzoyl ester using benzoyl chloride in pyridine, which was replaced by the levulinoyl group at the end of the synthesis of the glucose block. The protection of the C-2 hydroxyl with the benzoyl ester ensured a successful reductive ring opening of the benzylidene acetal of **13** to give compound **14**. A selective ring opening reaction of 4,6-*O*-benzylidene acetal in **13** was performed by using triethylsilane (Et_3SiH) and dichlorophenyl borane ($PhBCl_2$) to afford 4-*O*-benzyl-6-hydroxy derivative **14**.[21] The carbonyl functionality in the levulinoyl group was also reduced, when subjected to these conditions. This problem was successfully tackled by



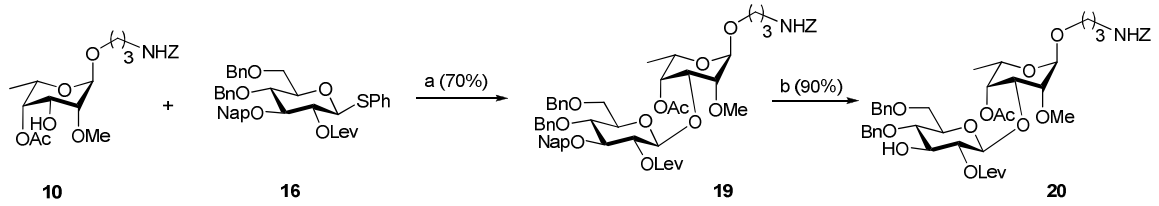
Scheme 3.3. Reagents: a) i) Bu_2SnO, MeOH, 65 °C, ii) 2-bromomethyl naphthalene, CsF, DMF, rt; b) BzCl, py, 0 °C - rt; c) Et_3SiH, $PhBCl_2$, CH_2Cl_2, 4 Å MS, - 78 °C; d) BnBr, NaH, DMF, 0 °C - rt; e) i) 1 M NaOMe/MeOH, 60 °C, ii) levulinic acid, DCC, DMAP, CH_2Cl_2, 0 °C - rt; f) i) NBS, TfOH, CH_2Cl_2:H_2O (10:0.1), ii) CCl_3CN, DBU, CH_2Cl_2.

protecting group swapping, the benzoyl group in this case. However, there was a need to replace the benzoyl ester by the levulinoyl group that would not only ensure the formation of β-linkage in subsequent glycosylation with the 6dTal*p* residue, but also could selectively be removed in the presence of the acetyl group, which is present in the 6dTal*p* residue in the final compound (**1**). The C-6 hydroxyl in **14** was benzylated using benzyl bromide and NaH in DMF at 0 °C to afford **15** in a reasonable yield. The C-2 benzoyl ester was cleaved by treatment of **15** with NaOMe/MeOH at 60 °C, followed by treatment of the crude product with levulinic acid, 1,3-dicyclohexylcarbodiimide (DCC), and 4-(dimethylamino) pyridine (DMAP) in CH_2Cl_2 to give the thioglycosyl donor **16**.[22] Hydrolysis of the thioglycoside donor by using *N*-bromosuccinimide (NBS)/TfOH in a mixture of CH_2Cl_2:H_2O (10:0.1)[23], followed by treatment of the resulting lactol with trichloroacetonitrile in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) provided the trichloroacetimidate (TCA) activated donor **17**.[24, 25]

Having synthesized all the building blocks, the attention was focused on the assembly of these monosaccharides to prepare disaccharide precursors **18** and **20** (Scheme 3.4 and 3.5). Coupling of the TCA-activated donor **17** with **8** in the presence of trifluromethanesulfonate (TMSOTf) in CH_2Cl_2 gave disaccharide **18** in 75% yield (Scheme 3.3). The acceptor **10** was glycosylated with **16** by using the NIS/TfOH promoter-activator system to afford disaccharide **19** in 70% yield. Next, the NAP ether of **19** was oxidatively cleaved[17, 18] by using 1,2-dichloro 4,5-dicyanoquinone (DDQ) in a mixture of CH_2Cl_2/H_2O to provide glycosyl acceptor **20** in an excellent yield (Scheme 3.5).



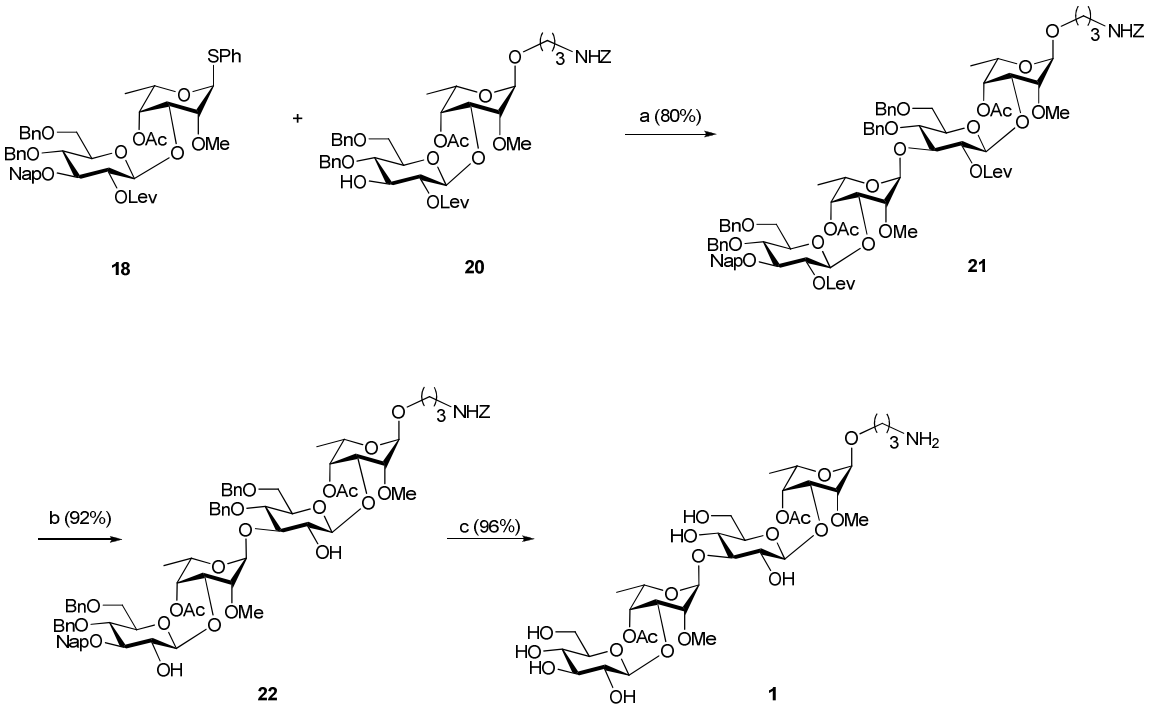
Scheme 3.4. Reagents: a) TMSOTf, CH_2Cl_2, -30 °C to rt.



Scheme 3.5. Reagents: a) NIS, TfOH, CH_2Cl_2, -10 °C to rt; b) DDQ, CH_2Cl_2:H_2O (10:1), rt.

Fully protected tetrasaccharide (**21**) was assembled in an excellent yield (80%) by NIS/TfOH mediated coupling of the disaccharide precursors **18** and **20**. The anomeric configuration [*6-deoxy-α-L-talopyranose-(1→3)-β-D-glucopyranose*] was confirmed from one-bond ^{13}C-^{1}H couplings ($^{1}J_{CH}$), since the difference in coupling between the two anomeric configurations is generally 10 Hz with the higher value for equatorial ^{13}C-^{1}H coupling, i.e., the α-anomer. The mean $^{1}J_{CH}$ values for α- and β-anomers are 170 and 160, respectively. $^{1}J_{CH}$ = 174 & 171 and $^{1}J_{CH}$ = 162 & 162 in **21** indicated the α-configuration of 6-deoxy talopyranose and the β-configuration of glucopyranose, respectively. The levulinoyl groups in **21** were selectively removed, without affecting the acetyl esters, by treatment with hydrazine acetate to give **22**.[22] Finally, concomitant cleavage of the benzyl and the 2-methylnaphthyl ethers and benzyloxycarbamate by catalytic hydrogenation over $Pd(OH)_2$ in a mixture of *t*-BuOH/H_2O /AcOH afforded compound **1** (Scheme 3.6). The presence of the C-4 acetyl esters and the C-2 methyl

ethers as well as the anomeric configurations in **1** were confirmed by using ^{1}H- and ^{13}C-NMR spectroscopy. The multiplicities and small $J_{4,5}$ coupling values (2.6 & 2.9 Hz) confirmed the *talo* configuration.



Scheme 3.6. Reagents: a) NIS, TfOH, CH_2Cl_2, -10 to 0 °C; b) NH_2NH_2•AcOH, MeOH, CH_2Cl_2, rt; c) $Pd(OH)_2$, H_2 (g), *t*-BuOH/H_2O/AcOH (40:1:1), rt.

3.4 Conclusion

We have developed a convergent total synthesis of a *B. pseudomallei* O-PS tetrasaccharide antigen ready for conjugation to carrier proteins. We have demonstrated a successful glycosylation at the sterically challenging C-3 hydroxyl position of the 6dTal*p* residue. Immunological studies are underway. Ongoing studies will demonstrate

the potential of this cellular carbohydrate epitope for use as vaccine component and/or diagnostic marker.

3.5 Experimental

General

^{1}H NMR spectra were recorded in $CDCl_3$ or D_2O on Varian Merc-300 or Varian Inova-500 spectrometers equipped with Sun workstations at 300 K. TMS (δ_H 0.00) or D_2O (δ_H 4.67) was used as the internal reference. ^{13}C NMR spectra were recorded in $CDCl_3$ or D_2O at 75 MHz on a Varian Merc-300 spectrometer, respectively, using the central resonance of $CDCl_3$ (δ_C 77.0) as the internal reference. COSY, HSQC, HMBC and TOCSY experiments were used to assist signal assignment of the spectra. Mass spectra were obtained on Applied Biosystems Voyager DE-Pro MALDI-TOF (no calibration) and Bruker Daltonics 9.4T (FTICR, external calibration with BSA). Optical rotatory power was obtained on Jasco P-1020 polarimeter at 300 K. Chemicals were purchased from Aldrich or Fluka and used without further purification. CH_2Cl_2, acetonitrile and toluene were distilled from calcium hydride; THF from sodium; and MeOH from magnesium and iodine. Aqueous solutions are saturated unless otherwise specified. Molecular sieves were activated at 350 ℃ for 3 h in vacuo. All reactions were performed under anhydrous conditions under argon and monitored by TLC on Kieselgel 60 F_{254} (Merck). Detection was by examination under UV light (254 nm) and by charring with 10% sulfuric acid in methanol. Silica gel (Merck, 70-230 mesh) was used for chromatographies. Iatrobeads 6RS-8060 was purchased from Bioscan.

Phenyl 6-deoxy-3,4-*O*-isopropylidene-1-thio-α-L-talopyranoside (5)

Oxalyl chloride (6.12 mL, 70.18 mmol) was added slowly to a solution of DMSO (4.99 mL, 70.18 mmol) in CH_2Cl_2 (125 mL) at -78 °C. The mixture was agitated for 15 minutes, and then a solution of **3** (16 g, 53.99 mmol) in CH_2Cl_2 (125 mL) was added dropwise over 15 minutes. The reaction mixture was stirred at -78 °C for 30 minutes, and then TEA (16.60 mL, 118.77 mmol) was added over 5 minutes. The cloudy mixture was kept at -78 °C for 15 minutes, and then the reaction mixture was allowed to warm to room temperature over 45 minutes. The mixture was diluted with diethyl ether (500 mL) and washed with water (2 x 250 mL) and brine (2 x 250 mL). The solution was dried over $MgSO_4$ and was evaporated to afford a yellow oily residue (15.57g, 98%) of phenyl 4,6-dideoxy-2,3-*O*-isopropylidene-1-thio-α-L-*talo*-hex-4-ulopyranoside (**4**).

The crude ketone **4** (15 g, 50.96 mmol) was dissolved in 1:1 CH_2Cl_2-IPA (250 mL), and the solution was cooled to 0-5 °C. $NaBH_4$ (2.89 g, 76.43 mmol) was added gradually, and the reaction mixture was stirred for 4 h at 5-15 °C. After complete consumption of ketone was observed on TLC, the reaction mixture was quenched by addition of ice pieces. The solution was diluted with diethyl ether (500 mL) and washed with water (2 x 250 mL). The dried solution was evaporated to give colorless oil. Purification by silica gel column chromatography (hexane:EtOAc 1:1) afforded compound **5** (12.08 g, 80%) as clear oil. *Rf* = 0.69 (hexane/EtOAc 1:1); ^{1}H NMR (300 MHz $CDCl_3$) δ 7.55 – 7.24 (m, 5H, H_{arom}), 5.31 (d, *J* = 7.8 Hz, 1H, H-1), 4.56 (dd, *J* = 3.0, 7.5 Hz, 1H, H-3), 4.11 (dd, *J* = 1.8, 7.5 Hz, 1H, H-4), 3.84 (qd, *J* = 1.8, 6.3 Hz, 1H, H-5), 3.78 (dd, *J* = 3.6, 7.2 Hz, 1H, H-2), 1.51 (s, 3H, C*H*$_3$ - *i*Pr), 1.36 (s, 3H, C*H*$_3$ - *i*Pr), 1.23 (d, *J* = 6.6 Hz, 3H, H-6); ^{13}C NMR (75 MHz, $CDCl_3$) δ [134.23, 132.36, 131.75,

128.86, 128.78, 127.49, 127.36 (C_{arom})], 110.42 [-*C*$(CH_3)_2$], 85.71 (C-1), 76.9 (C-2), 76.14 (C-4), 71.76 (C-3), 66.25 (C-5), [26.23, 25.58 C(*C*$H_3)_2$], 15.45 (C-6); HR-MALDI-TOF/TOF: calcd for $C_{16}H_{22}O_4SNa$ [M+Na^+] 319.1082, found 319.1071.

Phenyl 6-deoxy-3,4-*O*-isopropylidene-2-*O*-methyl-1-thio-α-L-talopyranoside (6)

NaH (3.25 g, 67.63 mmol, 60% in mineral oil) was added to a solution of **5** (3.0 g, 33.81 mmol) in DMF (90 mL). The reaction mixture was stirred at 0 ℃ for 1 h, and then methyl iodide (4.21 mL, 67.62 mmol) was added dropwise. The reaction mixture was stirred at room temperature for 6 h, and then poured into ice water. The solution was extracted with CH_2Cl_2 (100 mL) and washed with water (100 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated to dryness. Purification of the crude product by column chromatography on silica gel (hexane/EtOAc 4:1) afforded the desired product 6 as colorless oil (8.66 g, 99 %). R_f = 0.74 (hexane/EtOAc 2:1); ^{1}H NMR (300 MHz $CDCl_3$) δ 7.55 – 7.24 (m, 5H, H_{arom}), 5.33 (d, *J* = 8.1 Hz, 1H, H-1), 4.69 (dd, *J* = 2.9, 7.6 Hz, 1H, H-3), 4.11 (dd, *J* = 2.0, 7.6 Hz, 1H, H-4), 3.81 (qd, *J* = 1.9, 6.5 Hz, 1H, H-5), 3.57 (s, 3H, OC*H*$_3$), 3.33 (dd, *J* = 2.9, 8.0 Hz, 1H, H-2), 1.49 (s, 3H, C*H*$_3$ - *i*Pr), 1.37 (s, 3H, C*H*$_3$ - *i*Pr), 1.19 (d, *J* = 6.4 Hz, 3H, H-6); ^{13}C NMR (75 MHz, $CDCl_3$) δ [134.23, 132.36, 131.75, 128.86, 128.78, 127.49, 127.36 (C_{arom})], 110.48 [-*C*$(CH_3)_2$], 85.79 (C-1), 77.93 (C-2), 76.07 (C-4), 71.87 (C-3), 66.32 (C-5), 58.86 (O*C*H_3), [26.25, 25.54 C(*C*$H_3)_2$], 15.52 (C-6); HR-MALDI-TOF/TOF: calcd for $C_{16}H_{22}O_4SNa$ [M+Na^+] 333.1239, found 333.1228.

Phenyl 6-deoxy-2-*O*-methyl-1-thio-α-L-talopyranoside (7)

The isopropylidene acetal **6** (2.5 g, 8.05 mmol) was stirred with 90% aq AcOH (20 mL) at 80 ℃ for 2 h. After completion on TLC, the reaction mixture was

concentrated to dryness under reduced pressure. The residue was co-evaporated with toluene in order to remove traces of water and AcOH. No further purification was required and the diol **7** (2.16 g, quant.) was obtained as colorless oil. *Rf* = 0.37 (hexane/EtOAc 1:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.41 – 7.10 (m, 5H, H_{arom}), 5.59 (s, 1H, H-1), 4.32 (q, *J* = 6.4 Hz, 1H, H-5), 3.71 – 3.64 (m, 2H, H-3, H-2), 3.53 (d, *J* = 8.7 Hz, 1H, H-4), 3.41 (s, 3H, OC*H*$_3$), 2.98 (d, *J* = 8.5 Hz, 1H, C-3 OH), 2.72 (d, *J* = 11.7 Hz, 1H, C-4 OH), 1.25 (d, *J* = 6.5 Hz, 1H, H-6); ^{13}C NMR (75 MHz, $CDCl_3$) δ [134.20, 131.26, 131.22, 129.12, 129.01, 128.21, 127.50 (C_{arom})], 84.37 (C-1), 81.87 (C-2), 73.07 (C-4), 68.29 (C-5), 67.10 (C-3), 58.58 (O*C*H_3), 16.37 (C-6); HR-MALDI-TOF/TOF: calcd for $C_{13}H_{18}O_4SNa$ [M+Na^+] 293.0926, found 293.0917.

Phenyl 4-*O*-acetyl-6-deoxy-2-*O*-methyl-1-thio-α-L-talopyranoside (8)

The diol **7** (1.0 g, 3.70 mmol) and 1,1,1-triethoxyethane (0.54 g, 0.56 mL, 4.44 mmol) were dissolved in 20 mL of DMF and stirred for 1 h at 50 °C. DMF was removed under reduced pressure. The residue was dissolved in AcOH/H_2O (4:1, v/v) and stirred for 15 min at room temperature. Acetic acid was removed in vacuo and the crude product was isolated as a mixture of regio-isomers, Phenyl 4-*O*-acetyl-6-deoxy-2-*O*-methyl -1-thio-α-L-talopyranoside (**8**) and Phenyl 3-*O*-acetyl-6-deoxy-2-*O*-methyl -1-thio-α-L-talopyranoside (**9**). Separation of regio-isomers **8** and **9** was achieved by column chromatography (hexane:EtOAc 1:1) to give compound **8** (0.52 g) and compound **9** (0.57 g) as colorless oil in a combined yield of 90%.

*Compound **8***

Rf = 0.40 (hexane/EtOAc 1:1); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.42 – 7.19 (m, 5H, H_{arom}), 5.73 (s, 1H, H-1), 5.11 (d, *J* = 3.9 Hz, 1H, H-4), 4.43 (q, *J* = 6.6 Hz, 1H, H-5), 3.94 – 3.90 (m, 1H, H-3), 3.53 (d, *J* = 4.1 Hz, 1H, H-2), 3.44 (s, 3H, OC*H*$_3$), 2.78 (d, *J* = 10.4 Hz, 1H, OH), 2.13 (s, 3H, OOCC*H*$_3$), 1.13 (d, *J* = 6.6 Hz, 3H, H-6); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.29 (OO*C*CH$_3$), [134.04, 131.27, 130.58, 129.13, 127.54, 127.24 (C_{arom})], 85.04 (C-1), 80.02 (C-3), 70.91 (C-2), 69.92 (C-4), 68.74 (C-5), 58.95 (O*C*H$_3$), 21.12 (OOC*C*H$_3$), 16.29 (C-6); HR-MALDI-TOF/TOF: calcd for $C_{15}H_{20}O_5SNa$ [M+Na$^+$] 335.1031, found 335.1022.

*Compound **9***

Rf = 0.46 (hexane/EtOAc 1:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.44 – 7.19 (m, 5H, H_{arom}), 5.58 (s, 1H, H-1), 4.94 (t, *J* = 3.2 Hz, 1H, H-3), 4.34 (q, *J* = 6.5 Hz, 1H, H-5), 3.75 (dt, *J* = 1.4, 3.0 Hz, 1H, H-2), 3.71 – 3.62 (m, 1H, H-4), 3.43 (s, 1H, OC*H*$_3$), 3.31 (d, *J* = 11.0 Hz, 1H, OH), 2.12 (s, 1H, OOCC*H*$_3$), 1.24 (d, *J* = 6.5 Hz, 1H, H-6); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.29 (OO*C*CH$_3$), [134.04, 131.27, 129.13, 127.54 (C_{arom})], 85.04 (C-1), 80.02 (C-2), 70.91 (C-4), 69.92 (C-3), 68.74 (C-5), 58.95 (O*C*H$_3$), 21.12 (OOC*C*H$_3$), 16.29 (C-6); HR-MALDI-TOF/TOF: calcd for $C_{15}H_{20}O_5SNa$ [M+Na$^+$] 335.1031, found 335.1022.

3-[(*N*-Benzyloxycarbonyl)amino]propyl 4-*O*-acetyl-6-deoxy-2-*O*-methyl-1-thio-α-L-talopyranoside (10)

The glycosyl donor **8** (340 mg, 1.09 mmol) and 3-(*N*-benzyloxycarbonyl)aminopropanol (456 mg, 2.18 mmol) were premixed in CH_2Cl_2 (20

ml) and agitated with 4 Å powdered molecular sieves (800 mg) at room temperature for 30 minutes. NIS (269 mg, 1.2 mmol) was added to the reaction mixture at room temperature. The mixture was cooled to -10 °C and TfOH (19 µL, 0.22 mmol) was added. The mixture was stirred at -10 °C for 1 h and filtered through celite; then diluted with CH_2Cl_2. The filtrate was washed with 1 M $Na_2S_2O_3$, saturated $NaHCO_3$, and brine solutions; dried over $MgSO_4$; and concentrated in vacuo. Purification of the crude product by silica gel column chromatography (hexane:EtOAc 1:1) gave compound **10** as white solid (314 mg, 70%). *Rf* = 0.17 (hexane/EtOAc 1:3);^{1}H NMR (300 MHz, $CDCl_3$) δ 7.36 – 7.26 (m, 5H, H_{arom}), 5.09 (s, 3H, C*H*$_2$ – Z, H-4), 4.95 (s, 1H, H-1), 4.85 (bs, 1H, NH), 3.97 – 3.91 (m, 2H, H-3, H-5), 3.78 – 3.71 (m, 1H, OC*H*HCH$_2$CH$_2$NHZ), 3.51 – 3.45 (m, 4H, OC*H*$_3$, OCH*H*CH$_2$CH$_2$NHZ), 3.34 – 3.27 (m, 3H, OCH$_2$CH$_2$C*H*$_2$NHZ, H-2), 2.73 (d, *J* = 10.4, 1H, OH), 2.18 (s, 3H, OOCC*H*$_3$), 1.84 – 1.76 (m, 2H, OCH$_2$C*H*$_2$CH$_2$NHZ), 1.17 (d, *J* = 6.6, 3H, H-6); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.32 (OO*C*CH$_3$), 156.33 (NH*C*OOCH$_2$Ph, NHZ), [136.49, 128.55, 128.52, 128.17, 128.13 (C_{arom})], 97.10 (C-1), 78.00 (C-2), 71.76 (C-4), 65.62 (NHCOO*C*H$_2$Ph, NHZ), 65.20 (O*C*H$_2$CH$_2$CH$_2$NHZ), 64.55 (C-3, C-5), 59.80 (O*C*H$_3$), 38.64 (OCH$_2$CH$_2$*C*H$_2$NHZ), 29.64 (OCH$_2$*C*H$_2$CH$_2$NHZ), 20.98 (OOC*C*H$_3$), 16.36 (C-6); HR-MALDI-TOF/TOF: calcd for $C_{20}H_{29}NO_8Na$ [M+Na$^+$] 434.1893, found 434.1884.

Phenyl 4,6-*O*-benzylidene-3-*O*-(2-naphthylmethyl)-1-thio-*β*-D-glucopyranoside (12)

Dibutyltin oxide (11.05 g, 44.39 mmol) was added to a solution of the diol (16 g, 44.39 mmol) in dry MeOH (350 mL). The reaction mixture was refluxed until the solution became clear. After cooling to room temperature, the reaction mixture was concentrated to dryness. 2-Napthylmethyl bromide (14.72 g, 66.59 mmol) and CsF (8.09 g, 53.27

mmol) were added to a solution of the residue in DMF (150 mL). The reaction mixture was stirred at room temperature overnight, and then quenched with water. A solution was extracted with ethyl acetate and the extracts were washed with water. The organic phase was dried over $MgSO_4$ and concentrated to dryness. Purification of the crude product by crystallization in hexane/EtOAc (6:1) afforded the desired product **12** as white solid (14.45 g, 65%). *Rf* = 0.58 (hexane/EtOAc 3:1); $[\alpha]_D^{27}$ = + 1.44 (*c*=1 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.82 – 7.31 (m, 17H, H_{arom}), 5.59 (s, 1H, PhC*H*), 5.03 (dd, *J* = 11.9, 41.6 Hz, 2H, C*H*$_2$ - naphthylmethyl), 4.63 (d, *J* = 9.7 Hz, 1H, H-1), 4.39 (dd, *J* = 4.9, 10.5 Hz, 1H, H-6_a), 3.84 – 3.66 (m, 3H, H-6_b, H-3, H-4), 3.59 – 3.48 (m, 2H, H-2, H-5), 2.57 (d, *J* = 2.2 Hz, 1H, OH); ^{13}C NMR (75 MHz, $CDCl_3$) δ [137.19, 135.57, 133.25, 133.19, 133.05, 131.26, 129.05, 128.38, 128.28, 127.93, 127.66, 126.94, 126.06, 125.99, 125.92 (C_{arom})], 101.35 (Ph*C*H), 88.53 (C-1), [81.44, 81.08 (C-3 & C-4)], 74.80 (*C*H_2 – naphthylmethyl), [72.36, 70.76 (C-2 & C-5)], 68.63 (C-6); HR-MALDI-TOF/MS: calcd for $C_{30}H_{28}O_5SNa$ [M+Na^+] 523.1657, found 523.1646.

Phenyl 2-*O*-benzoyl-4,6-*O*-benzylidene-3-*O*-(2-naphthylmethyl)-1-thio-*β*-D-glucopyranoside (13)

Benzoyl chloride (6.5 mL, 55.93 mmol) was added to a cooled solution of compound **12** (14 g, 27.97 mmol) in pyridine (50 mL) at 0 ˚C. The mixture was stirred at room temperature for 4 h and then poured onto ice water. The cloudy solution was extracted with ethyl acetate (3 x 100 mL), dried over $MgSO_4$ and concentrated in vacuo. The crude product was crystallized in ethylacetate:hexane (1:6) to give compound **13** (16.8 g, quant) as white solid. *Rf* = 0.69 (hexane/EtOAc 3:1); $[\alpha]_D^{27}$ = + 71.74 (*c*=1 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.96 – 7.18 (m, 22H, H_{arom}), 5.63 (s, 1H, PhC*H*),

5.31 (dd, *J* = 8.5, 10.0 Hz, 1H, H-2), 4.98 – 4.81 (m, 3H, C*H*$_2$ – naphthylmethyl, H-1), 4.42 (dd, *J* = 4.9, 10.5 Hz, 1H, H-6$_a$), 3.97 – 3.82 (m, 3H, H-3, H-4, H-6$_b$), 3.61 – 3.53 (m, 1H, H-5); HR-MALDI-TOF/MS: calcd for $C_{37}H_{32}O_6SNa$ [M+Na$^+$] 627.1920, found 627.1909.

Phenyl 2-*O*-benzoyl-4-*O*-benzyl-3-*O*-(2-naphthylmethyl)-1-thio-*β*-D-glucopyranoside (14)

To a solution of **13** (3.5 g, 5.79 mmol) in CH_2Cl_2 (30 mL) at room temperature was added 4 Å molecular sieve (3.5 g). After stirring for 1 h at room temperature, the mixture was cooled to -78 °C, and then to the stirred solution, Et_3SiH (1.34 mL, 8.68 mmol) and $PhBCl_2$ (1.29 mL, 9.84 mmol) were added successively. After 1 h of agitation at -78 °C, Et_3N (13.6 mL) and MeOH (13.6 mL) were added successively. The mixture was diluted with $CHCl_3$ (200 mL), filtered through Celite®, washed with aqueous $NaHCO_3$ (2 x 100 mL), dried over $MgSO_4$, and concentrated in vacuo. The crude product was purified by silica gel column chromatograhphy (hexane:EtOAc 2:1) to afford **14** (2.8 g, 80%) as white solid. *Rf* = 0.24 (hexane:EtOAc 3:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.89 (d, *J* = 7.2 Hz, 2H), 7.61 – 7.13 (m, 20H), 5.21 (t, *J* = 9.8 Hz, 1H, H-2), 4.82 [d, *J* = 11.4 Hz, 1H, CH*H*(naphthylmethyl)], 4.77 [d, *J* = 10.9 Hz, 1H, PhC*H*H], 4.72 [d, *J* = 11.4 Hz, 1H, C*H*H(naphthylmethyl)], 4.78 (d, *J* = 9.9 Hz, 1H, H-1), 4.61(m, 1H, PhCH*H*, PhC*H*$_2$), 3.87 – 3.81 (m, 2H, H-3, H-6$_a$), 3.69 – 3.62 (m, 2H, H-4, H-6$_b$), 3.47 – 3.41 (m, 1H, H-5). ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.20 [O*C*(O)Ph], [137.71, 135.62, 135.08, 133.90, 133.20, 133.06, 132.90, 132.60, 132.45, 129.76, 129.68, 128.95, 128.86, 128.54, 128.35, 128.15, 128.07, 128.03, 128.00, 127.83, 127.61, 126.86, 126.02, 125.93, 125.80 (C$_{arom}$)], 86.18 (C-1), 83.90 (C-3), 79.59 (C-5), 77.62 (C-4),

75.38 (*C*H_2 – naphthylmethyl), 75.21 (Ph*C*H_2), 72.40 (Ph*C*H_2), 72.31(C-2), 62.05 (C-6); MALDI-TOF/MS: calcd for $C_{44}H_{40}O_6SNa$ [M+Na^+] 629.2076, found 629.2064.

Phenyl 2-*O*-benzoyl-4,6-di-*O*-benzyl-3-*O*-(2-naphthylmethyl)-1-thio-*β*-D-glucopyranoside (15)

A solution of compound **14** (2.0 g, 3.3 mmol) in DMF (30 mL) was stirred with NaH (0.08 g, 3.33 mmol) and BnBr (0.8 mL, 6.59 mmol) at 0 ℃ for 2 h. The reaction was quenched by adding MeOH (1 mL). The organic layer was diluted with ethyl acetate (50 mL), washed with water (2 x 30 mL), dried over $MgSO_4$ and concentrated in vacuo. The crude product was purified by silica gel column chromatography (hexane:EtOAc 4:1) to give compound **15** (1.6 g, 70%) as white solid. *Rf* = 0.59 (hexane/EtOAc 3:1); $[\alpha]_D^{27}$ = + 47.54 (*c*=1 in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.89 (d, *J* = 7.2 Hz, 2H), 7.64 – 7.09 (m, 25H), 5.23 (t, *J* = 9.5 Hz, 1H, H-2), 4.82 [d, *J* = 11.4 Hz, 1H, CH*H*(naphthylmethyl)], 4.77 [d, *J* = 10.9 Hz, 1H, PhC*H*H], 4.72 [d, *J* = 11.4 Hz, 1H, C*H*H(naphthylmethyl)], 4.70 (d, *J* = 10 Hz, 1H, H-1), 4.58 – 4.47 (m, 3H, PhCH*H*, PhC*H*$_2$), 3.82 (t, *J* = 9.0 Hz, 1H, H-3), 3.76 (dd, *J* = 1.6, 10.9 Hz, 1H, H-6_a), 3.72 – 3.67 (m, 2H, H-4, H-6_b), 3.55 (ddd, *J* = 1.7, 4.7, 9.8 Hz, 1H, H-5). ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.15 [O*C*(O)Ph], [138.16, 137.86, 135.13, 133.11, 133.03, 132.88, 132.84, 132.47, 129.74, 128.77, 128.43, 128.35, 128.30, 128.09, 127.97, 127.85, 127.81, 127.73, 127.67, 127.58, 126.82, 126.02, 125.87, 125.74 (C_{arom})], 86.16 (C-1), 84.16 (C-3), 79.45 (C-5), 77.90 (C-4), 75.37 (*C*H_2 – naphthylmethyl), 75.12 (Ph*C*H_2), 73.48 (Ph*C*H_2), 72.31(C-2), 68.92 (C-6); MALDI-TOF/MS: calcd for $C_{44}H_{40}O_6SNa$ [M+Na^+] 719.8498, found 719.8491.

Phenyl 4,6-di-*O*-benzyl-2-*O*-levulinoyl-3-*O*-(2-naphthylmethyl)-1-thio-*β*-D-glucopyranoside (16)

Treatment of **15** (1.5 g, 2.15 mmol) with 1 M NaOMe/MeOH (pH = 10-11) at 60 ℃ gave the debenzoylated derivative of **15** in quantitative yield as white solid. Levulinic acid (0.62 ml, 6.07 mmol) was added to cooled solution of the resulting compound (1.2 g, 2.02 mmol) in CH_2Cl_2 (30 ml) at 0 ℃. A solution of DCC (1.25 g, 6.07 mmol) in CH_2Cl_2 (5 ml) and a catalytic amount of DMAP (0.01 g, 0.10 mmol) was added to the reaction mixture at 0 ℃. The mixture was stirred at room temperature for 2 h and then filtered through celite. The filtrate was washed with water (2 x 20 mL), dried over $MgSO_4$, and concentrated in vacuo. Purification of the crude product by silica gel column chromatography (hexane:EtOAc 2:1) gave compound **16** (1.34, 90%) as white solid. *Rf* = 0.51 (hexane/EtOAC 2:1); $[\alpha]_D^{27}$ = +23.84 (*c*=1 in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.95 – 7.12 (m, 22H, H_{arom}), 5.07 (t, *J* = 9.5 Hz, 1H, H-2), 4.93 (dd, *J* = 11.6, 39.5 Hz, 2H, C*H*$_2$-naphthylmethyl), 4.84 (d, *J* = 10.9 Hz, 1H, PhCH*H*), 4.68 – 4.53 (m, 4H, PhC*H*H, PhC*H*$_2$, H-1), 3.90 – 3.65 (m, 4H, H-3, H-4, H-6$_{a+b}$), 3.58 (dd, *J* = 3.2, 9.6 Hz, 1H, H-5), 2.69 – 2.32 [m, 4H, $CH_3C(O)CH_2CH_2C(O)O$ – Lev], 2.11 [s, 3H, $CH_3C(O)CH_2CH_2C(O)O$ – Lev]. ^{13}C NMR (75 MHz, $CDCl_3$) δ 206.08 [$CH_3C(O)CH_2CH_2C(O)O$ – Lev], 171.38 [$CH_3C(O)CH_2CH_2C(O)O$ – Lev], [138.16, 137.85, 135.64, 133.22, 132.93, 132.34, 128.80, 128.40, 128.32, 128.07, 127.92, 127.82, 127.73, 127.64, 127.63, 127.56, 126.58, 126.03, 125.97, 125.87 (C_{arom})], 86.09 (C-1), 84.40 (C-3), 79.37 (C-5), 77.76 (C-4), 75.30 (CH_2 – naphthylmethyl), 75.07 (PhCH_2), 73.45 (PhCH_2), 72.16 (C-2), 68.89 (C-6), 37.70 [$CH_3C(O)CH_2CH_2C(O)O$ –

Lev], 29.74 [*C*H_3C(O)CH_2CH_2C(O)O – Lev], 28.06 [CH_3C(O)CH_2*C*H_2C(O)O – Lev]; MALDI-TOF/MS: calcd for $C_{42}H_{42}O_7SNa$ [M+Na^+] 713.8437, found 713.8432.

4,6-di-*O*-benzyl-2-*O*-levulinoyl-3-*O*-(2-naphthylmethyl)-1-*O*-trichloroacetimidate-α-D-glucopyranoside (17)

Treatment of compound **16** (100 mg, 0.145 mmol) with NBS (20 mg, 0.179 mmol) and TfOH (1.3 µL, 0,015 mmol) in CH_2Cl_2/H_2O (5:0.1, 5.1 ml) at 0 °C gave a mixture of hydrolyzed anomers, which was purified by silica gel column chromatography (hexane:EtOAc 2:1) to achieve hemiacetal compound (67 mg). A solution of this compound in CH_2Cl_2 (5 ml) was treated with trichloroacetonitrile (22 µL, 2.19 mmol) and DBU (17 µL, 0.114 mmol). The reaction mixture was stirred at room temperature for 1 h and then concentrated to dryness in vacuo. The crude product was purified by silica gel column chromatography (hexane:EtOAc 1:1+0.5% TEA) to achieve the imidate donor 17 (86 mg, 80%) as pale yellow solid. *Rf* = 0.78 (hexane:EtOAc 1:1+0.5% TEA); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.48 (s, 1H, NH), 7.79 – 6.93 (m, 17H, H_{arom}), 6.43 (d, *J* = 3.5 Hz, 1H, H-1), 5.04 (dd, *J* = 3.5, 10.0 Hz, 1H, H-2), 4.90 (dd, *J* = 11.7 Hz, 35.3, 2H, C*H*$_2$ - naphthylmethyl), 4.79 (d, *J* = 10.6 Hz, 1H, PhC*H*H), 4.54 (dd, *J* = 11.3, 22.4 Hz, 2H, PhC*H*$_2$), 4.43 (d, *J* = 12.1 Hz, 1H, PhCH*H*), 4.08 (t, *J* = 9.6 Hz, 1H, H-3), 3.96 (d, *J* = 10.0 Hz, 1H, H-5), 3.83 (t, *J* = 9.6 Hz, 1H, H-4), 3.75 (dd, *J* = 3.3, 11.1 Hz, 1H, H-6$_a$), 3.63 (dd, *J* = 1.4, 11.0 Hz, 1H, H-6$_b$), 2.53 – 2.21 [m, 4H, CH_3C(O)C*H*$_2$C*H*$_2$C(O)O – Lev], 1.99 [s, 3H, C*H*$_3$C(O)CH_2CH_2C(O)O – Lev].

Phenyl 3-*O*-[4,6-di-*O*-benzyl-2-*O*-levulinoyl-3-*O*-(2-naphthylmethyl)-*β*-D-glucopyranosyl]- 4-*O*-acetyl-6-deoxy-2-*O*-methyl-1-thio-*α*-L-talopyranoside (18)

The trichloroacetimidate donor **17** (85 mg, 0.114 mmol) and the glycosyl acceptor **8** (29 mg, 0.093 mmol) were premixed in dry toluene and dehydrated. The residue was dissolved in CH_2Cl_2 (3 ml) and agitated with 4 Å powdered molecular sieves (100 mg) at room temperature for 30 minutes. TMSOTf (6 µL, 0.033 mmol) was added at -30 °C. The reaction mixture was stirred at this temperature for 1 h and then neutralized with TEA. The mixture was filtered through celite, diluted with CH_2Cl_2 and concentrated in vacuo. Purification by silica gel column chromatography (hexane:EtOAc 2:1) afforded compound **18** (26 mg, 75%) as white solid. *Rf* = 0.51 (hexane:EtOAc 2:1); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.74 – 7.12 (m, 22H), 5.60 (s, 1H, H-1), 5.13 (s, 1H, H-4), 4.98 (t, *J* = 8.4 Hz, 1H, H-2'), 4.84 (q, *J* = 11.6 Hz, 2H, C*H*$_2$ – naphthylmethyl), 4.76 (d, *J* = 10.9 Hz, 1H, PhC*H*H), 4.54 – 4.48 (m, 4H, PhCH*H*, PHC*H*$_2$, H-1'), 4.37 (q, 1H, H-5), 3.93 (t, *J* = 3.5 Hz, 1H, H-3), 3.72 (d, *J* = 3.1 Hz, 1H, H-2), 3.71 – 3.62 (m, 4H, H-3', H-4', H-6$_{a+b}$'), 3.50 (dd, *J* = 4.0, 9.0 Hz, 1H, H-5'), 3.42 (s, 3H, OC*H*$_3$), 2.58 – 2.37 (m, 4H, OOCC*H*$_2$C*H*$_2$COCH$_3$, Lev), 2.07 (s, 3H, OOCC*H*$_3$), 1.99 (s, 3H, OOCCH$_2$CH$_2$COC*H*$_3$, Lev), 1.08 (d, *J* = 6.5 Hz, 3H, H-6); ^{13}C NMR (75 MHz, $CDCl_3$) δ 206.36 (CH$_3$*C*OCH$_2$CH$_2$COO, Lev), 171.47 (CH$_3$COCH$_2$CH$_2$*C*OO, Lev), 171.08 (OO*C*CH$_3$), [138.00, 137.87, 135.72, 134.50, 133.25, 132.95, 130.28, 130.19, 129.11, 129.03, 128.64, 128.53, 128.43, 128.40, 128.13, 128.07, 127.95, 127.84, 127.67, 126.96, 126.53, 126.07, 126.01, 125.97, 125.84 (C$_{arom}$)], 100.27 (C-1'), 86.36 (C-1), 82.85 (C-3'), 79.21 (C-2), 77.76 (C-4'), 75.08 (*C*H$_2$, naphthylmethyl), 75.03 (PH*C*H$_2$), 75.01 (C-5'), 74.40 (C-3), 73.60 (PH*C*H$_{2)}$, 73.43 (C-2'), 69.23 (C-4), 68.94 (C-6'), 66.34 (C-5), 60.02

(O*C*H_3), 37.74 (CH_3CO*C*H_2CH_2COO, Lev), 29.73 (*C*$H_3COCH_2CH_2COO$, Lev), 27.94 (CH_3COCH_2*C*H_2COO, Lev), 20.90 (*C*H_3COO), 16.07 (C-6); HR-MALDI-TOF/MS: calcd for $C_{51}H_{56}O_{12}SNa$ [M+Na^+] 915.3492, found 915.3488.

3-[(*N*-Benzyloxycarbonyl)amino]propyl 4-*O*-acetyl-3-*O*-[4,6-di-*O*-benzyl-2-*O*-levulinoyl-3-*O*-(2-naphthylmethyl)-*β*-D-glucopyranosyl]-6-deoxy-2-*O*-methyl-*α*-L-talopyranoside (19)

The glycosyl donor **16** (195 mg, 0.282 mmol) and the glycosyl acceptor 10 (116 mg, 0.282 mmol) were premixed in CH_2Cl_2 (5 ml) and agitated with 4 Å powdered molecular sieves (300 mg) at room temperature for 30 minutes. NIS (76 mg, 0.339 mmol) was added to the reaction mixture at room temperature. The mixture was cooled to -10 ℃ and TfOH (13 µL, 0.147 mmol) was added. The mixture was stirred at -10 ℃ for 1 h and filtered through celite; then diluted with CH_2Cl_2. The filtrate was washed with 1 M $Na_2S_2O_3$, saturated $NaHCO_3$, and brine solutions; dried over $MgSO_4$; and concentrated in vacuo. Purification of the crude product by silica gel column chromatography (hexane:EtOAc 1:1) gave compound **19** as white solid (195 mg, 70%). *Rf* = 0.42 (hexane:EtOAc 1:1); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.80 – 7.08 (m, 22H, H_{arom}), 5.05 (s, 1H, H-4), 5.03 [dd, 2H, PhC*H*$_2$OC(O)], 4.93 (t, *J* = 8.2 Hz, 1H, H-2'), 4.83 (q, *J* = 11.6 Hz, 2H, C*H*$_2$ – naphthylmethyl), 4.77 – 4.70 (m, 3H, H-1, NH, PhC*H*H), 4.53 – 4.42 (m, 3H, PhCH*H*, PhC*H*$_2$), 4.40 (d, *J* = 7.7 Hz, 1H, H-1'), 3.90 (t, *J* = 3.3 Hz, 1H, H-3), 3.84 (q, 1H, H-5), 3.67 – 3.55 (m, 5H, H-3', H-4', H-6_{a+b}', OC*H*HCH_2CH_2NHZ), 3.47 (d, *J* = 1.4 Hz, 1H, H-2), 3.46 – 3.30 (m, 5H, OC*H*$_3$, OCH*H*CH_2CH_2NHZ, H-5'), 3.14 (m, 2H, OCH_2CH_2C*H*$_2$NHZ), 2.59 – 2.36 (m, 4H, $OOCC$*H*$_2C$*H*$_2COCH_3$, Lev), 2.05 (s, 3H, OOCC*H*$_3$), 1.99 (s, 3H, $OOCCH_2CH_2COC$*H*$_3$, Lev), 1.69 – 1.58 (m, 2H,

$OCH_2CH_2CH_2NHZ$), 1.06 (d, *J* = 6.5 Hz, 3H, H-6); ^{13}C NMR (75 MHz, $CDCl_3$) δ 206.38 ($CH_3COCH_2CH_2COO$, Lev), 171.46 ($CH_3COCH_2CH_2COO$, Lev), 171.18 ($OOCCH_3$), 156.27 ($NHCOOCH_2Ph$, NHZ), [138.12, 137.95, 136.53, 135.75, 133.25, 132.94, 128.59, 128.39, 128.36, 128.21, 128.13, 128.06, 127.95, 127.89, 127.79, 127.64, 127.54, 126.50, 125.99, 125.96, 125.82 (C_{arom})], 100.40 (C-1'), 99.69 (C-1), 82.83 (C-3'), 77.83 (C-4'), 77.20 (C-2), 75.04 (CH_2, naphthylmethyl), 74.94 ($PhCH_2$), 74.83 (C-5'), 74.30 (C-3), 73.48 (C-2'), 73.38 ($PhCH_2$), 69.40 (C-4), 69.01 (C-6'), 66.67 ($NHCOOCH_2Ph$, NHZ), 65.70 ($OCH_2CH_2CH_2NH$), 65.12 (C-5), 60.18 (OCH_3), 38.78 ($OCH_2CH_2CH_2NH$), 37.75 ($CH_3COCH_2CH_2COO$, Lev), 29.73 ($CH_3COCH_2CH_2COO$, Lev), 29.49 ($OCH_2CH_2CH_2NH$), 27.94 ($CH_3COCH_2CH_2COO$, Lev), 20.93 (CH_3COO), 16.11 (C-6); MALDI-TOF/MS: calcd for $C_{56}H_{65}NO_{15}Na$ [$M+Na^+$] 1014.4354, found 1014.7513.

3-[(*N*-Benzyloxycarbonyl)amino]propyl 4-*O*-acetyl-3-*O*-[4,6-di-*O*-benzyl-2-*O*-levulinoyl-*β*-D-glucopyranosyl]-6-deoxy-2-*O*-methyl-*α*-L-talopyranoside (20)

A solution of compound **19** (160 mg, 0.161 mmol) in CH_2Cl_2/H_2O (10:1 v/v, 10 mL) was stirred vigoursly with DDQ (48 mg, 0.211 mmol) in the dark at room temperature for 3 h. The reaction mixture was quenched with an aqueous mixture of citric acid, ascorbic acid, and NaOH (1.2%, 1 %, 0.92% w/v, 0.25 mL). The mixture was diluted with EtOAc and the solution was washed with saturated $NaHCO_3$ solution, dried over $MgSO_4$; and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane:EtOAc, 1:1) to give compound **20** (123 mg, 90%) as white solid. *Rf* = 0.31 (hexane:EtOAc 1:1); ^1H NMR (500 MHz, $CDCl_3$) δ 7.32 – 7.15 (m, 15H, H_{arom}), 5.07 (s, 1H, H-4), 5.01 (q, *J* = 12.2 Hz, 2H, CH_2Ph - Z), 4.84 (d, *J* = 11.2 Hz, 1H,

PhC*H*H), 4.78 – 4.72 (m, 3H, H-1, H-2', NH), 4.55 (d, *J* = 11.2 Hz, 1H, PhCH*H*), 4.52 – 4.40 (m, 3H, PhCH_2, H-1'), 3.92 (t, *J* = 3.5 Hz, 1H, H-3), 3.85 (q, *J* = 6.2 Hz, 1H, H-5), 3.72 (t, *J* = 7.8 Hz, 1H, H-3'), 3.65 – 3.56 (m, 3H, H-6_{a+b}', OC*H*HCH_2CH_2NHZ), 3.47 – 3.35 (m, 7H, H-2, OCH_3, OCH*H*CH_2CH_2NHZ, H-4', H-5'), 3.14 (m, 2H, OCH_2CH_2CH_2NHZ), 3.00 (s, 1H, OH), 2.87 – 2.49 (m, 4H, OOCCH_2CH_2COCH_3, Lev), 2.13 (s, 3H, OOCCH_3), 2.05 (s, 3H, OOCCH_2CH_2COCH_3, Lev), 1.64 (bs, 2H, OCH_2CH_2CH_2NHZ), 1.07 (d, *J* = 6.5 Hz, 3H, H-6); ^{13}C NMR (75 MHz, $CDCl_3$) δ 202.43 (CH_3*C*OCH_2CH_2COO, Lev), 172.45 ($CH_3COCH_2CH_2$*C*OO, Lev), 171.32 (OO*C*CH_3), 156.20 (NH*C*OOCH_2Ph, NHZ), [138.21, 136.46, 128.58, 128.41, 128.35, 128.10, 127.80, 127.62, 127.55 (C_{arom})], 99.90 (C-1'), 99.61 (C-1), 77.58 (C-4'), 77.20 (C-2), 75.97 (C-3'), 74.82 (C-5'), 74.71 (Ph*C*H_2), 74.31 (C-2'), 74.27 (C-3), 73.36 (Ph*C*H_2), 69.42 (C-4), 69.21 (C-6'), 66.61 (NHCOO*C*H_2Ph), 65.65 (O*C*$H_2CH_2CH_2$NH), 65.04 (C-5), 60.21 (O*C*H_3), 38.63 (OCH_2CH_2*C*H_2NH), 38. 59 (CH_3CO*C*H_2CH_2COO, Lev), 29.78 (*C*$H_3COCH_2CH_2$COO, Lev), 29.51 (OCH_2*C*H_2CH_2NH), 28.23 (CH_3COCH_2*C*H_2COO, Lev), 20.93 (*C*H_3COO), 16.10 (C-6); HR-MALDI-TOF/MS: calcd for $C_{45}H_{57}NO_{15}Na$ [M+Na^+] 874.3728, found 874.3719.

3-[(*N*-Benzyloxycarbonyl)amino]propyl 4-*O*-acetyl-3-*O*-{3-*O*-[4-*O*-acetyl-3-*O*-(4,6-di-*O*-benzyl-2-*O*-levulinoyl-3-*O*-2-naphthylmethyl-*β*-D-glucopyranosyl)-6-deoxy-2-*O*-methyl-*α*-L-talopyranosyl]-4,6-di-*O*-benzyl-2-*O*-levulinoyl-*β*-D-glucopyranosyl}-6-deoxy-2-*O*-methyl-*α*-L-talopyranoside (21)

The glycosyl donor **18** (33 mg, 0.037 mmol) and the glycosyl acceptor **20** (32 mg, 0.037 mmol) were dissolved in CH_2Cl_2 (3 ml) and stirred with 4 Å powdered molecular sieves (70 mg) at room temperature for 30 minutes. NIS (10 mg, 0.045 mmol) was

added to the reaction mixture at room temperature. The mixture was cooled to -10 °C and TfOH (1.6 µL, 0.018 mmol) was added. The mixture was stirred at -10 °C for 1 h and filtered through celite; then diluted with CH_2Cl_2. The filtrate was washed with 1 M $Na_2S_2O_3$, saturated $NaHCO_3$, and brine solutions; dried over $MgSO_4$; and concentrated in vacuo. Purification of the crude product by silica gel column chromatography (hexane:EtOAc 1:3) gave tetrasaccharide **21** as white solid (49 mg, 80%). *Rf* = 0.39 (toluene:EtOAc 1:3); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.74 – 7.09 (m, 32H, H_{arom}), 5.06 – 4.97 (m, 5H, C*H*$_2$Ph – Z, H-1”, H-4, H-2’”), 4.94 (t, *J* = 9.0 Hz, 1H, H-2’), 4.89 – 4.72 (m, 6H, C*H*$_2$– naphthylmethyl, NH, H-1, H-4”, PhC*H*H), 4.58 – 4.38 (m, 8H, PhCH*H*, 3 x PhC*H*$_2$, H-1’”), 4.28 (d, *J* = 7.8 Hz, 1H, H-1’), 3.93 (q, *J* = 6.1 Hz, 1H, H-5), 3.87 – 3.83 (m, 3H, H-3”, H-5”, H-3), 3.76 (t, *J* = 8.9 Hz, 1H, H-3’), 3.70 – 3.58 (m, 7H, H-3’”, H-6$_{a+b}$’, H-6$_{a+b}$’”, H-4’”, OC*H*HCH$_2$CH$_2$NHZ), 3.53 – 3.33 (m, 12H, H-2, H-2”, OC*H*$_3$, OC*H*$_3$, OCH*H*CH$_2$CH$_2$NHZ, H-4’, H-5’, H-5’”), 3.22 – 3.10 (m, 2H, OCH$_2$CH$_2$C*H*$_2$NHZ), 2.54 – 2.36 (m, 8H, 2 x OOCC*H*$_2$C*H*$_2$COCH$_3$, Lev), 2.06 (s, 3H, OOCC*H*$_3$), 2.01 (s, 3H, OOCC*H*$_3$), 1.99 (s, 3H, OOCCH$_2$CH$_2$COC*H*$_3$, Lev), 1.97 (s, 3H, OOCCH$_2$CH$_2$COC*H*$_3$, Lev), 1.68 – 1.63 (m, 2H, OCH$_2$C*H*$_2$CH$_2$NHZ), 1.07 (d, *J* = 6.5 Hz, 3H, H-6”), 0.68 (d, *J* = 6.5 Hz, 3H, H-6); ^{13}C NMR (75 MHz, $CDCl_3$) δ 206.24 (CH$_3$*C*OCH$_2$CH$_2$COO, Lev), 205.70 (CH$_3$*C*OCH$_2$CH$_2$COO, Lev), 171.41 (CH$_3$COCH$_2$CH$_2$*C*OO, Lev), 171.29 (CH$_3$COCH$_2$CH$_2$*C*OO, Lev), 171.05 (OO*C*CH$_3$), 171.04 (OO*C*CH$_3$), 156.25 (NH*C*OOCH$_2$Ph, NHZ), [138.16, 137.89, 137.86, 137.72, 136.45, 135.76, 133.23, 132.91, 128.64, 128.48, 128.42, 128.39, 128.36, 128.16, 128.03, 127.95, 127.93, 127.87, 127.80, 127.71, 127.62, 127.58, 127.55, 127.53, 127.07, 126.42, 125.98, 125.89, 125.80 (C_{arom})], 100.64 (C-1’), 99.56 (C-1), 99.49 (C-1”), 99.19 (C-1’”), 82.91 (C-

3'''), 77.82 (C-4'''), 77.58 (C-2), 77.32 (C-2''), 76.67 (C-4'), 76.03 (C-3'), 75.17 (C-5' or C-5'''), 75.05 (*C*H_2, naphthylmethyl), 75.01 (Ph*C*H_2), 74.79 (C-5' or C-5'''), 74.78 (C-3), 74.70 (Ph*C*H_2), 74.37 (C-2'), 73.49 (C-2'''), 73.39 (Ph*C*H_2), 73.33 (Ph*C*H_2), 73.23 (C-3''), 69.49 (C-4), [68.87, 66.84 (C-6' & C-6''')], 68.67 (C-4''), 66.71 (NHCOO*C*H_2Ph), 65.92 (O*C*$H_2CH_2CH_2$NH), 65.21 (C-5''), 65.05 (C-5), [60.33, 60.03 (2 x O*C*H_3)], 38.94 (OCH_2CH_2*C*H_2NH), [37.74, 37.68 (2 x CH_3CO*C*H_2CH_2COO, Lev)], [29.69, 29.63 (2 x *C*$H_3COCH_2CH_2$COO, Lev)], 29.46 (OCH_2*C*H_2CH_2NH), [28.07, 27.89 (2 x CH_3COCH_2*C*H_2COO, Lev)], [20.91, 20.87 (2 x OOC*C*H_3)], 16.09 (C-6''), 15.67 (C-6); HR-MALDI-TOF/MS: calcd for $C_{90}H_{107}NO_{27}Na$ [M+Na^+] 1656.7030, found 1656.7023.

3-[(*N*-Benzyloxycarbonyl)amino]propyl 4-*O*-acetyl-3-*O*-{3-*O*-[4-*O*-acetyl-3-*O*-(4,6-di-*O*-benzyl-3-*O*-2-naphthylmethyl-*β*-D-glucopyranosyl)-6-deoxy-2-*O*-methyl-*α*-L-talopyranosyl]-4,6-di-*O*-benzyl-*β*-D-glucopyranosyl}-6-deoxy-2-*O*-methyl-*α*-L-talopyranoside (22)

A solution of hydrazine acetate (1.1 mg, 0.012 mmol) in dry MeOH (0.5 M) was added to a solution of compound **21** (20 mg, 0.012 mmol) in CH_2Cl_2 (10 mL) and the reaction mixture was stirred at room temperature for 1 hr. After completion on TLC, reaction was terminated by adding acetonyl acetone (1.5 µL, 0.012 mmol) to the mixture; and concentrated to dryness. The residue was dissolved in CH_2Cl_2; washed with water; and dried over $MgSO_4$. The crude product was purified by column chromatography (hexane:EtOAc 1:3) to afford compound **22** as colorless oil (16.5 mg, 92%). *Rf* = 0.17 (toluene:EtOAc 1:3); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 – 7.11 (m, 32H, H_{arom}), 5.58 (s, 1H, H-1''), 5.16 (d, *J* = 11.4 Hz, 1H, C*H*H– naphthylmethyl), 5.07 – 4.95 (m, 3H, C*H*$_2$ – Z, H-4''), 4.89 (d, *J* = 11.4 Hz, 1H, CH*H*– naphthylmethyl), 4.85 – 4.79

(m, 2H, PhC*H*H, H-1), 4.74 (m, 2H, H-4, NH), 4.55 – 4.44 (m, 7H, PhCH*H*, 3 x PhCH_2), 4.29 (d, *J* = 7.6 Hz, 1H, H-3'"), 4.21 (d, *J* = 7.6 Hz, 1H, H-1'), 4.02 (q, *J* = 6.3 Hz, 1H, H-5), 3.89 (m, 3H, H-5", H-3, H-3"), 3.68 – 3.60 (m, 7H, H-3', H-6_{a+b}', H-6_{a+b}'", H-3'", OC*H*HCH_2CH_2NHZ), 3.55 – 3.34 (m, 15H, H-2", H-2'", H-4'", OCH_3, OCH_3, H-2, OCH*H*CH_2CH_2NHZ, H-4', H-5', H-5'", H-2'), 3.19 (dt, *J* = 6.5, 12.7 Hz, 2H, OCH_2CH_2CH_2NHZ), 2.10 (s, 3H, OOCCH_3), 2.08(s, 3H, OOCCH_3), 1.71 – 1.69 (m, 2H, OCH_2CH_2CH_2NHZ), 1.12 (d, *J* = 6.5 Hz, 3H, H-6"), 0.84 (d, *J* = 6.5 Hz, 3H, H-6); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.04 (OO*C*CH_3), 172.87 (OO*C*CH_3), 156.29 (NH*C*OOCH_2Ph, NHZ), [138.30, 138.23, 138.11, 137.90, 136.44, 136.38, 133.32, 132.92, 128.62, 128.48, 128.37, 128.34, 128.33, 128.29, 128.15, 127.94, 127.92, 127.88, 127.66, 127.64, 127.61, 127.55, 127.48, 127.22, 126.60, 126.28, 125.84, 125.65 (C_{arom})], 104.60 (C-1'), 104.13 (C-1'"), 99.08 (C-1"), 98.62 (C-1), 84.45 (C-3'"), 78.36 (C-3'), 78.06 (C-2), 77.84 (C-2"), 77.20 (C-2'"), 77.17 (C-2'), 75.84 (C-3), 75.81 (C-3"), 75.79 (C-4'"), [75.32, 75.15, 75.11 (C-4', C-5' & C-5'")], 75.06 (Ph*C*H_2), 75.00 (*C*H_2, naphthylmethyl), [74.82, 73.42, 73.38 (3 x Ph*C*H_2)], 70.88 (C-4"), 70.57 (C-4), [69.18, 69.01 (C-6' & C-6'")], 66.73 (NHCOO*C*H_2Ph), 65.76 (O*C*H_2CH_2CH_2NH), 64.76 (C-5"), 64.55 (C-5), [60.22, 59.95 (2 x O*C*H_3)], 38.70 (OCH_2CH_2*C*H_2NH), 29.69 (OCH_2*C*H_2CH_2NH), [21.33, 21.29 (2 x OOC*C*H_3)], 16.18 (C-6"), 15.93 (C-6); HR-MALDI-TOF/MS: calcd for $C_{80}H_{95}NO_{23}Na$ [M+Na^+] 1460.6295, found 1460.6288.

3-Aminopropyl 4-*O*-acetyl-3-*O*-{3-*O*-[4-*O*-acetyl-3-*O*-*β*-D-glucopyranosyl)-6-deoxy-2-*O*-methyl-*α*-L-talopyranosyl]-*β*-D-glucopyranosyl}-6-deoxy-2-*O*-methyl-*α*-L-talopyranoside (1)

The partially deprotected compound **22** (8 mg, 5.6 µmol) was stirred overnight with a $Pd(OH)_2/C$ (15 mg) in *tert*-butanol/H_2O/AcOH (2 mL/0.05 mL/0.05 mL) under an atmosphere of hydrogen. The reaction mixture was filtered through polytetrafluoroethylene (PTFE) filter and the residue was washed with acetic acid (2 mL). The combined filtrates were concentrated in vacuo and the residue was purified over itrobeads to give compound **1** as white solid. (5 mg, 96%). R_f = 0.40 (CH_3CN/H_2O/AcOH 4:2:0.1); ^{1}H NMR (500 MHz, D_2O) δ 5.42 (s, 1H, H-1”), 5.38 (d, *J* = 2.4 Hz, 1H, H-4”), 5.36 (d, *J* = 2.9 Hz, 1H, H-4), 5.09 (s, 1H, H-1), 4.60 (t, *J* = 7.6 Hz, 2H, H-1’, H-1’”), 4.46 (q, *J* = 6.5 Hz, 1H, H-5”), 4.38 (t, *J* = 3.7 Hz, 1H, H-3”), 4.33 (t, *J* = 3.8 Hz, 1H, H-3), 4.16 (q, *J* = 6.0 Hz, 1H, H-5), 3.94 (d, *J* = 12.3 Hz, 2H, H-6_a’, H-6_a’”), 3.87 – 3.82 (m, 1H, OC*H*HCH$_2$CH$_2$NHZ), 3.76 – 3.73 (m, 3H, H-6_b’, H-6_b’”, H-2”), 3.67 (d, *J* = 3.6 Hz, 1H, H-2), 3.66 – 3.61 (m, 2H, OCH*H*CH$_2$CH$_2$NHZ, H-3’), 3.51 – 3.38 (m, 12H, H-3’”, H-4’, H-4’”, H-5’, H-5’”, 2 x OC*H*$_3$, H-2’), 3.30 (dd, *J* = 8.1, 9.1 Hz, 1H, H-2’”), 3.17 – 3.08 (m, 2H, OCH$_2$CH$_2$C*H*$_2$NHZ), 2.19 (s, 6H, 2 x OOCC*H*$_3$), 2.00 (dt, *J* = 7.2, 14.4 Hz, 2H, OCH$_2$C*H*$_2$CH$_2$NHZ), 1.14 (d, *J* = 6.5 Hz, 3H, H-6), 1.11 (d, *J* = 6.6 Hz, 3H, H-6”); ^{13}C NMR (75 MHz, D_2O) δ [174.05, 173.95 (2 x OO*C*CH$_3$)], 100.85 (C-1’), 100.39 (C-1’”), 97.88 (C-1”), 96.83 (C-1), 82.50 (C-3”), 77.83 (C-2”), 77.61 (C-2), [76.16, 76.11, 75.50 (C-3’”, C-4’, C-4’”)], 73.74 (C-5’ or C-5’”), 73.09 (C-2’”), 71.80 (C-3), 71.10 (C-3”), 69.76 (C-2’), 69.58 (C-4), 69.44 (C-4”), 68.02 (C-5’ or C-5’”), 65.62 (C-5”), 65.48 (C-5), 65.34 (O*C*H$_2$CH$_2$CH$_2$NH), [60.90, 60.87 (C-6’ & C-6’”)], [58.34, 58.09 (2 x O*C*H$_3$)], 37.53

($OCH_2CH_2\mathit{C}H_2NH$), 26.80 ($OCH_2\mathit{C}H_2CH_2NH$), [20.56, 20.53 (2 x $OOC\mathit{C}H_3$)], 15.33 (C-6), 15.29 (C-6"); HR-MALDI-TOF/MS: calcd for $C_{33}H_{57}NO_{21}Na$ [$M+Na^+$] 826.3423, found 826.3430.

3.6 References

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Chapter IV

Conclusion

Bacillus anthracis, *Burkholderia pseudomallei* and *Burkholderia mallei*, etiological agents of anthrax, melioidosis, and glanders, respectively, are believed to have potential for use in biological warfare and bioterrorism. These pathogens contain unique carbohydrate epitopes that are specific to these bacterial species. The capsular polysaccharides-based vaccines have been developed in the recent past and successfully administered to prevent diseases. We envisioned that the cell-surface oligosaccharides derived from these pathogens could be interesting targets for development of the carbohydrate-conjugate vaccines for anthrax, melioidosis, and glanders. Additionally, neutralizing antibodies can be raised against these antigens those could be used as diagnostics in rapid detection of these pathogens before they become fatal.

In order to evaluate the antigenicity of these oligosaccharides, we decided to chemically synthesize these carbohydrate antigens. And thus, we developed convergent syntheses of trisaccharide derived from the highly immunogenic glycoprotein, BclA, of the *B. anthracis* spore exosporium containing a unique monosaccharide unit, trivially named as anthrose. The structural analogs of this trisaccharide were also synthesized in order to determine the immunodominant components of this oligosaccharide. In our study, we demonstrated that the anthorse-

containing trisaccharide of BclA is antigenic and exposed on the surface of *B. anthracis* Sterne $34F_2$ spores. We have also located an important antigenic component in the terminal 3-methyl-butyryl moiety of the anthrose residue and confirmed its specificity using synthetic trisaccharide analogs. Our observations provide an important proof-of-concept step in development of spore-specific reagents for the early detection and targeting of non-protein structures in *B. anthracis*.

During the course of this study, we also synthesized the tetrasaccharide *O*-antigen fragment derived from *B. pseudomallei*. While constructing this fragment, we also developed a convergent synthetic approach to achieve a challenging glycosylation at the sterically hindered C-3 hydroxyl of the 6-deoxy-talopyranoside. Protein-conjugate of this carbohydrate epitope will facilitate the immunological evaluation of this fragment. In future, removal of the 4-*O*-acetyl substitution from this tetrasaccharide by treatment with a base will provide a carbohydrate epitope characteristic of *B. mallei*.

Ongoing study will determine the potential of these oligosaccharides for use in development of vaccines and/or diagnostics for anthrax, melioidosis, and glanders.