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Synthesis and Evaluation of Structural Analogs of Escherichia coli Lipid A for Application Towards CD14-Targeting Glycotherapeutics

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by

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Abstract

Synthesis and Evaluation of Structural Analogs of Escherichia coli Lipid A for Application Towards CD14-Targeting Glycotherapeutics

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Doctor of Philosophy

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Prof. Alexei V. Demchenko, Chairperson

Lipid A is a glucosamine-based phospholipid that anchors carbohydrate molecules to the outer membrane of most Gram-negative bacteria. During severe infection, this amphiphilic macromolecule invoke a strong and acute pro-inflammatory response that leads to sepsis and ultimately to septic shock, organ failure and death. Thus, following the convention that the best antidote is often a derivative of the parent agonistic molecule, researchers have actively pursued the idea of creating an analog of Lipid A that can block the cellular receptor for this molecule and thereby arrest the cascade of events that lead to sepsis. Consequently, we became interested in creating monosaccharide and disaccharide analogs of Lipid A, anticipating that our targeted design may exhibit the desired antagonistic effect without being harmful to the host cell. Since our target compounds share a structural motif prevalent in biologically relevant carbohydrates – the presence of 1,2-trans linked residues of 2-amino-2-deoxysugars - we investigated the application of our novel thioimidoyl methodology to the synthesis of 1,2-trans glycosides of 2-amino-2-deoxysugars. We reported previously that, along with providing a flexible approach to the

synthesis 2-amino-2-deoxyglucopyranosides, our method allowed for the chemoselective assembly of oligosaccharides containing multiple residues of 2-amino-2-deoxyglycoses. Herein, the application of the aforementioned thioimidoyl methodology to the selective activation approach to oligosaccharide assembly is described. More importantly, this dissertation will detail the synthesis of Lipid A analogs that contain three interesting structural features – the aminosugar core, (R)-3-hydroxy-fatty acids, and amino acids. This unique structural configuration has never before been synthesized and tested for antagonistic activity against enteric Lipid A. Therefore, it is to be anticipated that the synthetic approaches described herein could pave the way for expanding the range of antiendotoxic compounds prepared to date.

This dissertation is dedicated to my parents with

love and respect, Alex and Ofelia

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List of abbreviations

Å.....Angstrom

Ac.....Acetyl

Bn.....Benzyl

Bz.....Benzoyl

CSA.....(±)10-Camphorsulfonic acid

d.....Doublet

DCE.....1,2-Dichloroethane

DCM.....Methylene chloride

dd.....Double of doublets

DMF.....*N,N*-Dimethylformamide

Et.....Ethyl

FAB-MS.....Fast atom bombardment mass spectroscopy

h.....hour(s)

Hz.....Hertz

m.....Multiplet

min.....minute

m/z.....Mass to charge ratio

Me.....Methyl

MS.....Molecular sieves (in schemes)

NIS.....*N*-iodosuccinimide

NMR.....Nuclear magnetic resonance

Ph.....Phenyl

Phth ..... Phthalimido

ppm ..... Parts per million

R_f ..... Retention factor

s ..... Singlet

t ..... Triplet

TFA ..... Trifluoroacetyl

Tf ..... Trifluoromethanesulfonyl (triflate)

THF ..... Tetrahydrofuran

TLC ..... Thin layer chromatography

TMS ..... Trimethylsilyl

Troc 2,2,2-Trichloroethoxycarbonyl

Ts ..... *p*-Toluenesulfonyl

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Chapter 1

Introduction

1.1. Bacteria and Endotoxins

Interactions between the animal and microbial worlds are intimate, complex, and vital to the good health of both.[1] Over the course of almost a billion years, the mutual accommodation between microorganism and multi-cellular host has enabled both to survive and adapt to a changing environment. Without intracellular microbial parasites, the evolution of multicellular organisms would not have been possible. Indeed, the basic processes of cellular respiration in eukaryotes are possible only because of the presence of a microbial parasite in the cell called the mitochondrion.[2] As the endosymbiotic theory purports, mitochondria are descended from ancient proteobacteria engulfed by eukaryotic cells billions of years ago.

This intricate, symbiotic relationship between humans and microbes, however, has had its darker side. From parasitic and acute infections to chronic illnesses such as peptic ulcer disease,[3] cancer, and coronary heart disease,[4] microorganisms have triggered a plethora of human diseases. In response, the innate immunity in humans has evolved into a complex system that enables it to respond to a microbial threat and achieve a survival advantage. The latter is often accomplished by exploiting features unique to the threat, just as the microbes have used the same defensive and subversive strategies to circumvent our immune system.[1] Evidence of this evolutionary transformation also manifests in our genome as genetic mutations, i.e. thalassemia have permitted local populations to survive in areas where malaria is endemic.[5]

Amongst the billions of microorganisms present on earth, bacteria are the most diverse and abundant group. Based on their response to a staining procedure developed by Christian Gram in 1884, bacteria are classified into two broad groups – Gram-positive

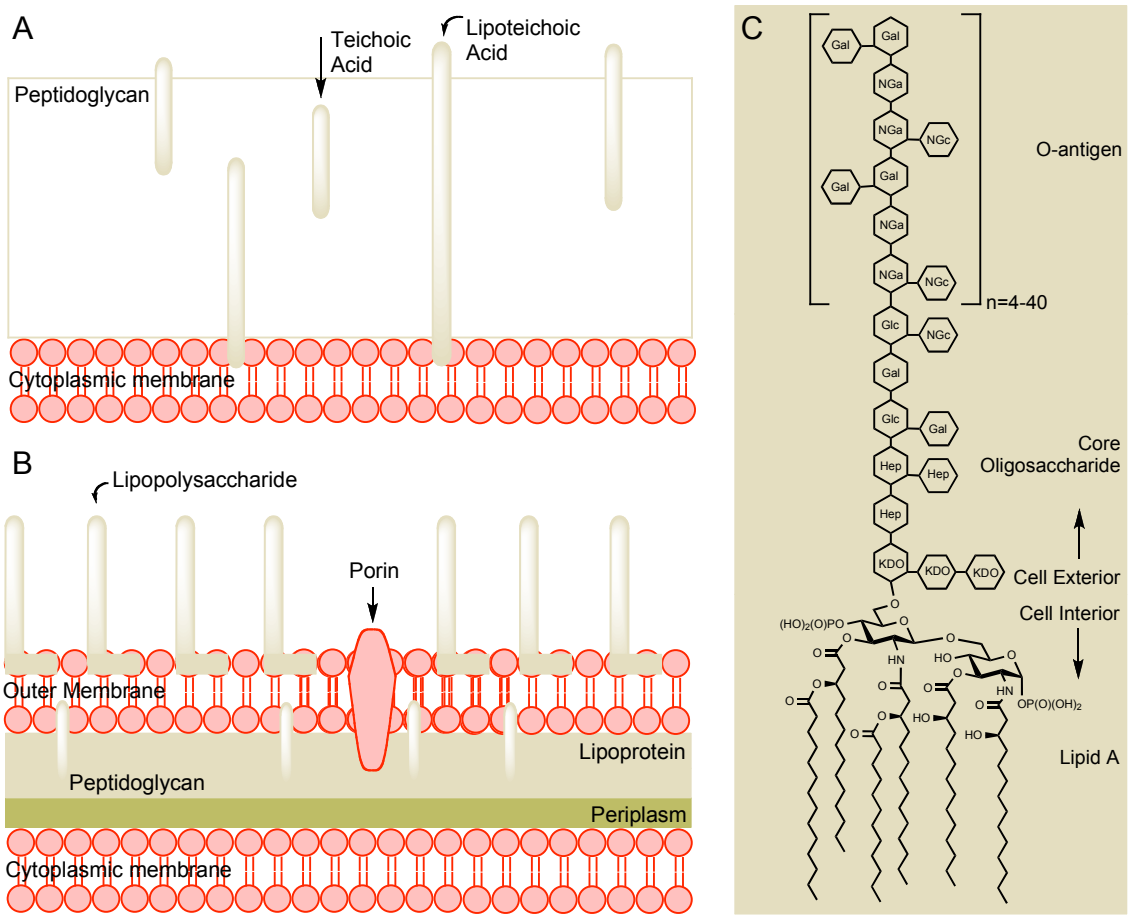

Figure 1.1 Representation of the Bacterial Cell Wall and Lipopolysaccharide: a) Gram-positive bacterial cell wall, b) Gram-negative cell wall, c) Lipopolysaccharide of *Escherichia coli*.

and Gram-negative.[6] This procedure is reflective of the fundamental differences in the composition and organization of the bacteria's cell wall.[7] Accordingly, Gram-positive bacteria have a multi-layered cross-linked polymer of peptidoglycan surrounding its plasma membrane while Gram-negative bacteria only have a monolayer of peptidoglycan (Figure 1.1.a, b). Furthermore, the latter are further encapsulated with a second membrane exterior to its peptidoglycan, whereas no comparable structure is observed in the former.

Given the marked difference in the structure of the cell envelope, the Gram-stain reaction also tends to correlate with the susceptibility of the bacteria to a variety of antibacterial agents. In general, Gram-negative bacteria are intrinsically more resistant to antibiotics and antibacterial compounds. This observation is perhaps counterintuitive when we consider that Gram-positive bacteria have thicker cell walls. Apparently, while the peptidoglycan surrounding the latter is mechanically strong, it offers little resistance to the diffusion of small molecules such as antibiotics because its meshwork is quite porous.[8] As such, the higher intrinsic resistance of Gram-negative bacteria can only be rationalized in terms of the permeability restrictions imposed by their outer membranes.[9]

The Gram-negative outer membrane is an asymmetric lipid bi-layer interspersed with proteins. Like the plasma membrane, its inner leaflet is composed of glycerophospholipids; i.e. phosphatidylethanolamine, with smaller amounts of phosphatidylglycerol and cardiolipin. The outer leaflet, in contrast, is comprised almost exclusively of lipopolysaccharide (LPS) molecules. LPSs are amphiphilic glycolipids whose unique structural features primarily dictate the selective permeability and barrier function of the outer membrane.[6] Interest in LPS as a target for the development of novel antimicrobial compounds stems both from its essential role in outer membrane stability (and consequently, cell viability) and from the realization that this complex structure was responsible for the diverse immunological and biological effects attributed to "endotoxins".

In 1892, Pfeiffer and Centanni independently described a heat-stable pyrogenic toxin intrinsic to *Vibrio cholerae* and *Salmonella typhi*.[10] Pfeiffer initially called it "endotoxin" and it was not until the 1930s that Boivin was able to extract this endotoxin

using the trichloroacetic acid technique.[11] Endotoxin purified through this method, however, was essentially a crude fraction containing many cell wall proteins. This made any progress towards understanding the biological impact of this molecule slow and inconclusive. After a decade or so, Westphal and Luderitz were finally able to develop a method for obtaining pure active fractions of this endotoxin, which was shown to be lipopolysaccharides (LPSs).[12]

LPSs are composed of three genetically, structurally, and antigenically distinct regions (Figure 1.1c) namely: 1) a hydrophobic membrane anchor called Lipid A, 2) a short chain of sugar residues with multiple phosphoryl substituents referred to as the core oligosaccharide, and 3) a structurally diverse, serospecific polymer composed of oligosaccharide repeats called the O-antigen. The LPS of *Escherichia coli (E. coli), Salmonella enterica (S. enterica),* members of the family of *Enterobacteriaceae*, and important pathogens including representatives of the families *Vibrionaceae* and *Pseudomonadaceae* all share the same aforementioned structural organization. In contrast, the LPSs of many mucosal pathogens lack the typical long-chain, repeating O-antigen unit i.e. the genera *Haemophilus* and *Neisseria*. Instead, the latter possess short, stereospecific oligosaccharides extending from the core called lipooligosaccharides (LOSs). In general however, the basic tripartite LPS-framework holds true for all characterized Gram-negative bacteria.

Lipid A, the covalently linked lipid component of LPS, is composed of six or more fatty acid residues linked to two phosphorylated glucosamine residues (Fig. 1.1c and elaborated later).[13] Four of the fatty acids have a (R)-3-hydroxyl group and the other two are devoid of it. Each Gram-negative bacterial species has a unique Lipid A

configuration and the structural features that differentiate each molecule are as follows. First, the acylation pattern on each glucosamine residue can have either a symmetric (3+3) or an asymmetric (4+2) arrangement. Second, 3 or 4 different fatty acids can be present in the molecule, with a length that can be anywhere between 10-16 carbons. Finally, a 4-amino-deoxy-L-arabinose and or phosphonoethanolamine can be linked to the C-1 axial or C-4' phosphate groups on the glucosamine residues.

The second part of the LPS molecule, the core region is a short chain of polysaccharides with moderate inter-bacterial variability. The inner core consists of 2 or more 2-keto-3-deoxyoctonic acids (KDO) linked to C-6' of the glucosamine residues on one side and 2 or 3 L-glycero-D-manno-heptose residues on the other (Fig. 1.1c).[14] KDO and L-glycero-D-manno-heptose are unique to bacterial species.[15] Under natural conditions, the smallest LPS produced by Gram-negative bacteria is Re-LPS - it consists of Lipid A with 1 or 2 KDO sugars – but longer LPS are more common.[16] The Rd1- and Rd2-LPS serotypes contain a complete inner core and an inner core without 2 heptose sugars, respectively.[17] The outer core, on the other hand, consists of common sugars and is more variable than the inner core. It is normally 2 to 3 residues long with one or more covalently bound polysaccharides as side chains.[18-21] LPS consisting of the Lipid A and the complete inner and outer core are denoted Ra-LPS, whereas the Rb- and Rc-LPS serotypes only contain a part of the outer core.

The last part of the LPS molecule, the antigenic outer core, is the most variable part and consists of repeating units of oligosaccharides. Attached to the terminal sugar of the inner core, this portion extends from the bacterial surface and is highly immunogenic.[22-25] The chemical composition and structure of the O-antigen can be

strain-specific (inter-strain LPS heterogeneity) or it can vary within one bacterial strain (intra-strain LPS heterogeneity).[26] The inter- and intra-strain heterogeneity is characterized by variations at different levels. The first variation can occur through non-stoichiometric modification of the O-polysaccharides with sugar moieties, i.e. with glucosyl and fucosyl residues. The second variation occurs via addition of non-carbohydrate substituents – i.e. acetyl or methyl groups – to the O-antigen. This may arise with regularity but in most cases, these modifications are also non-stoichiometric. The length of the O-antigen may vary from 0 to as many as 40 repeating units, but it generally consists of 20 to 40 repeating units. If the O-antigen is present, colonies of the bacteria have a smooth (S) appearance on a Petri dish culture. Absence of the O-antigen, in contrast, gives the bacterial colony a rough (R) appearance on a Petri dish culture.

As mentioned, LPS participate in physiological membrane functions and are therefore essential for bacterial growth and viability.[27] They contribute to low membrane permeability and enhance the resistance towards hydrophobic agents. LPS is not toxic while it remains incorporated in the bacterial outer membrane. When released from the bacterial surface – either following cell division or death, as a consequence of antibacterial action of the immune system, or interaction with antibacterial agents – LPS may form aggregates and interact with the cells of the immune system. Following this interaction, LPS elicit multiple acute pathophysiological effects such as fever, toxicity, Schwartzman reactivity, macrophage and B-lymphocyte activation, among others.[28] In 1954, it was proposed that the Lipid A portion alone is responsible for the endotoxic properties of LPS and the polysaccharide portion is dispensable.[29] After Shiba and co-workers completed the first target synthesis of *Escherichia coli* Lipid A,[30] comparative

experiments between the natural LPS and the synthetic Lipid A confirmed that Lipid A represents the toxic principle of LPS.[31-33]

1.2. Immune Response to Lipopolysaccharides

The immune system of an organism consists of homeostatic mechanisms that protect against disease by identifying and killing pathogens and tumor cells. It detects a wide variety of agents – from viruses and bacteria to parasitic worms and fungi – and discriminates them from the organism's own healthy cells and tissues. Basic immune mechanisms evolved in ancient eukaryotes and remain in their modern descendants, such as plants, fish, reptiles, and insects. These mechanisms include the use of antimicrobial peptides (defensins), phagocytosis, and activation of the complement system. With the evolution of vertebrates, more sophisticated mechanisms have developed. The immune systems of vertebrates such as humans consist of many types of proteins, cells, organs, and tissues, which interact in an elaborate and dynamic network. Over time, this more complex immune response has also allowed the vertebrate system to recognize and adapt to particular pathogens more efficiently. The ease by which pathogens can adapt and evolve new means to infect the host organism has made the recognition event crucial and complicated. On the other hand, the immunological memories created by the process of adaptation have allowed more effective protection in future encounters with the pathogen.

The immune system is typically divided into two categories that are not mutually exclusive: the more primitive innate immune system and acquired or adaptive immune system of vertebrates. The innate immunity is an ancient part of host defense mechanisms and has evolved over time. It features similar molecular modules as those found in plants, suggesting its existence during the period before the plant and animal kingdoms had

split.[34] The innate immune response is always the first line of defense against microbial pathogens.[35-37] It lies behind most inflammatory responses triggered in the first instance by macrophages, polymorphonuclear leukocytes (PMN), and mast cells through their innate immune receptors. The adaptive immunity, in contrast, is a relative newcomer on the evolutionary landscape and is further divided into the humoral line and the cellular line. What it adds to the innate immune system is specific recognition of proteins, carbohydrates, lipids, nucleic acids, and pathogens using the same activated but not antigen-specific cells generated by innate immune recognition. The basic features of the innate and adaptive immunity are summarized in Table 1.1.[35]

Table 1.1. Characteristics of Innate and Adaptive Immunity

Property	Innate Immune System	Adaptive Immune System
Receptors	Fixed in genome Rearrangement is not necessary	Encoded in gene segments Rearrangement necessary
Distribution	Non-clonal All cells of a class identical	Clonal All cells of a class distinct
Recognition	Conserved molecular patterns (LPS, LTA, mannans, glycans)	Details of molecular structure (proteins, peptides, glycoconjugate)
Self-Nonself Discrimination	Perfect: selected over evolutionary time	Imperfect: selected in individual somatic cells
Response time	Immediate activation of effectors	Delayed activation of effectors
Response	Co-stimulatory molecules Cytokine (IL-1β, IL-6) Chemokines (IL-8)	Clonal expansion or anergy IL-2 Effector cytokines: (IL-4, IFNγ)

Overall, the immune system protects organisms from infection with layered defenses of increasing specificity. Most simply, physical barriers prevent pathogens such as bacteria and viruses from entering the organism. If a pathogen breaches these barriers, the innate immune system provides an immediate, but non-specific response. In the event

that pathogens successfully evade the innate response, vertebrates put in motion a third layer of protection, the adaptive immune system, activated in turn by the innate response. As mentioned earlier, the adaptive immune system confronts the pathogen with two lines of defense – the humoral line and the cellular line. The humoral line consists of the complement cascade, antibodies, and acute-phase proteins (the class of proteins whose serum concentrations increase or decrease in response to inflammation). The cellular line of defense, on the other hand, consists of mononuclear cells (such as monocytes and macrophages) and neutrophils. These cells may recognize bacterial cell wall constituents directly or indirectly, after the complement proteins bind to the bacterium and its consitutents.[38-40] After the host fails to control the invasion and proliferation of the microorganism, the pathological process of a disease begins. During the initial stages of tissue invasion, host factors that contribute to microbial growth – i.e. deficiencies in complement, antibody, or other immunological components – will determine whether the initial localized infection is allowed to disseminate and produce bacteraemia, fungimia, or parasitemia.

Infection is defined as a pathologic process caused by invasion of normally sterile tissue, fluid, or body cavity by pathogenic or potentially pathogenic microorganisms.[41] If not treated efficiently, infection may evolve into bacteraemia or the presence of a high volume of bacterial population in the bloodstream. As a result of bacteraemia, a high dose of bacterial antigens comes in contact with serum components of the immune system. Endotoxin or LPS, mentioned earlier, is the best-characterized bacterial antigen. Whereas LPS itself is chemically inert, recognition of LPS by extracellular receptors is the first step in the development of endotoxemia. Under normal homeostatic conditions, the

immune response to LPS provides protection against bacterial infection and is under stringent control of specific inhibitors. During severe infection, however, LPS can set off a cascade of exaggerated host responses – i.e the excessive production of intrinsic mediators of systemic inflammation – leading to systemic inflammatory response syndrome (SIRS). In the context of a bacterial infection, SIRS is also known as sepsis.[41]

Sepsis is a clinical disorder defined by the presence of both infection and systemic inflammation.[41] The disruption of homeostatic balance as a consequence of sepsis leads to massive production of pro-inflammatory mediators and dysregulation of the anti-inflammatory mechanisms. Sepsis, therefore, can be described as a pro- and anti-inflammatory disequilibrium syndrome.[42] The uncontrolled, overwhelming, abrupt systemic inflammatory response to sepsis ultimately manifests clinically in the frequently fatal syndrome called septic shock. The latter is characterized by endothelial damage, coagulopathy, loss of vascular tone, myocardial dysfunction, tissue hypoperfusion, and multiple organ failure.[43] Sepsis and its accompanying multi-organ dysfunction syndrome (MODS) remains as a leading cause of mortality in intensive care units.[44,45] A study by Angus *et al.* linking 1995 state hospital discharge records from the seven large states in the United States identified 192,980 cases of severe sepsis out of the 6,621,559 patients admitted.[46] This yields national estimates of 751,000 cases (3.0 cases per 1,000 population and 2.26 cases per 100 hospital discharges), of whom 383,000 (51.1%) received intensive care and 130,000 (17.3%) were ventilated in an intermediate care unit or cared for in a coronary care unit. They also found that incidence increased greater than 100-fold with age (0.2/1,000 in children to 26.2/1,000 in those 85 yrs old and older). Mortality was 28.6% or 215,000 deaths nationally and also increased with age, from 10%

in children to 38.4% in those greater than 85 yrs old. Women were found to have a lower age-specific incidence and mortality, an observation that is easily accounted for by the differences in underlying disease and site of infection. The average costs per case were $22,100 and it adds to annual total costs of $16.7 billion nationally. Costs were higher in infants, non-survivors, intensive care unit patients, surgical patients, and patients with more organ failure. Angus *et al*. projects that the incidence of sepsis will increase by 1.5% per annum. Similarly, Martin *et al*. found that from 1979 to 2000, the incidence of sepsis and the number of sepsis-related deaths are increasing, although the overall mortality rate among patients with sepsis is declining.[47]

In recent years, pathogenesis-oriented supportive therapy has been proven to reduce morbidity and mortality of sepsis. These include early and vigorous fluid replacement, strict blood glucose control, low-dose corticosteroid reposition, protective mechanical ventilation and activated protein-C.[48] The need to spread the importance of sepsis to the average population, train medical teams and measure the adherence to prescribed therapies formed the basis for launching the Surviving Sepsis Campaign. Thus, in October 2002, this campaign was launched during the European Society of Intensive Care Medicine annual congress.[49] The campaign issued a "Barcelona Declaration" – a document imploring critical care providers, governments, health agencies and lay people to join the fight against sepsis. The aim of the campaign was to reduce the sepsis mortality rate by 25% within 5 years (this deadline has been extended from 2007 to 2009). The policy, goals and strategies of this campaign are outlined by Silva et al. in an article.[49]

Despite efforts of the campaign, along with more than two decades of extensive research, the heterogeneity of the sepsis syndrome has provided serious limitations to the development of an anti-sepsis agent.[50-53] Recently, a recombinant human activated protein C, Drotrecogin-α (Xigris), was launched on the market.[53,54] Thus, based on a total of 1690 randomized patients treated (840 in the placebo group and 850 in the Drotrecogin-α activated group), it was found that Drotrecogin-α significantly reduced mortality rates in septic patients from 30.8% (placebo group) to 24.7% (Drotrecogin-α activated group).[54] The observed mortality difference between Drotrecogin-α and placebo, however, was limited to patients with a high risk of death. In addition, since Drotrecogin-α increases the risk of bleeding, it is contraindicated in patients with active internal bleeding and recent intracranial surgery.[55] ***To this end, there is still a major unmet medical need for more effective and safer anti-sepsis agents***.

1.3. Molecular Basis for the Pathogenesis of Sepsis: Extra-cellular Signaling Pathways for Microbial Antigens

Before an effective anti-sepsis agent can be designed, every aspect of the pathophysiological role of LPS in endotoxemia must first be understood. Information on how LPS is processed, recognized, and signaled by the immune system is necessary so that the agent will treat the underlying cause and not the symptoms. It is well known that the infection of a host by bacteria is a complex process and the outcome is usually the elimination of bacteria. Indeed, all species can rely on a myriad of elimination strategies to deal with the severity of a particular infection. Accordingly, a host can use a very sensitive and aggressive method of elimination, using every counter-measure available.

The drawback of this quick and full-blown reaction, however, is the large collateral damage of surrounding tissue. As such, a very mild immune response, wherein the host only uses a limited repertoire of mechanisms, also exists. Such a response leads to a smaller damage of surrounding tissue, but the bacteria is allowed to remain longer in the host and impair the host's health. The choice of elimination strategy depends on the different signaling pathways available to the host and the manner by which the invading entity activates it. ***It is therefore vital to elucidate the signaling pathways by which LPS activates the immune system in order to control the latter's exaggerated response to LPS.***

Under physiological conditions, the immune cells are continuously exposed to low levels of LPS derived from gastrointestinal bacteria. This LPS is taken up by macrophages and may be essential to maintain a basal level of attentiveness of the immune system. It was originally believed that LPS activated immune cells through a non-specific mechanism that involved intercalation of Lipid A into the mammalian lipid bilayer.[56] In the early 1980s, however, reports emerged suggesting that the biological actions of LPS were facilitated many fold by its binding to endogenous proteins. Indeed, the response of a host cell to LPS is highly dependent on whether it encounters the latter in free or bound form.[57]

Over the past 20 years, one of the major aims in LPS research has been to elucidate the exact sequence of events from when the LPS binds to the cell to when it elicits a response from the cell. As mentioned earlier, distinct plasma membrane proteins mediate the initial interaction between LPS (Lipid A) and phagocytes (monocytes, macrophages, polymorphonuclear leukocytes). Some of these interactions may be solely

involved in the removal and eventual degradation of LPS whereas others may play a critical role in trans-membrane signaling.

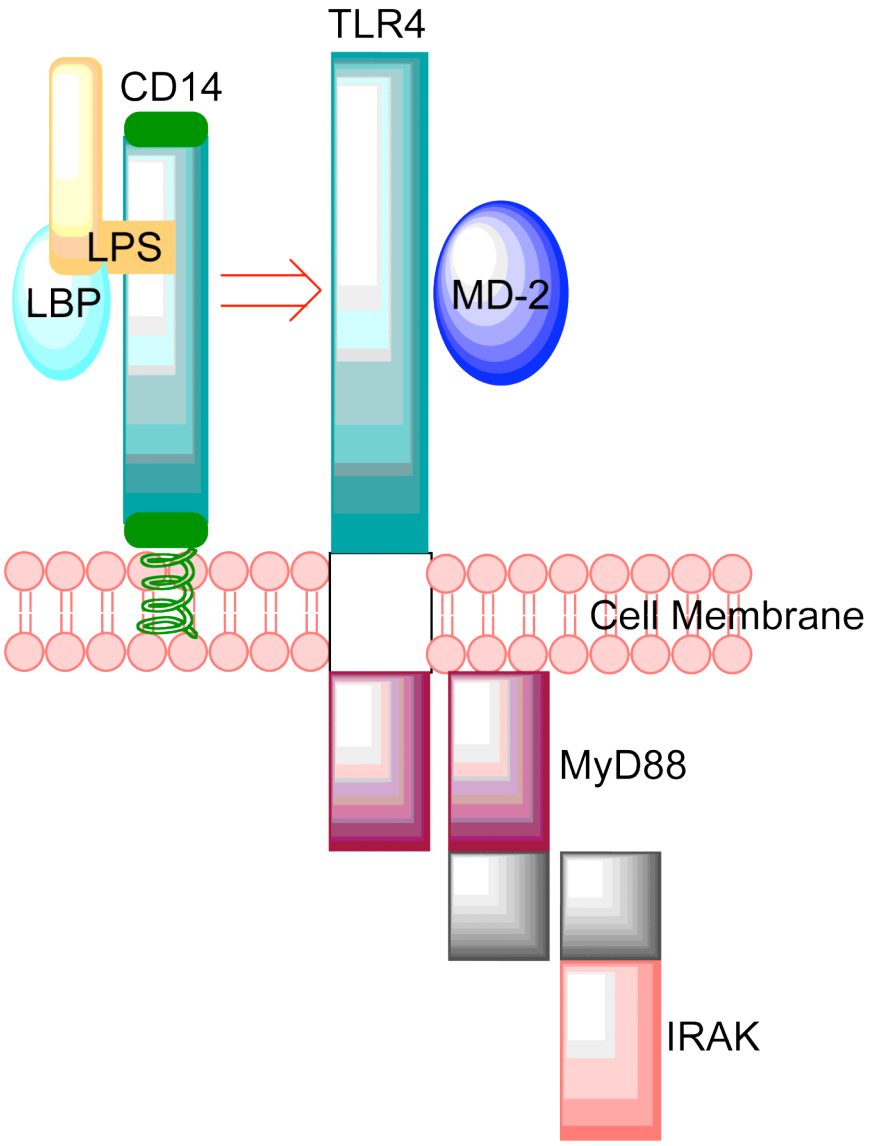

Figure 1.2. Recognition of LPS on the surface of phagocytes. LPS is opsonized by lipopolysaccharide-binding protein (LBP), and the LPS-LBP complex is recognized by cluster of diffrentiation 14 (CD14). CD14 is incapable of generating a transmembrane signal and subsequently, the LPS–LBP–CD14 ternary complex activates toll-like receptor 4 (TLR4). TLR4, in turn, signals through the Myeloid differentiation primary response gene (88) (MyD88) and the interleukin-1 receptor-associated kinase (IRAK). Myeloid differentiation 2 (MD-2) is a secreted protein that binds to the extracellular domain of TLR4 and is an important component of its signaling pathway.[36]

For interactions that are limited to facilitating the removal of LPS, the lipoprotein scavenger receptor cluster of differentiation 18 (CD18) appears to be the receptor of choice. The R-form LPS, lipid A, and partial lipid A structures (e.g. lipid IVa) are the preferred ligands of this receptor; S-form LPS appears not to interact with these membrane proteins.[58] The receptors involved in trans-membrane signaling (Figure 1.2) and the resulting release of proinflammatory cytokines, on the other hand, will be discussed in detail below.

1.3.a. Lipopolysaccharide Binding Protein (LBP)

While studying LPS binding to high-density lipoprotein (HDL) in normal and acute-phase rabbit serum, Tobias and co-workers observed that LPS was mainly complexed to a protein in the acute-phase serum.[59] Isolation of this protein from the rabbit serum led to the discovery of the lipopolysaccharide binding protein (LBP). LBP was recovered as a 58- and 60.5-kDa glycoprotein, wherein the difference in molecular mass reflect different degrees of glycosylation.[59,60] It is synthesized primarily by hepatocytes and released into the bloodstream after glycosylation.[61] Other sources of LBP include epithelial cells of the skin, the lung, the intestine and human gingival tissues as well as the small muscle cells of the lung arteries, heart muscle cells and renal cells.[62,63]

LBP is a member of the lipid transfer/lipopolysaccharide binding protein (LT/LBP) family along with bactericidal/permeability increasing (BPI) protein, cholesteryl ester-binding protein (CETP) and phospholipid transfer protein (PLTP).[64] More distant homologues of LBP have also been identified in the upper airway epithelia (palate, lung, nasal epithelium carcinoma associated proteins or PLUNC) and are collectively termed the BPI/PLUNC superfamily.[65] Similarly with LBP, BPI, and PLTP,

PLUNC genes are also located on chromosome 20, suggesting that the latter may also participate in host defense.[65]

Human LBP consists of 452 amino acids and has the typical 25-amino-acid signal sequence characteristic of secreted proteins.[61] Its amino acid sequence revealed a sequence homology to BPI, CETP, and PLTP of 45%, 23%, and 25%, respectively and suggested a similarity in their tertiary structure. After the three-dimensional structure of BPI was determined by X-ray crystallography, it provided a useful framework for modeling the three-dimensional structure of the LT/LBP family.[66] BPI appears as a boomerang-shaped molecule and consists of two symmetrical barrel domains connected by a proline-rich linker region. Each domain is composed of an anti-parallel β-stranded layer twisted around a long α-helix and forms a hydrophobic pocket that can incorporate one phosphatidylcholine molecule. From these observations, Beamer *et al.* proposed a simulated three-dimensional model for LBP that is very similar to the structurally and functionally related BPI.[67] Analogously with BPI, the LPS-binding domain of LBP is located at the N-terminal region. Indeed, the three-dimensional LBP model showed that the cationic cluster of the LPS binding site is fully exposed at the N-terminal tip.[67]

To verify the veracity of the claim that the LPS-binding site of LBP is at the N-terminus, Lamping *et al.* performed mutagenesis experiments.[68] LBP mutants with amino acid exchanges within the N-terminal region were expressed and tested in five different functional assays – binding to immobilized LPS, facilitation of binding of LPS aggregates to monocytes, transfer of LPS monomers from aggregates to other LPS receptors, transfer of LPS monomers to high density lipoprotein (HDL), and enhancement of LPS-induced cell activation. The double mutant glutamic acid 94/95 was

completely lacking LPS binding, transfer, and cell stimulatory activity, indicating that the integrity of amino acids 94 and 95 is required for LBP function.[68] While mutations of amino acids Arginine 94 or Lysine 95 into alanine reduced the LPS binding activity of LBP dramatically, the ability to facilitate binding of LPS aggregates to membrane-bound cluster of differentiation 14 (mCD14) at the cell surface was retained. These findings emphasize the distinction between binding of LPS aggregates to cells and binding of LPS monomers to CD14 – the former is not associated with cell stimulation and the latter leads to cell stimulation. The role of CD14 in LPS recognition will be discussed in detail later.

Consequently, structure/function studies were aimed at identifying the functional domains and elucidating the mechanism of action of LBP, BPI and CETP.[67-69] On the one hand, several studies showed that a peptide fragment at the N-terminal region (tethered by a disulfide bond) has endotoxin-neutralizing activity and forms a hairpin.[70-72] One the other hand, Taylor and co-workers identified overlapping 15-mer peptides corresponding to residues 91-108 of human LBP that specifically bound the lipid A moiety of LPS with high affinity.[70] Along with inhibiting the binding of LPS to LBP, these peptides also inhibited chromogenic Limulus amebocyte lysate reaction and blocked release of tumor necrosis factor alpha (TNFα) following LPS challenge *in vitro* and *in vivo*. It is strongly believed then that residues 91-108 of LBP form part of the LPS binding site. In contrast, Bosshart *et al.* showed arginine-rich polypeptides can stimulate monocyte activation and act synergistically with LPS; this indirectly supports the role of cationic residues of LBP in destabilizing the LPS aggregates.[73]

Studies show that LBP has a dual role in interactions with LPS. At low LPS concentrations, LBP enhances LPS signaling by extracting it from the bacterial membranes (LPS monomerization) and transferring the LPS monomers to CD14 at a rate of 150 molecules/minute.[74,75] At high concentrations, it inhibits the LPS signaling by shuttling the LPS to the serum lipoproteins and by forming aggregates with LPS.[76,77] Indeed, the increased secretion of LBP as a result of LPS stimulation serves as an inhibitor of excessive response to LPS in the serum of septic patients.

The two domains of LBP have different functions. Similarly for LBP and its homologue BPI, the amino-terminal domain has a high affinity for LPS.[78] LBP binds to smooth and rough LPS, Lipid A, and Lipid IV_A. With a stoichiometry of 1:1 and K_d's varying from 1 to 58 nM, LBP has a high binding affinity for Lipid A. [79,80] Since Lipid A is the endotoxically active part of LPS, it is possible then that the N-terminal residues of LBP, between 91 and 108, are binding Lipid A. The C-terminal domain, on the other hand, is required for the interaction with CD14 or the cell membrane.[81] LBP intercalates into the cellular membranes suggesting that the C-terminal domain may be important for cell activation.[77]

Adding LBP to serum-free cell system enhances the LPS-mediated stimulation of CD14-positive cells by 100- to 1000-fold.[61,82] In addition, LBP also transfers LPS to soluble CD14 (sCD14) resulting in the activation of mCD14-negative cells (endothelial and epithelial cells). Thus, the ability of LBP to transfer disaggregated LPS to both mCD14 and sCD14 supports the view that LBP has a central role in mediating LPS responses. It was proposed that a single LBP molecule is able to transport hundreds of LPS molecules to sCD14 and that LBP is not consumed by this reaction.[83] Consequently,

Yu and Wright demonstrated first order kinetics for this enzymatic transfer and were further able to define catalytic constants for this reaction.[75,83] To explain the catalytic reaction mechanism for the transfer of LPS to sCD14, two models were proposed. The "binary complex" model details that the initial step in the transfer involves a bimolar reaction between LBP and an LPS micelle. Following dissociation from the micelle with one molecule of LPS bound, LBP then binds to sCD14.[83] The "ternary complex" model, on the other hand, suggests a simultaneous interaction amongst LBP, LPS micelles, and sCD14.[84]

Finally, it must be noted that LBP binding to LPS is not restricted to the molecular level; LBP also binds viable bacteria. Thus, LBP-binding to *Salmonella spp.* and *Klebsiella pneumoniae* results in phagocytosis and clearance of these microorganisms.[85,86] In a respiratory tract infection model, it has been demonstrated that the binding of LBP to one colony of non-typeable *Haemophilus influenzae* already induced strong signaling in cells.[87]

1.3.b. Cluster of Differentiation 14 (CD14)

While it was clear that the CD18 complex interacts with LPS by bridging bacteria to the surface of phagocytes,[58] it was not clear whether this interaction triggers cellular responses. Studies show that the CD18 complex recognizes the presence of *E. coli* by binding LPS embedded in the outer membrane of *E. coli*.[40] Macrophages also bind to LPS (purified) inserted into the membranes of erythrocytes (ELPS), and down-modulation of the CD18 complex with anti-CD18 antibodies eliminates the capacity of macrophages to bind both *E. coli* and ELPS. Accordingly, cells from patients genetically deficient in the CD18 complex fail to bind either *E. coli* or ELPS.[88] Thus, to elucidate the

exact role of CD18 in cellular activation by LPS, Wright *et al*. performed experiments on mononuclear cells from CD18-deficient patients.[86,89] They observed, however, that these cells can still bind LPS and that the binding event resulted in cellular activation. Clearly, additional receptors must be present on the surface of macrophages and polymorphonuclear leukocytes (PMN).

In a series of experiments, Wright and co-workers identified this unknown receptor to be CD14 – a differentiation antigen of monocytes.[90] Based on their report, CD14 binds complexes of LPS and LBP and the blockade of CD14 with anti-CD14 antibodies prevents further binding of LPS-coated erythrocytes to macrophages. With the absence of a binding event, macrophages are unable to produce an LPS-induced inflammatory response. Golenbock *et al*. corroborated these findings by demonstrating that LPS-induced responsiveness can be transferred to a heterologous non-responder cell-type by expression of a single leukocyte-specific gene product.[91] Thus, transfection of human CD14 into Chinese hamster ovary (CHO) fibroblasts and treatment of CD14-bearing CHO cells with LPS led to a macrophage-like responsiveness in otherwise LPS-unresponsive cells. Similarly, Lee and co-workers showed that CD14-bearing 70Z/3 cells bind LPS and when LPS is complexed with LBP, the binding activity is even higher.[92] Consequently, Kirkland *et al*. determined the binding affinity of the LPS-LBP complex to CD14-transfected CHO cells and THP-1 cells and found K_d values of 2.7×10^{-8} to 4.8×10^{-8} M.[93]

CD14, a serum/cell-surface glycoprotein and the first described pattern recognition receptor, is usually found in two forms: membrane bound (mCD14) and soluble (sCD14).[90,94,95] Since sCD14 lacks the glycosyl phosphatidylinositol (GPI)

anchor, mCD14 and sCD14 have molecular masses of 53 and 48 kDa, respectively.[96,97] To determine the amino acid composition of CD14, Ferrero and Goyert cloned the CD14 gene and revealed a transcript encoding a 356-amino acid protein.[98] It was also found to have a high leucine content (15.5%) and four putative N-glycosylation sites.[98,99] The site involved with LPS-binding, as well as the sites involved in the interaction of human CD14 with supposed accessory receptors, have been identified in the N-terminal part of CD14.[100,101] This was determined by generating and transfecting 23 mutants in the N-terminal 152 amino acids of human CD14.[102] In each mutant, a block of 5 amino acids was substituted with Ala. Thus, Stelter *et al*. found that the region between amino acids 39 and 44 forms an essential part of the LPS-binding site of human CD14.[102] Moreover for human sCD14, two other regions were found to be essential for eliciting LPS-induced responses from endothelial and smooth muscle cells: aa 9 to 13 and aa 91 to 101.[103,104]

CD14 is expressed by various cells i.e. cells of the myeloid lineage (monocytes, macrophages, PMN), B cells, liver parenchymal cells, gingival fibroblasts, and microglial cells.[105-110] Each source expresses CD14 differentially: peritoneal and pleural macrophages exhibit a high level of constitutive CD14 expression while (murine) Kupffer cells, alveolar macrophages, monocytes, and PMN have a low level of constitutive CD14 expression.[105,111-113] In addition, LPS and TNF-α induce the release of sCD14 by mononuclear cells and PMN in a dose-dependent manner, whereas interferon-γ (IFN-γ) and interleukin 4 (IL-4) inhibit the release of sCD14.[114,115] In the steady state, human serum contains 2 to 6 μg/mL of sCD14.[116] This level increases in response to the presence of LPS and consequently, Landmann *et al*. suggested the use of sCD14 levels as a diagnostic marker in patients with severe infections.[117] The level of sCD14 in human

milk also explains why newborn infants are innately immune to bacteria with their hitherto sterile intestines. Thus, Labeta and co-workers found that the concentration of sCD14 is 10-fold higher in human milk than that in serum.[118]

Binding of LPS to a cell does not result in immediate response – a time lapse of 15 to 30 minutes is usually observed between LPS binding and LPS-induced cellular responses. Detmers *et al*. along with Lichtman *et al.* suggested that monomeric LPS is internalized in vesicles, and uptake may be required for signaling.[119,120] Indeed, several studies have revealed that blocking the internalization or endosome fusion also blocks LPS-induced signaling.[119-122] Although the precise mechanisms of this blocking event are not completely understood, it has been shown that monomeric LPS is transported into the cell to the Golgi complex and activates the cell from thereon.[123] To determine if mCD14 directs the movement of LPS to the Golgi apparatus, Vasselon *et al.* used an mCD14 chimera containing enhanced green fluorescent protein (mCD14-EGFP) to follow trafficking of mCD14 in stable transfectants.[124] Thus, it was found that monomeric LPS is transferred out of mCD14 at the plasma membrane and traffics within the cell independently of mCD14 involvement. In contrast, particulate (bacterium) and aggregated (micelles) LPS were internalized to the lysosomes via a CD14-dependent pathway called macropinocytosis – a process resembling that of phagocytosis.[122] After internalization, LPS induces mononuclear phagocytes (MPs) to produce three groups of powerful mediators; the reactive oxygen intermediates (O_2, H_2O_2, ·OH, and singlet oxygen), the pro-inflammatory cytokines, and a number of arachidonic acid metabolites, including prostaglandins and leukotrienes.

Since CD14 is a glycophosphatidylinositol-linked receptor that lacks a trans-membrane domain, it was anticipated that it requires an accessory molecule for signal transduction.[125] This hypothesis was confirmed using different anti-CD14 antibodies that either blocked LPS binding to CD14, or did not block LPS binding while preventing LPS-induced cell activation.[84,126] [126] This accessory receptor has been identified as a member of the TLR family.

1.3.c. Toll-like Receptor 4 (TLR4)

Approximately 8 years after the importance of LBP and CD14 was initially delineated in seminal discoveries, the next main advance in understanding the mechanisms of innate immunity emerged – the identification of the putative transmembrane protein that acted with CD14 to generate a transmembrane signal for LPS-induced cell activation. Two highly original and influential discoveries gave the impetus for this advance. First, it was found that Toll-like receptors (TLRs) play an important role in the innate immune response of Drosophila flies.[127,128] Second, a TLR homolog was identified as the gene responsible for LPS responses in two natural mouse mutants.[129-131] These results formed the basis for understanding how the innate immune system regulate responses to infection and how plasma membrane receptors control adaptive immune responses.[36]

It was well known that despite the lack of an adaptive immune system, Drosophila flies are very resistant to microbial infections. The only rationale for this attribute, at the time, had been its demonstrated ability to synthesize potent antimicrobial peptides. Then, in the early 1980s, Anderson *et al*. conducted a mutagenesis screen for genes involved in dorso-ventral patterning of the Drosophila embryo.[132] Their studies revealed a mutant

gene that had an unusual appearance. Consequently, the authors named this gene "Toll", meaning weird. The Toll gene, which encodes a single-pass transmembrane receptor, became highly important after it was found that it activates the signaling pathways that induce the synthesis of drosomycin, an anti-fungal peptide in Drosophila flies.[127,132]

Twelve years after Anderson's discovery, Williams *et al*. showed that 18-wheeler – another TLR gene found in Drosophila – could induce the release of attacin.[128] Attacin is one of the potent antibacterial peptides synthesized by Drosophila. As a result, it was established that the activation of a proteolytic cascade that produces peptidic ligands for the TLRs leads to the induction of these antimicrobial responses. Whether or not this mechanism is unique to Drosophila or has been conserved in mammalian cells remains unanswered. What was remarkably inferred from these results, however, was that Drosophila TLRs were capable of discriminating between fungi and bacteria and consequently, of inducing an appropriate and distinct antimicrobial response. Subsequently, Imler and colleagues showed that the activation of TLR-induced pathways in Drosophila initiates an intracellular kinase cascade that ultimately produces a translocation of transcription factors, Dif and Relish, from cytoplasm to nucleus.[133] Dif and Relish are homologous to nuclear factor-kappa B (NF-kB), a transcription factor known to activate inflammatory mediators in humans, thereby linking Drosophila TLRs to the study of LPS biology.[132]

The apparent importance of TLRs as well as the observation that the Toll gene shares a certain homology with the human IL-1 receptor provided impetus for the field of Toll biology to move beyond flies.[134] Thus, in the mid-1990s, Janeway and colleagues began a search for dToll-related proteins in mammalian gene sequences. As a result of

their efforts, the first human homolog of Drosophila Toll, initially termed human Toll and subsequently termed TLR4, was identified.[135] Human TLR is an 841-amino acid protein with a molecular mass of 92kDa.[135] After cloning and characterization, human Toll was found to be a type I transmembrane protein, the cytoplasmic domain of which bears a structural homology to human interleukin (IL)-1 receptor. Janeway and colleagues also determined that similarly with Drosophila Toll, human Toll could induce activation of NF-κB and subsequently, induce the expression of NFκB-controlled genes for the inflammatory cytokines. Finally, their observation that TLR4 could induce members of the B7 family – molecules that are required for the activation of naive T cells by antigen-presenting cells – provided a potentially important link between pathogen detection and induction of the adaptive immune response.

Other compelling evidence on the importance of TLRs in LPS-induced responses came when TLRs addressed the issue of why some strains of mice were unresponsive to LPS. For years, LPS has been known to be a very active mediator of inflammation in most mammalian system.[136] It was found, however, that LPS is relatively ineffective at inducing responses in the C3H/HeJ or C57BL/10ScCr strains of mice.[137] Then in 1998, Poltorak *et al*. showed via positional cloning techniques that mutations of a gene termed the "LPS gene" selectively reduced the ability of C3H/HeJ and C57BL/10ScCr mice to sense LPS. The co-dominant Lps^d allele of the C3H/HeJ strain was a result of a mis-sense mutation in the third exon of TLR4, a mutation that was predicted to result in a Pro712→His substitution.[129,130] When this mutation was introduced into wild-type TLR4, the receptor was converted into a dominant-negative mutant that inhibited LPS-dependent responses in a transfected macrophage cell line.[138] Similarly, Hoshino *et al*. demonstrated

that C3H/HeJ mice have a single point mutation of the amino acid that is conserved among the Il-1/Toll receptor family.[131] Using genetically modified mice in which the TLR4 gene was deleted, the latter showed that TLR4 was essential for sensing LPS and that mutations in this gene explained the lack of responsiveness in C3H/HeJ mice. Together, these seminal publications provided the first direct connection between TLRs and the physiological responses to LPS. Heine and co-workers provided further proof of this connection by showing that Chinese hamsters respond normally to LPS even though they carry a null allele for TLR2.[139] Their results implied that expression of TLR2 is sufficient but not essential for mammalian responses to endotoxin. Finally, it was shown that a dominant-negative mutant of TLR2 did not effect LPS responsiveness in transfected macrophages.[138]

By 1998, five human TLR homologues had been identified.[140] Presently, at least ten TLRs have been found in humans and thirteen in mice.[141] Humans and mice share TLRs 1-9 but TLR 10 is only found in humans and TLR11 is functional only in mice.[142] Subsequent studies provided a detailed analysis of the structural features that link these proteins to Drosophila Toll and to the IL-1 receptor family.[140] Thus, Rock *et al.* highlighted the general structural features of the TLR family – an ectodomain characterized by multiple leucine-rich repeats and a Toll-homology domain found in the cytoplasmic tail.[140]

1.3.d. Myeloid Differentiation Antigen 2 (MD-2)

Despite the fact that several groups have already shown evidence of LPS-induced signal transduction through Toll-like receptors, direct binding of LPS to the latter is yet to be demonstrated.[143,144] Moreover, it was found that *in vitro* transfection of TLR4 cDNA

did not confer LPS responsiveness on two LPS-unresponsive cell lines: human embryonic kidney-derived and a mouse IL-3-dependent pro-B cell line Ba/F3.[145,146] Then in 1999, Shimazu and co-workers reported and characterized a novel LPS binding protein called myeloid differentiation antigen-2 (MD-2).[146] In a series of experiments, they showed that MD-2 is physically associated with TLR4 on the cell surface and confers responsiveness to LPS. In a similar fashion, da Silva Correia determined that LPS binds directly to each of the members of a tripartite LPS receptor complex.[144] Using modified and radioiodinated LPS, they showed that LPS is cross-linked specifically to TLR4 and MD-2 when co-expressed with CD14. Thus, maximal cellular activation by LPS must be a cascade of events that likely involves transferring of LPS by LBP to CD14 and then to TLR4-MD-2. Moreover, although CD14 and LBP enhance cellular activation, activation of TLR4 by LPS was found to absolutely require MD-2.[147]

MD-2 is 20-25-kDa extracellular glycoprotein that belongs to the MD-2-related lipid recognition family of lipid-binding receptors.[148] Since MD-2 lacks a transmembrane domain that would anchor it to the cell membrane, several groups performed studies to verify the nature by which MD-2 associates with TLR4 – is it an intracellular soluble protein that binds to TLRs in the endoplasmic reticulum (ER) or is it first secreted into the medium and then binds to TLRs on the cell surface? To this end, Visintin *et al*. found that in some cells, MD-2 is synthesized in large excess to TLR4 and it saturates all available TLR4 molecules in the ER. The excess MD-2 is then secreted into the medium.[149] Although proper glycosylation and trafficking of TLR4 to the cell surface requires intracellular association with MD-2,[150,151] functional TLR4 can be presented on the cell surface without MD-2 in both transfected[152] and human airway epithelial[153] cells.

For reporter cells that expressed TLR4, but not MD-2, secreted MD-2 (sMD2) was found to restore LPS responsiveness.[149] Thus, even at concentrations as low as 50 pM, Visintin *et al*. showed that MD-2 significantly enhances LPS reactivity and suggested that TLR4 has a functional affinity constant for MD-2 in the range of 50–500 pM.[149]

Human MD-2 contains 160 amino acid residues, prominent regions of which are the 17-amino-acid sequence at the N-terminus, 7 cysteine residues, and 2 N-glycosylation sites.[154] To identify the regions of functional importance on human and mouse MD-2, common analytical methods – i.e. analysis of peptide fragments,[155] mutation analysis,[156-161] and computational modeling[162] – have been utilized. Computer modeling suggests that MD-2 is capable of forming a barrel-like structure with a hydrophobic cavity sufficient to accommodate the fatty acid moieties of lipid A.[162,163] In addition, Visintin *et al*. reported that a positively charged region flanking the hypothetical hydrophobic cavity of MD-2 is required for stable binding to LPS.[164] On the other hand, site-directed mutagenesis identified the regions of human MD-2 that are involved in TLR binding and consequently, in conferring LPS responsiveness.[159] Thus, Re *et al*. found that MD-2 binding to TLR4 took place via Cys95 and Cys105, probably through the formation of an inter-molecular disulfide bond.[159] Several studies predict that Cys95 is located on the surface of the hypothetical barrel, along with the other Cys residues except for Cys133.[149,165,166] This prediction is consistent with the idea that MD-2 is capable of forming covalently bound oligomers, but it does not preclude the existence of a monomeric form. Indeed, monomeric MD-2 has been reported to preferentially bind to a recombinant soluble TLR4 ectodomain.[167] Hydrophilic and charged residues surrounding this area, such as R90, K91, D100, and Y102, also contributed to the formation of the

TLR4-MD-2 complex.[159] Re *et al*. found, however, that a different region of MD-2 was responsible for conferring LPS responsiveness.[159] This region is not involved in TLR4 binding and is rich in basic and aromatic residues, several of which contribute to LPS responsiveness and might represent a LPS binding site. Consequently, mutations in the lysine residues of this region are correlated with the loss of LPS binding and as a result, the loss of activity.

Finally, it was found that binding of MD-2 by lipid A was greatly enhanced by serum components that have long been known to enhance LPS responses, i.e. sCD14 and LPS-binding protein (LBP).[152,165,168] MD-2 is unstable at 37°C, but the binding of LPS to MD-2 has been reported to dramatically stabilize its activity.[169] Overall, the evidence supports a model in which LPS interacts with the MD-2/TLR4 surface heterodimer. The interaction of LPS with the receptor complex occurs with high affinity, the K_d is estimated to be 3–10 nM.[170,171] The binding of LPS to MD-2 is then responsible for the aggregation of TLR4 and the recruitment of intracellular signal transducers.

1.4. Host-derived Mediators and the Pathogenesis of Sepsis

Once TLR4 binds to its LPS ligand, two possible pathways of cellular activation can occur – either through the myeloid differentiation factor 88 (MyD88) or through the TLR-domain-containing adapter-inducing interferon-β (TRIF) pathway.[172,173] In each pathway, signaling events lead to the sequential activation of specific tyrosine and threonine/serine kinases. This signaling cascade ultimately results in phosphorylation, ubiquitination, and degradation of inhibitory kappa-B (IκB) and other transcriptional activators. IκB degradation leads to translocation of nuclear factor-kappa B (NF-κB) into

the nucleus. Once NF-κB is translocated into the nucleus, it binds to specific DNA sequences located in the promoter regions and participates in the activation of a large variety of genes including cytokines, chemokines, stress response proteins, and anti-microbial and anti-apoptotic peptides.[174] As a result, the outpouring of inflammatory cytokines and other inflammatory mediators after LPS exposure contributes to generalized inflammation, procoagulant activity, tissue injury, and septic shock.[175-177]

In macrophages, lipid A activation of TLR4 triggers the biosynthesis of diverse mediators of inflammation and activates the production of costimulatory molecules required for the adaptive immune response.[178] Once activated, macrophages are the fundamental secretory cells of the immune system.[179] To date, more than one hundred macrophage products have been identified – with molecular weights ranging from 32 Da (superoxide anion) to 440,000 Da (fibronectin).[180] Amongst these, inflammatory cytokines such as TNF-α, IL-1β, and IL-6 are the most studied.

1.4.a. The Cytokine Networks

As mentioned above, monocytes/macrophages, neutrophils, and other inflammatory cells respond to LPS by secreting an array of microbial effectors and immunoregulatory mediators. The resulting autocrine (secretion of a substance that stimulates the secretory cell itself) and paracrine (secretion of cells into adjacent cells or surrounding tissue) activation results in synergistic potentiation of the inflammatory response and is critical in controlling the growth of pathogenic microorganisms. However, excessive production of these mediators is harmful to the host and can even be fatal, i.e. it can lead to the deleterious condition known as sepsis, or the dysregulation of regular hemodynamic and metabolic balances.[181] Amongst the well-known macrophage

mediators are the pro-inflammatory cytokines, chemokines, prostanoids, and reactive oxygen and nitrogen species.

Table 1.2. Pro- and anti-inflammatory mediators in sepsis.[182]

Pro-inflammatory Mediators	Tumor necrosis factor-α, lymphotoxin-a Interferon gamma Interleukins-1, -2, -8, -12, -18 Soluble CD14, MD2 Complement components, mannose-binding lectin Platelet-activating factor, leukotriene B4 Bradykinin Nitric oxide, other reactive nitrogen intermediates Reactive oxygen species Granulocyte macrophage colony-stimulating factor Macrophage inhibitor factor High mobility group box I Histamine, thrombin, factor X, tissue factor: factor VII Triggering receptor expressed on myeloid cells (TREM-1) Necrotic cells
Anti-inflammatory Mediators	Interleukin-1 receptor antagonist Soluble tumor necrosis factor or interleukin-1 Type II interleukin-1 receptor Transforming growth factor-b Interleukins-4, -6, -10, -11, -13 Prostaglandin E2a Granulocyte colony stimulating factor Endogenous antioxidants, glutathione, selenium, vitamin E Anticoagulants (antithrombin, activate protein C, tissue factor pathway inhibitor, soluble thrombomodulin) Interferon alpha, interferon beta Glucocorticoids Epinephrine, other catecholamines Cholinergic agonists Apoptotic cells

Indeed, the multitude of pro-inflammatory molecules present in the blood of septic patients is matched by an equally astounding array of anti-inflammatory mediators (Table 1.2). The network of cytokines and inflammatory molecules transmit signals between neutrophils, monocytes, macrophages, and endothelial cells. Much of the inflammatory response is localized and compartmentalized in the primary region of initial

inflammation (e.g. lung, kidney, or the gastrointestinal tract). Once the inflammatory process is released into the systemic circulation, its sustained production ensues a generalized reaction that culminates in diffuse endothelial injury, coagulation activation, and septic shock.

During the first 12 to 24 hours of sepsis, the pro-inflammatory mediators predominate locally. Thus, monocyte/macrophage-derived cytokines upregulate the expression of endothelial adhesion molecules such as ICAM-1, ICAM-2, and the P- and E-selectins.[183] These surface proteins, in turn, mediate neutrophil attachment, recruitment, and persistence at inflammatory sites. On the other hand, the anti-inflammatory mediators prevail in the later phases of sepsis.[184,185] Consequently, the loss of regulation by lymphocyte-derived cytokines and INFs ultimately determines the fate of patients with prolonged illness and septic shock.[186]

The cytokine TNFα, an endogenous monocyte/macrophage-derived protein, is one of the most important soluble mediators of inflammation. It is mainly synthesized by activated monocytes/macrophages and is responsible for a wide range of signaling events within cells. In response to an LPS challenge, TNFα is synthesized very quickly and the production peaks in a matter of 1.5 h.[187] Secretion of this molecule triggers a proinflammatory response in neutrophils and endothelial cells and leads to cell damage.[188-190] TNFα exerts most of its effects by binding, as a trimer, to either a 55kDa cell membrane receptor called TNFR-1 or to the 75 kDa cell membrane receptor TNFR-2; both are members of the TNF receptor superfamily.[191] In animal studies, the administration of tumor necrosis factor-α (TNFα), has been shown to have lethal consequences.[192] In human volunteers, dramatic hemodynamic, metabolic, and

hematologic changes are observed after administration of TNFα.[193] Perhaps the most dramatic demonstration of the pathophysiologic significance of systemic cytokine release was observed recently in a phase I study of an experimental anti-CD 28 monoclonal antibody.[175] The antibody was well tolerated in animal studies but was found to be markedly toxic to humans. Within a few hours of receiving the antibody, all six healthy human volunteers developed shock, disseminated intravascular coagulation (DIC), and multiorgan failure. The "cytokine storm" that often accompanies septic shock was clearly demonstrated by the striking elevations in IL-1, TNF, IL-8, IFN-g and other cytokines and chemokines that were released almost immediately into the patient's bloodstream.

Another important cytokine in host defense during sepsis is the interleukin-1 (IL-1) gene family.[194] This family consists of three members: IL-1α, IL-1β (both agonists with proinflammatory character) and the IL-1 receptor antagonist (IL-1ra, anti-inflammatory counterpart). While IL-1β is solely active in its processed and secreted form, IL-1α is active either in its intracellular precursor, membrane-associated, or secreted form.[195-198] The activation of numerous cell types by IL-1α and IL-1β leads to diverse proinflammatory events.[199]

The relative contribution of IL-1 to the inflammatory cascade in sepsis, however, is still unclear. Although the plasma levels of IL-1β are enhanced in patients suffering from septic shock,[200] systemic administration of LPS in IL-1β-deficient mice does not lead to changes in IL-1α, IL-6 and TNFα. Thus, on the basis of the response of LPS-treated mice with normal genetic background, IL-1β is not essential for systemic response to LPS.[201] Further examples for equivocal response within the IL-1 family are shown in IL-1ra deficient mice, which are highly susceptible to endotoxin-induced

death.[202] However, the treatment of sepsis syndrome mice with recombinant human IL-1ra did not lead to a statistically significant increase in survival time.[203]

Both IL-1 and TNFα act synergistically in the initiation of the inflammatory cascade in sepsis, leading to the expression of further factors.[204] These factors include other proinflammatory cytokines (IL-12, IL-18),[131,205] and chemokines (IL-8, monocyte chemoattractant protein-1/MCP-1).[206,207] The chemokines IL-8 and MCP-1 are key factors in chemotaxis – IL-8 is involved in neutrophil chemotaxis while MCP-1 is involved in the chemotaxis of monocytes. IL-8 also causes neutrophils to degranulate and cause tissue damage.[208] In addition, inflammatory cytokines upregulate the expression of endothelial adhesion molecules such as intercellular adhesion molecule-1 and -2 (ICAM-1, ICAM-2) as well as the P- and E-selectins.[209-212] These surface proteins, along with the large variety of genes described herein as a consequence of a dysregulated inflammatory response, regulates the sequence of events that define sepsis.

1.4.b. The Coagulation Cascade

Activation of the coagulation cascade has traditionally been synonymous with the need for hemostasis (stoppage of bleeding) at sites of injury. Over the past several decades, however, it has been increasingly recognized that initiation of coagulation is an integral and consistent element of the local and systemic response to inflammatory stimuli. The precise mechanism whereby coagulation contributes to the full expression of inflammation is an area of active study.

Tissue factor (TF) expression on the surface of endothelial cells and monocytes – induced by the presence of endotoxins or inflammatory cytokines – initiates the coagulation process.[213,214] Thus, as demonstrated below (see Figure 1.4), TF on the cell

surface activates factor VII, and the resulting complex of factor VIIa and TF converts factor X to factor Xa. In concert with factor Va, factor Xa converts prothrombin to thrombin, which in turn results in the cleavage of fibrinogen to fibrin. Fibrin deposition plays a critical role in hemostasis and in the localization of microorganisms within an abscess cavity. This process, however, can impede oxygen delivery to tissues and can induce further inflammatory injury indirectly through the response to hypoxia (lack of oxygen) and directly through signals delivered to the thrombin receptor. Engagement of the thrombin receptor activates the nuclear transcription factor NF-κB,[215] causing the transcription of a broad array of proinflammatory gene products and resulting in nitric oxide[216] release. The thrombin receptor is not a unique mechanism through which an inflammatory response is amplified. Clustering of tissue factor has also been shown to initiate gene expression for proinflammatory cytokines, including TNF.[217]

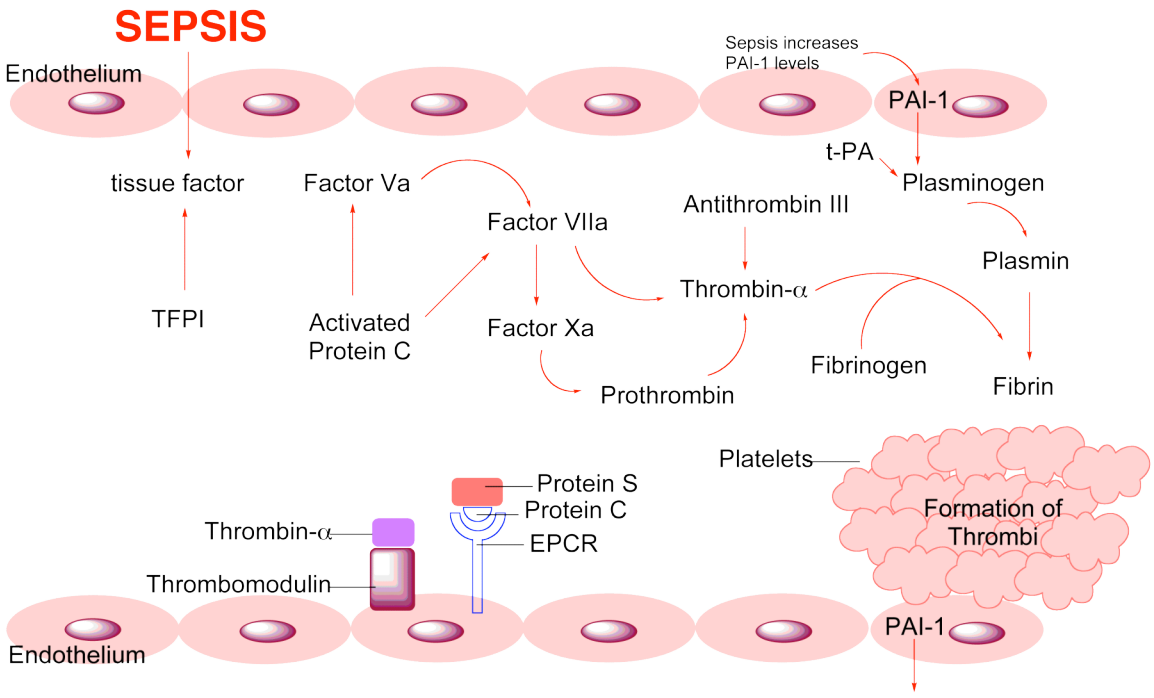

Figure 1.3. The impact of sepsis on the physiological network regulating thrombosis.

Two lines of evidence suggest that expression of TF is centrally important to initiation of the coagulation cascade during sepsis. First, TF expression is tightly regulated and is absent from the bloodstream during non-inflammatory conditions. TF is constitutively expressed by cells outside the bloodstream and therefore, it acts as an "extravascular envelope," triggering coagulation whenever the vascular endothelial integrity is breached.[218] Within the bloodstream only monocytes and the endothelium can be induced to express TF and have been demonstrated to do so both *in vitro* and *in vivo* in response to *E. coli*, LPS, and various cytokine mediators of the host response to infection.[219-221] Second, various strategies directed toward the neutralization of TF or the TF–VIIa complex have demonstrated efficacy in preventing organ damage associated with endotoxemia or bacteremia in experimental models, including the use of anti-TF antibody,[222,223] anti-factor VIIa,[224] and administration of tissue factor pathway inhibitor (TFPI).[225,226] In addition, a multitude of cytokines and inflammatory mediators known to play a role in local and systemic inflammatory responses — including TNFα, IL-1, IL-2, C5a, IL-6, platelet-activating factor (PAF) — have been demonstrated *in vitro* to either up-regulate or prime for TF expression. Moreover, these responses have been demonstrated to activate the coagulation cascade and contribute to septic mortality in primate models.[227-229]

In general, the activation of the coagulation pathway induces anticoagulant mechanisms that function to limit progression of the coagulation cascade. Indeed, there are three major pathways by which inhibition of coagulation can occur. In the first pathway, antithrombin III (ATIII) – a member of the serine protease inhibitor (serpin) family – covalently binds to and inactivates all of the serine protease coagulation factors,

including TF–VIIa.[230] Through binding to endothelial cells, ATIII–coagulation factor complexes will also initiate the release of prostacyclin, an inhibitor of platelet aggregation.[231] The second pathway involves the synthesis of tissue factor pathway inhibitor (TFPI) by endothelial cells. The complex formed between TFPI and factors such as TF or blood protease factors leads to inhibition of thrombin generation and fibrin formation.[232] Finally, the third pathway – the protein C pathway – is activated through the binding of thrombin to thrombomodulin on the endothelial surface. Protein C is a component of the protein C/protein S/C4bBP inhibitory axis. The endothelial surface protein thrombomodulin binds Xa, changing its specificity and leading to the activation of protein C.[233] The effect of activated protein C (APC) is amplified by complexing with protein S. The APC–protein S complex efficiently degrades factors Va and VIIIa, thereby inhibiting coagulation; APC alone also promotes fibrinolysis.[234]

During sepsis, however, an imbalance of the procoagulant and anticoagulant systems occur, resulting in a sustained hypercoagulable state. The specific abnormalities of the coagulation system that occur following endotoxemia and cytokinemia have been documented in detailed studies involving human volunteers and septic patients. Thus, in human volunteers injected with small doses of TNFα or lipopolysaccharide (LPS), there is gradual activation of coagulation as evidenced by increases in thrombin–antithrombin (TAT) complexes, prothrombin activation fragments, and fibrinopeptide A.[235-238] This process begins by the 2nd hour, peaks at 4 to 5 hours, and persists for 6 to 12 hours. There is also an early increase in plasma fibrinolytic activity (1–2 hours) due to the presence of plasminogen activators, which leads to plasmin generation. The anticoagulant effect of the latter, however, is rapidly neutralized by an increase in the amount of antifibrinolytic

plasminogen activator inhibitor-1 protein (PAI-1) released into the bloodstream. Both ATIII and protein C are also rapidly consumed during the septic state.[239,240] Additionally, the down-regulation of thrombomodulin due to both local and systemic release of cytokines, such as TNF and IL-1, results in impaired activation of the anticoagulant APC–protein S complex.[241]

The increased procoagulant activity, reduced anticoagulant activity, and impaired fibrinolysis in septic patients leads to the development of the clinical syndrome called disseminated intravascular coagulation (DIC). DIC is clinically defined as an overexuberant systemic clotting that depletes coagulation proteins and platelets from the blood and leads to bleeding complications.[219,242-248] It becomes increasingly common as patients advance from sepsis (SIRS) to septic shock.[249] The microvascular thrombosis that develops concomitantly results in organ injury, partly on an ischemic basis. As such, while DIC is considered in terms of bleeding complications, the clinical outcome is ultimately decided by the accompanying microvascular thrombosis and end-organ damage.

In summary, multiple and diverse pathways lead to activation of the cytokine networks and the coagulation cascade. To alleviate the fatal outcome of sepsis and septic shock, many therapeutic interventions have been targeted towards the later stages of endotoxin response: a) blocking cytokine synthesis/release by interfering with the transduction of cell-surface signals,[250-254] b) neutralizing released cytokines by passive immunization[181,255-257] and soluble receptors,[258] and c) blocking cytokine cell-surface receptors with a specific receptor antagonist.[259-261] The majority of these approaches have demonstrated efficacy in both in vitro and animal models but none has proven to be

effective at treating human sepsis. Due to the large diversity and quantity of cytokines released by activated cells, it is likely that the approach of blocking only a single cytokine may be inadequate. Since no single therapeutic agent has proven to be unequivocally beneficial for managing the abnormalities of sepsis, it has become increasingly clear that the therapeutic path to sepsis doesn't lie on the treatment of the downstream events. ***Under the assumption that end-organ damage ensues from an exuberant or hyperactivated immunological response that becomes unresponsive to supervening counter-regulatory mechanisms, the approach to sepsis treatment lies in interrupting the cascading inflammatory response by blocking the initial signaling events.*** Current progress towards this goal will be discussed below.

1.5. Approaches to Therapy: Anti-endotoxin Strategies

What we understand, thus far, is that endotoxin is essentially a signaling molecule that alerts the vertebrate host to the presence of an invasive Gram-negative bacterium within the body. It warns the host of the necessity to activate appropriate innate and adaptive immune defenses to effect clearance of the pathogen. This physiological defense mechanism has evolved into a well-coordinated survival strategy to localise, contain and eradicate invading bacterial pathogens. At the same time, the very same responses can precipitate a generalized and potentially fatal systemic inflammatory process once the infection enters the bloodstream. Thus, while the endotoxin molecule itself is not intrinsically toxic, the exaggerated host response to endotoxin accounts for septic shock from Gram-negative bacterial organisms.

To summarize from above, endotoxin mediates its injurious effects through systemic activation of host-derived inflammatory mediators including: (a) the

proinflammatory cytokine networks; (b) neutrophil, monocyte and endothelial cell activation; (c) the complement system; (d) the extrinsic coagulation cascade and the fibrinolytic system; (e) platelet activating factor; (f) the kinins; (g) the prostaglandins and leukotrienes; (h) reactive oxygen intermediates; (i) nitric oxide; and (j) probably other systems as yet undetermined. As such, endotoxemia is an archetypal example of a self-regulating complex system – it features multiple cascading nonlinear interactions and feedbacks that are mostly irreversible. Since the mechanisms regulating these events are amplifiable at the molecular level, the effects are allowed to expand from the source to the whole body and then combine to precipitate endotoxic shock.

However, while the evolution of organ dysfunction in septic shock is a complex, highly variable and multifactorial process involving many mediators, the past few decades have seen enormous advances towards understanding the cellular and molecular basis of the initial events in this process. The thorough characterization of these events has allowed researchers to design rational therapies directed against endotoxins. As summarized in Figure 1.5, there are seven potentially therapeutic points of intervention during the initial events of the LPS signaling cascade:

1. Inhibition of LPS synthesis,
2. Neutralization of LPS with antibodies directed at the core glycolipid region,
3. Binding and neutralization LPS with bactericidial/permeability-increasing (BPI) protein or endotoxin-binding columns,
4. Absorbance and clearance of endotoxins with high density lipoprotein (HDL),
5. Use of LPS analogues such as E-5531 and E5564 to antagonize LPS,
6. Binding CD14 – the LPS receptor on monocytes and neutrophils – with monoclonal antibodies, and
7. Neutralization of TLR4 and its accessory proteins to inhibit intracellular signaling pathways.

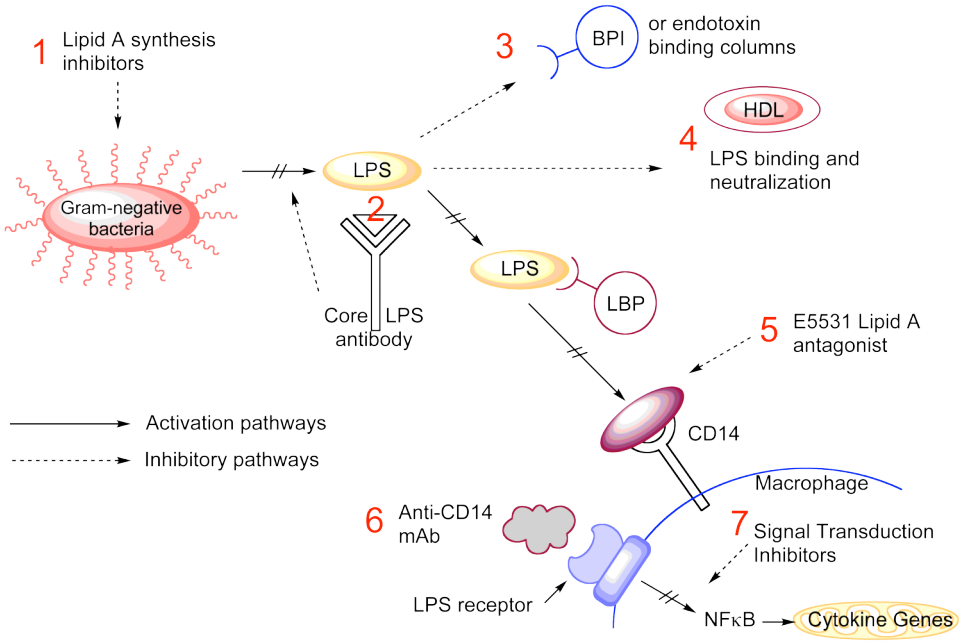

Figure 1.4. Schematic representation of various pathways of endotoxin activity and the seven potentially therapeutic points of intervention. Abbreviations: LBP = lipopolysaccharide binding protein; mAb = monoclonal antibodies; MAPK = mitogen-activated protein kinase; NFkB = nuclear factor k for B cells (important signal transduction protein for cytokine genes in monocytes and neutrophils); TK = tyrosine kinase.

Table 1.3. Current anti-endotoxin therapies.[262]

1. Enhanced LPS clearance
    - Anti-LPS antibodies
    - Haemoglobin derivatives
    - Direct removal of LPS through filtration
2. Direct neutralization of circulating LPS
    - Anti-LPS antibodies
    - LPS neutralizing proteins (ENP, BPI, defensins)
    - Polymyxin B
3. Inhibition of LPS-LBP and/or LPS-sCD14 interactions
    - Lipid A analogues (E5531, E5564)
    - Anti-LBP antibodies, anti-CD14 antibodies
    - BPI
4. Blocking cellular LPS receptors
    - Lipid A analogues (E5531, E5564)
    - Anti-CD14 antibodies
5. Inhibition of cell signal transduction
    - Tyrosine kinase or protein kinase C inhibitors

It is quite clear then that there are various avenues by which one can approach the design of therapeutic interventions against the inflammatory effects of LPS in the circulation. Table 1.3 summarizes a few examples of therapeutic agents that are under evaluation for each category. We believe, however, that ***the central role played by the Lipid A moiety of LPS in activating cells clearly indicates that blocking the cellular receptor for Lipid A may halt the chain of events leading to septic shock***. Amongst the various receptors discussed above, we believe that targeting CD14 is the most promising. In fact, there is plenty of evidence to support this idea. First, the formation of the CD14/LPS complex significantly reduces (100 to 1000 fold) the concentration of LPS required for activation of macrophages when compared to LPS alone.[263] Second, CD14-deficient mice are highly resistant to LPS-induced shock and monocytes derived from CD14-deficient mice are insensitive to LPS.[264] Finally, while CD14 seems to have the ability to discriminate between bacterial products and direct their signals to different TLRs, it does not have the fine binding specificity to discriminate Lipid A structures.[265,266]

It is a widely accepted convention in the pharmaceutical industry that a pharmacological receptor antagonist is often best derived from modification of the parent agonistic molecule. Since Lipid A represents the toxic principle of LPS,[31,267] synthetic endotoxin antagonists are structurally based on natural Lipid A and their biosynthetic precursors. To date, there has been a considerable effort expended towards modifying Lipid A from a variety of bacterial species as LPS antagonists. There are two observations that serve as a guiding principle for the synthesis of modified Lipid A substructures: 1) Lipid A of *Escherichia coli* is the most toxic amongst its class, and 2)

naturally derived Lipid A from bacteria such as *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* are less toxic and can potently antagonize the agonistic activity of *E. coli* Lipid A.[268,269]. Studies toward elucidating structure-activity relationships on the synthetic Lipid A substructures have already provided valuable insight into the significance of key portions of the molecule. Broadly classified into either monosaccharides or disaccharides, the insights gleaned from each structural lead will be discussed below.

1.5.a. Monosaccharide Antagonists

Concurrent with the classical target synthesis of *E. coli* Lipid A by Shiba and Kusumoto,[30] seminal studies by Raetz and co-workers led to the identification[270,271] and subsequent synthesis[272,273] of Lipid X (**1**, see Figure 1.5). Lipid X (2,3 diacyl glucosamine 1-phosphate) is a monosaccharide precursor in the biosynthetic pathway of lipid A.[274,275] This compound has been shown to possess the immunomodulating properties of LPS but not its toxicity.[276-278] As such, Lipid X antagonizes LPS activity *in vitro*[279] and has been reported to protect mice[280] and sheep[281] from LPS-induced lethality. In contrast, Lipid X has been shown to be ineffective *in vivo* in a canine sepsis model.[282] The structural similarity between lipid X and lipid A suggests that lipid X might be mediating its protective effect by specifically blocking endotoxin-triggered events, and possibly even competing directly with LPS for cellular binding sites, a hypothesis that can be tested in an in vitro system. However, more recent studies have shown that the beneficial immunomodulatory effects of lipid X were due to minor disaccharide contaminants.[283,284]

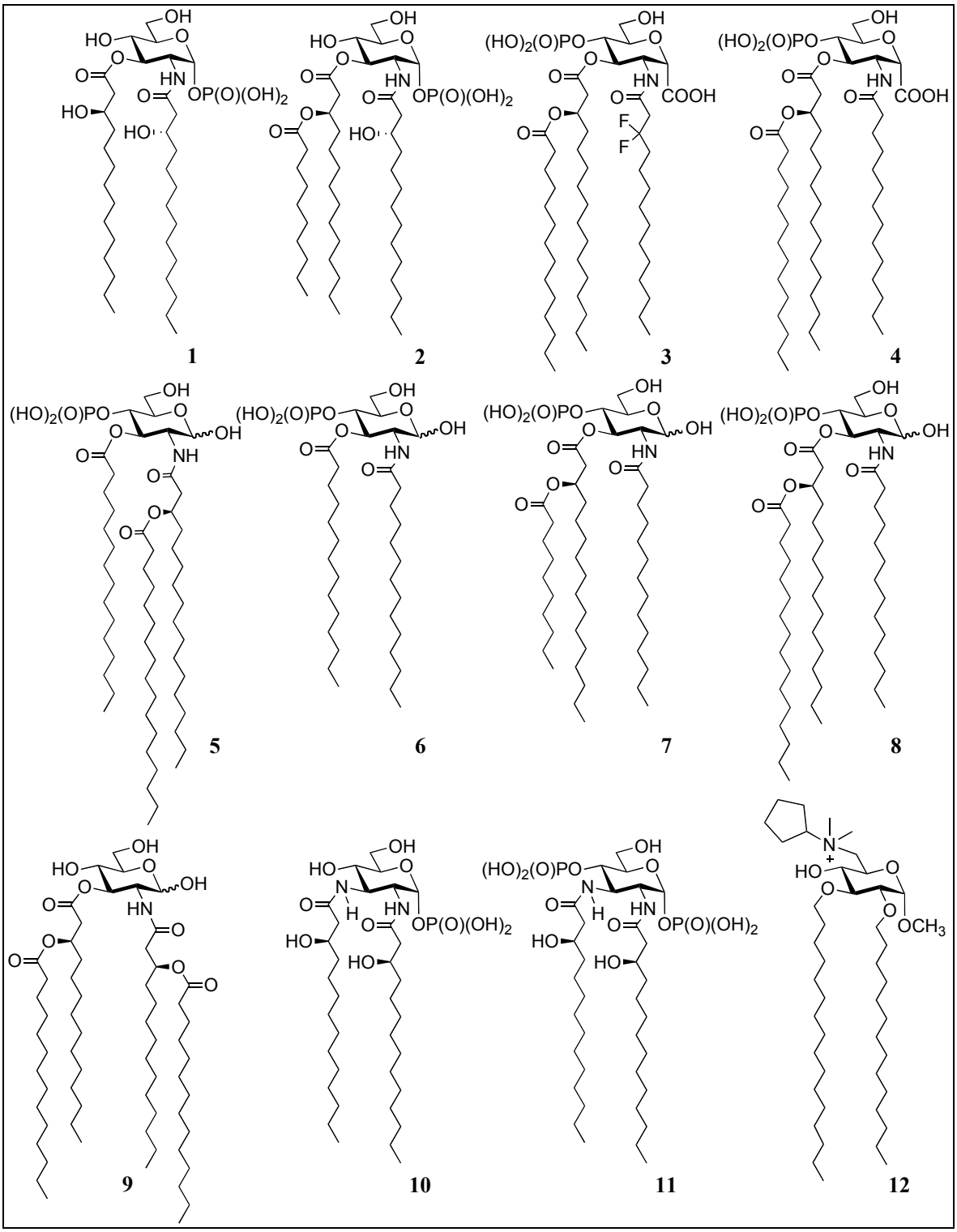

Figure 1.5. Structures of monosaccharide Lipid A analogs.

On the other hand, Rossignol *et al*. mentioned in a review that their efforts to develop monosaccharide-based endotoxin antagonists met with limited success, yielding compounds with low antagonistic potency.[285] However, amongst the compounds they tested, they found that ERI-1 (**2**, see Figure 1.5) demonstrates good activity in *in vitro* assays utilizing cultured cells or primary cultures of monosaccharides containing low concentrations of plasma.[286] When the compound was tested *in vivo* in the mouse endotoxin challenge or *in vitro* such as in whole blood assays, the activity of **2** was attenuated.

Two similar tri-acyl monosaccharides containing an anomeric carboxylic acid and O-4-phosphate (**3** and **4,** see Figure 1.5) were recently reported by researchers at Sankyo.[287,288] These compounds were shown to be potent inhibitors of LPS-induced TNFα production in monocytes at 5 and 17 nM, respectively. The fascinating aspect of this work is that it corroborates findings by researchers at Sandoz that the use of 2-keto carboxylic acids prevents the formation of disaccharide contaminants that are agonistic.[283,284] The *in vivo* activities of these potentially antagonistic monosaccharides, however, have not been reported.

Matsuura *et al*. believed that further exploration of the beneficial applications of monosaccharide analogues to sepsis therapy requires a deeper understanding of the characteristic features of human cells that allow for the recognition of lipid A-like structures.[289] Consequently, this group conducted more detailed studies of the effects of monosaccharide analogues on human cells in relation to their structures. Thus, a series of structurally related monosaccharide lipid A analogues were examined for their ability to activate human monocytic cells to induce cytokines such as TNF-α and IL-6, in

comparison with their ability to activate murine monocytic cells. To this end, they found that the monosaccharide analogue GLA-58 (**5**, see Figure 1.5), which has been found to be an LPS antagonist in murine[290,291] cells, also exhibited antagonistic activity in human[289] cells. In addition, they found that while monosaccharide analogues GLA-26, GLA-47, GLA-89, and GLA-64 (**6**, **7**, **8**, and **9**, respectively, Figure 1.5) do not induce cytokine release in human monocytic cell lines, they showed significant potency to activate murine monocytic cells. More interestingly, however, despite the agonistic activity exhibited by compounds **6-9** in murine cells, they were found to have antagonistic activity to LPS-induced activation of human monocytic cells. To summarize their results, they found that the structure of lipid A analogues recognized as LPS agonists by human monocytic cells comprises D-glucosamine, phosphoryl groups, and acyl groups with defined carbon chain lengths of C14 and C12 in a ratio proportional to 1:1:3. Moreover, human cells have stricter requirements toward recognizing elements of Lipid A substructures compared to that of murine cells. As such, Lipid A-like structures, recognized by the murine cells as LPS agonists and antagonists, are recognized as being LPS antagonists by human cells.

Building on the hypothesis that derivative **1** might be mediating its protective effect by specifically blocking endotoxin-triggered events, and possibly even competing directly with LPS for cellular binding sites, Danner *et al*. evaluated several derivatives of **1** for anti-endotoxin activity.[286] Thus, lipid X and its diamino analog, 3-aza-lipid X (**10**, Figure 1.5),[279] were evaluated for their ability to either prime or prevent LPS-mediated priming of human neutrophils. To this end, they found that compound **10** demonstrated marked inhibition of endotoxin-induced priming at low concentrations but an endotoxin-

like priming effect at high concentrations. However, when a phosphate group was added at C-4 of compound **10**, the resulting compound (**11**, see Figure 1.5) exhibited pure antagonistic and increased anti-endotoxic[292] activity.

Thus far, we find that in order for a monosaccharide analogue to exhibit anti-endotoxin activity, it has to bear some structural similarity with that of *E. coli* Lipid A. Recently, however, it was shown that substances with chemical structures totally unrelated to Lipid A such as taxol,[293] flavolipin,[293] and vitamin D[294] have LPS-like activity towards TLR4. Moreover, Jerala *et al*. projected that the structural motif that can be recognized by LPS receptors can be essentially reduced from Lipid A to a structure with anionic groups and a large hydrophobic moiety.[295] In an attempt to explore the verity of these observations, Peri *et al*. presented the synthesis and biological activity of a glucose-derived ammonium species (**12**, Figure 1.5).[296] To this end, they found that despite the lack of a glucosamine backbone, ester- and amide-linked fatty acids, and phosphate residues, compound **12** inhibits lipid A-induced cytokine production in MT2 macrophages in a dose-dependent manner. Interestingly, this seminal publication highlights that the presence of a five-membered ring and a positive charge on the nitrogen atom at C-6 was fundamental to the activity of compound **12**.

1.5.b. Disaccharide Antagonists

Reports describing that Lipid A from *R. capsulatus* (**14**, see Figure 1.6)[268] and *R. sphaeroides* (**15**, Figure 1.6)[269,287,288] were non-toxic compared to Lipid A from *E. coli* (**13**, Figure 1.6) verified the potential utility of disaccharide-based endotoxin antagonists. The proposed structures of nontoxic compounds **14** and **15** differ from **13** in the number of acyl chains on the reducing end, the difference in length of the individual acyl chains,

and the presence of unsaturation on the acyloxyacyl moiety of **14** and **15**. Compound **14** also possess two β-ketoacyl chains at the C-2 and C-2' positions whereas only one β-ketoacyl chain is present at the C-2 position on **15**.

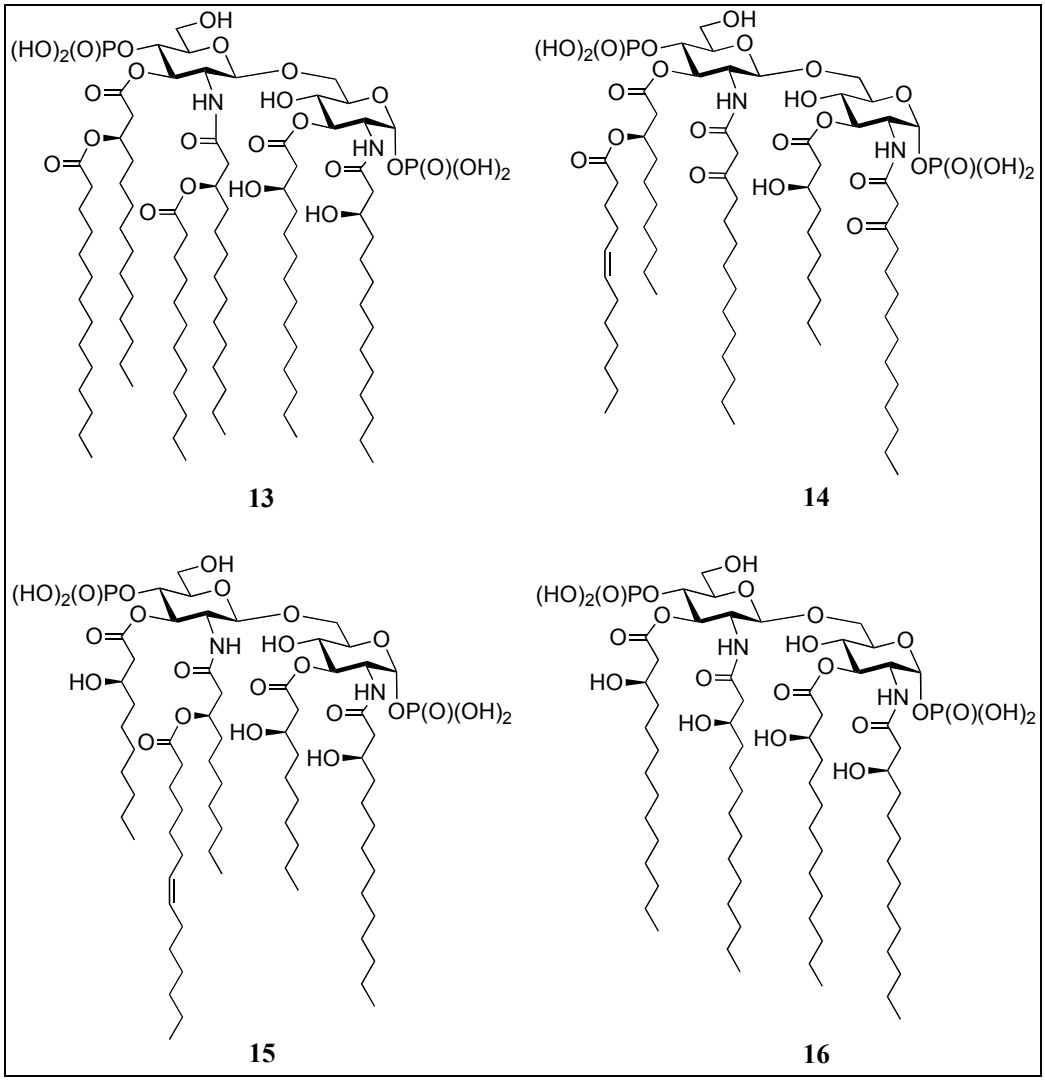

Figure 1.6. Structures of Lipid A derived from various bacterial species (**13**-**15**) and the biosynthetic precursor of *E. coli* Lipid A (**16**).

Lipid IV_A (**16**, Figure 1.6) is the biosynthetic precursor of LPS and is a potent LPS antagonist in human *in vitro* assays but is an agonist in murine systems.[297] Since the structure of **16** presents a synthetically simpler disaccharide, various groups began

investigating the feasibility of using it as a lead structure. To this end, synthetic analogues of **16** were generated in order to investigate the structural constraints of the antagonistic activity of **16**, to eliminate its agonistic activity, and to determine potential stabilization of the compound for pharmaceutical development.[285] However, they found that similarly with **16**, biological activity of these derivatives strongly depended on the assay system used.

Both *in vitro* and *in vivo*, derivative **14** possesses an anti-endotoxin potency that is desirable for a pharmaceutical antagonist.[269] While the synthetic version of **14** appears to retain the same antagonistic activity ascribed to the bacterially derived molecule, it was found that it cannot be readily synthesized and purified to pharmaceutical standards, especially on a scale necessary for development.[285] To address this problem, Rossignol and co-workers decided to investigate O-3 and O-3' ethers in place of esters.[285] Although Shiozaki *et al*.[298] have shown that stabilization of compound **13** with the generation of O-3 and O-3' alkyl chains produced an analogue without anti-endotoxin properties, conversion of derivative **14** to ERI-6 (**17**, Figure 1.7) yielded a potent LPS antagonist both *in vitro* and in mice. During the synthesis of this molecule, however, small amounts of impurities arose from nucleophilic attack of the free C-6' hydroxy group toward the adjacent C-4' phosphate and rendered final purification difficult. Since natural Lipid A is connected to the core region and O-antigen portion of the LPS molecule at the C-6' position, alteration or "capping" of this position seemed to be a logical modification of derivative **17**. Consequently, methylation of the C-6' hydroxyl group not only eliminated the formation of impurities, it also blocked the formation of a cyclic phosphate. The resulting molecule, E5531 (**18**, Figure 1.7), is a fully stabilized endotoxin antagonist with

no detectable agonistic activity. Lack of toxicity and mutagenicity, along with the availability of a stable formulation, established **18** as a candidate for clinical development.[299-301]

Concurrent with the clinical development of **18**, Rossignol *et al*. also performed additional studies to determine the structural requirements for a second-generation anti-endotoxin candidate possessing a much simpler structure.[285] Thus, they found that methylation of the C-4 hydroxyl led to an approximate two-fold enhancement of the antagonistic activity in the resulting molecule ERI-7 (**19**, Figure 1.7). On the other hand, changing the configuration of the γ-carbon of the C-3 alkyl chain to the (S)-configuration to give ERI-18 (**20**, Figure 1.7) enhanced the antagonistic activity approximately fivefold. Finally, the role of the phosphate functionalities on C-1 and C-4' towards antagonistic activity was also evaluated. Unfortunately, dephosphorylation either gave a compound that was insoluble in the assay medium or devoid of a measurable antagonistic activity. The phosphate functionalities on the parent compound seemed necessary for biological activity.

The lipid A from *R. sphaeroides* (**15**, see Figure 1.6) was also found to possess potent antagonistic activity in both human and murine systems.[302-304] While the synthetic version was as potent as the bacterially derived material in terms of antagonizing LPS in human monocytes, human whole blood, and murine macrophages, it lacked the weak agonistic activity present in the latter.[305] Attempts at developing a clinically acceptable

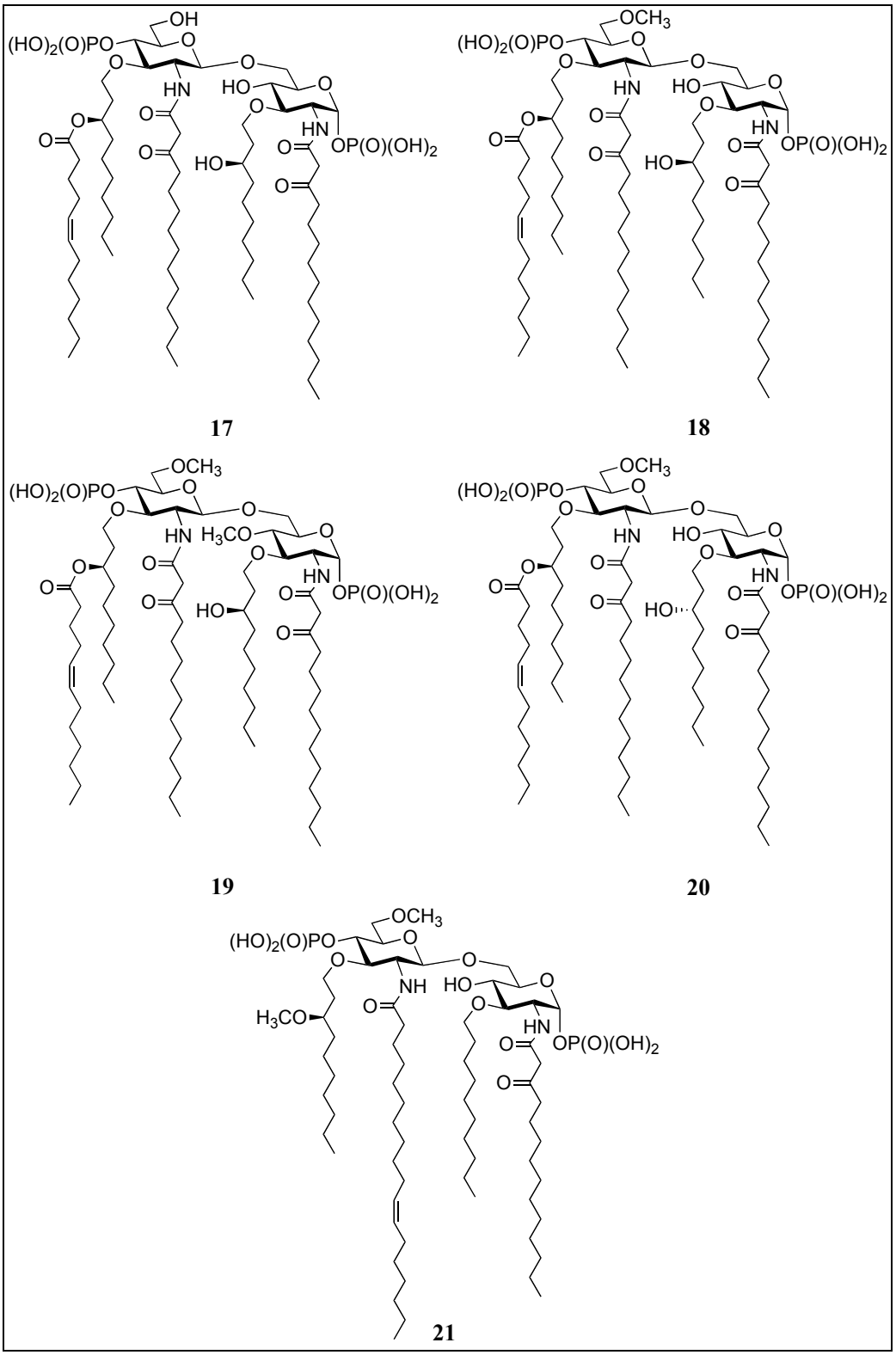

Figure 1.7. Lipid A analogues derived from *R. capsulatus* and *R. sphaeroides* Lipid A.

version of **15** were similarly focused towards obtaining a pure, stable material with high potency. Thus, the C-3 and C-3' acyl chains were converted to alkyl chains and the C-6' hydroxyl was methylated, as in compound **18**.[285]

Rossignol *et al*. were also able to simplify the C-2' acyloxy chain into a single acyl chain 18 carbons in length containing the (Z)-unsaturation at an approximated position. In addition, they found that removal of the γ–hydroxyl moiety at C-3 increased the activity significantly and made the molecule more synthetically accessible. Finally, methylation of the hydroxyl moiety on the C-3' alkyl chain optimized the series for both biological activity and synthetic ease. The resulting molecule, E5564 (**21**, Figure 1.7), is stable, more readily synthesized, and more potent than E5531. Consequently, **21** is being developed by the Eisai company as a second-generation antisepsis drug and is currently undergoing clinical trials.

It was mentioned earlier that the phosphate moieties are essential to the biological activity of Lipid A derivatives. Boons and co-workers, however, have found that a "phosphate-free" derivative of *Rhizobium sin-1* Lipid A (**22**, see Figure 1.8) lack the proinflammatory effects of *E. coli* LPS.[306] Furthermore, compound **22** was found to emulate the ability of heterogeneous *R. sin-1* LPS to antagonize enteric LPS, providing evidence for the critical role of the gluconolactone moiety in the antagonistic activity of *R. sin-1* LPS.

Finally, Nielsen *et al*. determined that LPS derived from *Helicobacter pylori* also exhibits no proinflammatory activity.[307,308] This prompted Fukase and co-workers to explore derivatives of *H. pylori* Lipid A for possible antagonistic activity. Previously, this group had designed and synthesized monosaccharide analogues containing acidic amino

acid residues at C-6 and observed a switch in endotoxic or antagonistic activity based on the type of anionic residue present.[309] To this end, they decided to synthesize *H. pylori* Lipid A derivatives containing 3-deoxy-D-manno-2-octurosonic acid (Kdo) at C-6. Since natural Lipid A of *E. coli* is generally connected to the rest of the LPS domain via the Kdo residue, it was reasoned that attaching the acidic Kdo unit may aid in the recognition event that the anionic phosphate residue usually mediates. Indeed, it was found that compound **23** (Figure 1.8) antagonize E. coli LPS-induced immunostimulation.[310]

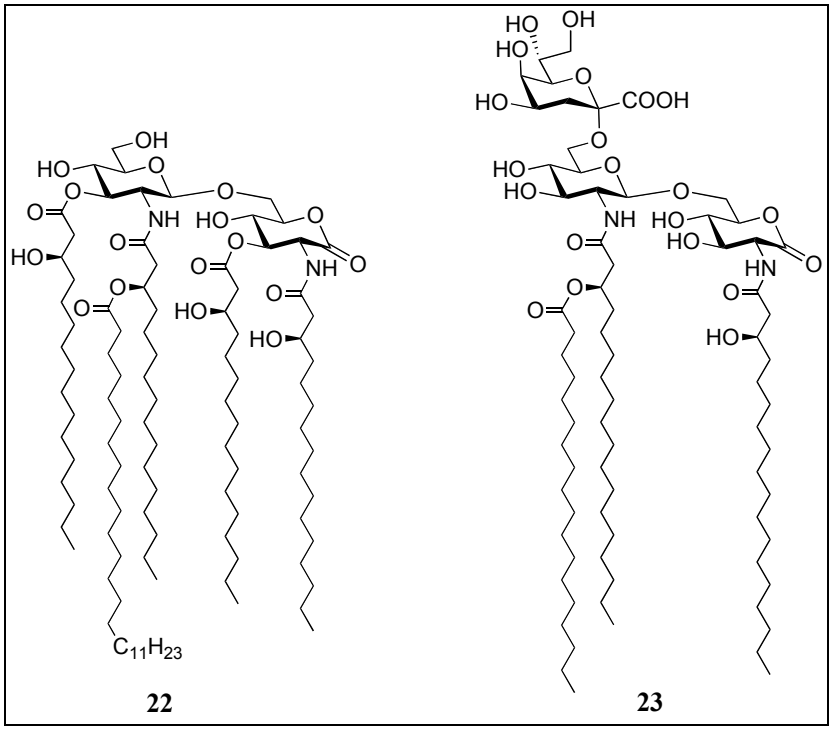

Figure 1.8. Lipid A derivatives from *Rhizobium sin-1* and *Helicobacter pylori*.

1.6. Statement of Research Goals

As mentioned, there ***is still a major unmet medical need for more effective and safer anti-sepsis agents***. Indeed, we raised several points regarding a feasible approach to sepsis therapy. First, the multiple and diverse pathways regulating the cytokine networks

and the coagulation cascade make it likely that blocking the downstream events following LPS-induced cellular activation will be completely inadequate. Unfortunately, this is the only strategy that has been approved and applied in hospital settings to date.[50,193] Second, under the assumption that end-organ damage ensues from an exuberant immunological response that becomes unresponsive to supervening counter-regulatory mechanisms, it is reasonable to suppose that the approach to sepsis treatment lies in interrupting the inflammatory cascade by blocking the initial signaling events. Third, the central role played by the Lipid A moiety of LPS in activating cells clearly indicates that blocking the cellular receptor for Lipid A may halt the chain of events leading to septic shock. Finally, for compelling reasons that were elaborated earlier, it is rational to conclude that CD14 presents the most promising target amongst the cell-surface receptors discussed above. Thus, to address the need for a single therapeutic agent that is unequivocally beneficial for managing the abnormalities of sepsis, the goals of this dissertation are as follows:

1. **Design monosaccharide and disaccharide analogs of Lipid A that will potentially bind with high affinity to the cell receptor CD14.**
2. **Develop and optimize high-yielding, convergent, facile, scalable, and novel methods for the synthesis of the Lipid A analogues**
3. **Evaluate the synthetic analogues for antagonistic activity towards LPS-induced release of TNFα in human macrophage cells *in vitro***

While considering our targeted design for Lipid A analogues that will bind with high affinity to CD14, we took into consideration the lessons gleaned from the analogues described in Section 1.5. Since it has been well proven that disaccharide analogs usually provide more appreciable antagonism towards LPS for obvious reasons of structural

similarities, we were inclined to pursue the design and synthesis of disaccharide analogs. Subsequently, we found it essential to verify the idea presented by Jerala[295] that the structural motif that can be recognized by LPS receptors can be condensed to the arrangement of anionic groups and a large hydrophobic moiety. Apparently, this type of arrangement provides a more likely complement to the cationic residues and the large hydrophobic binding site of the LPS receptors. To this end, we decided to build on the structural simplicity of Lipid IVA (**16,** see Figure 1.6) by having four hydrophobic chains attached to the disaccharide core. Since previous research showed a distinct ambivalence towards the type of hydrocarbon chain necessary for antagonistic activity, we decided to try all three types – the simple alkyl chain, the acyloxy chain, and the acyl chain. Finally, to provide the anionic character to our design, we decided to attach an acidic amino acid on C-6. Therefore, our targeted design for the disaccharide analogues **24-26** are illustrated in Figure 1.9.

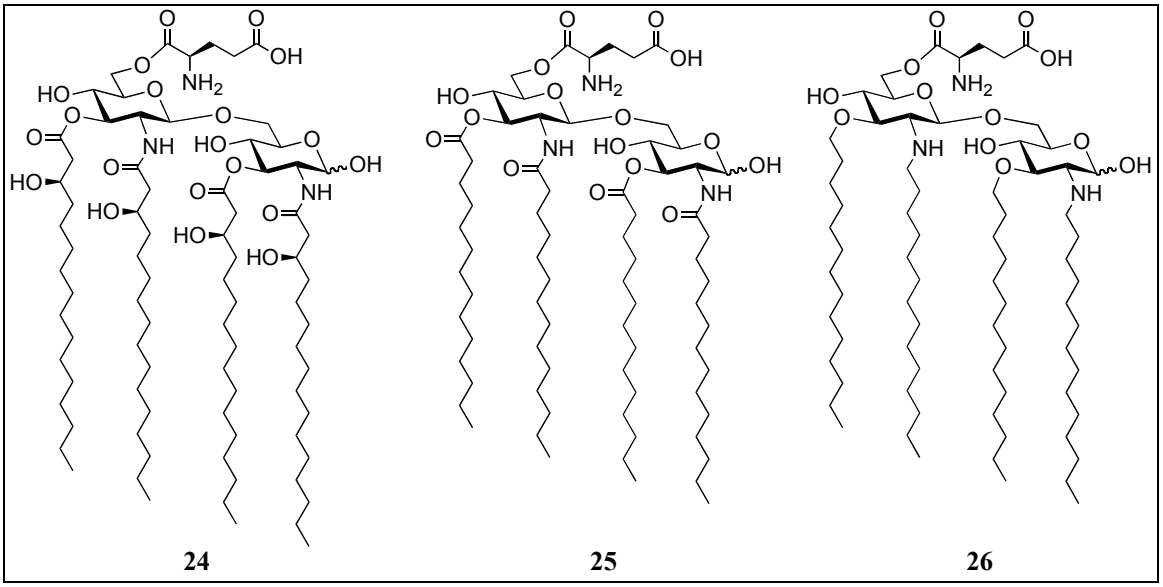

Figure 1.9. Targeted design for the disaccharide analogues.

Moreover, since there are scarce examples of Lipid A analogues that lack the disaccharide core yet still maintain potent antagonistic activity, we felt it was essential to explore the design and synthesis of potent monosaccharide antagonists. To this end, the observation by Peri and coworkers that compounds structurally unrelated to Lipid A can still exhibit potent antagonistic activity was considered.[296]

In this connection, the development of similar glucose-derived ammonium species (containing hydrophobic alkyl chains in place of the ester- and amide-linked acyl chains) would possibly offer a fruitful venue for further studies. Nonetheless, we found ourselves particularly interested in pursuing a different structural lead. Thus, we decided to investigate a structural motif that: 1) preserves the native glucosamine residue and the ester- and amide-linked (R)-3-hydroxy fatty acid chains, 2) lacks any unstable phosphate residue, and 3) contains a cationic residue on C-6. Basically, we wanted to explore the ramifications of Peri's idea on a structure that is analogous to Lipid X (**1**, see Figure 1.5). In addition, following in the footsteps of Fukase *et al*., we wanted to utilize amino acids to provide the cationic character.[309,310] Thus, our goal is to synthesize compounds **27-32** (see Figure 1.10) and evaluate these targeted designs for possible antagonism towards LPS-induced proinflammatory activity.

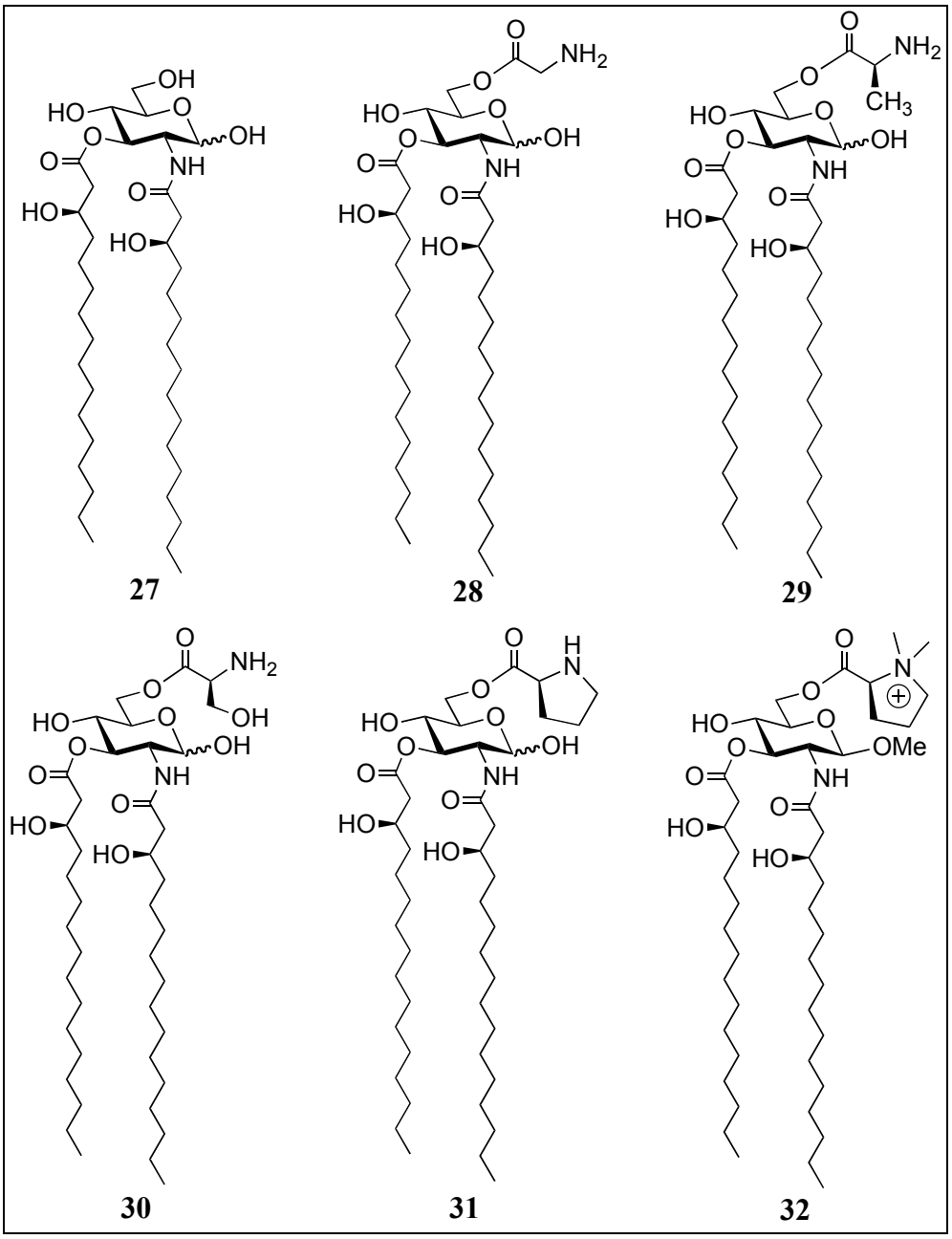

Figure 1.10. Targeted design of monosaccharide Lipid A analogues.

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Chapter 2

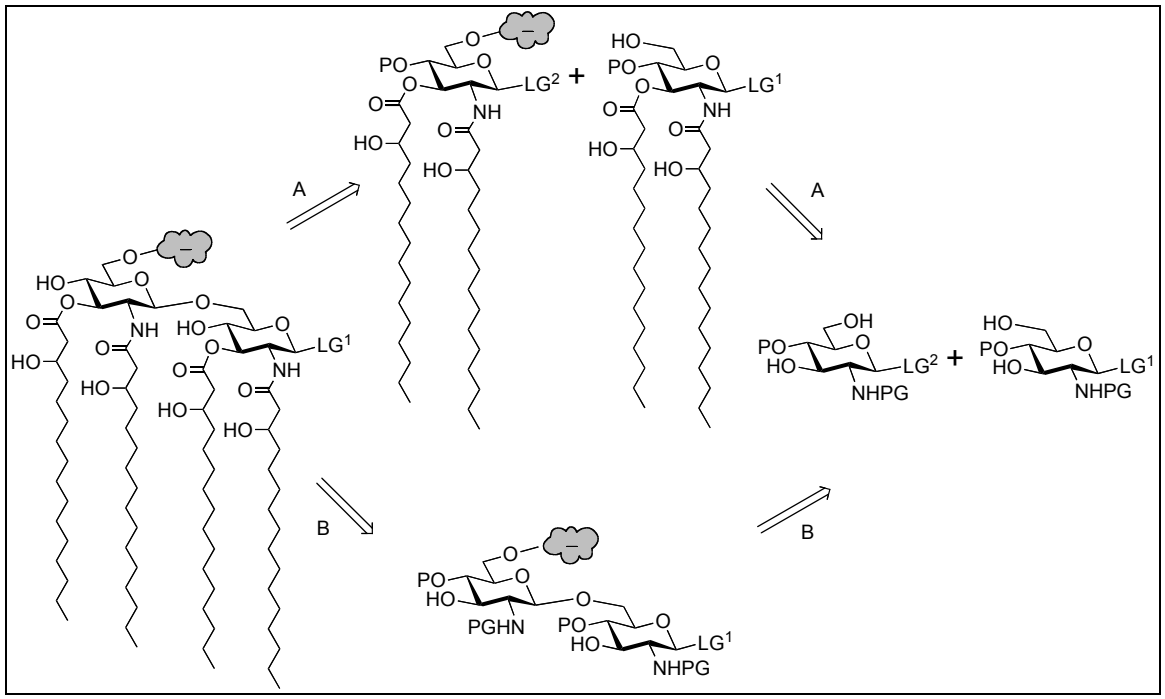
Method Development

Bongat, A. F. G.; Demchenko, A. V., "Recent trends in the synthesis of O-glycosides of 2-amino-2-deoxysugars," *Carbohydr. Res*., **2007**, *342*, 374-406.

Bongat, A. F. G.; Kamat, M. N.; Demchenko, A. V., "Chemoselective Synthesis of Oligosaccharides of 2-Deoxy-2-aminosugars," *J. Org. Chem*., **2007**, *72*, 1480-1483.

2.1. Retrosynthetic Scheme

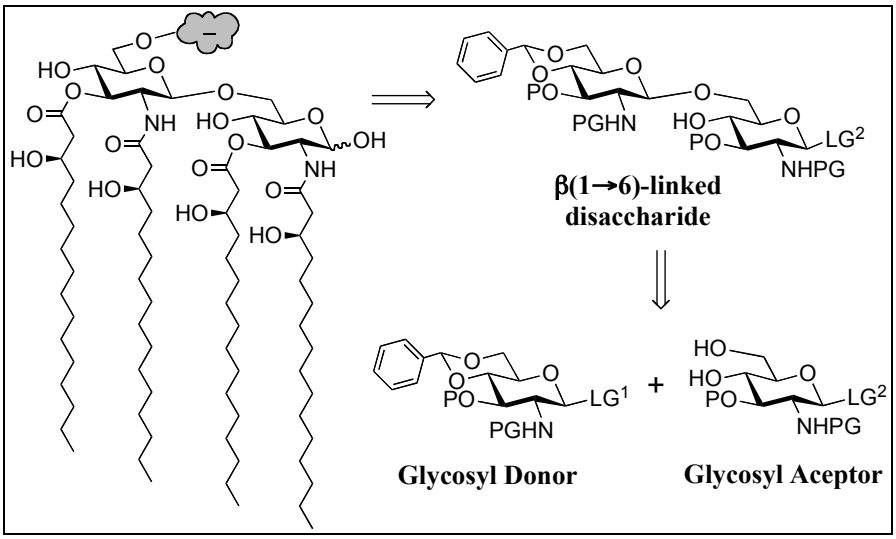
Currently, the synthesis of disaccharide Lipid A derivatives and their biosynthetic precursors follow two common approaches. In the first approach, the monosaccharides are first functionalized with lipid chains and then coupled to form the beta-(1→6) linked disaccharide (route A, Scheme 2.1). This approach was utilized by Shiba and co-workers in the first reported synthesis of the proposed structure of *E. coli* Lipid A.[1-3] In the second approach, the disaccharide core is formed first and then selectively functionalized with lipids (route B, Scheme 2.1).



Scheme 2.1. Strategic approaches to the synthesis of Lipid A analogues. P = Protecting Group; PG = Participating Group; LG = Leaving Group.

While the first approach has the advantage of having fewer linear steps, a significant amount of synthetic work in this area has preferentially employed the second pathway.[4,5] Amongst obvious reasons for these are: (1) the difficulty in obtaining analytical data as

the molecule gets more complex, (2) the abysmal yield usually obtained in glycosylating monosaccharides with bulky substituents, and more importantly (3) the formation of a stable by-product (oxazoline) during the glycosylation step. Hence, as shown in Scheme 2.2, the retrosynthetic scheme for the target synthesis of our disaccharide analogues follows the second approach:

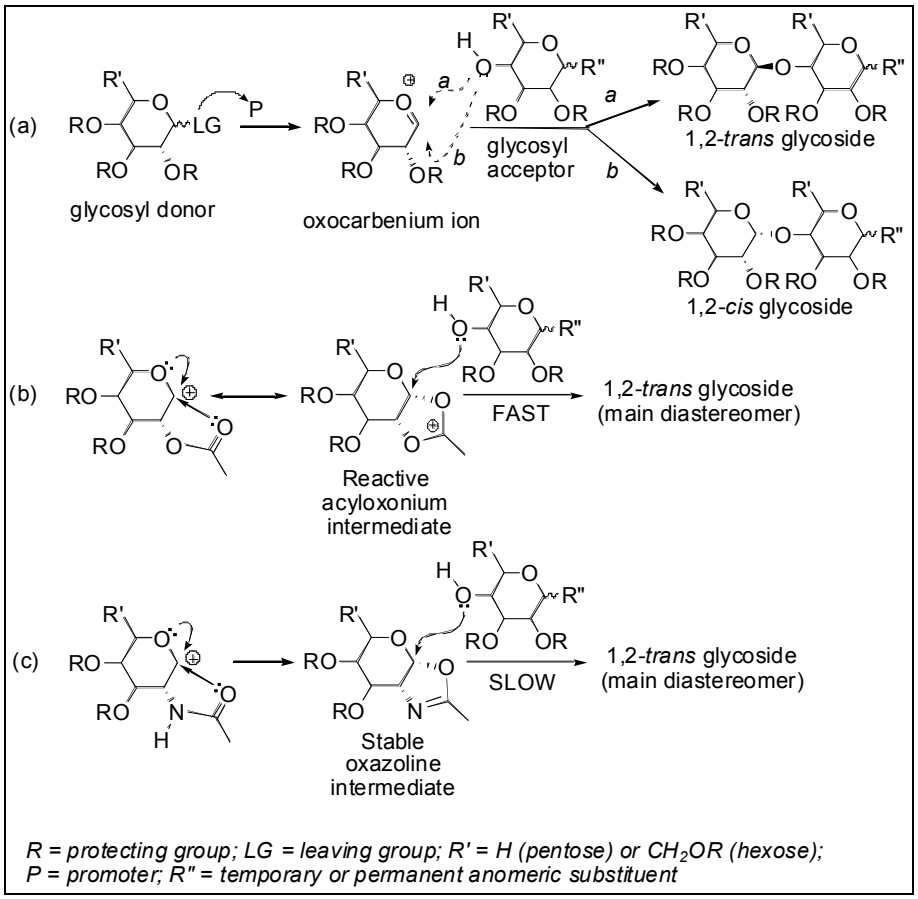


Scheme 2.2. Retrosynthetic scheme the synthesis of Lipid A analogs. P = Protecting Group; PG = Participating Group; LG = Leaving Group; R = Alkyl Group

As illustrated above, our plan is to synthesize the beta-(1→6)-linked disaccharide from a differently protected glycosyl donor and acceptor. Once the disaccharide is formed, it will be selectively deprotected and functionalized into our target compounds. The monosaccharide analogues, on the other hand, will be synthesized from the same building blocks used to synthesize the disaccharides. Thus, based on the retrosynthetic scheme and the necessity of forming a glycosidic linkage with high stereocontrol, our initial concern in planning the synthesis was to choose a suitably derivatized 2-amino-2-deoxy glycoside.

Glycosides of 2-amino-2-deoxy sugars are present in the most important classes of glycoconjugates and naturally occurring oligosaccharides, in which they are connected to other residues via either 1,2-cis (α) or, more frequently, 1,2-trans (β) glycosidic linkage.[6-8] In particular, 2-N-acetamido-2-deoxyglycosides, most commonly of the D-gluco and D-galacto series, are widely distributed in living organisms as glycoconjugates (glycolipids, lipopolysaccharides, glycoproteins),[6] or glycosaminoglycans (heparin, heparin sulfate, dermatan sulfate, chondroitin sulfate),[9] and in blood group oligosaccharides,[10] etc. Aminosugars on cell surfaces play an important role as receptor ligands for protein molecules such as enzymes,[11] antibodies,[12] and lectins,[13] and participate in antibody-antigen interactions.[14] As appreciation for the biological importance of 2-amino sugars has increased, so have efforts to develop chemical methods for the synthesis of oligosaccharides containing these residues. Special efforts for the synthesis of glycosyl donors of 2-amino-2-deoxy sugars have been focusing on the development of simple, efficient, regio-, and stereoselective procedures.

Nowadays, a variety of synthetic approaches to the synthesis of 2-amino-2-deoxyglycosides have been developed and the progress in this area has been previously reviewed.[15-17] The synthesis starts either directly from a glycosamine or by introduction of a nitrogen functionality to glycose or glycal derivatives. To this end, various glycosamine donors with modified functionalities have been investigated, in particular, those bearing an N-2 substituent capable of either efficient participation via acyloxonium, but not (2-methyl)oxazoline, intermediate for 1,2-trans glycosylation. The reason for this is illustrated below.



Scheme 2.3. Synthesis of 1,2-cis and 1,2-trans 2-amino-2-deoxy glycosides.

Thus, promoter-assisted departure of the leaving group of regular (O-2) glycosyl donors results in the formation of the glycosyl cation, which is stabilized by resonance from O-5 via flattened oxocarbenium ion (Scheme 2.3.a). Hence, the nucleophilic attack is almost equally possible from either the top (*trans,* β- for the D-gluco series) or the bottom face (*cis,* α-) of the ring. Even though the α-product is thermodynamically favored (anomeric effect), a substantial amount of the kinetic β-linked product is often obtained. Various factors such as temperature, protecting groups, conformation, solvent, promoter, steric hindrance, or leaving groups may influence the glycosylation outcome.[18,19] If the use of a base-labile ester-protecting group is permitted, 1,2-*trans* glycosides can be reliably prepared with the assistance of a neighboring participating

group at C-2.[20] These glycosylations proceed primarily via a reactive bicyclic acyloxonium ion intermediate directing the nucleophilic attack mainly to the top face of the ring and allowing stereoselective formation of a 1,2-*trans* glycoside (Scheme 2.3.b). Many traditional glycosyl donors such as halides, thioglycosides, or O-trichloroacetimidates provide excellent stereoselectivity and high yields.[21,22]

Since a vast majority of naturally-occurring 2-amino-2-deoxy sugars are N-acetylated, it is synthetically desirable to utilize a 2-acetamido-2-deoxy substituted glycosyl donor and thereby minimize protecting group manipulations. For this type of glycosyl donors however, the oxocarbenium ion rearranges rapidly into an oxazoline intermediate (Scheme 2.3.c). Even under harsh Lewis acid catalysis, this highly stable oxazoline intermediate does not exert strong glycosyl donor properties. As a result, the use of N-acetylated donors is often impractical. On one hand, glycosidation of such donors often leads to the formation of relatively unreactive oxazoline intermediate that often remains as a major by-product. On the other hand, high nucleophilicity of the lone pair of electrons on nitrogen of the acetamido group also presents a complication by attracting electrophilic species (such as promoters of glycosylation) that often results in decreased reactivity and/or additional by-products.

A common way to decrease the reactivity of the amino group it is to temporarily convert it into an electron-withdrawing amide, carbamate, or imide. The carbonyl group(s) effectively withdraw the electron density from the nitrogen atom and renders it less reactive. The drawback of this indirect approach is in the necessity to perform a number of additional steps that are required for the synthesis of the glycosyl donor and the conversion of the temporary substituent into the N-acetamido moiety. These

additional synthetic manipulations significantly decrease the over-all efficiency and yields.

The choice of an amine protecting group for C-2 of aminosugars is also influenced by the fact that the protecting group at C-2 can directly influence the stereochemical outcome of the glycosylation reactions (see Scheme 2.3). Thus, for a 1,2-trans glycosidic linkage, it would be desirable if the amine function on C-2 would be capable of providing anchimeric assistance to the oxocarbenium ion that forms. Overall, the ideal amine protecting group should be stable to a wide range of reaction conditions while also being able to impart sufficient reactivity, stereoselectivity, and high yield in glycosylation reactions. Moreover, it should be readily removed under mild reaction conditions. Thus, in order to determine the type of amine protecting group to install on our glycosyl donor and acceptor, we surveyed the literature for the most suitable class of 2-amino-2-deoxy glycoside to use for our purposes.[17] Following the general theory that neighboring group participation promotes efficient formation of 1,2-trans-linked glycosides, we narrowed our search to 2-amino substituents that are capable of providing anchimeric assistance. Adapting from our published review on the subject,[17] protecting groups of this type that have been successfully employed in glycosylation reactions are summarized below.

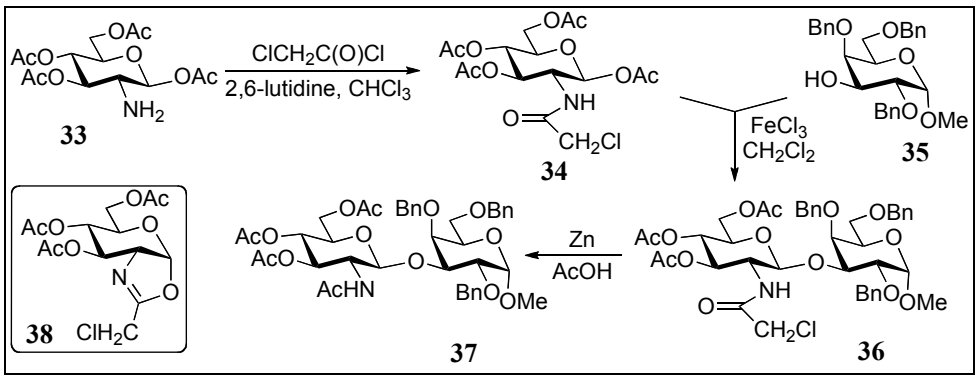
2.2. Monosubstituted 2-Amino-2-Deoxy Glycosides: Haloacetamido Derivatives

Simple amides are generally prepared from the acid chloride or the anhydride. Many amides are stable toward mild acidic or basic hydrolysis and are classically hydrolyzed by heating in strongly acidic or basic solutions.[23] Among simple amides, hydrolytic stability increases from formyl to acetyl to benzoyl. Lability of the haloacetyl

derivatives to mild acid hydrolysis increases with substitution: acetyl < chloroacetyl < dichloroacetyl < trichloroacetyl < trifluoroacetyl.[24]

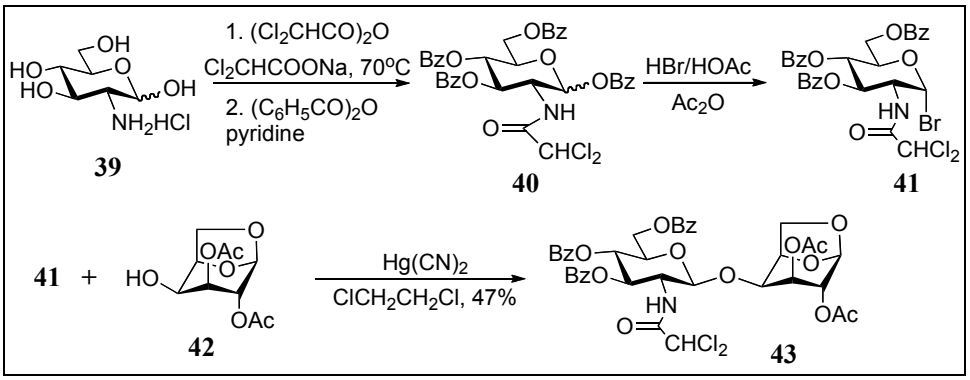
The use of 2-chloroacetamido-2-deoxy glycopyranosyl donors was introduced by Kiso and Anderson in 1985, who reasoned that halogen-substituted 2-acetamides will form weaker, and therefore more reactive, oxazoline intermediate (see compound **38**, Scheme 2.4) by virtue of electron withdrawal.[25] As a result, glycosyl donors of this type would be better electrophiles, and hence, more reactive in glycosidation. To this end, 2-chloroacetamido derivative **34** was synthesized from **33** using chloroacetyl chloride in the presence of 2,6-lutidine in $CHCl_3$ (Scheme 2.4). To demonstrate the effectiveness of monochloroacetyl derivatives as glycosyl donors, **34** was then glycosidated with various primary and secondary glycosyl acceptors.[24,26]

Thus, $FeCl_3$-promoted glycosidation of **34** with glycosyl acceptor **35** gave the disaccharide **36** in 80% yield. When acetate **34** was treated with $FeCl_3$ in CH_2Cl_2 in the absence of the glycosyl acceptor, the oxazoline derivative **38** was isolated as proof that the glycosylation reaction proceeds via an oxazoline intermediate. It was demonstrated that the treatment of **36** with zinc and acetic acid in refluxing oxolane reduces the N-chloroacetyl group (see the synthesis of **37**, Scheme 2.4).



Scheme 2.4. 2-Chloroacetamido-2-deoxy glucopyranosyl derivatives.

2-Deoxy-2-dichloroacetamido derivatives were introduced as glycosyl donors to provide a solution to one of the biggest issues concerning the use of 2-acetamido derivatives as glycosyl donors. Thus, glycosyl donor 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-glucopyranosyl bromide was found to be easily transformed into a relatively unreactive 1-acetyl-2-amino-2-deoxy hydrobromide derivative by an N→O acyl migration via the oxazoline.[27] Consequently, it was hypothesized that the powerful electron-attracting dichloroacetyl group may have the appropriate structure to address this challenge.[28] For this purpose, glucosamine hydrochloride **39** was reacted with dichloroacetic anhydride and sodium dichloroacetate followed by O-benzoylation to afford the 2-deoxy-2-dichloroacetamido sugar **40** in 68% yield (Scheme 2.5). Bromination of **40** with HBr/HOAc gave the glycosyl donor **41**, which was reacted with the glycosyl acceptor **42** under Helferich conditions to afford the disaccharide **43** in 47% yield.[28,29] Hydrolysis of the amide group was effected by adding 1N methanolic barium methoxide and water to a solution of **43** in methanol.[28]



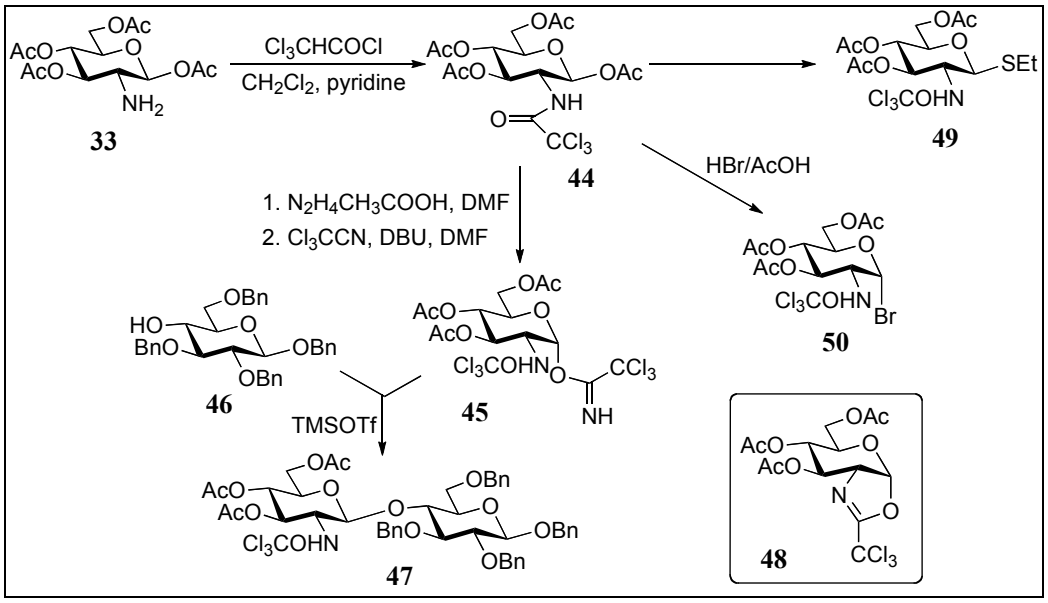
Scheme 2.5. 2-Deoxy-2-dichloroacetamido glucopyranosyl derivatives.

2-Deoxy-2-trichloroacetamido derivatives have been also investigated, initially for its applicability towards the synthesis of nucleosides under acidic conditions.[30] Unfortunately, it was found that a stable trichloromethyl oxazoline was the main product of this glycosylation method and as a result, further studies were abandoned. Three decades later, Beau and co-workers noted that, as a matter of fact, (2-trichloromethyl)oxazoline resembles an intramolecular trichloroacetimidate.[31] Since glycosyl trichloroacetimidates are excellent glycosyl donors, this bicyclic derivative could serve as a potentially reactive glycosyl donor for the synthesis of 1,2-trans-2-amino-2-deoxyglycosides.[32]

To this end, 1,3,4,6-tetra-O-acetyl-2-deoxy-2-trichloroacetamido-β-D-glucopyranose **44** was prepared from D-glucosamine tetraacetyl derivative **33** as illustrated in Scheme 2.6.[30,33] Anomeric deacetylation of **44** with hydrazine acetate in DMF followed by reaction with trichloroacetonitrile in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) afforded the trichloroacetimidate derivative **45** in 79% yield. The glycosyl donor **45** then was then glycosidated with **46** in the presence of TMSOTf to afford 1,2-trans-linked disaccharide **47** in 82% yield. The complete stereoselectivity achieved supports the intermediacy of an oxazolinium species. This

conclusion was confirmed when treatment of **45** with TMSOTf followed by workup furnished oxazoline **48** as the major product (Scheme 2.6). Thus, it seems that the electron-withdrawing effect of the trichloromethyl group in the intermediary oxazolinium ion greatly increases the electrophilic character of the anomeric carbon, as compared to its methyloxazoline counterpart.

Other classes of glycosyl donors with the 2-deoxy-2-trichloroacetamido moiety have been investigated.[31,34-36] Amongst derivatives investigated, glycosyl donors **49** and **50** serve as a clear illustration of the versatility of 2-trichloroacetamido derivatives in terms of their application to oligosaccharide synthesis. In this connection, Donohoe *et al.* found that 2-(trichloroacetyl)oxazoline derivatives of allosamines and tallosamines are also excellent glycosyl donors.[37]



Scheme 2.6. 2-Deoxy-2-trichloroacetamido glucopyranosyl derivatives.

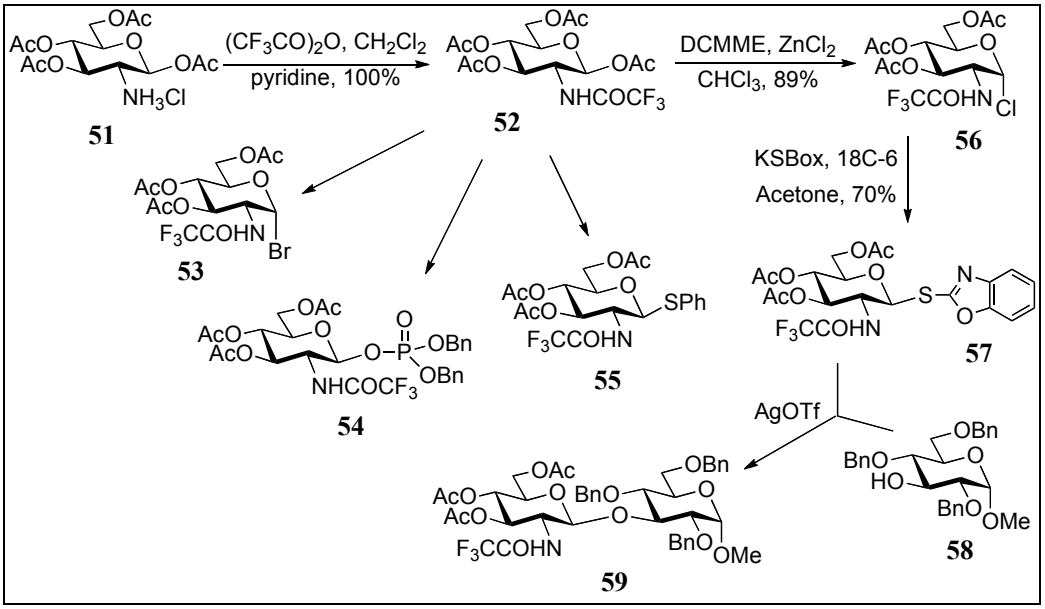
Another advantage to the use of N-trichloroacetyl derivatives is the ease of their deprotection. This can be achieved under a variety of reaction conditions, amongst which

are tributylstannane-azobisisobutyronitrile (AIBN) in refluxing benzene,[31] hydrogenolysis (H_2, Et_3N, PtO, AcOEt, 55 psi),[38] or cleavage with NaOH followed by reacetylation.[37,39]

The early application of 2-deoxy-2-trifluoroacetamido (TFA) derivatives was to the synthesis of steroid glycosides using glycosyl bromides as donors.[30,40,41] The precursor for this synthesis, derivative **51**, was obtained in three steps from D-glucosamine via sequential treatment with anisaldehyde in 1N NaOH, acetylation with acetic anhydride, and hydrolysis of the N,N-anisylidene moiety with 5N HCl.[42,43] Reaction of **51** with trifluoroacetic anhydride gave the N-trifluoroacetyl derivative **52** quantitatively (Scheme 2.7). A variety of glycosyl donors with a suitable anomeric leaving group have been prepared. Amongst the reported glycosyl donors bearing the N-trifluoroacetyl moiety on C-2 are the acetates (such as **52**),[44,45] bromides (**53**),[30,41,46-51] phosphates (**54**),[52-55] thioglycosides (**55**) and their corresponding sulfoxides,[56] 4-tertbutylthiophenyl glycosides,[57] phenylselenides,[58] etc. Very recently, the anomeric acetyl moiety was converted into chloride **56** and then into S-benzoxazolyl (SBox) glycosyl donor **57** (Scheme 2.7),[59] The latter derivative showed potent glycosyl donor properties; for example, glycosylation of the glycosyl acceptor **58** in the presence of AgOTf provided 1,2-trans-linked disaccharide **59** in 89% yield.

To convert the N-trifluroacetyl moiety back to the free amine, various methods have been employed. Amongst these are the use of HCl in MeOH,[60] NaOH in Acetone-H_2O,[49] or LiOH[56] in THF-MeOH. It should be noted that these conditions allow for the orthogonal deprotection of the N-trifluoroacetyl group over other amine protecting groups.[56] Glycosyl donors of this class have been employed in the synthesis of chiro-

inositols,[46] didemnin B analogues,[61] C-glycosyl phosphonates,[52] globotriosylceramide,[62] diosgenin derivatives,[50] bacterial polysaccharide fragments,[63] and Lipid A[64] analogues.



Scheme 2.7. 2-Deoxy-2-trifluoroacetamido glucopyranosyl derivatives.

2.3. Monosubstituted 2-Amino-2-Deoxy Glycosides: Alkoxycarbamoyl Derivatives

2-Alkoxycarbamoyl-2-deoxy derivatives have found widespread use as glycosyl donors due to their ease of formation and the orthogonality or chemoselectivity of N-deprotection.[15] Protection is usually accomplished by condensation of the free amine and the appropriate chloroformate in the presence of a mild base.[65] Conditions for removal, however, depend on the carbamate moiety employed, i.e. benzyl carbamate (NHCbz or NHZ) is cleaved by catalytic hydrogenolysis; allyl carbamate (NHAlloc) is readily cleaved by Pd-catalyzed isomerization; while trichloroethyl carbamate (NHTroc) is readily cleaved by acidic hydrolysis or reduction with Zn.[23] Carbamates such as N-

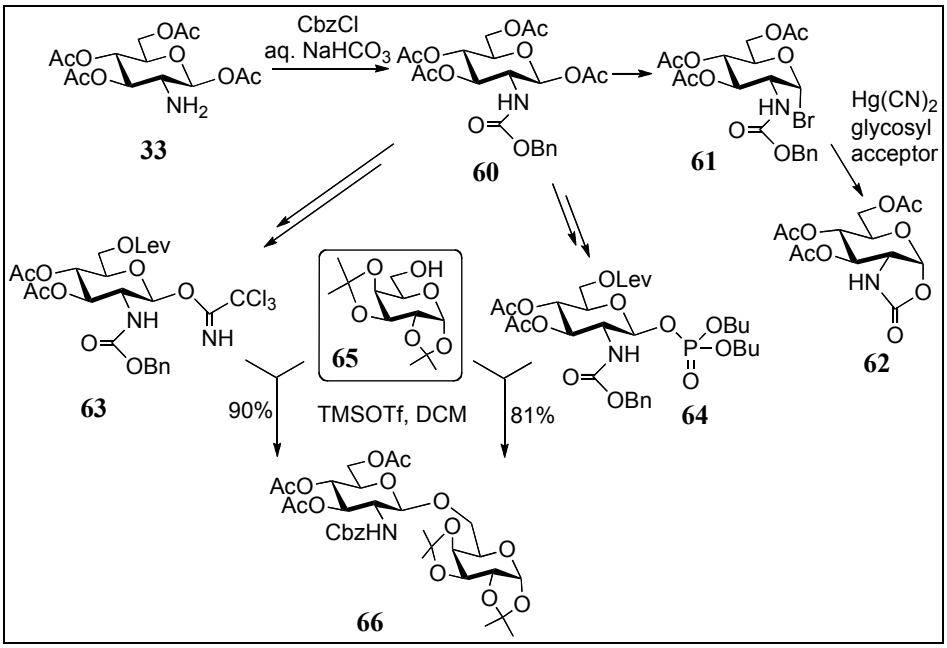
methoxycarbonyl,[66-71] N-(tert-butyl)oxycarbonyl (tBoc),[65] and N-(p-nitrobenzyl)oxy-carbonyl (PNZ)[72] have been used in oligosaccharide synthesis.

Dating back to the 1930's,[42,73] the use of benzylchloroformate (CbzCl) in the presence of a suitable base is still the method of choice for installing the N-benzyloxycarbonyl moiety on the free amine function.[65,74-78] The tetraacetate **60** can be easily prepared from either unprotected glucosamine[73] or from 2-amino tetraacetate **33**.[65] The latter approach is preferred if the synthesis of **60** in anomerically pure β-form is required (Scheme 2.8). It has been effectively used in the synthesis of glycopeptide[79] and glycolipid[80] derivatives. Cbz-protected glycosamines can also be obtained by consequent reprotection of azido or another carbamate-protected glucosamine.[74,81]

The first reported glycosylations with 2-N-benzyloxycarbonyl amino sugars involved activation of bromides.[82,83] Unfortunately, the $Hg(CN)_2$-promoted reaction of glycosyl bromide **61** with glycosyl acceptors gave the oxazolidinone derivative **62** with migration of the benzyl group to the aglycone.[82] Silver salt-promoted condensation of simple alcohols with 2-NHCbz chloride was more successful. Yet, β-glycosides were isolated only in moderate yields (42-46%) despite the use of a large excess of alcohol.[84] When applied to the synthesis of simple aminosugar-pyrimidine nucleosides, however, 2-NHCbz chlorides gave the 4-methoxy-pyrimidinone nucleoside in 84% yield.

Over the years, more potent glycosyl donors bearing the 2-NHCbz moiety have been developed including dimethylphosphinothioates,[85] phosphorodiamidimidothioate,[86] trichloroacetimidates,[87] 4-pentenyl glycosides,[86] and phosphates.[86] For example, glycosylation of glycosyl acceptor **65** with glycosyl donors **63** or **64** in the presence of TMSOTf provided the disaccharide **66** in 90 and 81% yield, respectively (Scheme 2.8).[86]

These glycosyl donors were prepared in a few steps using conventional synthetic manipulations via stable O-pentenyl moiety as a temporary anomeric substituent.

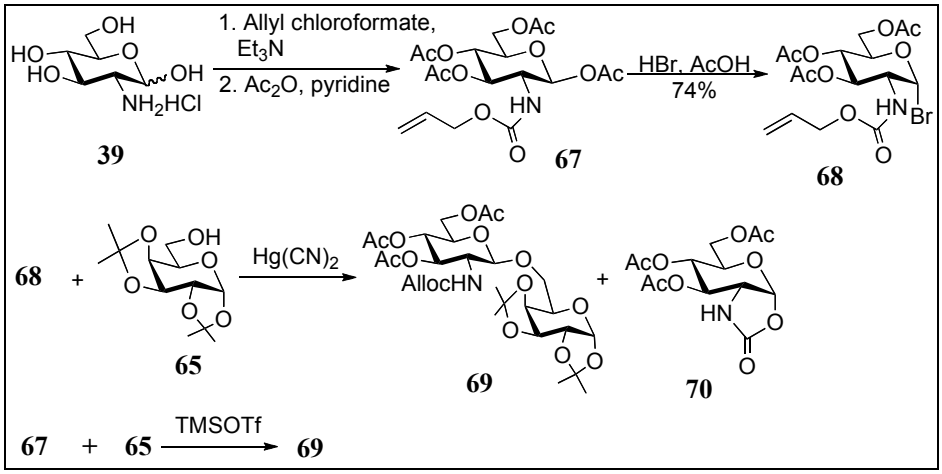


Scheme 2.8. 2-Deoxy-2-Benzyloxycarbamoylglucopyranosides.

As mentioned, catalytic hydrogenolysis cleaves the carbobenzyloxy moiety to afford the free amine. Reported deprotection conditions include H_2/Pd in EtOH,[87] H_2/Pd in dioxane/water,[75] and H_2/Pd in MeOH/H_2O,[88,89] along with the chemoselective N-deprotection reported by Hasegawa[90] using H_2/Pd in the presence of MeOH, HBr, and dioxane. The efficacy of these glycosyl donors have been demonstrated in the synthesis of nucleosides[91] inner core of lipopolysaccharides,[92] heparin derivatives,[88,93-95] dibekacin,[96] Lipid A derivatives,[2] neoglycoconjugates,[74] and antibacterial amino glycosides,[97] among others.

Versatile applications of the allyloxycarbamoyl (Alloc) protective group[98,99] prompted Boullanger *et al*. to investigate its participating properties in 1,2-trans

glycosylation reactions.[100] For this purpose, the glucosyl bromide **68** was prepared in 74% overall yield from D-glucosamine hydrochloride **39** by sequential treatment with allylchloroformate and Et_3N at 50 ºC, followed by acetylation (Ac_2O, pyridine) and bromination with 33% HBr in AcOH (Scheme 2.9).[101] The reaction of 2-N-allyloxycabonyl bromide **68** with galactosyl acceptor **65** promoted by $Hg(CN)_2$ gave the β-linked disaccharide **69** in 86% yield along with the oxazolidinone derivative **70**.[101] In contrast, glycosidation of β-D-acetate **67** with glycosyl acceptor **29** in the presence of trimethylsilyl triflate (TMSOTf) provided disaccharide **69** in 82% yield with no side product **70** formation.[100]



Scheme 2.9. 2-Allyloxycarbamoyl-2-deoxyglucopyranosides.

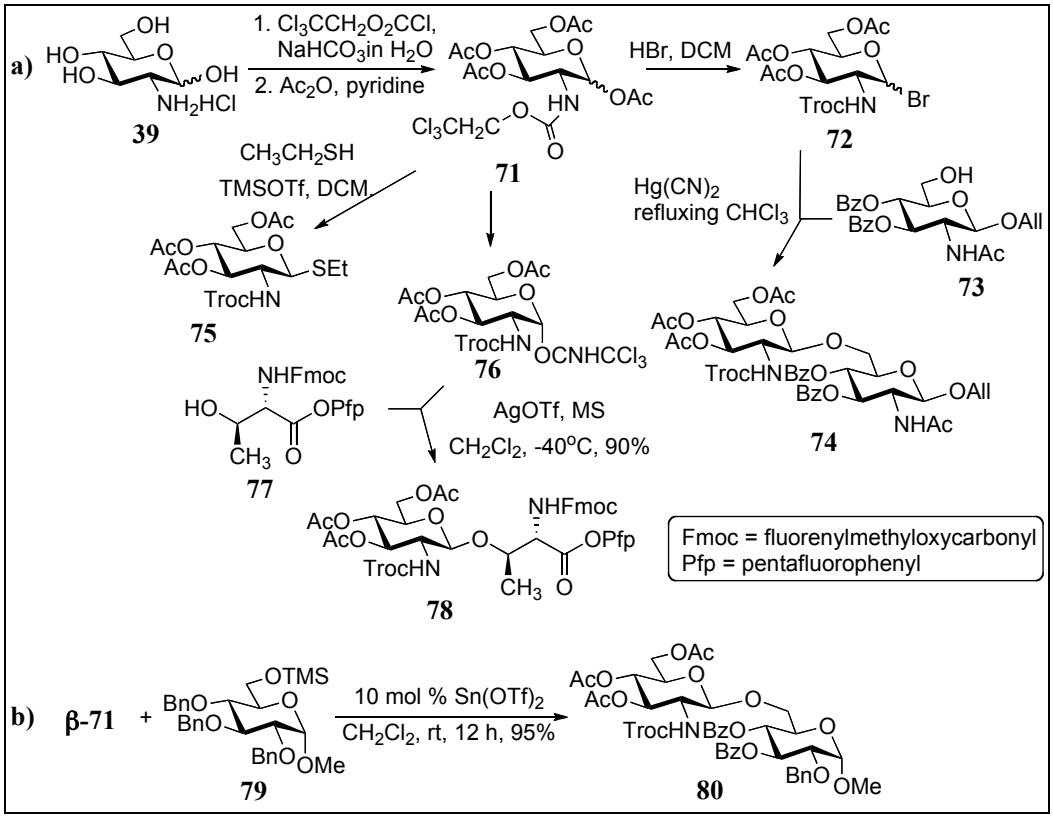
In an attempt to improve the reactivity as well as to expand the scope of applications, a variety of 2-allyloxycarbamoyl-2-deoxyglycopyranosyl derivatives have been developed over the years; for example, trichloroacetimidates,[102] and thioglycosides.[103] Derivatives of 2-N-allyloxycarbonyl sugars have been applied to the synthesis of peptidoglycan partial structures,[104] glycopeptide assemblies,[102,105] 1,6-anhydro derivatives,[106] neoglycolipid analogues,[107] N-acetylneuraminic acid

derivatives,[103] and surfactants.[108] The deprotection of the N-allyloxycarbonyl group is achieved using any of the following methods: reductive cleavage,[109] treatment with $Ni(CO)_4$,[110] basic hydrolysis,[103] homogeneous palladium catalysis using $Pd(PPh_3)_4$,[100,104,111-113] or Pd-catalyzed hydrogenolytic cleavage with formic acid in the presence of an allyl acceptor.[114] The allyl acceptor can be dimedone,[111] 2-ethylhexanoic acid,[112] tributyl tin hydride,[102,113] dimethyl malonate,[100,107] or AcOH.[104]

The use of 2-N-trichloroethoxycarbonyl (Troc) derivatives in aminoglycoside synthesis was first introduced by Kusumoto *et al*. in 1985.[115] In their report, the N-Troc bromide **72** was synthesized from commercially available compound **39** in quantitative yield over three steps (Scheme 2.10). Glycosylation of glucosyl acceptor **73** with bromide **72** gave the disaccharide **74** in good yield and complete β-selectivity. This method was used extensively in the synthesis of *Escherichia coli* Lipid A and analogues thereof, as well as in the synthesis of O- and S-linked glycopeptides.[3,116-123] Another typical procedure for the preparation of the NHTroc derivatives is via temporary N,N-anisylidene protection.[65] The resulting intermediate **71** can serve as a suitable precursor for the preparation of a range of excellent glycosyl donors.

Schultz and Kunz converted the intermediate β-**71** into thioglycoside donor **75**, thiophilic activation of which with DMTST gave the desired β-linked glycopeptides.[105] Trichloroacetimidate **76**, obtained from the acetate **71** via conventional two-step protocol,[124] provided high yields in the synthesis of glycopeptides. For example AgOTf-promoted reaction of **76** with glycosyl acceptor **77** provided 1,2-trans glycoside **78** (Scheme 2.10a).[125] Moreover, Mukaiyama and Matsubara showed that tetraacetate β–**71** can be useful in tin(II) trifluoromethanesulfonate-catalyzed glycosylations with alkyl

trimethylsilyl ethers.[126,127] Under these conditions, β-glycosides were typically obtained in 80-99% yields. For example, the synthesis of disaccharide **80** was accomplished in 95% yield from building blocks β–**71** and **79** (Scheme 2.10b).



Scheme 2.10. 2-Deoxy-2-Trichloroethoxycarbamoylglucopyranosides.

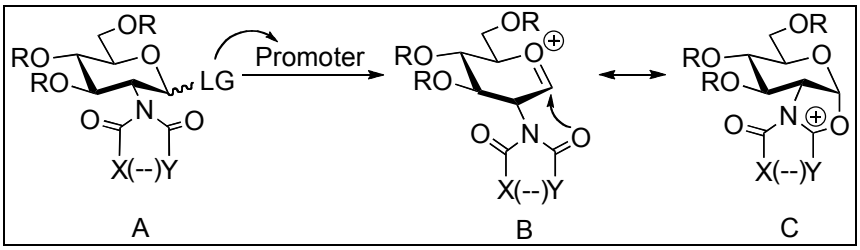
Over the years, other classes of 2-NHTroc glycosyl donors have been found useful in glycoside and oligosaccharide synthesis. These include trichloroacetimidates,[104,125,128-152] fluorides,[130,153,154] chlorides,[155] thioglycosides,[131,134,156-174] sulfoxides,[70,161] and more recently, thioimidates,[59] among others.

The Troc group has been found to be stable under a wide range of standard conditions typically used in carbohydrate synthesis.[156] Its sensitivity to alcoholysis under basic conditions permits convenient transformations into other carbamates. Removal of the Troc group to liberate the primary amine is typically accomplished by reductive

elimination using Zn in AcOH,[105,128,130,131,133,139,140,175] Zn-Cu alloy in AcOH,[104,129,136,137,145] Zn and acidic buffer,[141,176] Cd-Pb in AcOH,[173,177,178] or more recently, using $(Bu_3Sn)_2$[179] in DMF. The use of Zn-N-methylimidazole,[180] in particular, allows selective deprotection of Troc in the presence of reducible and acid-sensitive functionalities.

2.4. Disubstituted 2-Amino-2-Deoxy Glycosides

As mentioned above, the most attractive means for the establishment of a 1,2-trans-glycosidic linkage would be through the formation of cationic species from a derivative of the sugar with participation of the neighboring substituent at C-2 (Scheme 2.3). The latter substituent should be so chosen that its engagement does not lead to products other than the desired 1,2-trans-β-glycoside, i.e. the formation of stable 1,2-oxazolines or oxazolidinones seen above. In this regard, blocking the amino function with two monovalent protecting groups or a bivalent cyclic group seems to be a reasonable recourse. Thus, electrophilic activation of the bivalently protected glycosyl donor **A** yields an oxocarbenium ion **B** which can form an oxazolinium intermediate **C** (Scheme 2.11). The reactive intermediate **C** can only be attacked from the β-face by a nucleophile, and more importantly, cannot form a stable oxazoline. Various 2-amino-2-deoxy glycosyl donors of this type have been developed over the years. Their preparation, activation, N-deprotection and model glycosidations will be discussed hereafter.



Scheme 2.11. Charge delocalization via bivalently-protected aminosugars.

In 1954, Baker and co-workers prepared 1,3,4,6-tetra-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranose and observed that treatment of this compound with HBr/HOAc gave 3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl bromide.[181] Six years later, Akiya and Osawa utilized the latter in glycosylations of simple alcohols and obtained the desired β-glycosides in high yields.[182] Hence in 1976, Lemieux *et al*. proposed the use of 2-phthalimido glycopyranosyl halides for the development of a generally useful preparation of 2-amino-2-deoxy oligosaccharides.[183] It was anticipated that engagement of the phthalimido group in charge delocalization at the anomeric center to form a strongly delocalized intermediate **C** (Scheme 2.11) will strongly influence the formation of a 1,2-*trans* linkage. Since then, 2-deoxy-2-phthalimido-protected glycosyl donors have been extensively studied and arguably became the method of choice for the preparation of 1,2-trans-linked glycosides and oligosaccharides of 2-amino-2-deoxy sugars.[15]

Preparation of 2-deoxy-2-phthalimido glycopyranosyl derivatives is usually accomplished by treatment of the corresponding 2-amino-2-deoxy sugar precursor **39** with phthalic anhydride in the presence of a base. Acetylation of the monosubstituted intermediate **81** results in the formation of the per-acetylated derivative **82** that was obtained by Kochetkov *et al*. (conditions A)[184] or Lemieux *et al*. (conditions B)[183] in 69%

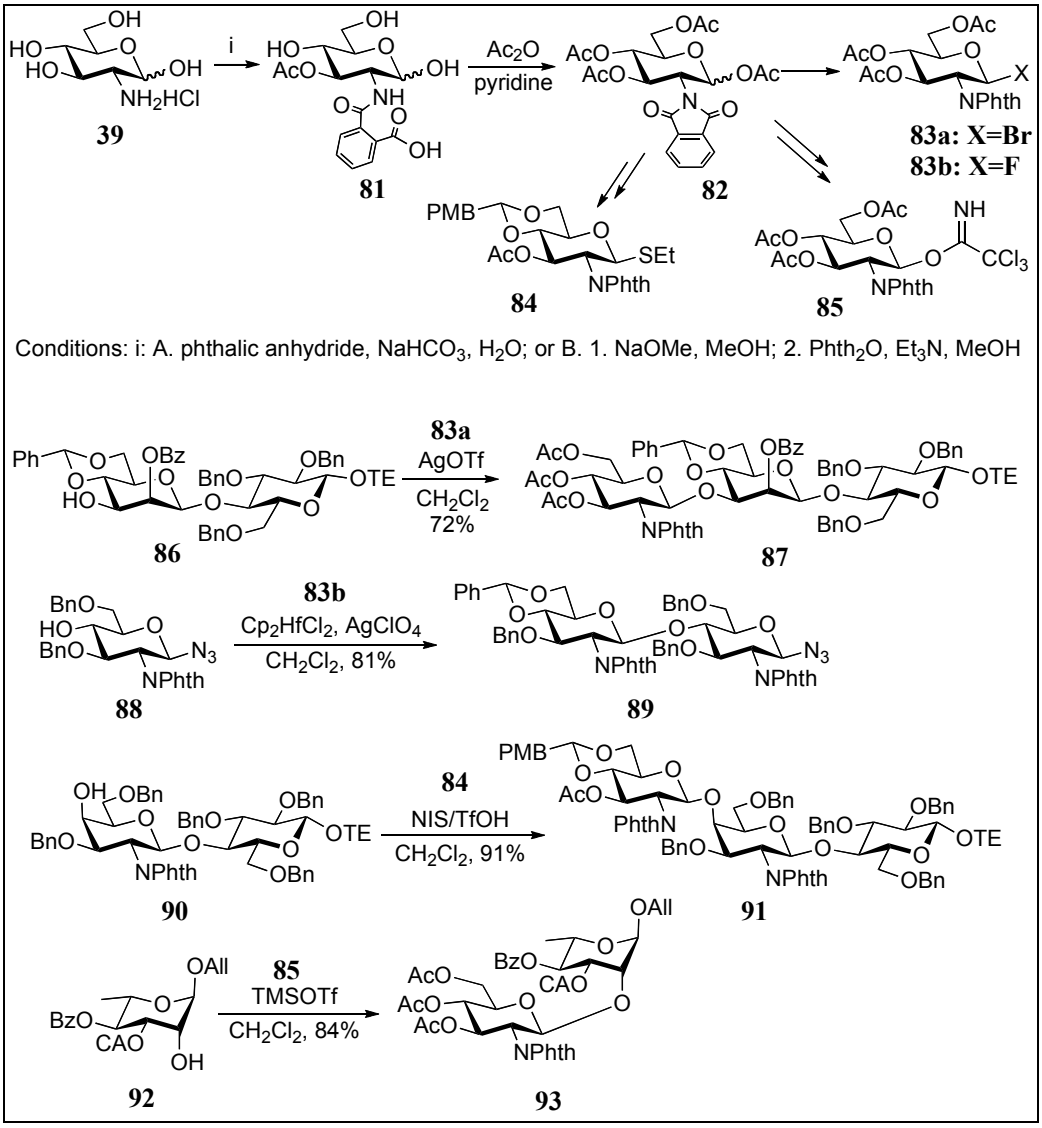
and 79% yields, respectively (Scheme 2.12). Starting from precursor **82**, a range of glycosyl donors, including halides, trichloroacetimidates, thioglycosides, and thioimidates have been synthesized. The progress in this area since a comprehensive report by Banoub *et al*. (1992)[15] will be discussed herein.

The chemical synthesis and properties of both the anomeric forms of 3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-D-glucopyranosyl halides, for example **83a**, as well as their 1-chloro and 1-iodo counterparts, have been described by Lemieux and contrary to the premise of the anomeric effect, they found that the β-form was more stable.[183] Initially, as described in the synthesis of O-specific polysaccharides of *Shigella flexneri*,[185,186] asialo-GM1 and –GM2,[187] 1,6-anhydromuramyl petides,[92] and cell-surface glycans,[188] 2-phthalimido bromides were more commonly used in glycosylations under Koenigs-Knorr or Helferich conditions.

A representative example is shown in Scheme 2.12; thus, reaction of the bromide donor **83a** with glycosyl acceptor **86** resulted in the formation of the trisaccharide **87** in 72%.[189] The greater stability of the chloride counterpart, however, started a change in the trend and resulted in a number of syntheses employing this building block; for example, blood-group antigens,[190] peptidoglycans,[191] and disaccharide analogues of meonomycin.[192]

Highly stable 2-phthalimidoglycosyl fluorides also found application in aminosugar synthesis.[193] For example, 2-phthalimidoglucopyranosyl fluoride **83b**, obtained from **82** by a protecting group sequence followed by treatment with dimethylaminosulfur trifluoride (DAST) in THF, was reacted with glycosyl acceptor

88.[194] Amongst the activating conditions developed, Cp_2HfCl_2-$AgClO_4$ (Suzuki's protocol)[195] was chosen, providing the disaccharide **89** in 81% yield.[194]



Scheme 2.12. 2-Deoxy-2-phthalimidoglucopyranosides.

2-Phthalimido thioglycosides have been also widely employed in oligosaccharide synthesis. Amongst these: *methyl thioglycosides*, used in the synthesis of novel sulfated GM1b analogues,[196,197] ganglioside GD2 and GQ1b,[198-200] and galactosyl globosides;[201] *ethyl thioglycosides*, used in the synthesis of blood group antigens,[202-204] capsular

polysaccharides of bacterial pathogens,[205,206] glycoprotein[207] residues, and peptidoglycan[208,209] residues; *phenyl thioglycosides*, used in the synthesis of blood group determinants and antigens,[210-212] chitin oligosaccharides,[213] lactosamine[160] donors, glycan residues,[214] oligosaccharides from respiratory mucins,[215] selectin ligands,[72,216] and bacteriohopanetetrol[217] derivatives; *p-methylphenyl thioglycosides*, used in the synthesis of α-Gal epitopes,[218,219] synthetic aminoglycosides,[220] and synthetic heparan sulfate[221] ligands; and *isopropyl thioglycosides*, used in the synthesis of immunostimulants[222] buffalo milk pentasaccharide derivatives,[223] and anticancer agents.

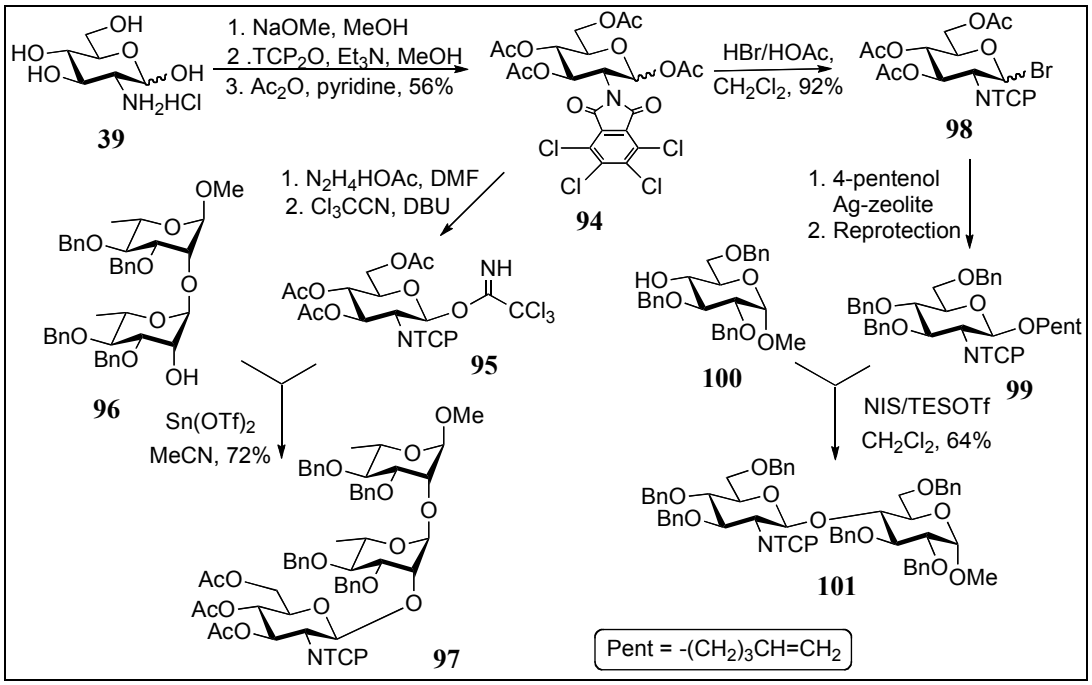
The introduction of the thioalkyl/aryl leaving group is generally accomplished by condensation of the appropriate acetate precursor with the corresponding thiol,[72,156,222,224,225] stannane,[188,226] or silyl ether[227-229] in the presence of Lewis Acids such as BF_3OEt_2,[72,156,222,224] $TiCl_4$,[230] TMSOTf,[227,228] $FeCl_3$,[225] ZnI_2,[229] and $SnCl_4$.[188,231] Nucleophilic displacement of an anomeric halide with the salt of the thiol is also a viable route.[232-234] Glycosidation of thioglycosides can be achieved with the use of a variety of acceptors.[235] For example, NIS/TfOH-promoted reaction of glycosyl donor **84** with glycosyl acceptor **90** afforded the oligosaccharide **91** in 91% yield (Scheme 2.12).[236]

The use of 2-deoxy-2-phthalimido trichloroacetimidates in aminosugar synthesis was introduced by Grundler and Schmidt in 1983.[237] Thus, the β-imidate **85** was obtained by reaction of carbohydrate hemiacetals with trichloroacetonitrile in the presence of a base (Scheme 2.12). Typical glycosylations using trichloroacetimidates are performed in CH_2Cl_2 as solvent, using a sub-stoichiometric amount of the promoter, usually BF_3OEt_2[238-242] or TMSOTf.[132,243-246] For example, the synthesis of the disaccharide **93** from glycosyl donor **85** and glycosyl acceptor **92** was accomplished in 84% yield.[244] To

date, this method has been applied to the synthesis of bacterial antigenic polysaccharides,[238,239,244,245] tumor-associated antigens,[247] chitosan polysaccharides,[248] buffalo milk polysaccharides,[223] mucin-type[240] core units,[132] hyaluronic acid,[241] and E-selectin ligands,[242] among others.

While the above glycosyl donors have proved to be potent and efficient, other glycosyl donors, such as phenylselenides,[249,250] trifluoroacetimidates,[251,252] and thioimidates,[59] have also been investigated. Deprotection of the phthaloyl group can be achieved with hydrazine hydrate in MeOH or EtOH,[197,253] alcoholic methylamine,[199,254] ethylenediamine in BuOH or EtOH,[194,202,205,207,255,256] ammonia in MeOH,[257] sodium borohydride,[225,258] hydrazine acetate,[259] or hydroxylamine,[260] forming a free amine.

The strongly basic conditions described and the high temperature required to carry out the cleavage of N-phthaloyl moiety presents the biggest drawback of the phthalimido procedure in target synthesis. This excludes its application to the synthesis of a broad range of targets, including various families of glycopeptides and glycolipids where numerous base-sensitive groups such as esters and α-amino acid residues exist.[16] Thus, Fraser-Reid[261] and Schmidt[262] reasoned that the presence of electron-withdrawing groups on the aromatic ring would enable cleavage under less demanding conditions. It was pointed out, however, that the remote withdrawal of electron density should not inhibit neighboring group participation.[261] This lead to the introduction of readily available tetrachlorophthalimido (TCP) derivatives as building blocks for aminosugar synthesis. Application of the 4,5-dichlorophthalimido moiety to glycoside and oligosaccharide synthesis has also been investigated.[263,264]



Scheme 2.13. 2-Deoxy-2-tetrachlorophthalimidoglucopyranosides.

The TCP derivatives can be readily prepared using Lemieux's classic procedure for the synthesis of 2-phthalimido derivatives.[183] Thus, starting from precursor **39**, the tetra-acetyl 2-tetrachlorophthalimido derivative **94** is obtained in 56% yield over three steps (Scheme 2.13).[16] The precursor **94** can then be modified accordingly into potent glycosyl donors such as bromides,[50,265,266] flourides,[264] pentenyl ethers,[261,267-269] trichloroacetimidates,[262,270] or thioglycosides.[271-273] Herein, the synthesis and application of trichloroacetimidate **95** and O-pentenyl glycoside **99** synthesized via bromide **98**, will be discussed. Thus, reaction of trichloroacetimidate **95** with glycosyl acceptor **96** in the presence of tin(II) triflate afforded trisaccharide **97** in 72% yield.[270] Glycosylation of glycosyl acceptor **100** with O-pentenyl glycoside **99** lead to the corresponding disaccharide **101** in a yield of 64% (Scheme 2.13).[261]

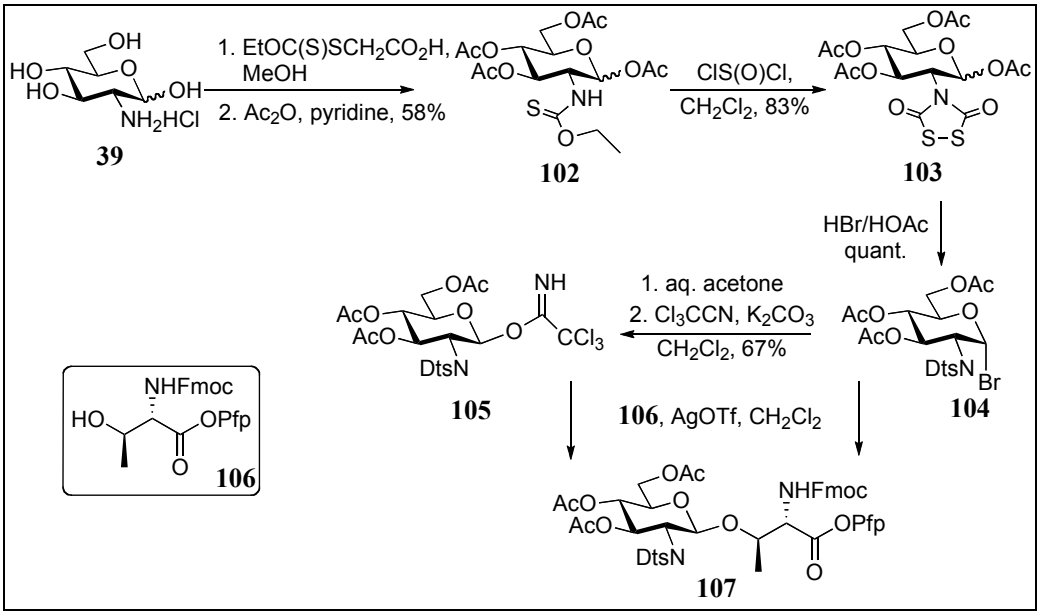
Among other applications, TCP glycosyl donors have been used for the synthesis of nodulation factors,[268] Lipid A derivatives,[269] saponins,[50] mucin oligosaccharide

chains,[272] and blood group antigens.[172] Removal of the TCP group either by ethylene diamine,[261,262,267,269] $NaBH_4$ in isopropanol,[262] followed by acetylation affords the corresponding N-acetyl derivative.

As mentioned, the relatively harsh conditions required for the removal of the phthalimido moiety have limited its use for β-selective coupling.The high temperature and strongly basic conditions are incompatible with base-labile groups such as esters and α-amino acid residues. Hence, for glycopeptide synthesis, an orthogonal amine protecting group that can be easily removed is highly desirable. One such candidate was the N-dithiasuccinoyl (Dts) function, which was found to be compatible with the Fmoc/Pfp protecting group strategy for the effective synthesis of glycopeptides and readily removed under mild conditions by thiolysis or other reductive methods.[274-276] Originally developed by Barany and Merrifield,[274] NDts derivatives had been heavily applied in peptide synthesis for many years before it was utilized as an orthogonally removable N-amino-protecting group of D-glucosamine derivatives.[277]

Following Barany and Merrifield's approach,[274] NDts derivatives can be prepared by treating ethoxythiocarbonyl intermediates with anhydrous chlorocarbonylsulfenyl chloride. To this end, the ethoxythiocarbonyl glucosamine intermediate **102** was obtained by treatment of **39** with S-carbomethyl O-ethyl dithiocarbonate in methanol[277] or bis(ethoxythiocarbonyl) sulfide in aqueous ethanol,[278] followed by per-acetylation with acetic anhydride in pyridine (Scheme 2.14). Upon condensation of the intermediate **102** with (chlorocarbonyl)sulfenyl chloride, a rapid and clean cyclization occurs to form the N-dithiasuccinoyl derivative **103**. The latter can then be transformed into a suitable glycosyl donor, such as the bromide **104**[277,278] or the trichloroacetimidate **105**.[277,279]

Reaction of either glycosyl donor **104** or **105** with the threonine acceptor **106** in the presence of AgOTf afforded stereoselectively the β-glycoside **107** in 85 or 71% yield, respectively.[277,278]

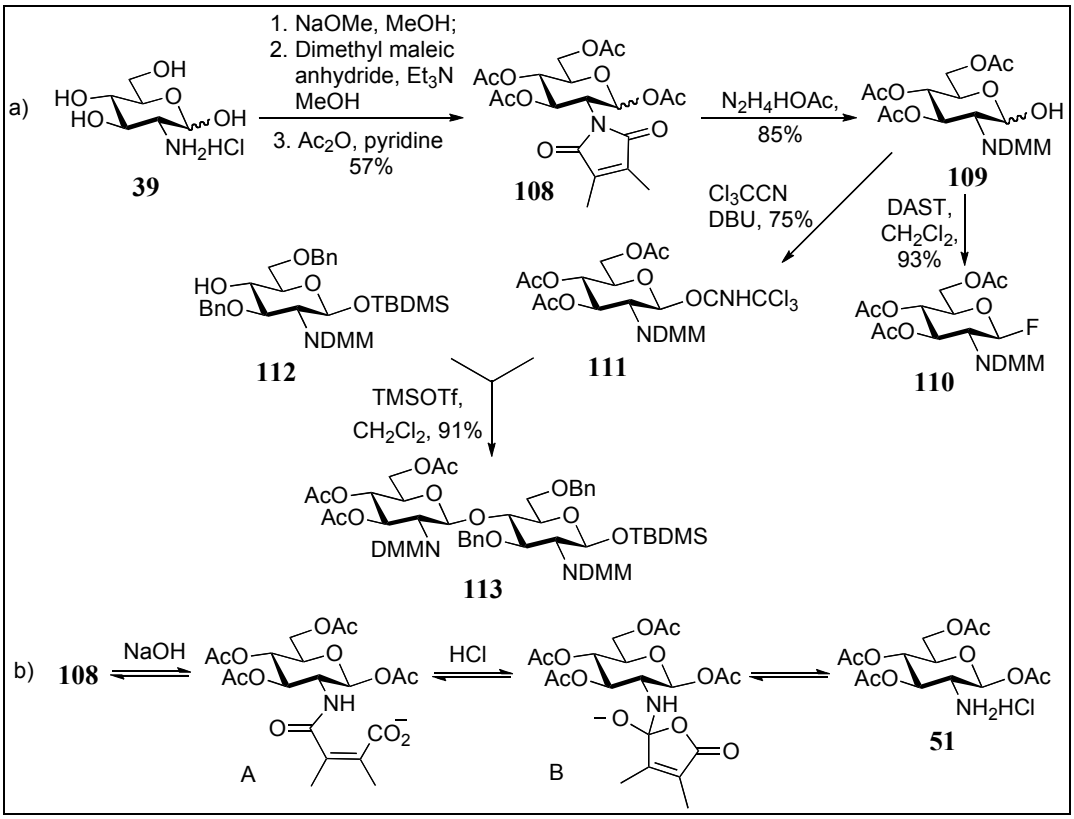


Scheme 2.14. 2-Deoxy-2-Dithiasuccinoylglucopyranosides.

The Dts group can be removed by thiols through an open-chain carbamoyl disulfide intermediate,[16] which reacts further to give the free amine. To date, N-Dts derivatives have been successfully transformed into the free amine using β-mercaptoethanol (BME), N-methyl-mercaptoacetamide (MAc), and dithiothreitol (DTT).[277,278] Moreover, addition of tertiary amines like N,N-diisopropylethylamine was also found to greatly accelerate the thiolytic cleavage of the Dts group.

In 1998, Schmidt *et al.* introduced the use of N-dimethylmaleoyl (DMM) derivatives for the synthesis of 2-amino glycosides.[280] Aside from its reported ease of introduction and cleavage under weakly aqueous basic then acidic conditions, the DMM moiety also provides anchimeric assistance to enforce β linkage. More importantly, its

stability to acids and non-nucleophilic bases makes it compatible with a wide array of functional group interconversions.[281] Furthermore, the cyclic DMM group is symmetric – a definite advantage for structural assignments by NMR.



Scheme 2.15. 2-Deoxy-2-N-dimethylmaleoylglucopyranosides.

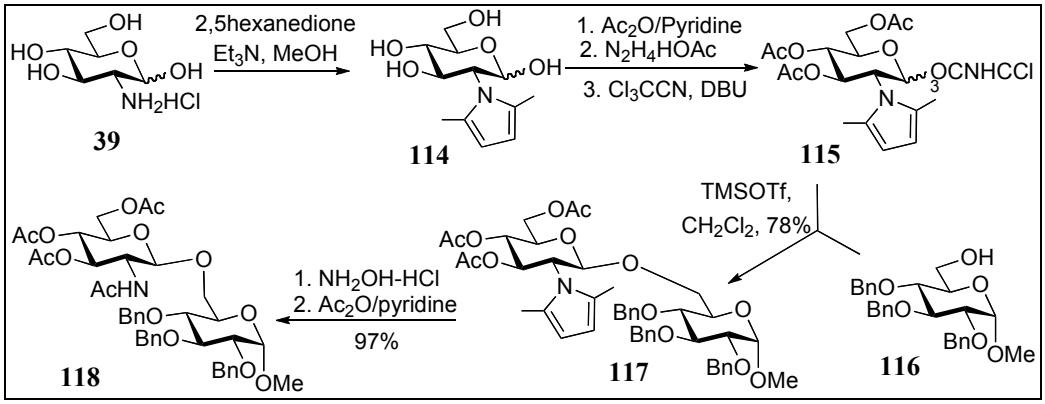
Adopting Lemieux's classic procedure for the synthesis of 2-phthalimido derivatives,[183] 2-NDMM derivatives can be easily synthesized from glucosamine hydrochloride **39** in three steps, as shown in Scheme 2.15.[281] The first step liberates the free amine while the next step forms the amide bond upon nucleophilic attack of the amine on the carbonyl of the anhydride. Acetylation by acetic anhydride in pyridine protects all the hydroxyls and cyclizes the amide into the dimethylmaleoyl moiety to afford the tetraacetyl 2-NDMM derivative **108**. The anomeric acetate can then be readily

removed by treatment with hydrazine acetate to form the hemiacetal **109**, which can be converted accordingly into glycosyl donors, i.e. fluorides, such as **110**, iodides, and more commonly, trichloroacetimidates (**111**).[280,282,283]

Glycosyl donors bearing the 2-NDMM moiety have been applied to the synthesis of N-glycan derivatives,[282,284] lacto-N-hexaose analogues,[285,286] glycopeptides,[287,288] glucosamine-glycerophospholipids,[289] and human milk[290] oligosaccharides. For example, TMSOTf-promoted glycosidation of trichloroacetimidate **111** with glycosyl acceptor **112** afforded chitodisaccharide **113** in 91% yield.[281] The cleavage of the DMM moiety in **108** is outlined in Scheme 2.15b.[281] Thus, base hydrolysis leads to ring-opened intermediate **A**, which, in the presence of an acid, is in equilibrium with butenolide **B**. Protonation of the basic nitrogen atom of the amide acetal moiety leads to generation of DMMA and the amine hydrochloride **51**, which can then be transformed into the 2-acetamido derivative by N-acetylation.

Boons and co-workers developed the 2,5-dimethylpyrrole group, which was found to be compatible with many reaction conditions employed in carbohydrate chemistry.[280] It was installed by the reaction of D-glucosamine **39** with 2,5-hexanedione in the presence of Et_3N in methanol to afford the intermediate **114**, which was then conventionally converted into the trichloroacetimidate derivative **115** (Scheme 2.16). Glycosylation of the acceptor **116** with glycosyl donor **115** in the presence of TMSOTf was entirely stereoselective affording the disaccharide **117** in 78% yield. Since the dimethylpyrrole moiety is not capable of anchimeric assistance, the high 1,2-trans diastereoselectivity of this glycosylation was attributed to a hindered access to the bottom face of the ring resulting from its steric bulk. It was also demonstrated that the

dimethylpyrrole moiety in **117** can be converted into the natural 2'-acetamido derivative **118** by treatment with hydroxylamine followed by acetylation. Importantly, the dimethylpyrrole moiety was found to be stable toward conditions for the 2-phthalimido group removal.



Scheme 2.16. 2-Deoxy-2,5-dimethylpyroleglucopyranosides.

In summary, it should be noted that the discovery of new methods and strategies for stereoselective glycoside synthesis and convergent oligosaccharide assembly is critical for the area of glycosciences. However, as evident from above, a universal approach for the synthesis 2-amino-2-deoxy sugars has not yet been developed despite recent progress in the field. While a variety of excellent participating moieties are available for the synthesis of 1,2-trans glycosides, the N-phthaloyl moiety is arguably the most commonly employed. However, in recent years, new excellent participating groups (especially NTroc) that can be removed under mild reaction conditions have been rapidly emerging. Amongst the amides, the trifluoroacetyl moiety (TFA) also looks promising. Indeed, these compounds deliver excellent properties to the glycosyl donor, yet cumbersomeness of their preparation often results in lengthy synthetic sequences. In

addition, it should be noted that virtually all leaving groups that have been employed in glycosidations with neutral sugars have also been employed in the synthesis of the glycosides of 2-amino-2-deoxy sugars. Amongst these leaving groups, the trichloroacetimidoyl, alkyl/arylthio, acetyl, or halogeno moieties are the most commonly used. While anomeric halides or acetates offer the most reliable glycosyl donors for the preparation of glycosides of simple alcohols, there is arguably a lack of an efficient, universal method for the synthesis of complex oligosaccharides. Moreover, we noticed that in contrast to the enormous effort expended on the development of suitable amine protecting groups, the development of potent glycosylating agents of 2-amino-2-doxy sugars have been sorely neglected.

2.5. Chemoselective Synthesis of Oligosaccharides of 2-Deoxy-2-Aminosugars

In response to the observations above, we decided to initiate studies on improving current methods for the introduction of 2-deoxy-2-N-substituted-β-D-glucopyranosyl residues into complex oligosaccharides. We assumed that this goal could be achieved by applying novel thioimidoyl glycosidation methodology, the development of which has been the primary focus of our research program. Amongst thioimidates investigated, our laboratory has already reported that 1-S-benzoxazolyl (SBox) derivatives make excellent glycosyl donors due to their accessibility, stability, and high stereoselectivity and yields in glycosylations achieved under mild activation conditions.[291,292] However, application of this particular methodology to 2-amino-2-deoxy glycosides is yet to be explored. Indeed, the difference in valencies between oxygen and nitrogen precludes the interchangeability of glycosidation methods between neutral sugars and aminosugars. Details from our recently published report are reported herein.[59] Furthermore, while the

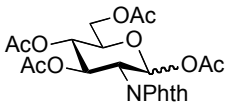
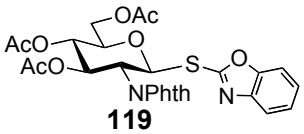
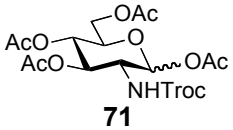
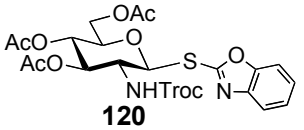
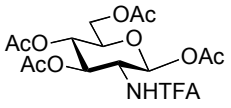
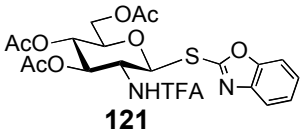
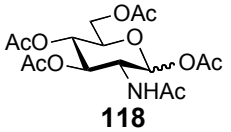
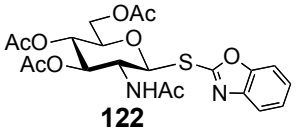
majority of oligosaccharides are synthesized in an expeditious selective or chemoselective fashion[293] nowadays, persistent attempts to apply these methods to 2-aminosugars have yet to emerge. As a result, the ultimate goal of our study was to develop an efficient strategy for the synthesis of oligosaccharides containing multiple sequential residues of 2-amino-2-deoxysugars. It is our belief that the discovery outlined hereafter provides the first essential step towards this meritorious aim.

With these intentions in mind, we employed acetate precursors containing an N-protecting group from each class surveyed above – trifluoroacetamide (TFA) from the amides, trichloroethoxycarbamoyl (Troc) from the carbamates, and pthalimido (Phth) from the imide – and subsequently, synthesized four novel N-substituted SBox glycosides **119-122** (Table 2.1). It should be noted that the 2-acetamido donor **122** was mainly intended for comparative studies. As reported previously, there are two methods by which the SBox glycosides are synthesized.[291,292] The first involves reaction between the glycosyl acetates and 2-mercaptobenzoxazole (HSBox) in the presence of a Lewis acid. The second method involves conversion of the acetate precursor into a glycosyl halide followed by nucleophilic displacement of the halide with 2-benzoxazolethione potassium salt (KSBox) in the presence of 18-crown-6.

Thus, 2-pthalimido tetra-acetate precursor **82** (Scheme 2.12)[183,184] can be transformed into the SBox glycoside **119** either by the reaction between **82** and HSBox or by conversion of **82** into the corresponding bromide **83a**[183] followed by displacement of the bromide using KSBox. The latter was accomplished in 82% yield and complete anomeric β-stereoselectivity (entry 1, Table 2.1). The former, on the other hand, can be accomplished with a variety of Lewis acids. The best method, however, was the reaction

between **82** and HSBox in the presence of $BF_3 \cdot Et_2O$ which gave the β anomer of **119** in 85% yield (entry 2, Table 2.1).

Table 2.1. Synthesis of 2-amino-2-deoxy SBox glycosides.

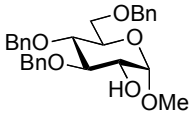
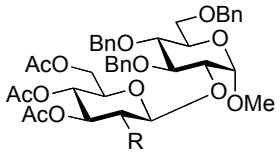
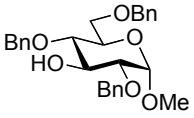
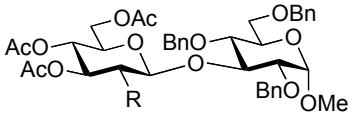
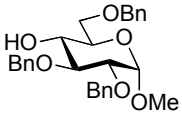
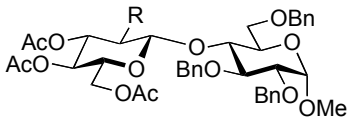
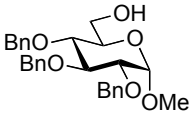
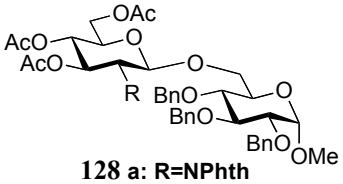
Entry	Starting Material	Conditions	Yield	Stereo-selectivity	Product
1	 **82**	HBr/HOAc, CH_2Cl_2, 1h then KSBox,18C-6, Acetone, 16h	82%	β only	 **119**
2		HSBox, $BF_3 \cdot Et_2O$, 3ÅMS, DCM, 16 h	85%	β only	
3		HSBox, $AlCl_3$, 3ÅMS, DCM, 1.5h	95%	1:10 α:β	
4		HSBox, $ZrCl_4$, 3ÅMS, DCM, 16h	75%	1:16 α:β	
5		HSBox, TMSOTf, 3ÅMS, DCM, 1.5h	85%	1:8 α:β	
6	 **71**	HBr/HOAc, DCM, 1.5h then KSBox, 18C-6, Acetone, 1.5h	73%	β only	 **120**
7	 **52**	DCMME, $ZnCl_2$, $CHCl_3$, 1h then KSBox, 18C-6, Acetone, 1h	71%	β only	 **121**
8	 **118**	$TiCl_4$, $CHCl_3$, 1h, rt then KSBox, 18C-6, Acetone, 1h	78%	β only	 **122**

The 2-Troc precursor **71** (Scheme 2.10)[65,115] was converted to SBox glycoside **120** via bromide **72**[115] followed by nucleophilic displacement of the bromine using KSBox (entry 6, Table 2.1). The nucleophilic displacement step was accomplished in 1.5 hours and gave **120** in 73% yield and complete β-selectivity. Despite reports stating that **71** can be transformed into a thioglycoside via reaction between **71** and a thiol in the

presence of a Lewis acid,[65] we found that this method of less efficiency in our particular case. Likewise, we found that transformation of **52** (Scheme 2.7)[42,43] into SBox glycoside **122** was best accomplished using the same two-step route. To this end, **52** was converted to the corresponding chloride **56** using an adapted method from a published procedure by Pavliak and Kovac.[294] Nucleophilic displacement of the chlorine using KSBox gave **122** in 71% yield and complete β-selectivity. Finally, commercially available 2-acetamido-2-deoxy-1,3,4,6-tetra-O-acetyl-β-D-glucopyranose **118** was first transformed into the corresponding chloride using an adapted method from a published procedure by Hesek *et al*.[295] Again, the nucleophilic displacement of the chlorine using KSBox in the presence of 18-crown-6 gave **122** in 78 % yield and complete β- selectivity.

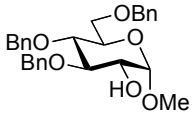
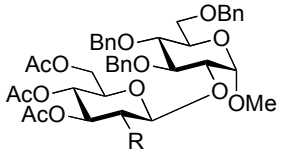
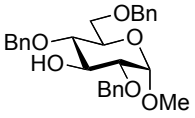
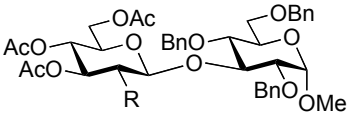
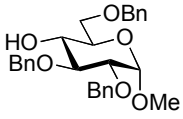
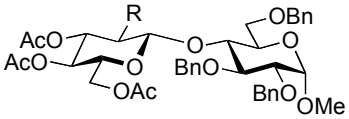
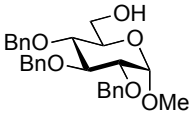
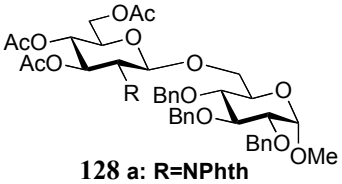
Having obtained SBox glycosides **119-122**, which were found to be stable crystalline or amorphous solids, we turned our attention to investigating their glycosyl donor properties in reactions with common glycosyl acceptors **58, 116**, **123, and 124**.[296-298] Based on previous reports from our group, SBox derivatives of neutral sugars can be activated with mildly electrophilic promoters such as silver trifluoromethanesulfonate (AgOTf), methyl trifluoromethanesulfonate (MeOTf), copper trifluoromethanesulfonate ($Cu(OTf)_2$), or the combination of N-iodosuccinimide and trifluoromethanesulfonic acid (NIS/TfOH).[292,299]

Table 2.2. MeOTf-promoted synthesis of 1,2-trans-linked disaccharides 125-128.

Entry	Acceptor	Donor	Product	Reaction time	Reaction yield
1	 **123**	**119**	 **125 a: R=NPhth** **b: R=NHTroc** **c: R=NHTFA**	2 h	92%
2		**120**		15 min	92%
3		**121**		1.5 h	80%
4	 **58**	**119**	 **126 a: R=NPhth** **b: R=NHTroc** **c: R=NHTFA**	1h	87%
5		**120**		1h	88%
6		**121**		1h	84%
7	 **124**	**119**	 **127 a: R=NPhth** **b: R=NHTroc** **c: R=NHTFA** **d: R=NHAc**	30 min	84%
8		**120**		15 min	89%
9		**121**		3 h	78%
10	 **116**	**119**	 **128 a: R=NPhth** **b: R=NHTroc** **c: R=NHTFA** **d: R=NHAc**	2 h	92%
11		**120**		15 min	88%
12		**121**		1h	89%
13		**122**		16 h	79%*

* based on recovered acceptor.

Table 2.3. AgOTf-promoted synthesis of 1,2-trans-linked disaccharides 125-128.

Entry	Acceptor	Donor	Product	Reaction time	Reaction yield
1	 **123**	**119**	 **125 a: R=NPhth** **b: R=NHTroc** **c: R=NHTFA**	15 min	90%
2		**120**		15 min	88%
3		**121**		15 min	84%
4	 **58**	**119**	 **126 a: R=NPhth** **b: R=NHTroc** **c: R=NHTFA**	15 min	85%
5		**120**		15 min	90%
6		**121**		15 min	89%
7	 **124**	**119**	 **127 a: R=NPhth** **b: R=NHTroc** **c: R=NHTFA** **d: R=NHAc**	15 min	86%
8		**120**		15 min	88%
9		**121**		45 min	86%
10	 **116**	**119**	 **128 a: R=NPhth** **b: R=NHTroc** **c: R=NHTFA** **d: R=NHAc**	15 min	90%
11		**120**		15 min	91%
12		**121**		15 min	93%
13		**122**		16 h	87%*

* based on recovered acceptor.

In this instance, we found that glycosidations of 2-N-substituted SBox glycosides are best activated with either AgOTf or MeOTf. The corresponding MeOTf-promoted and AgOTf-promoted glycosidations between glycosyl donors **119-122** and glycosyl acceptors **58, 116, 123**, and **124** are summarized in Tables 2.2 and 2.3, respectively.

As shown above, MeOTf-promoted glycosidations of glycosyl donors **119-121** proceeded fairly quickly (30 minutes to 3 hours) and gave the corresponding disaccharides in high yields ranging from 78% to 92% (entry 1-12, Table 2.2). Moreover, in line with our expectations, the disaccharides were obtained with complete 1,2-trans selectivity. In contrast, glycosidation of 2-acetamido derivative **122** (entry 13, Table 2.2) was sluggish and incomplete even in prolonged experiments (16-48 h). As anticipated, the reaction was negatively affected by the formation 1,2-oxazoline intermediate which was relatively unreactive under the reaction conditions applied.

On the other hand, the AgOTf-promoted glycosidations of glycosyl donors **119-121** proceeded even faster than the analogous MeOTf-promoted reactions and were generally complete after 15 minutes. The corresponding disaccharides **125-128** were obtained in yields ranging from 84% to 93% and complete 1,2-trans selectivity (entry 1-12, Table 2.3). Again, glycosidation of 2-acetamido derivative **122** was sluggish and incomplete even in prolonged experiments (16-48 h).

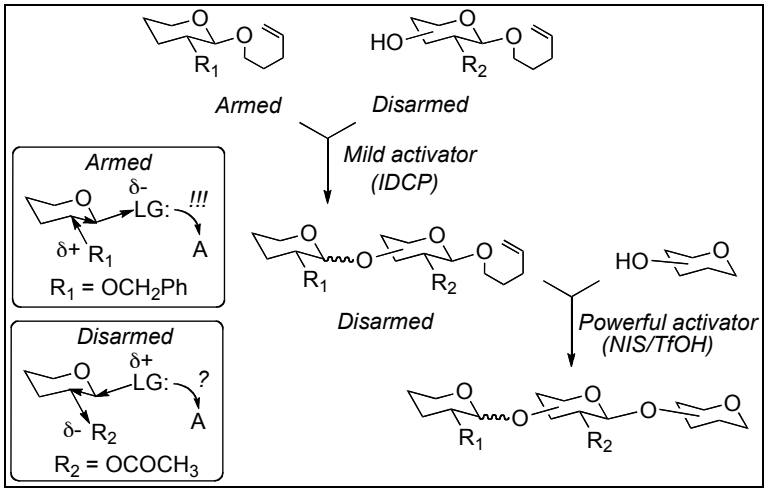
At this point, it should be noted that while AgOTf – promoted glycosidations of **119-121** were complete in less than 15 minutes, significantly different results have been recorded in MeOTf-promoted glycosylations. Accordingly, we observed that under essentially the same reaction conditions, glycosidation of 2-NPhth (**119**) and 2-NHTFA (**121**) typically required at least 1-2 h whereas NHTroc glycosyl donor (**120**) could be

glycosidated in a matter of minutes (<5-15 min). Thus, we anticipated that this observation could give rise to a complementary glycosylation approach for chemoselective glycosidation of 2-aminosugars similar to that discovered by Fraser-Reid[300] and explored by others[296,301,302] for the neutral sugars.

The chemoselective approach and its variations make use of only one class of leaving group for both the glycosyl donor and glycosyl acceptor. This leaving group is either activated (armed) or deactivated (disarmed), respectively, by the influence of the protecting groups. Thus, depending on the type of protecting groups installed in both reaction components, a direct coupling can be achieved wherein the armed glycosyl donor is chemoselectively activated over the disarmed derivative (bearing the same type of LG) in the presence of a mild promoter (Scheme 2.17). The disaccharide obtained can then be used for subsequent direct glycosylation in the presence of a more powerful promoter capable of activating the disarmed leaving group. In some applications, the same promoter used in step 1 could be used for step 2 provided that the reactions are performed at elevated temperatures.

Chemoselective strategies have become valuable techniques for expeditious oligosaccharide synthesis. These strategies allow efficient oligosaccharide assembly without the necessity of performing additional synthetic steps between the glycosylation steps. The classic armed–disarmed approach, developed by Fraser-Reid, has created a solid basis for extensive studies and applications and all chemoselective strategies devised thus far are related to (or derived from) this elegant concept. The effect of protecting groups on leaving-group ability has been known for long time, but it was Fraser-Reid and co-workers who explored this phenomenon and developed it into a new

strategy for chemoselective oligosaccharide synthesis.[303,304] This concept utilizes the fact that the protective groups present on the sugar molecule dictate its reactivity. It was discovered that armed glycosides, sugar molecules that are protected with an ether moiety at C-2, can be readily activated by mild promoters. On the other hand, disarmed glycosides that are protected by an ester group at C-2 require stronger promoters. This allows for direct coupling according to which a benzylated (electronically activated, armed) glycosyl donor is chemoselectively activated over the acylated (electronically deactivated, disarmed) derivative bearing the same type of LG in the presence of a mild promoter (Scheme 2.17).



Scheme 2.17. Armed-disarmed approach.

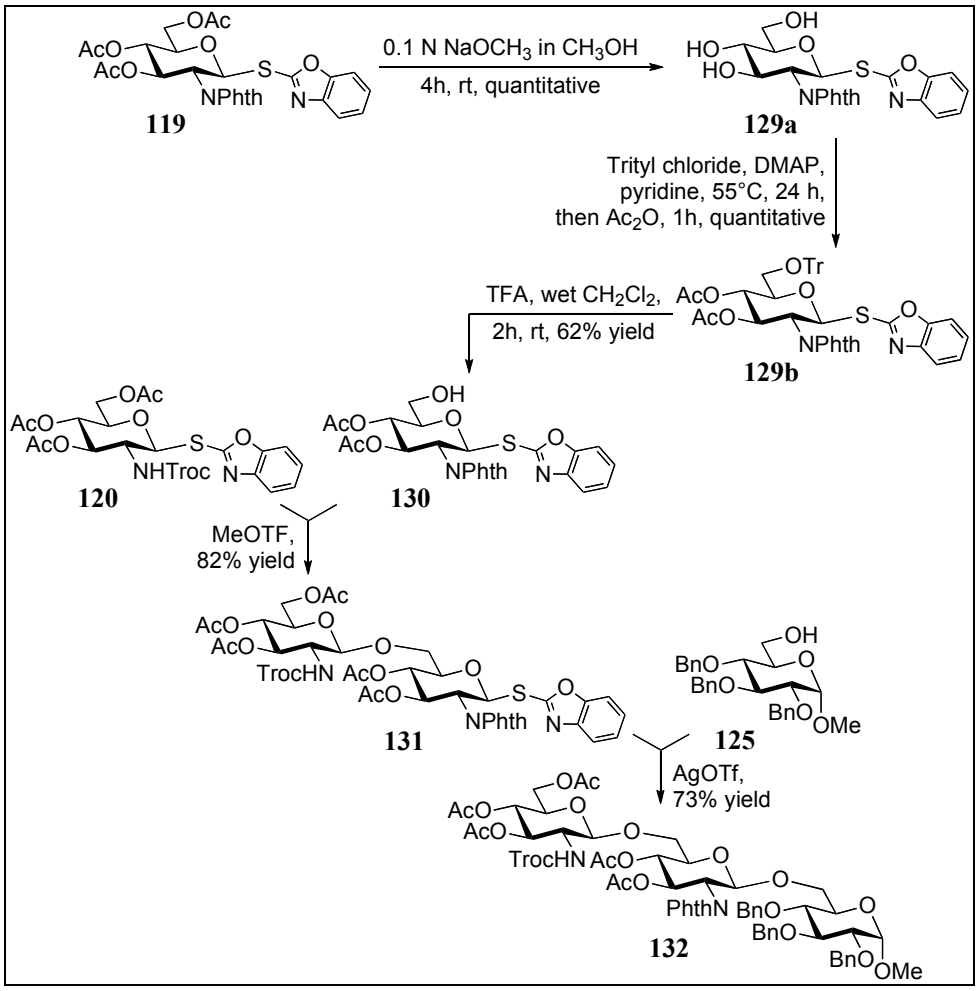
The key factor for the armed–disarmed activation to take place is the availability of a suitable promoter that differentiates between the armed and disarmed building blocks. Consequently, we believe that the difference in reaction rates we observed during MeOTf- and AgOTf-promoted reactions of 2-amino-2-deoxy SBox glycosides makes these promoters suitable for the development of a chemoselective strategy for

aminosugars. Moreover, while a significant disparity in reaction rates between NPhth- and NHTroc-protected derivatives have been previously reported,[131,156] no systematic studies have yet become available. The possibility of chemoselective activation of NTroc-protected donor over NPhth-protected acceptor for convergent oligosaccharide synthesis has only been used once by Baasov's group in sequential one-pot oligosaccharide synthesis employing tolyl thioglycosides.[305] Another relevant report explores the chemoselective activation of 2-NPhth-protected glycosyl donor over 2-azido acceptor. Unfortunately, only a modest yield of 31 % was achieved.[205]

To explore this opportunity, we needed to transform glycosyl donor **119** into a suitable glycosyl acceptor. Thus, deacetylation of **119** under Zemplen conditions was completed in 4h and gave precursor **129a** in essentially quantitative yields (Scheme 2.18). A sequential tritylation-acetylation sequence was then performed on **129a** to obtain **129b** in quantitative yield. Finally, removal of the trityl protecting moiety on C-6 of **129b** gave the glycosyl acceptor **130** in 62% yield after these three steps. We would like to note that the SBox moiety remained stable during the basic (deacetylation step) and acidic (detritylation step) conditions employed for the conversion of glycosyl donor **119** into glycosyl acceptor **130**.

Consequently, glycosyl acceptor **130** was then glycosylated with glycosyl donor **120**, as illustrated in Scheme 2.18. We determined that this coupling is best accomplished at reduced temperature (5ºC); under these reaction conditions, the disaccharide **131** was obtained in complete β-stereoselectivity and a high yield of 82%. Finally, to reiterate the potency of SBox 2-aminoglycosides as glycosyl donors, subsequent AgOTf-promoted glycosidation of disaccharide **131** with glycosyl acceptor **125** at room temperature gave

trisaccharide **132** in 73% yield, also with complete β−selectivity. In a similar fashion, we believe that based on the significant difference in the glycosylation rates, 2-TFA or 2-acetamido-protected glycosyl acceptors could also be glycosidated with NTroc glycosyl donor **120**.



Scheme 2.18. SBox glycosides of aminosugars in chemoselective strategies.

2.6. Summary and Conclusions

In summary, we investigated the application of the SBox glycosyl donors to the stereoselective and high yielding synthesis of 1,2-trans glycosides of 2-amino-2-deoxysugars. In addition, we discovered that 2-NTroc-protected SBox glycosides can be chemoselectively activated over 2-NPhth SBox glycosyl acceptor. This two-step sequential activation leads to the formation of trans-trans-linked oligosaccharides. It should be noted that our results differ significantly from Fraser-Reid's classic armed-disarmed approach that was developed for neutral sugars, according to which a cis linkage should be introduced first. It is to be expected that the strategy developed would significantly complement Baasov's previously reported approach. Since the SBox glycosides can be selectively activated over the *S*-alkyl/aryl moiety, a longer oligosaccharide sequences could be obtained by the application of chemoselective and selective activation protocols.

2.7. Experimental Section

2.7.a. General

Column chromatography was performed on silica gel 60 (EM Science, 70-230 mesh), reactions were monitored by TLC on Kieselgel 60 F_{254} (EM Science). The compounds were detected by examination under UV light and by charring with 10% sulfuric acid in methanol. Solvents were removed under reduced pressure at < 40 ^{o}C. CH_2Cl_2, $ClCH_2CH_2Cl$, CH_3CN, $CHCl_3$, and toluene were distilled from CaH_2 directly prior to application. Methanol was dried by refluxing with magnesium methoxide, distilled and stored under argon. Pyridine was dried by refluxing with CaH_2 and then

distilled and stored over molecular sieves (3 Å). Acetone was dried by refluxing with K_2CO_3 and stored over 3 Å molecular sieves. Anhydrous DMF (EM Science) was used as is. Molecular sieves (3 Å), used for reactions, were crushed and activated *in vacuo* at 390 °C during 8 h in the first instance and then for 2-3 h at 390 °C directly prior to application. AgOTf (Acros) was co-evaporated with toluene (3 x 10 mL) and dried *in vacuo* for 2-3 h directly prior to application. Optical rotations were measured at 'Jasco P-1020' polarimeter. Unless noted otherwise, ^{1}H-n.m.r. spectra were recorded in $CDCl_3$ at 300 MHz (Bruker Avance), ^{13}C-NMR spectra and two-dimensional experiments were recorded in $CDCl_3$ at 75 MHz (Bruker Avance) or at 125 MHz (Bruker ARX-500). HRMS determinations were made with the use of JEOL MStation (JMS-700) Mass Spectrometer.

2.7.b. Preparation of the SBox Glycosides

Method A. *Typical procedure for the preparation from glycosyl halides:* Crown ether (18-crown-6, 0.2 mmol) and salt (KSBox, 3.0 mmol) were added to a stirred solution of a glycosyl halide (1.0 mmol) in dry acetone (10 mL) under argon. The reaction mixture was stirred for 1-16 h at rt. Upon completion, the mixture was diluted with CH_2Cl_2 (30 mL) and washed with 1% aq. NaOH (15 mL) and water (3 x 10 mL). The organic phase was separated, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (ethyl acetate - hexane gradient elution) to afford the corresponding SBox glycoside.

Method B. *Typical procedure for the preparation from glycosyl acetates:* The solution of a glycosyl acetate (0.128 mmol), 2-mercaptobenzoxazole (0.256 mmol) and

activated molecular sieves 3Å (100 mg) in CH_2Cl_2 (1.0 mL) was stirred under argon for 30 min at rt. The Lewis acid (BF_3OEt_2, $AlCl_3$, $ZrCl_4$, or TMSOTf, 0.256 mmol) was then added dropwise and the reaction mixture was kept for 45 min at rt. After that, another portion of 2-mercaptobenzoxazole (0.256 mmol) and Lewis acid (0.256 mmol) were added and the reaction mixture was kept for 1.5-16 h at rt. Upon completion, the mixture was diluted with CH_2Cl_2 (10 mL), the solid was filtered-off and the residue was washed with CH_2Cl_2 (2 x 10 mL). The combined filtrate (30 mL) was washed with 1% aq. NaOH (15 mL) and water (3 x 10 mL). The organic layer was separated, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (ethyl acetate-hexane gradient elution) to afford the corresponding SBox glycoside.

3,4,6-tri-O-acetyl-2-deoxy-2-trifluoroacetamido-α-D-glucopyranosyl chloride (**56**). This chlorination method was adopted and modified from a procedure previously reported by Pavliak and Kovac.[294] To a mixture of 1,3,4,6-tetra-O-acetyl-2-deoxy-2-trifluoroacetamido-β-D-glucopyranose (4.43 g, 10 mmol) and zinc chloride (~300 mg) in alcohol-free chloroform (15 mL) was added α,α-2,2-dichloromethyl methyl ether (DCMME, 15 mL). The mixture was stirred at 50ºC for an hour, wherein TLC showed complete conversion of the starting material into a faster moving product. After concentration of the mixture and co-evaporation with toluene, the residue was purified by column chromatography on silica gel (ethyl acetate – toluene gradient elution) to afford 4 in white foam (3.74 g, 89 %). R_f = 0.53 (hexanes-ethyl acetate, 3/2, v/v); $[\alpha]_D^{25}$ 32.8º (c = 1.0, $CHCl_3$); ^{1}H-n.m.r.: δ, 2.04, 2.05, 2.10 (3s, 9H, COC*H*$_3$), 4.14 (dd, 1H, $J_{5,6a}$ = 3.0 Hz, $J_{6a,6b}$ = 13.6 Hz, H-6a), 4.30-4.35 (m, 2H, H-5, 6b), 4.50-4.56 (m, 1H, H-2), 5.23 (dd, 1H, $J_{4,5}$ = 9.7 Hz, H-4), 5.43 (dd, 1H, $J_{3,4}$ = 10.1 Hz, H-3), 6.23 (d, 1H, $J_{1,2}$ = 3.8 Hz, H-1),

7.07 (d, 1H, N*H*) ppm, ^{13}C-n.m.r.: δ, 20.54, 20.63, 20.82, 54.17, 61.17, 66.95, 69.97, 71.22, 92.02, 115.57, 157.57, 169.32, 170.70, 171.71 ppm; HR-FAB MS $[M+H]^+$ calcd for $C_{14}H_{18}ClF_3NO_8$ 420.0673, found 420.0668.

Benzoxazolyl 3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside (119) obtained by Method B as an amorphous solid in 85% yield: R_f = 0.53 (toluene-ethyl acetate, 3/2, v/v); $[\alpha]_D^{25}$ 145.4° (c = 1.0, $CHCl_3$); ^{1}H-n.m.r.: δ, 1.89, 2.06, 2.07 (3s, 9H, 3 x COC*H*$_3$), 4.23-4.12 (m, 2H, $J_{5,6b}$ = 2.1 Hz, $J_{6a,6b}$ = 12.4 Hz, H-6b, H-5), 4.36 (dd, 1 H, $J_{5,6a}$ = 4.6 Hz, H-6a), 4.68 (dd, 1H, $J_{2,3}$ = 10.5 Hz, H-2), 5.29 (dd, 1H, $J_{4,5}$ = 9.7 Hz, H-4), 5.98 (dd, 1H, $J_{3,4}$ = 9.6 Hz, H-3), 6.52 (d, 1H, $J_{1,2}$ = 10.9 Hz, H-1), 7.86-7.26 (m, 8H, aromatic) ppm, ^{13}C-n.m.r.: δ, 20.6, 20.8, 20.9, 53.6, 62.0, 68.6, 71.5, 76.6, 77.4, 81.3, 110.3, 119.3, 124.1 (x 2), 124.7 (x 2), 129.2 (x 2), 131.6, 134.7, 141.8, 152.1, 160.0, 169.7, 167.4, 170.2, 170.6 ppm; HR-FAB MS $[M+H]^+$ calcd for $C_{27}H_{25}N_2O_{10}S$ 569.1230, found 569.1219.

Benzoxazolyl 3,4,6-tri-O-acetyl-2-deoxy-2-trichloroethoxycarbamoyl-1-thio-β-D-glucopyranoside (120) was obtained as a white amorphous solid in 73% yield. R_f = 0.55 (toluene-ethyl acetate, 3/2, v/v); $[\alpha]_D^{25}$ 19.3° (c = 1.0, $CHCl_3$); ^{1}H-n.m.r.: δ, 1.96, 2.00, 2.01 (3s, 9H, 3 x COC*H*$_3$), 3.86-3.91 (m, 1H, H-5), 4.05-4.12 (m, 2H, H-2, 6b), 4.22 (dd, 1H, $J_{5,6a}$ = 4.8 Hz, $J_{6a,6b}$ = 12.5 Hz, H-6a), 4.63 (s, 2H, C*H*$_2$CCl$_3$), 5.12 (dd, 1H, $J_{3,4}$ = 9.7 Hz, H-4), 5.28 (dd, 1H, $J_{2,3}$ = 9.8 Hz, H-3), 5.68 (d, 1H, $J_{1,2}$ = 10.7 Hz, H-1), 5.69 (d, 1H, N*H*), 7.20-7.53 (m, 4H, aromatic) ppm; ^{13}C-n.m.r.: δ, 20.8, 20.8, 20.9, 55.9, 62.0, 68.2, 73.5, 74.7, 76.9, 84.8, 95.4, 110.4, 118.9, 124.8, 128.8, 141.5, 152.1, 154.5, 161.9,

169.6, 170.9, 171.1 ppm, HR-FAB MS $[M+H]^+$ calcd for $C_{22}H_{24}Cl_3N_2O_{10}S$ 613.0217, found 613.0217.

Benzoxazolyl 3,4,6-tri-O-acetyl-2-deoxy-2-trifluoroacetamido-1-thio-β-D-glucopyranoside (121) was obtained as an off-white amorphous solid in 70 % yield. R_f = 0.51 (toluene-ethyl acetate, 1/1, v/v); $[\alpha]_D^{25}$ 21.3° (c = 1.0, $CHCl_3$); ^{1}H-n.m.r.: δ, 2.01, 2.03, 2.09 (3s, 9H, 3 x COC*H*$_3$), 3.95-4.00 (m, 1H, H-5), 4.15 (dd, 1H, $J_{5,6b}$ = 2.2 Hz, $J_{6a,6b}$ = 12.6 Hz, H-6b), 4.27 (dd, 1H, $J_{5,6a}$ = 4.9 Hz, H-6a), 4.50 (dd, 1H, $J_{2,3}$ = 10.2 Hz, H-2), 5.19 (dd, 1H, $J_{4,5}$ = 9.8 Hz, H-4), 5.46 (dd, 1H, $J_{3,4}$ = 9.8 Hz, H-3), 5.76 (d, 1H, $J_{1,2}$ = 10.7 Hz, H-1), 7.21-7.56 (m, 4H, aromatic), 7.73 (d, 1H, N*H*) ppm, ^{13}C-n.m.r.: δ, 20.6, 20.8, 20.9, 62.1, 68.0, 73.8, 77.2, 84.0, 110.6, 113.8, 117.6, 118.7, 125.0, 125.0, 141.2, 152.2, 157.8, 161.8, 169.5, 170.9, 171.8 ppm, HR-FAB MS $[M+H]^+$ calcd for $C_{21}H_{22}F_3N_2O_9S$ 535.0998, found 535.1000.

Benzoxazolyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-1-thio-β-D-glucopyranoside (122) was obtained as a white crystalline solid in 70 % yield. R_f = 0.53 (acetone-toluene, 1/1, v/v); $[\alpha]_D^{25}$ 4.3° (c = 1.0, $CHCl_3$); ^{1}H-n.m.r.: δ, 1.92, 2.02, 2.06, 2.07 (4s, 12H, COC*H*$_3$), 3.89-3.94 (m, 1H, H-5), 4.14 (dd, 1H, $J_{5,6b}$ = 2.2 Hz, $J_{6a,6b}$ = 12.6 Hz, H-6b), 4.27 (dd, 1H, $J_{5,6a}$ = 4.8 Hz, H-6a), 4.45 (dd, 1H, $J_{2,3}$ = 10.0 Hz, H-2), 5.19 (dd, 1H, $J_{4,5}$ = 9.6 Hz, H-4), 5.26 (dd, 1H, $J_{3,4}$ = 9.7 Hz, H-3), 5.68 (d, 1H, $J_{1,2}$ = 10.7 Hz, H-1), 6.02 (d, 1H, N*H*), 7.27-7.61 (m, 4H, aromatic) ppm, ^{13}C-n.m.r.: δ, 20.81, 20.88 (x 2), 23.34, 53.56, 62.06, 68.05, 73.96, 77.43, 85.20, 110.46, 118.81, 124.74, 124.84, 141.59, 152.14, 162.50, 169.44, 170.52, 170.88, 171.48 ppm, HR-FAB MS $[M+H]^+$ calcd for $C_{21}H_{24}N_2O_9SNa$ 503.1100, found 503.1093.

Benzoxazolyl 3,4-di-*O*-acetyl-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside (130). To a suspension of the SBox glycoside **1** (0.57 g, 1.0 mmol) in methanol (10 mL) was added a 0.1 N solution of $NaOCH_3$ in methanol to pH 7.5. The reaction mixture was kept for 4 h at rt, then Dowex (H^+) was added until neutral pH. The resin was filtered off and washed with methanol (5 x 15 mL). The combined filtrate was concentrated *in vacuo* and dried. A crude residue containing benzoxazolyl 2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside (44 mg, 1.0 mmol), was dissolved in dry pyridine (5 mL) and 4-Dimethylaminopyridine (0.24 g, 2 mmol) along with triphenylmethyl chloride (1.1 g, 4 mmol) was added. The reaction mixture was refluxed at 55^{o}C for 24 h, then cooled to rt and acetic anhydride (0.95 mL, 10 mmol) was added. The reaction mixture was stirred at rt for 1 h and then quenched with methanol (10 mL). Volatile solvents were evaporated *in vacuo*, the residue was diluted with CH_2Cl_2 (100 mL), and the organic layer was washed with water (20 mL), saturated aq. $NaHCO_3$ (20 mL), water (20 mL), 1M HCl (2 x 20 mL), and water (3 x 20 mL). The organic phase was separated, dried over $MgSO_4$, and concentrated *in vacuo*. The crude intermediate was then dissolved in CH_2Cl_2 containing trifluoroacetic acid, and water (1/94/5, v/v/v). The reaction mixture was kept for 2 h at rt, then diluted with CH_2Cl_2 (100 mL) and washed with water (20 mL), sat. $NaHCO_3$ (20 mL), and water (3 x 20 mL). The organic phase was separated, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (ethyl acetate - hexane gradient elution) to allow **13** as an amorphous solid in 62% yield**.** R_f = 0.56 (toluene-ethyl acetate, 2/3, v/v); $[\alpha]_D^{25}$ 43.0^o (c = 1.0, $CHCl_3$); ^{1}H-n.m.r.: δ, 1.90, 2.10 (2s, 6H, 2 x COC*H*$_3$), 3.68 (dd, 1H, $J_{5,6a}$ = 5.1 Hz, $J_{6a,6b}$ = 12.5 Hz, H-6a), 3.84 (dd, 1H, H-6b), 3.95-4.01 (m, 1H, H-5), 4.66 (dd,

1H, $J_{2,3}$ = 10.5 Hz, H-2), 5.23 (dd, 1H, $J_{4,5}$ = 9.7 Hz, H-4), 6.01 (dd, 1H, $J_{3,4}$ = 10.0 Hz, H-3), 6.50 (d, 1H, $J_{1,2}$ = 10.7 Hz, H-1), 7.26-7.86 (m, 8H, aromatic) ppm, ^{13}C-n.m.r.: δ, 20.69, 20.93, 53.83, 61.71, 69.18, 71.47, 77.47, 79.18, 81.24, 110.35 (x 2), 119.46 (x 2), 124.11 (x 4), 124.87 (x 2), 134.78 (x 2), 141.82, 152.10, 170.32, 170.37 ppm, HR-FAB MS $[M+H]^+$ calcd for $C_{25}H_{23}N_2O_9S$ 527.1124, found 527.1119.

2.7.c. Preparation of the di- and trisaccharides

Method A. *Typical AgOTf-promoted glycosylation procedure (activation of the SBox glycosides):* A mixture the glycosyl donor (0.11 mmol), glycosyl acceptor (0.10 mmol), and freshly activated molecular sieves (3Å, 200 mg) in $ClCH_2CH_2Cl$ (2 mL) was stirred under argon for 1.5 h. Freshly conditioned AgOTf (0.22 mmol) was added and the reaction mixture was stirred for 1-2 h at rt. The mixture was diluted with CH_2Cl_2, filtered to remove the solids, and the residue was washed with CH_2Cl_2. The combined filtrate (30 mL) was washed with 20% aq. $NaHCO_3$ (15 mL) and water (3 x 10 mL). The organic phase was separated, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (ethyl acetate - hexane gradient elution) to afford a di- or an oligosaccharide derivative.

Method B. *Typical MeOTf-promoted glycosylation procedure (activation of SBox glycosides).* A mixture the glycosyl donor (0.11 mmol), glycosyl acceptor (0.10 mmol), and freshly activated molecular sieves (3Å, 200 mg) in $ClCH_2CH_2Cl$ (2 mL) was stirred for 2 h under argon. MeOTf (0.33 mmol) was added and the reaction mixture was stirred for 1-2 h at room temperature. Triethylamine (0.5 mL) was added to neutralize the reaction and the mixture was diluted with CH_2Cl_2 (30 mL), the solids were filtered off,

and the residue was washed with CH_2Cl_2. The combined filtrate was washed with water (4 x 10 mL) then the organic phase was separated, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (ethyl acetate - hexane gradient elution) to yield the corresponding di- or oligosaccharide.

Methyl O-(3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-(1→2)-2,4,6-tri-O-benzyl-α-D-glucopyranoside (**126a**) was obtained from **119** and **123** as an amorphous solid in 92% yield. R_f = 0.50 (toluene-ethyl acetate, 3/2, v/v); $[\alpha]_D^{25}$ 57.8º (c = 1.0, $CHCl_3$); ^{1}H-n.m.r.: δ, 1.74, 1.96, 2.05 (3s, 9H, 3 x COC*H*$_3$), 3.33 (s, 3H, OC*H*$_3$), 3.48-3.65 (m, 5H, H-2, 4, 5, 6a, 6b), 3.77 (dd, 1H, $J_{3,4}$ = 9.3 Hz, H-3), 3.80-3.84 (m, 1H, H-5'), 4.15-4.18 (m, 2H, H-6a', 6b'), 4.30 (dd, 2H, ^2J = 10.7 Hz, C*H*$_2$Ph), 4.31 (s, 2H, C*H*$_2$Ph), 4.41-4.44 (m, 1H, H-2'), 4.46 (dd, 2H, ^2J = 12.2 Hz, C*H*$_2$Ph), 4.94 (d, 1H, $J_{1,2}$ = 3.3 Hz, H-1), 5.08 (dd, 1H, $J_{4,5}$ = 9.6 Hz, H-4'), 5.56 (d, 1H, $J_{1',2'}$ = 8.5 Hz, H-1'), 5.66 (dd, 1H, $J_{3',4'}$ = 9.2 Hz, H-3'), 6.73-7.40 (m, 19H, aromatic) ppm, ^{13}C-n.m.r.: δ, 20.6, 20.8, 21.0, 54.8, 56.3, 62.6, 68.5, 69.0, 70.1, 71.0, 72.2, 73.6, 74.8, 75.1, 77.8, 80.5, 83.1, 99.3, 100.0, 123.5, 126.1 (x 2), 126.7, 127.7, 127.9, 128.0 (x 4), 128.1 (x 6), 128.3 (x 2), 128.5 (x 2), 131.0, 134.2 (x 2), 138.1 (x 2), 138.7, 169.7, 170.3, 170.8 ppm, HR-FAB MS $[M+H]^+$ calcd for $C_{48}H_{51}NO_{15}Na$ 904.3156, found 904.3130.

Methyl O-(3,4,6-tri-O-acetyl-2-deoxy-2-trichloroethoxycarbamoyl-β-D-glucopyranosyl)-(1→2)-3,4,6-tri-O-benzyl-α-D-glucopyranoside (**126b**) was obtained from **120** and **123** as an amorphous solid in 92% yield. R_f = 0.49 (toluene-ethyl acetate, 3/2, v/v); $[\alpha]_D^{25}$ 43.3° (c = 1.0, $CHCl_3$); ^{1}H-n.m.r.: δ, 1.90, 1.94, 2.01 (3s, 9H, 3 x COC*H*$_3$), 3.32 (s, 3H, OC*H*$_3$), 3.56-3.70 (m, 7H, H-2, 2', 4, 5, 5', 6a, 6b), 3.89 (dd, 1H,

$J_{3,4}$ = 9.3 Hz, H-3), 4.07-4.16 (m, 2H, H-6a', 6b'), 4.24 (dd, 2H, ^2J = 12.2 Hz, CH_2CCl$_3$), 4.49 (dd, 2H, ^2J = 12.1 Hz, CH_2Ph), 4.50 (dd, 2H, ^2J = 10.8 Hz, CH_2Ph), 4.71-4.79 (m, 4H, H-1', N*H*, CH_2Ph), 4.85 (d, 1H, $J_{1,2}$ = 3.6 Hz, H-1), 4.94 (dd, 1H, $J_{4',5'}$ = 9.7 Hz, H-4'), 5.14 (dd, 1H, $J_{3',4'}$ = 9.9 Hz, H-3'), 6.95-7.29 (m, 15H, aromatic) ppm, ^{13}C-n.m.r.: δ, 20.8 (x 2), 21.0, 29.9, 55.4, 56.5, 62.4, 68.5, 68.9, 70.2, 72.1 (x 2), 73.7, 75.2, 75.3, 77.4, 78.2, 81.3, 81.4, 95.5, 99.5, 101.9, 127.1, 127.8, 127.9, 128.0, 128.1 (x 4), 128.5 (x 2), 128.6 (x 2), 128.7 (x 2), 138.1, 138.2, 139.1, 154.0, 169.7, 170.5, 170.8 ppm, HR-FAB MS $[M+H]^+$ calcd for $C_{43}H_{50}Cl_3NO_{15}Na$ 948.2144, found 948.2172.

Methyl O-(3,4,6-tri-O-acetyl-2-deoxy-2-trifluoroacetamido-β-D-glucopyranosyl)-(1→2)-3,4,6-tri-O-benzyl-α-D-glucopyranoside (**126c**) was obtained from **121** and **123** as an amorphous solid in 84% yield. R_f = 0.45 (toluene-ethyl acetate, 1/1, v/v); $[\alpha]_D^{25}$ 8.6º (c = 1.0, $CHCl_3$); ^{1}H-n.m.r.: δ, 1.95, 2.02, 2.08 (3s, 9H, 3 x COCH_3), 3.37 (s, 3H, OCH_3), 3.60-3.75 (m, 6H, H-2, 4, 5, 5', 6a, 6b), 3.93 (dd, 1H, $J_{3,4}$ = 9.2 Hz, H-3), 4.06 (dd, 1H, $J_{2',3'}$ = 8.8 Hz, H-2'), 4.14-4.24 (m, 2H, H-6a', 6b'), 4.52 (dd, 2H, ^2J = 10.6 Hz, CH_2Ph), 4.54 (dd, 2H, ^2J = 11.7 Hz, CH_2Ph), 4.74 (dd, 2H, ^2J = 10.6 Hz, CH_2Ph), 4.88 (d, 1H, $J_{1',2'}$ = 7.9 Hz, H-1'), 4.90 (d, 1H, $J_{1,2}$ = 3.2 Hz, H-1), 5.06 (dd, 1H, $J_{4',5'}$ = 9.6 Hz, H-4'), 5.20 (dd, 1H, $J_{3',4'}$ = 9.9 Hz, H-3'), 6.33 (d, 1H, N*H*), 6.98-7.32 (m, 15H, aromatic) ppm, ^{13}C-n.m.r.: δ, 20.5, 20.8, 21.0, 55.2, 55.4, 62.2, 68.5, 70.1, 72.1, 72.3, 73.7, 75.1, 75.2, 77.4, 78.3, 81.0 (x 2), 99.5, 101.2, 113.6, 117.4, 127.0 (x 2), 127.7, 127.9, 128.1 (x 2), 128.5 (x 4), 128.6 (x 2), 128.7 (x 2), 138.0, 138.1, 138.7, 157.4, 169.5, 170.8, 171.0 ppm, HR-FAB MS $[M+H]^+$ calcd for $C_{42}H_{48}F_3NO_{14}Na$ 870.2925, found 870.2911.

Methyl O-(3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-(1→3)-2,4,6-tri-O-benzyl-α-D-glucopyranoside (**127a**) was obtained from **119** and **58** as a colorless syrup in 87% yield. R_f = 0.55 (toluene-ethyl acetate, 3/2, v/v); $[\alpha]_D^{25}$ +2.0º (c = 1.0, $CHCl_3$); ^{1}H-n.m.r.: δ, 1.88, 1.98, 2.02 (3s, 9H, 3 x COC*H*$_3$), 3.07 (s, 3H, OC*H*$_3$), 3.19 (dd, 1H, $J_{2,3}$ = 9.6 Hz, H-2), 3.45-3.60 (m, 4H, H-4, 5, 6a, 6b), 3.76-3.86 (m, 1H, H-5'), 4.02 (dd, 1H, $J_{5',6a'}$ = 1.8 Hz, $J_{6a',6b'}$ = 12.2 Hz, H-6a'), 4.09 (d, 1H, $J_{1,2}$ = 3.6 Hz, H-1), 4.25-4.39 (m, 3H, H-2, 3, 6b'), 4.22 (dd, 2H, ^2J = 12.6 Hz, C*H*$_2$Ph), 4.43 (dd, 2H, ^2J = 12.3 Hz, C*H*$_2$Ph), 4.71 (dd, 2H, ^2J = 10.9 Hz, C*H*$_2$Ph), 5.19 (dd, 1H, $J_{4',5'}$ = 9.6 Hz, H-4'), 5.81 (d, 1H, $J_{1',2'}$ = 8.4 Hz, H-1'), 5.97 (dd, 1H, $J_{3',4'}$ = 9.1 Hz, H-3'), 7.05-7.85 (m, 19H, aromatic) ppm, ^{13}C-n.m.r.: δ, 20.6, 20.9 (x 2), 55.0, 55.5, 62.1, 68.6, 69.3, 69.6, 70.7, 71.5, 73.6, 74.1, 74.8, 76.0, 78.4, 81.0, 97.7, 98.2, 123.7, 127.6, 127.9, 128.1 (x 4), 128.2, 128.3 (x 2), 128.4 (x 4), 128.5 (x 4), 128.6 (x 2), 131.7, 134.5, 138.0, 138.4, 138.9, 167.7, 169.8, 170.3, 170.9 ppm, HR-FAB MS $[M+H]^+$ calcd for $C_{48}H_{51}NO_{15}Na$ 904.3156, found 904.3193.

Methyl O-(3,4,6-tri-O-acetyl-2-deoxy-2-trichloroethoxycarbamoyl-β-D-glucopyranosyl)-(1→3)-2,4,6-tri-O-benzyl-α-D-glucopyranoside (**127b**) was obtained from **120** and **58** as a colorless syrup in 90% yield. R_f = 0.52 (toluene-ethyl acetate, 3/2, v/v); $[\alpha]_D^{25}$ +9.1° (c = 1.0, $CHCl_3$); ^{1}H-n.m.r.: δ, 1.89, 1.91, 1.93 (3s, 9H, 3 x COC*H*$_3$), 3.24 (s, 3H, OC*H*$_3$), 3.46-3.65 (m, 6H, H-2, 5, 5', 6a, 6b, 6b'), 3.76 (dd, 1H, $J_{2',3'}$ = 9.7 Hz, H-2'), 3.93 (dd, 1H, $J_{3,4}$ = 12.3 Hz, H-3), 4.12-4.20 (m, 2H, H-4, 6a'), 4.46 (dd, 2H, ^2J = 12.1 Hz, C*H*$_2$Ph), 4.59 (d, 2H, C*H*$_2$CCl$_3$), 4.61 (dd, 2H, ^2J = 10.6 Hz, C*H*$_2$Ph), 4.61 – 4.78 (m, 4H, H-1, 1', 3', N*H*), 4.74 (dd, 2H, ^2J = 8.6 Hz, C*H*$_2$Ph), 4.98 (dd, 1H, $J_{4'5'}$ = 9.6 Hz, H-4'), 7.10-7.37 (m, 15H, aromatic) ppm, ^{13}C-n.m.r.: δ, 20.8 (x 2), 20.9, 55.3,

56.6, 62.3, 68.6, 70.0, 71.9, 72.9, 73.2, 73.8, 74.9, 75.1, 75.5, 77.5, 80.1, 81.5, 95.8, 97.2, 101.7, 127.8, 128.0, 128.2 (x 2), 128.4 (x 6), 128.6 (x 2), 128.8 (x 2), 129.2, 137.8, 138.1, 138.7, 154.6, 169.6, 170.7, 171.0 ppm, HR-FAB MS $[M+H]^+$ calcd for $C_{43}H_{50}Cl_3NO_{15}Na$ 948.2144, found 948.2186.

Methyl O-(3,4,6-tri-O-acetyl-2-deoxy-2-trifluoroacetamido-β-D-glucopyranosyl)-(1→3)-2,4,6-tri-O-benzyl-α-D-glucopyranoside (**127c**) was obtained from **3** and **7** as a colorless syrup in 89% yield. R_f = 0.46 (ethyl acetate-hexane, 3/2, v/v); $[\alpha]_D^{25}$ 14.3° (c = 1.0, $CHCl_3$); ^{1}H-n.m.r.: δ, 1.91, 1.92, 1.96 (3s, 9H, 9 x COC*H*$_3$), 3.20 (s, 3H, 3 x OC*H*$_3$), 3.41-3.61 (m, 6H, H-2, 5', 6a, 6a', 6b, 6b'), 3.96 (dd, 1H, $J_{5,6a}$ = 2.1 Hz, $J_{6a,6b}$ = 12.3 Hz, H-6a), 4.10 (dd, 1H, $J_{2',3'}$ = 9.2 Hz, H-2'), 4.18 (dd, 1H, $J_{3,4}$ = 9.1 Hz, H-3), 4.20 (dd, 1H, H-6a'), 4.44 (dd, 2H, ^2J = 12.2 Hz, C*H*$_2$Ph), 4.53 (s, 2H, C*H*$_2$Ph), 4.57 (d, 1H, $J_{1,2}$ = 3.4 Hz, H-1), 4.60 (dd, 2H, ^2J = 11.0 Hz, C*H*$_2$Ph), 4.90 (dd, 1H, $J_{3',4'}$ = 10.6 Hz, H-3'), 4.95 (d, 1H, $J_{1',2'}$ = 8.8 Hz, H-1'), 5.05 (dd, 1H, $J_{4',5'}$ = 9.6 Hz, H-4'), 6.09 (d, 1H, N*H*), 7.10-7.38 (m, 15H, aromatic) ppm, ^{13}C-n.m.r.: δ, 20.77, 21.00, 21.05, 55.19, 55.42, 62.31, 68.42, 68.68, 70.19, 72.32, 72.66, 72.85, 73.96, 75.30, 75.73, 77.65, 79.95, 81.53, 97.38, 101.10, 127.77 (x 2), 127.99, 128.17, 128.42 (x 2), 128.54 (x 2), 128.56 (x 2), 128.83 (x 2), 128.86, 129.30 (x 2), 138.06, 138.25, 138.86, 157.67, 169.70, 171.14, 171.32 ppm, HR-FAB MS $[M+H]^+$ calcd for $C_{42}H_{48}F_3NO_{14}Na$ 870.2925, found 870.2941.

Methyl O-(3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-(1→4)-2,3,6-tri-O-benzyl-α-D-glucopyranoside (**128a**) was obtained from **119** and **124** as an amorphous solid in 86% yield. R_f = 0.56 (toluene-ethyl acetate, 3/2, v/v); $[\alpha]_D^{25}$

11.3º (c = 1.0, $CHCl_3$); ^{1}H-n.m.r.: δ, 1.84, 2.00 (x 2) (3s, 9H, 3 x COC*H*$_3$), 3.28 (s, 3H, OC*H*$_3$), 3.34-3.39 (m, 1H, H-5'), 3.45-3.50 (m, 3H, H-2, 6a, 6b), 3.55-3.60 (m, 1H, H-5), 3.82 (dd, 1H, $J_{5',6b'}$ = 2.0 Hz, $J_{6a',6b'}$ = 10.5 Hz, H-6b'), 3.90 (dd, 1H, $J_{3,4}$ = 9.1 Hz, H-4), 4.00 (dd, 1H, $J_{4,5}$ = 9.30 Hz, H-3), 4.10 (dd, 1H, $J_{5',6a'}$ = 3.7 Hz, H-6a'), 4.28 (dd, 1H, $J_{2',3'}$ = 8.4 Hz, H-2'), 4.36 (s, 2H, C*H*$_2$Ph), 4.52 (d, 1H, $J_{1,2}$ = 3.6 Hz, H-1), 4.64 (dd, 2H, ^2J = 12.2 Hz, C*H*$_2$Ph), 4.98 (dd, 2H, ^2J = 11.8 Hz, C*H*$_2$Ph), 5.13 (dd, 1H, $J_{4',5'}$ = 9.6 Hz, H-4'), 5.65 (d, 1H, $J_{1',2'}$ = 8.4 Hz, H-1'), 5.71 (dd, 1H, $J_{3',4'}$ = 9.2 Hz, H-3'), 7.26-7.82 (m, 19H, aromatic) ppm, ^{13}C-n.m.r.: δ, 20.6, 20.8, 20.9, 55.4, 55.5, 61.7, 68.4, 68.8, 69.5, 71.0, 71.8, 73.0, 73.6, 74.8, 75.7, 77.4, 79.6, 80.4, 97.5, 98.3, 123.8, 127.0 (x 2), 127.3, 127.5 x2), 127.6, 128.0, 128.2 (x 2), 128.4 (x 8), 128.5 (x 2), 131.6, 134.5, 138.4, 138.5, 139.7, 169.6, 170.4, 170.9 ppm, HR-FAB MS $[M+H]^+$ calcd for $C_{48}H_{51}NO_{15}Na$ 904.3156, found 904.3141.

Methyl O-(3,4,6-tri-O-acetyl-2-deoxy-2-trichloroethoxycarbamoyl-β-D-glucopyranosyl)-(1→4)-2,3,6-tri-O-benzyl-α-D-glucopyranoside (**128b**) was obtained from **120** and **124** as an amorphous solid in 89% yield. R_f = 0.55 (toluene-ethyl acetate, 3/2, v/v); $[\alpha]_D^{25}$ -26.3° (c = 1.0, $CHCl_3$); ^{1}H-n.m.r.: δ, 1.93, 1.98, 2.02 (3s, 9H, 3 x COC*H*$_3$), 3.35 (s, 3H, OC*H*$_3$), 3.36-3.72 (m, 6H, H-2, 2', 4, 5', 6a, 6b'), 3.79-3.81 (m, 4H, H-3, 5, 6b, C*H*$_2^a$CCl$_3$), 4.11-4.23 (m, 2H, H-6a', C*H*$_2^b$CCl$_3$), 4.58 (d, 1H, $J_{1,2}$ = 4.0 Hz, H-1), 4.60 (dd, 2H, ^2J = 12.2 Hz, C*H*$_2$Ph), 4.59 – 5.03 (m, 8H, H-1', 3', 4', NH, 2 x C*H*$_2$Ph), 7.14-7.54 (m, 15H, aromatic) ppm, ^{13}C-n.m.r.: δ, 20.8 (x 3), 55.5, 56.4, 62.0, 67.4, 68.82, 69.3, 71.4, 72.5, 73.7, 73.9, 74.6, 75.4, 77.4, 77.5, 78.8, 80.4, 95.8, 98.6, 100.8, 127.3 (x 3), 128.0, 128.2 (x 2), 128.3 (x 2), 128.5 (x 2), 129.3 (x 2), 129.4, 129.7,

137.6, 138.4, 139.8, 154.0, 169.6, 170.5, 170.8 ppm, HR-FAB MS $[M+H]^+$ calcd for $C_{43}H_{50}Cl_3NO_{15}Na$ 948.2144, found 948.2167.

Methyl O-(3,4,6-tri-O-acetyl-2-deoxy-2-trifluoroacetamido-β-D-glucopyranosyl)-(1→4)-2,3,6-tri-O-benzyl-α-D-glucopyranoside (**128c**) was obtained from **121** and **124** as an amorphous solid in 86% yield. R_f = 0.48 (toluene-ethyl acetate, 3/2, v/v); $[\alpha]_D^{25}$ - 45.4° (c = 1.0, $CHCl_3$); ^{1}H-n.m.r.: δ, 1.94, 1.99, 2.03 (3s, 9H, 3 x COC*H*$_3$), 3.36 (s, 3H, OC*H*$_3$), 3.36-3.39 (m, 1H, $J_{5',6a'}$ = 4.2 Hz, H-5'), 3.44-3.62 (m, 4H, H-2, 5, 6a, 6b), 3.81-3.93 (m, 4H, H-2', 3, 4, 6b'), 4.14 (dd, 1H, $J_{6a',6b'}$ = 12.4 Hz, H-6a'), 4.28 (d, 1H, $J_{1',2'}$ = 8.3 Hz, H-1'), 4.57 (d, 1H, $J_{1,2}$ = 3.2 Hz, H-1), 4.59 (dd, 2H, ^2J = 12.1 Hz, C*H*$_2$Ph), 4.65 (dd, 2H, ^2J = 12.3 Hz, C*H*$_2$Ph), 4.81 (dd, 1H, $J_{3',4'}$ = 9.9 Hz, H-3'), 4.86 (dd, 2H, ^2J = 10.9 Hz, C*H*$_2$Ph), 4.99 (dd, 1H, $J_{4',5'}$ = 9.7 Hz, H-4'), 5.82 (d, 1H, N*H*), 7.25 –7.49 (m, 15H, aromatic) ppm, ^{13}C-n.m.r.: δ, 20.5, 20.7, 20.8, 55.1, 55.6, 61.9, 67.7, 67.8, 68.2, 69.3, 71.6, 72.0, 72.2, 73.6, 73.9, 75.3, 77.4, 78.9, 80.0, 98.5, 99.6, 115.7, 127.4 (x 3), 129.0, 128.3 (x 2), 128.5 (x 2), 129.1 (x 2), 129.3 (x 3), 137.5, 138.3, 139.6, 157.2, 169.4, 170.8, 170.9 ppm, HR-FAB MS $[M+H]^+$ calcd for $C_{42}H_{48}F_3NO_{14}Na$ 870.2925, found 870.2907.

Methyl O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-2,3,6-tri-O-benzyl-α-D-glucopyranoside (**128d**) was obtained from **122** and **124** as an amorphous solid in 95% yield. R_f = 0.46 (ethyl acetate-hexane, 3/2, v/v); $[\alpha]_D^{25}$ 14.3° (c = 1.0, $CHCl_3$); ^{1}H-n.m.r.: δ, 1.91, 1.92, 1.96 (3s, 9H, 3 x COC*H*$_3$), 3.20 (s, 3H, 3 x OC*H*$_3$), 3.41-3.61 (m, 6H, H-2, 4, 5, 5', 6b, 6b'), 3.96 (dd, 1H, $J_{5,6a}$ = 2.1 Hz, $J_{6a,6b}$ = 12.3 Hz, H-6a), 4.10 (dd, 1H, $J_{2',3'}$ = 9.2 Hz, H-2'), 4.18 (dd, 1H, $J_{3,4}$ = 9.1 Hz, H-3), 4.20

(dd, 1H, H-6a'), 4.44 (dd, 2H, ^2J = 12.2 Hz, CH_2Ph), 4.53 (s, 2H, CH_2Ph), 4.57 (d, 1H, $J_{1,2}$ = 3.4 Hz, H-1), 4.60 (dd, 2H, ^2J = 11.0 Hz, CH_2Ph), 4.90 (dd, 1H, $J_{3',4'}$ = 10.6 Hz, H-3'), 4.95 (d, 1H, $J_{1',2'}$ = 8.8 Hz, H-1'), 5.05 (dd, 1H, $J_{4',5'}$ = 9.6 Hz, H-4'), 6.09 (d, 1H, N*H*), 7.10-7.38 (m, 15H, aromatic) ppm, ^{13}C-n.m.r.: δ, 20.77, 21.00, 21.05, 55.19, 55.42, 62.31, 68.42, 68.68, 70.19, 72.32, 72.66, 72.85, 73.96, 75.30, 75.73, 77.65, 79.95, 81.53, 97.38, 101.10, 127.77 (x 2), 127.99, 128.17, 128.42 (x 2), 128.54 (x 2), 128.56 (x 2), 128.83 (x 2), 128.86, 129.30 (x 2), 138.06, 138.25, 138.86, 157.67, 169.70, 171.14, 171.32 ppm, HR-FAB MS $[M+H]^+$ calcd for $C_{42}H_{48}F_3NO_{14}Na$ 870.2925, found 870.2941.

Methyl O-(3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-(1→6)-2,3,4-tri-O-benzyl-α-D-glucopyranoside (**129a**) was obtained from **119** and **125** as an amorphous solid in 92% yield. R_f = 0.53 (toluene-ethyl acetate, 3/2, v/v); $[\alpha]_D^{25}$ 14.3^o (c = 1.0, $CHCl_3$); ^{1}H-n.m.r.: δ, 1.82, 1.99, 2.05 (3s, 9H, 3 x COCH_3), 3.13 (s, 3H, OCH_3), 3.20 (dd, 1H, $J_{4,5}$ = 8.9 Hz, H-4), 3.35 (dd, 1H, $J_{2,3,}$ = 9.7 Hz, H-2), 3.59-3.65 (m, 2H, H-5, 6b), 3.80 (dd, 1H, $J_{3,4}$ = 9.3 Hz, H-3), 3.81-3.86 (m, 1H, H-5'), 4.03-4.16 (m, 3H, H-6a, 6b', CH_2^aPh), 4.26-4.40 (m, 4H, H-1, 2', 6a', CH_2^bPh), 4.61 (dd, 2H, ^2J = 12.1 Hz, CH_2Ph), 4.72 (dd, 2H, ^2J = 10.8 Hz, CH_2Ph), 5.14 (dd, 1H, $J_{4',5'}$ = 9.6 Hz, H-4'), 5.40 (d, 1H, $J_{1',2'}$ = 8.5 Hz, H-1'), 5.76 (dd, 1H, $J_{3',4'}$ = 9.1 Hz, H-3'), 6.97-7.51 (m, 19H, aromatic) ppm, ^{13}C-n.m.r.: δ, 20.6, 20.8, 20.9, 54.6, 55.1, 60.5, 62.2, 68.9, 69.1, 69.3, 70.9, 72.1, 73.5, 74.9, 75.8, 77.7, 79.8, 82.0, 98.1, 98.5, 123.6 (x 2), 127.7, 127.8, 127.9 (x 2), 128.1 (x 3), 128.2 (x 2), 128.5 (x 4), 128.6 (x 2), 131.8 (x 2), 134.3 (x 2), 137.9, 138.2, 138.8, 167.8, 169.6, 170.3, 170.9 ppm, HR-FAB MS $[M+H]^+$ calcd for $C_{48}H_{51}NO_{15}Na$ 904.3156, found 904.3157.

Methyl O-(3,4,6-tri-O-acetyl-2-deoxy-2-trichloroethoxycarbamoyl-β-D-glucopyranosyl)-(1→6)-2,3,4-tri-O-benzyl-α-D-glucopyranoside (**129b**) was obtained from **120** and **125** as an amorphous solid in 91% yield. R_f = 0.46 (ethyl acetate-hexanes, 3/2, v/v); $[\alpha]_D^{25}$ 22.89° (c = 1.0, $CHCl_3$); ^{1}H-n.m.r.: δ, 1.93, 1.95, 1.97 (3s, 9H, 3 x COC*H*$_3$), 3.29 (s, 3H, OC*H*$_3$), 3.36-3.43 (m, 2H, H-2, 4), 3.51-3.69 (m, 4H, $J_{5',6a'}$ = 4.2 Hz, H-2, 5, 5', 6a), 3.91 (dd, 1H, $J_{3,4}$ = 9.1 Hz, H-3), 3.98-4.06 (m, 2H, H-6b, 6b'), 4.15 (dd, 1H, $J_{6a',6b'}$ = 12.1 Hz, H-6a'), 4.32 (d, 1H, $J_{1',2'}$ = 8.7 Hz, H-1'), 4.45 (s, 2H, C*H*$_2$CCl$_3$), 4.52 (d, 1H, $J_{1,2}$ = 3.5 Hz, H-1), 4.65 (dd, 2H, ^2J = 11.4 Hz, C*H*$_2$Ph), 4.65 (dd, 2H, ^2J = 12.8 Hz, C*H*$_2$Ph), 4.79 (d, 1H, N*H*), 4.82 (dd, 2H, ^2J = 10.8 Hz), 4.95 (dd, 1H, $J_{4',5'}$ = 9.6 Hz, H-4'), 5.12 (dd, 1H, $J_{3',4'}$ = 9.4 Hz, H-3'), 7.19-7.30 (m, 15H, aromatic) ppm, ^{13}C-n.m.r.: δ, 20.8 (x 2), 20.9, 55.5, 56.2, 62.2, 68.2, 68.8, 69.7, 72.0, 72.1, 73.6, 74.6, 74.7, 76.0, 77.4, 79.9, 82.3, 95.4, 98.3, 101.0, 127.9, 128.1 (x 2), 128.2 (x 4), 128.4 (x 2), 128.6 (x 2), 128.7 (x 2), 128.8 (x 2), 138.2, 138.8, 138.9, 154.0, 169.6, 170.8, 170.9 ppm, HR-FAB MS $[M+H]^+$ calcd for $C_{43}H_{50}Cl_3NO_{15}Na$ 950.2126, found 950.2116.

Methyl O-(3,4,6-tri-O-acetyl-2-deoxy-2-trifluoroacetamido-β-D-glucopyranosyl)-(1→6)-2,3,4-tri-O-benzyl-α-D-glucopyranoside (**129c**) was obtained from **121** and **125** as an amorphous solid in 93% yield. R_f = 0.48 (toluene-ethyl acetate, 1/1, v/v); $[\alpha]_D^{25}$ 3.51° (c = 1.0, $CHCl_3$); ^{1}H-n.m.r.: δ, 2.02, 2.04, 2.06 (3s, 9H, 3 x COC*H*$_3$), 3.34 (s, 3H, OC*H*$_3$), 3.35 (dd, 1H, $J_{4,5}$ = 9.3 Hz, H-4), 3.49 (dd, 1H, $J_{2,3}$ = 9.5 Hz, H-2), 3.57 (dd, 1H, $J_{5,6a}$ = 5.1 Hz, $J_{6a,6b}$ = 10.5 Hz, H-6a), 3.63-3.68 (m, 1H, $J_{5',6a'}$ = 4.3 Hz, H-5'), 3.74-3.79 (m, 1H, H-5), 3.99 (dd, 1H, $J_{3,4}$ = 9.2 Hz, H-3), 3.97-4.16 (m, 3H, H-2', 6b, 6b'), 4.25 (dd, 1H, $J_{6a',6b'}$ = 12.2 Hz, H-6a'), 4.34 (d, 1H, $J_{1',2'}$ = 8.3 Hz, H-1'), 4.44 (dd, 2H, ^2J = 8.3 Hz, C*H*$_2$Ph), 4.55 (d, 1H, $J_{1,2}$ = 1.6 Hz, H-1), 4.70 (dd, 2H, ^2J=

12.1 Hz, CH_2Ph), 4.71 (dd, 2H, ^2J = 12.1 Hz, CH_2Ph), 5.08 (dd, 1H, $J_{4',5'}$ = 9.5 Hz, H-4'), 5.20 (dd, 1H, $J_{3',4'}$ = 10.1 Hz, H-3'), 6.42 (d, 1H, N*H*), 7.25-7.39 (m, 15H, aromatic) ppm, ^{13}C-n.m.r.: δ, 20.6, 20.8, 20.9, 54.9, 55.3, 68.4, 68.6, 69.5, 71.9, 72.2, 73.6, 74.7, 75.8, 77.4, 80.1, 82.2, 98.1, 100.5, 127.8 (x 2), 128.0 (x 2), 128.1 (x 6), 128.4 (x 2), 128.6 (x 2), 128.7 (x 4), 138.3, 138.5, 138.9, 169.5, 170.9, 171.3 ppm, HR-FAB MS $[M+H]^+$ calcd for $C_{42}H_{48}F_3NO_{14}Na$ 870.2925, found 870.2911.

Methyl O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→6)-2,3,4-tri-O-benzyl-α-D-glucopyranoside (**129d**) was obtained from **122** and **125** as an amorphous solid in 93% yield. R_f = 0.56 (acetone - toluene, 1/1, v/v); $[\alpha]_D^{25}$ - 20.1° (c = 1.0, $CHCl_3$); ^{1}H-n.m.r.: δ, 1.74, 1.94 (x 3) (4s, 12H, 3 x COCH_3), 3.29 (s, 3H, OCH_3), 3.40 (dd, 1H, $J_{4,5}$ = 9.3 Hz, H-4), 3.44 (dd, 1H, $J_{2,3}$ = 9.7 Hz, H-2), 3.58-3.82 (m, 4H, H-2', 5, 5', 6b), 3.91 (dd, 1H, $J_{3,4}$ = 9.2 Hz, H-3), 3.97-4.05 (m, 2H, H-6a, 6b'), 4.14 (dd, 1H, $J_{5',6a'}$ = 4.9 Hz, $J_{6a',6b'}$ = 12.4 Hz, H-6a'), 4.52 (d, 1H, $J_{1,2}$ = 3.0 Hz, H-1), 4.61 (d, 1H, $J_{1',2'}$ = 8.1 Hz, H-1'), 4.63 (dd, 2H, ^2J = 10.5 Hz, CH_2Ph), 4.64 (dd, 2H, ^2J = 12.2 Hz, CH_2Ph), 4.81 (dd, 2H, ^2J = 10.8 Hz, CH_2Ph), 4.97 (dd, 1H, $J_{4',5'}$ = 9.7 Hz, H-4'), 5.20 (dd, 1H, $J_{3',4'}$ = 10.4 Hz, H-3'), 5.36 (d, 1H, N*H*), 7.18-7.29 (m, 15H, aromatic) ppm, ^{13}C-n.m.r.: δ, 20.80, 2.86 (x 3), 23.46, 54.85, 55.36, 62.34, 67.83, 68.81, 69.65, 72.03, 72.45, 73.52, 74.77, 75.88, 77.57, 79.92, 82.17, 98.22, 100.73, 127.77, 127.92 (x 2), 128.05, 128.11 (x 2), 128.32 (x 2), 128.55 (x 2), 128.63 (x 2), 128.67 (x 2), 138.27, 138.48, 138.91, 169.55, 170.12, 170.86, 171.00 ppm, HR-FAB MS $[M+H]^+$ calcd for $C_{42}H_{51}NO_{14}Na$ 816.3207, found 816.3209.

Benzoxazolyl *O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-trichloroethoxycarbamoyl-β-D-glucopyranosyl)-(1→6)-3,4-di-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (**131**) was obtained by Method B from **120** and **130** as an amorphous solid in 82% yield: R_f = 0.57 (toluene-ethyl acetate, 2/3, v/v); $[\alpha]_D^{25}$ 61.2° (c = 1.0, $CHCl_3$); ^{1}H-n.m.r.: δ, 1.87, 1.93, 1.96, 2.07, 2.09 (5s, 15H, 5 x COCH_3), 3.54-3.61 (m, 1H, H-5'), 3.78-3.96 (m, 3H, H-2', 6a, 6b), 4.07-4.26 (m, 3H, H-5, 6a', 6b'), 4.54 (dd, 1H, $J_{2,3}$ = 10.6 Hz, H-2), 4.79 (d, 1H, $J_{1',2'}$ = 8.5 Hz, H-1'), 4.84-4.92 (m, 3H, H-3', CH_2CCl$_3$), 4.97-5.10 (m, 2H, H-4, 4'), 5.90 (dd, 1H, $J_{3,4}$ = 9.7 Hz, H-3), 6.41 (d, 1H, $J_{1,2}$ = 10.9 Hz, H-1), 6.84 (d, 1H, N*H*), 7.26-7.88 (m, 8H, aromatic) ppm, ^{13}C-n.m.r.: δ, 20.59, 20.75, 20.79, 20.85, 20.98, 29.91, 53.56, 55.90, 62.11, 67.93, 68.86, 69.02, 71.16, 72.04, 72.56, 74.77, 77.43, 79.75, 81.67, 95.94, 101.71, 110.47 (x 2), 119.51 (x 2), 124.14 (x 2), 124.87, 125.28, 134.78, 141.29, 151.98, 155.40, 160.84, 166.57, 169.64, 169.90, 170.09, 170.23, 170.95 ppm, HR-FAB MS $[M+H]^+$ calcd for $C_{40}H_{40}Cl_3N_3O_{18}SNa$ 1012.0972, found 1012.0935.

Methyl O-(3,4,6-tri-O-acetyl-2-deoxy-2-trichloroethoxycarbamoyl-β-D-glucopyranosyl)-(1→6)-O-(3,4-di-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-(1→6)-2,3,4-tri-O-benzyl-α-D-glucopyranoside (**132**) was obtained by Method A from **125** and **131** as an amorphous solid in 73% yield: R_f = 0.56 (toluene-ethyl acetate, 2/3, v/v); $[\alpha]_D^{25}$ 7.3° (c = 1.0, $CHCl_3$); ^{1}H-n.m.r.: δ, 1.76, 1.94, 1.95, 1.97, 2.03 (5s, 15H, 5 x COCH_3), 3.18 (s, 3H, OCH_3), 3.27 (dd, 1H, $J_{4A,5A}$ = 9.4 Hz, H-4A), 3.36 (dd, 1H, $J_{2A,3A}$ = 9.6 Hz, H-2A), 3.55-3.82 (m, 7H, H-2C, 3A, 5A, 5B, 5C, 6B, 6C'), 3.93 (dd, 1H, $J_{5B,6B'}$ = 1.9 Hz, $J_{6B,6B'}$ = 10.9 Hz, H-6B'), 4.00-4.10 (m, 3H, H-6A', 6C, CH_2^aPh), 4.20-4.36 (m, 3H, H-2B, 6A, CH_2^bPh), 4.50-4.71 (m, 4H, H-1A, 1C, CH_2CCl$_3$), 4.61 (d, 2H, ^2J = 15.8 Hz, CH_2Ph), 4.70 (dd, 2H, ^2J = 10.9 Hz, CH_2Ph), 4.93-5.02 (m, 2H,

H-4B, 4C), 5.10 (dd,1 H, $J_{3C,4C}$ = 9.9 Hz, H-3C), 5.38 (d, 1H, $J_{1B,2B}$ = 8.5 Hz, H-1B), 5.56 (d, 1H, N*H*), 5.68 (dd, 1H, $J_{3B,4B}$ = 10.7 Hz, H-3B), 6.84-6.90 (m, 15H, aromatic) ppm; ^{13}C-n.m.r.: δ, 20.63, 20.83, 20.87, 20.92, 20.98, 29.92, 54.65, 55.30, 56.35, 62.14, 68.41, 68.97, 69.37, 69.61, 69.78, 70.89, 72.06, 72.68, 73.17, 73.80, 74.84, 75.79, 77.44, 79.61, 81.97, 95.72, 98.11, 98.51, 101.75, 123.71 (x 2), 127.71 (x 2), 127.83 (x 2), 127.89 (x 2), 128.10 (x 3), 128.31 (x 2), 128.47 (x 2), 128.50 (x 3), 128.67 (x 2), 131.30, 134.35 (x 2), 137.94, 138.33, 138.96, 154.55, 169.66, 170.02, 170.31, 170.71, 170.91 ppm; HR-FAB MS $[M+H]^+$ calcd for $C_{40}H_{40}Cl_3N_3O_{18}SNa$ 1323.3098, found 1325.3164.

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Chapter 3

Attempted Synthesis of Disaccharide Analogues

3.1. Introduction

As mentioned in Chapter 1, disaccharide analogues usually provide more appreciable antagonism towards LPS due to their structural similarity with native Lipid A. To this end, various structure-activity studies have been performed on bacterial Lipid A and its derivatives.[1] However, recent findings on the structure and binding site of the LPS receptors has led Jerala to propose that the structural motif of a possible LPS antagonist can be condensed to the arrangement of anionic groups and a large hydrophobic moiety.[2] Apparently, this arrangement provides a more likely complement to the cationic residues and the large hydrophobic binding site of the LPS receptors.

Since Lipid IV_A – the biosynthetic precursor of E. coli Lipid A (**16**, see Chapter 1) – is known to antagonize LPS, we built our targeted design for the disaccharide analogues on the relative structural simplicity of this molecule.[3-6] To this end, we maintained the di-glucosamine β-(1→6)-linked disaccharide core and the four identical hydrophobic chains. Moreover, since previous research showed a distinct ambivalence towards the type of hydrocarbon chain necessary for antagonistic activity,[7] we decided to try all three types namely: the simple alkyl chain (**26**, Figure 4.1), the β-hydroxyacyl chain (**24**, Figure 4.1), and the acyl chain (**25**, Figure 4.1). We believe that these structures exhibit the hydrophobic character recommended by Jerala.[2]

With the hydrophobic moiety in place, we turned our attention to finding a suitable anionic group to install on C-6' of the disaccharide. Previous research by Fukase *et al*. introduced the novel idea of employing acidic amino acid on C-6 of their monosaccharide analogues.[8] They reasoned that acidic amino acids provide a natural complement to the basic amino acid residues present in the binding sites of LPS

receptors. In our case, we believe that the tendency of amino acids to assume a zwitterionic character under physiological conditions converts acidic amino acids into a suitable anionic group. Thus, our targeted designs for the disaccharide analogues, mentioned earlier in Chapter 1, are reiterated below (Figure 4.1).

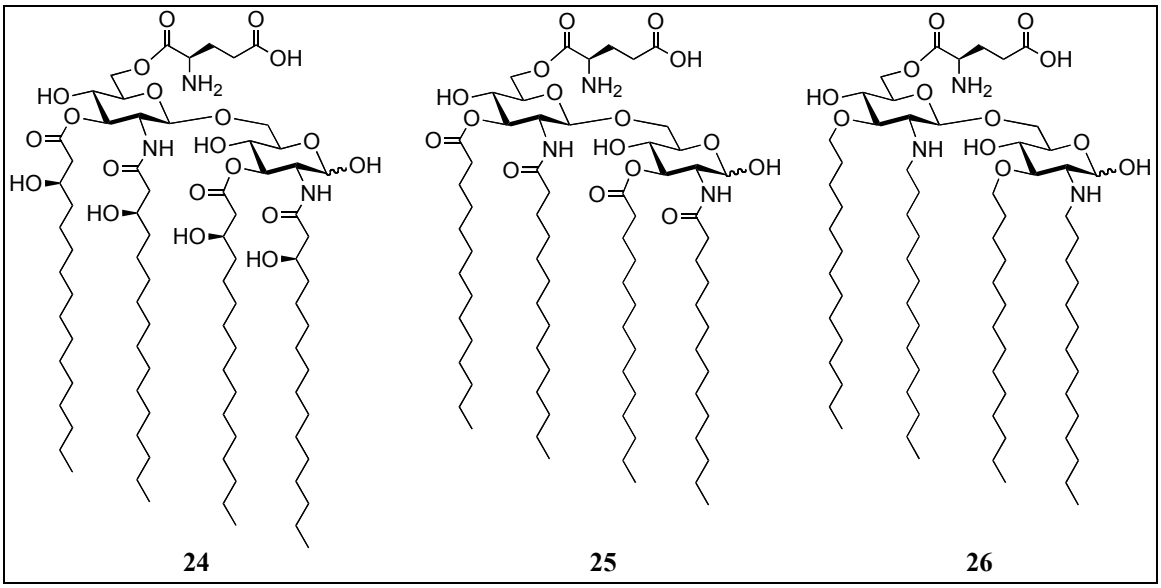
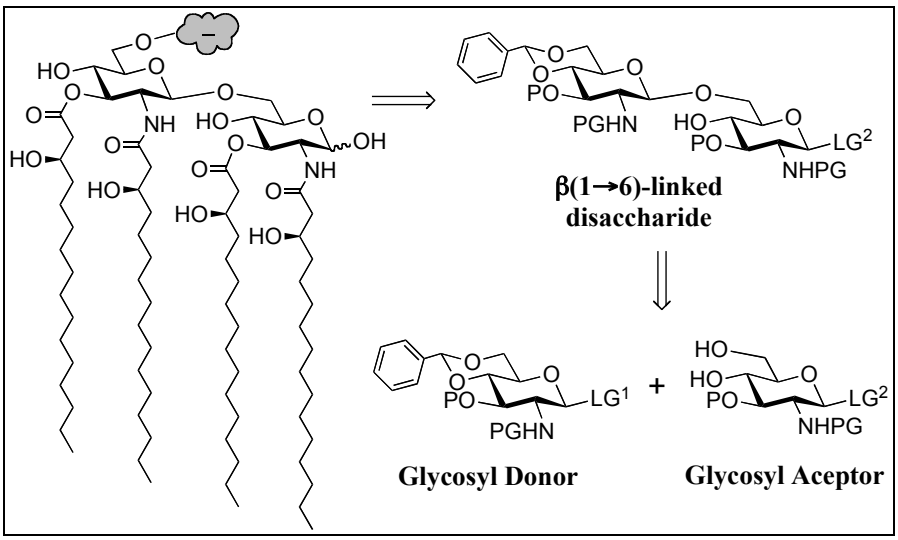


Figure 3.1. Targeted design for the disaccharide analogues.

The first part of Chapter 2 mentions the retrosynthetic scheme for the synthesis of our disaccharide analogues. As illustrated below (Scheme 4.1), our plan is to synthesize the beta-(1→6)-linked disaccharide from a differently protected glycosyl donor and acceptor. Once the disaccharide is formed, it will be selectively deprotected and functionalized into our target compounds. To this end, we set out to synthesize various differently protected glycosyl donors and acceptors that would allow a more efficient construction of the disaccharide core.

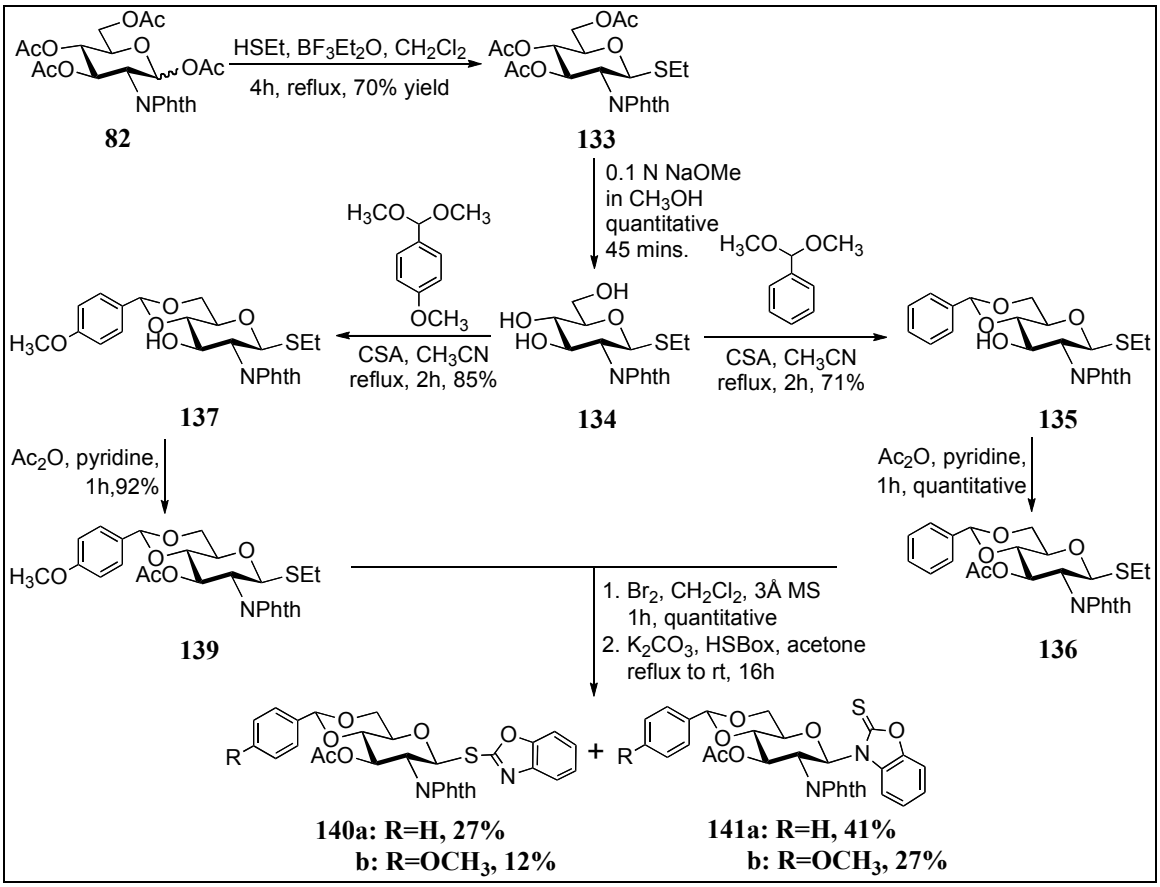


Scheme 3.1. Retrosynthetic scheme the synthesis of Lipid A analogs. P = Protecting Group; PG = Participating Group; LG = Leaving Group; R = Alkyl Group

3.2. Synthesis of the Differently Protected Glycosyl Donors

Aside from the 2-deoxy-2-N-substituted SBox glycosides we synthesized earlier (Chapter 2), we also decided to prepare other 2-deoxy-2-N-substituted SBox donors for comparison. These donors will feature the appropriate protecting group pattern necessary to reduce the number of linear steps involved in the construction of the disaccharide analogues. The synthesis of the glycosyl donors began with the conversion of tetra-acetate precursor **82** into the corresponding thioglycoside **133**. Thioglycosides are known to be stable towards a wide range of reaction conditions and more importantly, it can be easily hydrolyzed into the corresponding hemiacetal. Thus, the reaction between **82** and ethanethiol, in the presence of $BF_3 \cdot Et_2O$ and CH_2Cl_2 under reflux, gave **133** in 70% yield after 4 hours (Scheme 3.2).[9] Deacetylation of **133** under Zemplen conditions followed by protection of the 4,6-diol in **134** as a benzylidene acetal gave **135** in 71 % yield. Our choice of protecting the C-4 and C-6 hydroxyls as a benzylidene acetal was a result of two main considerations – this protecting group can be easily deprotected under mildly

acidic conditions or opened regioselectively into a C-4 or C-6 hydroxyl. As illustrated in Scheme 3.2, conversion of **134** to **135** was accomplished using benzaldehyde dimethyl acetal and catalytic camphorsulfonic acid in acetonitrile. This procedure was adapted from a published account which used a different acid source and solvent (p-toluenesulfonic acid in DMF).[10]



Scheme 3.2. Synthesis of glycosyl donors.

After a quantitative acetylation of the C-3 hydroxyl, the thioglycoside moiety in **136** was converted into the more potent thioimidate moiety in two steps. The first step involved the conversion of the thioglycoside into a glycosyl bromide while the second consists of a nucleophilic displacement of the bromide 2-benzoxazolethione potassium

salt (KSBox) in the presence of 18-crown-6. Herein, we observed the formation of the N-linked isomer **141a** as the major product. Unfortunately for our purposes, this isomer is known to be unreactive during glycosidation reactions.[11] Thus, the known thioglycoside **136**[12] was converted into the S-benzoxazolyl glycoside **140a** in 27% yield.

As a result of our unsuccessful attempts discussed above, we decided to protect the 4,6-diol in **134** as a p-methoxybenzylidene acetal instead. Our decision was based on a well-accepted convention that benzylidene acetals deactivate the sugar ring towards substitution reactions.[13] To this end, the reaction between **134** and p-anisaldehyde dimethyl acetal, in the presence of catalytic CSA and CH_3CN, gave the known thioglycoside **137**[14] in 85% yield after 2 hours (see Scheme 3.2 above). Following the acetylation of the C-3 hydroxyl in **137** as an acetate, the thioglycoside **139**[15] was converted into the corresponding thioimidate using the same reaction sequence described for **136**. Unfortunately, we obtained an even lower yield of the corresponding S-benzoxazolyl glycoside **140b** (12% yield) that was forming alongside the unreactive N-linked isomer **141b** (27% yield).

We would like to note, at this point, that our unsuccessful synthesis of glycosyl donors **140a** and **140b** has discouraged us to pursue it further. Consequently, simplified glycosyl donor **119** (see Table 2.1 in Chapter 2) was our donor of choice henceforth.

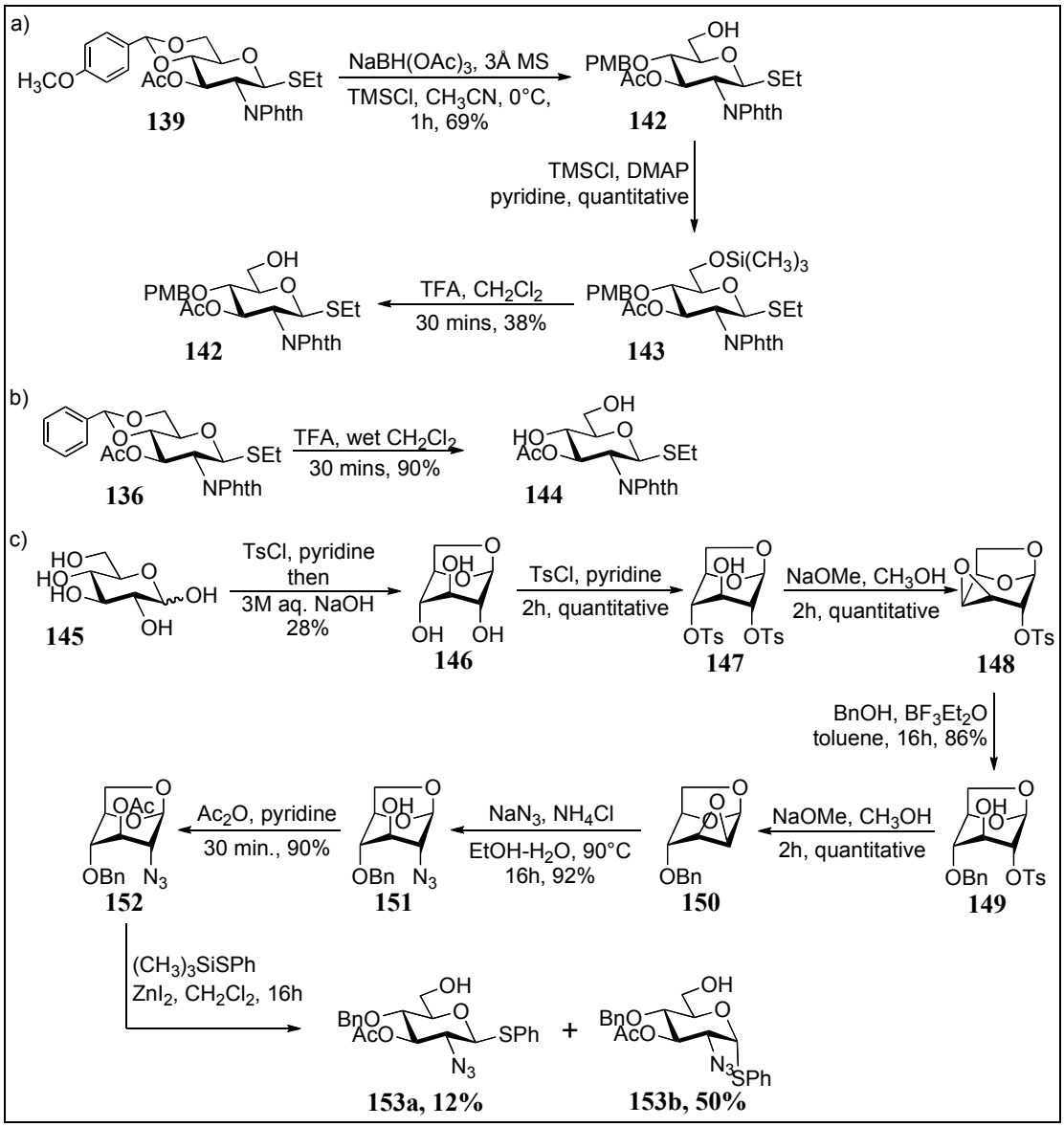
3.3. Synthesis of the Differently Protected Glycosyl Acceptors

As illustrated in Scheme 3.1, we also needed a differently protected glycosyl acceptor to synthesize the β-(1→6)-linked disaccharide. Our initial considerations in designing the glycosyl acceptor included: 1) the C-2 and C-3 protecting group moieties on the glycosyl acceptor should be compatible with the C-2 and C-3 protecting groups on

the glycosyl donors in that we should be able simultaneously deprotect all four positions in one step; 2) the C-4 protecting group should remain stable throughout the subsequent steps but it should be easily removable during the final, global deprotection step; and most importantly, 3) we should be able to chemoselectively activate the anomeric moiety on the glycosyl donor over the anomeric moiety on the glycosyl acceptor.

Thus, starting from precursor **139**, cleavage of the *p*-methoxybenzylidene acetal using $NaBH(OAc)_3$ and $(CH_3)_3SiCl$ in CH_3CN gave 69% of glycosyl acceptor **142** (see Scheme 3.3a).[14] Our initial attempts to glycosylate this acceptor, however, were unsuccessful. We realized that the presence of trace amounts of $NaBH(OAc)_3$ were negatively affecting the outcome of the glycosylation. After our repeated attempts to purify **142** by column chromatography proved futile, we decided to convert the C-6 hydroxyl into a silyl ether. We reasoned that the difference in R_f between **142** and **143** would give us a better chance of removing the impurities from the previous step. After removal of the silyl protecting group, the glycosyl acceptor **142** was obtained in a disappointing yield of 39%. This is very likely due to the marginal stability of p-methoxybenzylidene acetals in the acidic medium.

At this point, we have also realized that the lithium aluminum hydride-aluminum chloride method to regioselectively open benzylidene acetals to a C-6 hydroxyl[16] will lead to the undesired opening of the imide protecting group in **136**. Since there are currently no reported methods to effect this transformation, we resolved to form the 4,6-diol instead by simple acid hydrolysis of the benzylidene acetal in the presence of trifluoroacetic acid (TFA). Accordingly, acceptor **144**[17] was obtained from **136**[12] in 90% yield after 30 minutes (Scheme 3.3b).



Scheme 3.3 Synthesis of glycosyl acceptors.

We were still quite concerned, however, about the possible ramifications of leaving the C-4 hydroxyl unprotected during the subsequent steps. Obviously, the formation of unwanted products will make the elaboration of the disaccharide into our target compound too complicated. In order to address this dilemma, we decided to synthesize our acceptor via the levoglucosan route. Levoglucosan (or 1,6-anhydroglucopyranoses) has enjoyed a long and interesting history in the preparation of

biologically important and structurally diverse natural products[18-25] and modified[26-28] sugars. Its [3.2.l] bicyclic framework has been known to elicit a remarkable range of stereo- and regioselectivities during transformations. Moreover, since the pyranose ring is locked in the $^{1}C_4$ conformation, substituents adapt axial orientations as opposed to the equatorial orientations encountered with the conventional $^{4}C_1$ counterparts. In addition to these topological properties, the presence of an internal acetal reduces the number of protecting groups that are required. Accordingly, we decided to take advantage of the attractive properties of this molecule for the synthesis of our glycosyl acceptor.

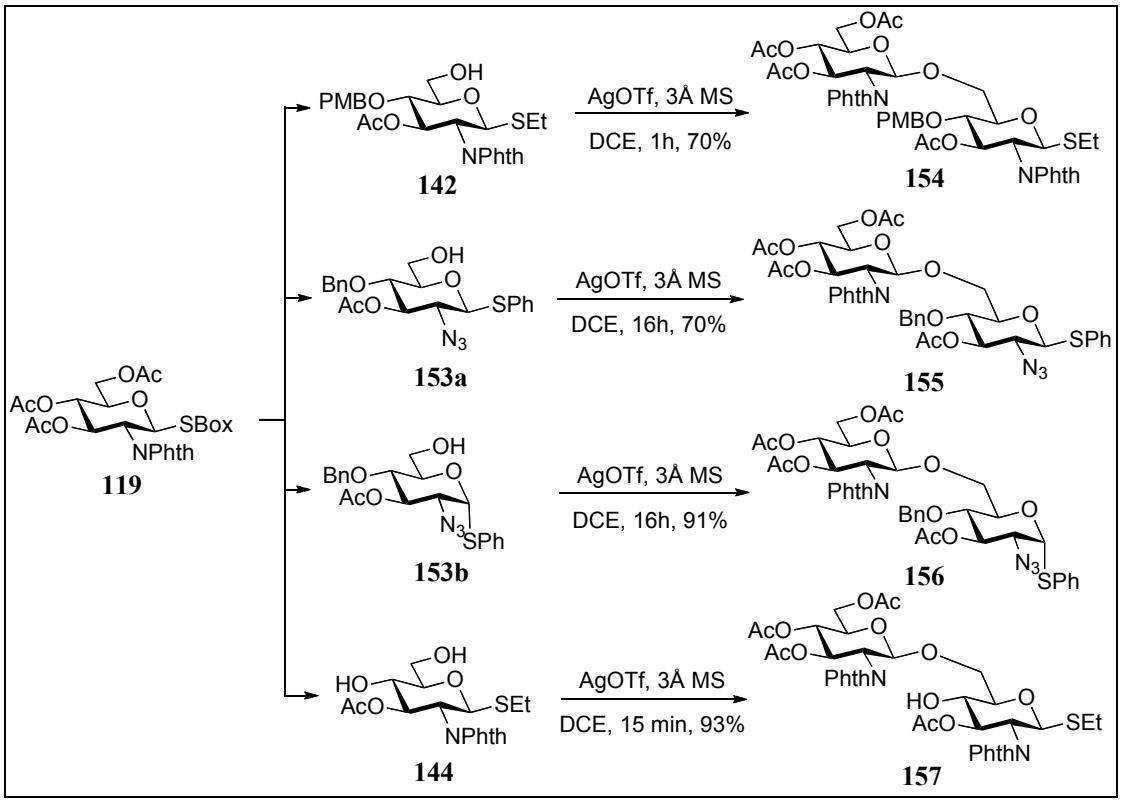
The synthesis began with the transformation of D-glucose **145** into levoglucosan **146** in 28% yield (Scheme 3.3c). Thus, as described by Fraser-Reid *et al*.,[29] the primary hydroxyl group of **145** was selectively tosylated under carefully controlled conditions. This was followed, *in situ*, by the intramolecular displacement of the C-6 tosylate by the anomeric hydroxyl group on treatment of the reaction mixture with aqueous sodium hydroxide. After **146** was obtained, the C-2 and C-4 hydroxyl was selectively tosylated using tosyl chloride in pyridine to give **147** in essentially quantitative yields.[30] Intramolecular displacement of the C-4 tosylate was then achieved by treatment of **147** with sodium methoxide in methanol to form the 1,6:3,4-dianhydrogalactopyranose **148** in quantitative yields.[31] Remarkably, this particular configuration allows selective introduction of a nucleophile onto C-4. Thus, selective opening of the 3,4-oxirane ring in **148** with benzyl alcohol, in the presence of BF_3Et_2O and toluene, was achieved after stirring overnight to give **149**[31,32] in 86% yield.

To introduce the amino moiety, we decided to take advantage of the chemistry of Cerny epoxides.[33] To this end, precursor **149** was quantitatively transformed into the

Cerny expoxide **150** via intramolecular displacement of the C-2 tosylate, in the presence of $NaOCH_3$ in MeOH.[34,35] Selective opening of the 2,3-oxirane with sodium azide then proceeded smoothly to give **151** in 92% yield.[36] After acetylation of the **151**,[37] the 1,6-anhydride bond was then selectively opened using $(CH_3)_3SiSPh$ and ZnI_2 to give glycosyl acceptor **153** as a separable mixture of anomers.[38]

3.4. Formation of the Disaccharide Core

With the glycosyl donors and acceptors in hand, we then proceeded to determine the best building blocks to construct our disaccharide core. Glycosidations with the glycosyl acceptors **142**, **144**, **153a**, and **153b** (Scheme 3.4) were all performed in the presence of AgOTf, and 3Å molecular sieves (MS) in dichloroethane (DCE) to afford the corresponding disaccharides with complete β-stereoselectivity. Glycosylations with glycosyl acceptors **153a** and **153b** proceeded much slower which, in connection with the low-yielding synthesis of these acceptors (mentioned earlier), made us abandon this route. The moderate yield achieved in glycosylation of **142** made us believe that the best glycosyl donor and glycosyl acceptor for our purposes were **119** and **144**, respectively. The resulting disaccharide **157** was obtained in 93% yield. Moreover, it should be noted that the deactivating effect of the C-2 phthalimido moiety on C-4, along with the increased steric hindrance around the C-4 hydroxyl has allowed us to regioselectively form the β-(1→6) linked product, without any traces of the β-(1→4)-linked by-products.

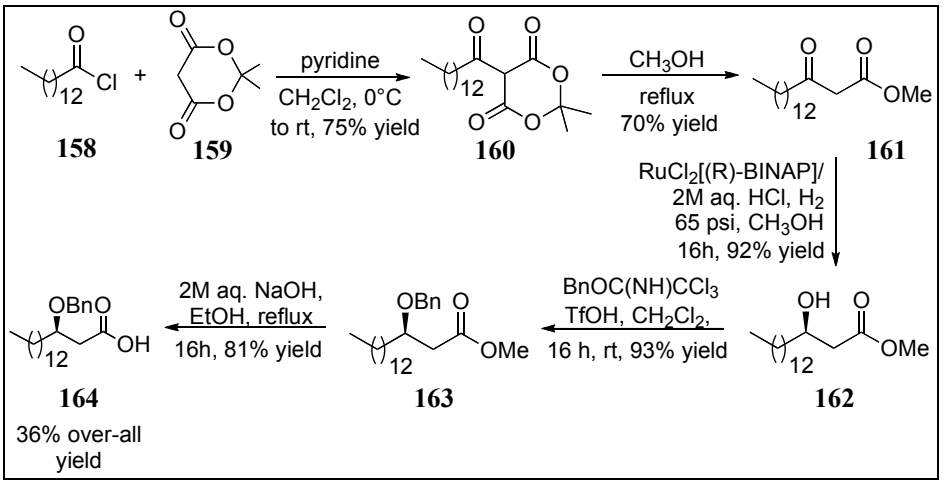


Scheme 3.4. Glycosidation reactions with triacetate glycosyl donor **119**.

3.5. Synthesis of the (R)-3-Hydroxy Hexadecanoic Acid

In line with the synthetic effort towards preparing Lipid A analogues that possess minimal (if not completely absent) endotoxic properties and beneficial immunostimulant activities, several methods have been developed for the synthesis of (R)-3-hydroxy fatty acids. Indeed, synthesis of the latter has been achieved by both chemical[39-43] and enzymatic[44-46] means, giving a wide range of truly practical methods for large scale preparations. Along with the progress in developing scalable methods to synthesize 3-hydroxy fatty acid, various methods to determine the enantiomeric purity have also been developed to date. These include NMR analysis of Mosher MTPA ester derivatives[39,45] chiral NMR shift studies[42,43] and HPLC Chiralpak® AS analysis[47] of p-bromophenacyl

esters. Deriving from these methods, the synthesis of (R)-3-benzyloxyhexadecanoic acid **164** is described below (see Scheme 3.2.).



Scheme 3.5. Synthesis of (R)-3-benzyloxyhexadecanoic acid.

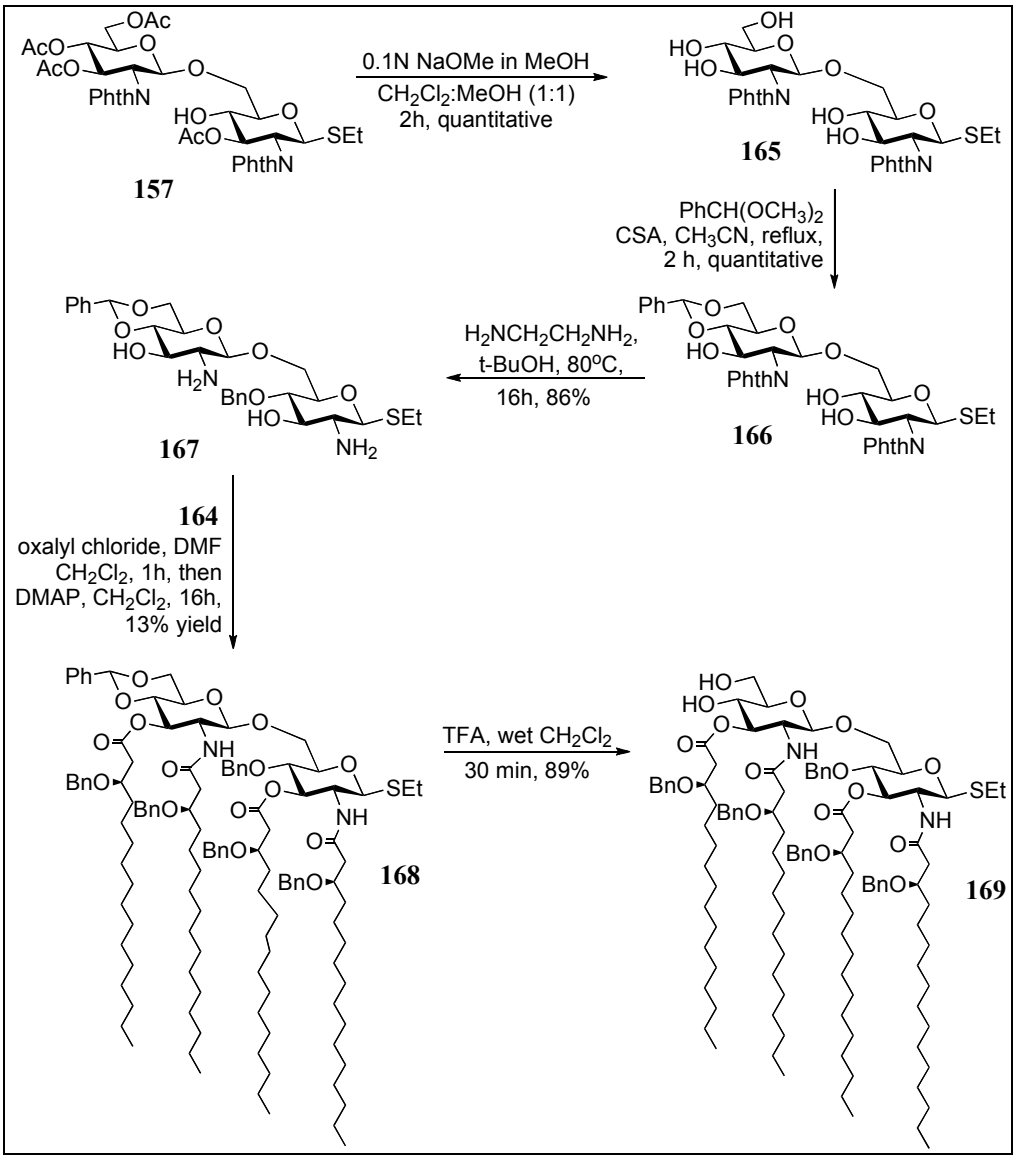
The synthesis began with the acylation of commercially available Meldrum's acid **159** (2,2-dimethyl-1,3-dioxane-4,6-dione) with myristoyl chloride **158** by the Yonemitsu procedure.[48,49] The crude myristylated Meldrum's acid **160**, formed in 75% yield in the presence of pyridine in CH_2Cl_2, was subjected to acetal removal followed by decarboxylation. This was accomplished in boiling methanol for 2 hours to afford the desired methyl 3-oxohexadecanoate **161**[50] in 70% yield.[42,48] It should be noted that a simple filtration on a pad of silica gel was sufficient to purify compound **161** for the subsequent step. The introduction of the stereocenter at the β-carbon in enantiomerically pure form was then achieved via Noyori's catalytic hydrogenation.[51] Thus, following a procedure developed by Taber *et al*.,[52] the asymmetric hydrogenation of **161** in methanol using catalytic dichloro[(R)-(+)-2,2'-bis(diphenylphosphino)-1,1'-binaphthyl]ruthenium (II) or ($RuCl_2$[(R)-BINAP]) gave methyl (R)-3-hydroxyhexadecanoate **162**[50] in 92% yield after 16 hours. This reduction was performed in the presence of 0.1 mol % HCl

using a standard Parr apparatus at 60 psig hydrogen, as described by King and co-workers.[53] Noyori's asymmetric hydrogenation was particularly convenient since the BINAP-Ru(II) catalyst is commercially available in both enantiomeric forms, has very high catalytic efficiency, and gives products of predictable absolute configuration.[54]

The enantiomeric excess of **162** was then identified by ^{1}H NMR of the mixture in the presence of tris[3-(heptafluoro-1-hydroxybutylidene]-(+)-camphoratoeuropium (III) [Eu(hfc)$_3$] in $CDCl_3$.[55,56] The singlet around 3.61 ppm associated with C(O)C*H*$_3$ was separated into two singlets – 3.88 and 3.92 ppm for the (R) and (S) forms, respectively – and made it possible to identify 6% of the (S) form. Then, benzylation of the β-hydroxyl using benzyl 2,2,2-trichloroacetimidate in CH_2Cl_2, in the presence of catalytic trifluoromethanesulfonic acid, gave **163** in 93% yield.[38] Finally, base hydrolysis of the methyl ester using 2M aqueous NaOH in tetrahydrofuran (THF)[57] afforded (R)-3-benzyloxyhexadecanoic acid **164**[38] in 81% yield, or 36 % overall yield from the starting material **158**.

3.6. Synthesis of the Disaccharide Template

After obtaining disaccharide **157**, we then turned our attention to selectively functionalizing key positions (see Scheme 3.6). Thus, deacetylation of **157** in the presence of $NaOCH_3$ in methanol gave **165** in quantitative yield after 2 hours. The 4,6-diol in intermediate **165** was then selectively protected as a benzylidene acetal using benzaldehyde dimethyl acetal in the presence of CSA and CH_3CN. Compound **166** was subsequently obtained in quantitative yield after 2 hours.



Scheme 3.6. Synthesis of the disaccharide template.

Deprotection of the phthalimido moieties was then performed in the presence of ethylene diamine in butanol, and gave intermediate **167** in 86% yield. The (R)-3-hydroxy fatty acids were then installed simultaneously via a-chloroenamines and gave **168** in a disappointing yield of 13%. Finally, the disaccharide template **169** was obtained in 89% yield after stirring **168** in the presence of CF_3COOH and wet CH_2Cl_2 for 30 mins. Unfortunately, our attempts to install the butoxycarbonyl-protected glutamic acid moiety

on C-6' proved unsuccessful. Despite our best intentions, we were unable to synthesize our target disaccharide analogues. Regardless, our efforts toward this end are still ongoing.

3.7. Summary and Conclusions

As shown above, we have revisited classic reactions in carbohydrate chemistry in our effort to optimize conditions for the synthesis of the disaccharide core. In particular, the levoglucosan route had shown the remarkable versatility of carbohydrate synthons. Based on the disappointing yields we obtained from the synthesis of glycosyl donors **161** and **162**, we have also proved the verity of our initial claim that the unique chemistry of aminosugars precludes any illusion that methods developed for neutral sugars will be equally effective for aminosugars. Thus, considering the prevalence of this motif in biologically important substrates, the necessity of improving the array of glycosylating agents for aminosugars is of particular importance.

3.8. Experimental Section

3.8.a. General (see Section 2.7, page 125)

3.8.b. Preparation of the Glycosyl Donors

Typical procedure for preparation of SBox glycosides from thioglycosides: The solution of the thioglycosides **136** and **139** (0.13 mmol) and activated molecular sieves 3Å (70 mg) in CH_2Cl_2 (2 mL) was stirred under argon for 1 h. A freshly prepared solution of Br_2 in CH_2Cl_2 (1.2 mL, 1/165, v/v) was then added and the reaction mixture

was kept for 5 min at rt. After that, CH_2Cl_2 was removed under reduced pressure at rt. The crude residue was then treated with KSBox (0.51 mmol) in dry acetonitrile (1 mL) under argon for 2 h at rt. Upon completion, the mixture was diluted with toluene, the solid was removed by filtration and the residue was washed with toluene. The combined filtrate (30 mL) was washed with 1% aq. NaOH (15 mL) and water (3x10 mL), the organic layer was separated, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (acetone-toluene gradient elution) to afford the SBox glycosides **140a** and **140b**.

Benzoxazolyl 3-O-acetyl-2-deoxy-4,6-O-benzylidene-2-phthalimido-1-thio-β-D-glucopyranoside (140a) was obtained as an amorphous solid in 27% yield. R_f = 0.69 (hexanes-ethyl acetate, 1/1, v/v); ^{1}H-n.m.r.: δ, 1.84 (s, 3H, C*H*$_3$CO), 3.77 (dd 1H, $J_{6a,6b}$ = 10.2 Hz, H-6a), 3.81 (dd, 1H, $J_{4,5}$ = 9.4 Hz, H-4), 3.90-3.95 (m, 1H, H-5), 4.40 (dd, 1H, $J_{5,6b}$ = 4.9 Hz, $J_{6a,6b}$ = 10.5 Hz, H-6b), 4.58 (dd, 1H, $J_{2,3}$ = 10.5 Hz, H-2), 5.50 (s, 1H, C*H*Ph), 5.98 (dd, 1H, $J_{3,4}$ = 9.4 Hz, H-3), 6.49 (d, 1H, $J_{1,2}$ = 10.7 Hz, H-1), 7.17-7.78 (m, 13H, aromatic) ppm, ^{13}C-n.m.r.: δ, 20.7, 54.2, 68.6, 70.5, 71.0, 79.0, 81.8, 102.0, 110.3, 119.4, 124.0, 124.7, 124.8, 126.5, 126.6, 128.5, 129.2, 129.4, 129.9, 134.7, 137.0, 141.7, 152.0, 159.9, 170.3 ppm; HR-FAB MS $[M+H]^+$ calcd for $C_{30}H_{24}N_2O_8SNa$ 595.1151, found 595.1149.

Benzoxazolyl 3-O-acetyl-2-deoxy-4,6-O-*p*-methoxybenzylidene-2-phthalimido-1-thio-β-D-glucopyranoside (140b) was obtained as an amorphous solid in 12% yield. ^{1}H-n.m.r.: δ, 2.25 (s, 3H, C*H*$_3$CO), 3.65-3.79 (m, 5H, H-4, H-6a, OC*H*$_3$), 4.20 (dd, 1H, $J_{5,6b}$ = 4.9 Hz, $J_{6a,6b}$ = 10.4 Hz, H-6b), 4.30 (dd, 1H, $J_{5,6a}$ = 5.0 Hz, $J_{4,5}$ = 9.7

Hz, H-5), 4.97 (dd, 1H, $J_{2,3}$ = 11.5 Hz, H-2), 5.43 (s, 1H, PMPC*H*), 6.48 (dd, 1H, $J_{3,4}$ = 9.4 Hz, H-3), 6.59 (d, 1H, $J_{1,2}$ = 5.7 Hz, H-1), 6.79 (d, 2H, J = 8.7 Hz, PMP), 7.03-7.77 (m, 13H, aromatic), ppm.

3.8.c. Preparation of Disaccharides

Typical AgOTf-promoted glycosylation procedure (activation of the SBox glycosides): A mixture the glycosyl donor **119** (0.11 mmol), glycosyl acceptors **142**, **144**, **153a**, or **153b** (0.10 mmol), and freshly activated molecular sieves (3Å, 200 mg) in $ClCH_2CH_2Cl$ (2 mL) was stirred under argon for 1.5 h. Freshly conditioned AgOTf (0.22 mmol) was added and the reaction mixture was stirred for 1-2 h at rt. The mixture was diluted with CH_2Cl_2, filtered to remove the solids, and the residue was washed with CH_2Cl_2. The combined filtrate (30 mL) was washed with 20% aq. $NaHCO_3$ (15 mL) and water (3 x 10 mL). The organic phase was separated, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (ethyl acetate - hexane gradient elution) to afford the disaccharide.

Phenyl O-(3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-(1→6)-3-O-acetyl-2-azido-4-O-benzyl-2-deoxy-1-thio-α-D-glucopyranoside (**156**) was obtained as an amorphous solid in 91% yield. ^{1}H-n.m.r.: 2.01, 2.04, 2.17, 2.20 (s, 12H, 4 x C*H*$_3$CO), 3.61 (t, 1H, $J_{4,5}$ = 9.5 Hz, H-4), 3.89-4.05 (m, 3H, H-2, H-5', H-6a'), 4.14 (dd, 1H, $^3J_{5',6b'}$ = 1.0 Hz, $^2J_{6a',6b'}$ = 10.8 Hz, H-6b'), 4.20 (d, 1H, ^2J = 10.7 Hz, PhCH_2^a), 4.27-4.33 (m, 1H, H-6a), 4.33 (d, 1H, ^2J = 10.7 Hz, PhCH_2^b), 4.40-4.49 (m, 2H, H-5, H-6b), 4.58 (dd, 1H, $J_{2',3'}$ = 10.5 Hz, H-2'), 5.31 (t, 1H, $J_{4',5'}$ = 9.6 Hz, H-4'), 5.36-5.46 (m, 1H, H-3), 5.60 (d, 1H, $J_{1',2'}$ = 8.5 Hz, H-1'), 5.65 (d, 1H, $J_{1,2}$ = 5.5 Hz, H-1),

5.95 (dd, 1H, $J_{3',4'}$ = 9.2 Hz, H-3'), 7.06-7.14 (m, 2H, aromatic), 7.27-7.49 (m, 6H, aromatic), 7.60 (dd, 2H, ^3J = 1.4 Hz, ^3J = 7.6 Hz, aromatic), 7.72-7.80 (m, 2H, aromatic), 7.82-7.92 (m, 2H, aromatic) ppm, ^{13}C-n.m.r.: δ, 20.4, 20.58, 20.64, 20.7, 54.4, 62.0, 62.1, 67.6, 69.0, 70.6, 72.1, 73.0, 74.3, 76.1, 86.9, 98.0, 123.5, 127.5, 127.6, 127.9, 128.2, 128.3, 129.0, 129.1, 131.1, 131.8, 133.3, 134.3, 137.0, 167.6 (× 2), 169.3, 169.4, 170.1, 170.6 ppm.

Ethyl O-(3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-(1→6)-3-O-acetyl-2-deoxy-2-phthalimido-1-thio-β-D-glucopyranoside (**157**) was obtained as an amorphous solid in 93% yield. ^{1}H-n.m.r.: δ, 0.96 (t, 3H, ^{3}J = 7.3 Hz, C*H*$_3$CH$_2$S), 1.75 (s, 3H, C*H*$_3$CO), 1.78 (s, 3H, C*H*$_3$CO), 1.95 (s, 3H, C*H*$_3$CO), 2.04 (s, 3H, C*H*$_3$CO), 2.20-2.48 (m, 2H, CH$_3$C*H*$_2$S), 3.13 (d, 1H, 3J = 6.4 Hz, O*H*), 3.29-3.44 (m, 1H, H-4), 3.63 - 3.77 (m, 2H, H-5, H-6a), 3.85 (m, 1H, H-5'), 4.04 (t, 1H, $^3J_{2,3}$ = 10.4 Hz, H-2), 4.08-4.19 (m, 2H, H-6b, H-6a'), 4.22-4.35 (m, 2H, H-6b', H-2'), 5.12 (dd, 1H, $^3J_{4,5}$ = 9.6 Hz, H-4'), 5.37 (d, 1H, $^3J_{1,2}$ = 10.5 Hz, H-1), 5.46 (d, 1H, $^3J_{1',2'}$ = 8.5 Hz, H-1'), 5.56 (dd, 1H, $^3J_{3,4}$ = 9.5 Hz, H-3), 5.73 (dd, 1H, $^3J_{3',4'}$ = 9.9 Hz, H-3'), 7.62-7.80 (m, 8 H, aromatic) ppm, ^{13}C-n.m.r.: δ, 14, 20.2, 20.4, 20.4, 20.5 (x 4), 23.7, 53.5, 54.3, 61.6, 68.6, 69.1, 69.9, 70.6, 71.7, 73.9, 78.7, 80.1, 98.2, 123.4, 130.8, 131.1, 131.2, 131.3, 134.0, 134.1, 134.3, 167.1, 167.5 (x 2), 169.3, 169.9, 170.5, 170.9 (x 4) ppm.

3.8.c. Preparation of Disaccharide Template

Ethyl O-(4,6-benzylidene-2-[(R)-3-benzyloxy-hexadecan]amido-3-O-[(R)-3-benzyloxyhexadecanoyl]-2-deoxy)-(1→6)-3-O-acetyl-2-[(R)-3-benzyloxy-hexadecan]amido-3-O-[(R)-3-benzyloxy-hexadecanoyl]-2-deoxy-1-thio-β-D-

glucopyranoside (**168**). To a stirred solution of **164** (6.0 mmol) in CH_2Cl_2 (30 mL) was added oxalyl chloride (6.0 mmol) and the reaction was stirred at 0°C under Argon. Catalytic amounts of DMF (0.006 mmol) were then added and the reaction was allowed to warm up to room temperature. After a TLC of the reaction showed complete conversion of the carboxylic acid to a higher running spot, the reaction mixture was evaporated to dryness under reduced pressure. The crude residue was then redissolved in CH_2Cl_2 (ca. 15 mL) and added to a stirred solution of the sugar (1 mmol) and DMAP (1 mmol) in CH_2Cl_2 (20 mL). The reaction mixture was stirred for 16 hours, diluted with CH_2Cl_2 (30 mL), and washed with the following: 0.5 N aq. $KHSO_4$ (2 x 20 mL), saturated aq. $NaHCO_3$ (20 mL), and brine (20 mL). The organic phase was separated, dried with anhydrous $MgSO_4$, and concentrated to dryness under reduced pressure. The residue was purified by column chromatography on silica gel (acetone - toluene gradient elution) to afford **168** as an amorphous solid in 13% yield. R_f = 0.38 (CH_2Cl_2-methanol, 9/1, v/v); ^{1}H-n.m.r.: δ, 0.76 - 0.87 (m, 12H, 4 × CH_3 lipid), 1.08-1.13 (m, 3H, C*H*$_3$CH$_2$S), 1.13-1.30 (m, 88H, 44 × CH_2 lipid), 1.32-1.58 (m, 8H, 4 × C*H*$_2$CHOBn), 2.19-2.30 (m, 4H, 2 × CH_2CO), 2.31-2.45 (m, 2H, 2 × CH$_2^a$CO), 2.50-2.63 (m, 4H, 2 × CH$_2^b$CO, CH$_3$C*H*$_2$S), 3.07-3.15 (m, 1H, H-5'), 3.29-3.36 (m, 2H, H-4', H-5), 3.45-3.52 (m, 1H, H-6a'), 3.61-3.74 (m, 6H, H-4, H-6a, 2 × CHOBn, PhCH$_2$), 3.78-3.90 (m, 3H, H-6b', PhCH$_2$), 3.91-4.01 (m, 3H, H-2, H-2', PhCH$_2^a$), 4.26 - 4.33 (m, 2H, H-6b, PhCH$_2^b$), 4.39-4.51 (m, 2H, PhCH$_2$), 4.67 (dd, 1H, $^3J_{3',4'}$= 8.9 Hz, H-3'), 5.12 (dd, 1H, $^3J_{3,4}$ = 10.0 Hz, H-3), 5.35 (s, 1H, PhCH), 6.13 (d, 1H, $^3J_{1',2'}$ = 9.0 Hz, H-1'), 6.30 (d, 1H, $^3J_{1,2}$ = 9.0 Hz, H-1), 7.14 - 7.32 (m, 25H, aromatic) ppm, ^{13}C-n.m.r.: δ, 14.1 (x 4), 14.9, 22.7 (x 4), 23.2, 25.1, 29.3, 29.7, 31.9, 33.9, 34.1, 34.4, 39.59, 39.62, 41.3, 41.5 (x 48), 51.8, 54.2, 66.5, 68.5, 69.0,

69.3, 69.7, 71.0, 71.7, 75.4, 75.7, 76.3 (x 14), 78.4, 78.6, 83.8, 101.3, 102.2, 126.0, 127.4, 127.5, 127.6, 127.7, 127.8, 128.1, 128.2, 128.3, 128.37, 128.42, 128.5, 128.6, 128.7, 128.79, 128.84, 128.9, 129.0, 136.7, 138.2, 138.3, 138.4, 138.6, 171.1, 171.4, 171.7, 172.1 (x 4) ppm. HR-FAB MS $[M+H]^+$ calcd for $C_{113}H_{176}N_2O_{16}SNa$ 1872.2638, found 1872.2593.

Ethyl O-(2-[(R)-3-benzyloxy-hexadecan]amido-3-O-[(R)-3-benzyloxy-hexadecanoyl]-2-deoxy)-(1→6)-3-O-acetyl-2-[(R)-3-benzyloxy-hexadecan]amido-3-O-[(R)-3-benzyloxy-hexadecanoyl]-2-deoxy-1-thio-β-D-glucopyranoside (**169**). To a solution of 168 (1 mmol) in wet CH_2Cl_2 (10 mL CH_2Cl_2, 0.1 mL H_2O) was added CF_3COOH dropwise, simultaneously monitoring the reaction progress by TLC upon each addition. Once TLC showed complete conversion of the starting material (ca. after 30 min), the reaction was diluted with CH_2Cl_2 (50 mL) and washed with the following: saturated aq. $NaHCO_3$ (3 x 20 mL), brine (20 mL). The organic phase was separated, dried with anhydrous $MgSO_4$, and concentrated to dryness under reduced pressure. The residue was purified by column chromatography on silica gel (acetone - toluene gradient elution) to afford **169** as an amorphous solid in 89% yield. R_f = 0.43 (CH_2Cl_2-methanol, 9/1, v/v). ^{1}H-n.m.r.: δ, 0.76 - 0.87 (m, 12H, 4 × C*H*$_3$ lipid), 1.08-1.13 (m, 3H, C*H*$_3$CH$_2$S), 1.13-1.30 (m, 88H, 44 × C*H*$_2$ lipid), 1.32-1.58 (m, 8H, 4 × C*H*$_2$CHOBn), 2.19-2.30 (m, 4H, 2 × C*H*$_2$CO), 2.31-2.45 (m, 2H, 2 × C*H*$_2^a$CO), 2.50-2.63 (m, 4H, 2 × C*H*$_2^b$CO, CH$_3$C*H*$_2$S), 3.07-3.15 (m, 1H, H-5'), 3.29-3.36 (m, 2H, H-4', H-5), 3.45-3.52 (m, 1H, H-6a'), 3.61-3.74 (m, 6H, H-4, H-6a, 2 × C*H*OBn, PhC*H*$_2$), 3.78-3.90 (m, 3H, H-6b', PhC*H*$_2$), 3.91-4.01 (m, 3H, H-2, H-2', PhC*H*$_2^a$), 4.26 - 4.33 (m, 2H, H-6b, PhC*H*$_2^b$), 4.39-4.51 (m, 2H, PhC*H*$_2$), 4.67 (dd, 1H, $^3J_{3',4'}$= 8.9 Hz, H-3'), 5.12 (dd, 1H, $^3J_{3,4}$ = 10.0 Hz,

H-3), 5.35 (s, 1H, PhC*H*), 6.13 (d, 1H, $^{3}J_{1',2'}$ = 9.0 Hz, H-1'), 6.30 (d, 1H, $^{3}J_{1,2}$ = 9.0 Hz, H-1), 7.14 - 7.32 (m, 25H, aromatic) ppm, ^{13}C-n.m.r.: δ, 14.1 (x 4), 14.9, 22.7 (x 4), 23.2, 25.1, 29.3, 29.7, 31.9, 33.9, 34.1, 34.4, 39.59, 39.62, 41.3, 41.5 (x 48), 51.8, 54.2, 66.5, 68.5, 69.0, 69.3, 69.7, 71.0, 71.7, 75.4, 75.7, 76.3 (x 14), 78.4, 78.6, 83.8, 101.3, 102.2, 126.0, 127.4, 127.5, 127.6, 127.7, 127.8, 128.1, 128.2, 128.3, 128.37, 128.42, 128.5, 128.6, 128.7, 128.79, 128.84, 128.9, 129.0, 136.7, 138.2, 138.3, 138.4, 138.6, 171.1, 171.4, 171.7, 172.1 (x 4) ppm. HR-FAB MS $[M+H]^+$ calcd for $C_{106}H_{172}N_2O_{16}SNa$ 1784.2325, found 1784.2288.

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Chapter 4

Synthesis of Monosaccharide Analogues

Bongat, A. F. G.; Demchenko, A. V., "Targeted design and synthesis of monosaccharide Lipid A analogues for potential application towards CD14-targeting therapeutics", in preparation.

4.1. Introduction

As mentioned in Chapter 1, the main goal of this dissertation is to design Lipid A analogues that will bind with high affinity to CD14. Consequently, since there are scarce examples of Lipid A analogs that lack the disaccharide core yet still maintain potent antagonistic activity, we felt the necessity to focus our efforts toward designing potent monosaccharide antagonists.

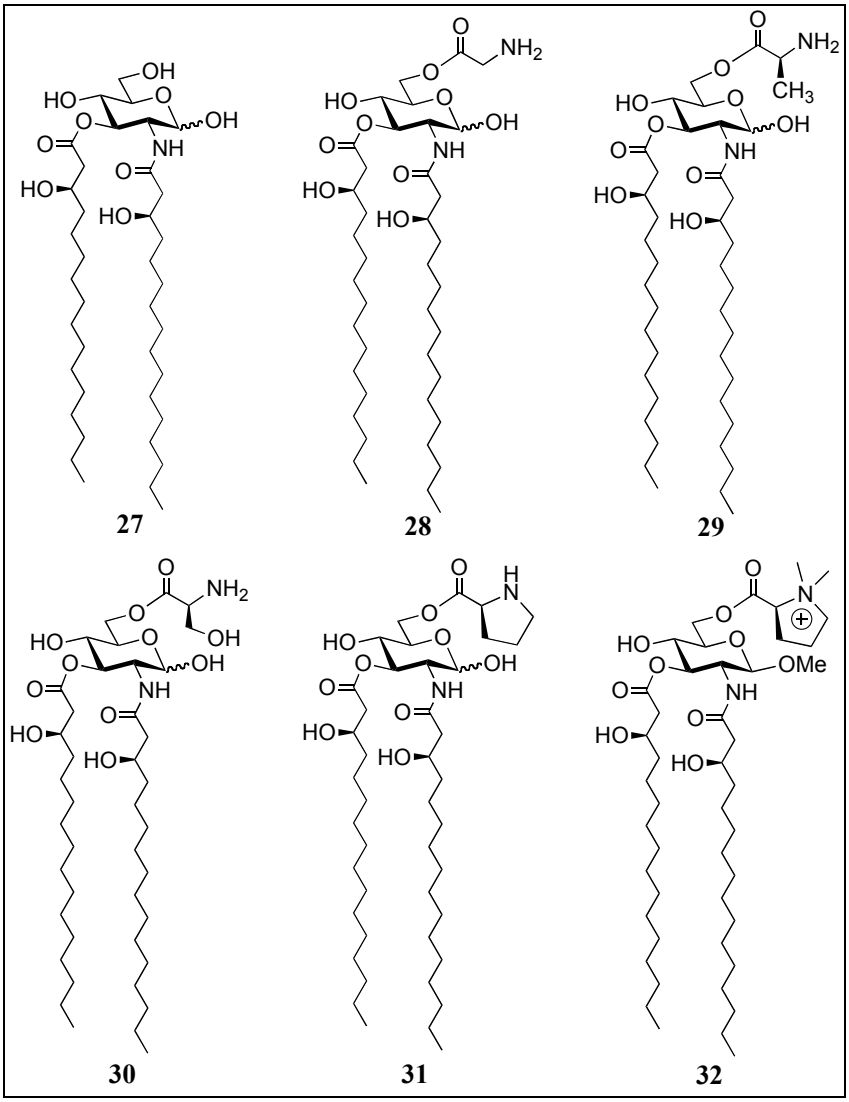


Figure 4.1. Targeted design for monosaccharide Lipid A analogues.

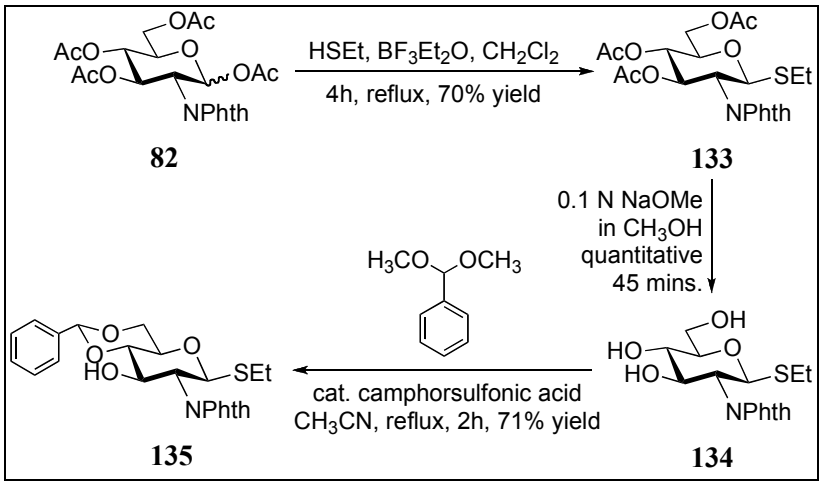
Our first consideration in designing our monosaccharide targets was the observation by Peri[1] and colleagues that compounds structurally unrelated to Lipid A can still exhibit potent antagonistic activity; for example, flavolipin and Vitamin D.[2,3] Following their findings, our natural course of action should have been to develop other glucose-derived ammonium species containing hydrophobic alkyl chains, in place of the natural ester- and amide-linked acyl chains. Instead, we found ourselves interested in pursuing a structural lead that: 1) preserves the native glucosamine residue and the ester- and amide-linked (R)-3-hydroxy fatty acid chains, 2) lacks any unstable phosphate residue, and 3) contains a cationic residue on C-6. Basically, we wanted to explore the ramifications of Peri's idea on a structure that is analogous to Lipid X (**1**, see Figure 1.5 in Chapter 1). In addition, Fukase *et al*.'s approach of utilizing amino acids to provide the cationic character also seems quite fitting to include in our design.[4,5] Therefore, our goal is to synthesize compounds **27**-**32** (Figure 4.1) and evaluate these molecules for possible antagonism towards LPS-induced proinflammatory activity.

As seen above, compounds **27**-**32** all contain a glucosamine residue and amide- (C-2) and ester-linked (C-3) (R)-3-hydroxy fatty acids. The basic difference between the analogs we designed is the type of amino acid installed on C-6. Target **27**, which has a C-6 hydroxyl is synthesized as a negative control – in essence, we expect that this compound will not induce nor antagonize an LPS-induced release of pro-inflammatory cytokines *in vitro*. Monosaccharide analogs **28**-**30**, on the other hand, are designed to contain ester linked amino acid moieities namely Glycine, Alanine, and Serine, respectively. We expect that following the natural tendency of amino acids to adopt a zwitterionic form under physiological conditions, the free amine on these moieties will be

protonated (NH_3^+) and provide a cationic character to our analogs. Finally, for analogs **31** and **32**, we chose to install a proline moiety on C-6 since its heterocyclic motif closely resembles the C-6 moiety on Peri's most promising monosaccharide antagonist. In contrast to Peri's design, we reasoned that an ester-linked proline moiety will be more stable in the conditions required for the biological assays. Moreover, in order to observe the effect of a fully cationic character, we also decided to form an ammonium species by methylating proline as per Peri's published procedure. To eliminate any intramolecular destabilization of the salt by proton exchange, the anomeric hydroxyl on analog **32** was protected as a methyl glycoside.

4.2. Synthesis of the Differently Protected Glucosamine Core

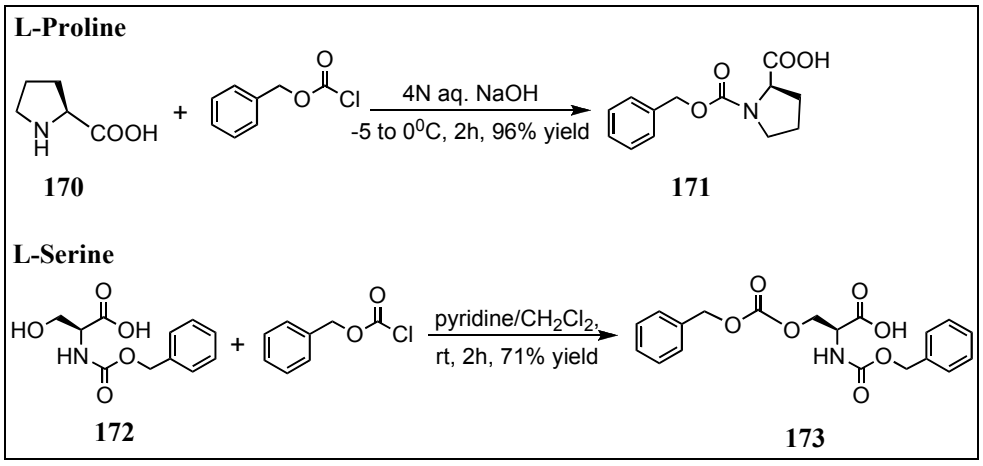
In order to synthesize our monosaccharide analogs, we first needed to obtain a differently protected glucosamine building block. Henceforth referred to as the glucosamine core, this precursor should contain a protecting group pattern that will allow us to selectively deprotect and functionalize key positions of the molecule into the desired configuration. Consequently, the synthesis of the glucosamine core began with the conversion of 1,3,4,6-tetra-O-acetyl-2-deoxy-2-phthalimido-α,β-D-glucopyranose (**82**) into thioglycoside **133** (Scheme 3.1). As described earlier, deacetylation of thioglycoside **133** under Zemplen conditions, followed by protection of the 4,6-diol in **134** as a benzylidene acetal gave **132** in 71 % yield.



Scheme 4.1. Synthesis of the glucosamine core.

4.3. Derivatization of the Amino Acids

As mentioned earlier, our plan is to install amino acids on the C-6 hydroxyl of the glucosamine core anticipating that these moieties will provide the cationic character to our analogs. Since amino acids contain multiple functionalities, we needed to obtain derivatives where the carboxylic function is free and the rest of the functionalities are masked. To this end, we found that N-(benzyloxycarbonyl)glycine and N-(benzyloxycarbonyl-L-alanine are commercially available. In contrast, N-(benzyloxy-carbonyl)-(S)-proline **171** and 2-(benzyloxy-carbonyl-amino)-3-(benzyloxycarbonyloxy)-propanoic acid **173** had to be synthesized. To this end, compound **171**[6] was synthesized from L-proline **170** by reaction with benzyl chloroformate in 2M aqueous sodium hydroxide at 0-5°C, in 96% yield (Scheme 4.2.).[7] On the other hand, reaction of commercially available N-(benzyloxycarbonyl)-L-serine **172** with benzyl chloroformate, in pyridine at room temperature, gave compound **173** in 71% yield.[8]

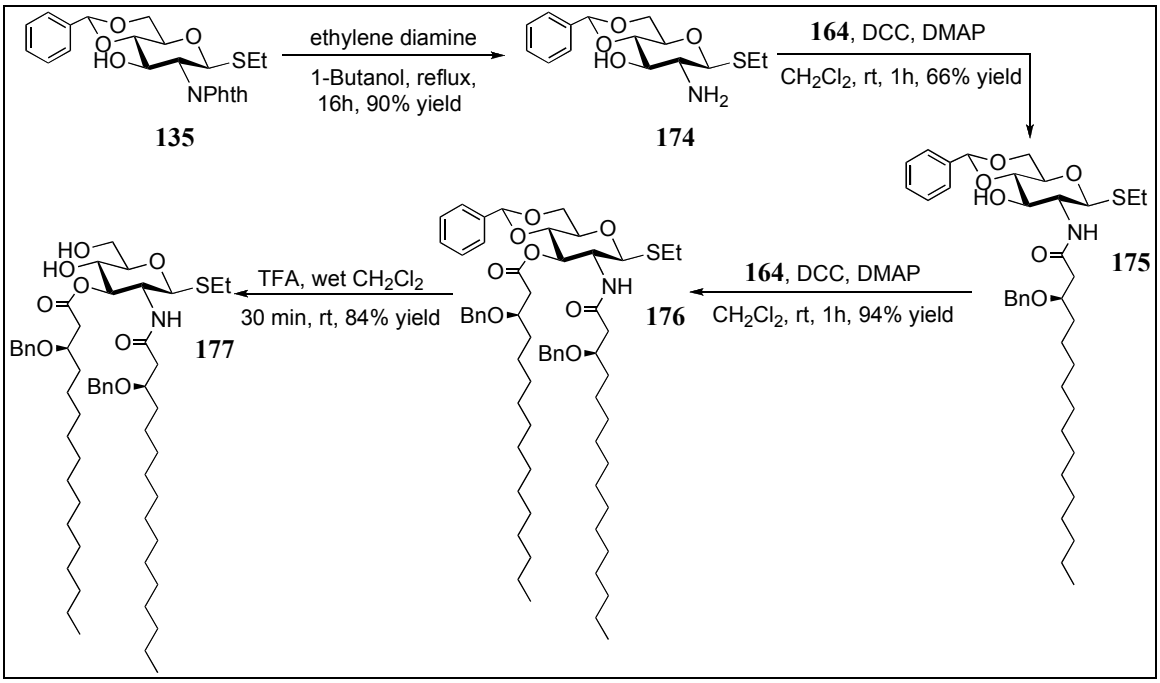


Scheme 4.2. Derivatization of L-serine and L-proline.

4.4. Synthesis of the 2,3-Lipidated Monosaccharide Template

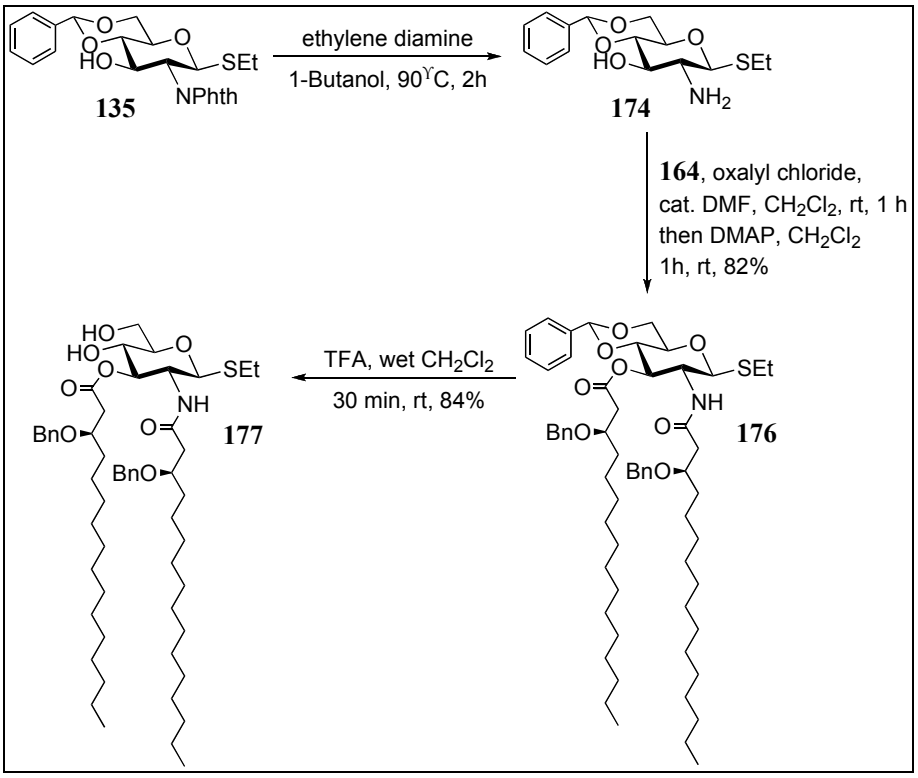
The synthesis of the monosaccharide template, suitable for the introduction of amino acid residues, began with the removal of the phthalimido protecting group in compound **135**. Thus, treatment of **135** with ethylene diamine in refluxing 1-butanol overnight gave intermediate **174** in 90% yield (see Scheme 4.3).[9] Selective N-acylation of intermediate **174** with (R)-3-benzyloxy-hexadecanoic acid **164** was then achieved via dicyclohexylcarbodiimide (DCC)-mediated coupling, in the presence of 4-dimethylaminopyridine (DMAP) in CH_2Cl_2.

Consequently, we found that the optimum formation of the "activated" ester, O-acylisourea, can be achieved after leaving **164** to stir overnight in the presence of DCC and DMAP in CH_2Cl_2. Addition of a solution of **174** to this pre-activated reagent gave **175** in 66% yield after 1 hour. It should be noted that our attempts to perform O- and N-acylation in one step proved to be extremely inefficient. Thus, while N-acylation was achieved in 30 minutes to an hour, attempts to O-acylate by adding more reagents were extremely futile.



Scheme 4.3. Synthesis of monosaccharide template via DCC-mediated coupling.

Between the weak reactivity of the C-3 hydroxyl of amino sugars and the competing rearrangement of O-acylisourea to the more stable N-acylurea, it became more effective to perform the reaction in two steps with chromatographic separation in between. Then, following the same overnight pre-activation procedure of **164**, as established for the synthesis of **175**, O-acylation of intermediate **175** gave **176** in 94% yield. Finally, the monosaccharide template **177**, suitably configured for subsequent conjugation with amino acid building blocks, was obtained in 84% yield by treatment of **176** with trifluoroacetic acid in wet CH_2Cl_2.



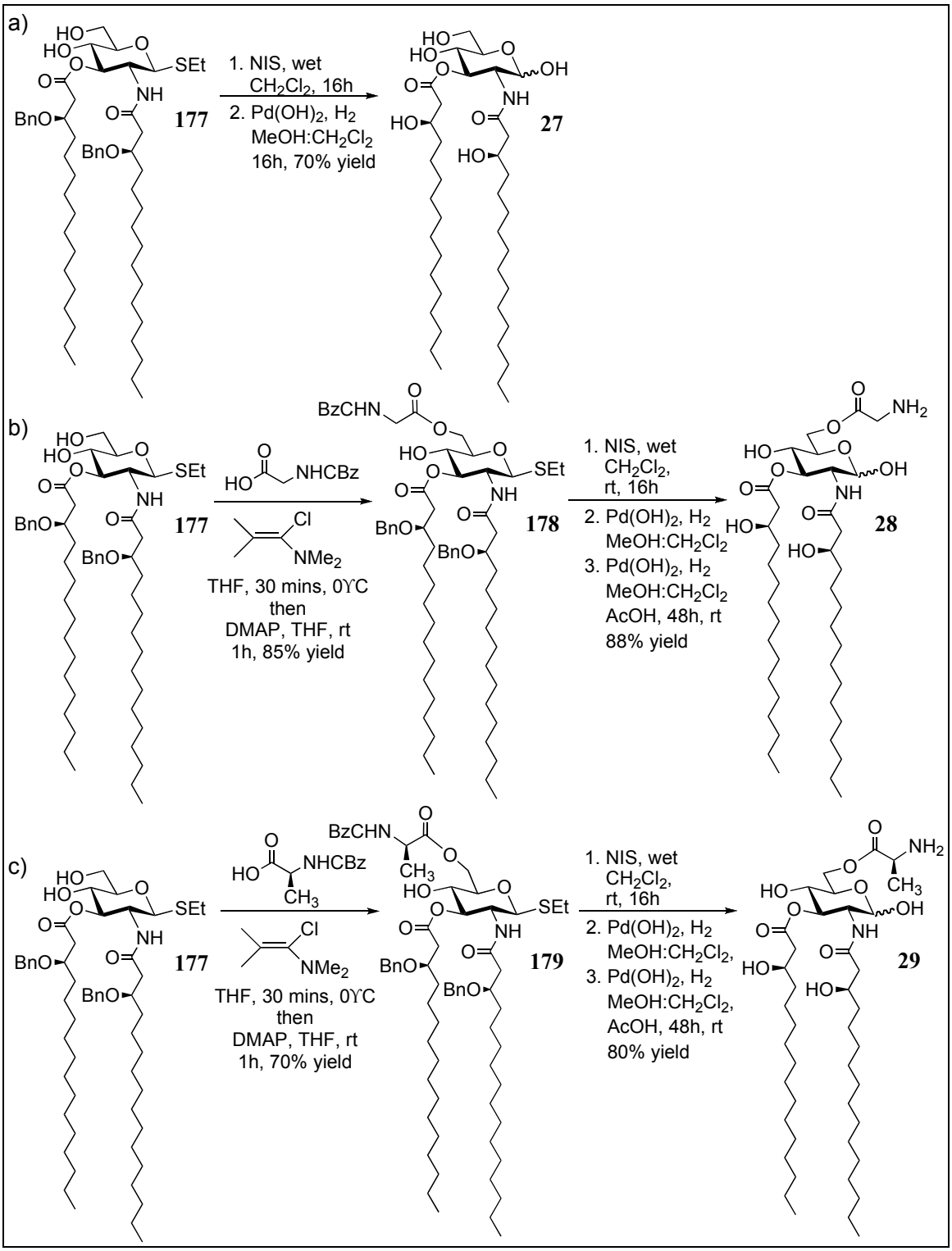
Scheme 4.4. Synthesis of monosaccharide template via acyl chloride.

The inefficiency of performing N- and O-acylation simultaneously via DCC-mediated coupling in the presence of DMAP, along with the inherent difficulty of separating the byproduct N,N'-dicyclohexylurea from the product, led us to explore other avenues by which the fatty acids can be more effectively installed. To this end, we found that *in situ* conversion of **164** into an acyl chloride provides a more efficient reaction intermediate with which to perform the N- and O-acylation concomitantly. Formation of the acyl chloride was quickly achieved by reacting **164** with oxalyl chloride, in the presence of catalytic DMF and CH_2Cl_2.[10] After evaporating the excess reagent off, the acyl chloride was redissolved in CH_2Cl_2 and added to a solution of **174** and DMAP in CH_2Cl_2 to give **176** in 82% yield. Finally, the desired monosaccharide template **177** was obtained as described above in Scheme 4.4.

4.5. Synthesis of the Monosaccharide Amino Acid Conjugates

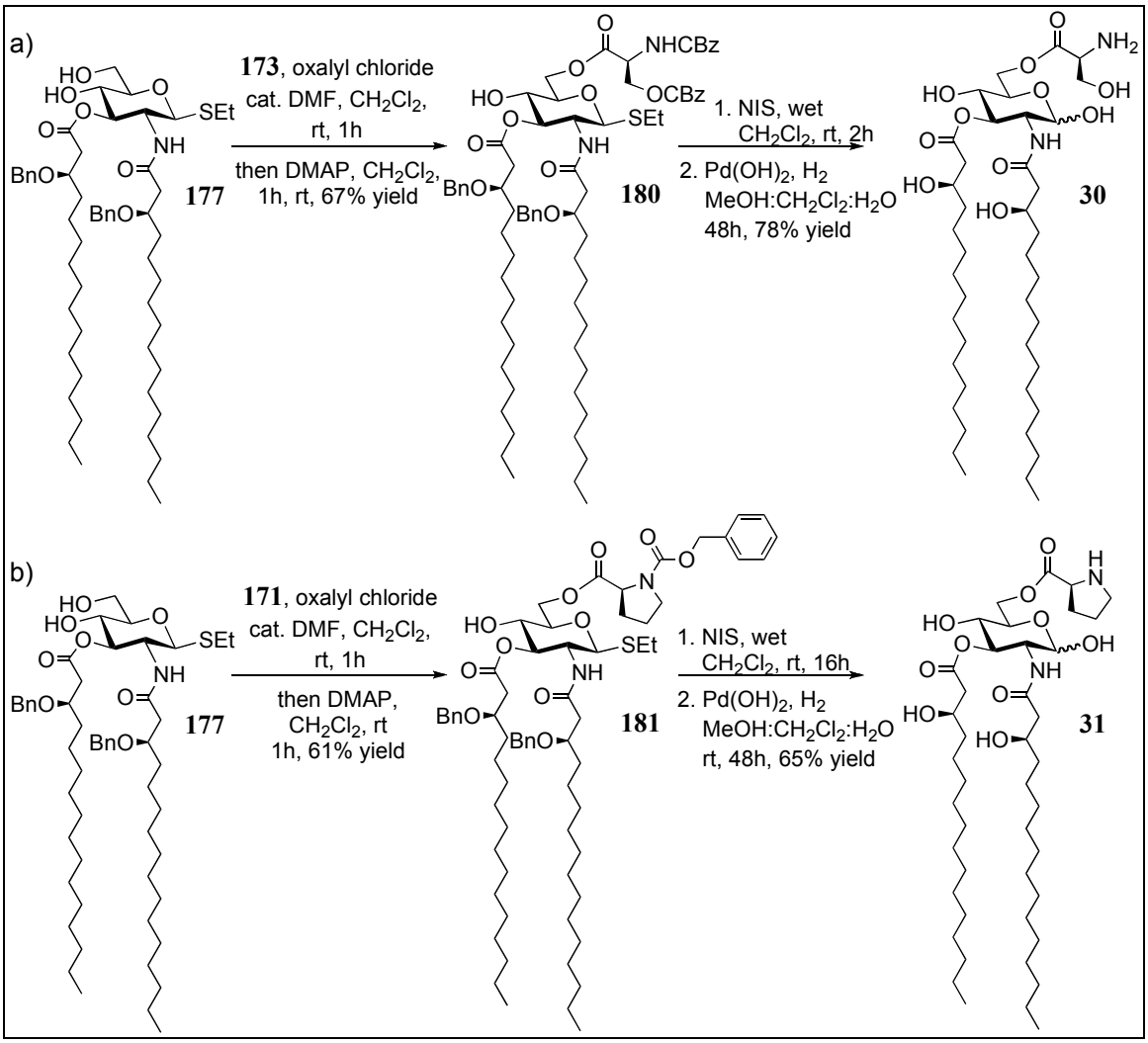
To provide a basis for comparison (anticipated negative control) and to determine any residual toxicity of the monosaccharide template, we conducted the synthesis of analogue **24**. Thus hydrolysis of the thioglycoside[11] in **177** was performed in the presence of NIS in wet CH_2Cl_2. This step was immediately followed by removal of the benzyl protecting groups via catalytic hydrogenolysis in the presence of $Pd(OH)_2$ on charcoal (Pearlman's catalyst),[12] to give **27** in 70% yield over two steps (Scheme 4.5a).

On the other hand, attempts to install the amino acid moiety on C-6 of **177** via DCC-mediated coupling were ineffective in our hands. Instead, we found that commercially available N-(benzyloxy-carbonyl)glycine can be converted to an acyl chloride by reacting the corresponding carboxylic acid with 1-chloro-*N,N*,2-trimethyl-1-propen-1-amine[13] using a procedure described by Schmidt *et al*.[14] The solution of the amino acid chloride in THF was then added to a stirred solution of **177** and DMAP in THF and subsequently, compound **178** was formed in 85% yield after 1 hour (Scheme 4.5b). While hydrolysis of the thioglycoside in **178** proceeded quantitatively in the presence of NIS and wet CH_2Cl_2, deprotection of the benzyloxycarbonyl (CBz) and benzyl protecting groups proved to be quite tricky. Thus, we found that after the CBz moiety has been removed, the resulting free amine poisons Pearlman's catalyst and makes it very unreactive.[15,16] This problem was solved by adding a fresh batch of catalyst and 0.1 mol % of AcOH.[15] As a result, analogue **28** was obtained in 88% yield after stirring the reaction mixture under H_2 for 2 days.



Scheme 4.5. Synthesis of monosaccharide analogues **27**-**29**.

Similarly, acylation of **177** with N-(benzyloxycarbonyl)-L-alanine was accomplished using 1-chloro-*N,N*,2-trimethyl-1-propen-1-amine[13] to give **179** in 70% yield (Scheme 4.5). After performing the same thioglycoside hydrolysis and deprotection sequence described for analogue **28**, the monosaccharide analogue **29** was obtained in 80% yield.



Scheme 4.6. Synthesis of monosaccharide analogues **30** and **31**.

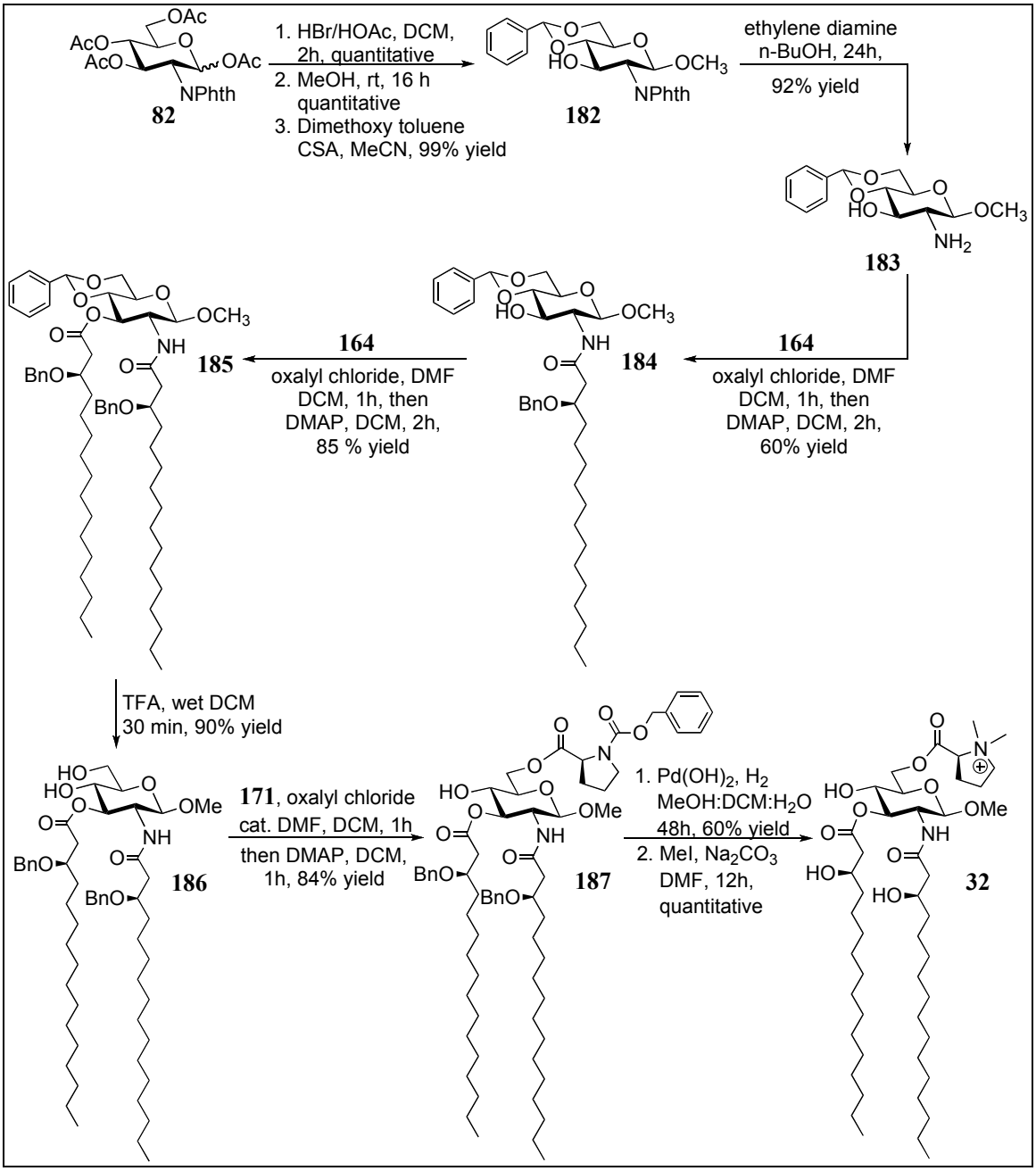
While the amino acid chloride method by Schmidt and colleagues[14] has worked well in synthesis of **178** and **179**, it was practically ineffective in converting derivatives

171 and **173** (see Scheme 4.2) into the corresponding acid chloride. In this case, *in situ* formation of the α-chloroenamine via reaction of **171** or **173** with oxalyl chloride and catalytic DMF provided a more effective solution to this problem.[17] After the corresponding acid chlorides of **171** and **173** were formed, the excess reagents were evaporated off and the crude residue was redissolved in CH_2Cl_2. These solutions were then added to a stirred solution **177** and DMAP in CH_2Cl_2 to give **181** and **180** in 61% and 67% yield, respectively (Scheme 4.6). Moreover, while hydrolysis of thioglycosides **178, 179** and **181** took 16 hours to complete (NIS, wet CH_2Cl_2), the same reaction set-up with **180** was complete in 2 hours.

With regards to the final step, the cleavage of the CBz and benzyl groups by catalytic hydrogenolysis, we found that the amino acid moiety in **180** is more acid labile than that of **178** and **179** and tends to hydrolyse in a slightly acidic medium. Fortunately, the addition of water to the solvent mixture of MeOH:CH_2Cl_2 (1:1) proved to be effective in this case. Thus, catalytic hydrogenation with Pearlman's catalyst was performed in a solvent mixture of MeOH:CH_2Cl_2:H_2O (2:2:0.2) for 2 days to give **30** in 78% yield.[15] Similarly, since the proline moiety on **181** remains a secondary amine after CBz deprotection and is thereby not as poisonous to the catalyst, the hydrogenation conditions used the synthesis of **30** was also effective in the synthesis of analog **31** and gave the later in 65% yield.

At this point, since proline closely resembles the C-6 moiety on Peri's most promising monosaccharide analog[1] (see **12**, Figure 1.6), we found ourselves interested in synthesizing the ammonium counterpart of **32**. Subsequently, we realized that the close proximity of a hemiacetal to the ammonium moiety may be lead to the collapse of the salt

via proton exchange. Thus, following Peri's lead, we decided to protect the anomeric hydroxyl as a methyl glycoside. The synthesis of compound **32** began with the three-step conversion of **82** to **182** in 99% yield (Scheme 4.7), as described by Kochetkov et al.[18]



Scheme 4.7. Synthesis of analogue **32**.

Cleavage of the phthalimido group in **182** was then performed using ethylene diamine in butanol to give compound **183** in 92% yield.[9] However, despite the *in situ* conversion of the fatty acid **164** to the corresponding acyl chloride, a two step acylation sequence also proved to be necessary in this case. Hence, as described earlier, fatty acid **164** was converted into an acyl chloride using oxalyl chloride and catalytic DMF and then added to a stirred solution of **183** and DMAP in CH_2Cl_2 to give **184** in 60% yield.[10] After repeating the same acylation sequence on **184**, bis-acylated compound **185** was obtained in 85% yield. Removal of the benzylidene acetal was accomplished through the slow addition of TFA to a stirred solution of **185** in wet CH_2Cl_2 to give **186** in 90% yield.

With compound **186** in hand, the proline moiety was installed as described for the synthesis of **181** (see Scheme 4.6) and gave **187** in 84% yield (Scheme 4.7). Following a the deprotection of the CBz and benzyl groups via catalytic hydrogenolysis, and reaction with methyl iodide in the presence of sodium carbonate, the ammonium analogue **32** was finally obtained in 60% yield.

4.6. Summary and Conclusions

The compounds synthesized above represents an innovative approach to the targeted design of monosaccharide Lipid A analogues. Expanding on Peri's hypothesis that a cationic moiety is responsible for the surprising anti-endotoxic activity of **12**, we designed and synthesized analogs that possess the configuration of Lipid X and contain an amino acid moiety on C-6. We were convinced that the observed tendency of amino acids to adopt a charge at physiological pH will furnish the cationic character to our compounds. Moreover, should any of these compounds show antagonistic activity towards LPS-induced proinflammatory activity and be possibly considered as a drug candidate, clearance from the body after administration should not be an issue due to the fact that all of the components in our design are native to the human body.

On the other hand, the syntheses described herein illustrate novel applications of well-established reactions. Thus, we showed the usefulness of the *in situ* conversion of carboxylic acids to acid chlorides – using oxalyl chloride or commercially available α-chloroenamines – in esterification and amidation reactions involving sugar substrates. We have also demonstrated ways by which to circumvent problems associated with the presence of free amines during catalytic hydrogenolysis. Finally, we have provided an interesting insight on the determination of the enantiomeric purity of methyl (R)-3-hydroxyhexadecanoate using chiral shift reagents. While the latter approach has been previously described, the relevant details are initially presented herein.

The compounds synthesized herein are currently being evaluated for antagonistic activity by our collaborators.

4.7. Experimental Section

4.7.a. General. See Section 2.7, page 125.

4.7.b. Preparation of the Amino Acid Derivatives

2-(Benzyloxycarbonylamino)-3-(benzyloxycarbonyloxy)propanoic acid (173). The preparation of **173** was adapted from a published procedure by Ramesh and colleagues.[8] Thus, N-benzyloxycarbonyl-L-serine (**172**, 1 mmol) was dissolved in freshly distilled CH_2Cl_2 (5 mL) and the solution was cooled to 0°C. After stirring for 5 minutes, pyridine (2.5 mmol, 0.2 mL) was added dropwise. The solution was allowed to stir for another 5 minutes and then 1.1 mmol of benzylchloroformate was added over a period of 30 minutes. The reaction was allowed to warm up to room tempearature and stirred for 2 hours. Once a TLC of the reaction mixture showed no changes between starting material and product ratios, the crude reaction mixture was diluted with CH_2Cl_2 (50 mL). The CH_2Cl_2 extract was then washed twice with citric acid solution (20 mL), once with water (20 mL) and finally with brine (20 mL). The organic phase was separated, dried over anhydrous $MgSO_4$, and concentrated to dryness under reduced pressure. The crude residue was purified by column chromatography on silica gel (ethyl acetate – hexane gradient elution) to afford **173** as a colorless oil (0.27 g, 71 %). HR-FAB MS $[M+H]^+$ calcd for $C_{18}H_{19}NO_5Na$ 352.1163, found 352.1159.

4.7.c. General Acylation Protocols

Method A. *Typical procedure for DCC-mediated acylation:* The carboxylic acid (4 mmol), 4-dimethylaminopyridine (4 mmol) and *N,N′*-Dicyclohexyl-Carbodiimide (4

mmol) was stirred in CH_2Cl_2 (10 mL) overnight under argon for 16h at room temperature. Subsequently, this reaction mixture was added to solution of the monosaccharide derivatives (1 mmol) in CH_2Cl_2 (10 mL). The resulting mixture was stirred under Argon for 1 hour at room temperature then diluted with CH_2Cl_2 (50 mL) and washed with the following: 5% aq. oxalic acid (2 x 20mL), saturated aq. $NaHCO_3$ (2 x 20mL), brine (20 mL). The organic phase was separated, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (ethyl acetate - toluene gradient elution) to afford the acylated product.

Method B. *Typical procedure for acylation using oxalyl chloride:* To a stirred solution of the carboxylic acid (2 mmol) in CH_2Cl_2 (10 mL) was added oxalyl chloride (2 mmol) and the reaction mixture was stirred for 5 minutes at 0°C under Argon. A catalytic amount of DMF (0.002 mmol) was then added and the reaction was allowed to warm to rt and kept for an additional 55 minutes. The volatile components were then evaporated to dryness under reduced pressure. The crude residue was redissolved in CH_2Cl_2 (ca. 5 mL) and added to a stirred solution of the monosaccharide derivative (1 mmol) and DMAP (1 mmol) in CH_2Cl_2 (10 mL). After stirring the reaction mixture under Argon for 1 hour, the reaction was diluted with CH_2Cl_2 (30 mL) and washed with the following: 0.5 N aq. $KHSO_4$ (2 x 15 mL), saturated aq. $NaHCO_3$ (15 mL), and brine (15 mL). The organic phase was separated, dried with anhydrous $MgSO_4$, and concentrated to dryness. The residue was purified by column chromatography on silica gel (ethyl acetate - toluene gradient elution) to afford the acylated product.

Method C. *Typical procedure for acylation via α-chloroenamines:* To a stirred solution of the amino acid (2 mmol) in CH_2Cl_2 (10 mL) was added 1-chloro-N,N,2-trimethyl-1-propen-1-amine (2.2 mmol) and the reaction was stirred at 0°C under Argon for 30 minutes. The reaction was then warmed up to room temperature and added to a stirred solution of the monosaccharide derivative (1 mmol) and DMAP (1 mmol) in CH_2Cl_2 (10 mL). The resulting reaction mixture was stirred at rt for 1 hour, under Argon. After that, it was diluted with CH_2Cl_2 (30 mL) and washed with the following: 0.5 N aq. $KHSO_4$ (2 x 15 mL), saturated aq. $NaHCO_3$ (15 mL), and brine (15 mL). The organic phase was separated, dried with anhydrous $MgSO_4$, and concentrated to dryness under reduced pressure. The residue was purified by column chromatography on silica gel (ethyl acetate - toluene gradient elution) to afford the amino acid ester.

Ethyl 4,6-benzylidene-2-[(R)-3-benzyloxy-hexadecan]amido-2-deoxy-1-thio-β-D-glucopyranoside (175) was obtained by Method A as an amorphous solid in 66% yield**.** ^{1}H-n.m.r.: δ, 0.80 (t, 3H, J = 6.4 Hz, CH_3 lipid), 1.12 (t, 3H, J = 7.5 Hz, CH_3CH$_2$S), 1.17 (s, 12H, 11 × CH_2 lipid), 1.43-1.69 (m, 2H, CH_2CHOBn), 2.37 (dd, 1H, ^3J = 7.0 Hz, ^2J = 15.3 Hz, CH_2^aCO), 2.43-2.60 (m, 3H, CH_2^bCO, CH$_3$CH_2S), 3.25-3.37 (m, 1H, H-4), 3.47 (dd, 1H, $J_{5,6a}$ = 9.0 Hz, H-5), 3.60-3.82 (m, 3H, H-2, H-6a, CHOBn), 4.23 (dd, 1H, $J_{4,5}$ = 9.5 Hz, H-4), 4.32 (dd, 1H, $J_{6a,6b}$ = 9.6 Hz, H-6b), 4.46 (d, 1H, ^2J = 11.3 Hz, PhCH_2^a), 4.55 (d, 1H, ^2J = 11.3 Hz, PhCH_2^b), 5.45 (s, 1H, PhCH), 6.69 (d, 1H, $J_{1,2}$ = 6.6 Hz, H-1), 7.22- 7.32 (m, 8H, aromatic), 7.38-7.43 (m, 2H, aromatic) ppm, ^{13}C-n.m.r.: δ, 14.1, 14.8, 22.7, 23.9, 24.9, 25.1, 25.6, 29.3, 29.5, 29.6, 29.6, 31.9, 33.5, 33.9, 40.9, 49.1, 56.2, 68.5, 70.4, 71.1, 73.5, 81.4, 83.8, 101.8, 126.3, 127.6, 127.9, 128.2, 128.6, 129.1, 137.0, 138.0, 172.7 ppm;

Ethyl 4,6-benzylidene-2-[(R)-3-benzyloxy-hexadecan]amido-3-O-[(R)-3-benzyloxy-hexadecanoyl]-2-deoxy-1-thio-β-D-glucopyranoside (146) was obtained as an amorphous solid from **175** by Method A in 94% yield and from **174** by Method B in 82% yield. R_f = 0.35 (hexanes-ethyl acetate, 4/1, v/v); (c = 1.0, $CHCl_3$); ^{1}H-n.m.r.: δ, 0.80 (t, 6H, ^3J = 6.4 Hz, 2 × C*H*$_3$ lipid), 1.15-1.21 (m, 44H, 22 × C*H*$_2$ lipid), 1.31-1.54 (m, 4 H, 2 × C*H*$_2$CHOBn), 2.26 (d, 2H, ^3J = 5.5 Hz, C*H*$_2$CO), 2.37 (dd, 1H, ^3J = 5.6 Hz, ^2J = 15.0 Hz, C*H*$_2^a$CO), 2.60 (dd, 1H, ^3J = 6.3 Hz, ^2J = 15.0 Hz, C*H*$_2^b$CO), 3.22 (s, 3H, OC*H*$_3$), 3.34-3.43 (m, 1H, H-5), 3.57 (dd, 1H, $J_{4,5}$ = 9.4 Hz, H-4), 3.63-3.77 (m, 3H, H-6a, 2 × C*H*OBn), 3.98 (dd, 1H, $J_{2,3}$ = 9.1 Hz, H-2), 4.14 (d, 1H, ^3J = 8.3 Hz, NH), 4.22 (dd, 1H, $J_{5,6b}$ = 4.8 Hz, $J_{6a,6b}$ = 10.5 Hz, H-6b), 4.30 (d, 1H, ^2J = 11.9 Hz, PhC*H*$_2^a$), 4.41 (d, 2H, ^2J = 12.2 Hz, PhC*H*$_2^{a'}$, PhC*H*$_2^{b'}$), 4.49 (d, 1H, ^2J = 11.9 Hz, PhC*H*$_2^b$), 5.25 (dd, 1H, $J_{3,4}$ = 9.4 Hz, H-3), 5.33 (s, 1H, PhC*H*), 6.42 (d, 1H, $J_{1,2}$ = 9.1 Hz, H-1), 7.15-7.32 (m, 15H, aromatic) ppm, ^{13}C-n.m.r.: δ, 14.1 (x 2), 14.6, 22.6 (x 2), 23.8, 25.0, 25.1, 29.5 (x 2), 29.6 (x 4), 31.9, 33.9, 34.4, 39.6, 41.2 (x 24), 52.7, 68.5, 70.6, 71.0, 72.6, 73.0, 75.4, 76.4 (x 5), 78.7, 85.0, 101.2, 126.0, 127.3, 127.5, 127.6, 127.7, 128.1, 128.2, 128.4, 128.5, 128.6, 136.7, 138.4 (x 2), 171.3 (x 2) ppm;

Ethyl 6-O-[N-(benzyloxycarbonyl)glycine]-2-[(R)-3-benzyloxy-hexadecan]-amido-3-O-[(R)-3-benzyloxy-hexadecanoyl]-2-deoxy-1-thio-β-D-glucopyranoside (178) was obtained as an amorphous solid from **177** by Method C in 85% yield. R_f = 0.60 (toluene-acetone, 4/1, v/v); ^{1}H-n.m.r.: δ, 0.81 (t, 6 H, *J* = 6.2 Hz, 2 × C*H*$_3$ lipid), 1.08-1.13 (m, 3H, C*H*$_3$CH$_2$S), 1.14-1.27 (m, 44H, 22 × C*H*$_2$ lipid), 1.37-1.59 (m, 4H, 2 × C*H*$_2$CHOBn), 2.29 (d, 2H, *J* = 5.1 Hz, C*H*$_2$CO), 2.37-2.45 (m, 1H, C*H*$_2^a$CO), 2.46-2.64 (m, 3H, C*H*$_2^b$CO, CH3C*H*$_2$S), 2.89 (d, 1H, *J* = 3.2 Hz, O*H*), 3.25-3.49 (m, 2H, H-4, H-5),

3.65-3.75 (m, 1H, C*H*OBn), 3.76-3.84 (m, 1H, C*H*OBn), 3.85- 4.03 (m, 3H, H-2, CH_2 Gly), 4.18-4.28 (m, 2H, H-6a, H-6b), 4.38-4.49 (m, 3H, PhCH_2^a, PhCH_2), 4.56 (d, 1H, J = 11.7 Hz, PhCH_2^b), 4.80 (dd, 1H, $J_{3,4}$ = 8.9 Hz, H-3), 6.32 (d, 1H, $J_{1,2}$ = 8.5 Hz, H-1), 7.12-7.42 (m, 15H, aromatic) ppm.

Ethyl 6-O-[N-(benzyloxycarbonyl)-L-alanine]-2-[(R)-3-benzyloxyhexadecan]-amido-3-O-[(R)-3-benzyloxy-hexadecanoyl]-2-deoxy-1-thio-β-D-glucopyranoside (179) was obtained as an amorphous solid from **177** by Method C in 70% yield. R_f = 0.37 (toluene-acetone, 4/1, v/v); (c = 1.0, $CHCl_3$); ^{1}H-n.m.r.: δ, 0.77-0.85 (m, 6H, 2 × CH_3 lipid), 1.07-1.14 (m, 3H, CH_3CH$_2$S), 1.14-1.24 (m, 44H, 22 × CH_2 lipid), 1.26 (d, 3H, ^3J = 7.0 Hz, CH_3 Ala), 1.31-1.61 (m, 4H, 2 × CH_2CHOBn), 2.25-2.33 (m, 2H, CH_2CO), 2.40 (dd, 1H, ^3J = 4.8 Hz, ^2J = 14.8 Hz, CH_2^aCO), 2.46-2.68 (m, 3H, CH$_3$CH_2S, CH_2^bCO), 3.27-3.39 (m, 1H, H-4), 3.65-3.83 (m, 2H, PhCH_2), 3.85-4.04 (m, 1H, H-2), 4.25-4.35 (m, 2H, H-5, PhCH_2^a), 4.40-4.46 (m, 2H, 2 × C*H*OBn), 4.52-4.66 (m, 2H, C*H* Ala, PhCH_2^b), 4.77-4.84 (m, 1H, H-3), 5.01-5.04 (m, 3H, PhCH_2, H-6a), 5.23 (dd, 1 H, $^3J_{5,6}$ = 1.8 Hz, $^2J_{6a,6b}$ = 4.4 Hz, H-6b), 5.72-5.78 (m, 1H, N*H*), 6.34 (d, 1 H, $^3J_{1,2}$ = 9.2 Hz, H-1), 7.21-7.32 (m, 15H, aromatic) ppm, ^{13}C-n.m.r.: δ, 14.1 (x 2), 14.8, 18.9, 22.7 (x 2), 24.0, 25.1, 29.4, 29.6, 29.65, 29.69, 31.9, 33.8, 34.0, 35.7, 36.9 (x 24), 46.8, 49.7, 52.1, 64.3, 66.7, 67.0, 69.1, 71.0 (x 2), 75.9 (x 8), 84.4, 127.6, 127.7, 127.8, 127.9 (x 2), 128.0, 128.1, 128.2, 128.4, 128.5 (x 2), 128.6, 136.1, 136.5, 138.3, 155.5, 171.3, 172.2, 172.9 ppm;

Ethyl 6-O-[2-(benzyloxycarbonylamino)-3-(benzyloxycarbonyloxy)]propanoyl-2-[(R)-3-benzyloxy-hexadecan]amido-3-O-[(R)-3-benzyloxy-hexadecanoyl]-

2-deoxy-1-thio-β-D-glucopyranoside (180) was obtained as an amorphous solid from **177** by Method B in 60% yield. ^{1}H-n.m.r.: δ, 0.81 (t, 6H, ^{3}J = 6.6 Hz, 2 × CH_3 lipid), 1.10 (t, 3H, ^{3}J = 7.4 Hz, CH_3CH$_2$S), 1.14-1.32 (m, 44H, 22 × CH_2 lipid), 1.35-1.61 (m, 4H, 2 × CH_2CHOBn), 2.26-2.33 (m, 2H, CH_2CO), 2.37-2.45 (m, 1H, CH_2^{a}CO), 2.49-2.60 (m, 3H, CH$_3$CH_2S, CH_2^{b}CO), 3.18-3.24 (m, 1H, H-5), 3.50 (dd, 1H, $^{3}J_{3,4}$ = ^{3}J$_{4,5}$ = 9.5 Hz, H-4), 3.58-3.84 (m, 8H, H-6a, H-6b, CH_2 Ser, 2 × CHOBn, PhCH_2), 3.84-4.00 (m, 2H, H-2, PhCH_2^{a}), 4.08 (d, 1H, ^{2}J = 10.4 Hz, PhCH_2^{b}), 4.35-4.40 (m, 1H, CH Ser), 4.41-4.45 (m, 2H, PhCH_2), 4.83 (dd, 1H, $^{3}J_{2,3}$ = $^{3}J_{3,4}$ = 9.5 Hz, H-3), 5.01-5.10 (m, 2H, PhCH_2), 5.74-5.83 (m, 1H, NH), 6.41 (d, $^{3}J_{1,2}$ = 9.4 Hz, 1H, H-1), 7.20-7.39 (m, 20H, aromatic) ppm, ^{13}C-n.m.r.: δ, 14.1 (x 2), 14.7, 22.6 (x 2), 23.9, 25.1, 29.3, 29.58, 29.61, 29.63, 29.65, 29.67, 31.9, 33.9, 39.6, 41.2 (x 24), 52.2, 52.7, 62.5, 67.1 (x 2), 67.4, 69.5, 71.0, 71.0, 75.9, 76.5, 77.1, 79.3 (x 7), 84.3, 127.6, 127.7, 127.8, 128.0, 127.98, 128.06, 128.1, 128.2, 128.4, 128.50, 128.52, 128.6, 135.1, 136.1, 137.8, 138.3, 156.2, 170.4, 171.0, 171.4, 172.2 ppm; HR-FAB MS $[M+H]^{+}$ calcd for $C_{54}H_{89}NO_8SNa$ 934.6207, found 934.6237.

Ethyl 6-O-[N-benzyloxycarbonyl-L-proline]-2-[(R)-3-benzyloxyhexa-decan]-amido-3-O-[(R)-3-benzyloxy-hexadecanoyl]-2-deoxy-1-thio-β-D-glucopyranoside (181) was obtained as an amorphous solid from **177** by Method B in 60% yield. R_f = 0.64 (toluene-acetone, 4/1, v/v); ^{1}H-n.m.r.: δ, 0.78-0.85 (m, 6H, 2 × CH_3 lipid), 1.12 (t, 3H, ^{3}J = 7.5 Hz, CH_3CH_2S), 1.15-1.29 (m, 44H, 22 × CH_2 lipid), 1.34-1.56 (m, 4H, 2 × CH_2CHOBn), 1.56-1.64 (m, 2H, CH_2CH$_2$N), 2.02 (m, 2H, ^{3}J = 6.6 Hz, CH_2CHN), 2.24-2.34 (m, 2H, CH_2CO), 2.37-2.47 (m, 1H, CH_2^{a}CO), 2.47-2.66 (m, 3H, CH$_3$CH_2S, CH_2^{b}CO), 2.88 (d, 1 H, ^{3}J = 3.6 Hz, OH), 3.15-3.26 (m, 1H, H-5), 3.47 (dd, 1 H, $^{3}J_{3,4}$ =

9.5 Hz, $^{3}J_{4,5}$ = 3.3 Hz, H-4), 3.55-3.83 (m, 6H, H-6a, H-6b, 2 × C*H*OBn, C*H*N, PhCH_2^{a}), 3.84-3.99 (m, 3H, H-2, CH_2N), 4.00-4.08 (m, 1H, PhCH_2^{b}), 4.38-4.49 (m, 3H, PhC$H_2^{a'}$, PhCH_2), 4.52 - 4.61 (m, 1H, PhC$H_2^{b'}$), 4.73 - 4.86 (m, 1H, H-3), 6.35 (d, 1 H, $^{3}J_{1,2}$ = 9.2 Hz, H-1), 7.14 - 7.39 (m, 15H, aromatic) ppm, ^{13}C-n.m.r.: δ, 14.1 (x 2), 14.8, 18.9, 22.7 (x 2), 24.0, 25.1, 29.4, 29.6, 29.65, 29.69, 31.9, 33.8, 34.0, 35.7, 36.9 (x 24), 46.8, 49.7, 52.1, 64.3, 66.7, 67.0, 69.1, 71.0 (x 2), 75.9 (x 8), 84.4, 127.6, 127.7, 127.8, 127.9 (x 2), 128.0, 128.1, 128.2, 128.4, 128.5 (x 2), 128.6, 136.1, 136.5, 138.3, 155.5, 171.3, 172.2, 172.9 ppm; HR-FAB MS $[M+H]^+$ calcd for $C_{67}H_{102}N_2O_{11}Na$ 1165.7102, found 1165.7144.

Methyl 4,6-benzylidene-2-[(R)-3-benzyloxy-hexadecan]amido-2-deoxy-β-D-glucopyranoside (184) was obtained from **183** by Method B as an amorphous solid in 60% yield**.** R_f = 0.59 (toluene-acetone, 7/3, v/v); $[\alpha]_D^{25}$ 32.8º (c = 1.0, $CHCl_3$); ^{1}H-n.m.r.: δ, 0.81 (t, 3H, ^{3}J = 6.3 Hz, CH_3 lipid), 1.09-1.30 (m, 22H, 11 × CH_2 lipid), 1.30-1.67 (m, 2H, CH_2CHOBn), 2.36 (dd, 1H, ^{3}J = 7.0 Hz, ^{2}J = 15.1 Hz, CH_2^{a}CO), 2.48 (dd, 1H, ^{3}J = 3.6 Hz, ^{2}J = 15.1 Hz, CH_2^{b}CO), 3.26 (s, 3H, OCH_3), 3.31-3.40 (m, 1H, H-5), 3.40-3.53 (m, 2H, H-2, H-4), 3.64-3.81 (m, 2H, H-6a, C*H*OBn), 3.91 (dd, 1H, $^{3}J_{3,4}$ = 9.1 Hz, H-3), 4.25 (dd, 1H, $^{3}J_{5,6b}$ = 4.8 Hz, $^{2}J_{6a,6b}$ = 10.3 Hz, H-6b), 4.34 (d, 1H, ^{3}J = 8.3 Hz, NH), 4.45 (d, 1H, ^{2}J = 11.3 Hz, PhCH_2^{a}), 4.52 (d, 1H, ^{2}J = 11.3 Hz, PhCH_2^{b}), 5.47 (s, 1H, PhC*H*), 6.72 (d, 1H, ^{3}J1,2 = 5.8 Hz, H-1), 7.05 - 7.35 (m, 8H, aromatic), 7.35 - 7.48 (m, 2H, aromatic) ppm, ^{13}C-n.m.r.: δ, 14.1, 22.7, 25.2, 29.3, 29.5, 29.58, 29.61, 29.64, 31.9, 33.6, 41.1, 56.8, 58.6, 66.2, 68.6, 71.2, 71.7, 81.5, 101.7, 101.8, 126.3, 127.7, 127.9, 128.2, 128.5, 129.1, 137.1, 138.0, 173.2 ppm; HR-FAB MS $[M+H]^+$ calcd for $C_{37}H_{56}NO_7$ 626.4057, found 626.4058.

Methyl 4,6-benzylidene-2-[(R)-3-benzyloxy-hexadecan]amido-3-O-[(R)-3-benzyloxy-hexadecanoyl]-2-deoxy-β-D-glucopyranoside (185) was obtained as an amorphous solid from **154** by Method B in 85% yield. ^{1}H-n.m.r.: δ, 0.80 (t, 6H, ^{3}J = 6.4 Hz, 2 × CH_3 lipid), 1.15-1.21 (m, 44H, 22 × CH_2 lipid), 1.31-1.54 (m, 4 H, 2 × CH_2CHOBn), 2.26 (d, 2H, ^{3}J = 5.5 Hz, CH_2CO), 2.37 (dd, 1H, ^{3}J = 5.6 Hz, ^{2}J = 15.0 Hz, CH_2^{a}CO), 2.60 (dd, 1H, ^{3}J = 6.3 Hz, ^{2}J = 15.0 Hz, CH_2^{b}CO), 3.22 (s, 3H, OCH_3), 3.34-3.43 (m, 1H, H-5), 3.57 (dd, 1H, $^{3}J_{4,5}$ = 9.4 Hz, H-4), 3.63-3.77 (m, 3H, H-6a, 2 × C*H*OBn), 3.98 (dd, 1H, $^{3}J_{2,3}$ = 9.1 Hz, H-2), 4.14 (d, 1H, ^{3}J = 8.3 Hz, N*H*), 4.22 (dd, 1H, ^{3}J = 4.8 Hz, ^{2}J = 10.5 Hz, H-6b), 4.30 (d, 1H, ^{2}J = 11.9 Hz, PhCH_2^{a}), 4.41 (d, 2H, ^{2}J = 12.2 Hz, PhCH_2^{a}, PhCH_2^{b}), 4.49 (d, 1H, ^{2}J = 11.9 Hz, PhCH_2^{b}), 5.25 (dd, 1H, $^{3}J_{3,4}$ = 9.4 Hz, H-3), 5.33 (s, 1H, PhC*H*), 6.42 (d, 1H, ^{3}J = 9.1 Hz, H-1), 7.15 - 7.32 (m, 15H, aromatic) ppm, ^{13}C-n.m.r.: δ, 14.0, 22.6, 25.1, 25.1, 29.3, 29.56, 29.59, 31.8, 34.1, 34.3, 39.5, 41.7, 53.9, 56.7, 66.0, 68.5, 70.9, 71.1, 71.7, 75.4, 76.2, 78.9, 101.1, 102.6, 125.9, 127.3, 127.49, 127.52, 128.0, 128.1, 128.3, 128.7, 128.8, 136.8, 138.39, 138.42, 171.4, 171.5 ppm;

3.8.b. Deprotection Protocols

Method A. *Typical procedure for benzylidene deprotection***:** To a solution of starting material (1 mmol) in wet CH_2Cl_2 (CH_2Cl_2/H_2O, 100/1, v/v) was added CF_3COOH dropwise, simultaneously monitoring the progress of the reaction by TLC upon each addition. Once TLC showed complete conversion of the starting material (ca. after 30 min) after the addition of ca. 0.20 mL TFA, the reaction was diluted with CH_2Cl_2 (50 mL) and washed with 20 mL portions of the following: thrice with saturated aq. $NaHCO_3$, brine. The organic phase was separated, dried over anhydrous $MgSO_4$, and

concentrated to dryness under reduced pressure. The residue was purified by column chromatography on silica gel (ethyl acetate - toluene gradient elution) to afford the 4,6-diol derivative.

Method B. *Typical procedure for thioglycoside hydrolysis:* To a solution of the sugar (0.1 mmol) in CH_2Cl_2 (5 mL) was added N-iodosuccinimide (0.2 mmol) and H_2O (2 mmol) and the reaction was stirred at rt from a period of 2 hours to 16 hours. After that, the reaction mixture was diluted with CH_2Cl_2 (25 mL) and washed with the following: 0.1 N aq. $Na_2S_2O_3$ (2 x 10 mL), saturated aq. $NaHCO_3$ (10 mL), brine (10 mL). The organic phase was separated, dried over $MgSO_4$, and concentrated to dryness under reduced pressure to afford the corresponding hemiacetal.

Method C. *Typical procedure for catalytic hydrogenolysis under neutral, anhydrous conditions:* To a solution of the sugar (0.1 mmol) in MeOH: CH_2Cl_2 (4 mL, 1/1, v/v) was added Pearlman's catalyst ($Pd(OH)_2$, 0.02 mmol) and the reaction mixture was stirred under Argon for 30 minutes at room temperature. The argon was removed under vacuum and the reaction mixture was flushed repeatedly flushed with hydrogen under atmospheric pressure and stirred for 16 hours to 2 days at rt. After that, the reaction mixture was diluted with a solution of CH_2Cl_2:MeOH:H_2O (4.2 mL; 1/1/0.1, v/v), stirred for 30 minutes, and filtered over a pad of Florisil®. After washing the pad of florisil repeatedly with the CH_2Cl_2:MeOH:H_2O solvent system (20 mL), the combined filtrates were concentrated under reduced pressure to afford **24**.

Method D. *Typical procedure for catalytic hydrogenolysis under neutral, hydrous conditions:* To a solution of the sugar (0.1 mmol) in MeOH:CH_2Cl_2:H_2O (4.2 mL,

1/1/0.1, v/v) was added Pearlman's catalyst ($Pd(OH)_2$, 0.02 mmol) and the reaction mixture was stirred for 30 minutes under Argon at room temperature. The argon was removed under vacuum and the reaction mixture was flushed repeatedly flushed with hydrogen under atmospheric pressure and stirred for 16 hours to 2 days at rt. After that, the reaction mixture was diluted with a solution of CH_2Cl_2:MeOH:H_2O (4.2 mL; 1/1/0.1, v/v), stirred for 30 minutes, and filtered over a pad of Florisil®. After washing the pad of florisil repeatedly with the CH_2Cl_2:MeOH:H_2O solvent system (20 mL), the combined filtrates were concentrated under reduced pressure to afford **25** and **26**.

Method E. *Typical procedure for catalytic hydrogenolysis under slightly acidic conditions:* To a solution of the sugar (0.1 mmol) in MeOH:CH_2Cl_2 (4 mL, 1/1, v/v) was added Pearlman's catalyst ($Pd(OH)_2$, 0.02 mmol) and CH_3COOH (10 μL). the reaction mixture was stirred for 30 minutes under Argon at room temperature. The argon was removed under vacuum and the reaction mixture was flushed repeatedly flushed with hydrogen under atmospheric pressure and stirred for 16 hours at rt. After that, the reaction mixture was reaction mixture was neutralized with Et_3N, diluted with a solution of CH_2Cl_2:MeOH:H_2O (4.2 mL; 1/1/0.1, v/v), stirred for 30 minutes, and filtered over a pad of Florisil®. After washing the pad of florisil repeatedly with the CH_2Cl_2:MeOH:H_2O solvent system (20 mL), the combined filtrates were concentrated under reduced pressure to afford **27** and **28**.

Ethyl 2-[(R)-3-benzyloxy-hexadecan]amido-3-O-[(R)-3-benzyloxy-hexadeca-noyl]-2-deoxy-1-thio-β-D-glucopyranoside (177) was prepared from **176** using Method A and obtained as an amorphous solid in 84% yield**.** R_f = 0.52 (toluene-acetone, 7/3, v/v); ^{1}H-n.m.r.: δ, 0.78 - 0.83 (m, 6H, 2 × CH_3 lipid), 1.11 (t, 3H, ^3J = 7.4 Hz, CH_3CH$_2$S),

1.15 - 1.23 (m, 44H, 22 × CH_2 lipid), 1.43 - 1.64 (m, 4H, 2 × CH_2CHOBn), 2.29 (d, 2H, ^3J = 5.5 Hz, CH_2CO), 2.42 (dd, 1H, ^3J = 4.9 Hz, ^2J = 14.7 Hz, CH_2^aCO), 2.48 - 2.60 (m, 3H, CH_2^bCO, CH_3CH_2S), 3.03 (s, 1H, O*H*), 3.20 (dd, 1H, $^3J_{4,5}$ = 9.4 Hz, $^3J_{5,6}$ = 4.5 Hz, H-5), 3.48 (dd, 1H, $^3J_{3,4}$ = $^3J_{4,5}$ = 9.1 Hz, H-4), 3.58 - 3.67 (m, 1H, H-6a), 3.67 - 3.75 (m, 2H, H-6b, C*H*OBn), 3.76 - 3.87 (m, 1H, C*H*OBn), 3.89 - 3.98 (m, 1H, H-2), 4.04 (m, 1H, O*H*), 4.38 - 4.48 (m, 3H, PhCH_2, PhCH_2^a), 4.56 (d, 1H, ^2J = 11.7 Hz, PhCH_2^b), 4.80 (dd, 1H, $^3J_{2,3}$ = $^3J_{3,4}$ = 9.5 Hz, H-3), 6.37 (d, 1H, $^3J_{1,2}$ = 9.2 Hz, H-1), 7.20 - 7.34 (m, 10H, aromatic) ppm, ^{13}C-n.m.r.: δ, 14.1 (x 2), 14.7, 22.7 (x 2), 24.0, 25.1, 29.4, 29.59, 29.61, 29.65, 29.67, 29.70, 30.9, 31.9, 33.8 (x 2), 39.7, 41.2 (x 24), 52.0, 62.7, 69.7, 70.9, 71.0 (x 2), 75.9, 77.2, 79.2 (x 2), 84.5, 127.6, 127.8, 127.9, 128.0, 128.4, 128.6, 137.8, 138.3, 171.3, 172.3 ppm; HR-FAB MS $[M+H]^+$ calcd for $C_{54}H_{90}NO_8S$ 912.6387, found 912.6400.

Methyl 2-[(R)-3-benzyloxy-hexadecan]amido-3-O-[(R)-3-benzyloxy-hexadecanoyl]-2-deoxy-β-D-glucopyranoside (156) was prepared from **155** using Method A and obtained as an amorphous solid in 90% yield**.** R_f = 0.41 (toluene-acetone, 7/3, v/v); ^{1}H-n.m.r.: δ, 0.81 (t, 6H, ^3J = 6.6 Hz, 2 × CH_3 lipid), 1.14- 1.27 (m, 44H, 22 × CH_2 lipid), 1.33-1.61 (m, 4H, 2 × CH_2CHOBn), 2.25-2.31 (m, 2H, CH_2CO), 2.36-2.44 (m, 1H, CH_2^aCO), 2.50-2.59 (m, 1H, CH_2^bCO), 3.21-3.26 (m, 1H, H-5), 3.28 (s, 3H, OCH_3), 3.54 (dd, 1H, $^3J_{3,4}$ = $^3J_{4,5}$ = 9.2 Hz, H-4), 3.63-3.75 (m, 3H, H-6a, H-6b, C*H*OBn), 3.76-3.87 (m, 3H, C*H*OBn, H-2, O*H*), 4.14 (d, 1H, ^3J = 8.3 Hz, N*H*), 4.38-4.46 (m, 3H, PhCH_2, PhCH_2^a), 4.51 (d, 1H, 2J = 11.5 Hz, PhCH_2^b), 4.94 (dd, 1H, $^3J_{2,3}$ = $^3J_{3,4}$ = 9.5 Hz, H-3), 6.32 (d, 1H, $^3J_{1,2}$ = 9.0 Hz, H-1), 7.17-7.36 (m, 10H, aromatic) ppm, ^{13}C-n.m.r.: δ, 14.1 (x 2), 22.7, 25.1, 25.2, 29.3, 29.6 (x 2), 29.7 (x 3), 30.9, 31.9, 33.9, 39.6, 41.5 (x 26), 53.5,

56.6, 62.3, 69.6, 71.1 (x 3), 75.1, 75.9 (x 3), 76.4, 102.0, 127.6, 127.7, 127.8, 128.0, 128.4, 128.5, 137.8, 138.3, 172.4 (x 2) ppm.

3.9. References:

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