SYNTHESIS AND IMMUNOLOGICAL PROPERTIES OF *RHIZOBIUM SIN*-1 LIPID A AND AN OLIGOSACCHARIDE DERIVED FROM THE CELL WALL OF *BACILLUS ANTHRACIS*

by

MAHALAKSHMI VASAN

(Under the Direction of Geert-Jan Boons)

ABSTRACT

LPS from *Rhizobium sin*-1 (*R. sin*-1) can antagonize the production of tumor necrosis factor alpha (TNF-α) by *E. coli* LPS in human monocytic cells. Although these compounds provide interesting leads for the development of therapeutics for septic shock yet, the propensity of these compounds to undergo β-elimination to give biological inactive enone derivatives hampers detailed structure activity relationship studies. To address this problem, we have chemically synthesized in a convergent manner a *R. sin*-1 lipid A derivative in which the β-hydroxy ester at C-3 of the proximal sugar unit has been replaced by an ether linked moiety. The antagonizing ability against *E. coli* lipid A and the stability of the synthetic compound has been tested and compared with narural *R. sin*-1 lipid A.

Bacillus anthracis is a gram-positive, spore-forming bacterium that causes anthrax in humans and other mammals. The secondary cell wall of vegetative cells of Bacillus anthracis contains an unusual polysaccharide, which may represent an important target for vaccine and diagnostics development. The antigenic properties of this oligosaccharide have, however, not

been studied. We have synthesized two trisaccharides and tested for their affinity toward the antibodies produced from a live- and irradiated spore vaccine and polysaccharide linked to the carrier protein KLH.

Finally, to locate important antigenic components of the hexasaccharide we are synthesizing various oligosaccharide fragments. The oligosaccharides are synthesized with an aminopentyl spacer to facilitate conjugation to carrier proteins, which is required for immunization and ELISA.

INDEX WORDS: Sepsis, Septic Shock, Anthrax, *Bacillus anthracis*, *Rhizobium sin*-1, Lipid A, Lipopolysaccharide, Vaccine, Diagnostic Tool, ELISA, Glycoconjugates, Antagonists, Agonists, Oligosaccharides, BSA, KLH, Toll like Receptors, Glycosylation, Imidate, Thioethyl, Gram Negative Bacteria, Gram Positive Bacteria, Aminopentyl Linker, Therapeutics.

SYNTHESIS AND IMMUNOLOGICAL PROPERTIES OF *RHIZOBIUM SIN*-1 LIPID A AND AN OLIGOSACCHARIDE DERIVED FROM THE CELL WALL OF *BACILLUS ANTHRACIS*

by

MAHALAKSHMI VASAN

B.S., Bharathidasan University, India, 1997

B. Tech., Mumbai University, India, 2000

A Dissertation Submitted to the Graduate Faculty of the University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2008

SYNTHESIS AND IMMUNOLOGICAL PROPERTIES OF *RHIZOBIUM SIN*-1 LIPID A AND AN OLIGOSACCHARIDE DERIVED FROM THE CELL WALL OF *BACILLUS ANTHRACIS*

by

MAHALAKSHMI VASAN

Major Professor: Geert-Jan Boons

Committee: Russell W. Carlson
Vladimir V. Popik

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
December 2008

DEDICATION

This dissertation is dedicated to my parents and in-laws, husband Sivakumar Raghavan and my other family members for their unconditional support and encouragement.

ACKNOWLEDGEMENTS

I would like to thank Dr Geert Jan Boons for giving me an opportunity to work in his lab and for his support and supervision throughout my research. I would like to thank Dr. Margreet Wolfert for doing all the bioassays of my compounds without which my projects won't be complete and also explaining me all the basics of the assays. I would like to thank my committee members Dr. Carlson and Dr. Popik for their suggestions and help. I would like to thank Dr. John Glushka for helping me with the NMR instruments and clarifying doubts about the spectrum. It has been my greatest pleasure to work with Dr. Andre Venot, who is the most experienced person in our lab. I not only learnt how to carry out experiments but also how to judicially use various chemicals and keep the hood, surroundings clean, and follow safety precautions. I would like to thank Dr. Therese Buskas and Dr. Jana Rauvolfova for their help in preparing glycoconjugates without which my project would not be complete. I also want to express my appreciation toward all my lab mates who have been very friendly, helpful, resourceful, and supportive.

I would like to specially thank my husband Dr. Sivakumar Raghavan for his inspiration, encouragement, and support at all times of my PhD life without which it would not have been easy to accomplish this task. Also, I want to thank my parents and my parent in laws and my other family members for their constant support and encouragement.

Finally I would like to thank Saeid for his technical help with computer and other software related issues and CCRC staff members who have been very friendly and helpful.

TABLE OF CONTENTS

Page

ABBREVIATIONS

Å…….....Angstrom

Ac…….. Acetyl

Ac_2O……..... Acetic anhydride

AcOH…….. Acetic acid

ATCC…….....American type culture collection

AVA…….Anthrax vaccine adsorbed

B. anthracis……..... *Bacillus anthracis*

$BH_3 \cdot NHMe_2$…….. Dimethylamine borane

$BF_3 \cdot Et_2O$……..Borontrifluoride diethylether

Bn……Benzyl

BSA……...Bovine serum albumin

BuOH……Butanol

C…….Carbon

Cbz…….Carboxybenzyl

C. jejuni……...*Campylobacter jejuni*

C. trachomatis……..*Chlamydia trachomatis*

$C_6H_5CH\text{-}(OMe)_2$…….Benzaldehyde dimethylacetal

CD4/14…….....Cluster differentiation 4/14

CDAP……. Cyano dimethyl aminopyridinium

$CHCl_3$……..Chloroform

CHF……..... Congestive heart failure

CH_3CN……..... Acetonitrile

CSA……Camphor Sulfonicacid

CO_2……Carbon dioxide

$CuSO_4$……..... Copper sulfate

COSY……..Correlation spectrocopy

DCC…….....Dicylcohexyl carbidiimide

DCM/CH_2Cl_2…….Dichloromethane/Methylene chloride

DDQ…….Dichloro dicyano quinone

DMAP…….....*N, N*-Dimethylaminopyridine

DMF…….....*N*, *N*-dimethylamineformamide

DMSO…….....Dimethyl sulfoxide

pcDNA……...Deoxyribo nucleicacid

EC_{50}…….. Effective concentration

E. coli……..*Escherichia coli*

EDTA……...Ethylene diamine tetracetate

pELAM-Luc……...plasmid Endothelial cell leukocyte adhesion molecule-luciferase

ELISA…….....Enzyme linked immunosorbent assay

ET/EF……..Edema toxin/factor

Et_3SiH…… Triethylsilane

ERK……Extracellular signal regulated kinases

EtOH…….....Ethanol

Et_2O…….....Diethyl ether

FBS…….....Fetal bovine serum

Gal……..... Galactose

Glc……..... Glucose

H_2……... Hydrogen

HEK……..... Human embryonic kidney

HEPES…….....4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Hep……Heptose

HF…….Hydrogen fluoride

HIV…….....Human immunodeficiency virus

HMBC…….....Heteronulear multiple bond correlation

HPAEC-PAD……. …….....High performance anion exchange chromatography pulsed amperometric detection

HSQC……...Heteronuclear single quantum coherence

Hz……..Hertz

IC_{50}…….. Inhibition concentration

ICU……..... Intensive care unit

IgG……..... Immunoglobin G

IgM/IgA……..... Immunoglobin M/A

IL……. Interleukin

IRF…….....Interferon regulatory factor

INF-β…….Interferon Beta

IP-10…….....Inducible protein 10

JNK…….Jun *N*-terminal kinase

Kdo…….....3-deoxy-D-manno octulosonic acid

KLH……..Keyhole lymphocyte

LBP……. Lipid binding protein

LPS……..... Lipopolysaccharide

LT/LF…….....Lethal toxin/factor

M…….. Molar, Mega

MAbs…… Monoclonal antibodies

Man……..... Mannose

MAP/MAPK…….....Mitogen-activated protein/ kinase

MALDI-TOF-TOF……Matrix assisted laser desorption ionization spectroscopy time of flight

MeOH…… Methanol

$MgSO_4$……..... Magnesium sulfate

MHCII…….Major histocompatibility complex II

MM6……Mononuclear macrophages 6

MS……..... Molecular sieves

MyD88…….....Myeloid differentiating protein 88

NIS……..... *N*-iodosuccinimide

NaH……..... Sodium hydride

$NaHCO_3$……..... Sodium bicarbonate

NaN_3……..... Sodium azide

NaOH……..... Sodium hydroxide

NaOMe…….....Sodium methoxide

$Na_2S_2O_3$……..... Sodium bisulfite

NH_3……..... Ammonia

NO……..... Nitric oxide

NH_2NH_2…….....Hydrazine

NMR…….Nuclear magnetic resonance

NF-κB……...Necrosis Factor

OD……..... Optical density

Pd…….....Palladium

$Pd(OH)_2$……Palladium hydroxide

pRL-TK…….....Renilla luciferase

PS…….....Polysaccharide

PBS…….....Phosphate buffered saline

P. diminuta…….....*Pseudomonas diminuta*

R. capsulatus…….. *Rhodobacter capsulatus*

R. etli…….. *Rhizobium etli*

R. leguminosarum…….. *Rhizobium leguminosarum*

R. sphaeroides…… *Rhodobacter sphaeroids*

R. viridis…….....*Rhacophorus viridis*

STI-1……Sterne like vaccine

S. aureus……..... *Staphylococcus aureus*

S. enterica…….....*Salmonella enterica*

PA……..... Protective antigen

Pam_3CysSK_4……..Palmitic-cystein-lysine

PAMPs……...Pathogens associated molecular patterns

PCC……..... Pyridinim chlorochromate

PMe_3…… Trimethyl phosphine

PTFE……. Polytetrafluroethylene

TBDMS……..... Tert-butyl dimethylsilyl

TFA……. Trifluroacetic acid

Tf_2O……Triflic anhydride

TfOH……... Triflic acid

TLC……Thin layer chromatography

THF……..... Tetrahydrofuran

TIR……..Toll-interleukin receptor

TLR……... Toll like receptor

TMS…….. Trimethyl silyl

TMSOTf……... Trimethylsilyl trifluromethane sulfonate

TNFα……...Tumour necrosis factor alpha

TOCSY……..Total correlation spectroscopy

TRIF……..... Toll like interleukin factor

UV……Ultraviolet

Zn……... Zinc

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Carbohydrate-based Antibacterial Vaccines

Polysaccharide capsule, glycoproteins or glycolipids which cover the cell surfaces of many Gram negative and Gram positive bacteria are often distinct from those of their hosts. The lipid A of Gram negative bacteria and the cell surface polysaccharides of Gram positive bacteria can be a useful target for the development of carbohydrate-based diagnostics and vaccines. The development of vaccines based on carbohydrates has a long history. As early as 1923, Heidelberger[1],[2] and Avery described a soluble specific substance of pneumococci to consist most likely of polysaccharides (PSs) and being typical for a serotype. They also established that pneumococcal capsular polysaccharides could be used as vaccines, providing long lasting immunity. In 1983 a capsular polysaccharide vaccine Pneumovax^{TM} 23, which is derived from 14 pneumonia serotypes, was introduced. Subsequently, Pneumovax^{TM} 23 was developed containing isolated polysaccharides from 23 serotypes out of the about 90 known. This vaccine gives, in healthy adults, short term protection for about 90% of the infections by these microorganisms. However, polysaccharides are poorly immunogenic in persons of high-risk groups such as (i) neonates and children until the age of two; (ii) elderly and chronically ill people; (iii) splenectomised patients; (iv) immuno-compromised people such as HIV infected individuals. The age-related response to plain polysaccharides may also be structure dependent. For example, in contrast to other capsular polysaccharides, those of group A *Neisseria meningitidis* and pneumococci type 3 and 18 C are good immunogens in infants from

3 to 6 months as they induce protective IgG antibodies. In fact, vaccines have been prepared from capsular polysaccharides, *e.g.* vaccines containing capsular polysaccharides from the meningococcal types include A + C, A + C + W135 and A + C + Y + W135 are used against meningococcal infections. Several vaccines based on purified capsular polysaccharides or on neoglycoconjugates are now commercially available, such as vaccines against *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Haemophilus influenza* type b (Hib) and *Salmonella typhi*.

Polysaccharides are considered to give an immune response independent of T cells; they stimulate B-cells to produce antibodies without the involvement of T-cells. In contrast to polysaccharides, glycoproteins are T-cell dependent antigens, eliciting stronger immune response against the same antigens. Already in 1931, Avery and Goedel[3] reported that covalent attachment of carbohydrates to a suitable protein induced an enhanced immunogenicity compared to the polysaccharides as such. In general, capsular polysaccharides elicit type-specific protective immune responses in adults but not in infants, who do not respond with antibodies that confer protection. Immunization with neoglycoproteins consisting of capsule-derived carbohydrates coupled to an immunogenic protein can provide a long lasting protection to encapsulated bacteria for adults as well as for persons at high risk and young children.[4,5] However, it has recently been shown that some zwitterionic capsular polysaccharides are able to activate $CD4^+$ T cells. These polysaccharides are processed to low molecular weight carbohydrates by a nitric oxide-mediated mechanism and presented to T cells through the MHC II endocytic pathway.[6]

Traditionally, carbohydrate antigens for antibacterial vaccines have been isolated from biological sources. Recently, intense efforts have focused on the use of defined carbohydrate antigens that are synthesized rather than isolated. Improved analytical tools have helped to

identify the exact chemical structure of carbohydrate antigens and have aided the development of new vaccines. The procurement of defined oligosaccharides using improved solution- and solid-phase methods has become fast enough to be used reiteratively in drug-development efforts. Synthesis of well defined molecular entities renders possible, the relationship between saccharide chain length and/or their density on the carrier molecule and their immunological properites. A synthetic oligosaccharide-based conjugate vaccine is now used in Cuba, where the large-scale synthesis,[7] pharmaceutical development, and clinical evaluation of a conjugate vaccine composed of a synthetic capsular polysaccharide antigen of Hib was achieved. Long-term protective antibody titers compared favorably with products prepared with the Hib polysaccharide extracted from bacteria.

The focus in the subsequent chapters will be on development of carbohydrate based therapeutics from cell surface lipid A and polysaccharide for the treatment of Gram negative septicemia and anthrax, respectively. Currently there is no treatment available for treating septicemia. Of all the available strategies for the treatment of sepsis, the most promising method seems to be antagonizing the interaction of enteric lipopolysaccharide LPS with its cell-surface receptor and neutralizing their effects on the cell. *Rizobium sin*-1 (*R. sin*-1) lipid A has been shown to be a potent antagonist of *E. coli* LPS and doesn't induce any cytokine production by human macrophage cells.[8]

With respect to anthrax, the non-specific nature of the symptoms and the limitation associated with the current AVA vaccine to act on only germinated spores have led to a renewed interest toward the development of a diagnostic tools and vaccines. Carbohydrate antigens found on the cell surface of these bacteria could be a potential vaccine candidate by itself or in combination with PA, for treatment of anthrax or as a diagnostic tool for *Bacillus anthracis (B.*

anthracis). Recently a tetrasaccharide[9] and hexasaccharide[10] have been discovered on the surface of spores and vegetative cell wall respectively of the biowarfare agent *Bacillus anthracis*. The synthesis of these immunogenic surface oligosaccharide has been accomplished by different groups.[11-17] Synthesis of a species-specific tetrasaccharide antigen allowed the production of antibodies that specifically recognize *B. anthracis* in the presence of the closely related opportunistic human pathogen *Bacillus cereus*.[18]

Significance of Sepsis

Septicemia, commonly known as sepsis, is associated with a 40-60% mortality rate worldwide. It has been estimated that 1% of hospital patients and 20-30% of ICU patients develop sepsis and it is the leading cause of death in patients admitted to non-cardiac intensive care units.[19,20] According to the National Vital Statistics Report[21] sepsis is the tenth leading cause of death in the United States, as 750,000 people develop sepsis on an annual basis.[22] About 215,000 of affected Americans die of sepsis each year.

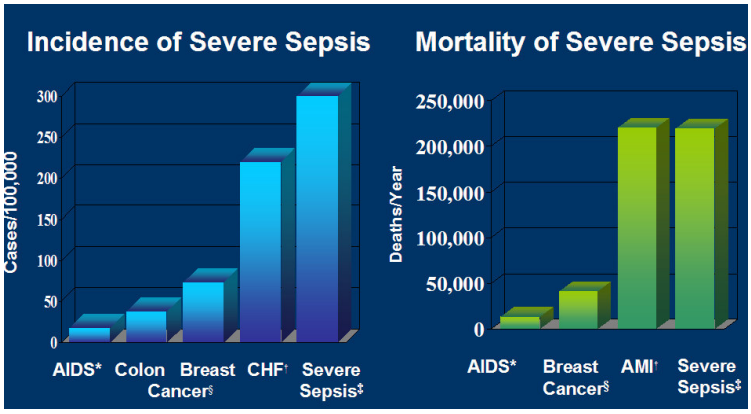


Figure 1.1. Sepsis in comparison with other major diseases.[23]

This number equals the number of Americans who die of coronary heart disease without hospital treatment (**Figure 1.1**). The incidence of sepsis is significantly greater than that of other major diseases such as congestive heart failure (CHF), colon and breast cancer.[23]

Endotoxin (lipopolysaccharide, LPS) from Gram-negative bacteria (*e.g. E. coli*) have been implicated as the major cause of sepsis and accounts for almost half (100,000 in the US) of deaths from the illness. The development of septicemia is often linked to a systemic inflammatory response to LPS in the blood of affected patients.[24-26] The presence of high LPS levels in the blood of affected patients strongly implicates endotoxemia as a potential critical factor in pathogenesis. If the Gram-negative bacteria release a moderate amount of endotoxins, the macrophage products help eradicate the immediate infection by generating a desirable, localized and controlled immune response. However, if an infection is severe, large amounts of endotoxin are released into the bloodstream, generating an overproduction of mediators by the macrophage, which leads to the undesirable outcome of septic shock. The biological activity of endotoxin is associated with the LPS and its toxicity is linked to the lipid component Lipid A. Lipid A, the hydrophobic anchor of LPS (endotoxin) is a major component of the outer membrane of Gram-negative bacteria. **(Figure 1.2)**

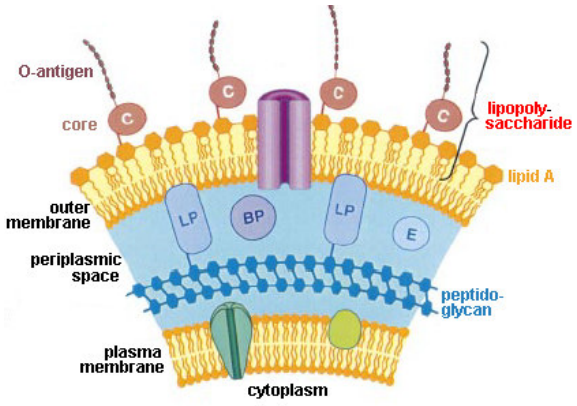


Figure 1.2. Cell envelope of a Gram-negative bacterium.[27]

Innate Immune Responses Toward Lipopolysaccharides

The innate immune system is an evolutionary ancient system of defense against microbial infections.[28] It responds rapidly to highly conserved families of structural patterns called pathogen associated molecular patterns (PAMPs), which are integral parts of pathogens and are perceived as danger signals by the host. Recognition of PAMPs is mediated by sets of highly conserved receptors[29] which then induce production of chemokines and cytokines to combat the invading pathogens. The discovery of TLRs less than a decade ago has advanced the understanding of early events in microbial recognition and response, and the development of an adaptive immune response.[30-32] LPS, which are structural components of the outer surface membrane of gram negative bacteria stimulate cellular responses through toll like receptor 4 (TLR4). LPS indirectly harms the body when massive amounts of the toxin are released during severe Gram-negative infections. It is the most potent pro inflammatory substance known, as its lipid A region initiates the production of multiple host derived inflammatory mediators such as cytokines (*e.g.*, tumor necrosis factor TNFα), arachidonic acid metabolites, and tissue factor.

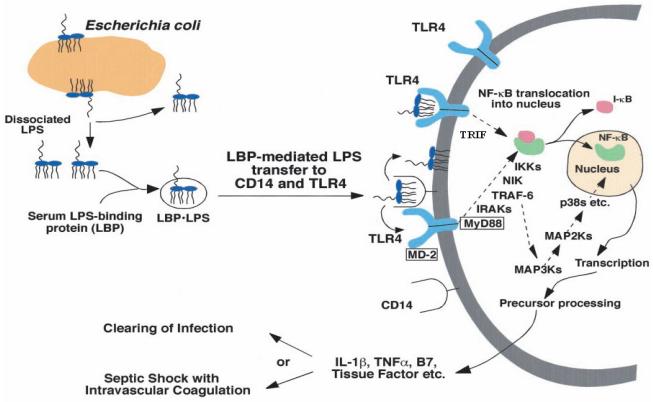


Figure 1.3. Mechanism of Septic Shock.[33]

Endotoxin (LPS) is shed in small amounts throughout the lifespan of the Gram-negative bacteria and is disseminated in large quantities upon cell death and lysis (**Figure 1.3**). It then binds to plasma binding protein produced by the body, creating a complex referred to as LPS-Lipid binding protein (LBP).[34] This complex is subsequently recogonized by CD14, a glycosylphosphatidylinositol (GPI) anchored protein. Alternately, it may interact with a soluble form of CD14 and activate cells lacking the membrane form of CD14.[35] CD14 lacks transmembrane and cytoplasmic domains, and is therefore unable to transmit LPS binding signals directly to the interior of the cell. The actual signal transduction is initiated after CD14 has transferred the LPS to TLR4, which is in turn complexed to an accessory protein MD2. TLR4 contains extracellular, transmembrane, and intracellular domains. TLR4 can initiate cell signaling by two cascades that involve recruitment of either the intracellular adaptor proteins MyD88 or TRIF.[36,37] This in turn leads to activation of the NF-κB and the MAP kinases. The end result is an up-regulation of more than 120 genes, producing TNF-α, IL-1β, and IL-6 through the MyD88 pathway and INF-β, IP-10, and NO through TRIF pathway. This condition stimulates an increase in the pro-inflammatory cytokine levels that are responsible for endotoxic shock or sepsis.

Structural Features of Lipopolysaccharide

The LPS of all Gram-negative bacteria consists of two main components, a hydrophilic polysaccharide and an *O*-polysaccharide.[38,39] The hydrophilic polysaccharide is covalently bound to the hydrophobic lipid A, thus creating an amphiphilic molecule (**Figure 1.4**). The *O*-polysaccharide composes the outermost part of the LPS of all Gram-negative bacteria and is therefore the primary antigen targeted by host antibody responses. The immune responses to the *O*-polysaccharide can be highly *O*-chain specific. Often referred to as the *O*-antigen, it consists

of repeating oligosaccharide subunits made up of 2-8 sugars. These subunits differ between strains by means of different sugar units, sequence and connectivity. The individual chains vary in length, the greatest length being equal to 50 repeating subunits. A single organism can produce a wide range of these lengths as a result of the incomplete synthesis of the chain. The *O*-polysaccharide is significantly longer than the core polysaccharide and maintains the hydrophilic domain of LPS. For example, *E. coli* contains only 5 unique core structures but more than 160 different *O*-chains.

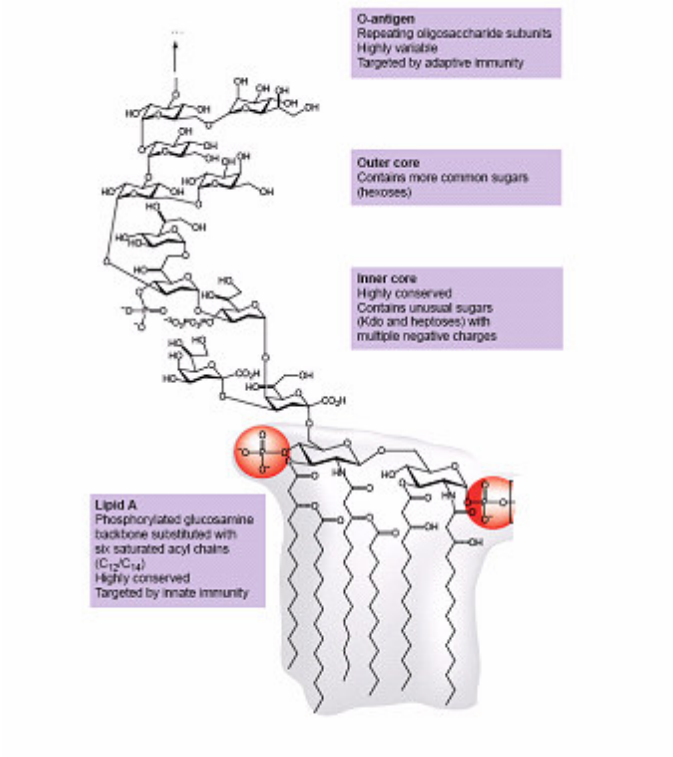


Figure 1.4. Lipopolysaccharide of *E. coli.*

The differences between the core polysaccharide and *O*-polysaccharide extend to sugar types. The outer core of the polysaccharide consists of common sugars such as glucose (Glc), galactose (Gal), *N*-acetylglucosamine (Glc*N*Ac), and *N*-acetyl galactosamine (Gal*N*Ac). The inner core consists of unusual sugars such as 3-deoxy-D-manno-octulosonic acid (Kdo) and L-glycero-D-manno-heptose (Hep). The Kdo unit is α-bound to the carbohydrate backbone of the

lipid A in all instances and very essential for bacteria viability. Hence, drugs targeting Kdo synthesis enzymes would represent a new class of antibiotics. The linkage between the first Kdo unit and the lipid A component is very acid labile, exhibiting a moderate pH of 4.4. The lipid A region is the hydrophobic and endotoxically active part of the LPS, and is covalently linked to the inner core by the Kdo residue. Structurally, lipid A consists of a β (1, 6) *N*-acetylglucosamine dimmer, carrying two phosphoryl groups at positions 1 and 4'. In many cases, the 4' phosphate group can be further substituted with ethanolamine, ethanolamine phosphate, Glc*N*, 4-amino-4-deoxy-L-arabino-pyranose and D-arabino-furanose. There are up to four acyl chains attached to this structure by ester and amide linkages and the chains may be substituted further by fatty acids that vary considerably within each species.

Lipid A: Structure *vs*. Function

In 1954 Otto Westphal and Otto Luderitz[40] postulated that the lipid A component of LPS was primarily responsible for its endotoxicity. Following several years of analytical studies beginning in 1954, the complete chemical structure of lipid A (*E. coli & S. enterica sv. Typhimurium*) was elucidated in 1983.[41] Analysis of the chemical structure of Lipid A continued in 1984 with the first total synthesis of *E. coli* by Tetsuo Shiba and Shoichi Kusumoto.[42] A biological analysis of the toxicity, pyrogenicity and activation of monocytes of synthetic lipid A showed that all test systems, were identical to the natural *E. coli* lipid A. These experiments successfully demonstrated that the endotoxic activity of the large LPS molecule was due to the lipid A component.[43] During the last decade, lipid A structures derived from several bacterial species have been elucidated and characterized in terms of immunoactivity. Although the lipid A region is often assigned as the highly conserved part of the LPS it has been found to express a

certain degree of structural diversity with respect to three structural elements: (i) Lipid A backbone; (ii) Polar group substituents; (iii) Acylation pattern

Even the lipid A derived from a single bacterial strain possesses a microheterogenic mixture of several chemical structures with respect to variations in the polar group substituents and acylation pattern.

i) Disaccharide or lipid A backbone: In a vast majority of the lipid A structures of Gram-negative bacteria characterized so far, the general structure of the glycosyl region consists of a β-(1, 6) linked disaccharide of D-glucosamine (Glc*pN*). Other naturally occurring backbone structures also include; 2,3-diamino-2,3-dideoxy-D-glucopyranose (Glc*pN*3*N*)-Glc*pN*3*N* disaccharide (*e.g. C. jejuni*), Glc*pN*3*N*-Glc*pN*3*N* disaccharide (*e.g. P. diminuta*) and Glc*pN*3*N* monosaccharide backbone (*e.g. R. viridis*).[44] Monosaccharide lipid A backbone generally lacks endotoxicity, suggesting that the disaccharide backbone is required for optimum recognition by for lipid A receptors.

ii) Polar group substituents: The backbone of a lipid A disaccharide contains, in general, two phosphate groups: one α-linked to the glycosylic hydroxyl group at C-1, and the other linked to the hydroxyl group present at C-4'. Structures containing one phosphate e.g. *B. fragilis* at either 1 or 4' are at least 1000 times less active than *E. coli* lipid A. However, alteration of the phosphates with phosphono-oxyethyl does not alter the activity of the compound, suggesting that charges play an important role in the restoration of activity. Other charged groups identified in naturally occurring lipid A analogs include ethanol amine, phosphoethanolamine,[45] L-4-amino-4-deoxy-arabinopyranose and D-galacturonic.

iii) Acylation Pattern: Of all the previously discussed modifications of the lipid A structure, the acylation pattern of the fatty acid is the most critical structural feature that determines endotoxcity. These fatty acids are in the form of (*R*)-3-hydroxy or (*R*)-3-hydroxyacyl chains that are linked to the backbone via ester and amide bonds at positions 2 and 3 as well as 2' and 3'. The number of fatty acid groups present in a molecule has a direct effect on its toxicity. The most common fatty acids in lipid A have 10-16 carbons although longer chains exist (e.g. C18 fatty acids in *H. pylori*,[46] C21 in *C. trachomatis*). Heterogeneity or variation in the degree of fatty acid substitution often results in more than three or four molecular species present in a single preparation due to mutations or defects in fatty-acid-transferases, leading to incomplete biosynthesis. These variations in the degree of fatty acid substitution have garnered much interest as it is now well recognized that fatty acids significantly influence their endotoxic potential when compared to the lipid A derived from wild-type strains. However, some of these compounds exhibit pronounced inhibitory effects. One such compound, compound 506, is a biological precursor of *E. coli* lipid A that has two acyl-substituted fatty acids removed. In a number of biological assays, this analogue not only lacks endotoxic activity, but is also an antagonist as it is able to inhibit normal lipid A signaling.

Strategies for Treatment of Sepsis

Based on the mechanism for the induction of sepsis by LPS, a variety of approaches have been examined for the treatment of sepsis. These approaches have focused on different points in the cascade of events that lead to severe sepsis. Although the majority of the approaches targeting the later stages of the endotoxin response have demonstrated efficacy both *in vitro* and in animal models, to date, none have proven to be effective in the treatment of human sepsis.[33]

Therefore, a more promising strategy for the treatment of sepsis may be to antagonize the interaction of LPS with its cell surface receptor. A number of approaches based on this strategy have been studied by using various antibodies directed against parts of the LPS molecule and the molecule as a whole so that this interaction would enhance LPS clearance or neutralize the ability of LPS to activate cells.[33]

LPS is a large and complex compound, so the use of antibodies directed against the entire LPS (in general the *O*-antigen region) would tend to be specific to the species from which the LPS was derived, and would limit its usefulness to countering infection by only a narrow range of bacterial stereotypes. In contrast, the lipid A region is more conserved. Thus, antibodies directed against lipid A should be a good candidate for antagonizing the interaction of LPS with its cell surface receptor and treating sepsis. As is often the case, efficacious pharmacological receptor antagonists are often derived by modifying a compound that has agonist activity. As mentioned before, lipid A is the endotoxical active part of LPS, so its structural analogs are promising candidates for actively antagonizing the effects of LPS.[33]

Certain lipid A analogs have been examined for their antagonistic properties. These analogs include naturally occurring lipid A precursors such as lipid X and lipid IVA, as well as a number of the synthetic analogs of these precursors. The best-studied derivatives are synthetic analogs derived from the lipid A of *R. sphaeroides* or *R. capsulatus*[47,48] (**Figure 1.5**), which have very similar lipid A structure. It has been shown that the *R. sphaeroides/R. capsulatus* lipid A lacks toxic effects and is an antagonist of enteric endotoxin.[49-52] Unfortunately, both natural *R. sphaeroides/R. capsulatus* lipid A and their synthetic analogs have ester-linked fatty acids to the glucosamine disaccharide backbone and can undergo degradation to 2,2'-di-ß-hydroxymyristyl-1,4'-bisphosphorylated glucosamine disaccharide that has agonistic properties.[53] To overcome

this problem, Christ and coworkers synthesized the analogs E5531[51] and E5564 (**Figure 1.5**) in which the fatty acid ester linkages to the disaccharide backbone were replaced by fatty alcohol ether linkages.

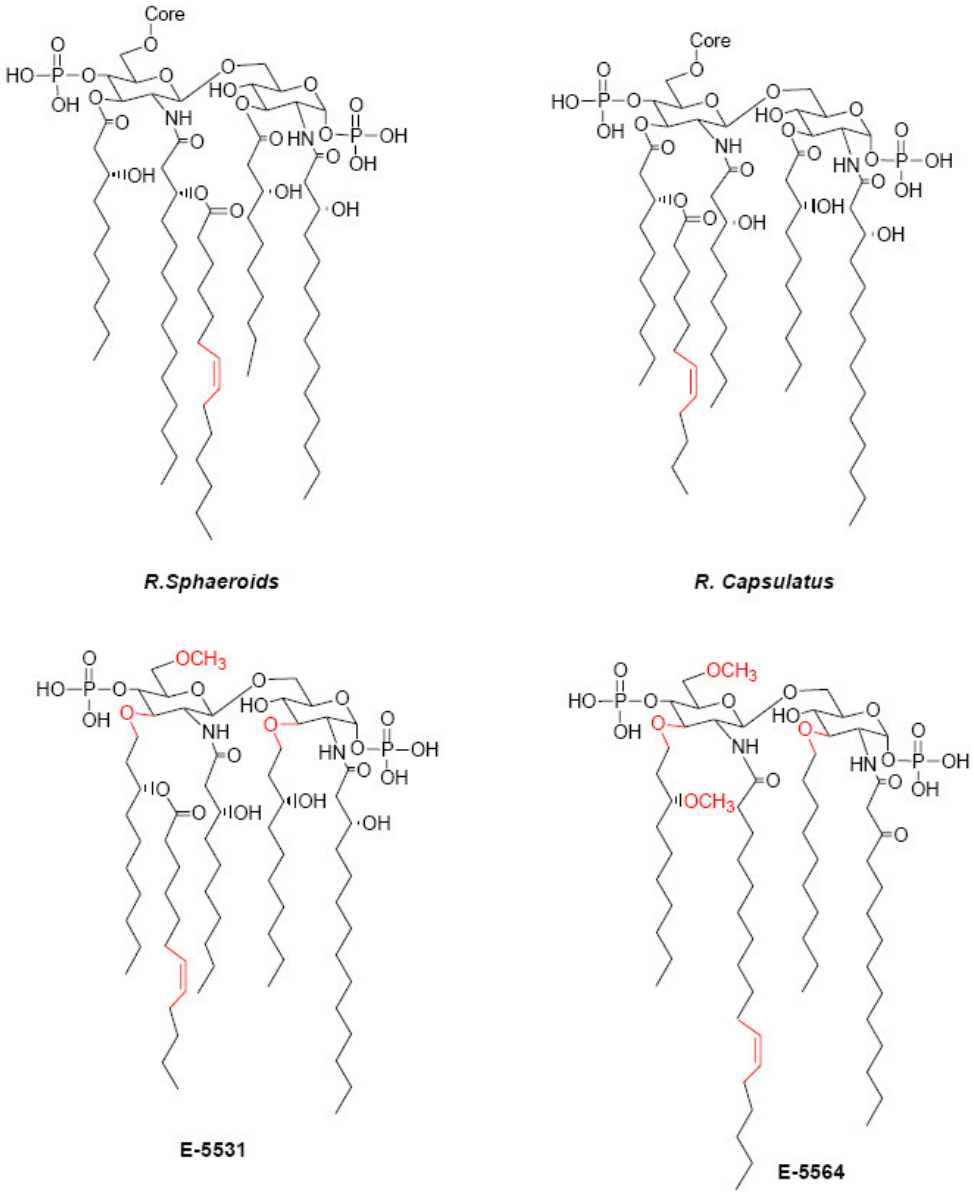


Figure 1.5. Lipid A structures of natural *R. Sphareroids, R. Capsulatus* and the synthetic lipid A, E5531 and E-5564.

It was discovered that E5531[8,51,54,55]and E5564[56] prevent the pyrogenic effects of enteric LPS in rabbits, protect against LPS-induced lethality in mice, and blocks the Toll-like receptor 4-

mediated NF-*k*B activation by LPS. However, the synthetic strategy is complex, specifically designed to produce these compounds and not amenable to the production of numerous lipid A analogs that can be examined for structure-activity relationship studies.

Lipid A from Rhizobial Species

Rhizobia refer collectively to the group of Gram-negative bacteria that belong to the rhizobiaceae family and form nitrogen-fixing symbioses with legume plants. Structural studies of *R. sin*-1 by Carlson and coworkers[42,57] have shown that its lipid A is very different from other species of rhizobiaceae family like *R. etli,*[56] *R. leguminosarum*[57] (**Figure 1.6**) and perhaps the most unusual lipid A reported to date. Most importantly, *R.* s*in*-1 LPS does not induce cytokine production in human monomac 6 (MM6) cells and prevents enteric LPS-induced cytokine production. The following are the common differences that were found in Lipid A of any rhizobial species: (i) the hydrophilic or disaccharide backbone is devoid of phosphate; (ii) the 4' phosphate has been replaced in some species by a galacturonosyl residue (iii) the lipid A backbone contains a 2-aminogluconolactone or 2-aminogluconate; (iv) the lipid-A contains an unusual long fatty acid referred to as the 27-hydroxyoctacosanoic acid which may be esterified by β-hydroxybutyrate.

As previously discussed, these species differ in all aspects including lipid A backbone and fatty acylation pattern. For example, the lipid A of *R. sin*-1 shows considerable microheterogeneity. The fatty acylation pattern is heterogeneous and consists exclusively of β-hydroxy fatty acids. The *N*-acyl groups can consist of β-hydroxymyristate, β-hydroxypalmitate, or β-hydroxystearate. The *O*-acyl groups are primarily β-hydroxymyristate, but occasionally can also include β-hydroxypentadecanoate. Furthermore, a significant percentage of *R. sin*-1 lipid A lacks a fatty acyl residue at the C-3 position. The biosynthetic mechanism for the synthesis of *R.*

sin-1 lipid A is not known. However, the similarities of its structure with *R. etli* and *R. leguminosarum* lipid-A suggests that the biosynthetic steps of *R. sin*-1 lipid-A synthesis would be similar to those reported for *R. etli* and *R. leguminosarum*. These steps would include all of the enzyme activities that convert UDP-*N*-acetylglucosamine into two residues of 3-deoxy-D-manno-2-octulsonic acid lipid-IVa as well as specific enzymes that process this common lipid A precursor into the mature lipid A structures.

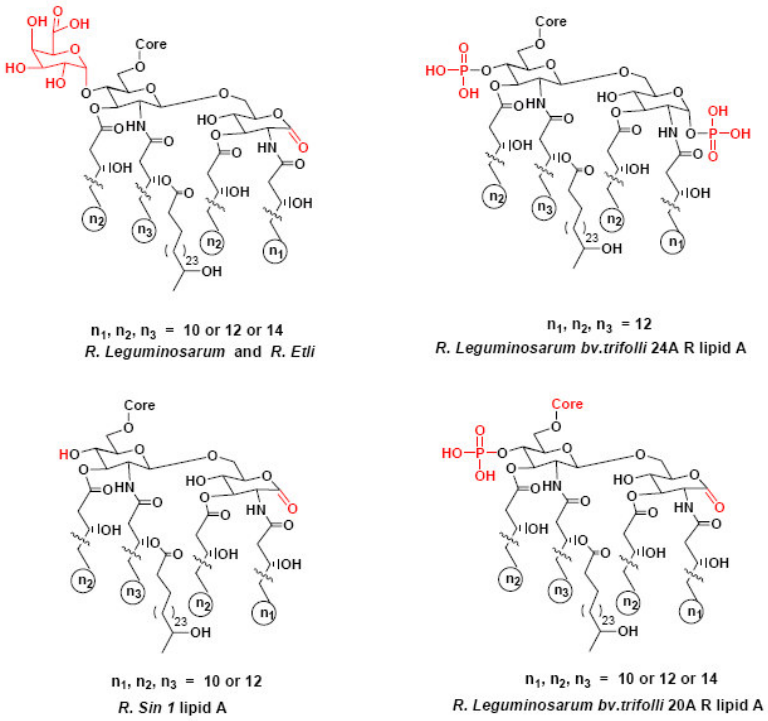


Figure 1.6. Lipid A structures for several *rhizobial* strains and their mutants AR24 and AR20.

These processing enzymes would be the 4' and 1-phosphatases, the glucosamine oxidase, the acyl carrier protein and transferase for 27-OHC28:0, and the acylase that removes the fatty acyl group from C-3. However, unlike *R. etli*, the *R. sin*-1 lipid-A structures suggest that this organism would lack the UDP-galacturonosyltransferase that adds galacturonic acid to C-4' and would possibly contain an additional acylase that removes, from a portion of the molecules, the fatty acyl group from C-3'. Due within any given rhizobial LPSs, it is difficult to be. The

inability to separate this heterogeneous forms limits the identification of specific structural features that makes *R. sin*-1 lipid A an antagonist as opposed to an agonist and developed as a therapeutic agent for Gram-negative septicemia.

Research Outline

Major issues concerning lipid A biosynthesis involves microheterogeneity and isolation of well-defined fragments. These factors limit detailed structure-activity relationship studies to identify the structural features that are responsible for its agonistic or antagonistic properties. Fortunately, recent advances in chemical synthesis of oligosaccharides and fatty acids make it possible to design and prepare pure lipid A derivatives containing a specific structure. Shiba *et al* described the first successful synthesis of an *E. coli* lipid A derivative.[42] Their approach, which involved incorporation of the appropriate lipid moieties at C-3 and C-3' and the C-4' phosphate group at the monosaccharide stage, yielded two monosaccharide building blocks. The monosaccharides were then coupled to produce a disaccharide derivative, which was then anomerically phosphorylated. Since then several strategies for the synthesis of lipid A derivatives have been described.[42,58-60] While the reported procedures for lipid A synthesis have established efficient methods for introducing *N*- and *O*- linked lipids and anomeric and non anomeric phosphate mono-esters, these synthetic routes are directed towards the preparation of specific individual lipid A analogs. Significant advances in chemical synthesis of oligosaccharides and fatty acids in recent years make it possible to synthesize a series of pure and well-defined lipid A structures ranging from *E. coli* to the rhizobial species. Recently, detailed structure-activity relationship studies of *R. sin*-1 conducted in our group has revealed the important structural features that are responsible for *R. sin*-1 antagonistic behavior.[61-65] Another major issue with the natural analog of *R. sin*-1 lipid A is the fact that the C-3 fatty acid chain at

the reducing end of the disaccharide eliminates even under neutral conditions to give an enone like derivative which does not have any activity. Microheterogeneity along with the instability of the natural *R. sin*-1 lipid A hampers the study of its biological activity. Hence, this project will mainly focus on (i) Development of a facile synthetic approach that enables synthesis of a wide range of *R. sin*-1 lipid A structures; (ii) Development of a stable analog of *R. sin*-1 lipid A with an ether linked lipid chain; (iii) Elucidation of the species specificity of natural and synthetic *R. sin*-1 lipid A

Significance of Anthrax

B. anthracis, the organism that causes anthrax, derives its name from the Greek word for coal, *B. anthrakis*, because of its ability to cause black, coal-like cutaneous eschars. Anthrax has been recognized since antiquity as a disease of humans and livestock. Some highlights of early research on anthrax include the confirmation of *B. anthracis* as the cause of the disease by Robert Koch in 1876;[66] the successful use of whole cell anthrax vaccines in 1880 and 1881 by Jean-Joseph Henri Toussaint, William Smith Greenfield and Louis Pasteur;[67] the discovery of a protective factor in anthrax edema fluid by Oskar Bail in 1904;[68] the discovery of protective antigen (PA) in culture filtrates in 1946 by G. P. Gladstone;[69] and the discovery of the anthrax toxins by Harry Smith and colleagues in the 1950s.[70] Concern about anthrax has resulted from its impact on domestic animals, wildlife and people occupationally exposed to infected animals or their products such as wool, hides/leather, and bone meal.[71] Vaccination efforts have reduced the threat and incidence of infections, but the disease persists in many areas of the world. Due to its exceptional virulence, ease of preparation and ability to form stable and environmentally resistant spores, *B. anthracis* has been developed as a biological weapon since World War I and, at least until recently, has been part of the offensive biological weapons programs of several

nations.[72,73] The potential of *B. anthracis* as a bioweapon is illustrated by several high-profile incidents occurring during the last 3 decades. In 1979, an apparent accidental release of spores at a military microbiology facility in Sverdlosk, Russian Republic, resulted in 96 reported cases of anthrax, including 68 deaths in people likely exposed to downwind.[74,75] In 1993, the Aum Shinrikyo doomsday cult sprayed *B. anthracis* from the top of a building in Tokyo, Japan.[76] This may be the first documented use of the bacterium as an aerosolized weapon by bioterrorists, but the strain used was subsequently found to be an attenuated variant surmised to be the Sterne vaccine strain.[77] In the autumn of 2001, the mailing of *B. anthracis* spores by unknown culprits to at least seven locations in the United States resulted in 22 confirmed cases of anthrax (11 cutaneous and 11 inhalational, including five deaths).[78] While none of these three incidents produced a sizeable medical impact, various projections have envisioned the potential of more devastating morbidity and mortality from a *B. anthracis* attack.[79,80] The potential use of antibiotic and/or vaccine resistant strains by bioterrorists heightens the urgency to develop anthrax vaccines that augment existing PA-based vaccines.

Modes of Entry

Anthrax takes one of three forms. By far the most common is cutaneous anthrax, which accounts for over 90% of all human cases and is acquired through a lesion on the skin. The other two forms are gastrointestinal anthrax and pulmonary, or inhalation, anthrax.

(i) Cutaneous Anthrax

After infection via an abrasion, cut, or possible insect bite, a small pimple or papule will develop within two to three days, although there are reports of incubation periods as short as 12 hours or as long as 19 days.[81] Over the next 24 hours a ring of vesicles develops, followed by ulceration of the central papule, which dries to form the classic black eschar, which in turn

enlarges to cover the drying vesicle. Pus will only be present if the lesion becomes secondarily infected with pyrogenic bacteria, such as *S. aureus*. The lesion, which is always painless, may be small or large and is always surrounded by oedema. Usually, by the fifth or sixth day a thick black eschar, firmly adherent to the underlying tissue develops. The bacilli remain localized to the lesion in uncomplicated cutaneous anthrax, although adenitis of the regional lymph nodes is not uncommon. Fever is rarely present. Ten days after the appearance of the original lesion, the eschar begins to resolve slowly over two to six weeks irrespective of treatment,[82] and resolution is usually completed with minimal scarring. In untreated anthrax, about 20% of patients may develop septicaemia and die, but with the use of appropriate antibiotics the mortality rate is < 1%. Cutaneous anthrax should always be considered when patients who have had contact with animals or animal products present with painless ulcers associated with vesicles and oedema.

(ii) Gastrointestinal Anthrax

This form of disease results from the ingestion of undercooked meat from animals with *B. anthracis*. The incubation period is two to five days. It has two clinical forms: abdominal and oro-oesophageal anthrax. In abdominal anthrax initial symptoms are nausea, vomiting, anorexia, and fever. As the disease progresses, severe abdominal pain resembling an acute abdomen, haematemesis, and bloody diarrhoea occur, followed by septicaemia and death.[83] The symptoms result from severe and widespread necrosis of the initial eschar, together with extreme oedema of intestines and mesentary, and enlargement of local mesenteric lymph nodes. In oro-oesophageal anthrax, the clinical manifestations include sore throat, dysphagia, fever, cervical lymphadenopathy, and oedema. If an early diagnosis is made then patients can be cured, but because of the non-specific presentation diagnosis is difficult, resulting in a high mortality rate.

(iii) Inhalation Anthrax

Until the recent events of 11 cases of inhalation anthrax following deliberate contamination of US mail,[84] inhalation anthrax had always been associated with industrial exposure to spores in textile or tanning industries. With improved industrial hygiene practice and immunization the numbers of cases have fallen dramatically.[85,86] The largest known outbreak of inhalation anthrax in the 20th century occurred in 1979 in the former soviet union.[87] The illness begins insidiously with "flu-like" symptoms of mild fever, fatigue, malaise, myalgia, and non-productive cough, usually two to five days after the initial exposure. This mild initial prodromal phase, which usually lasts about 48 hours, suddenly ends with the development of an acute illness characterized by acute dyspnoea, stridor, fever, and cyanosis. On examination at this time, the findings include fever, tachypnoea, cyanosis, tachycardia, moist rales, and evidence of pleural effusion. Terminally, the pulse becomes extremely rapid and faint, dyspnoea and cyanosis worsen, the patient becomes extremely disorientated, and this is quickly followed by coma and death.[88-90] Meningitis occurs in approximately 50% of patients. Until recently, the mortality rate was estimated to be > 95%; however, of the 11 known cases to date in the USA, six of the patients survived, providing a death rate of 45%. This lower figure may reflect the success of appropriate antibiotic treatment, together with full intensive care support, including draining of the pleural effusions. Jernigan *et al.* have reported on the first 11 US cases of inhalation anthrax associated with the recent bioterrorism event.[84] Epidemiological investigation indicated that the outbreak from October 4 to November 2, 2001 in the District of Columbia, Florida, New Jersey and New York resulted from the intentional delivery of *B. anthracis* spores through mailed letters or packages. The median age of the patients was 56 years (range, 43–73), seven were men, and except for one, all were known or believed to have processed, handled, or

received letters containing *B. anthracis* spores. The median incubation period from the time of exposure to onset of symptoms, when known, was four days (range, four to six). Symptoms at initial presentation included fever or chills, sweats, fatigue or malaise, minimal or non-productive cough, dyspnoea, and nausea. Nine patients had abnormal chest *x* rays; abnormalities included infiltrates, pleural effusion, and mediastinal widening. Computed tomography of the chest was performed on eight patients, and mediastinal lymphadenopathy was present in seven. With multi drug antibiotic regimens and supportive care, the survival of patients (60%) was much higher than previously reported.

The *B. anthracis* Genome

The genome of *B. anthracis* includes a single 5.2-megabase chromosome and two large virulence plasmids, pXO1 and pXO2 which contain 182 and 95 kilobases, respectively. Altogether, the genome has 5838 predicted protein-coding genes.[91] The chromosomal sequence and gene organization is quite similar to that of the closely related bacteria *B. cereus* and *B. thuringiensis*.[92] *B. anthracis* likely evolved from a single clone of *B. cereus* that acquired pXO1 and pXO2 from the environment by lateral genetic transfer. Genes required for virulence factor expression and regulation are located on the plasmids.[93] pXO1 contains a large pathogenicity island which encodes lethal and edema toxins (LT and ET),[93,94] while the biosynthetic enzymes of the poly-D-gamma glutamic acid capsule are encoded on a pathogenicity island on pXO2.[95,96] Loss of either plasmid significantly reduces virulence in most animal models.[97-101]

B. anthracis Toxins

The structure and mechanisms of action of the toxins have been intensely studied. The enzymatic effector proteins of the two toxins are called lethal factor (LF) and edema factor (EF).

Both LF and EF can bind a third protein, protective antigen (PA). PA is cleaved by mammalian serum and/or cell surface proteases and can bind to at least two specific receptors (TEM8 and CMG2) located on host cell membrane.[102,103] PA forms ring-shaped heptamers, and interacts with LF and EF,[104] which then enter the host cell by endocytosis.[105] Upon acidification of the endocytic vacuole, the PA heptamer apparently forms a pore through which the EF and LF moieties are translocated. Molecular targets within mammalian cells have been clearly identified for both toxins. LF is a zinc metalloprotease capable of inhibiting signal transduction through the mitogen-activated protein kinase (MAPK) cascade by cleaving most MAPK kinases (MAPKKs or MEKs), preventing the phosphorylation of MAPKs such as p38, ERK and JNK.[106-108] EF is a calcium/calmodulin-dependent adenylate cyclase that increases intracellular levels of cyclic AMP, leading to massive edema. LT and ET appear to impair both the innate and adaptive immune systems, having effects on multiple cell types, including macrophages, dendritic cells and neutrophils.[109] Although the precise mechanism is not yet well understood, this process results in the death of the host. Strains of *B. anthracis* deficient in EF remain pathogenic, whereas those that lack LF become attenuated. LF is therefore considered the dominant virulence factor of anthrax.

B. anthracis capsule and its Role in Virulence

The poly-glutamate capsule appears to be a fibrous structure in electron micrographs of the bacillus surface.[110] Early data show that the capsule consists entirely of poly-D-γ-glutamate.[111,112] One-dimensional and two-dimensional nuclear magnetic resonance (NMR) data recently confirmed that capsule has γ-carboxyl peptide linkages, and gas chromatography data recently confirmed that capsule appears to contain glutamic acid only of the D configuration.[113] Capsule synthesis is dependent on four proteins (CapA, B, C and E) encoded by an operon in

pXO2.[95,96,114-116] CapD (or DepA), another protein encoded by the *cap* operon, can degrade the capsule,[117] and the subsequent release of capsule fragments (low molecular weight capsule) has been linked to virulence.[118] A recent report not only confirms that CapD is required for full virulence in mice and that CapD can degrade capsule, but it also shows that CapD apparently covalently links the capsule to cell wall peptidoglycan.[119] Capsules may camouflage bacilli from the immune system by binding host proteins. As far back as the 1930s, investigators showed that capsule binds to basic serum proteins, such as lysozyme.[69,120] Recent evidence shows that it binds and deactivates antibacterial cationic peptides. It has been suggested that capsule fragments might bind to mediators of innate immunity, acting as a sink that drains immune modulators.[116] Complement binding by the capsule, perhaps in conjunction with S-layer proteins,[110] and capsule-mediated inhibition of anthracidal activity of normal horse serum and guinea pig leukocyte extracts, have also been reported.[111]

Other Virulence Factors

In addition to anthrax toxins, capsule and their regulators, a number of genes/proteins that have a measurable contribution to virulence and survival in mice or guinea pig models of infection have been identified. A select few of these, such as specific proteases,[121] may contribute directly to inflicting damage on the animal host; others, such as cell wall-modifying enzymes, may promote evasion of the innate immune system.[122] Most of the other genes/proteins known to affect virulence are not virulence factors *per se*, but appear to promote spore germination,[123] acquisition of key nutrients,[124-127] resistance to oxidative stress or coordination of an overall stress response during replication in the host environment.[125,128]

Treatment Strategies

(i) Antibiotics

Penicillin has long been considered the drug of choice and only rarely has penicillin resistance been found in naturally occurring strains. *In vitro B. anthracis* is susceptible to penicillins, fluoroquinolones, tetracycline, chloramphenicol, aminoglycosides, macrolides, imipenem/meropenem, rifampicin, and vancomycin. The organism is resistant to cephalosporins, trimethoprim, and sulfomanides. As the Ames strain which caused the recent infections in the USA, has shown the presence of constitutive and inducible β-lactamases, the treatment of systemic anthrax with penicillin or amoxicillin alone is not recommended now.[129] For mild cases of cutaneous anthrax, treatment with ciprofloxacin (500 mg twice daily), doxycycline (100 mg twice daily), or amoxicillin (500 mg three times daily) is recommended.[130] In the context of a bioterrorist attack, treatment should continue for 60 days, as opposed to seven to 10 days for naturally acquired disease. Post exposure prophylaxis is not recommended for asymptomatic people, unless public health or police authorities deem they have been exposed to a credible threat of anthrax spores. A long period (60 days) of prophylaxis is recommended because of the prolonged latency period that can elapse before germination of the inhaled spores occurs.[131] Ciprofloxacin is currently considered the prophylaxis of choice.

(ii) Vaccinations

Vaccination is the most cost effective form of mass protection. Although the first anthrax animal vaccine was developed by Pasteur in 1881, human vaccines did not emerge until the middle of the 20th century. Although the current vaccines available provide effective protection, they do suffer from several problems, namely, lack of standardization, the relatively high

expense of production, the requirement for repeated dosing, and the associated transient side effects.[132]

The former USSR and China use vaccination in humans with live spores, either by scarification or subcutaneous injection. The Russians use a strain, STI-1 analogous in its derivation to the Sterne 34_{F2} strain. Although analogous in many ways, current UK and US vaccines did develop along slightly different routes. The current UK vaccine (licence numbers 1511/0037 and 1511/0058) consists of an alum precipitated, cell free filtrate of an aerobic supernatant from the non-capsulated Sterne strain of *B. anthracis*.[133] In the production of the UK vaccine, a protein hydrolysate was preferred to synthetic 528 medium as used in US vaccine production.[134] Downstream processing consists of a filtration step to remove bacterial cells, along with OF and LF. Sterile material, now largely composed of PA, the essential protection immunogen, is alum precipitated at pH 6.0.[130] The UK vaccine was introduced for workers in their risk occupations in 1965,[135] and licensed for human use in 1979 after biological agents first fell under the European Directive75/319/EEC. At present, on empirical grounds, boosters are administered to people six months after the initial series of three doses (zero, three, and six weeks), and annually thereafter.

Anthrax vaccine adsorbed (AVA), also known as BioThrax since 2002, has been used to vaccinate against anthrax in the United States for over 30 years. AVA has been shown to be effective in preventing infection in several animal models, including non-human primates.[136] It consists of supernatant material from *B. anthracis* V770-NP1-R, which is pXO1+ and pXO2–, grown microaerophilically in a protein-free defined medium. This supernatant is filtered and adsorbed to aluminum hydroxide (Alhydrogel). The final product contains 1.2 mg/ml of aluminum, 25 μg/ml of benzethonium chloride and 100 μg/ml of formaldehyde. The current

vaccination regimen consists of six subcutaneous injections over 18 months followed by yearly boosters. AVA is similar to the vaccine used in United Kingdom. It is well documented that PA is the primary effective component of AVA,[137-139] and thus expression of PA is optimized for the vaccine manufacturing process.[140] While a vaccine preparation similar to AVA was shown to be safe and effective in a field trial,[141] and occurrence of systemic adverse events associated with AVA vaccination is rare,[142] concerns raised over manufacturing variability and reactogenicity have prompted development of a next-generation anthrax vaccine composed of rPA purified from an atoxigenic, asporogenic strain of *B. anthracis*. Comparative studies indicated that rPA with an aluminum-containing adjuvant conferred protection in a rhesus macaque aerosol model of anthrax.[143] In these studies, rhesus macaques were vaccinated once with either rPA or AVA and challenged 6 weeks later with spores of the virulent Ames strain. Both rPA formulated with Alhydrogel and AVA provided complete protection and elicited strong anti-PA immunoglobulin (Ig) G and IgM titers. Additionally, rPA-Alhydrogel and AVA elicited comparable toxin neutralization titers. In another study, rPA-Alhydrogel protected rhesus macaques against a target dose of 200 LD_{50} Ames spores in an aerosol challenge model, and antisera from rPA-immunized rhesus macaques protected A/J mice in passive transfer studies.[144] A contract to manufacture the rPA vaccine was awarded to VaxGen Inc. (Brisbane, CA) by the Department of Health and Human Services in 2004, and clinical trials are currently being conducted to determine its safety and immunogenicity.

In addition to developing a next-generation anthrax vaccine, researchers are currently examining alternative vaccine delivery routes. AVA is administered by subcutaneous injection, but new research is underway to determine whether intramuscular delivery will be less reactogenic and equally efficacious with fewer doses. While both AVA and rPA elicit high IgG

anti-PA titers in animal models, it may be that induction of both systemic and mucosal immunity would result in superior protection.[145] Strategies to elicit mucosal immunity to anthrax include oral vaccination with *Salmonella*[146,147] or *Lactobacillus*[148] expressing PA, nasal instillation with rPA,[149,150] nasal delivery of rPA associated with microspheres[149]or liposomes,[150] and oral spore vaccination with attenuated *B. anthracis* expressing rPA.[151] Coulson *et al.* demonstrated that an attenuated strain of *S. typhimurium* expressing PA could confer partial protection from challenge with *B. anthracis* Vollum 1B after oral vaccination, although PA-specific antibodies were not detected.[152] Galen *et al.* inoculated mice intranasally with *S. enterica* serovar Typhi CVD 908-htrA expressing domain 4 of PA and were able to detect serologic titers against PA.[147] Parenteral vaccination with a lysate of PA-expressing *Lactobacillus casei* resulted in a significant anti-PA response.[148] However, oral vaccination with the live strain failed to elicit a PA-specific response. These studies demonstrate the possibility of employing bacterial carriers to elicit PA immunity if technical hurdles can be overcome. Alternative adjuvants, such as cholera toxin[152] and soya phosphatidyl choline[153] have been used with mucosal PA administration. These approaches led to significant anti-PA IgG and IgA titers and strong Th2 cytokine responses. Another approach to mucosal vaccination was demonstrated with intranasal delivery of microencapsulated PA. Intranasal vaccination of mice with PA microencapsulated in poly-L-lactide 100 kDa microspheres resulted in a protective immune response against aerosol challenge equal to that elicited by AVA vaccine.[149] Sloat *et al.* demonstrated that vaccinating mice with PA carried by liposome-protamine-DNA (LPD) particles resulted in IgG and IgA anti-PA responses.[150] Protective efficacy of intradermal rPA delivery and intranasal administration of a powder form of rPA has also been investigated.[154] In these studies, rabbits given intradermal injections of rPA with micro needles were fully protected from aerosol challenge of *B. anthracis*. Finally, oral

vaccination of guinea pigs with spores of a ΔSterne strain (pXO1–, pXO2–) transformed with a PA-expressing plasmid resulted in IgG and IgA anti-PA responses and partial protection against subcutaneous challenge of 20 LD_{50} *B. anthracis* Vollum.[151] An advantage of an oral or nasal anthrax vaccine is the relative ease of delivery. With the current 6-dose initial vaccination regimen and yearly injections required for vaccinated individuals, such a vaccine may prove to be a more efficient alternative to traditional parenteral vaccination and may provide stronger immunity and a higher level of compliance.

Need for a New Vaccine

The threat of a natural isolate of *B. anthracis* that is vaccine resistant has led to determined efforts to identify novel vaccine targets other than the protective antigen. Since anthrax is asymptomatic until the bacterium reaches the blood, the development of antitoxin therapeutic compounds for preventive use, or for use in combination with antibiotics, is of high urgency. Alternatively, it would be highly beneficial if the developed material were bifunctional, with the ability to inactivate the released toxins and, in parallel, to function as an antibiotic. Carbohydrate antigens found on the cell surface of these bacteria could be a potential therapeutic candidate by itself or in combination with PA, for treatment of anthrax or as a diagnostic tool for *B. anthracis*.

Cohen *et al.*[155] reported that spore antigens might contribute to protection. They found that guinea pigs vaccinated with spores produced significant anti-spore antibody levels and were better protected against spore challenge than guinea pigs vaccinated with bacilli. Stepanov *et al.* reported similar findings with hamsters and rabbits[156]. Brossier *et al.* showed that spore antigens can elicit a protective immune response.[157] Finally, an exosporium glycoprotein, BclA, has proven to be highly immunogenic and might contribute to protection. [158,159]

Bacillus Anthracis Cell Wall Composition

Generally, the carbohydrate-containing components of the vegetative cell walls of Gram-positive bacteria consist of the extensive peptidoglycan layer, teichoic acids, lipoteichoic acids, capsular polysaccharides, and crystalline cell surface proteins known as S-layer proteins that are often glycosylated.[160] However, the *B. anthracis* cell wall differs in several aspects from this generalized description. First, *B. anthracis* cells are surrounded by a poly-γ-D-glutamate capsule and not by a polysaccharide (PS) capsule. Second, their cell walls do not contain teichoic acid, and last, their S-layer proteins are not glycosylated.[161-163] However, glycosyl composition comparisons of the cell walls of *B. anthracis*, *B. cereus*, and *B. thuringiensis* show that they do contain glycosyl residues and that they differ from one another in their glycosyl compositions.[164] Structures were determined for these polysaccharides from *B. anthracis* Ames, *B. anthracis* Pasteur, and *B. anthracis* Sterne 34F2. These structures were also compared with structures from the closely related *B. cereus* strain, ATCC 10987, and the *B. cereus* type strain, ATCC 14579. The results showed that all three *B. anthracis* strains contained the same PS structure that differed from that of *B. cereus* ATCC 10987, which, in turn, differed from that of *B. cereus* ATCC 14579. HF treatment releases wall polysaccharides covalently bound via a phosphate bond to the peptidoglycan of *B. anthracis* (Δ Sterne) (HF-PS)[10] containing Gal, Glc*N*Ac, and Man*N*Ac in an approximate ratio of 3:2:1. The HF-PSs from all of the *B. anthracis* isolates had an identical structure consisting of an 2-amino-2-deoxy sugar backbone of →6)-α-D-Glc*N*Ac-(1→4)-β- D-Man*N*Ac-(1→4)-β- D-Glc*N*Ac-(1→, in which the α- D-Glc*N*Ac residue is substituted with α-Gal and β-Gal at *O*-3 and *O*-4, respectively, and the β- D-Glc*N*Ac substituted with α-D-Gal at *O*-3. There is some variability in the presence of two of these three Gal substitutions.

Research Focus

B. anthracis carbohydrates have not been adequately investigated for development of diagnostic or vaccine antigens and is particularly important in order to identify anthrax in its early stages of infection and will, thus, be useful in the event of a large scale bioterrorism attack. The efficacy of current anthrax vaccines containing PA in combination with aluminum adjuvants licensed for use in humans has proven variable in animal models. It has, however, been shown that other *B. anthracis* components such as poly-γ-D-glutamate peptides or spore preparations increase the efficacy of such vaccines in animal models.[165] To date however, there are no reports of the efficacy of combining PA with purified spore-associated antigens or with spore/vegetative cell carbohydrates in particular. In this project, we will determine if HF-PS-protein conjugates can be used as antigens to generate specific antiserum against spore and/or vegetative cell forms of *B. anthracis*. We will also investigate the ability of these carbohydrate-protein conjugates (KLH and PA) to act as vaccine components for the prevention of anthrax using the mouse animal model. The polysaccharides by themselves are unable to activate helper T-cells and, therefore, do not induce immunological memory. Therefore, it is necessary to chemically conjugate these carbohydrates to protein carriers such as keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) so that helper T-cells and the immunological memory are activated. In some instances, this chemical processing of the bacterial polysaccharide destroys or removes structural epitopes that are essential for generating protective antibodies. Also a bacterial polysaccharide often possesses a great deal of microhetergeneity that can complicate the reproducibility of vaccine production; *e.g.* variation in non stoichiometric substitution of acetyl, phosphate, or sulfate groups, or branching glycosyl residues. Chemical synthesis can provide carbohydrate epitopes in high purity and in relatively large amounts for conjugation to a

carrier protein. In addition, synthetic carbohydrates can be prepared to (i) determine the minimal epitope required for a protective antibody response, (ii) map ligand requirements of monoclonal antibodies (MAbs) prepared against the natural polysaccharides, and (iii) prepare MAbs that can be used to identify the pathogen and determine which epitopes are expressed during various stages of its life cycle.

References

(1) Heidelberger, M.; Avery, O. T. *J. Exp. Med.* **1923**, *38*, 73-79.

(2) Heidelberger, M.; Dilapi, M. M.; Siegel, M.; Walter, A. W. *J. Immunol.* **1950**, *65*, 535-541.

(3) Avery, O. T.; Goebel, W. F. *J. Exp. Med.* **1931**, *54*, 437-447.

(4) Pozsgay, V. *Adv. Carbohydr. Chem. Biochem.* **2000**, *56*, 153-199.

(5) Lucas, A. H.; Apicella, M. A.; Taylor, C. E. *Clin. Infect. Dis.* **2005**, *41*, 705-712.

(6) Cobb, B. A.; Wang, Q.; Tzianabos, A. O.; Kasper, D. L. *Cell* **2004**, *117*, 677-687.

(7) Verez-Bencomo, V.; Fernandez-Santana, V.; Hardy, E.; Toledo, M. E.; Rodriguez, M. C.; Heynngnezz, L.; Rodriguez, A.; Baly, A.; Herrera, L.; Izquierdo, M.; Villar, A.; Valdes, Y.; Cosme, K.; Deler, M. L.; Montane, M.; Garcia, E.; Ramos, A.; Aguilar, A.; Medina, E.; Torano, G.; Sosa, I.; Hernandez, I.; Martinez, R.; Muzachio, A.; Carmenates, A.; Costa, L.; Cardoso, F.; Campa, C.; Diaz, M.; Roy, R. *Science* **2004**, *305*, 522-525.

(8) Vandenplas, M. L.; Carlson, R. W.; Jeyaretnam, B. S.; McNeill, B.; Barton, M. H.; Norton, N.; Murray, T. F.; Moore, J. N. *J. Biol. Chem.* **2002**, *277*, 41811-41816.

(9) Daubenspeck, J. M.; Zeng, H. D.; Chen, P.; Dong, S. L.; Steichen, C. T.; Krishna, N. R.; Pritchard, D. G.; Turnbough, C. L. *J. Biol. Chem.* **2004**, *279*, 30945-30953.

(10) Choudhury, B.; Leoff, C.; Saile, E.; Wilkins, P.; Quinn, C. P.; Kannenberg, E. L.; Carlson, R. W. *J. Biol. Chem.* **2006**, *281*, 27932-27941.

(11) Werz, D. B.; Seeberger, P. H. *Angewandte Chemie, International Edition in English* **2005**, *44*, 6315-6318.

(12) Mehta, A. S.; Saile, E.; Zhong, W.; Buskas, T.; Carlson, R.; Kannenberg, E.; Reed, Y.; Quinn, C. P.; Boons, G. J. *Chem. Eur. J.* **2006**, *12*, 9136-9149.

(13) Saksena, R.; Adamo, R.; Kovac, P. *Bioorg. Med. Chem.* **2007**, *15*, 4283-4310.

(14) Crich, D.; Vinogradova, O. *J. Org. Chem.* **2007**, *72*, 6513-6520.

(15) Guo, H.; O'Doherty, G. A. *Angew. Chem. Int. Ed. Engl.* **2007**, *46*, 5206-5208.

(16) Oberli, M. A.; Bindschadler, P.; Werz, D. B.; Seeberger, P. H. *Org. Lett.* **2008**, *10*, 905-908.

(17) Vasan, M.; Rauvolfova, J.; Wolfert, M. A.; Leoff, C.; Kannenberg, E. L.; Quinn, C. P.; Carlson, R. W.; Boons, G. J. *ChemBioChem* **2008**, *9*, 1716-1720.

(18) Tamborrini, M.; Werz, D. B.; Frey, J.; Pluschke, G.; Seeberger, P. H. *Angew. Chem. Int. Ed. Engl.* **2006**, *45*, 6581-6582.

(19) Roberts, F. J.; Geere, I. W.; Coldman, A. *Rev. Infect. Dis.* **1991**, *13*, 34-46.

(20) Pittet, D.; Tarara, D.; Wenzel, R. P. *JAMA* **1994**, *271*, 1598-1601.

(21) Heron, M. *Natl. Vital Stat. Rep.* **2007**, *56*, 1-95.

(22) Huang, D. T.; Clermont, G.; Dremsizov, T. T.; Angus, D. C. *Crit. Care Med.* **2007**, *35*, 2090-2100.

(23) Santhanam, B. Doctoral Dissertation, University of Georgia, 2004.

(24) Hurley, J. C. *J. Clin. Microbiol.* **1994**, *32*, 2120-2127.

(25) Hurley, J. C. *Clin. Microbiol. Rev.* **1995**, *8*, 268-292.

(26) Morrison, D. C.; Ryan, J. L. *Annu. Rev. Med.* **1987**, *38*, 417-432.

(27) Ritter, T. K.; Wong, C. H. *Angew. Chemie. Int. Ed.* **2001**, *40*, 3509-3533.

(28) Beutler, B. *Mol. Immunol.* **2004**, *40*, 845-859.

(29) Van Amersfoort, E. S.; Van Berkel, T. J.; Kuiper, J. *Clin. Microbiol. Rev.* **2003**, *16*, 379-414.

(30) Beutler, B.; Hoebe, K.; Du, X.; Ulevitch, R. J. *J. Leukoc. Biol.* **2003**, *74*, 479-485.

(31) Lien, E.; Ingalls, R. R. *Crit. Care Med.* **2002**, *30*, S1-11.

(32) Schmitz, F.; Mages, J.; Heit, A.; Lang, R.; Wagner, H. *Eur. J. Immunol.* **2004**, *34*, 2863-2873.

(33) Brade, H.; Opal, S. M.; Vogel, S. N.; Morrison, D. C. 1999, p 699-717.

(34) Tobias, P. S.; Ulevitch, R. J. *Immunobiology* **1993**, *187*, 227-232.

(35) Yu, B.; Hailman, E.; Wright, S. D. *J. Clin. Invest.* **1997**, *99*, 315-324.

(36) Akira, S.; Hoshino, K. *J. Infect. Dis.* **2003**, *187 Suppl 2*, S356-363.

(37) Kawai, T.; Takeuchi, O.; Fujita, T.; Inoue, J.; Muhlradt, P. F.; Sato, S.; Hoshino, K.; Akira, S. *J. Immunol.* **2001**, *167*, 5887-5894.

(38) Raetz, C. R.; Whitfield, C. *Annu. Rev. Biochem.* **2002**, *71*, 635-700.

(39) Caroff, M.; Karibian, D.; Cavaillon, J. M.; Haeffner-Cavaillon, N. *Microbes. Infect.* **2002**, *4*, 915-926.

(40) Luderitz, O.; Westphal, O. *Angew. Chem. Int. Ed. Engl.* **1966**, *5*, 198-210.

(41) Takayama, K.; Qureshi, N.; Mascagni, P. *J. Biol. Chem.* **1983**, *258*, 12801-12803.

(42) Imoto, M.; Yoshimura, H.; Sakaguchi, N.; Kusumoto, S.; Shiba, T. *Tetrahedron Lett.* **1985**, *26*, 1545-1548.

(43) Galanos, C.; Luderitz, O.; Rietschel, E. T.; Westphal, O.; Brade, H.; Brade, L.; Freudenberg, M.; Schade, U.; Imoto, M.; Yoshimura, H.; et al. *Eur. J. Biochem.* **1985**, *148*, 1-5.

(44) Mayer, H.; Krauss, J. H.; Urbanik-Sypniewska, T.; Puvanesarajah, V.; Stacey, G.; Auling, G. *Arch. Microbiol.* **1989**, *151*, 111-116.

(45) Krauss, J. H.; Seydel, U.; Weckesser, J.; Mayer, H. *Eur. J. Biochem.* **1989**, *180*, 519-526.

(46) Suda, Y.; Ogawa, T.; Kashihara, W.; Oikawa, M.; Shimoyama, T.; Hayashi, T.; Tamura, T.; Kusumoto, S. *J Biochem* **1997**, *121*, 1129-1133.

(47) Golenbock, D. T.; Hampton, R. Y.; Qureshi, N.; Takayama, K.; Raetz, C. R. *J. Biol. Chem.* **1991**, *266*, 19490-19498.

(48) Loppnow, H.; Libby, P.; Freudenberg, M.; Krauss, J. H.; Weckesser, J.; Mayer, H. *Infect. Immun.* **1990**, *58*, 3743-3750.

(49) Takayama, K.; Qureshi, N.; Beutler, B.; Kirkland, T. N. *Infect. Immun.* **1989**, *57*, 1336-1338.

(50) Christ, W. J.; McGuinness, P. D.; Asano, O.; Wang, Y.; Mullarkey, M. A.; Perez, M.; Hawkins, L. D.; Blythe, T. A.; Dubuc, G. R.; Robidoux, A. L. *J. Am. Chem. Soc.* **1994**, *116*, 3637-3638.

(51) Christ, W. J.; Asano, O.; Robidoux, A. L.; Perez, M.; Wang, Y.; Dubuc, G. R.; Gavin, W. E.; Hawkins, L. D.; McGuinness, P. D.; Mullarkey, M. A.; et al. *Science* **1995**, *268*, 80-83.

(52) Asai, Y.; Nozu, Y.; Ikeuchi, T.; Narazaki, R.; Iwamoto, K.; Watanabe, S. *Biol. Pharm. Bull.* **1999**, *22*, 432-434.

(53) Chow, J. C.; Young, D. W.; Golenbock, D. T.; Christ, W. J.; Gusovsky, F. *J. Biol. Chem.* **1999**, *274*, 10689-10692.

(54) Mullarkey, M.; Rose, J. R.; Bristol, J.; Kawata, T.; Kimura, A.; Kobayashi, S.; Przetak, M.; Chow, J.; Gusovsky, F.; Christ, W. J.; Rossignol, D. P. *J. Pharmacol. Exp. Ther.* **2003**, *304*, 1093-1102.

(55) Jeyaretnam, B.; Glushka, J.; Kolli, V. S.; Carlson, R. W. *J. Biol. Chem.* **2002**, *277*, 41802-41810.

(56) Forsberg, L. S.; Carlson, R. W. *J. Biol. Chem.* **1998**, *273*, 2747-2757.

(57) Bhat, U. R.; Forsberg, L. S.; Carlson, R. W. *J. Biol. Chem.* **1994**, *269*, 14402-14410.

(58) Imoto, M.; Yoshimura, H.; Kusumoto, S.; Shiba, T. *Proceedings of the Japan Academy Series B-Physical and Biological Sciences* **1984**, *60*, 285-288.

(59) Imoto, M.; Yoshimura, H.; Yamamoto, M.; Shimamoto, T.; Kusumoto, S.; Shiba, T. *Bull. Chem. Soc. Jpn.* **1987**, *60*, 2197-2204.

(60) Inage, M.; Chaki, H.; Kusumoto, S.; Shiba, T. *Tetrahedron Lett.* **1981**, *22*, 2281-2284.

(61) Demchenko, A. V.; Wolfert, M. A.; Santhanam, B.; Moore, J. N.; Boons, G. J. *J. Am. Chem. Soc.* **2003**, *125*, 6103-6112.

(62) Santhanam, B.; Wolfert, M. A.; Moore, J. N.; Boons, G. J. *Chem. Eur. J.* **2004**, *10*, 4798-4807.

(63) Santhanam, B.; Boons, G. J. *Org. Lett.* **2004**, *6*, 3333-3336.

(64) Lee, H. S.; Wolfert, M. A.; Zhang, Y.; Boons, G. J. *ChemBioChem* **2006**, *7*, 140-148.

(65) Zhang, Y.; Wolfert, M. A.; Boons, G. J. *Bioorg. Med. Chem.* **2007**, *15*, 4800-4812.

(66) Carter, K. C. *Med. Hist.* **1985**, *29*, 353-374.

(67) Geison, G. L. *The Private Science of Louis Pasteur*; Princeton University Press: Princeton, NJ, 1995.

(68) Bail, O. *Zentralb Bakteriol Parasitenk Infectionskr* **1904**, *47*, 270-272.

(69) Gladstone, G. P.; Johnston, H. H. *Br. J. Exp. Pathol.* **1955**, *36*, 363-372.

(70) Smith, H. *Int. J. Med. Microbiol.* **2002**, *291*, 411-417.

(71) Friedlander, A. M. *Curr. Clin. Top. Infect. Dis.* **2000**, *20*, 335-349.

(72) Christopher, G. W.; Cieslak, T. J.; Pavlin, J. A.; Eitzen, E. M., Jr. *JAMA* **1997**, *278*, 412-417.

(73) Riedel, S. *Proc (Bayl Univ Med Cent)* **2005**, *18*, 234-243.

(74) Abramova, F. A.; Grinberg, L. M.; Yampolskaya, O. V.; Walker, D. H. *Proc. Natl. Acad. Sci. U. S. A.* **1993**, *90*, 2291-2294.

(75) Meselson, M.; Guillemin, J.; Hugh-Jones, M.; Langmuir, A.; Popova, I.; Shelokov, A.; Yampolskaya, O. *Science* **1994**, *266*, 1202-1208.

(76) Takahashi, H.; Keim, P.; Kaufmann, A. F.; Keys, C.; Smith, K. L.; Taniguchi, K.; Inouye, S.; Kurata, T. *Emerg. Infect. Dis.* **2004**, *10*, 117-120.

(77) Keim, P.; Smith, K. L.; Keys, C.; Takahashi, H.; Kurata, T.; Kaufmann, A. *J. Clin. Microbiol.* **2001**, *39*, 4566-4567.

(78) Jernigan, D. B.; Raghunathan, P. L.; Bell, B. P.; Brechner, R.; Bresnitz, E. A.; Butler, J. C.; Cetron, M.; Cohen, M.; Doyle, T.; Fischer, M.; Greene, C.; Griffith, K. S.; Guarner, J.; Hadler, J. L.; Hayslett, J. A.; Meyer, R.; Petersen, L. R.; Phillips, M.; Pinner, R.; Popovic, T.; Quinn, C. P.; Reefhuis, J.; Reissman, D.; Rosenstein, N.; Schuchat, A.; Shieh, W. J.; Siegal, L.; Swerdlow, D. L.; Tenover, F. C.; Traeger, M.; Ward, J. W.; Weisfuse, I.; Wiersma, S.; Yeskey, K.; Zaki, S.; Ashford, D. A.; Perkins, B. A.; Ostroff,

S.; Hughes, J.; Fleming, D.; Koplan, J. P.; Gerberding, J. L. *Emerg. Infect. Dis.* **2002**, *8*, 1019-1028.

(79) Inglesby, T. V.; O'Toole, T.; Henderson, D. A.; Bartlett, J. G.; Ascher, M. S.; Eitzen, E.; Friedlander, A. M.; Gerberding, J.; Hauer, J.; Hughes, J.; McDade, J.; Osterholm, M. T.; Parker, G.; Perl, T. M.; Russell, P. K.; Tonat, K. *JAMA* **2002**, *287*, 2236-2252.

(80) Webb, G. F. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 4355-4356.

(81) Abdenour, D.; Larouze, B.; Dalichaouche, M.; Aouati, M. *J. Infect. Dis.* **1987**, *155*, 1083-1084.

(82) Turner, M. *Cent. Afr. J. Med.* **1980**, *26*, 160-161.

(83) Ndyabahinduka, D. G.; Chu, I. H.; Abdou, A. H.; Gaifuba, J. K. *Ann. Ist. Super. Sanita* **1984**, *20*, 205-208.

(84) Jernigan, D. B. *Emerg. Infect. Dis.* **2001**, *7*, 538.

(85) Brachman, P. S. *Ann. N. Y. Acad. Sci.* **1980**, *353*, 83-93.

(86) Shafazand, S.; Doyle, R.; Ruoss, S.; Weinacker, A.; Raffin, T. A. *Chest* **1999**, *116*, 1369-1376.

(87) Meselson, M.; Guillemin, J.; Hugh-Jones, M.; Langmuir, A.; Popova, I.; Shelokov, A.; Yampolskaya, O. *Science* **1994**, *266*, 1202-1208.

(88) Albrink, W. S.; Brooks, S. M.; Biron, R. E.; Kopel, M. *Am. J. Pathol.* **1960**, *36*, 457-471.

(89) Plotkin, S. A.; Brachman, P. S.; Utell, M.; Bumford, F. H.; Atchison, M. M. *Am. J. Med.* **1960**, *29*, 992-1001.

(90) Enticknap, J. B.; Galbraith, N. S.; Tomlinson, A. J.; Elias-Jones, T. F. *Br. J. Ind. Med.* **1968**, *25*, 72-74.

(91) Read, T. D.; Peterson, S. N.; Tourasse, N.; Baillie, L. W.; Paulsen, I. T.; Nelson, K. E.; Tettelin, H.; Fouts, D. E.; Eisen, J. A.; Gill, S. R.; Holtzapple, E. K.; Okstad, O. A.; Helgason, E.; Rilstone, J.; Wu, M.; Kolonay, J. F.; Beanan, M. J.; Dodson, R. J.; Brinkac, L. M.; Gwinn, M.; DeBoy, R. T.; Madpu, R.; Daugherty, S. C.; Durkin, A. S.; Haft, D. H.; Nelson, W. C.; Peterson, J. D.; Pop, M.; Khouri, H. M.; Radune, D.; Benton, J. L.; Mahamoud, Y.; Jiang, L.; Hance, I. R.; Weidman, J. F.; Berry, K. J.; Plaut, R. D.; Wolf, A. M.; Watkins, K. L.; Nierman, W. C.; Hazen, A.; Cline, R.; Redmond, C.; Thwaite, J. E.; White, O.; Salzberg, S. L.; Thomason, B.; Friedlander, A. M.; Koehler, T. M.; Hanna, P. C.; Kolsto, A. B.; Fraser, C. M. *Nature* **2003**, *423*, 81-86.

(92) Rasko, D. A.; Altherr, M. R.; Han, C. S.; Ravel, J. *FEMS Microbiol. Rev.* **2005**, *29*, 303-329.

(93) Okinaka, R. T.; Cloud, K.; Hampton, O.; Hoffmaster, A. R.; Hill, K. K.; Keim, P.; Koehler, T. M.; Lamke, G.; Kumano, S.; Mahillon, J.; Manter, D.; Martinez, Y.; Ricke, D.; Svensson, R.; Jackson, P. J. *J. Bacteriol.* **1999**, *181*, 6509-6515.

(94) Mikesell, P.; Ivins, B. E.; Ristroph, J. D.; Dreier, T. M. *Infect. Immun.* **1983**, *39*, 371-376.

(95) Green, B. D.; Battisti, L.; Koehler, T. M.; Thorne, C. B.; Ivins, B. E. *Infect. Immun.* **1985**, *49*, 291-297.

(96) Uchida, I.; Sekizaki, T.; Hashimoto, K.; Terakado, N. *J. Gen. Microbiol.* **1985**, *131*, 363-367.

(97) Ivins, B. E.; Ezzell, J. W., Jr.; Jemski, J.; Hedlund, K. W.; Ristroph, J. D.; Leppla, S. H. *Infect. Immun.* **1986**, *52*, 454-458.

(98) Pezard, C.; Berche, P.; Mock, M. *Infect. Immun.* **1991**, *59*, 3472-3477.

(99) Uchida, I.; Hashimoto, K.; Terakado, N. *J. Gen. Microbiol.* **1986**, *132*, 557-559.

(100) Welkos, S. L. *Microb. Pathog.* **1991**, *10*, 183-198.

(101) Welkos, S. L.; Friedlander, A. M. *Microb. Pathog.* **1988**, *4*, 53-69.

(102) Bradley, K. A.; Mogridge, J.; Mourez, M.; Collier, R. J.; Young, J. A. *Nature* **2001**, *414*, 225-229.

(103) Scobie, H. M.; Rainey, G. J.; Bradley, K. A.; Young, J. A. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 5170-5174.

(104) Mogridge, J.; Cunningham, K.; Lacy, D. B.; Mourez, M.; Collier, R. J. *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99*, 7045-7048.

(105) Abrami, L.; Liu, S.; Cosson, P.; Leppla, S. H.; van der Goot, F. G. *J. Cell Biol.* **2003**, *160*, 321-328.

(106) Duesbery, N. S.; Webb, C. P.; Leppla, S. H.; Gordon, V. M.; Klimpel, K. R.; Copeland, T. D.; Ahn, N. G.; Oskarsson, M. K.; Fukasawa, K.; Paull, K. D.; Vande Woude, G. F. *Science* **1998**, *280*, 734-737.

(107) Park, J. M.; Greten, F. R.; Li, Z. W.; Karin, M. *Science* **2002**, *297*, 2048-2051.

(108) Vitale, G.; Bernardi, L.; Napolitani, G.; Mock, M.; Montecucco, C. *Biochem. J.* **2000**, *352 Pt 3*, 739-745.

(109) Leppla, S. H. *Proc. Natl. Acad. Sci. U. S. A.* **1982**, *79*, 3162-3166.

(110) Mesnage, S.; Tosi-Couture, E.; Gounon, P.; Mock, M.; Fouet, A. *J. Bacteriol.* **1998**, *180*, 52-58.

(111) Zwartouw, H. T.; Smith, H. *Biochem. J.* **1956**, *63*, 437-442.

(112) Bruckner, V.; Kovacs, J.; Denes, G. *Nature* **1953**, *172*, 508.

(113) Joyce, J.; Cook, J.; Chabot, D.; Hepler, R.; Shoop, W.; Xu, Q.; Stambaugh, T.; Aste-Amezaga, M.; Wang, S.; Indrawati, L.; Bruner, M.; Friedlander, A.; Keller, P.; Caulfield, M. *J. Biol. Chem.* **2006**, *281*, 4831-4843.

(114) Makino, S.; Sasakawa, C.; Uchida, I.; Terakado, N.; Yoshikawa, M. *Mol. Microbiol.* **1988**, *2*, 371-376.

(115) Makino, S.; Uchida, I.; Terakado, N.; Sasakawa, C.; Yoshikawa, M. *J. Bacteriol.* **1989**, *171*, 722-730.

(116) Uchida, I.; Makino, S.; Sasakawa, C.; Yoshikawa, M.; Sugimoto, C.; Terakado, N. *Mol. Microbiol.* **1993**, *9*, 487-496.

(117) Makino, S.; Watarai, M.; Cheun, H. I.; Shirahata, T.; Uchida, I. *J. Infect. Dis.* **2002**, *186*, 227-233.

(118) Candela, T.; Fouet, A. *Mol. Microbiol.* **2005**, *57*, 717-726.

(119) Keppie, J.; Harris-Smith, P. W.; Smith, H. *Br. J. Exp. Pathol.* **1963**, *44*, 446-453.

(120) Leonard, C. G.; Thorne, C. B. *J. Immunol.* **1961**, *87*, 175-188.

(121) Popov, S. G.; Popova, T. G.; Hopkins, S.; Weinstein, R. S.; MacAfee, R.; Fryxell, K. J.; Chandhoke, V.; Bailey, C.; Alibek, K. *BMC Infect. Dis.* **2005**, *5*, 25.

(122) Fisher, N.; Shetron-Rama, L.; Herring-Palmer, A.; Heffernan, B.; Bergman, N.; Hanna, P. *J. Bacteriol.* **2006**, *188*, 1301-1309.

(123) Guidi-Rontani, C.; Pereira, Y.; Ruffie, S.; Sirard, J. C.; Weber-Levy, M.; Mock, M. *Mol. Microbiol.* **1999**, *33*, 407-414.

(124) Cendrowski, S.; MacArthur, W.; Hanna, P. *Mol. Microbiol.* **2004**, *51*, 407-417.

(125) Gat, O.; Mendelson, I.; Chitlaru, T.; Ariel, N.; Altboum, Z.; Levy, H.; Weiss, S.; Grosfeld, H.; Cohen, S.; Shafferman, A. *Mol. Microbiol.* **2005**, *58*, 533-551.

(126) Ivanovics, G.; Marjai, E.; Dobozy, A. *J. Gen. Microbiol.* **1968**, *53*, 147-162.

(127) Ivins, B. E.; Welkos, S. L.; Knudson, G. B.; Little, S. F. *Infect. Immun.* **1990**, *58*, 303-308.

(128) Fouet, A.; Namy, O.; Lambert, G. *J. Bacteriol.* **2000**, *182*, 5036-5045.

(129) *MMWR Morb Mortal Wkly Rep* **2001**, *50*, 941-948.

(130) Inglesby, T. V.; Henderson, D. A.; Bartlett, J. G.; Ascher, M. S.; Eitzen, E.; Friedlander, A. M.; Hauer, J.; McDade, J.; Osterholm, M. T.; O'Toole, T.; Parker, G.; Perl, T. M.; Russell, P. K.; Tonat, K. *JAMA* **1999**, *281*, 1735-1745.

(131) Friedlander, A. M.; Welkos, S. L.; Pitt, M. L.; Ezzell, J. W.; Worsham, P. L.; Rose, K. J.; Ivins, B. E.; Lowe, J. R.; Howe, G. B.; Mikesell, P.; et al. *J. Infect. Dis.* **1993**, *167*, 1239-1243.

(132) Baillie, L.; Read, T. D. *Curr. Opin. Microbiol.* **2001**, *4*, 78-81.

(133) Hambleton, P.; Carman, J. A.; Melling, J. *Vaccine* **1984**, *2*, 125-132.

(134) Wright, G. G.; Hedberg, M. A.; Slein, J. B. *J. Immunol.* **1954**, *72*, 263-269.

(135) *Br Med J* **1965**, *2*, 717-718.

(136) Fellows, P. F.; Linscott, M. K.; Ivins, B. E.; Pitt, M. L.; Rossi, C. A.; Gibbs, P. H.; Friedlander, A. M. *Vaccine* **2001**, *19*, 3241-3247.

(137) Friedlander, A. M.; Pittman, P. R.; Parker, G. W. *JAMA* **1999**, *282*, 2104-2106.

(138) Ivins, B. E.; Welkos, S. L.; Little, S. F.; Crumrine, M. H.; Nelson, G. O. *Infect. Immun.* **1992**, *60*, 662-668.

(139) Mahlandt, B. G.; Klein, F.; Lincoln, R. E.; Haines, B. W.; Jones, W. I., Jr.; Friedman, R. H. *J. Immunol.* **1966**, *96*, 727-733.

(140) Puziss, M.; Manning, L. C.; Lynch, J. W.; Barclaye; Abelow, I.; Wright, G. G. *Appl. Microbiol.* **1963**, *11*, 330-334.

(141) Brachman, P. S.; Gold, H.; Plotkin, S. A.; Fekety, F. R.; Werrin, M.; Ingraham, N. R. *Am. J. Public Health Nations Health* **1962**, *52*, 632-645.

(142) Pittman, P. R.; Gibbs, P. H.; Cannon, T. L.; Friedlander, A. M. *Vaccine* **2001**, *20*, 972-978.

(143) Ivins, B. E.; Pitt, M. L.; Fellows, P. F.; Farchaus, J. W.; Benner, G. E.; Waag, D. M.; Little, S. F.; Anderson, G. W., Jr.; Gibbs, P. H.; Friedlander, A. M. *Vaccine* **1998**, *16*, 1141-1148.

(144) Williamson, E. D.; Hodgson, I.; Walker, N. J.; Topping, A. W.; Duchars, M. G.; Mott, J. M.; Estep, J.; Lebutt, C.; Flick-Smith, H. C.; Jones, H. E.; Li, H.; Quinn, C. P. *Infect. Immun.* **2005**, *73*, 5978-5987.

(145) Boyaka, P. N.; Marinaro, M.; Vancott, J. L.; Takahashi, I.; Fujihashi, K.; Yamamoto, M.; van Ginkel, F. W.; Jackson, R. J.; Kiyono, H.; McGhee, J. R. *Am. J. Trop. Med. Hyg.* **1999**, *60*, 35-45.

(146) Coulson, N. M.; Fulop, M.; Titball, R. W. *Vaccine* **1994**, *12*, 1395-1401.

(147) Galen, J. E.; Zhao, L.; Chinchilla, M.; Wang, J. Y.; Pasetti, M. F.; Green, J.; Levine, M. M. *Infect. Immun.* **2004**, *72*, 7096-7106.

(148) Zegers, N. D.; Kluter, E.; van Der Stap, H.; van Dura, E.; van Dalen, P.; Shaw, M.; Baillie, L. *J. Appl. Microbiol.* **1999**, *87*, 309-314.

(149) Flick-Smith, H. C.; Eyles, J. E.; Hebdon, R.; Waters, E. L.; Beedham, R. J.; Stagg, T. J.; Miller, J.; Alpar, H. O.; Baillie, L. W.; Williamson, E. D. *Infect. Immun.* **2002**, *70*, 2022-2028.

(150) Sloat, B. R.; Cui, Z. *Pharm. Res.* **2006**, *23*, 262-269.

(151) Aloni-Grinstein, R.; Gat, O.; Altboum, Z.; Velan, B.; Cohen, S.; Shafferman, A. *Infect. Immun.* **2005**, *73*, 4043-4053.

(152) Boyaka, P. N.; Tafaro, A.; Fischer, R.; Leppla, S. H.; Fujihashi, K.; McGhee, J. R. *J. Immunol.* **2003**, *170*, 5636-5643.

(153) Gaur, R.; Gupta, P. K.; Banerjea, A. C.; Singh, Y. *Vaccine* **2002**, *20*, 2836-2839.

(154) Mikszta, J. A.; Sullivan, V. J.; Dean, C.; Waterston, A. M.; Alarcon, J. B.; Dekker, J. P.; Brittingham, J. M.; Huang, J.; Hwang, C. R.; Ferriter, M.; Jiang, G.; Mar, K.; Saikh, K. U.; Stiles, B. G.; Roy, C. J.; Ulrich, R. G.; Harvey, N. G. *J. Infect. Dis.* **2005**, *191*, 278-288.

(155) Cohen, S.; Mendelson, I.; Altboum, Z.; Kobiler, D.; Elhanany, E.; Bino, T.; Leitner, M.; Inbar, I.; Rosenberg, H.; Gozes, Y.; Barak, R.; Fisher, M.; Kronman, C.; Velan, B.; Shafferman, A. *Infect. Immun.* **2000**, *68*, 4549-4558.

(156) Stepanov, A. V.; Marinin, L. I.; Pomerantsev, A. P.; Staritsin, N. A. *J. Biotechnol.* **1996**, *44*, 155-160.

(157) Brossier, F.; Levy, M.; Mock, M. *Infect. Immun.* **2002**, *70*, 661-664.

(158) Hahn, U. K.; Boehm, R.; Beyer, W. *Vaccine* **2006**, *24*, 4569-4571.

(159) Welkos, S.; Friedlander, A.; Weeks, S.; Little, S.; Mendelson, I. *J. Med. Microbiol.* **2002**, *51*, 821-831.

(160) Messner, P. *Glycoconj. J.* **1997**, *14*, 3-11.

(161) Mesnage, S.; Fontaine, T.; Mignot, T.; Delepierre, M.; Mock, M.; Fouet, A. *EMBO J.* **2000**, *19*, 4473-4484.

(162) Mock, M.; Fouet, A. *Annu. Rev. Microbiol.* **2001**, *55*, 647-671.

(163) Molnar, J.; Pragai, B. *Acta Microbiol. Acad. Sci. Hung.* **1971**, *18*, 105-108.

(164) Fox, A.; Stewart, G. C.; Waller, L. N.; Fox, K. F.; Harley, W. M.; Price, R. L. *J. Microbiol. Methods* **2003**, *54*, 143-152.

(165) Schneerson, R.; Kubler-Kielb, J.; Liu, T. Y.; Dai, Z. D.; Leppla, S. H.; Yergey, A.; Backlund, P.; Shiloach, J.; Majadly, F.; Robbins, J. B. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 8945-8950.

CHAPTER 2

AGONISTIC AND ANTAGONISTIC PROPERTIES OF A *RHIZOBIUM SIN*-1 LIPID A MODIFIED BY AN ETHER-LINKED LIPID*

---

* Mahalakshmi Vasan, Margreet A. Wolfert, Geert-Jan Boons. *Org. Biomol. Chem.* **2007**, 5, 2087-2097. Reprinted here with permission of publisher.

Abstract

LPS from *Rhizobium sin*-1 (*R. sin*-1) can antagonize the production of tumor necrosis factor alpha (TNF-α) by *E. coli* LPS in human monocytic cells. Therefore these compounds provide interesting leads for the development of therapeutics for the prevention or treatment of septic shock. Detailed structure activity relationship studies have, however, been hampered by the propensity of these compounds to undergo β-elimination to give biological inactive enone derivatives. To address this problem, we have chemically synthesized in a convergent manner a *R. sin*-1 lipid A derivative in which the β-hydroxy ester at C-3 of the proximal sugar unit has been replaced by an ether linked moiety. As expected, this derivative exhibited a much-improved chemical stability. Furthermore, its ability to antagonize TNF-α production induced by enteric LPS was only slightly smaller than that of the parent ester modified derivative demonstrating that the ether-linked lipids affect biological activities only marginally. Furthermore, it has been shown for the first time that *R. sin*-1 LPS and the ether modified lipid A are also able to antagonize the production of the cytokine interferon-inducible protein 10, which arises from the TRIF-dependent pathway. The latter pathway was somewhat more potently inhibited than the MyD88-dependent pathway. Furthermore, it was observed that the natural LPS possesses much greater activity than the synthetic and isolated lipid As, which indicates that di-KDO moiety is important for optimal biological activity. It has also been found that isolated *R. sin*-1 LPS and lipid A agonize a mouse macrophage cell line to induce the production of TNF-α and interferon beta in a Toll-like receptor 4-dependent manner demonstrating species specific properties.

Introduction

The innate immune system is an evolutionary ancient system of defense against microbial infections.[1] It responds rapidly to highly conserved families of structural patterns, called pathogen associated molecular patterns (PAMPs), which are integral parts of pathogens and are perceived as danger signals by the host. Recognition of PAMPs is mediated by sets of highly conserved receptors,[2] each of which binds to a variety of PAMPs. Cellular activation by these receptors results in acute inflammatory responses that include the production of a diverse set of cytokines and chemokines, direct local attack against the invading pathogen and initiation of responses that activate and regulate the adaptive component of the immune response.

Lipopolysaccharides (LPSs) are structural components of the outer surface membrane of Gram-negative bacteria that trigger innate immune responses through Toll-like receptor 4 (TLR4), a member of the TLR family that participates in pathogen recognition. TLRs are transmembrane glycoproteins having an extracellular domain that contains multiple leucine-rich repeating motifs, a transmembrane domain and an intracellular signaling domain.[3,4] The intracellular domain serves as a docking site for a number of adaptor proteins,[5] which in turn recruit kinases to initiate specific down-stream processes, such as activation of mitogen-activated protein (MAP) kinases and transcription factors (NF-κB, AP-1 and interferon regulatory factor 3 (IRF-3). The end result is the up-regulation of hundreds of genes resulting in the production of a multitude of cytokines and chemokines.

TLR4 initiates cell-signaling by two cascades that involve recruitment of the intracellular TIR adaptor proteins MyD88 or TRIF.[3,4] Thus, the TIR domain of TLR4 can bind to the dimeric adapter protein MyD88, that then recruits and activates a number of kinases, subsequently leading to activation of the MAP kinases, such as p38, JNK and ERK1/2 and the transcription

factor NFκB. This *MyD88-dependent pathway* results in the synthesis of proinflammatory cytokines and chemokines including tumor necrosis factor alpha (TNF-α), interleukin 1beta (IL-1β) and IL-6. Another adaptor protein, called TRIF,[6] can also be recruited to the TIR domain leading to activation of the transcription factor IRF-3, NF-κB and the MAP kinase JNK. This *TRIF-dependent pathway* results in the synthesis of important inflammatory mediators, including interferon beta (IFN-β), interferon-inducible protein 10 (IP-10) and nitric oxide.

Although the initiation of acute inflammatory responses is important for the prevention of infections, over-activation of this response may lead to the clinical symptoms of septic shock. Septicemia is a serious world-wide health problem and is associated with mortality rates of 40–60%.[7,8] It has been estimated that 1% of hospital patients and 20–30% of ICU patients develop sepsis and that septic shock results in 100000 deaths annually in the United States.[7,8] A number of strategies for the prevention and treatment of sepsis have been directed against the lipid A region of LPS.[9] For example, structural analogs of lipid A have been examined for their ability to antagonize the effects of LPS. These antagonists include naturally occurring lipid A precursors,[10] as well as a number of synthetic analogs of these precursors.[11,12] The most widely studied analog is a synthetic analog based on the lipid A of *R. sphaeroides* or *R. capsulatus*, two species having very similar lipid A structures.[13-16] Although the lipid As of *R. sphaeroides/R. capsulatus* and *E. coli* have the same bis-1,4-phosphorylated glucosamine disaccharide backbone their fatty acyl patterns differ considerably. In this respect, *R. sphaeroides/R. capsulatus* lipid A consists of two 3-oxomyristic acid, two β-hydroxydecanoic acid and one dodecenoic acid residues.

Recently, we reported that LPS from *Rhizobium sin*-1 (*R. sin*-1), a nitrogen-fixing bacterial species, can prevent the induction of TNF-α by *E. coli* LPS in human monocytic cells.[17,18] Furthermore, another study showed that the biological properties of *R. sin*-1 LPS are

species specific and most notably it was found that it can agonize mouse macrophages in a TLR2-dependent manner.[19,20] The lipid A of *R. sin*-1 LPS is a structurally unusual lipid A differing in almost every aspect from those known to contribute to the toxicity of enteric LPS (**Figure 2.1**).[21] In particular, the disaccharide moiety of *Rhizobial* lipid A is devoid of phosphate and the glucosamine phosphate is replaced by 2-aminogluconolactone. It contains a very long chain fatty acid, 27-hydroxyoctacosanoic acid which, in turn, can be esterified by β-hydroxybutyrate. As is the case with other naturally occurring LPSs, the inherent microheterogeneity of the lipid A region of *R. sin*-1 makes it impossible to be developed as a therapeutic agent for Gram negative septicemia. Furthermore, the inability to separate the different species limits identification of specific structural features that makes *R. sin*-1 lipidA an antagonist as opposed to an agonist. To address these problems, we have synthesized a range of well defined lipid A derivatives based on the structure of *R. sin*-1 LPS for structure–activity relationship studies. We have already shown[18] that the synthetic compound **1** is able to antagonize *E. coli* LPS. In addition, we have demonstrated that the 2- aminogluconolactones can exist as a 2-aminogluconate.[22] The chemical synthesis and biological evaluation of a compound locked in the 2-aminogluconate established that this species possesses antagonistic properties.[23] Detailed biological evaluations of the synthetic *R. sin*-1 lipid As have been hampered by their chemical lability due to elimination to give the enone derivative **2**. To address this problem, we report here the chemical synthesis of compound **3**, which is derived from **1**, however, the β-hydroxy ester at C-3 of the proximal sugar unit has been replaced by an ether derivative. It was anticipated that this compound would be less prone to β-elimination due to the poor leaving group ability of the ether.[24-26] As a result, we have been able to investigate the ability of a *R-sin* 1 lipid A to antagonize cell-signaling events arising from the MyD88- and TRIF-dependent

pathways. Furthermore, species specific properties of **3** have been investigated by comparing biological properties of the compound exposed to human and mouse macrophages.

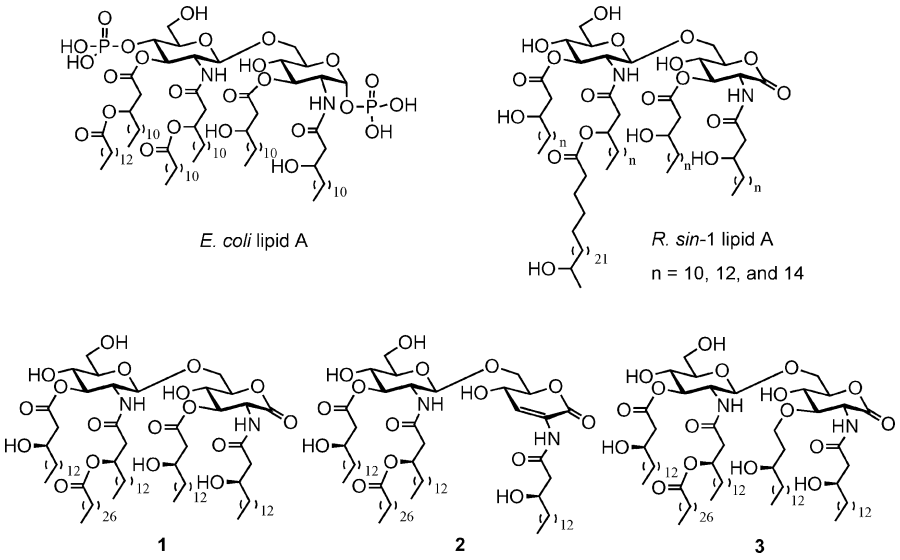


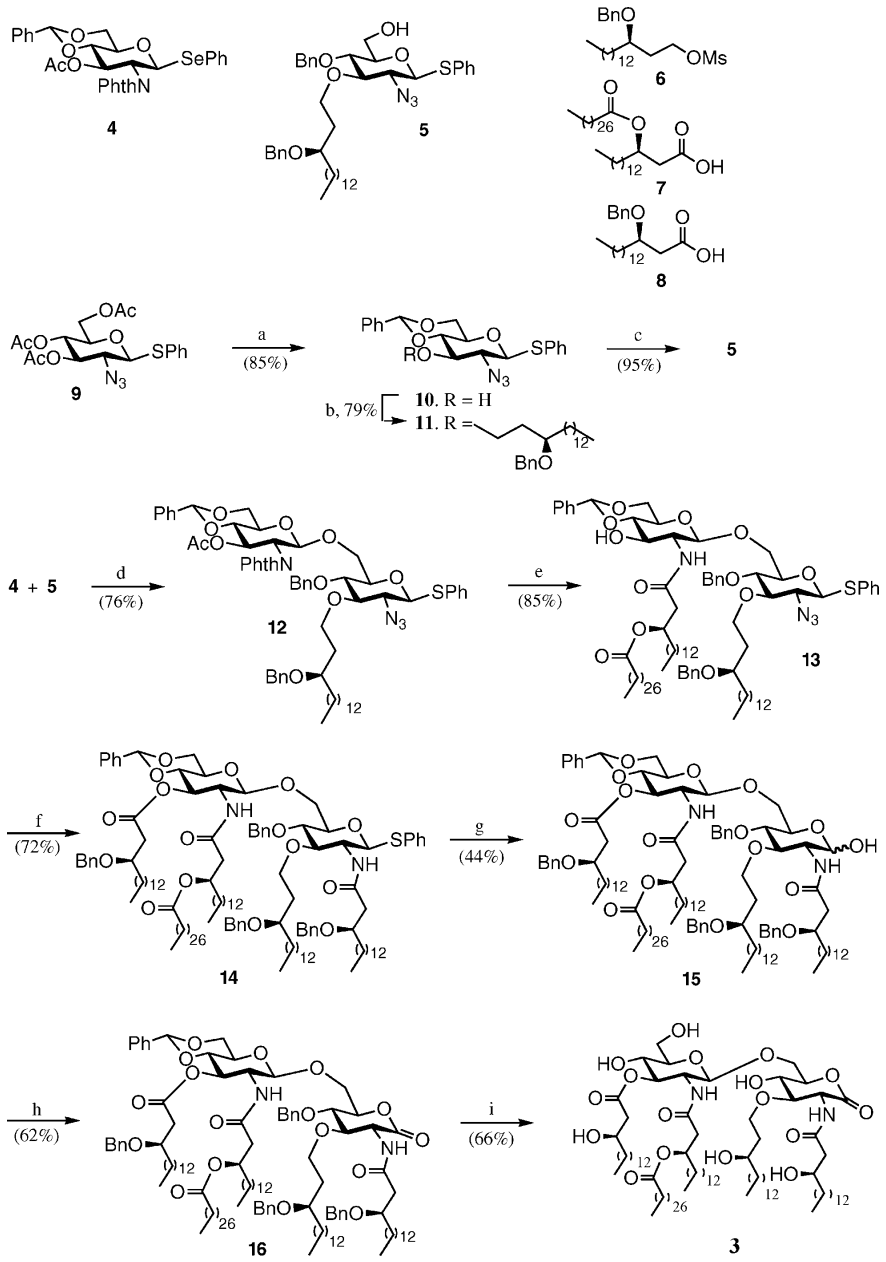
Figure 2.1. Structures of *E. coli* and *R. sin*-1 lipid A and synthetic *R. sin*-1 lipid A derivatives **1–3**.

Results and Discussion

Chemical Synthesis

It was envisaged that coupling of glycosyl donor **4** with glycosyl acceptor **5** would give disaccharide **12** (**Scheme 2.1**), which is appropriately protected for the selective introduction of β-hydroxyl fatty acids and oxidation of the C-1 position to lactone. Glycosyl acceptor **5** is modified by an ether linked γ-benzyloxy fatty acid, because it was anticipated that the harsh conditions required for its introduction would affect functionalities present in the disaccharide. Another feature of **5** is that its anomeric center is protected as a thioglycoside.[27,28] This functionality is stable under a wide range of chemical conditions, however, it can be hydrolyzed at a late stage of the chemical synthesis to give a lactol,which can then be oxidized to a lactone. Furthermore, the selenoglycoside of **4** was expected to be significantly more reactive towards activation with NIS–TMSOTf than the thioglycoside of **5**, and thus itwas expected that these

compounds could be employed in a chemoselective glycosylation to give **12**. The phthalimido and azido functions of **12** offer an attractive set of orthogonal protecting groups that allow selective derivatization of the two amino groups. Removal of the phthalimido group will result in cleavage of the *O*-acetyl ester. However, by exploiting the higher nucleophilicity of primary amines compared to hydroxyls it is possible to selectively acylate the amine. Glycosyl acceptor **5** was readily obtained from known derivative **9**.[29] Thus, the acetyl esters of **9** were cleaved by treatment with sodium methoxide in methanol and the resulting triol was selectively protected by reaction with benzylaldehyde dimethyl acetal in the presence of camphorsulfonic acid (CSA) in acetonitrile to give **10**. The C-3 hydroxyl of **10** was alkylated with sulfonate **6** by treatment with sodium hydride in DMF to give **11** in a good yield of 79%.[26] Next, the benzylidene acetal of **11** was regioselectively opened by reaction with $BH_3 \cdot NHMe_2$ and $BF_3 \cdot Et_2O$ in toluene at −30 °C to give **5** in an excellent yield. A number of other reaction conditions led to the formation of mixtures of regioisomeric benzyl ethers. For example, the use of $BH_3 \cdot NHMe_2$ in DCM, which is the conventional solvent for this reagent,[30] gave a mixture of products. Glycosyl donor **4**[31] and fatty acids **6**,[26] **7** and **8**[32,33] were prepared by reported procedures. Having glycosyl donor **4** and acceptor **5** at hand, attention was focused on the preparation of the disaccharide **12**, installment of the β- hydroxyl fatty acids and oxidation of the anomeric center. Thus, a NIS–TMSOTf mediated coupling of the glycosyl donor **4** with acceptor **5** in dichloromethane at −35 °C gave disaccharide **12** in a yield of 76%.[31,34,35] Only the β-anomer was formed due to neighboring group participation of the phthalimido group.



Scheme 2.1. *Reagents and conditions*: a) NaOMe, MeOH then C_6H_5CH-$(OMe)_2$, CSA, CH_3CN; b) **6**, NaH, DMF, 0 °C; c)$BH_3 \cdot NHMe_2$, $BF_3 \cdot Et_2O$, toluene, −30 °C; d) NIS, TMSOTf, MS 4Å , DCM, −35 °C; e) $NH_2NH_2 \cdot H_2O$, EtOH, Δ, then **7**, DCC, DCM; f) Zn, AcOH then **8**, DCC, DMAP, DCM; g) NIS, TMSOTf, DCM, H_2O, 0 °C; h) PCC, 3 ÅMS, DCM; i) Pd/C, H_2, *t*-BuOH, THF.

Next, the phthalimido moiety and acetyl ester of compound **12** were removed by treatment with hydrazine hydrate in refluxing ethanol[36] and the amine of the resulting compound was selectively acylated with alkanoyloxy fatty acid **7** in the presence DCC to give **13**. Reduction of the azido

moiety of **13** was easily accomplished by reaction with activated Zn in acetic acid and the amine and hydroxyl of the resulting compound were immediately acylated with **8** using 1,3-dicyclohexylcarbodiimide (DCC) and 4- dimethylaminopyridine (DMAP) as the activation reagents to afford **14**. It is important to note that selective *N*-acylation could be accomplished by performing the reaction in the absence of DMAP, thereby making it possible to synthesize derivatives having different substituents at C-2 amine and C-3 and C-3' hydroxyls. The thioglycoside of compound **14** was hydrolyzed by treatment with NIS–TMSOTf[37] in wet dichloromethane and the resulting lactol was oxidized with PCC in DCM to afford lactone **16**. Finally, the benzyl ethers and benzylidene acetal of **16** were removed by catalytic hydrogenation over Pd/C to give the target compound **3**. As expected, this derivative had an excellent shelf-life and after storage for three months at −20 °C no decomposition was observed. Under similar storage conditions compound **1** was decomposed.

It is important to note that alternative synthetic strategies, which employed either an allyl or TBDMS ether for protection of the anomeric center of the proximal sugar, led to failure. Thus, the anomeric TBDMS function was not compatible with the alkylation conditions required for the instalment of **6**. Furthermore, attempts to cleave an allyl glycoside at the final stage of the synthesis led either to recovery of starting material or decomposition. The use of a thioglycoside gave the best results for the preparation of the target compound.

Biological Evaluation

Based on the results of recent studies,[1-5] it is clear that enteric LPS induced cellular activation through TLR4 is complex as many signaling elements are involved. However, it appears that there are two distinct initiation points in the signaling process, one being a specific intracellular adaptor protein called MyD88 and the other an adaptor protein called TRIF, which

operates independently of MyD88. It is well established that TNF-α secretion is a prototypical measure for activation of the MyD88-dependent pathway, whereas secretion of IFN-β and IP-10 are commonly used as an indicator of TRIF-dependent cellular activation.

Compound **3** was tested over a wide concentration range for its ability to activate a human monocytic cell line (Mono Mac 6; MM6) to produce TNF-α and IP-10 protein and the resulting values were compared with similar data obtained for *E. coli* LPS and lipid A and *R. sin*-1 LPS and lipid A. Thus, MM6 cells were exposed to the isolated and synthetic compounds and after 5.5 hours, the supernatants were harvested and examined for human TNF-α and IP-10 using capture ELISAs. Potencies (EC_{50}, concentration producing 50% activity) and efficacies (maximal level of production) were determined by fitting the dose–response curves to a four parameter logistic equation using PRISM software. As can be seen in **Figure 2.2**, *E. coli* LPS and lipid A yielded clear dose–response curves for TNF-α and IP-10, whereas *R. sin*-1 LPS, *R. sin*-1 lipid A and synthetic compound **3** did not induce significant production of the cytokines. The EC_{50} values for *E. coli* 055:B5 LPS were significantly smaller than that of *E. coli* lipid A (**Table 1.1**) which is probably due to the di-KDO moiety of LPS, which is attached to the C-6 position of lipid A. In this respect, recent studies[38] have shown that meningococcal lipid A expressed by a strain defect in KDO biosynthesis has significantly reduced bioactivity compared to KDO containing *Meningococcal* lipooligosaccharides. It has also been shown that removal of the KDO moieties by mild acidic treatment reduces cellular responses.[18] It was observed that the EC_{50} values for TNF-α secretion were approximately three times smaller than that of IP-10 when *E. coli* LPS or *E. coli* lipid A was employed as an activator. Thus, it appears that the MyD88-dependent pathway is slightly more responsive than TRIF-mediated cellular activation. Based on its lack of proinflammatory effects, compound **3** was tested over a wide concentration range for

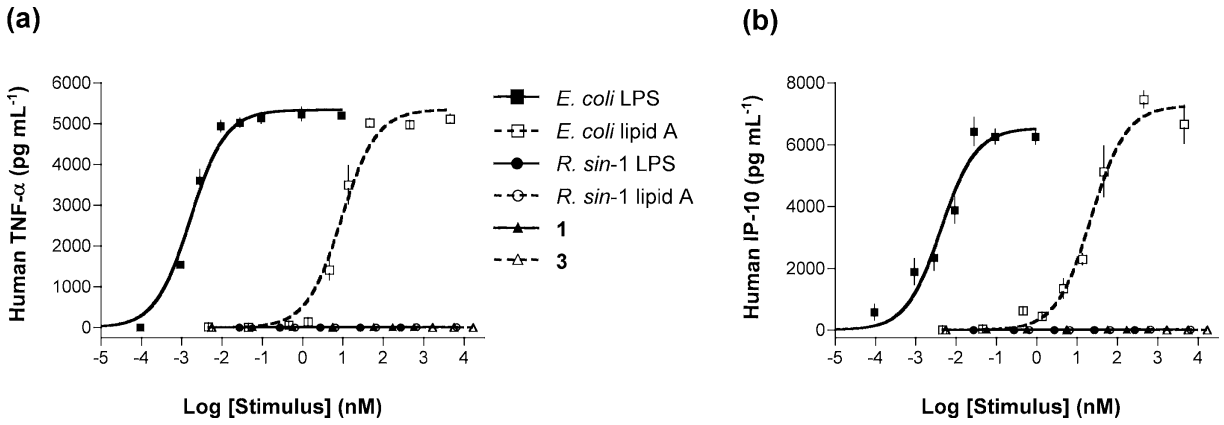


Figure 2.2. Concentration–response curves of *E. coli* LPS, *E. coli* lipid A, *R. sin*-1 LPS, *R. sin*-1 lipid A and synthetic compounds **1** and **3** in human monocytic cells. MM6 cells were incubated for 5.5 h at 37 °C with increasing concentrations of *E. coli* LPS, *E. coli* lipid A, *R. sin*-1 LPS, *R. sin*-1 lipid A and synthetic compounds **1** and **3** as indicated. TNF-α (a) and IP-10 (b) proteins in cell supernatants were measured using ELISAs. (Please note that *R. sin-1* LPS, *R. sin*-1 lipid A, **1** and **3** show background values and therefore overlap in the figure.) Treatment with *E. coli* LPS, *E. coli* lipid A, *R. sin*-1 LPS, *R. sin*-1 lipid A, **1** and **3** did not affect cell viability, as judged by cellular exclusion of trypan blue.

Table 1.1. EC_{50} values[a] (nM) of *E. coli* LPS and lipid A in MM6 cells.

	E. coli LPS	***E. coli* lipid A**
TNF-α	**0.0016 (0.0014–0.0019)**	**9.1 (7.3–11.4)**
IP-10	**0.0042 (0.0032–0.055)**	**22.2 (18.5–26.8)**
[a] EC_{50} values are reported as best-fit values and as minimum–maximum range (best-fit value ± std. error).		

its ability to antagonize TNF-α and IP-10 production by MM6 cells incubated with *E. coli* LPS (1 ng mL^{-1}) (**Figure 2.3**). An IC_{50} (concentration producing 50% inhibition) of 22 µM (38 µg mL^{-1}) was established for TNF-α inhibition by compound **3**. Similar inhibition experiments with *R. sin*-1 lipidA and compound **1** gave IC_{50} values of 2.0 µM (3.0 µg mL^{-1}) and 7.3 µM (13 µg mL^{-1}), respectively. As expected, *R. sin*-1 LPS was a much more potent inhibitor of TNF-α production than the corresponding lipid A and in this case an IC_{50} value of 6.5 nM (239 ng mL^{-1})

was determined. Thus, it is probable that the KDO moiety of LPS accounts for the higher inhibitory activity.

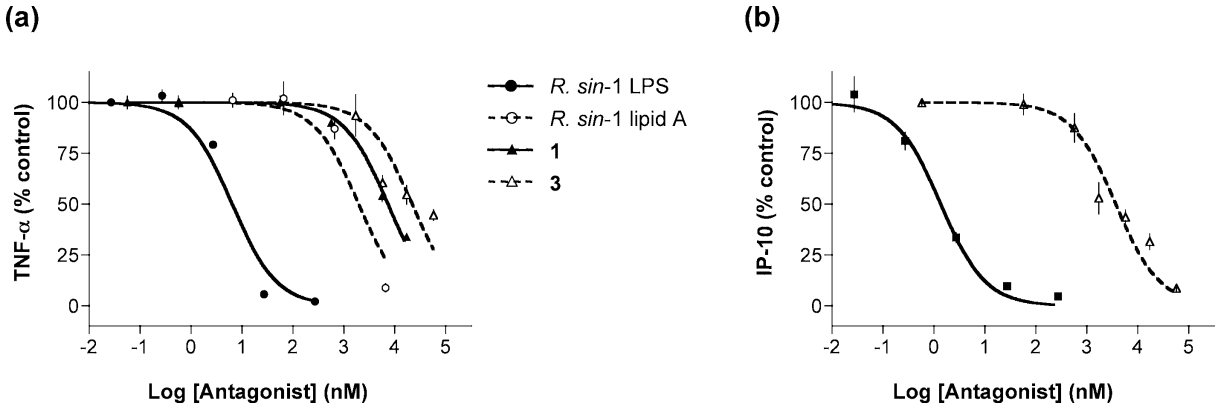


Figure 2.3. Antagonism of *E. coli* LPS by *R. sin*-1 LPS, *R. sin*-1 lipid A and synthetic compounds **1** and **3** in human monocytic cells. TNF-α (a) and IP-10 (b) concentrations were measured after preincubation of MM6 cells with increasing concentrations of *R. sin*-1 LPS, *R. sin*-1 lipid A, **1**, or **3** as indicated for 1 h at 37 °C, followed by 5.5 h of incubation with *E. coli* LPS (1 ng mL^{-1}). Results are expressed as percentage of cytokine concentration of control cells, which are incubated only with *E. coli* LPS.

Interestingly, for IP-10 secretion the IC_{50} values of *R. sin*-1 LPS and compound **3** were smaller than the corresponding values for TNFα (1.4 nM; 51 ng mL^{-1}) and 3.7 μM (6.5 μg mL^{-1}), respectively) and at the highest concentration tested compound **3** abolished all IP-10 biosynthesis induced by enteric LPS. Similar inhibition results were obtained when *E. coli* lipid A was employed as the agonist (Supplementary data†).

The results of the cellular activation studies show that the inhibitory activity of compound **3** is only slightly smaller than that of the parent compound **1** demonstrating that the ether linked fatty acid at C-3 of the proximal monosaccharide unit has only marginal effect on the biological activity. However, compound **3** has a much greater chemical stability than **1** making it a preferred compound for biological studies. Furthermore, the KDO moiety of *E. coli* and *R. sin*-1 LPS appears to significantly increase the agonistic and antagonistic properties, respectively. The data also reveal that the agonists and antagonists affect the MyD88 and TRIF

pathways slightly differently. Thus, the agonists (*E. coli* LPS and lipid A) display somewhat higher potencies for TNF-α compared to IP-10, whereas for the antagonists (*R. sin*-1 LPS and lipid A), IP-10 was more potently inhibited at lower concentrations compared to TNF-α.

There are several reports that indicate that structurally different lipid As may differentially induce proinflammatory responses.[39-42] However the heterogeneity in the structure of lipid A within a particular bacterial strain and possible contamination with other inflammatory components of the bacterial cell-wall complicated the dissecting of the biological responses to specific lipid As. Synthetic compounds may address this important issue.

Next, attention was focused on cellular activation studies using a mouse macrophage cell line (RAW 264.7 γNO(−) cells). Thus, secretion of TNF-α and IFN-β protein was measured after exposure of the cells for 5 h to a wide concentration range *E. coli* LPS and lipid A, *R. sin*-1 LPS and lipidA and compound **3** (**Figure 2.4**). Interestingly, *E. coli* and *R. sin*-1 LPS and lipid As activated the cells to produce TNF-α and IFN-β. No cytokine production was measured for compound **3** even when a very high concentration was employed (57 μM; 100 μg mL^{-1}). Furthermore, compound **3** was not able to antagonize the production of TNF-α or IFN-β induced by *E. coli* LPS. For each agonist, the potency for TNF-α secretion was higher by 5 to 7 fold compared to that of IFN-β (**Table 2.2**). Furthermore, for the *E. coli* derived compounds the EC_{50} values were significantly smaller than those derived from *R. sin*-1. As expected, the lipid As were less potent than their parent LPSs, however, the difference was much larger between *E. coli* LPS and lipid A (10000-fold) than between *R. sin*-1 LPS and lipid A (100-fold).

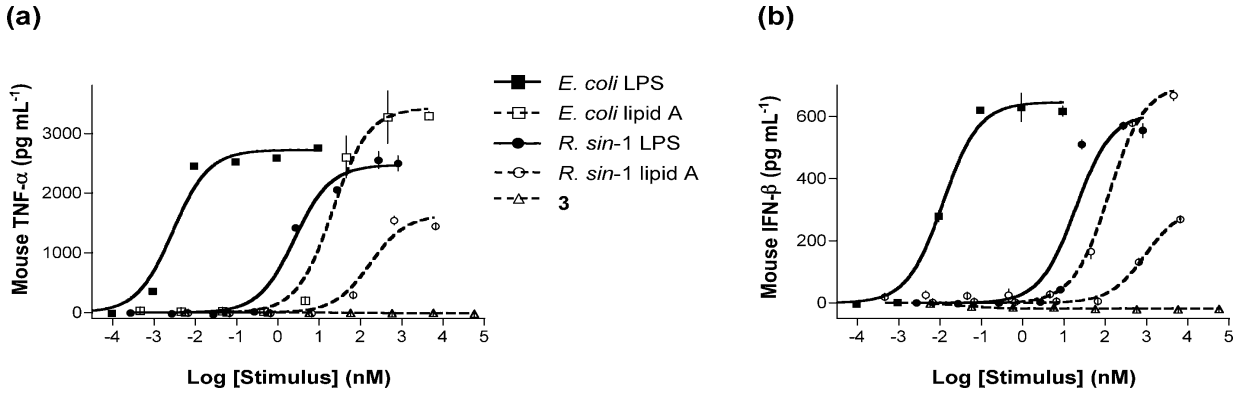


Figure 2.4. TNF-α and IFN-β production by murine macrophages after stimulation with *E. coli* LPS, *E. coli* lipid A, *R. sin*-1 LPS, *R. sin*-1 lipid A and synthetic compound **3**. Murine RAWγNO(–) cells were incubated for 5.5 h with increasing concentrations of *E. coli* LPS, *E. coli* lipid A, *R. sin*-1 LPS, *R. sin*-1 lipid A and **3** as indicated. TNF-α (a) and IFN-β (b) in cell supernatants were measured using ELISAs. Treatment with *E. coli* LPS, *E. coli* lipid A, *R. sin*-1 LPS, *R. sin*-1 lipid A and **3** did not affect cell viability, as judged by cellular exclusion of trypan blue.

Table 2.2. EC_{50} values[a] (nM) of *E. coli* and *R. sin*-1 LPS and lipid A in RAW cells.

	E. coli LPS	*E. coli* lipid A	*R. sin*-1 LPS	*R. sin*-1 lipid A
TNF-α	0.0028 (0.0020–0.0041)	21 (16–28)	2.5 (2.0–3.2)	171 (109–268)
IFN-β	0.0118 (0.0096–0.0145)	124 (105–147)	19.3 (11.3–32.8)	932 (816–1067)

[a] EC_{50} values are reported as best-fit values and as minimum–maximum range (best-fit value ± std. error).

Recent reports indicate that LPS of non-enterobacterial species such as *Porphyromonas gingivalis*, *Leptospira interrogans* and *R. sin*-1 are capable of signaling independent of TLR4, instead utilizing TLR2-mediated signal transduction.[19,20,43,44] However, TLR2 can only recruit the adaptor proteinMyD88 and as a result can only initiate the production of MyD88-dependent cytokines such as TNF-α, but not those TRIF-dependent cytokines such as IFN-β.[3,4] The fact that our results show that *R. sin*-1 can induce the production of IFN-β prompted us to investigate the TLR utilization of these compounds. Thus, *R. sin*-1 LPS and lipid A and *E. coli* LPS and lipid A were exposed at a range of concentrations to HEK 293T cells stably transfected with mouse

TLR2/TLR6 or TLR4/MD2 and transiently transfected with a plasmid containing the reporter gene pELAM-Luc (NFκB- dependent firefly luciferase reporter vector) and a plasmid containing the control gene pRL-TK (*Renilla* luciferase control reporter vector).

As a negative control, wild type HEK 293T cells transiently transfected with plasmids containing the reporter gene pELAM-Luc and control gene pRL-TK were used. After an incubation time of 4 h, the activity was measured using a commercial dual-luciferase assay. *E. coli* LPS and the lipopeptide Pam_3CysSK_4[45] were employed as positive controls for cellular activation by TLR4 and TLR2/6, respectively. As can be seen in **Figure 2.5**, *R. sin*-1 LPS and lipid A can induce cellular activation in a TLR4-dependent manner, whereas no activity was observed in cells transfected with TLR2/6. The TLR4-dependent NF-κB activation showed clear dose responses for *E. coli* LPS, *R. sin*-1 LPS and *R. sin*-1 lipid A (Supplementary data†). Previously it was established that the lipid A region of several *Rhizobiaceae* can stimulate bone marrow granulocytes of TLR4-deficient mice to induce the expression of CD14.[19]

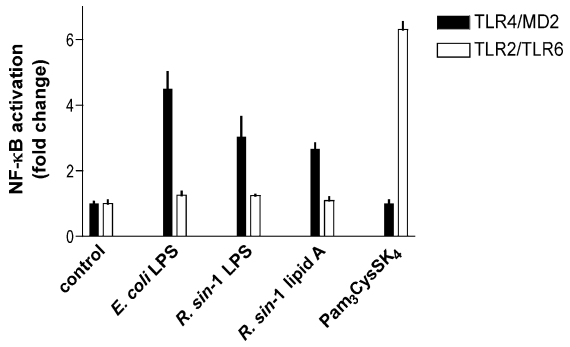


Figure 2.5. Response of HEK 293T cells expressing murine TLRs to *R. sin*-1 LPS and *R. sin*-1 lipid A. Induction of NF-κB activation was determined in triplicate cultures of HEK 293T cells stably transfected withmurineTLR4/MD2 or TLR2/TLR6 and transiently transfected with pELAM-Luc, pRL-TK and pcDNA3 plasmids. Forty-four h post-transfection, cells were treated with *E. coli* LPS (1 ng mL^{-1}), *R. sin*-1 LPS (1 μg mL^{-1}), *R. sin*-1 lipid A (1 μg mL^{-1}) and Pam_3CysSK_4 (1 μg mL^{-1}) or were left untreated (control). Forty-eight h post-transfection, NF-κB activation was determined by firefly luciferase activity relative to *Renilla* luciferase activity. In the transfection experiments shown, human TNF-α (10 ng mL^{-1}) induced 24.5 ± 0.6 and 21.8 ± 0.3-fold activation of NF-κB in HEK 293T cells stably transfected with TLR4/MD2 and TLR2/6, respectively.

Furthermore, no detectable levels of TNF-α were measured after mouse peritoneal macrophages were exposed to 100 ng mL^{-1} *R. sin*-1 LPS. Surprisingly, HEK cells transfected with TLR2/6 with an ELAM luciferease reporter plasmid showed activity at this concentration. Our results show clearly that at similar concentrations, *R. sin*-1 LPS and lipidA can induce the production of TNF-α and IFN-β in a TLR4-dependent manner.

The observation that synthetic compound **3** possessed no activity in the mouse cell line was surprising. LPS and lipid A isolated from *R. sin*-1 are composed of a complex mixture of compounds differing in fatty acid substitution. Probably, a compound with unique fatty acid composition is responsible for the TLR4 agonistic properties. A larger range of derivatives will need to be synthesized to establish which derivatives account for this activity. The synthetic approach reported here provides such an opportunity. Furthermore, the observation that TLR ligands exhibit species-specific properties should be considered when immuno modulators are being developed.

Conclusion

It has been shown that a derivative of *R. sin*-1 lipid A in which the C-3 fatty acid is replaced by an ether-linked moiety has a much improved chemical stability. Furthermore, this compound could antagonize cytokine production by a human monocytic cell line induced by enteric LPS with a similar potency to the natural ester-linked counter part. For the first time, it has been shown that such an antagonist can inhibit both MyD88- and TRIF dependent cell signaling events. *R. sin*-1 LPS and lipid A agonized mouse macrophages to produce TNF-α and IFN-β demonstrating species specific properties. For the agonists examined, the potency for TNF-α secretion was higher by 3–7 fold compared to that of IFN-β or IP-10. For, the antagonists, the IC_{50} values for IP-10 were smaller than the corresponding values for TNF-α. These data

indicate that the MyD88 and TRIF pathways are somewhat differently activated or inhibited by the examined compounds. Finally, the LPS agonist and antagonist were much more potent indicating that the KDO moiety of LPS is important for optimal biological properties.

Acknowledgement: This research was supported by the Institute of General Medicine of the National Institutes of Health (GM061761).

Experimental Section

Chemicals were purchased from commercial suppliers and used without further purification, unless otherwise noted. Dichloromethane (DCM) and toluene were distilled from calcium hydride under Argon. Tetrahydrofuran (THF) was distilled under argon from sodium directly prior to application. Dry *N*, *N*-dimethylamineformamide (DMF) was used without purification. Powdered molecular sieves (4 Å) were activated *in vacuo* at 390 °C for 8 h and cooled to room temperature *in vacuo* prior to application. Column chromatography was performed on silica gel 60 (EM Science, 70–230 mesh), size exclusion was performed on Sephadex LH-20 and eluted with a mixture of MeOH–CH_2Cl_2, (1:1, v/v). Reactions were monitored by thin layer chromatography (TLC) on kieselgel 60 F_{254} (EM Science) and compounds were visualized by examination under UV light and by charring with cerium sulfate–ammonium molybdate solution. Organic solvents were removed under reduced pressure at <40 °C. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Merc 300, Varian Inova 500 or Inova 600 equipped with Sun Workstations. ^{1}H NMR were recorded in $CDCl_3$ and referenced to residual $CHCl_3$ at 7.24 ppm. ^{13}C NMR spectra were referenced to the central peak of $CDCl_3$ at 77.0 ppm. Assignments were made by standard gCOSY and gHSQC. High resolution mass spectra were obtained on a Bruker model Ultraflex MALDI-TOF-TOF mass spectrometer. Optical rotations

were measured on a Jasco model P-1020 polarimeter. Signals marked with a subscript L belong to the ether-linked lipid at C-3, whereas signals marked with subscript LL belong to the lipid at C-2'. Signals marked with subscript LL' refer to the C-28 side chain. Signals marked with subscript LA belong to lipids at C-2, C-3'.

Phenyl 3-*O*-[(*R*)-3-benzyloxy-hexadecanoyl]-4,6-*O*-benzylidene-2-deoxy-2-azido-1-thio-β-D-glucopyranoside (11). NaH (55% oil dispersion, 0.12 g, 5.0 mmol) was added to a solution of compound **10** (0.34 g, 0.88 mmol) in DMF (6 mL) at 0 °C followed by addition of **6** (0.34 g, 0.79 mmol) dissolved in DMF (3 mL). The reaction mixture was allowed to reach room temperature and stirring was continued for 14 h. The reaction mixture was diluted with ethyl acetate (20 mL), quenched with water (1 mL) and subsequently washed with saturated aqueous $NaHCO_3$ (2 × 10 mL) and brine (2 × 10 mL). The combined organic layers were dried ($MgSO_4$), filtered and the filtrate was concentrated *in vacuo*. The residue was purified by silica gel column chromatography (10% ethyl acetate in hexane) to afford **9** as a colorless syrup (0.45 g, 79%, yield based on mesylate): R_f = 0.70 (20% ethyl acetate in hexane); $[a]^{25}{}_D$ = −55.94 (c = 1.0, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.27– 7.59 (m, 15H, aromatic), 5.52 (s, 1H, >C*H*Ph), 4.45–4.50 (m, 3H, H-1, H-3$_L$C*H*$_2$Ph), 4.38–4.03 (dd, 1H, $J_{5,6a}$ = 5.1 Hz, $J_{6a,6b}$ = 10.2 Hz, H-6a), 4.02–3.98 (m, 1H, H-1$_L$), 3.84–3.73 (m, 2H, H-1$_L$, H-6b), 3.58–3.39 (m, 4H, H-3$_L$, H-4, H-3, H-5), 3.30 (t, 1H, J = 10.2 Hz, J = 8.7 Hz, H-2), 1.82–1.80 (m, 2H, H-2$_L$), 1.50– 1.46 (m, 2H, H-4$_L$), 1.31–1.24 [m, 22H, H-(5$_L$–15$_L$)], 0.89 (t, 3H, H-16$_L$); ^{13}C NMR (300 MHz, $CDCl_3$): δ = 126.17–139.26 (aromatic), 101.46 (>*C*HPh), 86.91 (C-1), 82.07 (C-4), 81.42 (C-3), 76.19 (C-3$_L$), 71.16 (C-3$_L$ *C*H$_2$Ph), 70.73 (C-5), 70.47 (C-1$_L$), 68.70 (C-6), 65.08 (C-2), 35.02 (C-2$_L$), 34.24 (C-4$_L$), 32.1–22.93 [C-(5$_L$–15$_L$)], 14.37 (C-16$_L$); HRMS (*m*/*z*) for $C_{42}H_{57}N_3O_5S[M + Na]^+$: calcd 738.4019, found 738.4613.

Phenyl 3-*O*-[(*R*)-3-benzyloxy-hexadecanoyl]-4-*O*-benzyl-2-deoxy-2-azido-1-thio-β-D-glucopyranoside (5). To a solution of compound **11** (0.26 g, 0.36 mmol) in toluene (10 mL) was added $BH_3{\cdot}NHMe_2$ (0.11 g, 1.79 mmol). After cooling the reaction mixture (−30 °C), $BF_3{\cdot}OEt_2$ (0.31 g, 2.15 mmol) was added dropwise. The temperature was allowed to reach 0 °C over a period of 1 h after which TLC analysis indicated completion of the reaction. The reaction mixture was then quenched by the very slow addition of methanol (3 mL) followed by evaporation of the organic solvents *in vacuo*. The crude product was purified by silica gel column chromatography (20% ethyl acetate in hexane) to obtain compound **5** (0.25 g, 95%) as a white solid. R_f = 0.40 (20% ethyl acetate in hexane); $[a]^{25}{}_D$ = −33.56 (*c* = 1, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.19–7.46 (m, 15H, aromatic), 4.71 (d, 2H, $J_{a,b}$ = 10.8 Hz, H-3_L CH_aH$_b$Ph), 4.53 (d, 2H, $J_{b,a}$ = 10.8 Hz, H-3_LCH$_a$*H$_b$*Ph), 4.42 (bd, 2H, H-4 CH_2Ph), 4.35 (d, 1H, $J_{1,2}$ = 9.9 Hz, H-1), 3.91–3.76 (m, 3H, H-1_L, H-6a), 3.61–3.57 (m, 1H, H-6b), 3.47–3.43 (m, 1H, H-3_L), 3.39–3.33 (m, 1H, H-3), 3.28–3.13 (m, 3H, H-4, H-5, H-2), 2.63 (s, 1H, H-6 O*H*), 1.82–1.76 (m, 2H, H-2_L), 1.30–1.48 (m, 2H, H-4_L), 1.18 [m, 22H, H-(5_L–15_L)], 0.81 (t, 3H, H-16_L); ^{13}C NMR (300 MHz, $CDCl_3$): δ = 127.67–139.13 (aromatic), 86.28 (C-1), 85.56 (C-4), 79.76 (C-5), 77.50 (C-3), 76.46 (C-3_L), 75.26 (C-3_L *C*H_2Ph), 71.11 (C-4 *C*H_2Ph, C-1_L, C-6), 65.48 (C-2), 35.10 (C-2_L), 34.33 (C-4_L), 32.17–29.93 [C-(5_L–15_L)], 14.37 (C-16L);HRMS(*m*/*z*) for $C_{42}H_{59}N_3O_5S[M+Na]^+$: calcd 740.4175, found 740.4748.

Phenyl 3-*O*-acetyl-6-*O*-(3-*O*-acetyl-4,6-*O*-benzylidene-2-deoxy- 2-phthalimido-β-D-glucopyranosyl)-2-azido-3-*O*-[(*R*)-3-benzyloxyhexadecanoyl]- 4-*O*-benzyl-2-deoxy-1-thio-β-D-glucopyranoside (12). A suspension of **4** (0.59 g, 1.03 mmol) and **5** (0.61 g, 0.86 mmol) and molecular sieves (4 Å, 0.5 g) in DCM (10 mL) was stirred under an argon atmosphere for 2 h. The mixture was cooled (−35 °C) followed by the addition of NIS (0.23 g, 1.04 mmol) and

TMSOTf (0.01 g, 0.05 mmol). The reaction mixture was stirred for 45 min allowing it to slowly reach −10 °C after which TLC analysis showed complete consumption of the starting materials. The reaction mixture was quenched with pyridine (0.1 mL) and diluted with DCM (10mL). The molecular sieves were removed by filtration through a pad of Celite. The filtrate was then washed with aqueous $Na_2S_2O_3$ (2 × 20 mL, 15%) followed by water (20 mL). The organic phase was dried ($MgSO_4$) and concentrated *in vacuo*. The crude mixture was purified by a silica gel chromatography (25% ethyl acetate in hexane) to afford **12** (0.74 g, 76%, yield based on acceptor) as a white solid. R_f = 0.55 (30% ethyl acetate in hexane); $[a]^{25}{}_D$ = −19.28 (*c* = 1, $CHCl_3$). ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.70–7.07 (m, 24H, aromatic), 5.89 (t, 1H, $J_{3',2'}$ = 9.6 Hz, $J_{3',4'}$ = 10.2 Hz, H-3'), 5.57–5.56 (d, 2H, $J_{1',2'}$ = 9.0 Hz, H-1', >C*H*Ph), 4.49–4.39 (m, 5H, H-3_L, H-4 CH_2Ph, H-6'a), 4.38 (t, 1H, $J_{2',3'}$ = 9.6 Hz, $J_{1',2'}$ = 9.0 Hz, H-2'), 4.27–4.24 (m, 2H, H-1, H-3_L CH_2Ph), 4.06 (bd, 1H, H-6a), 3.87–3.79 (m, 4H, H- 6'b, H-4', H-1_L), 3.73–3.66 (m, 2H, H-5', H-6b), 3.47–3.45 (m, 1H, H-3_L), 3.33 (m, 1H, H-5), 3.19–3.18 (m, 2H, H-4, H-3), 3.12 (m, 1H, H-2), 1.91 (s, 3H, COCH_3), 1.79–1.76 (m, 2H, H-2_L), 1.51–1.42 (m, 2H, H-4_L), 1.32–1.24 [m, 22H, H-(4_L–15_L)], 0.86 (t, 3H, H-16_L). ^{13}C NMR (300 MHz, $CDCl_3$): δ = 170.41 (*C*=O), 123.80–139.11 (aromatic), 101.92 (*C*HPh), 98.79 (C-1'), 85.79 (C- 1), 85.39 (C-3), 79.58 (C-4'), 78.44 (C-4, C-5), 76.39 (C-3_L), 75.04 (C-3L *C*H_2Ph,C-4 *C*H_2Ph), 71.10 (C-6'), 71.05 (C-1_L), 70.09 (C-3'), 68.94 (C-6), 68.56 (C-5'), 66.56 (C-2), 55.49 (C-2'), 35.06 (C-2_L), 34.32 (C-4_L), 32.17–22.94 (C-5_L–15_L), 20.83 (CO*C*H_3), 14.38 (C- 16_L). HRMS (*m*/*z*) for $C_{65}H_{78}N_4O_{12}S$[M + Na]$^+$: calcd 1161.5337, found 1161.741.

Phenyl 2-azido-4-*O*-benzyl-6-*O*-(4,6-*O*-benzylidene-2-deoxy-2-[(*R*)-3-octacosanoyloxy-hexadecan]amido-β-D-glucopyranosyl)-3-*O*-[(*R*)-3-benzyloxy-hexadecanoyl]-2-deoxy-1-thio-β-D-glucopyranoside (13). To compound **12** (0.73 g, 0.64 mmol) in ethanol (20 mL) was

added hydrazine hydrate (1.5 mL). The reaction mixture was heated under reflux at 90 °C for 5 h, after which TLC analysis showed complete consumption of starting material. The reaction mixture was cooled to room temperature and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (5% MeOH in DCM) to afford free amine (0.59 g, 95% yield) as a white solid. R_f = 0.25 (2% methanol in DCM); $[a]^{25}{}_D$ = −23.76 (c = 1, $CHCl_3$). ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.57–7.30 (m, 20H, aromatic), 5.53 (s, 1H, >C*H*Ph), 4.81 (d, 1H, $J_{a,b}$ = 10.8 Hz, H-3$_L$ C*H*$_a$H$_b$Ph), 4.56 (d, 1H, $J_{a,b}$ = 11.4 Hz, H-3$_L$ CH$_a$*H*$_b$Ph), 4.51–4.46 (dd, 2H, J = 11.4 Hz, H-4 C*H*$_2$Ph), 4.44 (d, 1H, $J_{1,2}$ = 10.2 Hz, H-1'), 4.32–4.30 (dd, 1H, $J_{5',6a'}$ = 4.8 Hz, $J_{6a',6b'}$ = 10.2 Hz, H-6a'), 4.26 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1), 4.09 (bd, 1H, H-6a), 3.96–3.91 (m, 2H, H-1$_L$), 3.76 (t, 1H, $J_{5',6b'}$ = 10.2 Hz, $J_{6b',6a'}$ = 10.2 Hz, H-6b'), 3.63–3.48 (m, 5H, H-6b, H-3, H-4, H-3$_L$, H-5), 3.39 (m, 1H, H-5'), 3.32–3.24 (m, 3H, H-4', H-3', H-2'), 2.76 (t, 1H, $J_{2,3}$ = $J_{1,2}$ = 8.4 Hz, H-2), 1.86 (m, 2H, H-2$_L$), 1.47–1.55 (m, 2H, H-4$_L$), 1.34–1.24 [m, 22H, H-(5$_L$–15$_L$)], 0.88 (t, 3H, H-16$_L$); ^{13}C NMR (300 MHz, $CDCl_3$): δ = 126.49–139.10 (aromatic), 105.12 (C-1'), 102.15 (>*C*HPh), 86.17 (C-1), 85.70 (C-4'), 81.58 (C-3), 79.05 (C-3$_L$), 78.16 (C-3), 76.43 (C-5), 75.19 (C-3$_L$ *C*H$_2$Ph), 73.49 (C-4), 71.20 (C-1$_L$), 71.09 (C-4 *C*H$_2$Ph), 69.09 (C-6), 68.95 (C-6'), 66.66 (C-5'), 65.42 (C-2'), 58.22 (C-2), 35.09 (C-2$_L$), 34.31 (C-4$_L$), 32.15–22.92 (C 5$_L$–15$_L$), 14.36 (C-16$_L$); HRMS (*m*/*z*) for $C_{55}H_{74}N_4O_9S[M + Na]^+$: calcd 989.5177, found 989.6476. Lipid **7** (0.26 g, 0.39 mmol) was dissolved in DCM (10 mL) and DCC (92.5 mg, 0.45 mmol) was added. The mixture was stirred for 20 min and then the above free amine was added (0.28 g, 0.29 mmol). The reaction mixture was stirred for 16 h at room temperature after which TLC analysis indicated completion of the reaction. The urea was filtered off over a pad of Celite and the organic solvent was concentrated *in vacuo*. The residue was purified by silica gel column chromatography (15% ethyl acetate in toluene) to afford compound **13** (0.43 g, 89%) as a white

solid. R_f = 0.65 (30% ethyl acetate in toluene); $[a]^{25}{}_D$ = −15.96 (c = 1, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.57–7.26 (20H, m, aromatic), 6.05 (d, 1H, $J_{NH',2'}$ = 5.5 Hz, N*H'*), 5.57 (s, 1H, >C*H*Ph), 5.07–5.05 (m, 1H, H-3_{LL}), 4.79 (d, 1H, $J_{a,b}$ = 11.0 Hz, H-3_L CH_aH$_b$Ph), 4.75 (d, 1H, $J_{1',2'}$ = 8.5 Hz, H-1'), 4.54–4.46 (m, 4H, H-3_L CH$_a$*H*$_b$Ph, H-1, C-4 CH_2Ph), 4.36–4.33 (dd, 1H, $J_{5',6a'}$ = 5.0 Hz, $J_{6a',6b'}$ = 10.5 Hz, H-6a'), 4.13–4.11 (bd, 1H, H-6a), 4.08 (t, 1H, H-3'), 3.98–3.91 (m, 2H, H-1_L), 3.79 (t, 1H, $J_{5',6a'}$ = 10.5 Hz, $J_{6b',6a'}$ = 10.5 Hz, H-6b'), 3.63–3.56 (m, 3H, H-6b, H-5, H-4'), 3.53–3.44 (m, 3H, H-3_L, H-2', H-5'), 3.36 (t, 1H, $J_{2,3}$ = $J_{3,4}$ = 9.0 Hz, H-3), 3.26–3.21 (m, 2H, H-4, H-2), 2.25–2.31 (m, 4H, H-2_{LL}, H-$2_{LL'}$), 1.89–1.85 (dd, H, H-2_L), 1.60–1.46 (m, 6H, H-4_L, H-4_{LL}, H-$3_{LL'}$), 1.41–1.15 [m, 92H, H-(5_L–15_L, 5_{LL}–15_{LL}, $4_{LL'}$–$27_{LL'}$)], 0.89 (t, 9H, 16_L, 16_{LL}, $28_{LL'}$); ^{13}C NMR (300 MHz, $CDCl_3$): δ = 174.39, 172.03 (*C*=O), 126.60–139.08 (aromatic), 102.19 (*C*HPh), 101.41 (C-1'), 85.63 (C-1), 85.51 (C-3), 81.72 (C-4'), 79.09 (C-5), 78.45 (C-4), 76.41 (C-3_L), 75.24 (C-3_L *C*H$_2$Ph), 71.97 (C-3'), 71.62 (C-3_{LL}), 71.24 (C-1_L), 71.08 (C-4 *C*H$_2$Ph), 69.14 (C-6), 68.83 (C-6'), 66.67 (C-5), 65.09 (C-2), 59.48 (C-2'), 42.58 (C-2_{LL}, $2_{LL'}$), 35.08 (C-2_L), 34.30–34.79 (C-4_L, C-4_{LL}, C-$3_{LL'}$), 32.16–22.92 [C-(5_L–15_L, 5_{LL}–15_{LL}, $4_{LL'}$–$27_{LL'}$], 14.36 (16_L, 16_{LL}, $28_{LL'}$); HRMS (*m/z*) for $C_{99}H_{158}N_4O_{12}S[M + Na]^+$: calcd 1650.1597, found 1650.2234.

Phenyl 4-*O*-benzyl-6-*O*-(4,6-*O*-benzylidene-3-*O*-[(*R*)-3-benzyloxy-hexadecanoyl]-2-deoxy-2-[(*R*)-3-octacosanoyloxy-hexadecan]-amido-β-D-glucopyranosyl)-2-[(*R*)-3-benzyloxy-hexadecan]-amido-3-*O*-[(*R*)-3-benzyloxy-hexadecanoyl]-2-deoxy-1-thio-β-D-glucopyranoside (14). Compound **13** (0.26 g, 0.16 mmol) was dissolved in DCM (10 mL) and zinc dust (0.11 g, 1.67 mmol) was added followed by acetic acid (100 µL, 1.75 mmol). The reaction mixture was stirred for 2 h at room temperature after which TLC analysis showed completion of the reaction. The reaction mixture was washed with $NaHCO_3$ (10 mL), water (10

mL) and the organic layer was dried ($MgSO_4$) and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (2% MeOH in DCM) to afford a free amine (0.22 g, 84%) as a white solid. R_f = 0.45 (2% MeOH in DCM); $[a]^{25}{}_D$ = −9.24 (c = 1, $CHCl_3$). ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.56–7.28 (m, 20H, aromatic), 6.05 (d, 1H, $J_{NH',2'}$ = 6.6 Hz, N*H'*), 5.57 (s, 1H, >C*H*Ph), 5.11–5.09 (m, 1H, H-3_{LL}), 4.78 (d, 1H, $J_{a,b}$ = 11.0 Hz, H-3_L-C*H*$_a$H$_b$Ph), 4.74 (d, 1H, $J_{1',2'}$ = 8.5 Hz, H-1'), 4.56–4.44 (m, 4H, H-3_L-CH$_a$*H*$_b$Ph, H-1, H-4 C*H*$_2$Ph), 4.35–4.32 (dd, 1H, $J_{6a',5'}$ = 5.4 Hz, $J_{6a',6b'}$ = 12.6 Hz, H-6a'), 4.12–4.14 (m, 1H, H-6a), 4.08 (t, 1H, J = 10.8 Hz, J = 11.4 Hz, H-3'), 4.00–3.97 (m, 1H, H-1_L-C*H*$_1$H), 3.87–3.85 (m, 1H, H-1_L-CH*H*$_2$), 3.79 (t, 1H, $J_{6b',5'}$ = 12.0 Hz, $J_{6b',6a'}$ = 12.6 Hz, H-6b'), 3.66–3.61 (m, 2H, H-4, H-3_L), 3.60–3.43 (m, 4H, H-6b, H-4', H-2', H-5'), 3.32–3.24 (m, 2H, H-3, H-5), 2.75 (t, 1H, J = 10.8 Hz, J = 11.4 Hz, H-2), 2.27–2.23 [m, 4H, H-(2_{LL}, $2_{LL'}$)], 1.86–1.83 (dd, 2H, H-2_L), 1.58–1.45 [m, 6H, H-(4_L, 4_{LL}, $3_{LL'}$)], 1.39–1.14 [m, 92H, H-(5_L–15_L, 5_{LL}–15_{LL}, $4_{LL'}$–$27_{LL'}$)], 0.896 (t, 9H, H-16_L, 16_{LL}, $28_{LL'}$). ^{13}C NMR (300 MHz, $CDCl_3$): δ = 139.02–126.61 (aromatic), 102.17 (>*C*HPh), 101.48 (C-1'), 88.58 (C-1), 86.85 (C-4), 81.72 (C-4'), 79.31 (C-3), 78.98 (C-2), 76.36 (C-3_L), 75.07 (C-3_L *C*H$_2$Ph), 72.07 (C-3'), 71.49 (C-3_{LL}), 70.88 (C-1_L), 70.78 (C-4 *C*H$_2$Ph), 69.36 (C-6), 68.48 (C-6'), 66.67 (C-5'),59.52 (C-2'), 42.42 (C-2_{LL}, $2_{LL'}$), 35.04 (C-4_L), 34.13–34.78 (C-4_{LL}, $3_{LL'}$, 2_L), 22.92–32.16 (C-5_L–15_L, 5_{LL}–15_{LL}, $4_{LL'}$–$27_{LL'}$), 14.35 (16_L, 16_{LL}, $28_{LL'}$). HRMS (*m*/*z*) for $C_{99}H_{160}N_2O_{12}S[M + Na]^+$: calcd 1624.1692, found 1624.3170. Lipid **8** (0.23 g, 0.62 mmol) was dissolved in DCM (13 mL) followed by the addition of DCC (0.16 g, 0.75 mmol) and DMAP (0.046 g, 0.38 mmol). The reaction mixture was stirred for 20 min followed by addition of the above amino compound (0.20 g, 0.13 mmol). The reaction mixture was stirred for 16 h at room temperature after which TLC analysis indicated completion of the reaction. The urea was filtered off over a pad of Celite and the organic solvent was concentrated *in vacuo*. The crude product

was purified by silica gel chromatography (20% ethyl acetate in toluene) to afford compound **14** (0.25 g, 86% yield) as a white solid. R_f = 0.35 (20% ethyl acetate in toluene); $[a]^{25}{}_D$ = −34.48 (*c* = 1, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.23–7.49 (m, 30H, aromatic), 6.66 (d, 1H, $J_{NH',2'}$ = 9.0 Hz, N*H'*), 5.43 (s, 1H, >C*H*Ph), 5.37 (d, 1H, $J_{NH,2}$ = 9.0 Hz, N*H*), 5.26 (t, 1H, *J* = 9.5 Hz, *J* = 10 Hz, H-3'), 5.02–4.96 (m, 1H, H-3_L), 4.75 (d, 1H, $J_{a,b}$ = 11.0 Hz, H-3_L CH_aH$_b$Ph), 4.71 (d, 1H, $J_{1',2'}$ = 8.5 Hz, H-1'), 4.57 (d, 1H, $J_{b,a}$ = 11.0 Hz, H-3_L CH$_a$*H*$_b$Ph), 4.52–4.39 (m, 7H, H-1, H-4 C*H*$_2$Ph, H-3_{LA} × 2 C*H*$_2$Ph), 4.34–4.32 (dd, 1H, $J_{5',6a'}$ = 5.5 Hz, $J_{6a',6b'}$ = 11.0 Hz, H-6a'), 4.01 (d, 1H, $J_{a,b}$ = 11.0 Hz, H-6a), 3.86–3.62 (m, 9H, H-2, H-6b', H-3L, H-3_{LA} C*H* × 2, H-1_L, H-6b, H-2'), 3.54–3.40 (m, 5H, H-3, H-4, H-5, H-4', H-5'), 2.69–2.40 (m, 4H, H-2_{LA} × 2), 2.31–2.07 (m, 4H, 2_{LL}, $2_{LL'}$), 1.76–1.79 (m, 2H, 2_L), 1.68–1.39 [m, 10H, H-(4_L, 4_{LL}, $3_{LL'}$, 4_{LA}], 1.33–1.27 (m, 136H, 5_L–15_L, 5_{LL}–15_{LL}, $4_{LL'}$–$27_{LL'}$, 5_{LA}–15_{LA} × 2), 0.90 (t, 15H, 16_L, 16_{LL}, $28_{L'}$, 16_{LA} × 2); ^{13}C NMR (300 MHz, $CDCl_3$): δ = 174.00 (*C*=O), 171.44 (*C*=O), 171.32 (*C*=O), 169.70 (*C*=O), 139.22–126.33 (aromatic), 101.61 (>*C*HPh, C-1), 86.18 (C-1'), 83.68 (C-5), 79.79 (C-4'), 79.17 (C-4), 78.21 (C-5'), 76.35 (C-1_L), 75.82 (C-3), 74.84 (C-3_L *C*H$_2$Ph), 71.69 (C-3'), 71.42 (C-4 *C*H$_2$Ph), 71.32–70.65 (C-3_L, C-3_{LA} *C*H$_2$Ph), 70.25 (C-3_{LL}), 68.87 (C-6'), 68.56 (C-6), 55.21 (C-2), 55.02 (C-2'), 41.67, 41.53 (C-2_{LL}, $2_{LL'}$), 39.81 (C-2_{LA}), 35.09 (C-2_L), 34.80–34.29 [C-(4_{LL}, $3_{LL'}$ 4_L, 4_{LA} × 2)], 32.16–22.92 [C-(5_L–15_L, 5_{LL}–15_{LL}, $4_{LL'}$–$27_{LL'}$, 5_{LA}–15_{LA})], 14.34 (C-16_L, 16_{LL}, $28_{LL'}$, 16_{LA}); HRMS (*m*/*z*) for $C_{99}H_{158}N_4O_{12}S[M + Na]^+$: calcd 2312.7020, found 2312.8816.

4-*O*-Benzyl 6-*O*-(4,6-*O*-benzylidene-3-*O*-[(*R*)-3'-benzyloxy-hexadecanoyl]- 2-deoxy-2'-[(*R*)-3-octacosanoyloxy-hexadecan]-amido-β-D-glucopyranosyl)-2'-[(*R*)-3-benzyloxy-hexadecan]-amido-3-*O*-[(*R*)-3-benzyloxy-hexadecanoyl]-2-deoxy-α-D-glucopyranose (15). Compound **14** (0.05 g, 0.02 mmol) was dissolved in a mixture of DCM and water (3.0 mL, 100 :

1 v/v) and the resulting solution was cooled to 0 °C. NIS (0.03 g, 0.13 mmol) and TMSOTf (0.5 µL, 0.28 µmol) were added and after stirring for 30 min at 0 °C, TLC analysis indicated completion of the reaction. It was then quenched with pyridine (0.1 mL) and washed with $Na_2S_2O_3$ (8 mL, 15%) and water (8 mL). The organic layer was dried ($MgSO_4$) and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (1% MeOH in DCM) followed by size exclusion chromatography over LH-20 (MeOH–DCM, 1:1 v/v) to yield **15** (0.021 g, 44%) as a white solid. R_f = 0.40 (1% methanol–DCM); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.48–7.24 (m, 25H, aromatic), 6.51 (d, 1H, $J_{NH,2}$ = 9.5 Hz, N*H*), 5.91 (d, 1H, $J_{NH',2'}$ = 8.5 Hz, N*H'*), 5.45 (s, 1H, >C*H*Ph), 5.43–5.39 (m, 1H, H-3'), 5.16 (d, 1H, $J_{1',2'}$ = 8.5 Hz, H-1'), 5.09 (bs, 1H, H-1), 4.99–4.97 (m, 1H, H-3$_{LL}$), 4.81 (d, 1H, $J_{a,b}$ = 11 Hz, H-3$_L$ C*H*$_a$H$_b$Ph), 4.54–4.38 (m, 7H, H-4 C*H*$_2$Ph, H-3$_L$ CH$_a$*H*$_b$Ph, H-3$_{LA}$ C*H*$_2$Ph × 2), 4.38–4.34 (dd, 1H, $J_{5',6'}$ = 5.5 Hz, $J_{6a',6b'}$ = 11 Hz, H-6a'), 4.11–4.16 (m, 1H, H-2), 4.02–3.97 (m, 2H, H-1$_L$), 3.86–3.74 (m, 5H, H-6a, H-3L, H-3$_{LA}$ C*H* × 2, H-6b'), 3.68–3.62 (m, 5H, H-2', H-6b, H-5', H-4', H-3), 3.58–3.42 (m, 3H, H-5, H-4, H-1 O*H*), 2.67–2.49 (m, 4H, H-2 $_{LA}$ C*H*$_2$ × 2), 2.37–2.20 (m, 4H, H-2$_{LL}$, 2$_{LL'}$) 1.82–1.72 (m, 2H, H-2$_L$), 1.60–1.48 (10H, 4$_L$, 4$_{LL}$, 3$_{LL'}$, 4$_{LA}$C*H*$_2$ × 2), 1.39–1.27 [m, 136H, H-(5$_L$–15$_L$, 5$_{LL}$–15$_{LL}$, 4$_{LL'}$–27$_{LL'}$, 5$_{LA}$–15$_{LA}$ × 2], 0.89 (t, 15H, H-16$_L$, 16$_{LL}$, 28$_{LL'}$, 16$_{LA}$ × 2); HRMS (*m*/*z*) for $C_{139}H_{228}N_2O_{17}[M + Na]^+$: calcd 2220.6936, found 2220.9749.

4-*O*-Benzyl 6-*O*-(4,6-*O*-benzylidene-3-*O*-[(*R*)-3'-benzyloxy-hexadecanoyl]- 2-deoxy-2'-[(*R*)-3-octacosanoyloxy-hexadecan]-amido-β-D-glucopyranosyl)-2-[(*R*)-3-benzyloxy-hexadecanoyl]-amido-3-*O*-[(*R*)-3-benzyloxy-hexadecanoyl]-2-deoxy-α-D-glucono-1,5-lactone (16). Compound **15** (0.013 g, 5.92 µmol) was dissolved in DCM (2 mL) and molecular sieves (3Å, 0.02 g) were added and, after stirring the resulting suspension for 2 h under an atmosphere of argon, PCC (0.063 g, 29.6 µmol) was added. The reaction mixture was stirred for

2 h at room temperature after which TLC analysis indicated completion of the reaction. After concentration *in vacuo*, the crude product was purified by iatrobead column chromatography (20% ethyl acetate in toluene) to afford **16** (0.008 g, 62%) as a white solid. R_f = 0.60 (20% ethyl acetate in toluene); ^{1}H NMR (500 MHz, $CDCl_3$):δ = 7.48–7.24 (m, 25H, aromatic), 6.99 (d, 1H, $J_{NH,2}$ = 8.5 Hz, N*H*), 6.66 (d, 1H, $J_{NH',2'}$ = 7.5 Hz, N*H'*), 5.67 (t, 1H, *J* = 9.5 Hz, *J* = 9.0 Hz, H-3'), 5.39 (s, 1H, >C*H*Ph), 5.12 (m, 1H, H-3_{LL}), 5.00 (d, 1H, $J_{1',2'}$ = 8.0 Hz, H-1'), 4.74 (d, 1H, $J_{a,b}$ =11.0 Hz, H-3_L C$H_a$$H_b$Ph), 4.62–4.38 (m, 8H, H-2, C-4 CH_2Ph, H-3 L C$H_a$$H_b$Ph, H-3'$_{LA}$ CH_2Ph, H-2_{LA} CH_2Ph), 4.31–4.28 (dd, 1H, $J_{5',6a'}$ = 4.0 Hz, $J_{6a',6b'}$ = 10.5 Hz, H-6a'), 4.08 (d, 1H, *J* = 10.5 Hz, H-6a), 3.84–3.80 (m, 2H, H-1_L), 3.74–3.65 (m, 4H, H-6b', H-3_L, H-3_{LA} C*H* ×2), 3.63–3.47 (m, 7H, H-4', H- 5', H-6b, H-2', H-4, H-5, H-3), 2.69–2.46 (m, 4 H, H-2_{LA}, H-3'$_{LA}$), 2.42–2.24 (m, 4H, H-2_{LL}, $2_{LL'}$), 2.07–1.44 (m, 12H, H-2_L, 4_L, 4_{LL}, $3_{LL'}$, H-4_{LA}), 1.27–1.02 (m, 136H, 5_L–15_L, 5_{LL}–15_{LL}, $4_{LL'}$–$27_{LL'}$, 5_{LA}– 15_{LA}), 0.98–0.72 (t, 15H, 16_L, 16_{LL}, $28_{LL'}$, 16_{LA}); HRMS (*m*/*z*) for $C_{139}H_{226}N_2O_{17}$[M + Na]$^+$: calcd 2218.6779, found 2218.8311.

2-Deoxy-6-*O*-(2'-deoxy-3-*O*-[(*R*)-3'-benzyloxy-hexadecanoyl]-2'-[(*R*)-3-octacosanoyloxy-hexadecan]amido-β-D-glucopyranosyl)-2-[(*R*)-3-benzyloxy-hexadecan]-amido-3-*O*-[(*R*)-3-benzyloxy-hexadecanoyl]-2-deoxy-α-D-glucono-1,5-lactone (3). Pd/C (10 mg, 10 wt %) was added to compound **16** (4.5 mg, 2.05 µmol) dissolved in a mixture of THF–*t*-BuOH (2 mL, 1:1, v/v). The flask was degassed and filled with H_2 gas and then stirred for 12 h. The reaction progress was monitored by MALDI. After completion of the reaction, the catalyst was filtered off through a PTFE filter and washed with THF (0.5 mL × 3) The combined filtrates were concentrated *in vacuo* and the residue was purified by LH-20 size exclusion column chromatography (*i*-PrOH–DCM, 1 : 1, v/v) to yield compound **3** (2.3 mg, 66%) as a white solid. ^{1}H NMR (500 MHz, THF-D : $(CD_3)_2CDOD$ 1 : 1): δ = 5.12 (m, 1H, 3_{LL}), 5.02 (t, 1H, *J* = 10.0

Hz, J = 9.0 Hz, H-3'), 4.67 (d, 1H, $J_{1',2'}$ = 9.0 Hz, H-1'), 4.21 (m, 1H, H-2), 4.16 (dd, 1H, $J_{5',6'}$ = 2.0 Hz, $J_{6a',6b'}$ = 11.5 Hz, H-6a'), 4.05 (m, 1H, H-6a), 3.94 (m, 2H, H-1_L, 3.86–3.45 (m, 11H, H-6b', H-2', 3_L, H-3_{LA} × 2 C*H*, H-6b, H-5', H-4', H-3, H-4, H-5), 2.46–2.18 (m, 14H, H-2_{LA}, 2_{LL}, $2_{LL'}$, 2_L, 4_L), 2.06–1.94 (m, 6H, H-4_{LL}, $3_{LL'}$, 4_{LA}), 1.84–1.27 (bm, 136H, 5_L–15_L, 5_{LL}–15_{LL}, $4_{LL'}$–$27_{LL'}$, H-5_{LA}–15_{LA}), 1.65–0.67 (bm, 15H, 16_L, 16_{LL}, $28_{LL'}$, 16_{LA}); HRMS (*m*/*z*) for $C_{104}H_{198}N_2O_{17}[M + Na]^+$: calcd 1770.4588, found 1770.7673.

Reagents for Biological Experiments: *E. coli* 055:B5 LPS was obtained from List Biologicals, Pam_3CysSK_4 was obtained from Calbiochem and *R. sin*-1 LPS and lipid A were kindly provided by Dr R. Carlson (CCRC, Athens, GA). All data presented in this study were generated using the same batches of *E. coli* 055:B5 LPS and *R. sin*-1 LPS. The synthesis of *E. coli* lipid A has been reported elsewhere.[46] The *E. coli* lipid A was reconstituted in PBS with DMSO (10%) and stored at –80 °C. Synthetic compounds **1** and **3** were stored lyophilized at –80 °C and reconstituted in dry THF on the day of the experiment; final concentrations of THF in the biological experiments never exceeded 0.5% to avoid toxic effects.

Cell Maintenance: Mono Mac 6 (MM6) cells, provided by Dr H.W.L. Ziegler-Heitbrock (Institute for Inhalation Biology, Munich, Germany), were cultured in RPMI 1640 medium with L-glutamine (BioWhittaker) supplemented with penicillin (100 u mL^{-1})–streptomycin (100 µg mL^{-1}; Mediatech, OPI supplement (1%; Sigma; containing oxaloacetate, pyruvate and bovine insulin) and fetal calf serum (FCS; 10%; HyClone). New batches of frozen cell stock were grown up every 2 months and growth morphology evaluated. Before each experiment, MM6 cells were incubated with calcitriol (10 ng mL^{-1}; Sigma) for 2 days to differentiate into macrophage like cells. RAW 264.7 γNO(–) cells, derived from the RAW 264.7 mouse monocyte–macrophage cell line, were obtained from ATCC. The cells were maintained in RPMI 1640 medium (ATCC) with

L-glutamine (2 mM), adjusted to contain sodium bicarbonate (1.5 g L^{-1}), glucose (4.5 g L^{-1}), HEPES (10 mM) and sodium pyruvate (1.0 mM) and supplemented with penicillin (100 u mL^{-1}) streptomycin (100 µg mL^{-1}) and FBS (10%). Human embryonic kidney (HEK) 293T cells were grown in Dulbecco's modified Eagle's medium (ATCC) with L-glutamine (4 mM), glucose (4.5 g L^{-1}) and sodium bicarbonate (1.5 g L^{-1}) supplemented with penicillin (100 u mL^{-1}) streptomycin (100 µg mL^{-1}), Normocin (100 µg mL^{-1}; InvivoGen) and FBS (10%). Stably transfected HEK 293T cells with murine TLR4/MD2 and murine TLR2/TLR6 were obtained from InvivoGen and grown in the same growth medium as for HEK 293T cells supplemented with the appropriate selective agents HygroGold (50 µg mL^{-1}; InvivoGen) and blasticidin (10 µg mL^{-1}; InvivoGen). All cells were maintained in a humid 5% CO_2 atmosphere at 37 °C.

Cytokine Induction and ELISAs: On the day of the exposure assay differentiated MM6 cells were harvested by centrifugation and gently suspended (106 cells mL^{-1}) in prewarmed (37 °C) medium and RAW 264.7 γNO(–) cells were plated as 2×10^5 cells per well in 96-well tissue culture plates (Nunc). Cells were then incubated with different combinations of stimuli for 5.5 hours. Culture supernatants were then collected and stored frozen (–80 °C) until assayed for cytokine production. All cytokine ELISAs were performed in 96-well MaxiSorp plates (Nalge Nunc International). Concentrations of human TNF-α protein in culture supernatants were determined by a solid phase sandwich ELISA. Plates were coated with purified mouse anti-human TNF-α antibody (Pharmingen). TNF-α in standards and samples was allowed to bind to the immobilized antibody. Biotinylated mouse anti-human TNF-α antibody (Pharmingen) was then added, producing an antibody–antigen–antibody "sandwich". After addition of avidin–horseradish peroxidase conjugate (Pharmingen) and ABTS peroxidase substrate (Kirkegaard & Perry Laboratories), a green color was produced in direct proportion to the amount of TNF-α

present in the sample. The reaction was stopped by adding peroxidase stop solution (Kirkegaard & Perry Laboratories) and the absorbance was measured at 405 nm using a microplate reader (BMG Labtech). Cytokine DuoSet ELISA Development Kits (R & D Systems) were used for the cytokine quantification of human IP-10 and mouse TNF-α according to the manufacturer's instructions. The absorbance was measured at 450 nm with wavelength correction set to 540 nm. Concentrations of mouse IFN-β in culture supernatants were determined as follows. Plates were coated with rabbit polyclonal antibody against mouse IFN-β (PBL Biomedical Laboratories). IFN-β in standards and samples was allowed to bind to the immobilized antibody. Rat anti-mouse IFN-β antibody (USBiological) was then added, producing an antibody-antigen-antibody "sandwich". Next, horseradish peroxidase (HRP) conjugated goat anti-rat IgG (H + L) antibody (Pierce) and a chromogenic substrate for HRP 3,3',5,5'-tetramethylbenzidine (TMB; Pierce) were added. After the reaction was stopped, the absorbance was measured at 450 nm with wavelength correction set to 540 nm. All cytokine values are presented as the means ± SD of triplicate measurements, with each experiment being repeated three times.

Transfection and NF-κB Activation Assay: The day before transfection, HEK293T wild type cells and HEK 293T cells stably transfected with murine TLR2/TLR6 or murine TLR4/MD2 were plated in 96-well tissue culture plates (16000 cells per well). The next day, cells were transiently transfected using PolyFect TransfectionReagent (Qiagen) with expression plasmids pELAMLuc (NF-κB-dependent firefly luciferase reporter plasmid, 50 ng per well)[47] and pRL-TK (*Renilla* luciferase control reporter vector, 1 ng per well; Promega) as an internal control to normalize experimental variations. The empty vector pcDNA3 (Invitrogen) was used as a control and to normalize the DNA concentration for all of the transfection reactions (total DNA 70 ng per well). Fortyfour h post-transfection, cells were exposed to the stimuli in the presence of FCS

to provide sCD14 at the indicated concentrations for 4 h, after which cell extracts were prepared. The luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions and a combination luminometer–fluorometer microplate reader (BMG Labtech). Expression of the firefly luciferase reporter gene was normalized for transfection efficiency with expression of *Renilla* luciferase. The data are reported as the means ± SD of triplicate treatments. The transfection experiments were repeated at least twice.

Data Analysis: Concentration–response and inhibition data were analyzed using nonlinear least-squares curve fitting in Prism (GraphPad Software, Inc.). Concentration–response data were fit with the following four parameter logistic equation: $Y = E_{max}/(1 + (EC_{50}/X)^{Hill\ slope})$, where Y is the cytokine response, X is logarithm of the concentration of the stimulus, Emax is themaximumresponse and EC_{50} is the concentration of the stimulus producing 50% stimulation. The Hillslope was set at 1 to be able to compare the EC_{50} values of the different inducers. Inhibition data were fit with the following logistic equation: $Y = \text{Bottom} + (\text{Top} - \text{Bottom})/(1 + 10^{(X - \log IC50)})$, where Y is the cytokine response, X is the logarithm of the concentration of the inhibitor and IC_{50} is the concentration of the inhibitor that reduces the response by half.

References

(1) Beutler, B. *Mol. Immunol.* **2004**, *40*, 845-859.

(2) Van Amersfoort, E. S.; Van Berkel, T. J.; Kuiper, J. *Clin. Microbiol. Rev.* **2003**, *16*, 379-414.

(3) Akira, S.; Takeda, K. *Nat. Rev. Immunol.* **2004**, *4*, 499-511.

(4) Takeda, K.; Kaisho, T.; Akira, S. *Annu. Rev. Immunol.* **2003**, *21*, 335-376.

(5) McGettrick, A. F.; O'Neill, L. A. *Mol. Immunol.* **2004**, *41*, 577-582.

(6) Yamamoto, M.; Sato, S.; Hemmi, H.; Hoshino, K.; Kaisho, T.; Sanjo, H.; Takeuchi, O.; Sugiyama, M.; Okabe, M.; Takeda, K.; Akira, S. *Science* **2003**, *301*, 640-643.

(7) Roberts, F. J.; Geere, I. W.; Coldman, A. *Rev. Infect. Dis.* **1991**, *13*, 34-46.

(8) Pittet, D.; Tarara, D.; Wenzel, R. P. *JAMA* **1994**, *271*, 1598-1601.

(9) Pollack, M.; Ohl, C. A. *Curr. Top. Microbiol. Immunol.* **1996**, *216*, 275-297.

(10) Golenbock, D. T.; Hampton, R. Y.; Qureshi, N.; Takayama, K.; Raetz, C. R. *J. Biol. Chem.* **1991**, *266*, 19490-19498.

(11) Peri, F.; Marinzi, C.; Barath, M.; Granucci, F.; Urbano, M.; Nicotra, F. *Bioorg. Med. Chem.* **2006**, *14*, 190-199.

(12) Martin, O. R.; Zhou, W.; Wu, X.; Front-Deschamps, S.; Moutel, S.; Schindl, K.; Jeandet, P.; Zbaeren, C.; Bauer, J. A. *J. Med. Chem.* **2006**, *49*, 6000-6014.

(13) Krauss, J. H.; Seydel, U.; Weckesser, J.; Mayer, H. *Eur. J. Biochem.* **1989**, *180*, 519-526.

(14) Christ, W. J.; McGuinness, P. D.; Asano, O.; Wang, Y.; Mullarkey, M. A.; Perez, M.; Hawkins, L. D.; Blythe, T. A.; Dubuc, G. R.; Robidoux, A. L. *J. Am. Chem. Soc.* **1994**, *116*, 3637-3638.

(15) Shiozaki, M.; Watanabe, Y.; Iwano, Y.; Kaneko, T.; Doi, H.; Tanaka, D.; Shimozato, T.; Kurakata, S. *Tetrahedron* **2005**, *61*, 5101-5122.

(16) Rossignol, D. P.; Hawkins, L. D.; Christ, W. J.; Kobayashi, S.; Kawata, T.; Lynn, M.; Yamatsu, I.; kishi, Y.; Vol. 1, p 699-717.

(17) Vandenplas, M. L.; Carlson, R. W.; Jeyaretnam, B. S.; McNeill, B.; Barton, M. H.; Norton, N.; Murray, T. F.; Moore, J. N. *J. Biol. Chem.* **2002**, *277*, 41811-41816.

(18) Demchenko, A. V.; Wolfert, M. A.; Santhanam, B.; Moore, J. N.; Boons, G. J. *J. Am. Chem. Soc.* **2003**, *125*, 6103-6112.

(19) Girard, R.; Pedron, T.; Uematsu, S.; Balloy, V.; Chignard, M.; Akira, S.; Chaby, R. *J. Cell Sci.* **2003**, *116*, 293-302.

(20) Pedron, T.; Girard, R.; Jeyaretnam, B.; Carlson, R. W.; Chaby, R. *Immunology* **2000**, *101*, 262-270.

(21) Jeyaretnam, B.; Glushka, J.; Kolli, V. S.; Carlson, R. W. *J. Biol. Chem.* **2002**, *277*, 41802-41810.

(22) Santhanam, B.; Wolfert, M. A.; Moore, J. N.; Boons, G. J. *Chem. Eur. J.* **2004**, *10*, 4798-4807.

(23) Lee, H. S.; Wolfert, M. A.; Zhang, Y.; Boons, G. J. *ChemBioChem* **2006**, *7*, 140-148.

(24) Watanabe, Y.; Miura, K.; Shiozaki, M.; Kanai, S.; Kurakata, S.; Nishijima, M. *Carbohydr. Res.* **2003**, *338*, 47-54.

(25) Shiozaki, M.; Kobayashi, Y.; Arai, M.; Ishida, N.; Hiraoka, T.; Nishijima, M.; Kuge, S.; Otsuka, T.; Akamatsu, Y. *Carbohydr. Res.* **1991**, *222*, 69-82.

(26) Watanabe, Y.; Miura, K.; Shiozaki, M.; Kanai, S.; Kurakata, S.; Nishijima, M. *Carbohydr. Res.* **2001**, *332*, 257-277.

(27) Garegg, P. J. *Adv. Carbohydr. Chem. Biochem.* **1997**, *52*, 179-205.

(28) Boons, G. J. *Tetrahedron* **1996**, *52*, 1095-1121.

(29) Weidmann, H.; Zimmerman, H. K. *Justus Liebigs Ann. Chem.* **1959**, *620*, 4-7.

(30) Fukase, K.; Fukase, Y.; Oikawa, M.; Liu, W. C.; Suda, Y.; Kusumoto, S. *Tetrahedron* **1998**, *54*, 4033-4050.

(31) Mehta, S.; Pinto, B. M. *J. Org. Chem.* **1993**, *58*, 3269-3276.

(32) Hatakeyama, S.; Mori, H.; Kitano, K.; Yamada, H.; Nishizawa, M. *Tetrahedron Lett.* **1994**, *35*, 4367-4370.

(33) Keegan, D. S.; Hagen, S. R.; Johnson, D. A. *Tetrahedron-Asymmetry* **1996**, *7*, 3559-3564.

(34) Zuurmond, H. M.; Vandermarel, G. A.; Vanboom, J. H. *Recl. Trav. Chim. Pays-Bas* **1991**, *110*, 301-302.

(35) Mehta, S.; Pinto, B. M. *Tetrahedron Lett.* **1991**, *32*, 4435-4438.

(36) Kurihara, S.; Tsumuraya, T.; Fujii, I. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1179-1184.

(37) Veeneman, G. H.; Vanleeuwen, S. H.; Vanboom, J. H. *Tetrahedron Lett.* **1990**, *31*, 1331-1334.

(38) Zughaier, S. M.; Tzeng, Y. L.; Zimmer, S. M.; Datta, A.; Carlson, R. W.; Stephens, D. S. *Infect. Immun.* **2004**, *72*, 371-380.

(39) Zughaier, S. M.; Zimmer, S. M.; Datta, A.; Carlson, R. W.; Stephens, D. S. *Infect. Immun.* **2005**, *73*, 2940-2950.

(40) Netea, M. G.; Kullberg, B. J.; Joosten, L. A. B.; Sprong, T.; Verschueren, I.; Boerman, O. C.; Amiot, F.; van den Berg, W. B.; Van der Meer, J. W. M. *Eur. J. Immunol.* **2001**, *31*, 2529-2538.

(41) Mathiak, G.; Kabir, K.; Grass, G.; Keller, H.; Steinringer, E.; Minor, T.; Rangger, C.; Neville, L. F. *Int. J. Mol. Med.* **2003**, *11*, 41-44.

(42) van der Ley, P.; Steeghs, L.; Hamstra, H. J.; ten Hove, J.; Zomer, B.; van Alphen, L. *Infect. Immun.* **2001**, *69*, 5981-5990.

(43) Werts, C.; Tapping, R. I.; Mathison, J. C.; Chuang, T. H.; Kravchenko, V.; Saint Girons, I.; Haake, D. A.; Godowski, P. J.; Hayashi, F.; Ozinsky, A.; Underhill, D. M.; Kirschning, C. J.; Wagner, H.; Aderem, A.; Tobias, P. S.; Ulevitch, R. J. *Nat. Immunol.* **2001**, *2*, 346-352.

(44) Hirschfeld, M.; Weis, J. J.; Toshchakov, V.; Salkowski, C. A.; Cody, M. J.; Ward, D. C.; Qureshi, N.; Michalek, S. M.; Vogel, S. N. *Infect. Immun.* **2001**, *69*, 1477-1482.

(45) Aliprantis, A. O.; Yang, R. B.; Mark, M. R.; Suggett, S.; Devaux, B.; Radolf, J. D.; Klimpel, G. R.; Godowski, P.; Zychlinsky, A. *Science* **1999**, *285*, 736-739.

(46) Zhang, Y.; Gaekwad, J.; Wolfert, M. A.; Boons, G. J. *J. Am. Chem. Soc.* **2007**, *129*, 5200-5216.

(47) Chow, J. C.; Young, D. W.; Golenbock, D. T.; Christ, W. J.; Gusovsky, F. *J. Biol. Chem.* **1999**, *274*, 10689-10692.

CHAPTER 3

CHEMICAL SYNTHESIS AND IMMUNOLOGICAL PROPERTIES OF OLIGOSACCHARIDES DERIVED FROM THE VEGETATIVE CELL WALL OF *BACILLUS ANTHRACIS**

* Mahalakshmi Vasan, Jana Rauvolfova, Margreet A. Wolfert, Christine Leoff, Elmar L. Kannenberg, Conrad P. Quinn, Russell W. Carlson, Geert-Jan Boons. **2008**. *ChemBioChem.* 9, 1716 – 1720.
Reprinted here with permission of publisher.

Abstract

Sera from rabbits exposed either to live and irradiation-killed spores of *Bacillus anthracis* Sterne 34F2 or immunized with *B. anthracis* polysaccharide conjugated to keyhole limpet hemocyanin (KLH) were found to contain antibodies that recognized isolated polysaccharide (shown in scheme) and two synthetic trisaccharides. This provides proof-of-concept towards the development of vegetative and spore specific reagents for detection and targeting of nonprotein structures of *B. anthracis*.

Introduction

Bacillus anthracis is a Gram-positive, spore-forming bacterium that causes anthrax in humans and other mammals.[1,2] The relative ease by which *B. anthracis* can be weaponized and the difficulty associated with the early recognition of inhalation anthrax due to the unspecific nature of its symptoms were underscored by the deaths of five people who inhaled spores from contaminated mail.[3-5] As a result, there is a renewed interest in anthrax vaccines and early-disease diagnostics.[6] Anthrax vaccine adsorbed (AVA; BioThrax6, Emergent BioSolutions, Inc.) is currently the only licensed anthrax vaccine in the US.[7,8] The principal immunogen of AVA is anthrax toxin protective antigen (PA). Antibody responses against PA target and block the toxemia that is a necessary prerequisite of vegetative cell growth and bacteremia. Vaccines comprising additional *B. anthracis* specific antigens have been proposed as improvements to PA-only formulations as they have the potential to target inclusively the toxemia and the vegetative cell or infectious spore.[9-11] Recently described polysaccharides and glycoproteins of *B. anthracis* offer exciting new targets for these vaccine formulations and also for the development of improved diagnostics for *B. anthracis*.

For example, an unusual oligosaccharide derived from the collagen-like glycoprotein Bc1A of the exosporium of *B. anthracis* has been characterized,[12] chemically synthesized,[13-18] and immuno logically evaluated. The latter studies demonstrated that the oligosaccharide is exposed to the immune system[14] and has the ability to elicit relevant antibodies.[13] Recently, we reported the structure of a unique polysaccharide released from the vegetative cell wall of *B. anthracis*, which contains a →6)-α-D-Glc*N*Ac-(1→4)-β-D-Man*N*Ac-(1→4)-β-D-Glc*N*Ac-(1→) backbone and is branched at C-3 and C-4 of α-D-Glc*N*Ac with α-D-Gal and β-D-Gal residues, respectively, and the β-Glc*N*Ac substituted with α-Gal at C-3 (**Figure 3.1**).[19,20] These positions are, however, only partially substituted and this leads to microheterogeneity.

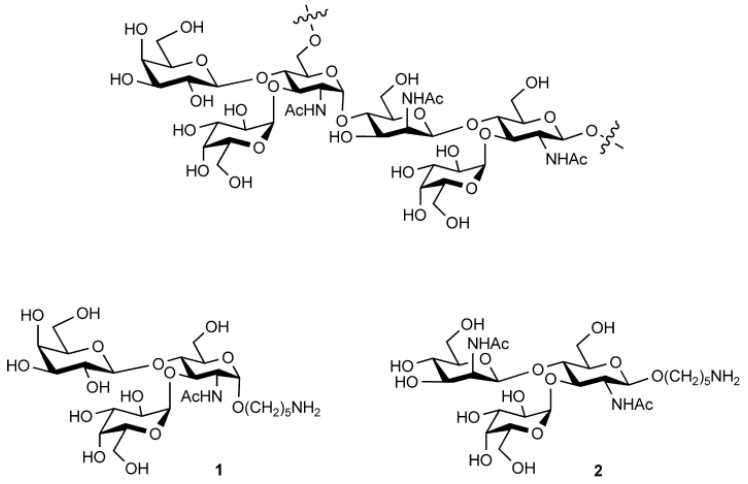


Figure 3.1. Structure of the secondary cell wall polysaccharide of *B. anthracis* and synthetic compounds **1** and **2**.

As part of a project to determine antigenic determinates of the polysaccharide of *B. anthracis* and to establish it as a diagnostic or vaccine candidate, we report here the chemical synthesis and immunologic al properties of trisaccharides **1** and **2** (**Scheme 1**). These compounds, which are derived from *B. anthracis* polysaccharide, contain a 5-aminopentyl spacer for selective conjugation to carrier proteins required for enzyme linked immunosorbent assays (ELISA). It has been found that sera from rabbits either exposed to live and irradiation-killed

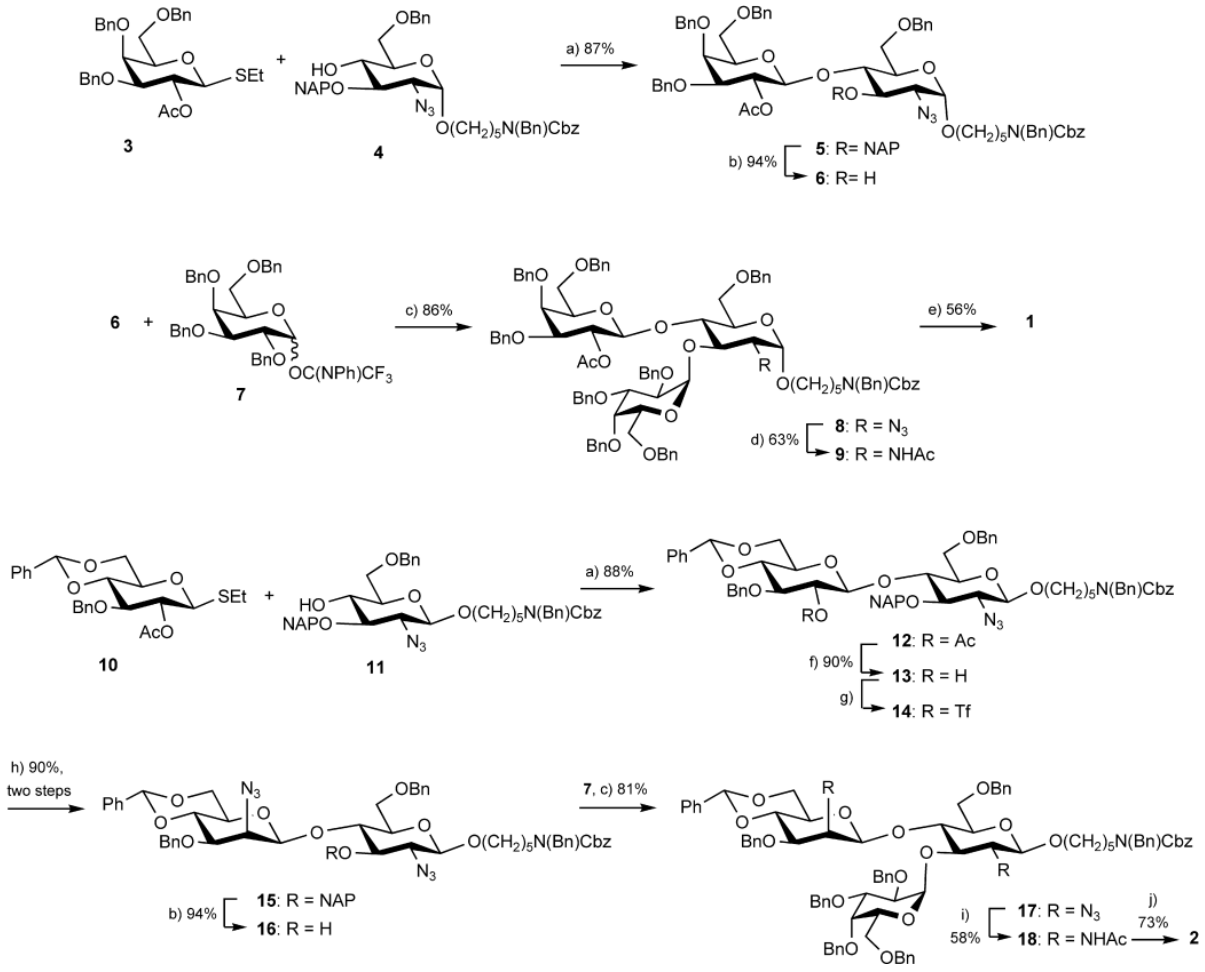
spores of *B. anthracis* Sterne 34F2 or immunized with polysaccharide conjugated to keyhole limpet hemocyanin (KLH) recognize the isolated polysaccharide and synthetic compounds **1** and **2**. The data provide proof-of-concept for the development of vegetative and spore- specific reagents for detection and targeting of nonprotein structures of *B. anthracis*.

Result and Discussion

Chemical Synthesis

Compound **1** was conveniently prepared from monosaccharide building blocks **3**,[21] **4**, and **7**.[22] Thus, a NIS/TMSOTf mediated glycosylation[23] of thioglycoside **3** with the C-4 hydroxyl of glycosyl acceptor **4** gave disaccharide **5** in a yield of 87% as only the β-anomer (**Scheme 3.1**). Interestingly, a lower yield of disaccharide was obtained when a glycosyl acceptor was employed that had a benzyloxycarbonyl-3-aminopropyl instead of a N-benzyl-N-benzyloxycarbonyl-5-aminopropyl spacer.[24] Next, the 2-naphthylmethyl ether[25,26] of **5** was removed by oxidation with DDQ in a mixture of dichloromethane and water to give glycosyl acceptor **6**, which was used in a TMSOTf mediated glycosylation with (N-phenyl) trifluoracetimidate (**7**)[27-29] to afford trisaccharide **8** in excellent yield as only the α-anomer. The use of a conventional trichloroacetimidate as glycosyl donor[30] led to a lower yield of product due to partial rearrangement to the corresponding anomeric amide. Target compound **1** was obtained by a three-step deprotection procedure that involved reduction of the azide to an acetamido moiety by treatment with $Zn/CuSO_4$[31] in a mixture of acetic anhydride, acetic acid, and THF, followed by saponification of the acetyl ester and reductive removal of benzyl ethers and benzyloxycarbamate by catalytic hydrogenation over Pd.

A challenging aspect of the preparation of target compound **2** is the installment of a β-mannosamine moiety.[27] A strategy was adopted in which a β-glucoside is initially installed by using a glucosyl donor that has a participating ester-protecting group at C-2 to control β-anomeric selectivity.[32] Next, the C-2 protecting group can be removed and



Scheme 3.1. *Reagents and conditions*: a) NIS/TMSOTf, DCM, 0 °C; b) DDQ, DCM, H_2O; c) TMSOTf, DCM, Et_2O, 50 °C; d) Zn/$CuSO_4$, AcOH, Ac_2O, THF; e) NaOMe, MeOH then $Pd(OH)_2$/C, H_2, AcOH, *t*BuOH, H_2O; f) NaOMe, MeOH; g) Tf_2O, pyridine, DCM, 0 °C; h) NaN_3, DMF, 50 °C; i) PMe_3, THF, H_2O then Ac_2O, pyridine; j) $Pd(OH)_2$/C, H_2, AcOH, *t*BuOH, H_2O.

the resulting hydroxyl triflated, which can then be displaced by an azide to give a 2-azido-β-D-mannoside. Another strategic aspect of the synthesis of **2** was the use of an acetyl ester and 2-naphtylmethyl ether[25,26] as a set of orthogonal-protecting groups, it possible to selectively modify

C-2' of the β-glucoside and install an α-galactoside at C-3 of 2-azido-glucoside moiety. Thus, a NIS/TMSOTf mediated glycosylation[23] of thioglycoside **10**[33] with **11** gave disaccharide **12** in excellent yieldas only the β-anomer. The acetyl ester of **12** was saponified by treatment with sodium methoxide in methanol to give **13**. Next, the alcohol of **13** was triflated by treatment with triflic anhydride in a mixture of pyridine and dichloromethane to afford triflate **14**, which was immediately displaced with sodium azide in DMF at 50°C to give mannoside **15**. The 2-naphthylmethyl ether of **15** was removed by oxidation with DDQ,[26] and the resulting glycosyl acceptor **16** was glycosylated with **7** in the presence of a catalytic amount of TMSOTf in a mixture of dichloromethane and diethyl ether to give anomerically pure trisaccharide **17**. Deprotection of **17** was accomplished by reduction of the azides with trimethyl phosphine[34] followed by acetylation of the resulting amine with acetic anhydride in pyridine, and then reductive removal of the benzyl ethers and benzyloxycarbamate by catalytic hydrogenation over Pd to give compound **2**.

Preparation of Carbohydrate-Protein Conjugates

For immunological evaluations, trisaccharides **1** and **2** were conjugated to bovine serum albumin (BSA) by treatment with S-acetylthioglycolic acid pentafluorophenyl ester to afford the corresponding thioacetate derivatives, which after purification by size-exclusion chromatography were de-S-acetylated by using 7% ammonia gas in DMF and conjugated to maleimide activated BSA (BSA–MI, Pierce Endogen, Inc.) in phosphate buffer (pH 7.2). After purification by using a centrifugal filter device with a nominal molecular weight cut-off of 10 kDa, neoglycoproteins were obtained with an average of eleven and nineteen molecules of **1** and **2**, respectively, per BSA molecule as determined by Bradford's protein assay and quantitative carbohydrate analysis

by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

Next, conjugates of KLH and BSA to the polysaccharide of *B. anthracis* were prepared for immunization of rabbits and examination of antisera for anti-polysaccharide antibodies, respectively. To this end, the polysaccharide was treated with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP)[35] to form reactive cyanyl esters, which were condensed with free amines of BSA and KLH to give, after rearrangement of isourea-type intermediate, carbamate-linked polysaccharides. The KLH– and BSA–polysaccharide conjugate solutions were purified by using centrifugal filter devices (Micron YM 30000 Da) and then lyophilized. Saccharide loadings of 0.3 mg per mg of BSA and 0.96 mg per mg of KLH were determined by using bicinchoninic acid (BCA; BSA conjugate) and Bradford's (KLH conjugate) protein assays and quantitative carbohydrate analysis by HPAEC-PAD. In addition, maltoheptaose was conjugated to BSA by using CDAP to obtain a control conjugate to examine for the possible presence of anti-linker antibodies.[36]

Biological Evaluation

Rabbits were inoculated intramuscularly four times at biweekly intervals with live- or irradiated spores (3x 10^6 total spores),[14] or polysaccharide–KLH conjugate, followed by the collection of terminal bleeds fourteen days after the last immunization. ELISA was used to examine the pre- and post immune sera for polysaccharide recognition. Microtiter plates were coated with the polysaccharide–BSA conjugate and serial dilutions of sera added. An anti-rabbit IgG antibody labeled with horseradish peroxidase was employed as a secondary antibody for detection purposes. High titers of anti-polysaccharide IgG antibodies had been elicited by the polysaccharide KLH conjugate (**Figure 3.2**, **Table 3.1**). Furthermore, inoculation with live and

irradiated spores resulted in the production of IgG antibodies that could recognize the polysaccharide. Antisera obtained from immunizations with polysaccharide–KLH conjugate showed recognition of maltoheptaose linked to BSA albeit at much lower titers than when polysaccharide–BSA was used as ELISA coating.

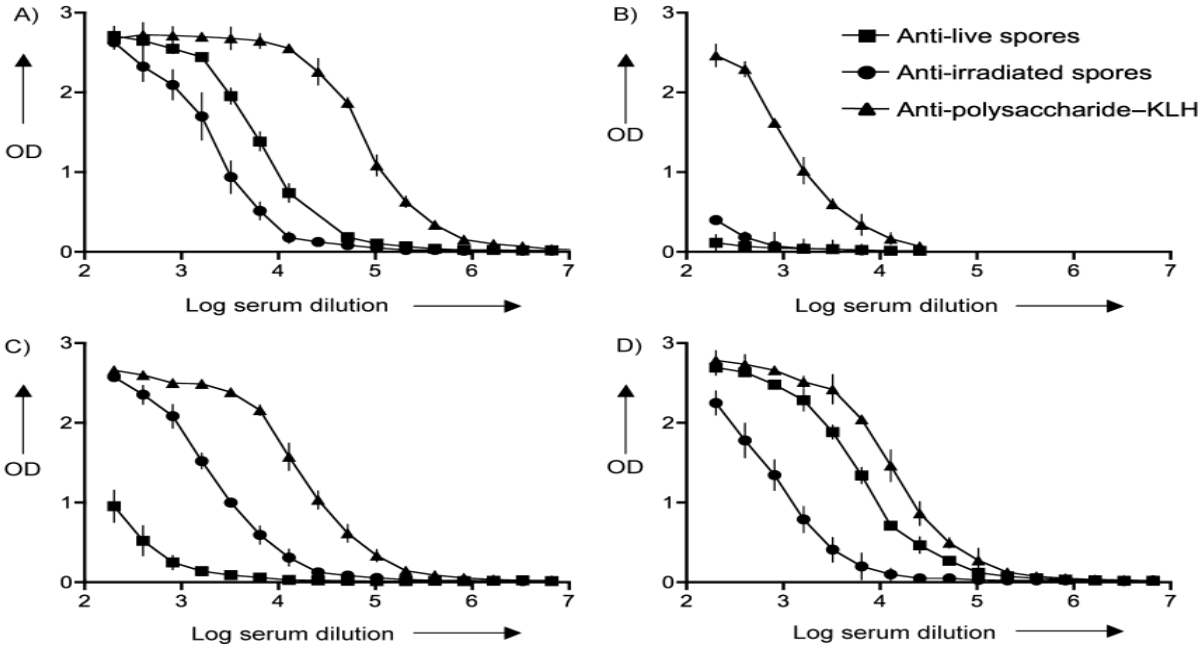


Figure 3.2. Immunoreactivity of polysaccharide and trisaccharides **1** and **2** to antisera elicited by *B. anthracis* Sterne live spores, irradiation-killed spores, and polysaccharide-KLH conjugate. Microtiter plates were coated with A) polysaccharide-BSA, B) maltoheptaose-BSA, C) **1**-BSA, and D) **2**-BSA conjugates (0.15 μg mL^{-1} carbohydrate). Serial dilutions of rabbit anti-live and anti-irradiated *B. anthracis* Sterne 34F2 spores antisera and rabbit anti-polysaccharide-KLH antiserum (starting dilution 1:200) were applied to coated microtiter plates. Serial dilutions of the preimmune sera from the rabbits (starting dilution 1:200) did not show any binding to polysaccharide-BSA (data not shown). Wells only coated with BSA at the corresponding protein concentration did not show binding to any sera (data not shown). The optical density (OD) values are reported as the means±SD of triplicate measurements.

This finding indicates that some anti-linker antibodies had been elicited.[36] As expected, antisera from rabbits immunized with live and irradiated spores showed no reactivity towards the maltoheptaose conjugate (**Figure 3.3**). Next, the specificity of the anti-polysaccharide antibodies was investigated by using synthetic trisaccharides **1** and **2** (**Figure 1**) linked to BSA.

Table 3.1. ELISA antibody titers after immunization with *B. anthracis* Sterne live spores, irradiation-killed spores, and polysaccharide-KLH. ELISA plates were coated with BSA conjugates (0.15 μg mL^{-1} carbohydrate) and titers were determined by linear regression analysis by plotting dilution versus absorbance. Titers are defined as the highest dilution that yielded an optical density of 0.5 or greater.

Immunization coating	**Live spores**	**Irradiated spores**	**Polysaccharide-KLH**
polysaccharide-BSA	18 500	6 100	239 700
maltoheptaose-BSA	0	0	3 600
1-BSA	400	6 800	57 300
2-BSA	18 700	2 600	46 700

Trisaccharides **1** and **2** were equally well recognizedby IgG antibodies that were elicitedby the polysaccharide–KLH conjugate and irradiation-killed spores (**Figure 3.3**, **Table 3.1**). Surprisingly, antisera obtained after inoculation with live spores recognized trisaccharide **2** much better than **1**. To further study the antigenic components of the various antisera, inhibition ELISAs were performed by coating microtiters plates with polysaccharide–BSA conjugate and by using **1**–BSA, **2**–BSA, and polysaccharide–BSA as inhibitors (**Figure 3.3**). As expected, for each antiserum, the polysaccharide–BSA inhibitor could completely block the binding of IgG antibodies to immobilized polysaccharide, whereas only partial inhibition was observed for **1**–BSA and **2**–BSA. Furthermore, antibodies elicited by the live spore vaccine recognized trisaccharide **2** much better than **1**, whereas the polysaccharide–KLH antiserum was better inhibited by **1**. Antibodies elicited by the irradiated spore inoculum recognized **1** and **2** equally well. The partial inhibition by the synthetic compounds indicates that heterogeneous populations of antibodies were elicited. Furthermore, the difference in antigenic component of the vaccines might be due to differences in presentation of the polysaccharide when it is part of vegetative cells, or attached to KLH, or when it is part of irradiation-killed spores. The results presented here show that both live- and irradiation-killed *B. anthracis* spore inoculae and polysaccharide

linked to the carrier protein KLH can elicit IgG antibodies that recognize isolated polysaccharide and the relatively small saccharides **1** and **2.**

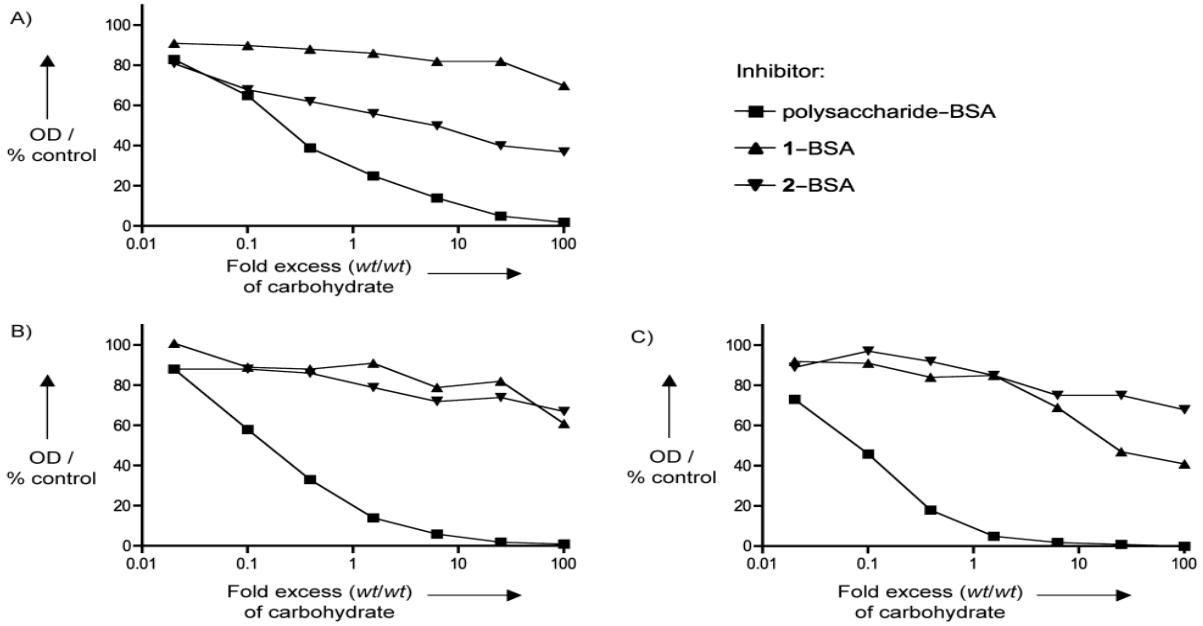


Figure 3.3. Competitive inhibition ELISA. Microtiter plates were coated with polysaccharide-BSA conjugate (0.15 μg mL^{-1} carbohydrate). Dilutions of A) rabbit anti-live, B) anti-irradiated *B. anthracis* Sterne 34F2 spores antisera, and C) rabbit anti-polysaccharide-KLH antiserum mixed with polysaccharide-BSA, **1**-BSA, and **2**-BSA (0-100-fold excess, wt/wt, based on carbohydrate concentration) were applied to coated microtiter plates. Maltoheptaose-BSA conjugate and unconjugated BSA at corresponding concentrations mixed with antisera did not display inhibition (data not shown). OD values were normalized for the OD values obtained in the absence of inhibitor (0-fold excess, 100 %).

Previously, the polysaccharide was identified as a component of the vegetative cell wall of *B. anthracis*, and thus, it was surprising that irradiation-killed spores could elicit anti-polysaccharide antibodies. It appears that not only vegetative cells but also *B. anthracis* spores express the polysaccharide. The implication of this finding is that a polysaccharide based vaccine could provide immunity towards vegetative cells as well as spores. In this respect, we hypothesize that immune responses to dormant *B. anthracis* spores at the mucosal surface might inhibit spore uptake across the mucosa and might also target the susceptible emergent vegetative cell; this would either prevent bacterial proliferation or enhance bacterial clearance. Highly

conserved integral carbohydrate components of the spore and vegetative cell structure are attractive vaccine candidate antigens because unlike capsules, they are not sloughed off the replicating cell. Finally, we have located important antigenic components of the various antisera using synthetic saccharides.

Conclusion

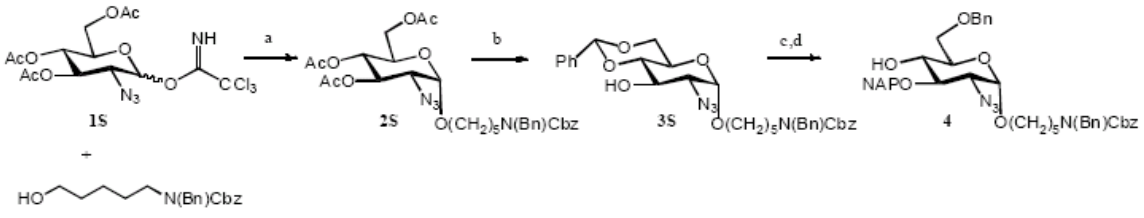
The data provide an important proof-of-concept step in the development of vegetative and spore-specific reagents for detection and targeting of nonprotein structures in *B. anthracis*. These structures might in turn provide a platform for directing immune responses to spore structures during the early stages of the *B. anthracis* infection process. Ongoing studies will demonstrate whether anti-polysaccharide antibodies can recognize *B. anthracis* spores, including the highly virulent *B. anthracis* Ames and *B. anthracis* cured of virulence plasmids (pXO1 and pXO2). Examination of the cross reactivity of the antisera with cell wall polysaccharides from various Bacillus species and determination of antigenic responses against the synthetic oligosaccharides are also underway.[37]

Acknowledgment: This research was supported by the Institute of General Medicine of the National Institutes of Health, grant M065248 (G.J.B.) and NIAID, grant AI059577 (R.W.C.). We wish to thank Dr. Therese Buskas for the preparation of the BSA conjugates and Dr. Elke Saile for assistance with rabbit immunizations. The findings and conclusions in this report are those of the author(s) and do not necessarily represent the views of the Centers for Disease Control and Prevention.

Experimental Section:

^{1}H NMR spectra were recorded in $CDCl_3$ or D_2O on a 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 Varian Merc-300 spectrometer, respectively by 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 assignment of the products. 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 rotary power was obtained on JASCO P-1020 polarimeter at 300 K. Chemicals were purchased from Aldrich or Fluka and used without further purification. DCM, 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 °C 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 chromatography. Iatrobeads 6RS-8060 was purchased from Bioscan. L denotes spacer.

Preparation of compound 4



Scheme 1S. a) DCM/Et_2O, TMSOTf, 0 °C, 80%; b) NaOMe, MeOH, then $C_6H_5CH(OMe)_2$, CSA, CH_3CN, 83%; c) 2-napthylmethyl bromide, NaH, DMF, 0 °C, 98%; d) Et_3SiH, TfOH, DCM, -78 °C, 87%.

***N*-Benzyl-*N*-benzyloxycarbonyl-5-aminopentyl 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-glucopyranoside (2S)**: A mixture of glucosyl donor **1S** (3.89 g, 8.2 mmol), *N*-benzyl-*N*-benzyloxy-carbonyl-5-aminopropanol (3.49 g, 10.0 mmol) was coevaporated with dry toluene (2 x 10 mL) and then dried in vacuo for 4 h. The dried compounds were dissolved in a mixture of DCM and diethyl ether (80 mL, 1:4, v/v) and 4Å MS was added. The mixture was stirred under an atmosphere of argon for 30 min and then cooled (0 °C). TMSOTf (74 µL, 0.41 mmol) was added and stirring was continued for 10 min and then the reaction mixture was quenched by the addition of pyridine (0.1 mL). The reaction mixture was filtered through celite and the filtrate concentrated in vacuo. The residue was purified by silica gel column chromatography hexane/ethyl acetate, 3:1, v/v) to give **2S** (4.2 g, 80%) as a clear oil. R_f = 0.3 (hexane/ethyl acetate, 3:1, v/v). ^{1}H (500 MHz, $CDCl_3$): δ = 7.38-7.19 (m, 10H, aromatic), 5.48 (t, 1H, $J_{2,3}$ = 10.5 Hz, $J_{3,4}$ = 10.0 Hz, , H-3), 5.21-5.17 (bd, 2H, CH_2, L_{Bn}), 5.05 (t, 1H, $J_{3,4}$ = $J_{4,5}$ = 10.0 Hz, H-4), 4.96-4.29 (bd, 1H, H-1), 4.51(bs, 2H, CH_2, L_{Bn}), 4.30-4.27 (m, 1H, H- 6a), 4.10-4.06 (m, 1H, H-6b), 4.00 (m, 1H, H-5), 3.70-3.65 (m, 1H, C*H*H- L), 3.49-3.41 (m, 1H, C*H*H- L), 3.29-3.26 (dd, 1H, $J_{1,2}$ = 3.5 Hz, $J_{2,3}$ = 10.5 Hz), 3.22 (m, 1H, CH_2-L), 2.1-2.04 (s, 9H, 3 COCH_3), 1.65-1.53 (m, 4H, 2 CH_2-L), 1.38-1.25 (m, 2H, C*H*2-L). ^{13}C (75 MHz, $CDCl_3$): δ = 170.79, 170.24, 170.21, 169.88, 169.82, 138.13, 128.76, 128.67, 128.15, 128.08, 127.53, 98.06 (C-1), 70.57, 68.87, 68.82, 68.67, 67.77, 67.39, 62.10, 61.03, 50.77, 50.50, 47.23, 46.35, 29.20, 23.52, 20.93, 20.83. HR-MALDI-TOF/MS (*m*/*z*) calcd for $C_{32}H_{40}N_4O_{10}$: 663.2642 $[M+Na]^+$; found: 663.2643.

***N*-Benzyl-*N*-benzyloxycarbonyl-5-aminopentyl-4,6-benzylidene-2-azido-2-deoxy-α-D-glucopyranoside (3S)**: Compound **2S** (2.2 g, 3.43 mmol) was dissolved in methanol (15 mL) and sodium metal (79.0 mg, 0.34 mmol) was added and the resulting reaction mixture was stirred for 2 h. The reaction mixture was then neutralized with weak acid resin (Amberlite IRC-50) and

filtered. The filtrate was concentrated under reduced pressure and dried in vacuo. The resulting crude product was dissolved in acetonitrile (20 mL) and benzaldehyde dimethylacetal (0.78 mL, 5.15 mmol) was added followed by camphorsulfonic acid (55.7 mg, 0.24 mmol). The reaction mixture was stirred for 11 h and then quenched by addition of Et_3N and concentrated in vacuo. The residue was purified by a silica gel column chromatography (hexane/ethyl acetate, 4:1, v/v) to give **3S** (1.6 g, 83%) as a clear oil. R_f = 0.25 (hexane/ethyl acetate, 4:1, v/v). ^{1}H (500 MHz, $CDCl_3$): δ = 7.51-7.19 (m, 15H, aromatic), 5.55 (s, 1H, >C*H*Ph), 5.21-5.18 (bd, 2H, C*H*$_2$, L_{Cbz}), 4.89-4.86 (bd, 1H, H-1), 4.51 (bs, 2H, C*H*$_2$, L_{Bn}), 4.27-4.22 (m, 2H, H-6a, H-3), 3.85 (m, 1H, H-5), 3.76-3.67 (t, 1H, $J_{5,6a} = J_{6a,6b}$ = 10.0 Hz, H-6b) 3.67 (m, 1H, C*H*H-L), 3.52 (t, 1H, $J_{3,4} = J_{4,5}$ = 9.5 Hz, H-4,), 3.45-3.39 (m, 1H, C*H*H-L), 3.30-3.23 (m, 3H, H-2, C*H*$_2$-L), 2.78 (s, 1H, O*H*), 1.66-1.59 (m, 4H, 2 x C*H*$_2$-L), 1.39-1.34 (m, 2H, C*H*$_2$-L). ^{13}C (75 MHz, $CDCl_3$): δ = 138.14, 137.13, 129.59, 129.32, 128.77, 128.68, 128.61, 128.14, 128.08, 127.53, 126.48, 102.29 (>CHPh), 98.80 (C-1), 82.13, 69.08, 68.89, 68.67, 67.41, 63.26, 62.67, 50.81, 47.30, 29.30, 28.08, 23.53. HRMALDI-TOF/MS (*m*/*z*) calcd for $C_{33}H_{38}N_4O_7$ $[M+Na]^+$: 625.2638; found: 625.2639.

***N*-Benzyl-*N*-benzyloxycarbonyl-5-aminopentyl 2-azido-6-O-benzyl-2-deoxy-3-*O*-(2-napthylmethyl)-α-D-glucopyranoside (4)**: Compound **3S** (0.5 g, 0.83 mmol) was dissolved in DMF (6 mL) and after cooling (0 °C), 60% NaH (60.0 mg, 1.5 mmol) was added and the resulting mixture was stirred under an atmosphere of argon for 20 min. 2-Naphthylmethyl bromide (0.24 g, 1.08 mmol) was added and the reaction mixture was stirred for 3 h and then quenched by the addition of methanol (0.5 mL). The reaction mixture was diluted with DCM (15 mL) and washed with aqueous solution of $NaHCO_3$ (sat., 10 mL) and brine (10 mL). The organic layer was dried ($MgSO_4$), filtered and the filtrate concentrated in vacuo. The residue was purified

by silica gel column chromatography (hexane/ethyl acetate, 4:1, v/v) to give *N*-benzyl-*N*-benzyloxycarbonyl- 5-aminopentyl 4,6-benzylidene-3-*O*-(2-napthylmethyl)-2-azido-2-deoxy-α-D-glucopyranoside (0.60 g, 98%) as a clear oil. R_f = 0.35 (hexane/ethyl acetate, 4:1, v/v). ^{1}H (500 MHz, $CDCl_3$): δ= 7.84-7.20 (m, 22H, aromatic), 5.64 (s, 1H, >C*H*Ph), 5.22-5.19 (bd, 2H, C*H*$_2$, L_{Cbz}), 5.13 (d, 1H, J_{HaHb} = 11.5 Hz, C*H*$_a$H$_b$, napthylmethyl), 5.00 (d, 1H, J_{HaHb} = 11.0 Hz, CH$_a$*H*$_b$, napthylmethyl), 4.90-4.87 (bd, 1H, H-1), 4.54-4.52 (bd, 2H, C*H*$_2$, L_{Bn}), 4.31-4.30 (m, 1H, H-6a), 4.15 (t, 1H, $J_{2,3} = J_{3,4} = 9.0$ Hz, H-3), 3.91 (m, 1H, H-5), 3.81-3.67 (m, 3H, H-6b, H-4, C*H*H-L), 3.50-3.46 (m, 1H, C*H*H-L), 3.41- 3.86 (dd, 1H, $J_{1,2}$ = 3.5 Hz, $J_{2,3}$ = 10.0 Hz, H-2), 3.31-3.23 (m, 2H, C*H*$_2$-L), 1.67-1.55 (m, 4H, 2 x C*H*$_2$-L), 1.42-1.29 (m, 2H, C*H*$_2$-L). ^{13}C (75 MHz, $CDCl_3$): δ= 138.14, 137.43, 135.63, 133.52, 133.31, 129.30, 128.77, 128.67, 128.55, 128.41, 128.20, 128.14, 128.07, 127.88, 127.51, 127.14, 126.30, 126.27, 126.21, 126.10, 101.72 (> *C*HPh), 98.78 (C-1), 83.08, 76.36, 75.27, 69.17, 68.60, 67.40, 63.29, 62.96, 50.80, 47.31, 29.28, 23.52. HR-MALDI-TOF/MS (*m*/*z*) calcd for $C_{44}H_{46}N_4O_7$: 765.3264 $[M+Na]^+$; found: 765.3262. The above compound (0.55 g, 0.74 mmol) was dissolved in DCM (7 mL) and 4 Å MS (1.0 g) was added and the resulting mixture stirred under an atmosphere of argon for 30 min. The mixture was cooled (-78 °C) and Et_3SiH (0.3 mL, 1.85 mmol) was added followed by TfOH (0.16 mL, 1.85 mmol). The reaction mixture was stirred for 30 min and then quenched with MeOH (1 mL) and Et_3N (1 mL) and diluted with DCM (7 mL). The reaction mixture was filtered through celite and the filtrate washed with aqueous solution of $NaHCO_3$ (sat., 7 mL) and brine (7 mL). The organic layer was dried ($MgSO_4$) and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 4:1, v/v) to give **4** (0.48 g, 87%) as a clear oil. R_f = 0.25 (hexane/ethyl acetate, 4:1, v/v). ^{1}H (500 MHz, $CDCl_3$): δ = 7.88-7.19 (m, 22 H, aromatic), 5.22-5.17 (bd, 2H, C*H*2, L_{Cbz}), 5.09 (d, 1H, J_{HaHb} = 11.0Hz, C*H*$_a$H$_b$,

napthylmethyl), 5.02-4.99 (m, 1H, CH_aH_b, napthylmethyl), 4.91-4.89 (bd, 1H, H-1), 4.64-4.51 (m, 4H, CH_2, OBn, CH_2, L_{Bn}), 3.93-3.91 (m, 1H, H-3), 3.80-3.71 (m, 5H, H-4, H-5, H-6a,b, CHH-L), 3.47-3.41 (m, 1H, CHH-L), 3.34-3.21 (dd, 1H, $J_{1,2}$ = 3.0 Hz, $J_{2,3}$ = 10.0 Hz, H-2), 3.28-3.23 (m, 2H, CH_2-L), 1.59-1.54 (m, 4H, 2 x CH_2-L), 1.42-1.28 (m, 2H, CH_2-L). ^{13}C (75 MHz, $CDCl_3$): δ = 138.09, 135.85, 133.56, 133.31, 128.78, 128.67, 128.22, 128.16, 128.05, 127.92, 127.90, 127.54, 127.06, 126.34, 126.19, 126.13, 98.14 (C-1), 80.02, 75.28, 73.91, 72.62, 70.48, 70.02, 68.27, 67.44, 63.01, 50.63, 29.14, 23.52. HR-MALDI-TOF/MS (*m*/*z*) calcd for C44H48N4O7: 767.3421 $[M+Na]^+$; found: 767.3450.

***N*-Benzyl-*N*-benzyloxycarbonyl-5-aminopentyl-2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-galacto-pyranosyl-(1→4)-2-azido-6-*O*-benzyl-2-deoxy-3-*O*-(2-naphthylmethyl)-α-D-glucopyrano-side (5)**: A mixture of galactosyl donor **3** (0.18 g, 0.35 mmol), glucosyl acceptor **4** (0.20 g, 0.27 mmol), and 4Å MS (0.4 g) in dichloromethane (5 mL) was stirred at room temperature under an atmosphere of argon for 30 min. The reaction mixture was cooled (0°C) and then NIS (78.7 mg, 0.35 mmol) and TMSOTf (7.0 μL, 0.035 mmol) were sequentially added. The reaction mixture was stirred for 10 min and then quenched with pyridine (50 μL). The reaction mixture was diluted with dichloromethane (5 mL) filtered through celite and washed with aqueous solution of $Na_2S_2O_3$ (15%, 10 mL), $NaHCO_3$ (sat., 7 mL), and water (7 mL). The organic layer was dried ($MgSO_4$), filtered and the filtrate concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 4:1, v/v) to give **5** (0.28 g, 87%) as a clear oil. R_f = 0.35 (hexane/ethyl acetate, 4:1, v/v). $[\alpha]^{25}{}_D$ = +46.3 (c 1.05, CHCl3); ^1H (500 MHz, $CDCl_3$): δ = 7.69-6.92 (m, 37H, aromatic), 5.28 (dd, 1H, $J_{1',2'}$ = 8.0 Hz, $J_{2',3'}$ = 8.5 Hz, H -2'), 5.19 (d, 1H, J_{HaHb} = 11.0 Hz, CH_aH_b, napthylmethyl), 5.11- 5.08 (bd, 2H, CH_2-L_{Cbz}), 4.86 (d, 1H, CHH, OBn), 4.74-4.72 (bd, 2H, CH_aH_b, napthyl- methyl, H-1), 4.63- 4.55 (dd, 2H, CH_2, OBn), 4.44-

4.42 (bd, 2H, CH_2-L_{Bn}), 4.37- 4.31 (dd, 3H, CH_2, OBn), 4.28 (d, 1H, $J_{1',2'}$ = 8.0 Hz, H-1'), 3.89-3.81 (m, 5H, H-4', H-3, H-4, CH_2, OBn), 3.69-3.67 (m, 1H, H-5'), 3.59-3.50 (m, 3H, H-5, H-6a,b), 3.23-3.14 (m, 6H, H-2, H-3', H -6'a,b, 3 x C*H*H-L), 3.04-3.02 (m, 1H, C*H*H-L), 1.88 (s, 3H, COCH_3), 1.52-1.48 (m, 4H, 2 x CH_2-L), 1.27-1.18 (m, 2H, CH_2-L). ^{13}C (75 MHz, $CDCl_3$): δ = 169.55, 138.97, 138.27, 138.19, 138.05, 136.62, 133.53, 133.10, 128.77, 128.68, 128.65, 128.52, 128.47, 128.28, 128.24, 128.15, 128.10, 128.07, 127.95, 127.92, 127.82, 127.80, 127.76, 127.51, 126.51, 125.82, 101.03 (C-1'), 97.99 (C-1), 80.70, 78.01, 77.68, 77.46, 77.26, 76.83, 75.21, 74.84, 73.81, 73.54, 73.35, 72.74, 72.17, 71.89, 70.82, 68.42, 68.04, 67.80, 67.39, 63.23, 29.24, 23.52, 21.32. HR-MALDI- TOF/MS (*m*/*z*) calcd for $C_{73}H_{78}N_4O_{13}$[*M*+Na]$^+$: 1241.5455; found: 1241.5457.

***N*-Benzyl-*N*-benzyloxycarbonyl-5-aminopentyl-2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-galacto-pyranosyl-(1→4)-6-*O*-benzyl-2-azido-2-deoxy-α-D-glucopyranoside (6)**: DDQ (49.0 mg, 0.21 mmol) was added to a solution of compound **5** (0.22 g, 0.18 mmol) in a mixture of dichloromethane and water (2.2 mL, 10:1, v/v). The reaction mixture was stirred vigorously at room temperature for 2 h in the dark and then quenched with an aqueous mixture of citric acid, ascorbic acid, and NaOH (0.1 mL, 1.2%, 1.0%, 0.92% w/v). The mixture was diluted with ethyl acetate (15 mL) and washed with aqueous $NaHCO_3$ (sat., 5 mL). The organic layer was dried ($MgSO_4$) and filtered, and the filtrate concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 4:1, v/v) to give **6** (0.18 g, 94%) as a clear oil. R_f = 0.30 (hexane/ethyl acetate, 4:1, v/v). $[\alpha]^{25}_{D}$ = +66.0 (c 0.87, CHCl3); ^{1}H (500 MHz, CDCl3): δ = 7.41-7.30 (m, 30H, aromatic), 5.38 (dd, 1H, $J_{1',2'}$ = 8.0 Hz, $J_{2',3'}$ = 8.5 Hz, H -2'), 5.20 (bd, 2H, CH_2 –L_{Cbz}), 4.93 (d, 1H, C*H*H –OBn), 4.83-4.81 (bd, 1H, H-1), 4.70-4.66 (dd, 2H, CH_2, OBn), 4.58-4.42 (m, 8H, 5 C*H*H, OBn, CH_2 -L_{Bn}), 4.34 (d, 1H, $J_{1',2'}$ = 8.0 Hz, H-1'), 4.11

(t, 1H, $J_{2,3}$ = 9.5 Hz, $J_{3,4}$ = 9.0 Hz, H-3), 3.89-3.88 (bd, 1H, H-4'), 3.73-3.60 (m, 7H, H-4, H-6a,b, H-5', H-6'a,b, H-5), 3.47-3.43 (m, 2H, H-3', C*H*H-L), 3.29-3.22 (m, 3H, 3 x C*H*H-L), 3.16-3.13 (dd, 1H, $J_{2,3}$ = 9.5 Hz, $J_{1,2}$ = 3.5 Hz, H-2,), 1.96 (s, 3H, COCH_3), 1.61-1.54 (m, 4H, CH_2-L), 1.36-1.27 (m, 2H, CH_2-L). ^{13}C (75 MHz, $CDCl_3$): δ = 169.45, 138.39, 138.18, 137.88, 137.55, 128.76, 128.73, 128.64, 128.54, 128.45, 128.22, 128.18, 128.07, 128.04, 127.94, 127.91, 127.72, 127.49, 101.80 (C-1'), 98.10 (C-1), 81.47, 80.47, 76.83, 74.71, 74.21, 74.00, 73.79, 72.40, 72.29, 71.40, 69.80, 69.56, 68.68, 68.40, 68.30, 67.36, 62.59, 29.26, 23.49, 21.21. HR-MALDI-TOF/MS (*m*/*z*) calcd for C62H70N4O13: 1101.4829 [*M*+Na]$^+$; found: 1101.4831.

***N*-Benzyl-*N*-benzyloxycarbonyl-5-aminopentyl-2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-galacto-pyranosyl-(1→4)-[2,3,4,6 -tetra-*O*-benzyl-α-D-galactopyranosyl-(1→3)]-6-*O*-benzyl-2-azido-2-deoxy-α-D-glucopyranoside (8)**: A mixture of **6** (0.098 g, 0.14 mmol) and **7** (0.1 g, 0.092 mmol) was co-evaporated with dry toluene (37 mL) and dried in vacuo for 4 h. The dried compounds were dissolved in a mixture of diethyl ether and dichloromethane (7 mL, 5:1, v/v) and 4Å MS (0.28 g) was added. The mixture was stirred under an atmosphere of argon for 30 min and then cooled (-50 °C). TMSOTf (2.5 μL, 0.014 mmol) was added and the reaction mixture was allowed to reach 0°C gradually over a period of 1 h. The reaction was then quenched by the addition of pyridine (20 μL), diluted with dichloromethane (7 mL), and filtered through celite. The filtrate was washed with aqueous $NaHCO_3$ (sat., 7 mL) and the organic layer was dried ($MgSO_4$) and filtered, and the filtrate was concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 4:1, v/v) to give **8** (0.13 g, 86%) as a clear oil. R_f = 0.35 (hexane/ethyl acetate, 4:1, v/v). $[\alpha]^{25}{}_D$ = +59.4 (c 1.72, $CHCl_3$); ^{1}H (500 MHz, $CDCl_3$): δ = 7.35-7.14 (m, 50H, aromatic), 5.76 (d, 1H, $J_{1'',2''}$ = 3.0 Hz, H-1''), 5.28 (t, 1H, $J_{2',3'}$ = 9.5 Hz, $J_{1',2'}$ = 8.0 Hz, H-2'), 5.18- 5.16 (bd, 2H, CH_2–L_{Cbz}), 4.89-4.84 (m, 2H,

C*H*H, OBn, H-1), 4.80-4.71 (dd, 3H, 3 x C*H*H, OBn), 4.68-4.62 (m, 5H, 3 x C*H*H, OBn, H-1'), 4.54-4.39 (m, 8H, 3 x CH_2, OBn, C*H*2 –L_{Bn}), 4.35-4.24 (m, 3H, C*H*H, OBn, H-3, H-5''), 4.18-4.04 (m, 5H, CH_2, OBn, H-4, H-3'', H-2''), 3.99 (bs, 1H, H-4''), 3.84 (bs, 1H, H-4'), 3.80-3.74 (m, 2H, H-6a, H-5), 3.65-3.51 (m, 4H, H-6b, H-6'a, H -6''a,b), 3.44-3.30 (m, 5H, CH_2-L, H -2, H-5', H-6'), 3.23-3.17 (m, 3H, CH_2-L, H-3'),1.90 (s, 3H, COCH_3), 1.51-1.46 (m, 4H, 2 x CH_2-L), 1.26-1.20 (m, 2H, CH_2-L). ^{13}C (75 MHz, $CDCl_3$): δ = 169.26, 139.36, 139.18, 138.70, 138.28, 138.24, 138.15, 138.12, 137.08, 128.77, 128.68, 128.63, 128.59, 128.48, 128.42, 128.37, 128.36, 128.34, 128.29, 128.05, 127.95, 127.93, 127.82, 127.80, 127.70, 127.60, 127.53, 127.41, 127.35, 127.30, 99.64 (C-1'), 97.63 (C-1), 96.22 (C-1''), 81.04, 78.64, 76.84, 76.51, 76.28, 75.54, 74.92, 74.68, 73.68, 73.58, 73.45, 73.23, 72.51, 72.40, 72.19, 71.96, 70.49, 70.16, 69.17, 68.76, 68.33, 68.22, 67.37, 62.64, 53.66, 29.13, 23.41, 21.26. HR-MALDI-TOF/MS (*m*/*z*) calcd for $C_{96}H_{104}N_4O_{18}$: 1623.7246 $[M+Na]^+$; found: 1623.7242.

***N*-Benzyl-*N*-benzyloxycarbonyl-5-aminopentyl-2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-galacto-pyranosyl-(1→4)-[2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl-(1→3)]-2-acetamido-6-*O*-benzyl-2-deoxy-α-D-glucopyranoside (9)**: Compound **8** (75 mg, 0.047 mmol) was dissolved in a mixture of THF, acetic anhydride, and acetic acid (2.0 mL/1.3 mL/0.7 mL, v/v/v). Zinc powder (40 mg, 0.61 mmol) was added followed by an aqueous solution of copper sulfate (sat., 60 μL) and the resulting reaction mixture was vigorously stirred for 20 min and then filtered through celite. The filtrate was concentrated in *vacuo* and the residue was purified by silica gel column chromatography (hexane/ethyl acetate 1:1, v/v) to give **9** (47 mg, 63%) as a clear oil. R_f = 0.6 (hexane/ethyl acetate 1:1, v/v). ^{1}H (500 MHz, $CDCl_3$): δ = 7.28-7.09 (m, 50H, aromatic), 6.29 (d, 1H, N*H*Ac, $J_{NHAc,2}$ = 8.5 Hz), 5.22-5.19 (m, 2H, H-1'', H-2'), 5.08-5.07 (bd, 2H, C*H*2–L_{Cbz}), 4.76 (bs, 1H, H-1), 4.66-4.54 (m, 6H, 3 x CH_2, OBn), 4.50-4.44 (m, 4H, C*H*H, OBn, CH_2 –L_{Bn},

H-1’), 4.40-4.27 (m, 9H, 9 x C*H*H, OBn), 4.17-4.08 (m, 2H, H-2, H-5’’), 3.96-3.95 (m, 2H, H-3, H-2’’), 3.88 (m, 1H, H-4), 3.80 (m, 2H, H-4’, H-4’’), 3.75-3.69 (m, 3H, H-5, H-3’’, H-6’’a), 3.63-3.59 (t, 1H, $J_{6a,6b} = J_{5,6a} = 9.5$ Hz, H-6a), 3.49-3.37 (m, 3H, H-6’a,b, H-6b, 6’’b), 3.29-3.26 (m, 1H, H-5), 3.21-3.19 (m, 2H, H-3’, C*H*H-L), 3.12-3.04 (m, 3H, 3 x C*H*H-L), 1.96 (s, 3H, COCH_3), 1.86 (s, 3H, NHCOCH_3), 1.5-1.42 (m, 4H, 2 x CH_2-L), 1.26-1.20 (m, 2H, CH_2-L). ^{13}C (75 MHz, $CDCl_3$): δ = 170.58, 169.78, 155.15, 151.23, 150.32, 149.83, 145.13, 142.29, 141.97, 140.39, 139.31, 138.94, 138.86, 138.60, 138.37, 138.24, 138.12, 138.02, 134.75, 134.47, 133.58, 131.44, 131.09, 129.98, 128.76, 128.65, 128.61, 128.60, 128.58, 128.46, 128.40, 128.38, 128.14, 128.11, 128.05, 128.03, 127.90, 127.87, 127.82, 127.74, 127.67, 127.49, 126.88, 126.06, 124.94, 100.05 (C-1’), 97.50 (C-1’’), 95.70 (C-1), 80.67, 78.20, 76.83, 75.91, 74.74, 73.97, 73.86, 73.77, 73.58, 73.44, 72.93, 72.50, 72.08, 71.22, 69.39, 68.56, 68.28, 67.37, 67.18, 51.83, 50.39, 47.27, 46.33, 29.93, 29.32, 23.47, 23.23, 21.31. HR-MALDI-TOF/MS (*m*/*z*) calcd for $C_{98}H_{108}N_2O_{19}$:1639.7436 $[M+Na]^+$; found:1639.7439.

5-Aminopentyl-β-D-galactopyranosyl-(1→4)-[α-D-galactopyranosyl-(1→3)]-2-acetamido-2-deoxy-α-D-glucopyranoside (1): Compound **9** (35 mg, 21.6 μmol) was dissolved in a mixture of methanol and dichloromethane (0.5 mL, 4:1, v/v). Sodium metal (1.0 mg) was added and stirred overnight. The reaction mixture was neutralized with weak acid resin (Amberlite IRC-50) and filtered. The filtrate was concentrated in vacuo and the residue purified by silica gel column chromatography (hexane/ethyl acetate 1:1, v/v) to give deacetylated product (13.0 mg, 90%) as a clear oil. R_f = 0.5 (hexane/ ethyl acetate, 1:1, v/v). ^{1}H (500 MHz, $CDCl_3$): δ = 7.24-7.06 (m, 50H, aromatic), 6.21 (bs, 1H, N*H*Ac), 5.48 (bs, 1H, H-1’’), 5.08-5.06 (bs, 2H, CH_2 –LCbz), 4.77-4.75 (m, 2H, H-1, C*H*H, OBn), 4.70-4.64 (m, 3H, 3 x C*H*H, OBn), 4.56-4.53 (m, 3H, H-1’, CH_2, OBn,), 4.47-4.34 (m, 10H, 4 x CH_2, OBn, CH_2 –L_{Bn}), 4.23-4.12 (m, 5H, CH_2,OBn, H-2, H-5’’,

H-3,), 4.02-4.01 (m, 2H, H-2'', 4''), 3.85-3.84 (m, 2H, H-4, H-6a), 3.76-3.56 (m, 3H, H-3'', H-2', H-4'), 3.62-3.56 (m, 3H, H-6''a, H-6b, H-5), 3.44-3.34 (m, 3H, H-6'a,b, H-6''b), 3,28-3.26 (m, 1H, H-5'), 3.15-3.02 (m, 4H, 2 CH_2-L), 2.83-2.80 (m, 1H, H-3'), 1.76 (s, 3H, NHCOCH_3), 1.37-1.28 (m, 4H, 2 x CH_2-L), 1.14-1.03 (m, 2H, CH_2-L). ^{13}C (75 MHz, $CDCl_3$): δ = 170.37, 139.25, 138.99, 138.81, 138.72, 138.18, 138.08, 128.77, 128.63, 128.60, 128.53, 128.49, 128.47, 128.34, 128.27, 128.19, 128.06, 128.03, 127.97, 127.90, 127.79, 127.65, 127.60, 127.46, 101.90 (C-1'), 97.69 (C-1''), 96.93 (C-1), 82.33, 82.19, 78.93, 76.82, 75.96, 75.87, 74.94, 74.71, 73.99, 73.87, 73.70, 73.52, 73.20, 73.04, 72.76, 72.56, 72.21, 70.99, 70.65, 69.98, 69.21, 68.57, 67.92, 67.39, 52.79, 50.42, 29.93, 29.19, 29.05, 23.48, 23.32. HR MALDI-TOF/MS: calcd for $C_{96}H_{106}N_2O1_8$: 1597.7331 $[M+Na]^+$; found: 1597.7336. The above compound (10.0 mg, 6.35 μmol) was dissolved in a mixture of AcOH, *t*-BuOH, and H_2O (0.64 mL, 0.3 mL, 0.06 mL, 10:5:1, *v/v/v*) and placed under argon atmosphere. $Pd(OH)_2$ /C (15.0 mg) was added and the reaction mixture was degassed and placed under H_2 atmosphere and stirred for 16 h. The reaction mixture was filtered through a PTFE (polytetrafluroethylene filter, Fischerbrand, 0.2 μm) filter and the residue washed with acetic acid (2.0 mL). The combined filtrates were concentrated in vacuo and the residue was purified over Iatrobeads (iPrOH/NH_4OH/H_2O, 3:2:1, v/v/v) to give **1** (2.5 mg, 63%) as a white solid. R_f = 0.25 (iPrOH/NH_4OH/H_2O, 3:2:1, v/v/v). ^{1}H (500 MHz, D_2O): δ = 5.33 (d, 1H, H-1'', $J_{1'',2''}$ = 3.5 Hz), 4.68 (d, 1H, H-1, $J_{1,2}$ = 3.0 Hz), 4.39 (d, 1H, $J_{1',2'}$ = 8.0 Hz, H-1'), 3.96-3.84 (m, 4H), 3.65-3.51 (m, 14H), 3.40-3.33 (m, 2H, H-2', C*H*H-L), 3.85 (t, 2H, CH_2-L), 1.90 (s, 3H, NHCOCH_3), 1.57-1.51(m, 4H, 2 x CH_2-L), 1.32- 1.28 (m, 2H, CH_2-L). ^{13}C (125 MHz, $CDCl_3$): δ = 102.95 (C-1''), 99.95 (C-1), 97.18 (C-1), 76.28, 76.17, 75.84, 74.78, 71.46, 71.24, 69.16, 69.71, 69.38, 68.94, 68.06, 61.27, 60.94, 60.07, 53.05 (C-2'), 39.67, 28.51,

27.15, 22.52, 22.38. HR-MALDI-TOF/MS (*m*/*z*) calcd for $C_{25}H_{46}N_2O_{16}$: 653.6255 $[M+Na]^+$; found: 653.6257.

***N*-Benzyl-*N*-benzyloxycarbonyl-5-aminopentyl-2-*O*-acetyl-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranosyl-(1→4)-2-azido-6-*O*-benzyl-2-deoxy-3-(2-napthylmethyl)-β-D-glucopyranoside (12)**: A mixture of galactosyl donor **10** (0.41 g, 0.93 mmol), glucosyl acceptor **11** (0.53 g, 0.72 mmol), and 4Å MS (1.0 g) in dichloromethane (10 mL) was stirred at room temperature under an atmosphere of argon for 30 min. The reaction mixture was cooled (0 °C) and then NIS (0.21 g, 0.93 mmol) and TMSOTf (16.0 μL, 0.09 mmol) were sequentially added. The reaction was stirred for 10 min and then quenched with pyridine (50 μL). The reaction mixture was diluted with dichloromethane (10 mL), filtered through celite, and washed with an aqueous solution of $Na_2S_2O_3$ (15%, 7 mL), $NaHCO_3$ (sat., 7 mL), and water (7 mL). The organic layer was dried ($MgSO_4$) and filtered, and the filtrate concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 4:1, v/v) to give **12** (0.69 g, 88%) as a white solid. R_f = 0.35 (hexane/ethyl acetate, 4:1, v/v). $[\alpha]^{25}{}_D$ = -15.4 (c 2.75, $CHCl_3$); ^{1}H (500 MHz, $CDCl_3$): δ = 7.87-7.81(m, 4H, aromatic), 7.55-7.16 (m, 33H, aromatic), 5.34 (s, 1H, >C*H*Ph), 5.18-5.15 (bd, 2H, CH_2-L_{Cbz}), 5.03 (d, 1H, $J_{Ha,Hb}$ = 11.0 Hz, CH_aH$_b$-napthylmethyl), 4.97 (dd, 1H, $J_{1',2'}$ = 8.0 Hz, $J_{2',3'}$ = 8.5 Hz, H-2'), 4.93 (d, 1H,, $J_{Ha,Hb}$ = 11.0 Hz, CH$_a$$H_b$- napthylmethyl), 4.84 (d, 1H, C*H*H, OBn), 4.72 (d, 1H, C*H*H, OBn), 4.62 (d, 1H, C*H*H, OBn) 4.52 (d, 1H, $J1_{',2'}$ = 8.0 Hz, H-1'), 4.49-4.47 (bd, 2H, CH_2-L_{Bn}), 4.42 (d, 1H, C*H*H, OBn), 4.15-4.12 (m, 1H, H-1), 4.10-4.07 (dd, 1H, $J_{6'a,6'b}$ = $J_{5,6a}$ = 10.0 Hz, H-6'a), 3.96 (m, 1H, H-4), 3.85-3.83 (m, 1H, C*H*H-L), 3.73-3.65 (m, 2H, H-6a,b), 3.59 (t, 1H, $J_{3',4'}$ = 9.0 Hz, $J_{4',5'}$ = 9.5 Hz, H -4'), 3.50 (t, 1H, $J_{2',3'}$ = 9.5 Hz, $J_{3',4'}$ = 9.0 Hz, H-3'), 3.40-3.25 (m, 7H, CH_2-L, H-2, H-4, H-3, H-5, H-6'b), 3.19 (m, 1H, C*H*H-L), 3.14-3.09 (m, 1H, H-5'), 1.93 (s, 3H, COCH_3), 1.58-1.51 (m,

4H, CH_2-L), 1.38-1.25 (m, 2H, L-CH_2). ^{13}C (75 MHz, $CDCl_3$): δ = 169.34, 138.52, 138.17, 37.99, 137.42, 136.08, 133.50, 133.26, 129.27, 128.76, 128.68, 128.57, 128.49, 128.29, 128.26, 128.17, 128.13, 128.05,1 27.98, 127.91, 127.50, 126.89, 126.89, 126.37, 126.30, 126.24, 126.04, 102.25 (C-1), 101.38 (>*C*HPh), 100.83 (C-1'), 81.83, 81.21, 78.68, 76.58, 75.64, 75.12, 74.27, 73.90, 73.55, 70.11, 68.69, 67.70, 67.37, 66.17, 66.07, 29.39, 23.41, 21.11. HR-MALDI-TOF/MS (*m*/*z*) calcd for C66H70N4O13: 1149.4837 [*M*+Na]$^+$; found: 1149.4839.

***N*-Benzyl-*N*-benzyloxycarbonyl-5-aminopentyl-3-*O*-benzyl-4,6-*O*-benzylidene-β-D-glucopyranosyl-(1→4)-2-azido-6-*O*-benzyl-2-deoxy-3-(2-napthylmethyl)-β-D-glucopyranoside (13)**: Compound **12** (0.48 g, 0.42 mmol) was dissolved in a mixture of methanol and dichloromethane (7 mL, 3:1, v/v) and sodium metal (10 mg) was added. The reaction mixture was stirred for 18 h and then neutralized with weak acid resin (Amberlite IRC-50) and filtered. The filtrate was concentrated in vacuo and the residue was purified by silica gel column chromatography (hexane/ethyl acetate, 4:1, v/v) to give **13** (0.41 g, 90%) as a clear oil. R_f = 0.30 (hexane/ethyl acetate, 4:1, v/v). $[\alpha]^{25}{}_D$ = -11.9 (c 6.85, $CHCl_3$); ^{1}H (500 MHz, $CDCl_3$): δ = 7.90-7.17 (m, 37H, aromatic), 5.40 (s, 1H, >C*H*Ph), 5.20-5.17 (bd, 2H, CH_2-N_{Cbz}), 5.06 (d, 1H, J_{HaHb} = 11.4 Hz, CH_aH$_b$-napthylmethyl), 4.98-4.93(dd, 2H, CH$_a$$H_b$- napthylmethyl, C*H*H, OBn), 4.76(d, 1H, C*H*H, OBn), 4.70-4.68 (d, 1H, C*H*H, OBn), 4.61 (d, 1H, $J_{1'2'}$ = 6.6 Hz, H-1'), 4.55-4.49 (m, 3H, CH_2-N_{Bn}, C*H*H, OBn), 4.22-4.21 (m, 1H, H-1), 4.06-4.03 (m, 2H, H-6'a, H-4), 3.99-3.97 (dd, 1H, $J_{6a,6b}$ = $J_{H5,6a}$ =10.8 Hz, H-6a), 3.94-3.86 (m, 1H, C*H*H-L), 3.77 (bd, 1H, H-6b), 3.56 (t, 1H, $J_{3',4'}$ = $J_{4',5'}$ = 9.0 Hz, H-4'), 3.52-3.42 (m, 7H, H-6'b, H-2',H-3, H-5, C*H*H-L, H-2, H-3'), 3.29-3.22 (m, 2H, CH_2-L), 3.15-3.09 (m, 1H, H-5'), 1.65-1.52 (m, 4H, 2 x CH_2-L), 1.39-1.33 (m, 2H, CH_2-L). ^{13}C (75 MHz, $CDCl_3$): δ = 138.68, 138,20, 137.93, 137.51, 136.10, 133.52, 133.26, 129.24, 128.79, 128.71, 128.70, 128.47, 128.30, 128.27, 128.23, 128.20, 128.17,

128.13, 128.09, 128.06, 128.03, 127.54, 126.41, 126.30, 126.10, 125.98, 103.50 (C-1'), 102.45 (C-1), 101.42 (>*C*HPh), 81.94, 81.52, 80.60, 75.47, 75.23, 74.90, 74.76, 73.82, 70.15, 68.82, 68.38, 67.41, 66.54, 66.36, 29.45, 23.46. HR-MALDI-TOF/MS (*m*/*z*) calcd for $C_{64}H_{68}N_4O_{12}$: 1107.4732 $[M+Na]^+$; found: 1107.4739.

***N*-Benzyl-*N*-benzyloxycarbonyl-5-aminopentyl 2-azido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-β-D-mannopyranosyl-(1→4)-2-azido-6-*O*-benzyl-2-deoxy-3-(2-napthylmethyl)-β-D-glucopyranoside (15)**: Compound **13** (0.23 g, 0.21 mmol) was dissolved in a mixture of dichloromethane and pyridine (7.2 mL, 5 :1, v/v). The mixture was cooled (0°C) and Tf_2O (0.18 mL, 1.06 mmol) was added slowly over 5 min. The reaction mixture was stirred under argon for 5 h, diluted with dichloromethane (10 mL) and washed with aqueous $NaHCO_3$ (sat., 10 mL). The organic layer was dried ($MgSO_4$) and filtered, and the filtrate concentrated to dryness and further dried in vacuo for 2 h. NaN_3 (60 mg, 0.92 mmol) was added to the crude product **14** dissolved in dry DMF (8 mL). The resulting mixture was heated at 50 °C for 6 h, after which it was cooled to room temperature, diluted with ethyl acetate (15 mL), and washed with water (7 mL). The organic layer was dried ($MgSO_4$), concentrated in vacuo and the residue was purified by silica gel column chromatography (hexane/ethyl acetate, 4:1 v/v) to give **15** (0.17 g, 70%) as a clear oil. R_f = 0.50 (hexane/ethyl acetate, 4:1 v/v). $[\alpha]^{25}{}_D$ = -43.8 (c 2.75, $CHCl_3$); ^1H (500 MHz, $CDCl_3$): δ = 7.87-7.15 (m, 37H, aromatic), 5.41 (s, 1H, >C*H*Ph), 5.17-5.14 (bd, 2H, CH_2-N_{Cbz}), 5.11 (d, 1H, J_{HaHb} = 10.5 Hz, C$H_a$$H_b$-napthylmethyl), 4.93 (d, 1H, J_{HaHb} = 10.5 Hz, C$H_a$$H_b$-napthylmethyl), 4.74 (d, 1H, C*H*H, OBn), 4.67-4.65 (d, 1H, C*H*H, OBn), 4.61-4.59 (d, 2H, H-1', C*H*H, OBn), 4.48-4.69 (bd, 2H, C*H*2-NBn), 4.39 (d, 1H, C*H*H, OBn), 4.19-4.18 (m, 1H, H-1), 4.00-3.95 (m, 2H, H-6'a, H-5'), 3.90 (t, 1H, $J_{1',2'}$ = 9.5 Hz, $J_{2',3'}$ = 9.5 Hz, H-2'), 3.87-3.83 (m, 1 H, C*H*H, L), 3.78 (m, 1H, H-4), 3.71- 3.64 (m, 2H, H-6a,b), 3.47-3.37 (m, 6H, H-3, C*H*H-L, H-

6'b, H-3', H-2, H-5), 3.26-3.19 (m, 2H, CH_2-L), 3.01-2.97 (m, 1H, H-4'), 1.61-1.50 (m, 4H, 2 x CH_2-L), 1.36-1.30 (m, 2H, C*H*2-L). ^{13}C (75 MHz, $CDCl_3$): δ = 138.18, 138.14, 137.83, 137.52, 136.03, 133.50, 133.29, 129.23, 128.84, 128.77, 128.71, 128.45, 128.39, 128.22, 128.16, 128.09, 128.07, 128.00, 127.74, 127.53, 127.00, 126.41, 126.29, 126.08, 102.37 (C-1), 101.73 (>*C*HPh), 100.19 (C-1'), 81.49, 78.63, 75.55, 74.51, 73.94, 73.06, 70.19, 68.67, 68.51, 67.44, 67.39, 66.23, 63.87, 29.41, 23.42. HR- MALDI-TOF/MS (*m*/*z*) calcd for $C_{64}H_{67}N_7O_{11}$[*M*+Na]$^+$: 1132.4797; found: 1132.4797.

***N*-Benzyl-*N*-benzyloxycarbonyl-5-aminopentyl 2-azido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-β-D-mannopyranosyl-(1→4)-2-azido-6-*O*-benzyl-2-deoxy- β -D-glucopyranoside (16)**: DDQ (22 mg, 0.09 mmol) was added to compound **15** (67.0 mg, 0.06 mmol) in a mixture of dichloromethane and water (3.3 mL, 10:1, v/v) and stirred vigorously in the dark for 2 h. The reaction mixture was then quenched with an aqueous mixture of citric acid, ascorbic acid, and NaOH (0.1 mL, 1.2%, 1.0%, 0.92% w/v). The reaction mixture was diluted with ethyl acetate (15 ml) and washed with aqueous $NaHCO_3$ (sat., 5 mL). The organic solvents were dried ($MgSO_4$) and filtered, and the filtrate concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 4:1, v/v) to give **16** (0.054 g, 94%) as a clear oil. R_f = 0.40 (hexane/ethyl acetate, 4:1, v/v). $[\alpha]^{25}_{D}$ = -20.2 (c 1.24, $CHCl_3$); ^{1}H (600 MHz, $CDCl_3$): δ = 7.40-7.09 (m, 30H, aromatic), 5.48 (s, 1H, >C*H*Ph), 5.11-5.08 (bd, 2H, CH_2-N_{Cbz}), 4.77 (d, 1H, C*H*H, OBn), 4.64-4.60 (m, 2H, CH_2, OBn), 4.43-4.41 (bd, 2H, CH_2-N_{Bn}), 4.33 (s, 1H, H -1'), 4.29 (d, 1H, C*H*H, OBn), 4.23-4.21 (dd, 1H, $J_{6'a,\ 6'b} = J_{5',6'}$ = 10.8 Hz, H-6'a), 4.17-4.15 (m, 1H, H -1), 3.88 (t, 1H, $J_{3'4'} = J_{4'5'}$ = 9.0 Hz, H -4'), 3.81- 3.78 (m,1H, C*H*H-L), 3.74 (t, 1H, $J_{6'a,\ 6'b} = J_{5',6'a}$ = 10.2 Hz, H-6'b), 3.63-3.58 (m, 3H, H -6a,b, H -4), 3.46-3.38 (m, 4H, H-2', H-3', H-3, H-5), 3.36 (m, 1H, C*H*H-L), 3.24-3.18 (m, 3H, H-2, H-5', C*H*H-L), 3.13 (m, 1H,

C*H*H-L), 1.56-1.44 (m, 4H, 2 x CH_2-L), 1.32- 1.23 (m, 2H, CH_2-L). ^{13}C (75 MHz, $CDCl_3$): δ = 138.14, 137.95, 137.90, 137.17, 129.35, 128.83, 128.76, 128.68, 128.52, 128.50, 128.20, 128.14, 128.06, 127.74, 127.51, 126.22, 102.29 (C-1), 101.88 (>*C*HPh), 100.96 (C-1'), 81.12, 78.35, 76.55, 3.82, 73.53, 73.41, 73.38, 70.30, 68.13, 67.53, 67.38, 65.52, 63.60, 29.39, 23.40. HR-MALDI-TOF/MS (*m*/*z*) calcd for $C_{53}H_{59}N_7O_{11}$: 992.4171 [*M*+Na]$^+$; found: 992.4174.

***N*-Benzyl-*N*-benzyloxycarbonyl-5-aminopentyl 2-azido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-β-D-mannopyranosyl-(1→4)-[2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl-(1→3)]-2-azido-6-*O*-benzyl-2-deoxy-β-D-glucopyranoside (17)**: A mixture of **7** (0.068 g, 0.096 mmol) and **16** (0.042 g, 0.043 mmol) was co-evaporated withndry toluene (3 5 mL) and then further dried in vacuo for 4 h. The mixture was dissolved in diethyl ether and dichloromethane (4 mL, 5:1, v/v) and 4Å MS (0.18 g) was added. The mixture was stirred under an atmosphere of argon for 30 min and then cooled (-50 °C). TMSOTf (1.7 μL, 4.6 μmol) was added and the reaction mixture was allowed to reach 0 °C gradually over a period of 1 h. The reaction was quenched by the addition of pyridine (20 μL), diluted with dichloromethane (7 mL) and filtered through celite. The filtrate was washed with aqueous $NaHCO_3$ (sat., 5 mL) and the organic layer was dried ($MgSO_4$) and filtered after which the filtrate was concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/ethyl acetate 4:1, v/v) to give **17** (0.052 g, 81%) as a clear oil. R_f = 0.35 (hexane/ethyl acetate 4:1, v/v). ^{1}H (500 MHz, $CDCl_3$): δ = 7.36-7.05 (m, 45H, aromatic), 5.57 (d, 1H, $J_{1'',2''}$ = 3.5 Hz, H-1''), 5.31 (s, 1H, >C*H*Ph), 5.10-5.07 (bd, 2H, CH_2-N_{Cbz}), 4.83 (d, 1H, C*H*H, OBn), 4.75- 4.38 (m, 10H, 7 x C*H*H, OBn, H-1', CH_2-N_{Bn}), 4.42-4.38 (m, 4H, 2 x CH_2, OBn), 4.09-4.08 (m, 1H, H-1), 4.04-4.00 (m, 2H, H-2'',H-4'') 3.97-3.89 (m, 4H, H-6'a, H-3, H-4, H-3'), 3.84-3.75 (m, 2H, C*H*H-L, H-4'), 3.68-3.65 (m, 3H, H-6a,b, H-2'), 3.61-3.59 (m, 2H, H-6''a,b), 3.52-3.36 (m, 4H, H-6'b, C*H*H-L, H-2, H-5), 3.29-3.27 (m, 2H,

H -5'', H-3''), 3.19-3.12 (m, 2H, CH_2-L), 2.80-2.75 (m, 1H, H-5'), 1.55-1.44 (m, 4H, 2 CH_2-L), 1.30- 1.21 (m, 2H, CH_2-L). ^{13}C (125 MHz, $CDCl_3$): δ = 128.98, 128.05, 126.26, 102.53 (C-1), 101.56 (>*C*HPh), 97.76 (C-1'), 95.99 (C-1''), 79.01, 78.48, 77.24, 76.62, 76.00, 75.65, 74.85, 75.03, 74.50, 73.88, 73.79, 73.53, 73.35, 73.44, 73.09, 72.82, 70.08, 69.72, 69.55, 69.49, 68.28, 67.43, 67.34, 65.57, 63.36, 50.53, 29.48, 23.54, 21.27. HRMALDI-TOF/MS (*m*/*z*) calcd for $C_{87}H_{93}N_7O_{16}$[*M*+Na]$^+$: 1514.6577; found: 1514.6578.

***N*-Benzyl-*N*-benzyloxycarbonyl-5-aminopentyl 2-acetamido-3-*O*-benzyl-4,6-*O* benzylidene-2-deoxy-β-D-mannopyranosyl-(1→4)-[2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl-(1→3)]-2-acetamido-6-*O*-benzyl-2-deoxy-β-D-glucopyranoside (18)**: Compound **17** (12.0 mgs, 8.04 μmol) was dissolved in THF (0.5 mL) and H_2O (30 μL) and then PMe_3 (1M in THF, 50 μL) was added. After stirring the reaction mixture for 4-5 h, the solvents were evaporated and the residue was dissolved in pyridine (1mL) and acetic anhydride (0.2 mL) and stirring was continued for 8 h. The solvents were then removed in vacuo and the residue purified by silica gel column chromatography (MeOH/DCM 1:100, v/v) to give **18** (7.0 mgs, 58%) as a clear oil. R_f = 0.35 (MeOH/DCM 1:100, v/v). ^{1}H (500 MHz, $CDCl_3$): δ = 7.39-7.12 (m, 45H, aromatic), 6.43-6.40 (bd, 1H, N*H*Ac), 5.69-5.67 (bd, 1H, N*H*'AC), 5.34 (s, 1H, >C*H*Ph), 5.09-5.07 (bd, 3H, CH_2-N_{Cbz}, H-1''), 4.82-4.80 (d, 2H, H-1', C*H*H, OBn), 4.70-4.57 (m, 6H, 2 x CH_2, OBn, H-2', H -1), 4.47-4.34 (m, 9H, 7 x C*H*H, OBn, CH_2-N_{Bn}), 4.08-3.83 (m, 7H, H-2'', H-2, H-6'a, H-3, H-4, H-4', H-5''), 3.72-3.45 (m, 8H, H-6a,b, H-2'', H-6'b, H-5'', H-3'', H-6''a,b), 3.40-3.26 (m, 2H, CH_2-L), 3.13-3.07 (m, 3H, CH_2-L, H-5'), 1.88 (s, 3H, NHCOCH_3), 1.73 (s, 3H, NH'COCH_3), 1.47-1.42 (m, 4H, 2 x CH_2-L), 1.23-1.18 (m, 2H, CH_2-L). ^{13}C (125 MHz, $CDCl_3$): δ = 128.43, 102.31 (>*C*HPh), 100.54 (C-1), 98.59 (C-1''), 98.03 (C-1'), 79.11, 78.84, 77.39, 76.34, 75.94, 75.19, 75.02, 75.04, 74.63, 74.35, 74.23, 73.87, 73.80, 73.52, 72.68, 72.13, 72.11, 70.00, 69.61,

68.68, 67.36, 67.39, 55.62, 51.23, 47.70, 46.65, 29.80, 29.02, 24.10, 23.58. HR-MALDI-TOF/MS (*m*/*z*) calcd for $C_{91}H_{101}N_3O_{18}$: 1546.6978 $[M+Na]^+$; found: 1546.6980.

5-Aminopentyl-2-acetamido-2-deoxy-β-D-mannopyranosyl-(1→4)-[α-D-galactopyranoside-(1→3)]-2-acetamido-2-deoxy-β-D-glucopyranoside (2): Compound **18** (8.5 mg, 5.6 μmol) was dissolved in a mixture of *t*-BuOH, AcOH, and H_2O (1.5 mL, 0.2 mL, 0.05 mL, 5:10:1, v/v/v) under an atmosphere of argon. $Pd(OH)_2$ /C (15.0 mg) was added and the mixture was degassed and placed under an atmosphere of H_2 and stirred for 16 h. The reaction mixture was filtered through a polytetrafluroethylene (PTFE) filter (Fischerbrand, 0.2 μm) and the residue was washed with acetic acid (3 mL). The combined filtrates were concentrated in vacuo and the residue was purified over Iatrobeads (iPrOH/NH_4OH/H_2O 3:2:1, v/v/v) to give **2** (2.7 mgs, 73%) as a white solid. R_f = 0.25 (iPrOH/NH_4OH/H_2O 3:2:1, v/v/v). ^1H (500 MHz, $CDCl_3$): δ = 5.43 (d, 1H, $J_{1'',2''}$ = 4.0 Hz, H -1''), 4.76 (s, 1H, H-1'), 4.42-4.41 (m, 2H, H-1, H-2'), 3.93 (t, 1H, H -5''), 3.86 (m, 1H, H-4), 3.80-3.60 (m, 9H, C*H*H-L, H-6'a, H-2, H-2'', H-6a,b, H-3, H-5, H-4''), 3.49-3.38 (m, 5H, C*H*H-L, H-6'b, H-6''a,b, H-3''), 3.28-3.25(m, 1H, H-5'), 2.85(t, 2H, CH_2-L), 1.96 (s, 3H, NHCOCH_3), 1.90 (s, 3H, NH'COCH_3), 1.57-1.45 (m, 4H, 2 CH_2-L), 1.29-1.23 (m, 2H, CH_2-L). ^{13}C (125 MHz, $CDCl_3$): δ = 101.18 (C-1), 98.79 (C-1'), 98.11 (C-1''), 76.87, 74.86, 72.01, 71.12, 70.37, 69.44, 69.25, 68.95, 66.70, 60.95, 60.35, 60.32, 60.16, 54.74, 53.39. HR-MALDI-TOF/MS (*m*/*z*) calcd for C $_{27}H_{49}N_3O_{16}$: 694.3011 $[M+Na]^+$; found: 694.3012.

Reagents for conjugation and immunological evaluation: 1-Cyano-4-dimethylaminopyridinium tetrafluroborate (CDAP), bovine serum albumine (BSA), galactose (Gal), *N*-glucosamine (Glc*N*Ac), *N*-acetylmannosamine (Man*N*Ac), glucosamine ($GluNH_2$), mannosamine ($ManNH_2$), acetonitrile (HPLC grade), triethylamine (TEA), and HEPES buffer were obtained from Sigma. Keyhole Limpet Hemocyanin (KLH) was purchased from Pierce Chemicals. Trifluoracetic acid

(TFA) was obtained from Aldrich. Nanopure water was obtained from B. Braun Medical, sodium hydroxide, 50% (w/w) solution was from J. T. Baker, sodium acetate anhydrous was from Fluka, Slide- A Lyzer Dialysis Cassette (MWCO 30,000; 1-3 mL) were from Thermo Scientific, centrifugal filter devices (Centriplus YM-30,000) were from Millipore, siliconized skirted bottom tubes with screw caps were from Fisher Scientific, and Sep-Pak®. PLUS C_{18} cartridge was from Waters. The polysaccharide from *Bacillus anthracis* Sterne was isolated as reported previously (B. Choudhury, C. Leoff, E. Saile, P. Wilkins, C.P. Quinn, E. L. Kannenberg, R. W. Carlson, *J. Biol. Chem*., **2006**, *281*, 27932-27941).

CDAP-polysaccharide activation: Polysaccharide and maltoheptaose (1 mg) were dissolved in HEPES buffer (90 µL, 0.15 M; pH 7.4) and a solution of CDAP (4 mg) in acetonitrile (90 µL) was slowly added while stirring to avoid precipitation. After 30 s, aqueous triethylamine solution (120 µL, 0.3 M) was added and after another 150 s, the pH was readjusted and protein (4 mg BSA or KLH) in PBS buffer (0.1 M, pH 7.4; 100 µL and 348µL, respectively) added. After stirring at 4 °C for 18 h, the reaction was quenched by the addition of 0.5 M ethanolamine in HEPES buffer (120 µL; 0.75 M; pH 7.4). No gelling was observed indicating that no excessive cross-linking of protein with polysaccharides had occurred. The polysaccharide-BSA, polysaccharide-KLH, and maltoheptaose-BSA conjugates were dialyzed against nanopure water (2 x 3 L) at 4 °C followed by isolation using centrifugal filter devices (Centriplus YM 30,000). Briefly, a solution of the polysaccharide-protein conjugate solution (3 mL) was transferred to a centrifugal filter tube with a cellulose membrane and centrifuged at 3,000 rpm at 4 °C for 1 h followed by addition of nanopure water (2 x2 mL) and centrifuged for 2 h at 4 °C. The filtrate was removed. The concentrate (polysaccharide-protein conjugate) remaining on a cellulose membrane in centrifugal filter tube was inverted to another assembly and further centrifugation

at 2,000 rpm at 4 °C for 4 min followed by lyophilization gave polysaccharide-BSA (3.2 mg), polysaccharide-KLH (4.3 mg), and maltoheptaose-BSA (4.0 mg) conjugates as white foams. Each conjugate was dissolved in PBS buffer at a concentration of 1 mg mL^{-1} and stored at 4 °C. The amount of polysaccharide in polysaccharide-protein conjugate products was determined by HPAEC-PAD. Thus, solutions of polysaccharide-BSA, polysaccharide-KLH, maltoheptaose-BSA, and trisaccharide conjugates (50 µL) in screw-capped siliconized skirted bottom tubes were treated with 2 M aqueous TFA (200 µL) and placed in a heating block at 100 °C for 4 h to cleave all glycosidic linkages. Next, the samples were cooled and the solvents removed by centrifugal vacuum evaporation (Speedvac) at 40 °C. During the acid hydrolysis Glc*N*Ac and Man*N*Ac are quantitatively de-*N*-acetylated giving $GlcNH_2$ and $ManNH_2$, respectively. Therefore, Gal, $GlcNH_2$, and $ManNH_2$ were employed as reference compounds and treated under the same condition as described for the conjugates. The dried samples were re-dissolved in nanopure water (500 µL) and passed through a SepPak® C_{18} cartridge. Briefly, before sample loading, a SepPak®. C_{18} cartridge was activated by subsequent washing with MeOH (5 mL), water (5 mL), and aqueous acetic acid (5%, 5 mL). The hydrolyzed samples (500 µL) were consequently loaded on activated SepPak®. C_{18} cartridges and eluted with nanopure water (3 mL). The concentrates containing respective hydrolyzed monosaccharides were lyophilized and re-dissolved in nanopure water (50µL) and the resulting solutions analyzed by 817 Bioscan Metrohm HPAEC-PAD equipped with a Metrohm-Peak Gradient 709 IC Pump Module, an 812 Valve Unit with a 50 µL Rheodym loop, a 762 IC interface and an analytical (4 x 250 mm) Dionex CarboPac PA10 column with a CarboPac PA10 guard column (3 x 30 mm). A flow rate of 0.9 mL min-1 at 32 °C and the following gradient program were used: t = 0 min, E1 = 97.5 %, E2 = 2.5%; t =10 min, E1 = 97.5 %, E2 = 2.5%; t = 25 min, E1 = 97.5 %, E2 = 2.5%; t = 27 min,

E1 = 0 %, E2 = 100%; *t* = 37 min, E1 = 0%, E2 = 100%; *t* = 39 min, E1 = 97.5 %, E2 = 2.5%, *t* = 50 min, E1 = 97.5 %, E2 = 2.5%. Eluent 1 (E1) is nanopure water and eluent 2 (E2) is 200 mM NaOH. All eluents were degassed before use for 1 h. (M. R. Hardy, R. R. Townsend *Methods in Enzymology* **1994**, *230*, 208-225).

General procedure for *S*-acetylthioglycolylamido derivatization of the aminopropyl spacer: The oligosaccharide **1** (2.0 mg, 3.17 μmol) was slurried in dry DMF (300 μL) and SAMA-OPfp (1.43 mg, 4.76 μmol) was added followed by addition of DIPEA (1.6 μL, 9.51 μmol). After stirring at room temperature for 1.5 h, the mixture was concentrated, co-evaporated twice with toluene and the residue purified by size exclusion chromatography (Biogel P2 column, eluted with H_2O containing 1% *n*-butanol) to give, after lyophilization, the corresponding thioacetate (1.98 mg, 84%) as a white powder. In a similar manner, the thioacetamido derivative of compound **2** was prepared in a yield of 86%.

General procedure for *S*-deacetylation: 7% NH_3 (g) in DMF solution (200 μL) was added to the thioacetate derivative corresponding to trisaccharide **2** (1.98 mg, 2.66 μmol) 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 dried under high-vacuum and the thiol derivatized trisaccharide was then further dried in *vacuo* for 30 min and immediately used 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 BSA), deprotected just prior to conjugation as described above, was dissolved in the conjugation buffer (sodium phosphate, pH 7.2 containing EDTA and sodium azide; 100 μL) and added to a solution of maleimide activated BSA (2.4 mg)

in the conjugation buffer (200 μL). The mixture was incubated at room temperature for 2 h and then purified by a D-Salt™ Dextran de-salting column (Pierce Endogen, Inc.), equilibrated, and eluted with sodium phosphate buffer, pH 7.4 containing 0.15 M sodium chloride. Fractions containing the glycoconjugate were identified using the BCA protein assay and combined to give glycoconjugates with a carbohydrate/BSA molar ratio of 11:1 for trisaccharide **1**, and 19:1 for trisaccharide **2** as determined by quantitative monosaccharide analysis by HPAEC/ PAD and Bradford's protein assay.

Preparation of *Bacillus anthracis* Sterne 34F2 spores: Spores of *B. anthracis* Sterne 34F2 were prepared from liquid cultures of PA medium grown at 37 °C, 200 rpm for six days. Spores were washed two times by centrifugation at 10 000 *g* in cold (4 °C) sterile deionized water, purified in a 50% Reno-60 (Bracco Diagnostics Inc.) gradient (10 000 *g,* 30 min, 4 °C) and washed 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 agar plates (BD BBL). 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 x 108 CFU, were irradiated in 200-mL Sarstedt freezer tubes (Sarstedt) in a gammacell irradiator with an absorbed dose of 2 million rads. Potential residual viability after irradiation was monitored by spread-plating 10 μL aliquots of irradiated spore suspension on BHI agar plates. The plates were incubated at 37 °C for 72 h and monitored for colony growth.

Preparation of antisera: 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, irradiated spore inoculum (3 x 10^6 total spores). Rabbits were

immunized at 0, 14, 28, and 42 days. Antiserum to *B. anthracis* polysaccharide-KLH conjugate was prepared by a primary injection with polysaccharide-KLH conjugate (500 μg) and the MPL, TDM, CWS adjuvant system (0.5 mL). Booster immunizations were administered at 14, 28, and 42 days using the polysaccharide-KLH conjugate (250 μg) and the MPL, TDM, CWS adjuvant system (0.5 mL). Terminal bleeds were collected 14 days after the last immunization. The CDC animal facilities are approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). 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 antisera to saccharide conjugates was performed by enzyme-linked immunosorbent assay (ELISA). Briefly, Immulon II-HB flat bottom 96-well microtiter plates (Thermo Labsystems) were coated overnight at 4 °C with 100 μL per well of polysaccharide-BSA, **1**-BSA, **2**-BSA, or maltoheptaose-BSA conjugate at a concentration of 0.15 μg mL^{-1} of carbohydrate content, or with the carrier protein BSA by itself at corresponding protein content in coating buffer (0.2 M borate buffer, pH 8.5 containing 75 mM sodium chloride). Plates were washed in wash buffer (3 x 0.05% Tween-20 in PBS, pH 7.4) using an automatic microplate washer (DYNEX Technologies, Inc.). After blocking the plate for 1 h with blocking buffer (PBS containing 1% BSA; 200 μL/well) and washing three times in wash buffer, serial dilutions in diluent buffer (PBS, pH 7.4 containing 1% BSA and 0.5% Tween-20) of either rabbit antisera from the terminal bleed or pre-immune sera were then added (100 μL/ well) and plates were incubated for 2 h. After incubation the plates were washed three times in wash buffer and a goat anti-rabbit IgG, Fc fragment specific, horseradish peroxidase conjugated antibody (Pierce Biotechnology) was added (0.16 μg mL^{-1}; 100 μL/well) for 2 h. Plates were then washed

three times in wash buffer and ABTS (2,2'-azido-di (3-ethylbenthiazoline-6-sulfonate)) peroxidase substrate was added (100 µL/well; KPL, Kirkegaard & Perry Laboratories, Inc). Color development was stopped after 25 min by addition of ABTS peroxidase stop solution (100 µL/well; KPL). Optical density (OD) values were measured at a wavelength of 410 nm (490 nm reference filter) using a microplate reader (BMG Labtech) and reported as the means ± SD of triplicate measurements. Titers are determined by linear regression analysis, plotting dilution versus absorbance. Titers are defined as the highest dilution yielding an optical density of 0.5 or greater. To explore competitive inhibition of the binding of sera to polysaccharide-BSA conjugate by polysaccharide-BSA, **1**-BSA, and **2**-BSA, rabbit antisera were diluted in diluent buffer in such a way that, without inhibitor, expected final OD values were ca. 1. For each well 60 µL of the diluted sera were mixed in an uncoated microtiter plate with either 60 µL diluent buffer or 60 µL BSA-conjugates (polysaccharide-BSA, **1**-BSA, and **2**-BSA and as controls maltoheptaose-BSA and unconjugated BSA) in diluent buffer with a final concentration corresponding to a 0.02, 0.1, 0.4, 1.6, 6.3, 25, or 100-fold weight excess of carbohydrate compared to carbohydrate used for coating. After incubation at room temperature for 2 h, 100 µL of the mixtures were transferred to a plate coated with polysaccharide-BSA. The microtiter plates were incubated and developed as described above.

References

(1) Priest, F. G.; Bacillus subtilis and Other Gram-Positive Bacteria: Biochemistry Physiology, and Molecular Biology: 1993, p 3-16.

(2) Mock, M.; Fouet, A. *Annu. Rev. Microbiol.* **2001**, *55*, 647-671.

(3) Jernigan, J. A.; Stephens, D. S.; Ashford, D. A.; Omenaca, C.; Topiel, M. S.; Galbraith, M.; Tapper, M.; Fisk, T. L.; Zaki, S.; Popovic, T.; Meyer, R. F.; Quinn, C. P.; Harper, S.

A.; Fridkin, S. K.; Sejvar, J. J.; Shepard, C. W.; McConnell, M.; Guarner, J.; Shieh, W. J.; Malecki, J. M.; Gerberding, J. L.; Hughes, J. M.; Perkins, B. A. *Emerg. Infect. Dis.* **2001**, *7*, 933-944.

(4) Jernigan, D. B.; Raghunathan, P. L.; Bell, B. P.; Brechner, R.; Bresnitz, E. A.; Butler, J. C.; Cetron, M.; Cohen, M.; Doyle, T.; Fischer, M.; Greene, C.; Griffith, K. S.; Guarner, J.; Hadler, J. L.; Hayslett, J. A.; Meyer, R.; Petersen, L. R.; Phillips, M.; Pinner, R.; Popovic, T.; Quinn, C. P.; Reefhuis, J.; Reissman, D.; Rosenstein, N.; Schuchat, A.; Shieh, W. J.; Siegal, L.; Swerdlow, D. L.; Tenover, F. C.; Traeger, M.; Ward, J. W.; Weisfuse, I.; Wiersma, S.; Yeskey, K.; Zaki, S.; Ashford, D. A.; Perkins, B. A.; Ostroff, S.; Hughes, J.; Fleming, D.; Koplan, J. P.; Gerberding, J. L.; Investi, N. A. E. *Emerg. Infect. Dis.* **2002**, *8*, 1019-1028.

(5) Webb, G. F. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 4355-4356.

(6) Bouzianas, D. G. *Expert Rev. Anti-Infect. Ther.* **2007**, *5*, 665-684.

(7) Friedlander, A. M.; Pittman, P. R.; Parker, G. W. *JAMA* **1999**, *282*, 2104-2106.

(8) Joellenbeck, L. M.; Zwanziger, L. L.; Durch, J. S.; Strom, B. L. *The Anthrax Vaccine: Is It Safe? Does It Work?*; National Academy Press, Washington, D.C., 2002.

(9) Schneerson, R.; Kubler-Kielb, J.; Liu, T. Y.; Dai, Z. D.; Leppla, S. H.; Yergey, A.; Backlund, P.; Shiloach, J.; Majadly, F.; Robbins, J. B. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 8945-8950.

(10) Chabot, D. J.; Scorpio, A.; Tobery, S. A.; Little, S. F.; Norris, S. L.; Friedlander, A. M. *Vaccine* **2004**, *23*, 43-47.

(11) Wang, J. Y.; Roehrl, M. H. *Med. Immunol.* **2005**, *4*, 4.

(12) Daubenspeck, J. M.; Zeng, H. D.; Chen, P.; Dong, S. L.; Steichen, C. T.; Krishna, N. R.; Pritchard, D. G.; Turnbough, C. L. *J. Biol. Chem.* **2004**, *279*, 30945-30953.

(13) Werz, D. B.; Seeberger, P. H. *Angewandte Chemie, International Edition in English* **2005**, *44*, 6315-6318.

(14) Mehta, A. S.; Saile, E.; Zhong, W.; Buskas, T.; Carlson, R.; Kannenberg, E.; Reed, Y.; Quinn, C. P.; Boons, G. J. *Chem. Eur. J.* **2006**, *12*, 9136-9149.

(15) Crich, D.; Vinogradova, O. *J. Org. Chem.* **2007**, *72*, 6513-6520.

(16) Guo, H.; O'Doherty, G. A. *Angew. Chem. Int. Ed. Engl.* **2007**, *46*, 5206-5208.

(17) Saksena, R.; Adamo, R.; Kovac, P. *Bioorg. Med. Chem.* **2007**, *15*, 4283-4310.

(18) Werz, D. B.; Adibekian, A.; Seeberger, P. H. *Eur. J. Org. Chem.* **2007**, 1976-1982.

(19) Choudhury, B.; Leoff, C.; Saile, E.; Wilkins, P.; Quinn, C. P.; Kannenberg, E. L.; Carlson, R. W. *J. Biol. Chem.* **2006**, *281*, 27932-27941.

(20) Leoff, C.; Saile, E.; Sue, D.; Wilkins, P.; Quinn, C. P.; Carlson, R. W.; Kannenberg, E. L. *J. Bacteriol.* **2008**, *190*, 112-121.

(21) Peters, T. *Liebigs Ann. Chem.* **1991**, 135-141.

(22) Tanaka, H.; Iwata, Y.; Takahashi, D.; Adachi, M.; Takahashi, T. *J. Am. Chem. Soc.* **2005**, *127*, 1630-1631.

(23) Veeneman, G. H.; Vanleeuwen, S. H.; Vanboom, J. H. *Tetrahedron Lett.* **1990**, *31*, 1331-1334.

(24) Mong, T. K. K.; Lee, H. K.; Duron, S. G.; Wong, C. H. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 797-802.

(25) Gaunt, M. J.; Yu, J. Q.; Spencer, J. B. *J. Org. Chem.* **1998**, *63*, 4172-4173.

(26) Xia, J.; Abbas, S. A.; Locke, R. D.; Piskorz, C. F.; Alderfer, J. L.; Matta, K. L. *Tetrahedron Lett.* **2000**, *41*, 169-173.

(27) Gridley, J. J.; Osborn, H. M. I. *J. Chem. Soc. Perkin Trans. 1* **2000**, *10*, 1471-1491.

(28) Yu, B.; Tao, H. *J. Org. Chem.* **2002**, *67*, 9099-9102.

(29) Yu, B.; Tao, H. C. *Tetrahedron Lett.* **2001**, *42*, 2405-2407.

(30) Schmidt, R. R.; Kinzy, W. *Adv. Carbohydr. Chem. Biochem.* **1994**, *50*, 21-123.

(31) Winans, K. A.; King, D. S.; Rao, V. R.; Bertozzi, C. R. *Biochemistry (Mosc).* **1999**, *38*, 11700-11710.

(32) Classon, B.; Garegg, P. J.; Oscarson, S.; Tiden, A. K. *Carbohydr. Res.* **1991**, *216*, 187-196.

(33) Misra, A. K.; Roy, N. *J. Carbohydr. Chem.* **1998**, *17*, 1047-1056.

(34) Alper, P. B.; Hendrix, M.; Sears, P.; Wong, C. H. *J. Am. Chem. Soc.* **1998**, *120*, 1965-1978.

(35) Shafer, D. E.; Toll, B.; Schuman, R. F.; Nelson, B. L.; Mond, J. J.; Lees, A. *Vaccine* **2000**, *18*, 1273-1281.

(36) Buskas, T.; Li, Y.; Boons, G. J. *Chem. Eur. J.* **2004**, *10*, 3517-3524.

(37) Oberli, M. A.; Bindschadler, P.; Werz, D. B.; Seeberger, P. H. *Org. Lett.* **2008**, *10*, 905-908.

CHAPTER 4

SYNTHESIS OF OLIGOSACCHARIDES

DERIVED FROM THE VEGETATIVE CELL WALL OF *BACILLUS ANTHRACIS**

* Mahalakshmi Vasan, Kanar Al-Mafraji, Geert-Jan Boons.
To be submitted to *Chemistry, a European Journal*.

Abstract

Recently reported HF-PSs isolated from the vegetative cell wall of *B. anthracis* were found to be heterogeneous. To determine antigenic determinates of the polysaccharide of *B. anthracis* and to establish it as a diagnostic or vaccine candidate, we here report the chemical synthesis of various oligosaccharide derived from this polysaccharide. The oligosaccharides are synthesized with an aminopentyl spacer to facilitate conjugation to carrier proteins, which is required for immunization and ELISA.

Introduction

B. anthracis is a spore forming Gram positive bacteria. The spores of these bacteria remain dormant for several years until they find the right condition for their germination. This nature of the bacteria has enabled its use as a bioterrorism agent.[1-6] When these spores 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. The difficulty in early recognition of inhalation anthrax due to the nonspecific nature of its symptoms has led to a renewed interest in development of anthrax vaccine and diagnostics.[7] Sterile cell free vaccines containing the protective antigen (PA) component of anthrax toxin have proven safe and effective but they target only the germinated spores.[8,9] So, a novel oligosaccharide based vaccine, that will enhance our immune responses towards the spore or cell surface carbohydrate of *B. anthracis* needs to be developed. Recently, Carlson and coworkers[10] isolated and characterized a polysaccharide from cell wall of *B. anthracis*. This is species specific and differs even from that of the closely related *B. cereus* strains. This polysaccharide has a repeating trisaccharide backbone of →6)-α-D-Glc*N*Ac-(1→4)-β-D-Man*N*Ac-(1→4)-β-D-Glc*N*Ac-(1→) and the α-D-Glc*N*Ac is substituted with α-D-Gal and β-D-Gal residues at C-3 and C-4 respectively, and the β-D-GlcNAc is substituted

with α-D-Gal at C-3. There is a considerable structural heterogeneity due to the number and location of terminal Gal residues attached to the trisaccharide amino sugar backbone.

To understand the importance of these Gal residues and to determine the minimal HF-PS structural feature needed to bind and generate protective *B. anthracis* antibodies various oligosaccharides will be chemically synthesized that will consist of various combinations of these terminal Gal residues. Recently, we synthesized and evaluated the immunological activity of two trisaccharide fragment of this polysaccharide.[11] In this chapter we show the synthetic scheme for synthesis of other oligosaccharide fragments.

Results and Discussion

Chemical Synthesis

It was envisaged that the assembly of target hexasaccharide **1** and other fragments including pentasaccharides **2**, **3**, and **4** and tetrasaccharides **5**, **6**, and **7** could be achieved by first synthesizing the protected trisaccharide backbone **8** consisting of appropriately substituted orthogonal protecting groups at R_1, R_2, R_3 (**Figure 4.1**)**.** Hence, a 2+1 strategy was followed to construct the trisaccharide backbone and accordingly, donor **10** was synthesized, which will provide us with R_1 = NAP, R_3 = OAc, R_2 = R_4 = >CHPh as a set of protecting groups. However, the glycosylation reaction between donor **10** and acceptor **9** using TMSOTf as a promoter was not successful. The trichloroacetimidate[12] along with the 4,6-benzylidene acetal ring in **10** probably made the donor unsuitable for glycosylation of a less reactive C-4 hydroxyl acceptor. Considering the challenges in the formation of the 1→4 α-linkage between the GlcNAc and ManNAc, we maintained flexibility in our synthetic approach by performing a series of glycosylations on acceptor **9** with different donors (**Figure 4.2**). So, we switched to a bromide donor **11**[13], and a AgOTf[14] mediated glycosylation between **9** and **11** led to formation of the

desired product, albeit in low yield, and an unidentified side product. Also, most of the acceptor remained unreacted and its R_f-value coincided with the product allowing only partial separation.

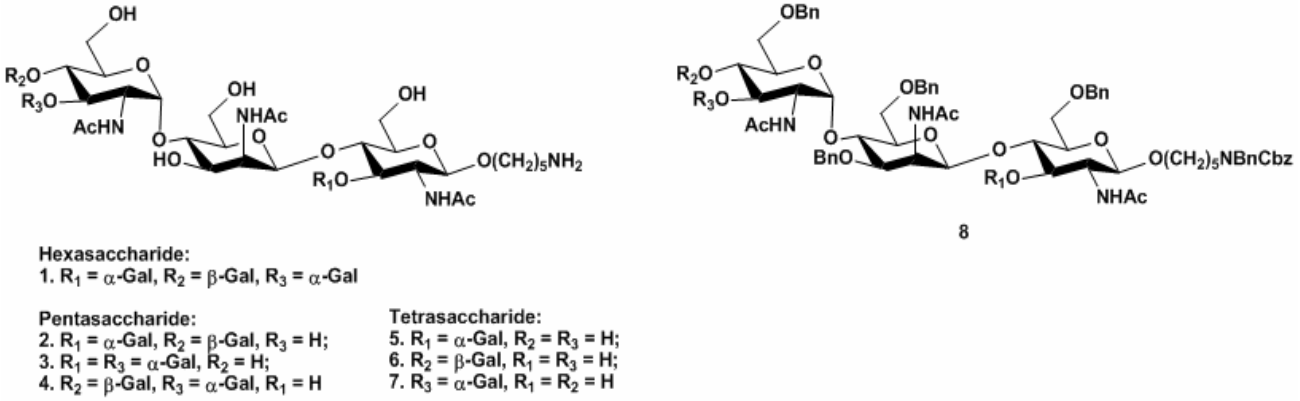


Figure 4.1. Structures of target hexasaccharide, pentasaccharides, tetrasaccharides and protected trisaccharide backbone.

Gin and cowrkers[15-17] and Van Boom and coworkers[18] has published a method involving dehydrative glycosylation for glycosylating C-4 hydroxyl of very unreactive acceptors. Our attempt to perform the dehydrative glycosylation with acceptor **9** and donor **12** with Ph_2SO and Tf_2O as promoter led to low yielding of product and, the unreacted acceptor was recovered. Again, the separation of product and the unreacted acceptor was challenging.

Various attempts to assemble the trisaccharide backbone **8** in a 2+1 strategy were not successful with the above donors. So, we then followed a 2+2 glycosylation strategy which will allow us to obtain tetrasaccharide **15** which then could be deprotected in four steps to get target compound **6**. Accordingly, we synthesized donor **13** and **14** from disaccharide **20** in two or three steps respectively (**Scheme 4.1**). A NIS-TMSOTf[19] mediated glycosylation between donor **18**[20,21] and acceptor **19** afforded disaccharide **20** in a 73 % yield.

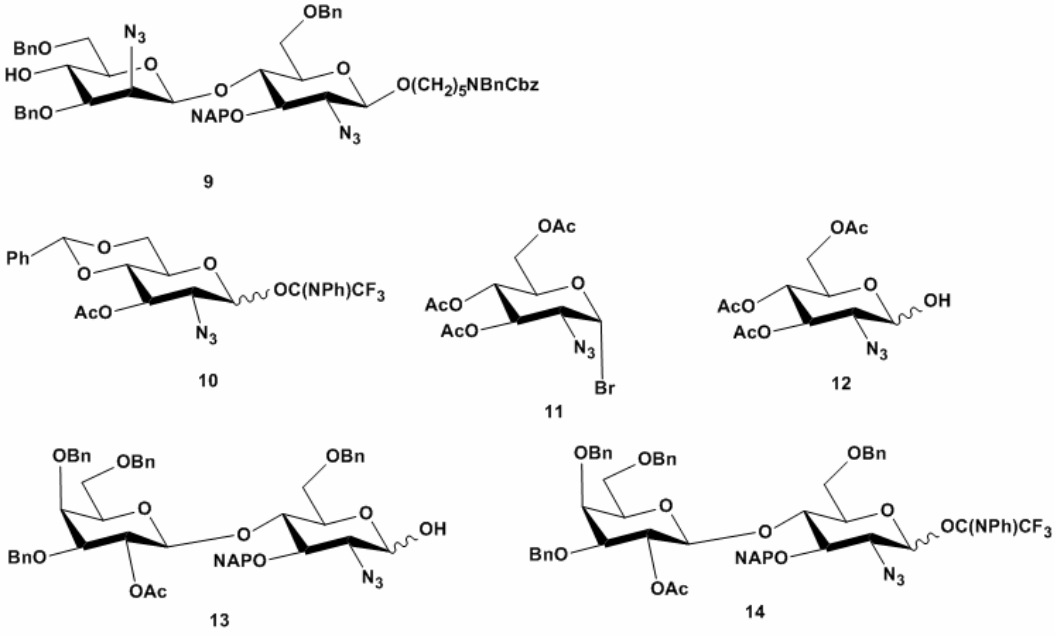
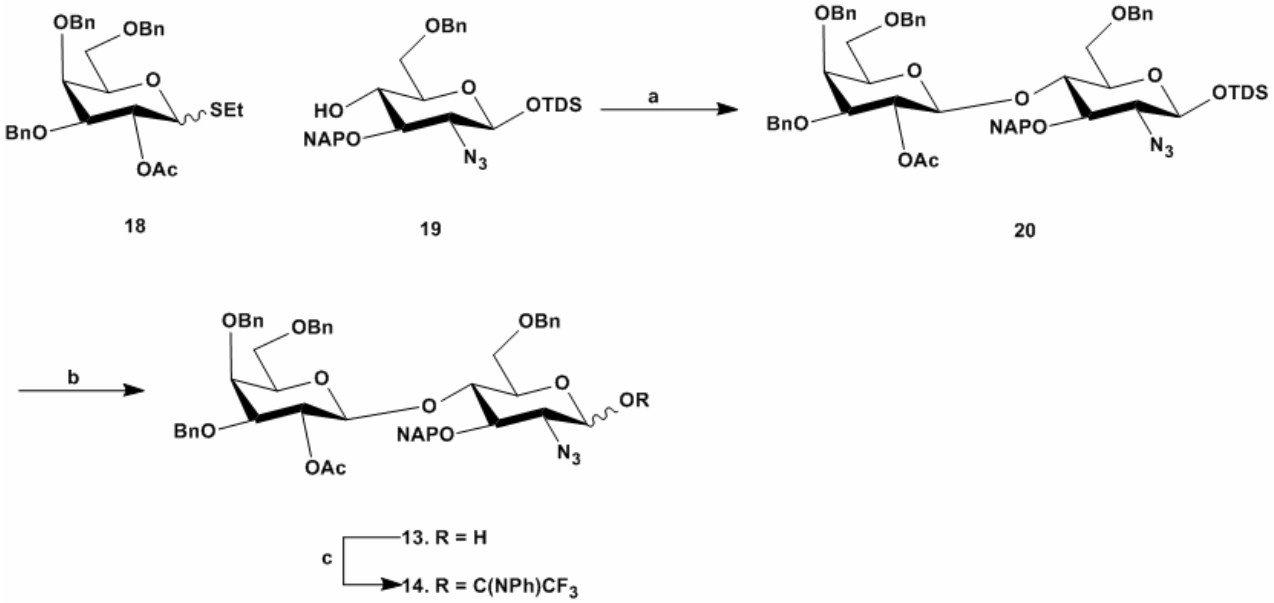


Figure 4.2. Structure of donors and acceptor.

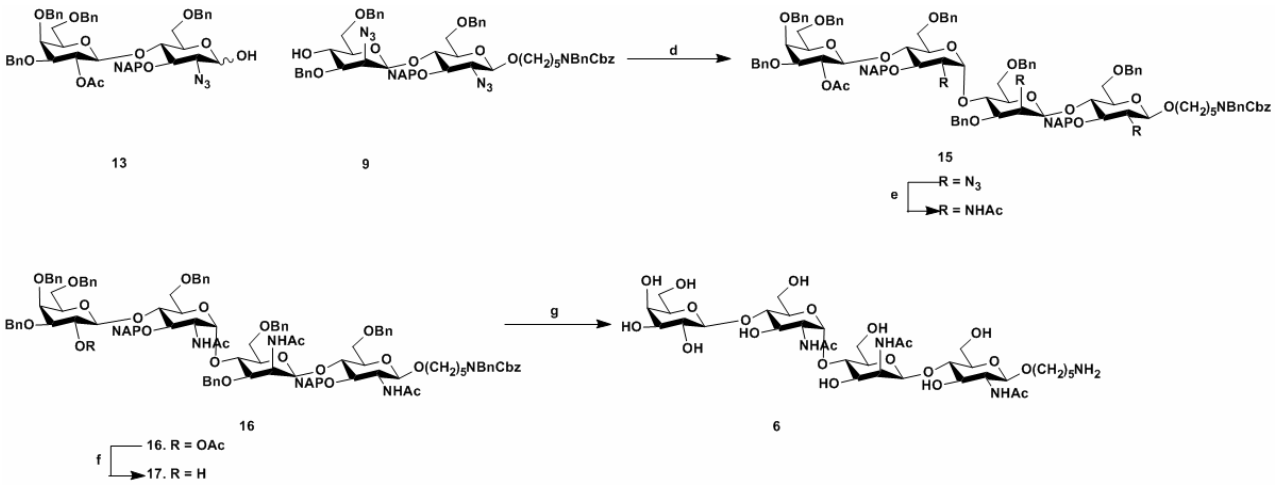
Then the anomeric TDS was removed using HF/Pyr to afford **13** in an excellent yield of 96%. The resulting compound **13** was then used as a donor for dehydrative glycosylation or converted to *N*-phenyl trifluroacetimidate **14** in 89% yields.



Scheme 4.1. *Reagents and conditions*: a) NIS, TMSOTf, 4Å MS, DCM, 0 °C, 73%; b) HF/Pyr, THF, 96%; c) $ClC(NPh)CF_3$, $CsCO_3$, DCM, 89%.

Our initial attempts to glycosylate acceptor **9** with disaccharide donor **14** in presence of TMSOTf or $BF_3.OEt_2$[22] as a promoter was unsuccessful. Most of the acceptor was recovered, probably due to the very reactive nature of donor **14** compared to the acceptor **9**. However, the use of donor **13** with acceptor **9** gave α-glycoside selectively in a modest yield of 30%, of the tetrasaccharide **15** (**Scheme 4.2**), although no acceptor or donor was recovered.

A scale up or replacing certain protecting groups in the donor might improve the yield of the glycosylation. Thus, the tetrasaccharide **15** was first converted to **16** using PMe_3[23] followed by acetylation of the free amine in a 70% yield. The acetylester of **16** was then saponified with sodium methoxide. The resulting compound **17** was then subjected to hydrogenation conditions to remove benzyl and naphthyl ethers to afford **6**. The synthesis of other oligosaccharides is in progress.



Scheme 4.2. *Reagents and conditions*: d) Ph_2SO, Tf_2O, DTBMP, DCM, 4Å MS, -60 °C-RT, 30%; e) PMe_3, NaOH, THF, then Ac_2O, Pyr, 70%; f) NaOMe, MeOH, 97%; g) $Pd(OH)_2$, t-BuOH, HOAc, H_2O, H_2, 85%.

Conclusion

We have successfully synthesized one of the tetrasaccharide **6** in moderate yield and we still need to find a convergent approach to synthesis the tetrasaccharides, pentasaccharides and hexasaccharide. Few more trials need to be performed to improve the existing yields of α-glycosylation. All the synthetic compounds will be equipped with an aminopentyl spacer to facilitate conjugation to the carrier proteins like Keyhole Limpet Hemocyanin (KLH) or bovine serum albumin (BSA). Serum antibodies of rabbits immunized with a live or irradiated spores of *B. anthracis* will be used to see whether they can recognize the synthetic compounds.

Acknowledgement**:** This research was supported by the Institute of General Medicine of the National Institutes of Health, grant M065248 (G.J.B.) and NIAID, grant AI059577 (R.W.C.).

Experimental Section

^{1}H NMR spectra were recorded in $CDCl_3$ or D_2O on a 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 Varian Merc-300 spectrometer, respectively by 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 assignment of the products. Mass spectra were obtained on Applied Biosystems Voyager DE-Pro MALDI-TOF (no calibration) and Bruker DALTONICS 9.4T (FTICR, external calibration with BSA). Chemicals were purchased from Aldrich or Fluka and used without further purification. DCM, toluene were distilled from calcium hydride. Aqueous solutions are saturated unless otherwise specified. Molecular sieves were activated at 350 °C 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 cerium ammonium molybdate solution. Silica gel (Merck, 70-230 mesh) was used for chromatography. L denotes spacer.

Dimethylthexylsilyl 2-*O*-acetyl-3,4,6-*O*-tribenzyl-β-D-galactopyranosyl-(1→4)-2-azido-6-*O*-benzyl-2-deoxy-3-(2-napthylmethyl)-β-D-glucopyranoside (20): Galactose donor (0.3 g, 0.56 mmol) and glucose acceptor (0.3 g, 0.52 mmol) were mixed and co-evaporated with dry toluene (5 mL) and dried under *vacuo* for 3 h. The dried compound was dissolved in dry DCM (5 mL) and 4Å MS was added. The above mixture was stirred under argon for 30 min and then cooled (0 °C). NIS (0.17 g, 0.56 mmol) was added followed by TMSOTf (13.6 µL, 0.056 mmol) were added and the reaction was stirred for 10 min. The reaction was then quenched with pyridine (50 µL), diluted with DCM (5 mL), and filtered through celite. The filtrate was washed with aqueous NaHCO3 (sat., 7 mL) and the organic layer was dried (MgSO4), filtered, and the filtrate was concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/ethyl acetate, 4:1, v/v) to give **8** (0.40 g, 73%) as a clear oil. R_f = 0.3 (hexane/ethyl acetate, 4:1, v/v). ^{1}H (500 MHz, $CDCl_3$) δ 7.59-6.85 (m, 27H, aromatic), 5.19 (t, 1H, H-$2_{\beta Gal}$, $J_{1,2}$ = $J_{2,3}$ = 9.25 Hz), 4.98 (d, 1H, C*H*H-naphthylmethyl), 4.76 (d, 1H, C*H*H-OBn), 4.67 (d, 1H, CH*H*-naphthylmethyl), 4.48 (t, 1H, 2 x C*H*H-OBn), 4.35-4.25 (m, 5H, 3 x C*H*H-OBn, H-1_{Gluc}, H-$1_{\beta Gal}$), 3.86-3.78 (dd, 2H, CH_2-OBn), 3.75-3.71 (m, 2H, H-4_{Gal}, H-3_{Glu}), 3.55-3.45 (m, 2H, H-6Glu), 3.2-3.10 (m, 5H, H-3_{Gal}, H-2_{Glu}, H-6_{Gal}, H-5_{Glu}, H-5_{Gal}), 3.02-2.98 (m, 1H, H-6_{Gal}), 1.78 (3H, COCH_3), 1.51-1.46 (m, 1H, C*H*-OTDS), 0.72-0.70 (m, 12H, 4 x CH_3), 0.0 (m, 6H, 2 x CH_3). ^{13}C (75 MHz, $CDCl_3$) δ 172.56, 141.95, 141.38, 141.23, 141.11, 139.54, 136.49, 136.11, 131.71, 131.61, 131.57, 131.52, 131.43, 131.25, 131.12, 130.94, 130.90, 130.88, 130.85, 130.80, 130.70, 130.51, 129.55, 129.47, 129.55, 128.86, 128.66, 104.07(C-1_{Gal}), 100.06 (C-1_{Glu}), 84.11,

83.69, 78.31, 78.27, 77.82, 76.78, 76.51, 76.40, 75.81, 75.23, 74.95, 71.76, 71.19, 71.10, 37.18, 28.06, 24.25, 23.25, 23.11, 21.72, 21.61, 1.12, 0.00. HR-MALDI-TOF/MS (*m*/*z*) calcd for $[C_{61}H_{73}N_3O_{10}SiNa]^+$ = 1058.4963, found = 1058.4967

***N*-Phenyl trifluoroacetimidate 2-*O*-acetyl-3,4,6-*O*-tribenzyl-β-D-galactopyranosyl-(1→4)-2-azido-6-*O*-benzyl-2-deoxy-3-(2-napthylmethyl)-β-D-glucopyranoside (14)**: Compound **20** (0.36 g, 0.34 mmol) was dissolved in THF (3 mL) and pyridine (0.5 mL) was added followed by HF/Pyridine (1.5 mL, 65-70%). The reaction mixture was stirred for 14 h and diluted with ethylacetate (6 mL), washed with $NaHCO_3$ (Sat. 7 mL) and water (7 mL). The organic layer was dried over $MgSO_4$, evaporated under *vacuo,* and the residue was purified by silicagel column chromatography (hexane/ethyl acetate, 3:1, v/v) to afford **13** (0.3 g, 96%) as a white solid. R_f = 0.2 (hexane/ethyl acetate, 3:1, v/v). To compound **13** (0.38, 0.42 mmol), in DCM (5 mL), $ClC(NPh)CF_3$ (0.27 g, 0.84 mmol) and $CSCO_3$ (0.27 g, 0.84 mmol) were added. The reaction mixture was stirred for 3 h at room temperature, diluted with DCM (5 mL) and filtered through celite. The filtrate was evaporated under *vacug* and the residue purified by silicagel column chromatography (hexane/ethyl acetate, 5:1, v/v) to afford **14** as a white solid (0.4 g, 89%). R_f = 0.5 (hexane/ethyl acetate, 3:1, v/v). ^{1}H (500 MHz, $CDCl_3$) δ 7.81-6.83 (m, 32H, aromatic), 5.44-5.31 (m, 2H, H-2, H-1_{Glu}), 5.24 (d, 1H, C*H*H-naphthylmethyl), 4.99-4.96 (m, 1H, C*H*H-OBn), 4.90-4.87 (m, 1H, C*H*H-naphthylmethyl), 4.73-4.66 (m, 2H, 2 x C*H*H-OBn), 4.55-4.43 (m, 4H, 3 x C*H*H-OBn, H-$1_{\beta Gal}$), 4.08-3.91 (m, 6H, CH_2-OBn, H-4', H-3), 3.82-3.50 (m, 3H, H-6_{Glu}, H-5), 3.36-3.32 (m, 3H, H-2, H-6_{Gal}, H-3'), 3.27-3.12 (m, 2H, H-6_{Gal}, H-5'), 1.97 (m, 3H, COCH_3). ^{13}C (150 MHz, $CDCl_3$) δ 128.86, 128.73, 128.59, 128.31, 128.23, 128.14, 127.98, 127.97, 127.91, 127.89, 127.81, 127.72, 127.53, 126.96, 126.62, 125.95, 119.59, 100.89 (C-1_{Gal}), 81.16, 80.67,

78.39, 76.05, 75.91, 75.68, 75.02, 73.85, 73.67, 73.58, 72.84, 72.26, 72.20, 68.10, 67.46, 67.30, 65.01, 62.73, 21.54

***N*-Benzyl-*N*-benzyloxycarbonyl-5-aminopentyl 2-azido-3-*O*-benzyl-6-*O*-benzyl-2-deoxy-β-D-mannopyranosyl-(1→4)-2-azido-6-*O*-benzyl-2-deoxy-3-(2-napthylmethyl)-β-D-glucopyranoside (9)**: To a solution of *N*-benzyl-*N*-benzyloxycarbonyl-5-aminopentyl 2-azido-3-*O*-benzyl-4,6-benzylidene-2-deoxy-β-D-mannopyranosyl-(1→4)-2-azido-6-*O*-benzyl-2-deoxy-3-(2-napthylmethyl)-β-D-glucopyranoside (0.64 g, 0.57 mmol) in dry DCM (7.0 mL), 4Å MS was added and the mixture stirred under argon for 30 min. The temperature was reduced (-78° C) followed by sequential addition of Et_3SiH (0.23 mL, 1.4 mmol) and TfOH (0.13 mL, 1.4 mmol). The reaction mixture was then quenched after 30 min with Et_3N (0.1 mL) and MeOH (1.0 mL) and filtered through celite. The mixture was washed with $NaHCO_3$ (Sat. 10.0 mL), water (10.0 mL) and the crude product was purified by silicagel chromatography (4/1 v/v hexane/ethylacetate) to afford **9** (0.5 g, 78%) as a colorless syrup. R_f = 0.25 (4/1 v/v hexane/ethylacetate). ^1H (500 MHz, $CDCl_3$) δ 7.86-7.13 (m, 32H, aromatic), 520-5.16 (m, 3H, C*H*$_2$-N_{Cbz}, C*H*H-naphthylmethyl), 4.97 (d, 1H, C*H*H-naphthylmethyl), 4.68 (d, 1H, C*H*H-OBn), 4.61 (s, 1H, H-$1_{\beta Man}$), 4.58-4.45 (m, 5H, C*H*$_2$-N_{Bn}, 3 x C*H*H-OBn), 4.23-4.18 (m, 3H, C*H*$_2$-OBn, H-$1_{\beta Gluc}$), 3.97 (t, 1H, $J_{3,4} = J_{4,5}$ = 9.25 Hz, H-4), 3.89-3.86 (m, 1H, C*H*H-L), 3.82-3.69 (m, 4H, H-2', H-4', H-$6_{\beta Glu}$), 3.48-3.38 (m, 6H, C*H*H-L, H-6'$_{\beta Man}$, H-2, H-3, H-3'), 3.3-3.28 (m, 1H, C*H*H-L), 3.23-3.15 (m, C*H*H-L, H-5, H-5'), 2.82 (s, 1H, C-4O*H*), 1.64-1.54 (m, 6H, 2 x C*H*$_2$-L), 1.38-1.34 (m, 2H, C*H*$_2$-L). ^{13}C (150 MHz, $CDCl_3$) δ 138.23, 138.16, 137.96, 137.87, 137.06, 136.29, 136.22, 133.50, 133.46, 133.19, 128.93, 128.80, 128.76, 128.68, 128.58, 128.52, 128.39, 128.36, 128.25, 128.23, 128.20, 128.16, 128.09, 128.06, 127.98, 127.91, 127.91, 127.90, 127.77, 127.71, 127.64, 127.51, 126.75, 126.69, 126.32, 126.20, 125.97, 102.35 (C-$1_{\beta Man}$), 99.75 (C-

$1_{\beta Glu}$), 81.41, 80.52, 75.21, 74.50, 74.42, 73.89, 73.77, 72.37, 71.19, 70.16, 69.45, 68.78, 67.38, 66.38, 61.70, 50.76, 50.45, 47.31, 46.39, 29.42, 28.08, 27.65, 23.42. HR-MALDI-TOF/MS (*m*/*z*) calcd for $[C_{64}H_{69}N_7O_{11}Na]^+$ = 1134.4953, found = 1134.4956

***N*-Benzyl-*N*-benzyloxycarbonyl-5-aminopentyl 2-*O*-acetyl-3,4,6-*O*-tribenzyl-β-D-galactopyranosyl-(1→4)-2-azido-6-*O*-benzyl-2-deoxy-3-(2-napthylmethyl)-α-D-glucopyranosyl-(1→4)-2-azido-3-*O*-benzyl-6-*O*-benzyl-2-deoxy-β-D-mannopyranosyl-(1→4)-2-azido-6-*O*-benzyl-2-deoxy-3-(2-napthylmethyl)-β-D-glucopyranoside (15)**: To a solution of donor **13** (53.2 mg, 0.06 mmol) in dry DCM (1.5 mL) was added 4Å MS, Ph_2SO (27.0 mg, 0.13 mmol) and DTBMP (23.8 mg, 0.16 mmol). The mixture was stirred under argon for 1.5 h and cooled (-60 °C) followed by addition of Tf_2O (11.7 µL, 0.07 mmol). The reaction mixture was stirred at this temperature for an hour and then allowed to reach -40 °C over a 20 min period followed by addition of a solution of acceptor **9** (50.0 mg, 0.05 mmol) in DCM (1.0 mL). The reaction mixture was then allowed to warm to room temperature, stirred overnight and quenched with NEt_3 (50µL). The mixture was diluted with DCM (5.0 mL), washed with $NaHCO_3$ (5.0 mL), water (5.0 mL). The organic layer was dried over $MgSO_4$, evaporated under *vacuo*, and the resulting crude mixture was purified by silicagel column chromatography (tolene/ethylacetate, 5/1 v/v) to afford (25 mg, 30%) **15** as a clear oil. R_f = 0.4 (tolene/ethylacetate, 5/1 v/v). ^1H (500 MHz, $CDCl_3$) δ 7.71-6.83 (m, 59H, aromatic), 5.54 (d, 1H, $J_{1,2}$ = 3.5 Hz, H-$1\alpha_{Glu}$), 5.24-5.19 (d, 3H, H-$2_{\beta Gal}$, 2 x C*H*H-naphthylmethyl), 5.11-5.08 (d, 3H, 2 x C*H*H-naphthylmethyl, CH_2-N_{Cbz}), 4.91-4.84 (d, 1H, C*H*H-OBn), 4.69 (d, 1H, C*H*H-OBn), 4.62 (d, 1H, , C*H*H-OBn), 4.55-4.53 (m, 2H, C*H*H-OBn, H-1_{Man}), 4.48-4.30 (m, 8H, 3 x CH_2-OBn, CH_2-N_{Bn}), 4.14-4.11(m, 3H, H-1_{Gal}, H-1_{Gluc}, C*H*H-OBn), 4.04-3.97 (m, 2H), 3.94-3.88 (m, 2H), 3.83-3.74 (m, 5H), 3.66-3.62 (m, 3H), 3.46-3.40 (m, 9H), 3.19-3.07 (m, 8H), 3.02-3.00 (dd, 1H),

1.72 (s, 3H, COCH_3), 1.55-1.45 (m, 4H, 2 x CH_2-L), 1.28-1.20 (m, 2H, CH_2-L). ^{13}C (150 MHz, $CDCl_3$) δ 168.17, 166.37, 137.74, 137.34, 137.04, 136.94, 136.89, 136.89, 136.78, 136.58, 135.90, 135.28, 134.94, 132.25, 132.17, 131.86, 131.82, 128.01, 127.66,127.38, 127.31, 127.25, 127.19, 127.16, 127.14, 127.08, 127.02, 126.92, 126.87, 126.83, 126.80, 126.75, 126.71, 126.67, 126.62, 126.54, 126.46, 126.39, 126.29, 126.17, 125.38, 125.32, 124.88, 124.87, 124.63, 124.56, 124.39, 124.27, 115.17, 101.08 (C-$1_{\beta\text{-Glu}}$), 99.51 (C-$1_{\beta\text{-Gal}}$), 98.14 (C-$1_{\beta\text{-Man}}$), 96.27 (C-$1_{\alpha\text{-Glu}}$), 81.47, 79.79, 79.38, 76.59, 75.84, 75.69, 75.46, 74.62, 73.98, 73.84, 73.55, 73.18, 72.75, 72.56, 72.40, 72.22, 72.07, 71.60, 70.79, 70.67, 70.65, 70.00, 68.87, 68.48 67.91, 67.57, 66.77, 66.13, 65.90, 65.17, 61.78, 60.12, 49.49, 49.20, 46.07, 45.13, 29.13, 28.17, 26.83, 26.38, 22.16, 19.85. HR-MALDI-TOF/MS (*m/z*) calcd for $[C_{117}H_{122}N_{10}O_{21}Na]^+$ = 2025.8684, found = 2025.8687

***N*-Benzyl-*N*-benzyloxycarbonyl-5-aminopentyl 2-*O*-acetyl-3,4,6-*O*-tribenzyl-β-D-galactopyranosyl-(1→4)-2-acetamido-6-*O*-benzyl-2-deoxy-3-(2-napthylmethyl)-α-D-glucopyranosyl-(1→4)-2-acetamido-3-*O*-benzyl-6-*O*-benzyl-2-deoxy-β-D-mannopyranosyl-(1→4)-2-acetamido-6-*O*-benzyl-2-deoxy-3-(2-napthylmethyl)-β-D-glucopyranoside (16)**: Compound **15** (15.0 mg, 7.5 μmol) was dissolved in THF (0.5 mL) and PMe_3 (0.5 mL, 1 M solution in THF) and 0.1 N NaOH (0.4 mL) were added. After stirring the reaction mixture for 5 h, the solvents were evaporated and the residue was dissolved in pyridine (1.5 mL) and acetic anhydride (1.0 mL) and stirring was continued for 8 h. The solvents were then removed in *vacuo* and the residue purified by silica gel column chromatography $CHCl_3$/MeOH 100:2, v/v) to give **16** (11.0 mg, 71%) as a clear oil. R_f = 0.2 ($CHCl_3$/MeOH 100:2, v/v). ^{1}H (500 MHz, $CDCl_3$) δ 7.75-6.92 (m, 59H, aromatic), 6.55 (d, 1H, $J_{\text{NHAc,2 }\alpha\text{-Glu}}$ = 9.5 Hz, N*H*Ac$_{\alpha\text{-Glu}}$), 5.90 (d, 1H, $J_{\text{NHAc,2}\beta\text{-Man}}$ = 10.0 Hz, N*H*Ac$_{\beta\text{-Man}}$), 5.69 (d, 1H, $J_{\text{NHAc,2 }\beta\text{-Glu}}$ = 7.5 N*H*Ac$_{\beta\text{-Glu}}$), 5.31 (dd, 1H, $J_{1,2}$ = $J_{2,3}$ = 8.25 Hz, H-2_{Gal}), 5.09-5.03 (m, 4H, CH_2-N_{Cbz}, 2 x C*H*H-naphthylmethyl), 4.92 (d, 2H, 2 x

CH*H*-naphthylmethyl), 4.85 (d, 1H, C*H*H-OBn), 4.79 (d, 1H, $J_{1,2}$ = 3.0 Hz, H-$1_{\alpha\text{-Glu}}$), 4.72-4.52 (m, 9H, H-2, H-$1_{\beta\text{-Man}}$, H-$1_{\beta\text{-Glu}}$, 6 x C*H*H-OBn), 4.46-4.31 (m, 9H, H-$1_{\beta\text{-Gal}}$, CH_2-N_{Bn}, 6 x C*H*H-OBn), 4.25 (d, 1H, C*H*H-OBn), 4.20 (dd, 1H, $J_{1,2}$ = $J_{2,3}$ = 3.5 Hz, H-$2_{\alpha\text{-Glu}}$), 4.09 (d, 1H, C*H*H-OBn), 3.89 (m, 2H), 3.89-3.80 (m, 4H), 3.69-3.53 (m, 8H), 3.47-3.42 (m, 3H), 3.28-3.17 (m, 5H), 3.19-3.03 (m, 4H), 2.90 (bd, 1H). 1.89 (s, 3H, COCH_3), 1.77 (bs, 3H, NHCOCH_3), 1.60-1.53 (bd, 6H, 2 x NHCOCH_3), 1.45-1.35 (m, 4H, 2 x CH_2-L), 1.26-1.17 (m, 2H, CH_2-L). ^{13}C (150 MHz, $CDCl_3$) δ 169.58, 169.52, 168.49, 137.78, 137.01, 136.99, 136.81, 136.74, 136.48, 135.91, 135.25, 132.28, 132.22, 131.80, 131.70, 128.56, 128.01, 127.68, 127.50, 127.44, 127.40, 127.38, 127.27, 127.19, 127.09, 127.00, 126.90, 126.87, 126.80, 126.77, 126.72, 126.67, 126.61, 126.57, 126.53, 126.51, 126.47, 126.28, 126.16, 125.23, 124.92, 124.72, 124.61, 124.58, 124.43, 124.31, 124.27, 99.97 (C-$1_{\alpha\text{-Glu}}$), 99.10 (C-$1_{\beta\text{-Glu, }\beta\text{-Man}}$), 98.35 (C-$1_{Gal}$), 79.32, 78.66, 77.90, 73.95, 73.53, 73.20, 73.03, 72.44, 72.30, 72.25, 72.11, 72.02, 71.43, 71.32, 71.13, 70.61, 70.46, 67.99, 67.18, 66.92, 66.72, 66.12, 51.87, 49.39, 49.23, 48.12, 46.22, 28.67, 28.06, 26.78, 26.22, 22.33, 22.14, 20.96, 20.08. HR-MALDI-TOF/MS (*m*/*z*) calcd for $[C_{123}H_{134}N_4O_{24}Na]^+$ = 2073.9286, found = 2073.9289

***N*-Benzyl-*N*-benzyloxycarbonyl-5-aminopentyl 3,4,6-*O*-tribenzyl-β-D-galactopyranosyl-(1→4)-2-acetamido-6-*O*-benzyl-2-deoxy-3-(2-napthylmethyl)-α-D-glucopyranosyl-(1→4)-2-acetamido-3-*O*-benzyl-6-*O*-benzyl-2-deoxy-β-D-mannopyranosyl-(1→4)-2-acetamido-6-*O*-benzyl-2-deoxy-3-(2-napthylmethyl)-β-D-glucopyranoside (17):** Compound **16** (11.0 mg, 5.4 μmol) was dissolved in a mixture of methanol and DCM (2 mL, 3:1, v/v) and sodium metal (2 mg) was added. The reaction mixture was stirred for 18 h and then neutralized with weak acid resin (Amberlite IRC-50) and filtered. The filtrate was concentrated in *vacuo* and the residue was purified by silica gel column chromatography ($CHCl_3$/MeOH 100:2, v/v) to give **17** (10.4 mg,

97%) as a clear oil. R_f = 0.2 ($CHCl_3$/MeOH 100:2, v/v). ^1H (500 MHz, $CDCl_3$) δ 7.71-6.98 (m, 59H, aromatic), 6.55 (d, 1H, N*H*Ac $_{\alpha\text{-Glu}}$), 5.97 (d, 1H, N*H*Ac $_{\beta\text{-Man}}$), 5.75 (d, 1H, N*H*Ac $_{\beta\text{-Glu}}$), 5.09-4.98 (m, 3H), 4.93-4.91 (bd, 1H), 4.79-4.77 (m, 2H), 4.73-4.58 (m, 7H), 4.55-4.34 (m, 10H), 4.25-4.21 (m, 2H), 4.03-3.97 (m, 4H), 3.87-3.84 (m, 2H), 3.80-3.77 (m, 2H), 3.71-3.58 (m, 5H), 3.48-3.44 (m, 2H), 3.38-3.30 (m, 2H), 3.25-3.07 (m, 6H), 2.97 (m, 1H), 2.87-2.86 (m, 1H), 1.76 (bs, 3H, NHCOCH_3), 1.61 (bs, 3H, NHCOCH_3), 1.54 (bs, 3H, NHCOCH_3),1.45-1.39 (m, 4H, 2 x CH_2-L), 1.21-1.12 (m, 2H, CH_2-L). ^{13}C (150 MHz, $CDCl_3$) δ 169.59, 169.46, 169.36, 137.86, 137.09, 136.82, 136.81, 136.75, 136.40, 135.67, 135.55, 135.28, 132.27, 132.22, 131.78, 131.72, 128.51, 127.69, 127.55, 127.51, 127.45, 127.43, 127.34, 127.28, 127.19, 127.15, 127.11, 126.97, 126.94, 126.92, 126.87, 126.85, 126.80, 126.79, 126.74, 126.70, 126.68, 126.66, 126.64, 126.61, 126.57, 126.49, 126.41, 126.28, 126.16, 125.27, 124.91, 124.75, 124.58, 124.42, 124.34, 102.64 (C-1_{Gal}), 99.27 (C-$1_{\alpha\text{-Glu}}$), 99.08 (C-$1_{\beta\text{-Glu}}$), 98.91 (C-$1_{\beta\text{-Glu}}$), 98.50 (C-1_{Man}), 80.93, 78.83, 78.74, 75.94, 74.55, 73.82, 73.50, 73.05, 73.02, 72.97, 72.30, 72.22, 72.19, 71.61, 71.07, 70.84, 70.52, 68.40, 68.00, 67.49, 67.03, 66.94, 66.12, 52.13, 49.38, 49.21, 48.06, 46.22, 45.03, 28.67, 28.63, 28.33, 28.08, 27.83, 26.76, 26.23, 22.35, 22.13, 21.66, 21.08. HR-MALDI-TOF/MS (*m/z*) calcd for $[C_{121}H_{132}N_4O_{23}Na]^+$ = 2031.9180, found = 2031.9181

5-Aminopentyl β-D-galactopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-β-D-mannopyranosyl-(1→4)-β-D-glucopyranoside (8): Compound **17** (10.4 mg, 5.2 μmol) was dissolved in a mixture of *t*-BuOH, AcOH, and H_2O (1.5 mL, 0.2 mL, 0.05 mL, 5:10:1, *v/v/v*) under an atmosphere of argon. $Pd(OH)_2$/C (18.0 mg) was added and the mixture was degassed and placed under an atmosphere of H_2 and stirred for 48 h. The reaction mixture was filtered through a polytetrafluroethylene (PTFE) filter (Fischerbrand, 0.2 μm) and the residue was washed with acetic acid (3 mL). The combined filtrates were concentrated in *vacuo* and the

residue was purified over Iatrobeads (iPrOH/NH_4OH/H_2O 3:2:1, v/v/v) to give **4** (4.0 mg, 85%) as a white solid. R_f = 0.25 (iPrOH/NH_4OH/H_2O 3:2:1, v/v/v). ^1H (500 MHz, $CDCl_3$) δ 5.17 (d, 1H, $J_{1,2}$ = 3.5 Hz, H-$1_{\alpha Glu}$), 4.77 (s, 1H, H-$1_{\beta Man}$), 4.39-4.33 (m, 3H, H-$1_{\beta Gal}$, H-$1_{\beta Glu}$, H-2), 3.96-3.93 (dd, 2H), 3.86-3.72 (m, 8H), 3.65-3.54 (m, 8H), 3.50-3.37 (m, 5H), 3.29 (s,1H), 3.03 (t, 1H, C*H*H-L), 2.86 (t, 1H, C*H*H-L), 1.94-1.91 (m, 9H, 3 x NHCOC*H*$_3$), 1.67-1.54 (m, 4H, 2 x CH_2-L), 1.30-1.27 (m, 2H, CH_2-L). ^{13}C (150 MHz, $CDCl_3$) δ 103.03, 101.32, 99.42, 97.91, 79.04, 78.64, 75.56, 75.24, 74.61, 73.18, 72.86, 72.63, 71.60, 71.12, 70.25, 69.86, 69.46, 68.75, 61.70, 61.23, 60.75, 60.54, 60.35, 60.04, 55.55, 55.45, 54.00, 44.78, 39.50, 28.12, 26.68, 22.37, 21.57. HR-MALDI-TOF/MS (*m*/*z*) calcd for $[C_{35}H_{62}N_4O_2Na]^+$ = 897.3805, found = 897.3807

References

(1) Christopher, G. W.; Cieslak, T. J.; Pavlin, J. A.; Eitzen, E. M., Jr. *JAMA* **1997**, *278*, 412-417.

(2) Abramova, F. A.; Grinberg, L. M.; Yampolskaya, O. V.; Walker, D. H. *Proc. Natl. Acad. Sci. U. S. A.* **1993**, *90*, 2291-2294.

(3) Takahashi, H.; Keim, P.; Kaufmann, A. F.; Keys, C.; Smith, K. L.; Taniguchi, K.; Inouye, S.; Kurata, T. *Emerg. Infect. Dis.* **2004**, *10*, 117-120.

(4) Jernigan, D. B.; Raghunathan, P. L.; Bell, B. P.; Brechner, R.; Bresnitz, E. A.; Butler, J. C.; Cetron, M.; Cohen, M.; Doyle, T.; Fischer, M.; Greene, C.; Griffith, K. S.; Guarner, J.; Hadler, J. L.; Hayslett, J. A.; Meyer, R.; Petersen, L. R.; Phillips, M.; Pinner, R.; Popovic, T.; Quinn, C. P.; Reefhuis, J.; Reissman, D.; Rosenstein, N.; Schuchat, A.; Shieh, W. J.; Siegal, L.; Swerdlow, D. L.; Tenover, F. C.; Traeger, M.; Ward, J. W.; Weisfuse, I.; Wiersma, S.; Yeskey, K.; Zaki, S.; Ashford, D. A.; Perkins, B. A.; Ostroff,

S.; Hughes, J.; Fleming, D.; Koplan, J. P.; Gerberding, J. L.; Investi, N. A. E. *Emerg. Infect. Dis.* **2002**, *8*, 1019-1028.

(5) Jernigan, J. A.; Stephens, D. S.; Ashford, D. A.; Omenaca, C.; Topiel, M. S.; Galbraith, M.; Tapper, M.; Fisk, T. L.; Zaki, S.; Popovic, T.; Meyer, R. F.; Quinn, C. P.; Harper, S. A.; Fridkin, S. K.; Sejvar, J. J.; Shepard, C. W.; McConnell, M.; Guarner, J.; Shieh, W. J.; Malecki, J. M.; Gerberding, J. L.; Hughes, J. M.; Perkins, B. A. *Emerg. Infect. Dis.* **2001**, *7*, 933-944.

(6) Webb, G. F. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 4355-4356.

(7) Bouzianas, D. G. *Expert Rev. Anti-Infect. Ther.* **2007**, *5*, 665-684.

(8) Friedlander, A. M.; Pittman, P. R.; Parker, G. W. *JAMA* **1999**, *282*, 2104-2106.

(9) Joellenbeck, L. M.; Zwanziger, L. L.; Durch, J. S.; Strom, B. L. *The Anthrax Vaccine: Is It Safe? Does It Work?*; National Academy Press, Washington, D.C., 2002.

(10) Choudhury, B.; Leoff, C.; Saile, E.; Wilkins, P.; Quinn, C. P.; Kannenberg, E. L.; Carlson, R. W. *J. Biol. Chem.* **2006**, *281*, 27932-27941.

(11) Vasan, M.; Rauvolfova, J.; Wolfert, M. A.; Leoff, C.; Kannenberg, E. L.; Quinn, C. P.; Carlson, R. W.; Boons, G. J. *ChemBioChem* **2008**, *9*, 1716-1720.

(12) Schmidt, R. R.; Kinzy, W. *Adv. Carbohydr. Chem. Biochem.* **1994**, *50*, 21-123.

(13) Paulsen, H.; Wulff, A.; Heitmann, A. C. *Liebigs Ann. Chem.* **1988**, 1073-1078.

(14) Paulsen, H. *Angew. Chemie. Int. Ed.* **1982**, *21*, 155-173.

(15) Garcia, B. A.; Poole, J. L.; Gin, D. Y. *J. Am. Chem. Soc.* **1997**, *119*, 7597-7598.

(16) Garcia, B. A.; Gin, D. Y. *J. Am. Chem. Soc.* **2000**, *122*, 4269-4279.

(17) Nguyen, H. M.; Poole, J. L.; Gin, D. Y. *Angew. Chemie. Int. Ed.* **2001**, *40*, 414-417.

(18) Codee, J. D.; Stubba, B.; Schiattarella, M.; Overkleeft, H. S.; van Boeckel, C. A.; van Boom, J. H.; van der Marel, G. A. *J. Am. Chem. Soc.* **2005**, *127*, 3767-3773.

(19) Veeneman, G. H.; Vanleeuwen, S. H.; Vanboom, J. H. *Tetrahedron Lett.* **1990**, *31*, 1331-1334.

(20) Boulineau, F. P.; Wei, A. *J. Org. Chem.* **2004**, *69*, 3391-3399.

(21) Boulineau, F. P.; Wei, A. *Org. Lett.* **2004**, *6*, 119-121.

(22) Toepfer, A.; Schmidt, R. R. *Carbohydr. Res.* **1990**, *202*, 193-205.

(23) Alper, P. B.; Hendrix, M.; Sears, P.; Wong, C. H. *J. Am. Chem. Soc.* **1998**, *120*, 1965-1978.

CHAPTER 5

CONCLUSIONS

LPS from *R. sin*-1 can antagonize the production of tumor necrosis factor alpha (TNF-α) by *E. coli* LPS in human monocytic cells. It has been shown that a derivative of *R. sin*-1 lipid A in which the C-3 fatty acid is replaced by an ether-linked moiety has a much improved chemical stability. Furthermore, this compound could antagonize cytokine production by a human monocytic cell line induced by enteric LPS with a similar potency to the natural ester-linked counter part. For the first time, it has been shown that such an antagonist can inhibit both MyD88- and TRIF dependent cell signaling events. *R. sin*-1 LPS and lipid A agonized mouse macrophages to produce TNF-α and IFN-β demonstrating species specific properties. For the agonists examined, the potency for TNF-α secretion was higher by 3–7 fold compared to that of IFN-β or IP-10. For, the antagonists, the IC_{50} values for IP-10 were smaller than the corresponding values for TNF-α. These data indicate that the MyD88 and TRIF pathways are somewhat differently activated or inhibited by the examined compounds. Furthermore, it was observed that the natural LPS possesses much greater activity than the synthetic and isolated lipid As, which indicates that di-KDO moiety is important for optimal biological activity.

We have shown that a live- and irradiated spore vaccine and polysaccharide linked to the carrier protein KLH can elicit IgG antibodies that recognize isolated polysaccharide and the synthetic trisaccharides. A surprising and important finding was that irradiated spores elicit anti-polysaccharide antibodies, and thus it appears that not only vegetative cells but also *B. anthracis*

spores express the polysaccharide. The implication of this finding is that a polysaccharide-based vaccine may provide immunity towards vegetative cells as well as spores. Finally, we have located important antigenic components of the various antisera using synthetic trisaccharides. The data provide an important proof-of-concept step in the development of vegetative and spore-specific reagents for detection and targeting of non-protein structures in *B. anthracis*. These structures may in turn provide a platform for directing immune responses to spore structures during the early stages of the *B. anthracis* infection process.

Finally, to locate important antigenic components of the hexasaccharide we are synthesizing various oligosaccharide fragments. We have successfully synthesized one of the tetrasaccharide **6** in moderate yield. However, few more trials need to be performed to improve the existing yields of α-glycosylation and we still need to find a convergent approach to synthesis the tetrasaccharides, pentasaccharides and hexasaccharide. All the synthetic compounds will be equipped with an aminopentyl spacer to facilitate conjugation to the carrier proteins like Keyhole Limpet Hemocyanin (KLH) or bovine serum albumin (BSA) to do the ELISA.