Synthesis and Immunological Evaluation of Oligosaccharide-Antigens as Vaccine Candidates for *Streptococcus pneumoniae* Serotypes 1 and 8

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Quidquid agis,
prudenter agas
et respice finem.

List of Publications

A) Scientific Publications and Reviews

1. Synthesis of conjugation-ready zwitterionic oligosaccharides by chemoselective thioglycoside activation. B. Schumann, R. Pragani, C. Anish, C. L. Pereira and P. H. Seeberger, *Chem. Sci.*, 2014, **5**, 1992-2002.

2. Chemical biology approaches to designing defined carbohydrate vaccines. C. Anish,* B. Schumann,* C. L. Pereira and P. H. Seeberger, *Chem. Biol.*, 2014, **21**, 38-50.

3. Carbohydrate Vaccines. B. Schumann, C. Anish, C. L. Pereira and P. H. Seeberger, in *Biotherapeutics: Recent Developments Using Chemical and Molecular Biology, RSC Drug Discovery Series No. 36*, eds. L. Jones and A. J. McKnight, RSC Publishing, Cambridge, 2013, ch. 3, pp. 68-104.

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B) Patents

1. Synthetic vaccines against Streptococcus pneumoniae. B. Schumann**,** C. Anish, C. L. Pereira, P. H. Seeberger, European Patent Application No. EP 13 196 568.3, 2013.

2. Synthetic vaccines against Streptococcus pneumoniae serotype 8. B. Schumann, S. G. Parameswarappa, H. S. Hahm, S. Govindan, C. Anish, C. L. Pereira, P. H. Seeberger, European Patent Application No. EP 14 186 597.2.

C) Scientific Conferences and Symposia

1. FEBS EMBO 2014 Conference, Paris, France (2014): "Elucidation of immune recognition of Zwitterionic Polysaccharides by synthetic oligosaccharides" (Poster).

2. International Carbohydrate Symposium, Bangalore, India (2014): "Synthesis of conjugation-ready oligosaccharides to study immunomodulation by Zwitterionic Polysaccharides" (Oral presentation).

3. International Glycan Forum, Berlin, Deutschland (2013): "Synthesis of oligosaccharides as tools to study the intracellular processing of Zwitterionic Polysaccharides" (Poster).

4. EMBO Conference Series: Chemical Biology, Heidelberg, Deutschland (2012): "Synthesis of oligosaccharides as tools to study the intracellular processing of Zwitterionic Polysaccharides" (Poster).

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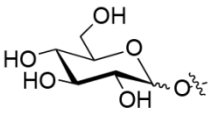
List of Abbreviations

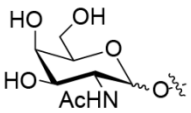
D-AAT	2-Acetamido-4-amino-2,4,6-trideoxy-D-galactose
APC	Antigen-presenting cell
BCR	B cell receptor
BOM	Benzyloxymethyl
BSA	Bovine serum albumin
CCV	Carbohydrate conjugate vaccine
CD	Cluster of differentiation
CDR	Complementarity determining region
CFA	Complete Freund's adjuvant
cfu	Colony-forming units
CPS	Capsular polysaccharide
CRM	Cross-reactive material
Da	Dalton
DIC	Differential interference contrast
DIPEA	*N*,*N*-Diisopropylethylamine
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DMTST	Dimethyl (methylthio) sulfonium trifluoromethanesulfonate
DSAP	Di-*N*-succinimidyl adipate
DTT	1,4-Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
ESI MS	Electrospray ionization mass spectrometry
equiv.	Equivalents
FELASA	Federation of European Laboratory Animal Science Associations
FITC	Fluorescein isothiocyanate
FS	Frameshift
HPLC	High performance liquid chromatography
HRP	Horseraddish peroxidase
IgM/IgG	Immunoglobulin M/G
IL	Interleukin
IFN	Interferon
iNKT	Invariant natural killer T cells
i.p.	Intraperitoneally
IPD	Invasive pneumococcal disease
IR	Infrared
Lev	Levulinoate
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MALDI-TOF MS	Matrix-assisted laser desorption/ionization with time-of-flight detection mass spectrometry
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex

NaPi	Sodium phosphate buffer
NHS	*N*-hydroxysuccinimide
NIS	*N*-iodosuccinimide
NMR	Nuclear magnetic resonance
NOESY	Nuclear Overhauser effect spectroscopy
OD	Optical density
OPKA	Opsonophagocytic killing assay
PBS	Phosphate-buffered saline
PCV	Pneumococcal conjugate vaccine
PMB	*p*-Methoxybenzyl
PSV	Polysaccharide vaccine
QC	Quality control
RU	Response units
SBAP	*N*-succinimidyl 3-(2-bromoacetamido)propionoate
s.c.	Subcutaneous
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPR	Surface plasmon resonance
ST1/3/8...	*Streptococcus pneumoniae* serotype 1/3/8...
TBAF	Tetra-n-butylammonium fluoride
TBS	*tert*-Butyldimethylsylil
TCEP	Tris(2-carboxyethyl)phosphine
TCR	T cell receptor
TDS	Thexyldimethylsilyl
TEMPO	(2,2,6,6-Tetramethylpiperidin-1-yl)oxyl
TES	Triethylsilane
TFA	Trifluoroacetic acid
Tf	Trifluoromethanesulfonate
THF	tetrahydrofuran
TLR	Toll-like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TMS	Trimethylsilyl
TrBS	Tris buffered saline
TrBS-T	Tris buffered saline with 0.1% Tween-20
*p*Ts	*p*-Toluolsulfonate
TTBPy	2,4,6-tri-*t*-butylpyridine
ZPS	Zwitterionic Polysaccharide

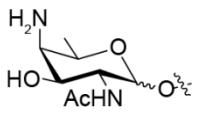
List of Monosaccharides



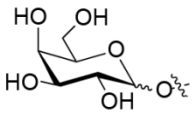
D-Glucose
(D-Glc)



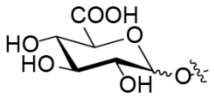
D-*N*-Acetylgalactosamine
(D-GalNAc)



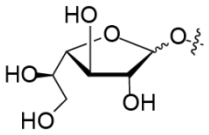
2-Acetamido-4-amino-
2,4,6-trideoxy-D-galactose
(D-AAT)



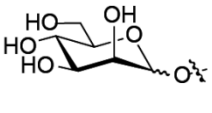
D-Galactose
(D-Gal)



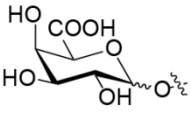
D-Glucuronic acid
(D-GlcA)



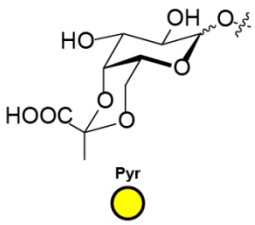
D-Galactofuranose
(D-Gal*f*)



D-Mannose
(D-Man)



D-Galacturonic acid
(D-GalA)



D-Galactose-4,6-pyruvate
(D-Gal-4,6-pyr.)

Summary

Carbohydrate-based vaccines have successfully prevented infectious diseases in the last decades. To date, several vaccines based on isolated capsular polysaccharides (CPSs) are marketed against pathogenic bacteria such as *Streptococcus pneumoniae*. The manufacture of these vaccines still suffers from shortfalls associated with the isolation of CPSs from natural sources. Furthermore, limited protectiveness of certain CPS antigens hampers vaccine efficiency. Synthetic, structurally defined oligosaccharides present an important alternative, with great potential to understand glycan immunology and rationally engineer efficacious antigens. To that end, strategies are needed to facilitate the streamlined design and generation of these antigens. Novel synthetic methods fuel oligosaccharide assembly. Strategies to reverse engineer antibodies enable the rational antigen design. Concomitantly, optimizing the vaccine formulation helps to fine-tune the immune responses elicited by oligosaccharide antigens. This work combines the development of strategies in organic chemistry and biochemistry with *in vivo* immunological evaluations to provide insight into the nature of optimized carbohydrate-based vaccines against *S. pneumoniae*.

Zwitterionic polysaccharides (ZPSs) have gained attention due to their immunomodulatory properties that may render these structures interesting vaccine components. The ZPS representative Sp1 is an attractive vaccine target against *S. pneumoniae* serotype 1 (ST1) that is, in turn, insufficiently covered by existing vaccines. The first part of this work (Chapter 2) emcompasses the development of a chemoselective thioglycoside activation method in the presence of thioethers that allowed for the first total syntheses of conjugation-ready ZPS fragments. Glycan microarray analysis of serum samples along with immunological evaluation in mice gave insights into the immune recognition of conjugation-ready ZPS fragments. Finally, the potency of a synthetic Sp1 trisaccharide as a vaccine antigen against ST1 was evaluated *in vivo*.

To facilitate the creation of next-generation vaccines, strategies are needed to rationally design oligosaccharide antigens based on first principles. Highly virulent *S. pneumoniae* serotype 8 (ST8) is not included in modern conjugate vaccine formulations,

and synthetic precedence on ST8-derived oligosaccharides is scarce. The second part of this work (Chapter 3) comprises the elucidation of an oligosaccharide substructure that confers protective immunity against ST8 CPS. A newly-designed, divergent synthetic route provided access to a collection of ST8-related oligosaccharides. These and other glycans were used to reverse-engineer a protective ST8 CPS-directed monoclonal antibody (mAb) by glycan microarray analysis. A distinct tetrasaccharide frameshift was found to harbor both protective and non-protective, immunodominant glycotopes, and a murine mAb raised against this frameshift protected mice from lethal pneumococcal infection. The molecular details that govern different *in vitro* phenotypes of bacterial recognition by a set of ST8 CPS-directed mAbs were investigated.

Despite the progress in the development of vaccine antigens based on synthetic oligosaccharides, several shortcomings are still associated with the generation of glycosidic bonds. Particularly, the 1,2-*cis*-stereoselective introduction of *N*-protected, amino alcohol-derived linkers at the reducing end of oligosaccharides remains a major challenge. Chapter 4 features the development of a linker that can be introduced into synthetic oligosaccharides with excellent 1,2-*cis*-selectivities. Lowering the *O*-nucleophilicity of a conventional, aliphatic linker by introducing two fluorine substituents leads to a striking increase of 1,2-*cis*-selectivities in glycosylation reactions. This effect is consistent over a wide range of reaction conditions and facilitates the generation of synthetic oligosaccharide antigens.

Zusammenfassung

Mittels kohlenhydratbasierter Impfstoffe konnte in den vergangenen Jahrzehnten eine Vielzahl von Infektionskrankheiten verhindert werden. Eine Reihe kommerziell erhältlicher Präparate beruht auf Kapselpolysacchariden pathogener Bakterien wie *Streptococcus pneumoniae*. Allerdings ist die Produktion dieser Impfstoffe oftmals aufwändig. Darüber hinaus sind bestimmte Polysaccharidantigene nicht in der Lage, einen ausreichenden Impfschutz hervorzurufen. Synthetische, strukturell definierte Oligosaccharide sind ein vielversprechender Ansatz, um einerseits die Immunreaktionen des Körpers gegen Zuckerstrukturen zu verstehen und andererseits hochwirksame Antigene gezielt darszustellen. Allerdings müssen sowohl Entdeckung als auch Herstellung dieser Antigene vereinfacht werden, was die Verbesserung bestehender Verfahren und die Entwicklung neuer Herangehensweisen auf mehreren Ebenen erfordert. Innovative Synthesemethoden sind zum einen essentiell zum Aufbau komplexer Oligosaccharide. Der zielgerichteten Antigenentwicklung dient zum anderen die Rekonstruktion von Antikörper-Kohlenhydrat-Interaktionen. Darüber hinaus ermöglichen neue Methoden der Impfstoffformulierung die Optimierung der Immunantwort gegen ein Oligosaccharidantigen. Diese Arbeit verbindet neuartige Strategien der organisch-chemischen Synthese und der Biochemie mit immunologischer Evaluierung synthetischer Antigene *in vivo*, um Aufschluss über die Charakteristika optimierter Kohlenhydratimpfstoffe gegen *S. pneumoniae* zu erhalten.

Zwitterionische Polysaccharide (ZPSs) sind auf Grund ihrer immunmodulierenden Eigenschaften für den Einsatz in Impfstoffen von Interesse. Das ZPS-Molekül Sp1 ist darüber hinaus eine wichtige Zielstruktur zur Impfstoffentwicklung gegen *S. pneumoniae* Serotyp 1 (ST1), einem Stamm, der von existierenden Impfstoffen nur unzureichend abgedeckt wird. Der erste Teil dieser Arbeit (Kapitel 2) behandelt die Entwicklung einer Methode zur chemoselektiven Aktivierung von Thioglycosiden in Anwesenheit von Thioethern. Mit Hilfe dieser Methode konnte die erste Totalsynthese konjugierbarer ZPS-Substrukturen realisiert werden. Durch Glycan-Microarray-Analyse sowie immunologische Evaluierung im Mausmodell konnte Aufschluss über die Erkennung der ZPS-Fragmente durch Komponenten des Immunsystems erhalten werden. Schließlich

wurde die Eignung eines synthetischen Sp1-Trisaccharids als Impfstoffkandidat gegen ST1 *in vivo* untersucht.

Zur Erforschung neuartiger Impfstoffe werden Methoden benötigt, um Oligosaccharidantigene zielgerichtet auf Basis weniger Grundprinzipien zu entwerfen. Obwohl der hochvirulente *S. pneumoniae* Serotyp 8 (ST8) kein Bestandteil kommerziell erhältlicher Kohlenhydrat-Protein-Konjugatimpfstoffe ist, behandeln nur sehr wenige Vorarbeiten die Synthese von ST8-basierten Oligosacchariden. Der zweite Teil dieser Arbeit (Kapitel 3) beschreibt die Entdeckung einer Oligosaccharidstruktur, die eine protektive Immunantwort gegen ST8 CPS hervorzurufen vermag („protektives Glycotop"). Mit Hilfe einer neu entworfenen, divergenten Synthesestrategie wurde eine Reihe ST8-abgeleiteter Oligosaccharide hergestellt und zusammen mit anderen verfügbaren Glycanstrukturen dazu verwendet, das Bindungsmuster eines protektiven, ST8-Kapselpolysaccharid-gerichteten, monoklonalen Antikörpers (mAk) zu rekonstruieren. Dabei stellte sich heraus, dass eine spezielle Tetrasaccharidstruktur sowohl protektive als auch immunodominante, nichtprotektive Glycotope enthält. Ein in Mäusen durch Immunisierung mit einem entsprechenden Glycokonjugat hergestellter mAk gegen dieses Tetrasaccharid schützte Mäuse vor einer tödlichen Infektionsdosis mit Pneumokokken. Außerdem wurden die molekularen Grundlagen untersucht, die zu Unterschieden in der Bakterienerkennung durch verschiedene mAks *in vitro* führen. Dieser Teil der Arbeit beschreibt eine gegenüber herkömmlichen Herangehensweisen stark vereinfachte Strategie zur zielgerichteten Entwicklung oligosaccharidbasierter Impfstoffantigene.

Trotz großer Fortschritte bei der Entwicklung von Impfstoffantigenen auf Basis synthetischer Oligosacccharide bestehen noch immer große Probleme mit deren organisch-chemischer Synthese, insbesondere mit der Knüpfung glycosidischer Bindungen. Vor allem die 1,2-*cis*-stereoselektive Einführung *N*-geschützter Aminoalkohole, die als Linker der späteren chemoselektiven Konjugation des ungeschützten Glycans dienen, stellt eine große synthetische Herausforderung dar. In Kapitel 4 wird die Entwicklung eines neuartigen Linkers beschrieben, der sich mit ausgezeichneten 1,2-*cis*-Stereoselektivitäten in synthetische Oligosaccharide einbringen lässt. Durch Einführung von zwei Fluorsubstituenten wurde die *O*-Nukleophilie eines herkömmlichen, aliphatischen Linkers stark herabgesetzt, was zu einer beachtlichen

Erhöhung der 1,2-*cis*-Selektivitäten in Glykosylierungsreaktionen führte. Dieser Effekt zeigte sich für eine große Bandbreite an Reaktionsbedingungen und sollte daher die Herstellung von konjugierbaren Oligosaccharidantigenen in Zukunft wesentlich vereinfachen.

1 Introduction

This chapter has been modified in part from the following articles:

Carbohydrate Vaccines. B. Schumann, C. Anish, C. L. Pereira and P. H. Seeberger, in *Biotherapeutics: Recent Developments Using Chemical and Molecular Biology, RSC Drug Discovery Series No. 36*, eds. L. Jones and A. J. McKnight, RSC Publishing, Cambridge, 2013, ch. 3, pp. 68-104.[1] http://dx.doi.org/10.1039/9781849737159-00068

Chemical biology approaches to designing defined carbohydrate vaccines. C. Anish, B. Schumann, C. L. Pereira and P. H. Seeberger, *Chem. Biol.*, 2014, **21**, 38-50.[2] http://dx.doi.org/10.1016/j.chembiol.2014.01.002

1.1 *Streptococcus pneumoniae*

1.1.1 Pneumococcal Disease

Streptococcus pneumoniae is a Gram-positive, facultative anaerobic bacterium that colonizes the upper respiratory tract in healthy humans.[3] Inflammatory diseases such as otitis media and pneumonia can arise after infection of normally sterile sites and turn into invasive pneumococcal diseases (IPD) that include meningitis and bacteremia. Risk groups for invasive disease include individuals with compromised immune systems, such as children, older adults and inhabitants of regions with poor health and hygiene standards. Accordingly, bacteremic pneumococcal pneumonia constitutes one of the leading causes of mortality and morbidity in HIV-infected individuals,[4] and co-infections with *S. pneumoniae* have been connected to fatal cases of the 2009 influenza pandemic.[5]

Children in developing countries are particularly susceptible to IPD. The global estimate by the World Health Organization (WHO) of pneumococcal diseases among children under the age of five years is 14.5 million, with more than 800000 deaths per year.[6] Overall, approximately two million deaths worldwide are caused by pneumococcal infections.[7] IPD is associated with a fatality rate of up to 10% even in the developed world, especially among the older population.[3] Furthermore, *S. pneumoniae* is the major cause of acute respiratory infections in children in developing countries.[6]

Up to one third of all healthy adults, and an even higher percentage of young children, carry pneumococci in their nasopharynx. Colonization of the respiratory tract precedes infection and is an important mechanism of horizontal spread of this bacterium in public communities such as day care centers.[8] Bloodstream invasion in immunocompromised individuals requires tight adherence to and subsequent damage of the bronchial epithelium and these processes are orchestrated by the interplay of multiple virulence factors.[9]

1.1.2 Pneumococcal Capsules as Virulence Factors

The pneumococcal surface displays a range of molecules that are important for infection. As a Gram-positive bacterium, *S. pneumoniae* is covered by invariant polymers that bear important physicochemical and regulative properties. The cell membrane is surrounded by several layers of peptidoglycan that are interspersed with lipoteichoic and teichoic acids, also known as F-antigen and C-polysaccharide, respectively (Fig. 1.1A).[10-12] The most indispensable virulence factor of pneumococci, however, is the capsule (Fig. 1.1B).

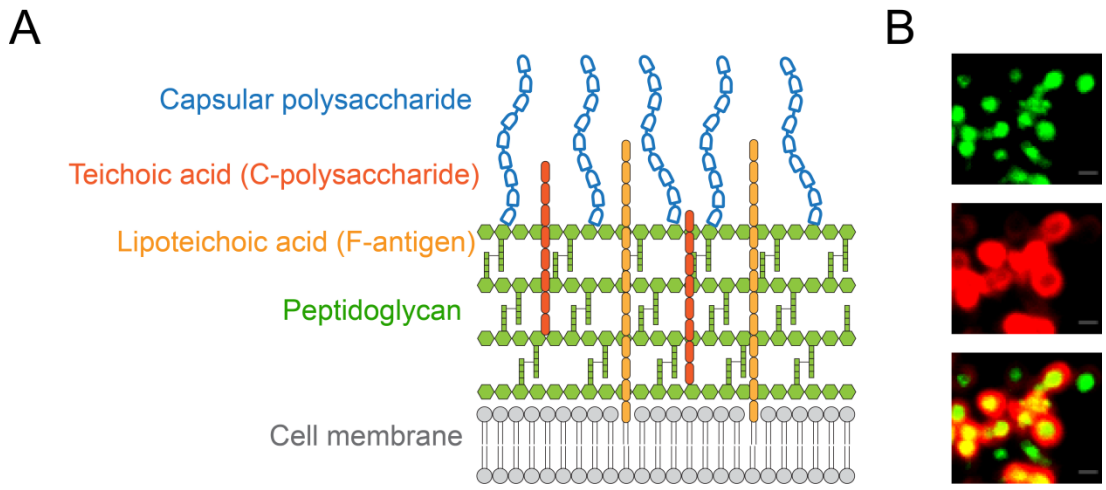


Figure 1.1. The pneumococcal cell surface. *A*, schematic representation. *B*, the pneumococcal capsule. Inactivated pneumococci are counter-stained (green fluorescence) and CPS is immuno-labeled (red fluorescence). The cellular coating provided by the capsule is clearly visible. Images were taken along with an immunofluorescence experiment in Chapter 3. Scale bar: 1 µm.

In the first decades of the 20th century, Heidelberger discovered that the immunodominant type-specific substance – the capsule – of *S. pneumoniae* is a polysaccharide.[13] The capsule has since become the best studied pneumococcal virulence factor and is an important tool in biomedical research. Capsules serve multiple purposes during infection, including the protection from desiccation, shielding of surface antigens

from complement deposition and prevention of interaction of surface-associated immunoglobulins with immune cells.[14, 15] The structures of capsular polysaccharides (CPSs) are the basis for the categorization of *S. pneumoniae* into more than 90 different serotypes (STs), and polysaccharide structures are highly heterogeneous.[16, 17] Proteins that mediate the biosynthesis of these polysaccharides are generally encoded in gene clusters and include glycosyltransferases, transporters and enzymes responsible for the biosynthesis of activated monosaccharides.[16, 18] Most pneumococcal CPSs are built up from pre-assembled oligosaccharide precursors, and hence consist of repeating units of different lengths.[15] The same polysaccharide can therefore be considered as a polymer of multiple frameshifts (FSs). These FSs, in turn, may harbor different immunological properties if presented as structurally defined oligosaccharides (*see* below).

Pneumococcal STs differ in their invasiveness: less virulent types are associated with higher rates and longer periods of asymptomatic carriage, while highly invasive types are found to cause IPD shortly after colonization.[19, 20] It has been reported that capsule structure determines virulence, and this connection has been traced back to capsule thickness and energy expense of repeating unit biosynthesis.[21] Interestingly, pneumococci exhibit reduced capsule thickness upon adherence to and invasion of the alveolar epithelium.[14, 22] However, ablation of CPS biosynthesis completely abrogates virulence.[14, 23]

1.2 Carbohydrate-based Vaccines

Pioneering the field of glycoimmunology, Avery and Goebel reported in the 1930s that saccharide-specific antibodies can be generated *in vivo* by conjugating monosaccharides to proteins.[24-30] By this time, the term "hapten" had already been defined by Landsteiner for any molecule that can induce a specific immune response upon attachment to a protein.[31] Francis and Tillett as well as Heidelberger discovered that immunization with CPSs from *S. pneumoniae* can induce protection from infections by this pathogen.[32, 33]

Despite the successful research into the immunogenicity of bacterial polysaccharides,[34] antibiotics rapidly gained importance and soon replaced vaccines as the most popular means to fight infectious diseases. However, the emergence of resistant

strains indicated that antibiotics would not be able to eradicate pathogenic bacteria,[3, 35-37] and the idea of vaccines based on CPSs was reconsidered. As a result of extensive research, polysaccharide vaccines (PSVs) against *Neisseria meningitidis* and *S. pneumoniae* were licensed in the 1970s. PSV formulations against pneumococci contained CPSs from 14 and, subsequently, 23 different serotypes. PSVs against other pathogenic bacteria followed.[38]

Although PSVs were highly successful in preventing invasive disease, limited efficiency was observed in very young children.[36] The classical approach of conjugating polysaccharides to proteins was thus reconsidered, furnishing carbohydrate conjugate vaccines (CCVs) that elicit a potent immune response even in infants.[39, 40] With the help of CCVs, the incidence of invasive diseases caused by pathogenic bacteria has been tremendously reduced.[41] In past decades, CCVs have developed into a multi-billion dollar market and are part of immunization programs recommended by health authorities.[42, 43]

1.2.1 Bacterial Capsular Polysaccharides as Vaccine Candidates

Vaccines can be generally subdivided into whole cell and subunit vaccines. The former class includes the first vaccines developed and is based upon either killed or attenuated organisms.[44] Despite the potential of whole-cell vaccines to confer long-lasting immunity against infections, culturing the respective pathogenic organisms in sufficient quantities may be cumbersome.[44-46] Subunit vaccines, in turn, comprise defined components of the target organisms, including cell surface or secreted proteins, virus-like particles and CPSs. Antigens employed in subunit vaccines can be obtained by recombinant expression.[44] Due to the low immunogenicity of these preparations, adjuvants are usually used in subunit vaccine formulations.[44, 46, 47] Proteins display a high immunogenic potential and are hence well-suited as antigens for subunit vaccines (*see* below). However, immunogenic proteins on the surface of pathogenic bacteria are often shielded by CPSs and thus inaccessible for components of the immune system.

Antibody responses can be raised against bacterial CPSs due to the recognition of these structures as “non-self” (non-host-derived) by the mammalian immune system. Unusual linkages, rare monosaccharides and modifications are structural determinants that contribute to this recognition.

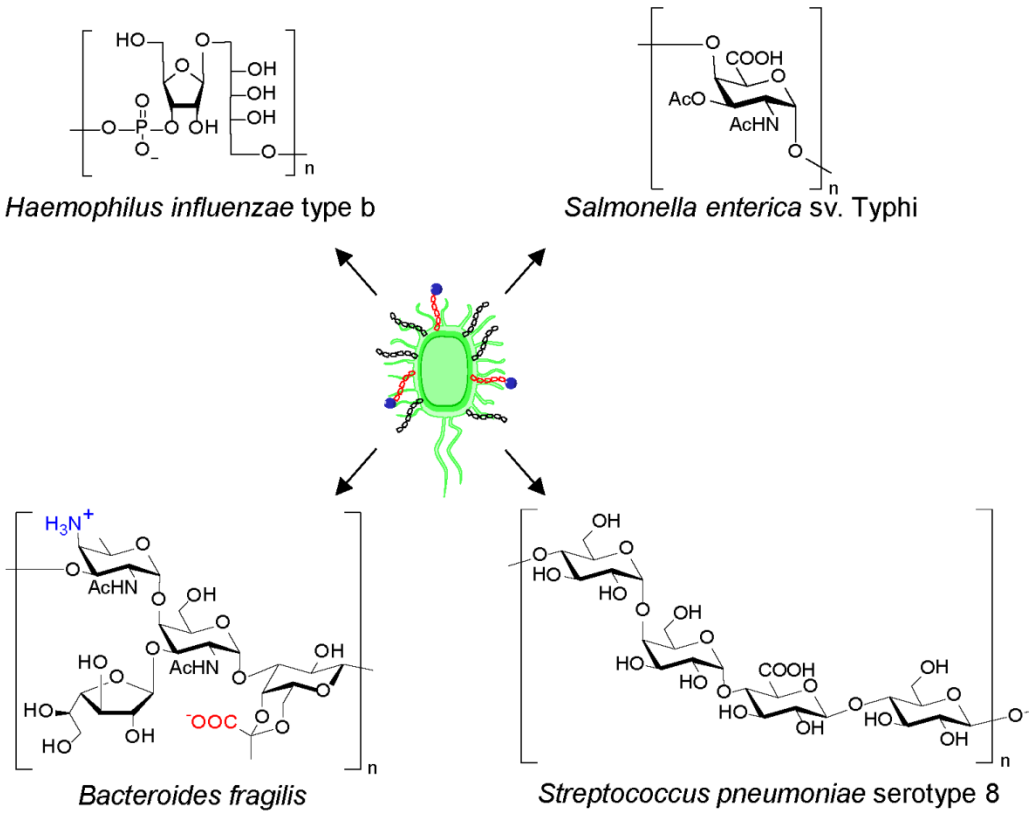


Figure 1.2. Examples of bacterial CPS structures. A plethora of unique functionalities is found due to the presence of phosphodiester linkages, rare monosaccharides, hydroxyl group modifications and unusual glycosidic linkages. Adapted from Schumann et al.[2]

CPSs of pathogenic bacteria are structurally extremely diverse (Fig. 1.2).[17, 48] The length of repeating units may range from mono- to octasaccharides.[17, 49] Polysaccharides are either linear or branched and contain common or rare sugars. While the human glycome is composed of just nine different monosaccharides (D-Glc, D-Gal, D-Man, L-Fuc, D-GlcNAc, D-GalNAc, D-Xyl, D-Sia and D-GlcA), the number of building blocks that make up the bacterial glycome easily exceeds one hundred.[50] Monosaccharides are present in either pyranose or furanose forms or as polyalcohols. Variability is further elevated by the presence of chemical modifications, including acetate, pyruvate and glycerate groups.[51] The multitude of structural peculiarities of bacterial CPSs influences the nature of immune responses directed against these glycans.

1.2.2 Immunology of Carbohydrate-based Vaccines

Antigens can invoke different types of immune responses and are categorized accordingly. The formation of immunological memory is a hallmark of thymus-dependent (T_D)

antigens that are mainly protein-based, while thymus-independent (T_I) antigens, usually polysaccharide- or lipid-based, do not elicit immunological memory.[52]

T_D antigens induce the immediate help of $CD4^+$ T cells (T-helper or T_H cells) to trigger antibody production. Pathogen-derived antigens are recognized by professional antigen-presenting cells (APCs). These include immunoglobulin-producing B cells, antigen-sampling dendritic cells and phagocytotically active macrophages. After recognition by an APC, antigens are internalized and degraded in endosomal compartments.[45] The peptide fragments resulting from protein breakdown are then loaded onto major histocompatibility complex (MHC) class II molecules and this complex is transported to the surface of the APC. The peptide-MHCII complex is recognized by the αβ-T cell receptor (TCR) found on a specific T_H cell. With the help of further APC-T cell interactions, cytokines such as interleukin(IL)-4 are released by the T cell. Cytokine release and cell-cell interactions synergistically stimulate affinity maturation and class switch of immunoglobulin (Ig) genes in B cells to give rise to highly antigen-specific antibodies of the IgG isotype. Concomitantly, B cells differentiate into specialized plasma cells that produce high levels of IgG to help fight invading pathogens (*see* below). Furthermore, memory B and T cells are produced that are re-activated upon re-encounter of the same T_D antigen. Immunological memory thus facilitates the generation of an efficient immune response upon re-infection.[45]

T_I antigens stimulate B cells to produce antibodies without directly engaging T cells and are subdivided into two different groups. T_I-1 antigens are B cell mitogens such as lipopolysaccharides (LPS) and activate even neonatal B cells.[52]

In contrast, T_I-2 antigens are polymers of high molecular weight, such as CPSs, and activate B cells by cross-linking membrane-bound B cell receptors (BCR).[53] T_I-2 antigens fail to induce antibody production in neonatal, immature B cells.[53] Furthermore, T_I-2 antigens do not invoke $CD4^+$ T cell help by classical MHC II-mediated antigen presentation.[54] Consequently, Ig class switching and affinity maturation are inefficient, and low-affinity IgM and IgG antbodies are produced.[53, 55] It has been suggested that functional Bruton's tyrosine kinase, a signaling molecule important for B cell development, is needed for antibody generation against T_I-2 antigens.[55-58] In addition,

other cell types, such as macrophages and natural killer cells, are required to induce the production of antibodies in response to T_I-2 antigens.[54, 59, 60]

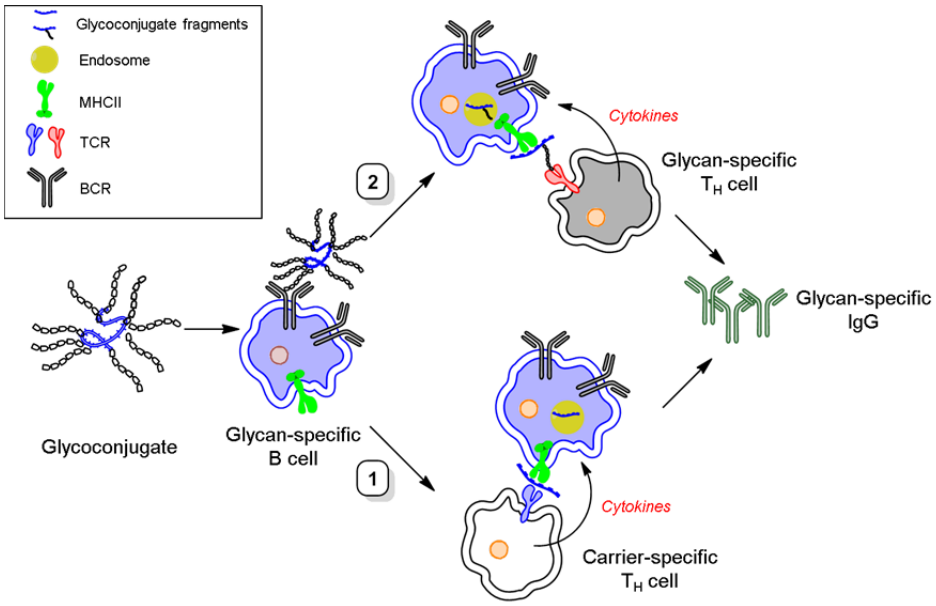


Figure 1.3. The mechanism of action of glycoconjugate vaccines. The glycan hapten is recognized by a specific BCR, internalized into endosomal compartments. After breakdown, carrier derived peptides (pathway 1) are presented on MHCII complexes to TCRs on the surface of specific TH cells. Additional cell-cell interactions (not shown) and the release of cytokines trigger B cell expansion, antibody class switch and affinity maturation to produce high-affinity IgG. Alternatively, glycopeptides can be presented on MHCII molecules (pathway 2) and recognized by glycan-specific T cells. Adapted from Schumann et al.[2]

Pathogenic bacteria harbor a plethora of T_I-1, T_I-2 and T_D antigens, and a potent immune response can be generated by the interplay of these stimuli upon infection. However, polysaccharide vaccines mainly display T_I-2 antigens. Therefore, PSV do not elicit a protective antibody response in infants,[61-63] and the pneumococcal polysaccharide vaccine PPV-23 (Pneumovax23®, Merck) is only recommended for children above two years of age.[64]

The conjugation of a polysaccharide to a carrier protein converts a T_I antigen into a T_D antigen. This leads to a significant enhancement in the immune response by invoking T cell help. Inactivated bacterial toxoids, such as denatured diphtheria and tetanus toxins (DT and TT, respectively), and the non-toxic DT mutant CRM197 are widely used carrier proteins due to their potency to induce T cell activation.[65, 66] Following recognition of the saccharide moiety by a glycan-specific BCR and internalization, carrier-derived peptides are presented to T_H cells to induce cellular and cytokine-mediated signals. As an alternative to this process, presentation of glycopeptides

can lead to the activation and expansion of glycan-specific T_H cells (Fig. 1.3).[67-71] High-affinity antibodies against the CPS moiety and immunological memory are then elicited even in young infants.[36, 72-74] Pneumococcal conjugate vaccines (PCV) cover a multitude of serotypes. Current formulations are 10- (Synflorix®, GSK) or 13-valent (Prevenar13®, Pfizer),[75] and cover serotypes responsible for more than 70% of global pneumococcal disease incidence.[76]

1.2.3 Antibodies and Their Role in Pneumococcal Disease

Antibodies orchestrate several important defense mechanisms of the humoral immune system against pathogenic organisms. Antibodies neutralize viruses and bacterial toxins, opsonize bacteria for phagocytosis and trigger cell-dependent immune responses against tumors and multicellular parasites.[45] A hallmark of the humoral immune system is the high diversity of proteins involved in antigen recognition and presentation, such as antibodies. Antibody diversity is further enhanced by genetic recombination and hypermutation mechanisms, especially of antigen-binding complementarity determining regions (CDRs).[45] Class switching and affinity maturation events (*see* above) tailor Ig isotype and strength of antigen binding to effectively defend the host against an infection.

CPSs are the immunodominant structures displayed by *S. pneumoniae* due to the abundance of unusual functionalities and their position as the outermost part of the pneumococcal cell surface. Antibodies raised by the host against the pneumococcal capsule protect from disease, and hence polysaccharides are the primary target antigens used in vaccines against that bacterium. The most important defense mechanism against pneumococci is opsonization by anticapsular antibodies with subsequent phagocytosis of antibody-bacteria-complexes by several effector cells such as macrophages and neutrophil granulocytes, although the precise mechanism of protection is still debated (*see* below).[8] The ability of antibodies to act as opsonins can be evaluated *in vitro* by assessing bacterial survival in the presence of phagocytes in an opsonophagocytic killing assay (OPKA). Since opsonophagocytosis titers of serum antibodies correlate with protection from disease,[77] OPKA is an important tool to determine the success of vaccinations against pneumococci.[78-80]

The development of techniques to generate monoclonal antibodies (mAbs) that originate from a single B cell clone has tremendously driven the understanding of immune recognition. MAbs bind to defined structural entities with often high affinities, and have been useful in various areas of biomedical research and treatment.[45] For instance, mAbs recognizing structures on the surface of pathogens can be applied in passive immunization experiments to treat disease, as seen in the experimental Ebola drug ZMapp.[81] MAbs that bind carbohydrate epitopes (glycotopes) have rapidly gained importance in the past decade. The ability to bind to the most exposed sites of a pathogen renders mAbs well-suited to be used as diagnostic tools, and precedence has been set by the mAb-mediated detection of the bio-warfare agents *Bacillus anthracis*[82] and *Yersinia pestis*.[83] Diagnostic kits to determine pneumococcal serotypes in clinical isolates are based on anticapsular antibodies that are not monoclonal, but cross-adsorbed against other serotypes.

MAbs raised against *S. pneumoniae* CPSs are protective against pneumococcal infection *in vivo*.[84-93] The precise mechanism of protection is not clear and may vary depending on determinants within the mAb and the antigen. For instance, mAbs that protect from infection with the same serotype may be effective opsonins or may rather cause agglutination of bacteria without promoting opsonophagocytosis *in vitro*, as observed for mAbs against ST3 and ST8.[89, 91] Furthermore, certain mAbs do not require a complement source for opsonophagocytic killing, while others do.[89] The mAb isotype has been shown to influence effectiveness during passive immunization against *Cryptococcus neoformans*,[94] and the same correlation may exist in anti-pneumococcal mAbs. Recently, antibodies raised against *Francisella tularensis* LPS have been distinguished based on their ability to recognize either internal or terminal glycotopes of the same polysaccharide,[95] introducing yet another layer of complexity into the mode of action of polysaccharide-specific antibacterial mAbs. The investigation of glycotopes recognized by protective mAbs can provide valuable information on putative antigens for vaccination.

Of note, antibodies against phosphocholine, the most immunogenic epitope in pneumococcal C-polysaccharide, exhibit limited protectiveness, highlighting the importance of CPSs as antigens of choice in anti-pneumococcal vaccines.[92] However,

certain shortfalls are associated with carbohydrate vaccines based on isolated polysaccharides.

1.2.4 Production and Manufacture of Glycoconjugate Vaccines

PCVs consist of multiple components from different origins and are thus unique medicinal products from a regulatory perspective. Quality control (QC) guidelines are to be met for each component prior to conjugation (Fig. 1.4).[80, 96-98] Polysaccharides are purified in a number of laborious steps and depolymerized into smaller fragments that are activated for conjugation.[92-94, 96] Two different concepts of conjugation are used. Methods of multipoint attachment introduce a number of reactive functionalities into CPS fragments, leading to the formation of cross-linked, "lattice-type" glycoconjugates (Fig. 1.4A). Single point attachment appends one reactive group per polysaccharide chain, usually at the reducing end of the glycan, and "neoglycoconjugates" are obtained. These procedures can result in the loss of labile modifications or the chemical derivatization of monosaccharides that are crucial for immunogenicity, leading to compromised PCV efficiency.[99] Additionally, varying amounts of co-isolated impurities such as the pneumococcal C-polysaccharide (*see* Fig. 1.1) are frequently found in CPS preparations, and the implications of these impurities during immunization experiments are not known.[100, 101]

To address the shortcomings associated with isolated polysaccharides, alternative strategies have been developed to produce immunogenic glycans.[102-105] Oligosaccharide synthesis has become the most promising alternative method.

1.3 Opportunities and Challenges of Vaccines Based on Synthetic Oligosaccharides

Many of the QC steps necessary for the preparation of glycoconjugates based on isolated polysaccharides can be circumvented by chemical synthesis. Synthetic oligosaccharides are well-defined, homogeneous compounds that can be characterized in detail by utilizing standard tools of organic chemistry. Furthermore, a linker for conjugation can be

incorporated at a specific site. The structural complexity of conjugation-ready glycans is thus drastically reduced, and a more defined glycoconjugate is produced (Fig. 1.4B).

In addition to their suitability as vaccine haptens, synthetic oligosaccharides allow for a detailed investigation of the mechanism of immunization. Defined glycan antigens can give insight into the immune recognition of individual functionalities and are key to the rational design of carbohydrate-based vaccines.

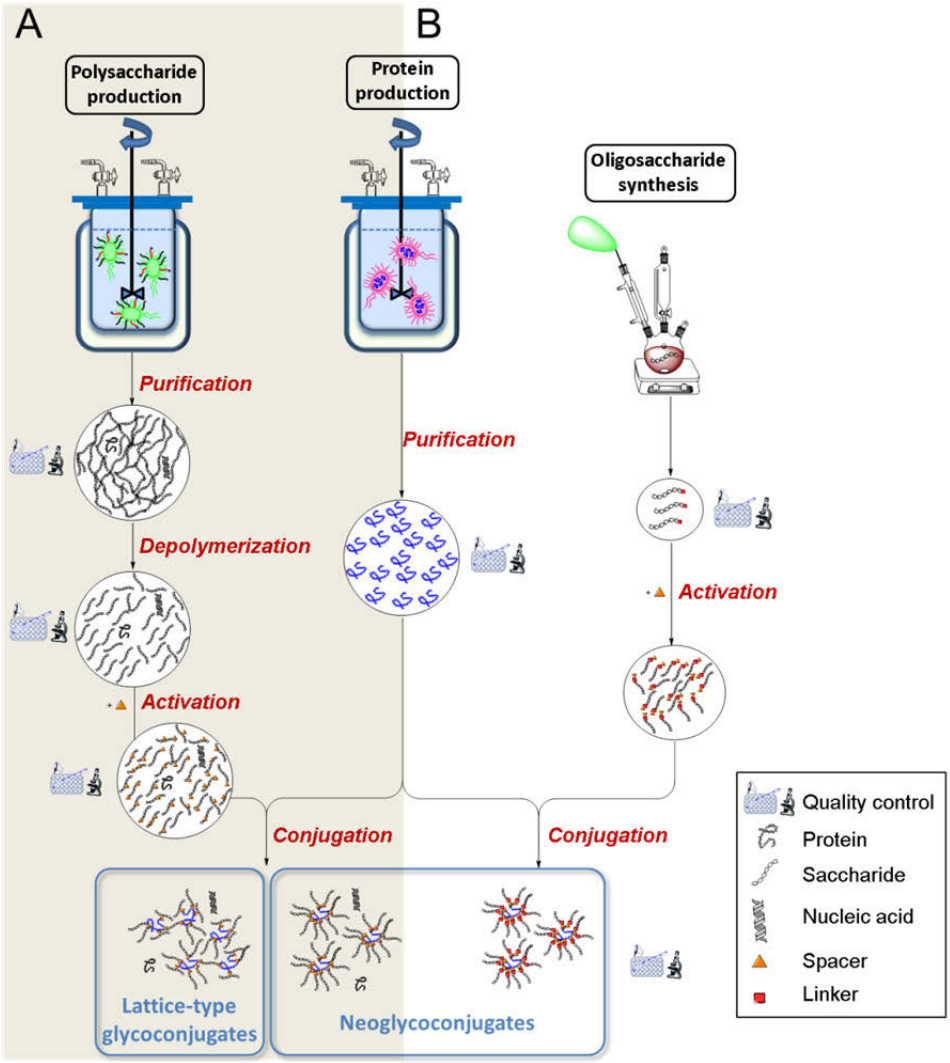


Figure 1.4. Manufacture of glycoconjugate vaccines. Carrier proteins are produced by heterologous expression and purified to homogeneity. *A*, bacterial polysaccharides are isolated from liquid culture and subjected to a number of purification, depolymerization and activation steps. Quality control is necessary to monitor each stage. Depending on the conjugation chemistry used, either polysaccharide-protein lattices or neoglycoconjugates are obtained. *B*, oligosaccharide synthesis provides uniform oligosaccharides that are ready for conjugation to carrier protein to generate neoglycoconjugates. Adapted from Schumann et al.[2]

1.3.1 Chemical Immunology of Synthetic Oligosaccharides

Due to the high structural heterogeneity of CPSs, the nature of glycotopes that confer a protective immune response ("protective glycotopes") varies on a case-by-case basis. Knowing the minimal protective glycotopes of a polysaccharide is essential to designing a modern CCV.

Classically, the antigenicity of oligosaccharides is determined by performing immunization trials with the respective glycoconjugates. The affinities of antisera towards synthetic oligosaccharides and native polysaccharides are measured,[106, 107] and the capacity of serum samples to bind pathogens and promote opsonophagocytosis is tested.[82, 108] Ideally, challenge studies are performed with live pathogenic bacteria to assess the potency of vaccination in immunized animals.[109] Alternatively, mice can be passively immunized with sera from vaccinated animals or mAbs generated against the respective oligosaccharide.[110] The latter approach offers the advantage that the variability of immune responses often observed by actively vaccinating mice is abrogated.

1.3.1.1 The Effect of Saccharide Length

Oligosaccharide synthesis can be significantly facilitated if insights into the minimal length of protective glycotopes are known. Two different structural concepts are at play. Sequential glycotopes are purely dependent upon the glycan sequence and can be covered by synthesizing small oligosaccharides. Certain *S. pneumoniae* serotypes, such as ST3,[109] ST6B[111] and ST14,[106, 112] have been shown to display sequential, protective glycotopes, and synthetic oligosaccharide haptens as small as tri- or tetrasaccharides have induced protective immune responses. In contrast, polysaccharides may display conformational epitopes only above a certain chain length, as seen in vaccine antigens against Group B *Streptococcus* (GBS) type III that require an optimal saccharide length of 40-70 monosaccharides.[71, 113, 114] The synthesis of glycans of that length is difficult to achieve by conventional methods. Therefore, the discovery of protective sequential epitopes is imperative to designing a synthetic carbohydrate-based vaccine on an industrial scale.

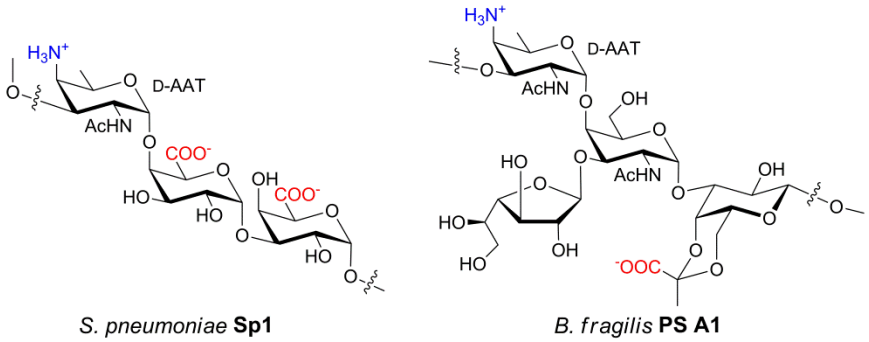
An oligosaccharide hapten can elicit a robust anti-glycan immune response even if it does not display protective glycotopes. The induction of antibodies that target these non-protective glycotopes is not desirable in vaccination.[113]

1.3.1.2 The Effect of Exposed Epitopes

The effectiveness of glycoconjugate vaccines requires immunogenic glycotopes to be exposed to the medium in order to bind to BCRs. It is therefore conceivable that the most exposed moiety of a hapten is most important for immune recognition.[115] If an unusual glycotope such as a rare bacterial monosaccharide is fashioned at an exposed position of a glycan chain, the recognition of this glycotope as "non-self" should manifest in enhanced immunogenicity. However, increased immunogenicity does not necessarily correlate with protection from disease, and the role of immunogenic glycotopes must be carefully assessed. It is thought that certain bacteria expose non-protective epitopes to components of the immune system as decoys.[116] On the other hand, CPSs may harbor epitopes that are not recognized as "non-self" by the host, contributing to immune evasion. Abrogating these epitopes may lead to increased vaccine efficacy, for example by removing sialic acid moieties from non-immunogenic GBS type V polysaccharide fragments.[117]

1.3.1.3 The Immunomodulatory Properties of Zwitterionic Polysaccharides

Most bacterial CPSs are negatively charged under physiological conditions, usually due to the presence of acidic uronic acid or phosphodiester groups. The repeating units of a particular class of CPSs display zwitterionic charge motifs, and positive charges are introduced through amine-containing monosaccharides such as the rare sugar 2-acetamido-4-amino-2,4,6-trideoxygalactopyranose (D-AAT, Scheme 1.1).[118] Zwitterionic polysaccharides (ZPSs) are displayed on the surface of certain commensal and symbiotic bacteria and bear important immunomodulatory properties.[119-123] The most prominent ZPSs are Sp1 found on the surface of *S. pneumoniae* ST1 and PS A1 on *Bacteroides fragilis* (Scheme 1.1) In resemblance to the processing of protein antigens, ZPSs are internalized by APCs, fragmented in endosomal compartments and loaded onto MHC class II molecules.[124-126] Together with the recognition of ZPSs by toll-like receptors (TLRs) on the surface of APCs, binding of MHC-presented ZPS fragments by specific TCRs leads to the expansion of certain T cell subsets and the modulation of immune processes, generating tolerance towards commensal bacteria.[122, 124, 127-130] Thus, ZPSs are the first glycans known to hijack the classical antigen presentation pathway of T_D antigens without the need of a carrier protein.



Scheme 1.1. Chemical structures of the ZPSs Sp1 and PS A1. The amino sugar D-AAT is found in the repeating units of both polysaccharides.

The immunomodulatory properties of ZPSs have been attributed to the unique secondary structure of these polysaccharides. ZPSs adopt right-handed helices in aqueous solution that are dependent upon the presence of the zwitterionic charge motifs.[131] A number of ZPS-derived oligosaccharides have been chemically synthesized (*see* below),[132-134] but have not been extensively studied in biochemical settings. Free of cellular contaminants, synthetic probes are essential to studying the immune recognition as well as the extent of immunomodulation of ZPSs.

The immunomodulatory activity of ZPSs has been exploited in vaccine design. It has been found that the incorporation of zwitterionic charge motifs into polysaccharides enhances their immunogenicity.[135-137] Furthermore, PS A1, a well-characterized ZPS representative found on the commensal bacterium *B. fragilis*, has been used as a carrier platform for the tumor-associated Tn antigen to generate an all-carbohydrate cancer vaccine formulation.[138, 139] In proof-of-principle studies, the Tn-PS A1 conjugate invoked a Tn-directed IgG response in mice without addition of an external adjuvant, and antisera showed a slightly better recognition of cancer cells than sera raised against unmodified PS A1.[138, 139] Despite the need to further optimize the formulation, the concept of all-carbohydrate vaccines is of high interest due to the biocompatibility and the enhanced stability of polysaccharide-based carriers in comparison to proteins.

1.4 Novel Developments in Vaccine Design

Based on the lessons learned after the introduction of glycoconjugate vaccines, numerous approaches have been followed to further improve vaccine efficacy by optimizing antigen

design, presentation and formulation. Concepts from different areas of biomedical research have been implemented in vaccine design, including immunology, structural biology and materials science.

1.4.1 Rational Vaccine Design

Finding the right antigen has always been an iterative process that is costly and time-consuming. Current efforts in vaccine design are invested to narrow down the number of potential antigens to those that can induce protective immunity.

Important precedence on the potential of rational vaccine design has been presented by Bundle and coworkers. Cell wall mannans found on the facultatively pathogenic yeast *Candida albicans* harbor β-(1→2)-linked oligo-D-mannosides.[140] A neutralizing mAb was screened for binding to a panel of synthetic oligosaccharides by inhibition experiments. It was found that small oligosaccharides (di- and trisaccharides) exhibited higher affinities to the mAb than longer glycans.[141] The synthesis of non-natural oligosaccharide congeners then allowed for a view into glycan recognition by the mAb.[142, 143] Nuclear magnetic resonance (NMR) spectroscopy and molecular modelling eventually revealed that the primary glycotope recognized by the mAb is a disaccharide.[144, 145] Longer oligosaccharides are not recognized by the antigen binding pocket unless these structures undergo conformational changes. Based on these results, a disaccharide conjugate vaccine was designed that induced protective immunity against *C. albicans* in rabbits.[145] While highly useful, the structural studies that led to the generation of a protective vaccine hapten took several years and were carried out to explain binding data obtained beforehand. Future vaccine design will likely make use of that knowledge and merge structural and design processes to enable faster antigen discovery.

Further examples on structure-based glycotope discovery include the rationalization of serotype specificity of anti-*Vibrio cholerae* antibodies[146] and the mapping of the glycotope bound by a protective mAb against *Shigella flexneri* serotype 2a.[147] Although this method of rational vaccine design is powerful, structure-guided antigen design is time-consuming and laborious. Furthermore, information on the

identity of a suitable ligand is crucial to enable co-crystallization experiments, and thus glycotope screening is a necessary prerequisite (*see* below).

1.4.2 Epitope Discovery as a Driving Force in Vaccine Development

Uncovering a potential antigenic glycotope of a polysaccharide by methods other than immunization can significantly reduce the number of animal trials. Information on the glycotopes that are recognized by the immune system is available in CPS-binding sera of infected or vaccinated individuals and protective mAbs. Recent years have seen progress in the development of methods to obtain this information. Conventional binding assays, such as enzyme-linked immunosorbent assay (ELISA) with immobilized CPSs, can be used to measure antibody titers, but give little insight into glycotope specificity. As an extension of classical binding assays, antibody-polysaccharide interactions can be inhibited with defined oligosaccharides to elucidate target glycotopes.[141, 144, 148] Novel methods have been developed to quantitatively determine carbohydrate-protein interactions with high throughput. The most important methods include glycan microarrays and surface plasmon resonance.

1.4.2.1 Glycan Microarrays

Glycan microarrays have greatly advanced the field of glycobiology.[149-154] Minute quantities of synthetic or isolated glycans are immobilized on surfaces either covalently or via non-covalent adsorption. Incubation with an antibody-containing sample results in specific antibody-glycan interactions that can be detected by utilizing fluorescence-labeled detection antibodies.[83, 155, 156] A large number of different glycans can be printed on the same microarray slide, including oligosaccharides with different chain lengths, FSs and oligosaccharide modifications. Carbohydrate microarrays are thus invaluable tools to study the interactions of antibodies with a multitude of synthetic oligosaccharides at the same time. Oligosaccharides produced through chemical synthesis usually harbor an amino group at the reducing terminus and can be coupled to glass slides functionalized with active esters (Fig. 1.5A).[149] Alternatively, maleimide-functionalized slides can be used to attach thiol-bearing glycans.[149, 157]

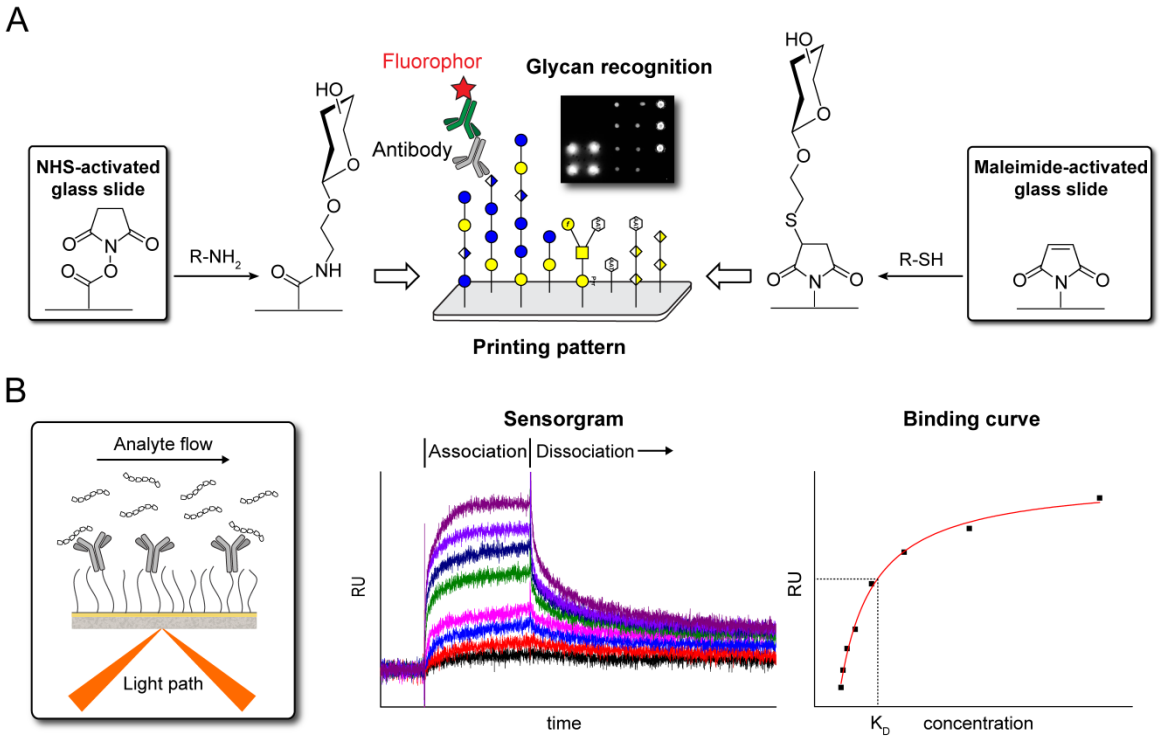


Figure 1.5. Methods to characterize carbohydrate-protein interactions. *A*, glycan microarrays. Multiple oligosaccharides can be immobilized on the same glass slide by virtue of reactive functionalities such as amino or thiol groups. *B*, surface plasmon resonance. Both kinetics and affinity of a carbohydrate-protein interaction can be quantified. SPR data were obtained along with an experiment in Chapter 3.

Glycan microarrays have become a standard tool in glycobiology and serve diverse purposes. Depending on the experimental setup, glycan microarray slides can be furnished either with large numbers of different oligosaccharides to map binding characteristics of a specific protein, or with few structures in repetitive wells to enable the screening of multiple biological samples.[158] Therefore, glycan microarrays are of particular importance to analyze sera of animals vaccinated with glycoconjugates harboring synthetic haptens.[155, 159, 160]

1.4.2.2 Surface Plasmon Resonance

While glycan microarray experiments are powerful tools to assess the fine specificities of carbohydrate-binding proteins in a high-throughput format, these measurements are usually biased towards tight binding events. Surface plasmon resonance (SPR) has emerged as a suitable complementary method to study both thermodynamic and kinetic parameters of carbohydrate-protein-interactions. Typically, a carbohydrate binding protein is immobilized on a gold chip and subjected to a flow of a glycan in solution (Fig.

1.5B). Binding events are associated with a change of the intensity of totally reflected light on the chip. Thereby, association and dissociation kinetics as well as affinities can be measured in a label-free fashion. Recent examples have revealed the potential of SPR analysis in glycotope mapping, for instance to study the mode of binding of mAbs to of *Yersinia pestis* LPS structures[161] and to characterize antibody furanoside recognition[162]. SPR has been used to determine whether the glycotopes bound by mAbs are found internally or on the non-reducing terminus of *Francisella tularensis* LPS.[95]

1.4.3 Liposomes as Novel Carrier Platforms

Immunogenic carrier proteins have been essential in the development of glycoconjugate vaccines. However, the high immunogenicity of commonly used carrier proteins as well as the cost associated with maintaining the cold chain have prompted the search for alternatives.[163, 164]

Novel carrier strategies feature the multivalent presentation of haptens. Encouraging vaccination results have been obtained with oligosaccharide haptens presented on virosomes, gold nanoparticles and, importantly, liposomes.[165, 166]

In contrast to carrier proteins, liposomal vaccine formulations are potentially traceless and may not interfere with the immune response against the carbohydrate antigen.[167-169] Thereby, an antigen harboring a lipophilic tag is incorporated into a phospholipid bilayer that is physically transformed to maintain a spherical shape. Liposomes can be generated in a defined fashion using purely synthetic components, rendering the manufacturing process more concise. In addition, liposomal membrane contents can be adjusted by simply varying the lipid composition, enabling a close control over the physical properties and antigen density that is displayed.[170] However, omitting the protein component may result in lower vaccine efficacy due to a lack of T cell help, and simple antigen-bearing liposomes are unlikely to invoke persistent immunological memory. Therefore, efforts have to be invested to increase immunogenicity and induce a T cell-dependent antibody response, for instance by co-formulating an immunostimulatory adjuvant. Multiple adjuvants with lipophilic properties are known that efficiently increase the potency of vaccine antigens, such as saponins,[171] monophosphoryl lipid A[172] or certain glycosphingolipids.[173] The latter group

has recently gained attention due to their interesting immunomodulatory properties. Certain glycosphingolipids, with an α-galactosylphytosphingolipid (KRN7000 or α-GalCer) originally isolated from a marine sponge as the most important representative, can activate invariant natural killer T (iNKT) cells after being presented by MHC I-like CD1d molecules found on antigen-presenting cells.[174, 175] Important progress has been brought forward by conjugating α-GalCer to isolated *S. pneumoniae* serotype 4 CPS.[85] The resulting conjugate significantly augmented the anti-polysaccharide immune response in mice. Co-administering a synthetically accessible glycosphingolipid derivative in conjunction with structurally defined oligosaccharide antigens holds great promise in the development of fully synthetic carbohydrate-based vaccines.

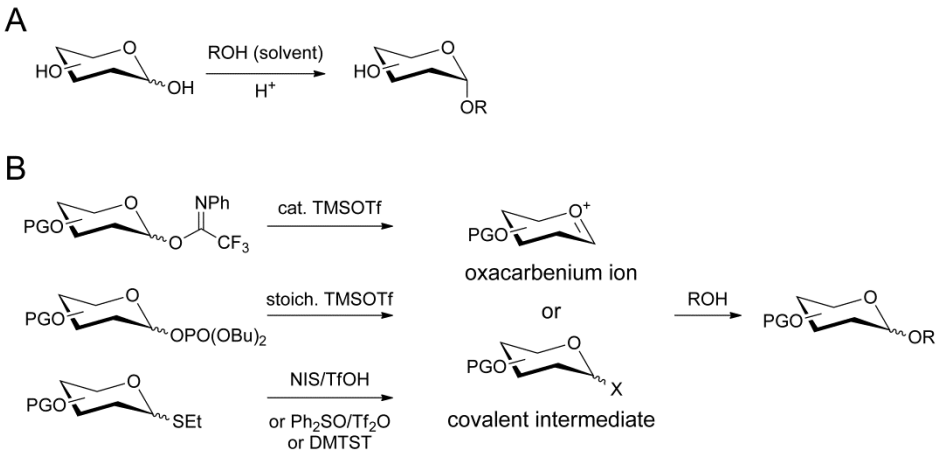
1.5 Organic Chemistry Approaches to Manufacturing Oligosaccharide-based Vaccines

1.5.1 The Glycosylation Reaction as a Key Transformation in Carbohydrate Synthesis

Although oligosaccharide synthesis is one of the oldest disciplines of organic chemistry, the details of glycosidic bond-forming reactions are still ill-defined. Multiple parameters determine the outcome of glycosylations, hence these reactions are typically the most time-consuming steps in oligosaccharide synthesis.

The glycosidic bond is a cyclic acetal that can be formally generated by condensation of a monosaccharide lactol at the anomeric center (C-1) with an alcoholic hydroxyl group (Scheme 1.2A). This simplest method of glycoside formation, the Fischer glycosylation,[176] is applicable only under special circumstances, for instance if the alcohol nucleophile can be used in large excess and if all reactants are compatible with harsh, acidic conditions. For all other glycosylation reactions, monosaccharides are equipped with reactive leaving groups to furnish glycosylating agents that can be activated under suitable conditions. The most commonly used leaving groups include thioglycosides that are susceptible towards oxidative or thiophilic activators, and glycosyl imidates and phosphates that can be activated using Lewis or Brønsted acids (Scheme 1.2B).[177] In

conventional kinetic considerations, all glycosylations employing these leaving groups proceed via the formation of an oxacarbenium ion that is then trapped with a nucleophilic alcohol. However, it has been found that isolated oxacarbenium ions are rare and energetically unfavored, and it is likely that ion pairs or covalent intermediates predominate depending on the leaving groups and activating agents used.[178] Thus, the choice of the leaving group can influence both reactivity- and stereoselectivity-determining steps of a glycosylation.



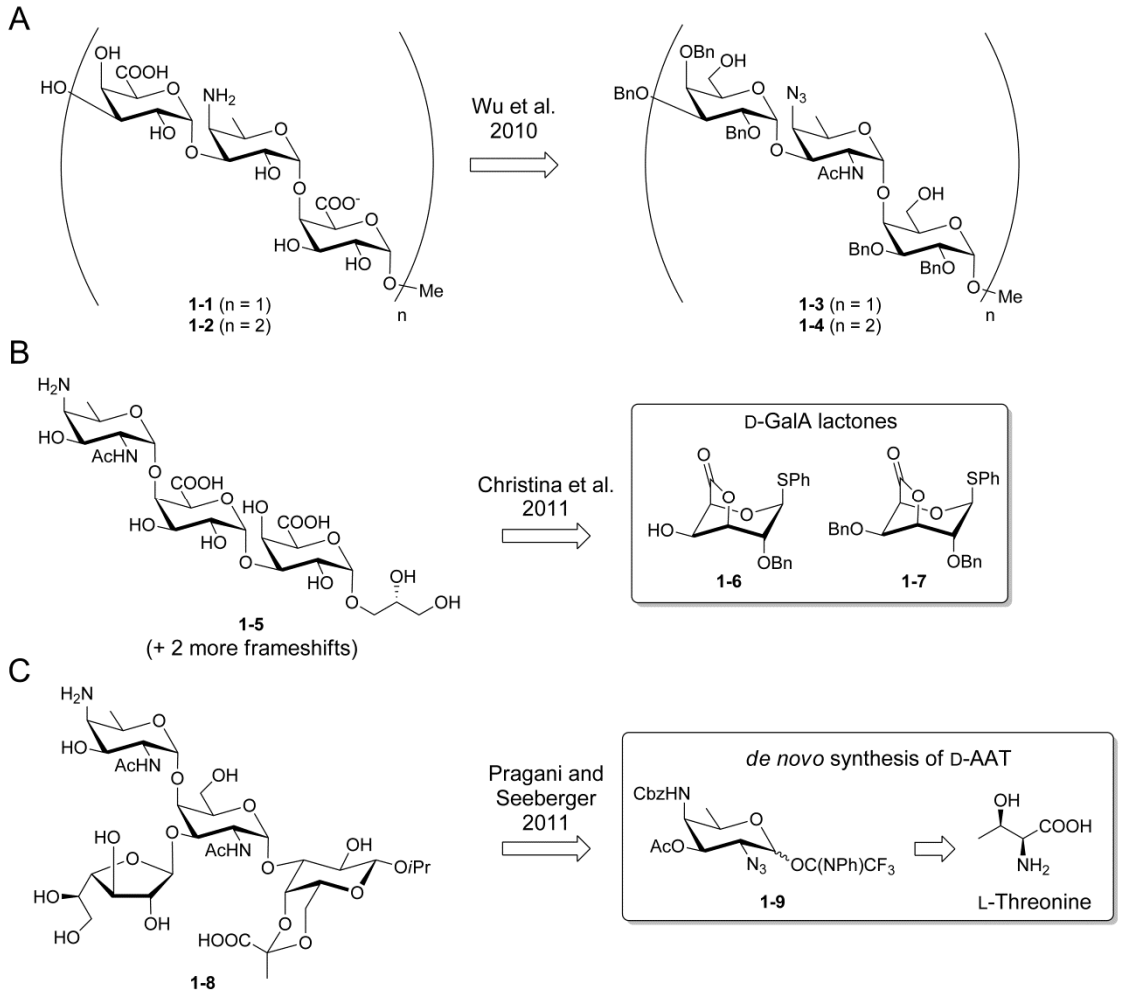
Scheme 1.2. Reaction pathways of chemical glycosylations. *A*, Fischer glycosylation. α-configured products are favored. *B*, glycosylation pathways using common leaving groups. Glycosylating agents employed in this work are shown, leading to product formation through different proposed intermediates.

Thioglycosides are particularly useful glycosylating agents due to their compatibility with the majority of chemical transformations used in building block synthesis. Furthermore, the reactivity of thioglycosides in glycosylation reactions can be altered by applying different activating agents. (Scheme 1.2B). Reactive activators include the Ph_2SO/Tf_2O and NIS/TfOH systems that can be used to activate unreactive glycosylating agents at low temperatures, while the mild thiophilic activator dimethyl (methylthio) sulfonium trifluoromethanesulfonate (DMTST) is suited to activate more reactive thioglycosides.

Differences in glycosylating agent reactivities are associated with the protecting group patterns found elsewhere in the molecule: the deactivating ("disarming") property of ester groups is contrasted by the activating ("arming") effect of ether groups.[177]

1.5.2 Challenges Associated with the Synthesis of Zwitterionic Polysaccharide Fragments

Synthetic carbohydrates are usually equipped with an amino group found at the reducing end of the glycan chain to facilitate chemoselective conjugation with reporter probes or carrier proteins (*see* above). Attention has to be paid when the target molecule itself harbors a monosaccharide with a free amine. The key structural feature of Zwitterionic Polysaccharides is the presence of both positive and negative charges in every repeating unit (Scheme 1.3A). Thereby, the positive charge is usually provided by the rare monosaccharide D-AAT.



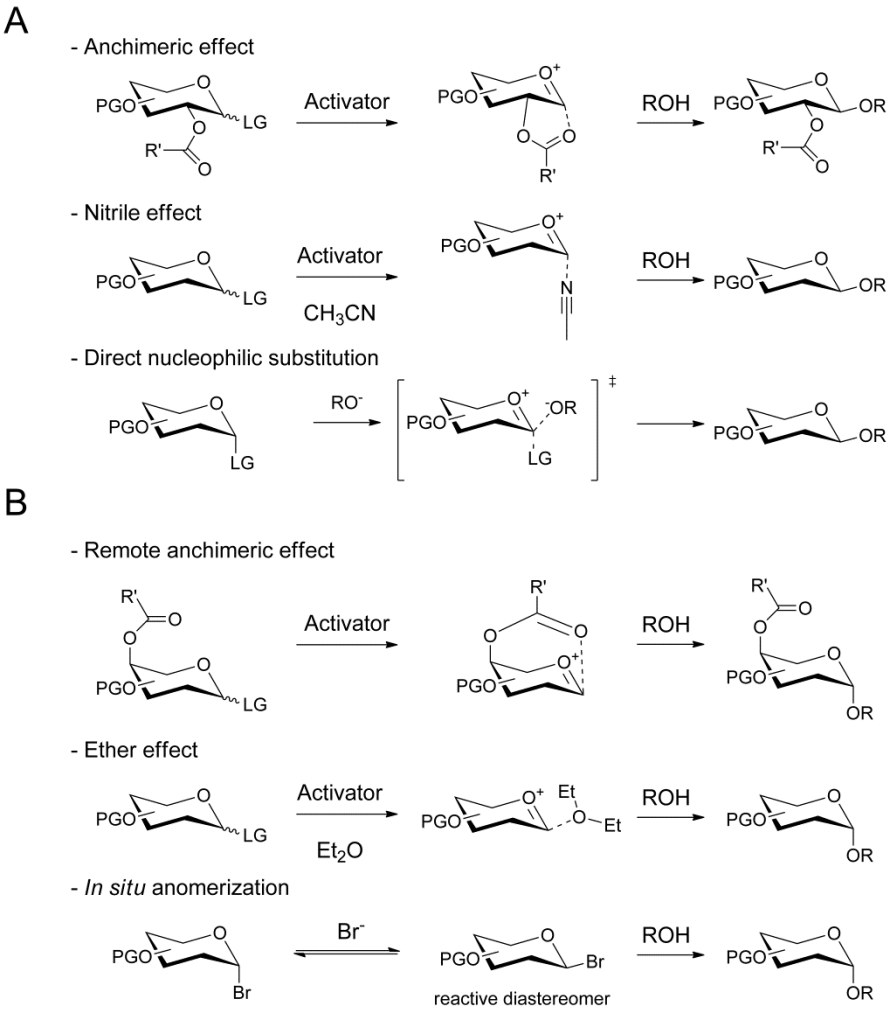
Scheme 1.3. Reported total syntheses of oligosaccharide representing the ZPSs Sp1 and PS A1. *A*, Bundle's synthesis of Sp1 tri- and hexasaccharide **1-1** and **1-2**, respectively.[133] *B*, Codee's synthesis of Sp1 trisaccharide **1-5** and two more FSs of the same polysaccharide, making use of galacturonic acid lactones **1-6** and **1-7**.[134] *C*, total synthesis of PS A1 repeating unit **1-8** utilizing D-AAT building block **1-9** by Pragani and Seeberger.[132, 179]

Several total syntheses have targeted the generation of ZPS fragments. The preparation of monomeric and dimeric repeating units of *S. pneumoniae* Sp1 (**1-1** and **1-2**, respectively) by Bundle and coworkers featured the late stage oxidation of multiple galactose moieties to the respective galacturonic acids in the precursors **1-3** and **1-4** (Scheme 1.3B),[133, 180] while Codee and colleagues employed galacturonic acid lactones **1-6** and **1-7** to furnish all three trisaccharide FSs of the same polysaccharide, such as **1-5** (Scheme 1.3C).[134] In an effort to generate repeating unit **1-8** of *B. fragilis* PS A1, the assembly of the tetrasaccharide backbone proved to be somewhat demanding.[181] The synthetic challenge was ultimately overcome by Pragani and Seeberger by carefully developing an appropriate synthetic strategy and employing *de novo*-accessible D-AAT building block **1-9** that partook in high-yielding glycosylation reactions (Scheme 1.3C).[132, 179]

None of these synthetic ZPS fragments is amenable for conjugation to reporter moieties without further modification. Due to the free amino group found on D-AAT, the use of commonly employed amine-containing linkers is precluded. Thus, the generation of conjugation-ready ZPS fragments should likely employ linker chemistry that is orthogonal to free amines.

1.5.3 Stereoselectivity of Glycosylation Reactions

A particular challenge of glycosidic bond formation is the construction of the desired stereoconfiguration at the anomeric carbon atom. 1,2-*trans* linkages (e.g. "β" in *gluco* and *galacto* series and "α" in *manno* series) can generally be produced with high stereoselectivities by providing anchimeric assistance at the 2-position of the same glycosylating agent (Scheme 1.4A). Alternatively, nitrile solvents significantly alter the 1,2-*trans*-stereoselectivities in many cases.[177] Axial leaving groups can be displaced in an S_N2-reaction by alkoxide nuclophiles to give β-configured 2-deoxy glycosides.[182]

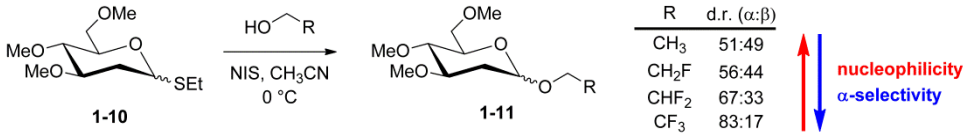


Scheme 1.4. Methods of stereoselective glycosylation. *A*, common strategies to increase β-selectivity (1,2-*trans* in *gluco* and *galacto* series). *B*, common strategies to increase α-selectivity (1,2-*cis* in *gluco* and *galacto* series).

The introduction of 1,2-*cis*-linkages is often accompanied by considerable amounts of the respective 1,2-*trans*-stereoisomer that lowers synthetic efficiency. A great number of concepts have been devised to optimize stereoselectivity. Similar to the anchimeric effect used to enhance 1,2-*trans*-stereoselectivity, remote anchimeric participation can be achieved by furnishing ester protecting groups at 3-, 4- or 6-positions of glycosylating agents (Scheme 1.4B).[183, 184] Furthermore, ethereal solvents are known to increase the α-selectivity of glycosylations using glucose and galactose glycosylating agents, for instance.[177] A classical strategy to improve 1,2-*cis*-selectivities is the *in situ*-anomerization of glycosyl halides. Thereby, an excess of a halide source is added to generate small amounts of the less stable, but more reactive anomeric halide in equilibrium that, in turn, reacts with an alcohol nucleophile in an S_N2-like reaction.[177] Similarly, anomeric triflates as intermediates in glycosylation reactions have been found

to significantly increase 1,2-*cis*-selectivities in certain cases, such as structurally confined 4,6-*O*-benzylidene mannose glycosylating agents.[185, 186] Recently, intermolecular hydrogen bonding has been introduced as a concept to increase 1,2-*cis*-selectivities, and was successfully applied in the total syntheses of α-glucans.[187]

A shortfall of most methods so far reported for the 1,2-*cis*-selective installation of glycosidic bonds is the low reproducibility for glycosylating agents with different monosaccharide configurations, protecting group patterns and leaving groups. Furthermore, alterations in the nature of the nucleophile are usually associated with unpredictable stereochemical outcomes. For instance, the 1,2-*cis*-selective introduction of protected amino alcohols as precursors of reducing-end linkers used for conjugation is a recurring obstacle in oligosaccharide synthesis, and conventional stereoselective glycosylation strategies often fail in these cases. In contrast to carbohydrate-borne hydroxyl groups that are classically used as nucleophiles to compare and optimize methodologies,[188-192] these primary alcohols differ in both steric demand and nucleophilicity, two major determinants to control stereoselectivities in glycosylations.



Scheme 1.5. Effect of alcohol nucleophilicity on the stereoselectivity of glycosylation reactions. Modified from Beaver et al.[193] NIS = *N*-iodosuccinimide.

The effect of nucleophilicity on the stereochemical outcome of glycosylation reactions has been the subject of elegant studies by Woerpel and colleagues.[193] By comparing ethanol with various 2-haloethanols as nucleophiles in glycosylation reactions, it has been found that stereoselectivity inversely correlates with nucleophilicity, and weakly nucleophilic alcohols produced high α-selectivities in 2-deoxyglucose series such as **1-10** (Scheme 1.5). It was proposed that reactions that proceed at the diffusion limit, such as those using highly nucleophilic alcohols, lead to an erosion of stereoselectivity while reactions with weaker nucleophiles are more susceptible to stereoelectronic factors in the reaction intermediate(s).[193-196] As an equivalent of the so-called anomeric effect that prefers α-glycosides in rarely-performed thermodynamically controlled glycosylations, a "kinetic anomeric effect" has been discussed and correlated to several stereoelectronic factors in the glycosylating agent.[176, 197-200] However, the precise

mechanisms that govern the increase of α-selectivity are yet to be determined. Furthermore, the effects of nucleophilicity on the outcome of a glycosylation reaction have not been harnessed to produce biologically active, conjugation-ready glycans thus far.

1.6 Aims of This Thesis

The objective of this work was to contribute to the improvement of synthetic oligosaccharide-based vaccine generation on multiple levels. To gain insight into the immune recognition of ZPSs, a synthetic strategy was devised that would yield ZPS substructures with an orthogonal functional group for conjugation despite the presence of a variety of unusual functionalities within these structures (Chapter 2). Immunological and biochemical evaluation of these glycans *in vivo* and *in vitro* would elucidate the mechanisms of ZPS immune recognition and reveal their potency as vaccine antigens.

The search for an optimal vaccine antigen against ST8 inspired the development of a straightforward synthetic route to produce a collection of ST8-related oligosaccharides (Chapter 3). These glycans would help to characterize available antibody samples to allow for the rational design of vaccine antigens. Antibodies raised against these antigens after immunization of mice could be compared to available mAb samples raised against the native polysaccharide to assess the details of polysaccharide recognition *in vitro* and protection *in vivo*.

Based on recent mechanistic findings that proposed a connection between alcohol nucleophilicity and glycosylation stereoselectivity, a novel linker was designed with an inherent propensity to give high 1,2-*cis*-selectivities during glycosylation reactions (Chapter 4). Screening the compatibility of this linker with a range of different reaction conditions and glycosylating agents would reveal its suitability for oligosaccharide synthesis. Overcoming the drawbacks of stereoselective glycosylation reactions would immensely facilitate the generation of future oligosaccharide haptens.

2 Synthesis and Immunological Evaluation of Conjugation-ready Zwitterionic Oligosaccharides

This chapter has been modified in part from the following article:

Synthesis of conjugation-ready zwitterionic oligosaccharides by chemoselective thioglycoside activation. B. Schumann, R. Pragani, C. Anish, C. L. Pereira and P. H. Seeberger, *Chem. Sci.*, 2014, **5**, 1992-2002.[201] http://dx.doi.org/10.1039/C3SC53362J

2.1 Introduction

2.1.1 Commensal Bacteria

Human mucosal surfaces are constantly exposed to a myriad of microbes. The gastrointestinal tract alone is colonized by 10^{14} bacteria of one thousand different species.[202, 203] While a fraction of these are potentially pathogenic, the vast majority of gut bacteria live in coexistence with the host in a commensal relationship. Commensal bacteria may support the host in a symbiotic fashion to degrade nutrients and suppress pathogenic organisms. Furthermore, these bacteria are crucial in maintaining the integrity of the gut epithelial barrier.[202, 204]

The host immune system must be tailored to distinguish between pathogenic and commensal organisms. Tolerance must be established towards the latter, while the former must be recognized and fought by both adaptive and innate immune systems. Any disturbance in this sensitive homeostasis may result in the activation of pro-inflammatory signal cascades by the host and result in chronic inflammatory states, such as Crohn's disease.[202, 205] The primary mediators of these processes are pro-inflammatory cytokines such as interferon (IFN)-γ, IL-17 or IL-23.[203] Commensal bacteria have

developed strategies to contribute to the maturation of the host immune system.[206, 207] Although the precise mechanisms of immunomodulation are complex and still ill-defined, certain molecules presented by commensal organisms have been found to play an important role in this process.

2.1.2 Zwitterionic Polysaccharides

Zwitterionic Polysaccharides (ZPSs) harbor both positive and negative charges in their repeating units. The most prominent and best-studied ZPS representatives are found on the surfaces of *Streptococcus pneumoniae* serotype 1 (ST1 CPS, also named "Sp1" polysaccharide) and *Bacteroides fragilis* ("PS A1" polysaccharide).[118] Both bacteria asymptomatically colonize mucosal surfaces of healthy individuals. *B. fragilis* is a gut commensal and induces sterile abscesses upon intra-abdominal lesions during surgery.[122] *S. pneumoniae* is found in the respiratory tract of healthy humans and can cause invasive disease upon colonization of otherwise sterile sites, especially in immunocompromised individuals.[208]

ZPSs are key players in the immunomodulatory function of commensal bacteria. Isolated ZPSs have the ability to correct abnormalities in the immune system of germ-free mice, such as reduced Peyer's patches and a dysbalance between two subsets of helper T cell responses, T_H1 and T_H2.[209] Furthermore, ZPSs can induce the expansion of $CD4^+$ T cells mediated by IL-12, a signaling molecule produced by stimulated dendritic cells.[209] These effects help the host to develop a mature immune system. A hallmark of ZPSs is the potency to induce anti-inflammatory immune responses, including the expansion of regulatory T cells that, in turn, protect from inflammatory disease.[123, 129, 210]

ZPSs are the first and only pure carbohydrates so far known to be presented on MHC class II molecules of APCs.[125, 211-214] After internalization by yet unknown receptors, ZPSs are depolymerized by nitric oxide in endosomal compartments. The polysaccharide fragments are loaded onto MHC class II molecules, and the resulting complex is transported to the APC surface.[125, 126, 211] An immunological synapse is then formed between APC and $CD4^+$ T cell, leading to T cell expansion and cytokine production.[124, 215]

Despite the progress in understanding the modes of action of ZPSs, the precise mechanisms of immunomodulation are still unclear. Mechanistic investigations have been complicated by the routine use of isolated, labeled polysaccharides that are structurally heterogeneous and potentially harbor contaminations by other cellular components.[100] Furthermore, the process of purification, fragmentation and labeling inevitably changes the structure of the glycan and may influence the immunological effects observed with ZPS probes. In addition, the role of individual monosaccharides in immunomodulation cannot be uncovered with isolated polysaccharides. Albeit the rare amino sugar D-AAT is part of the repeating units of both Sp1 and PS A1, the role of this monosaccharide for ZPS immune recognition has not been studied in detail. Thus, defined synthetic zwitterionic oligosaccharides are necessary tools to study immune recognition of ZPSs.

2.1.3 *Streptococcus pneumoniae* Serotype 1

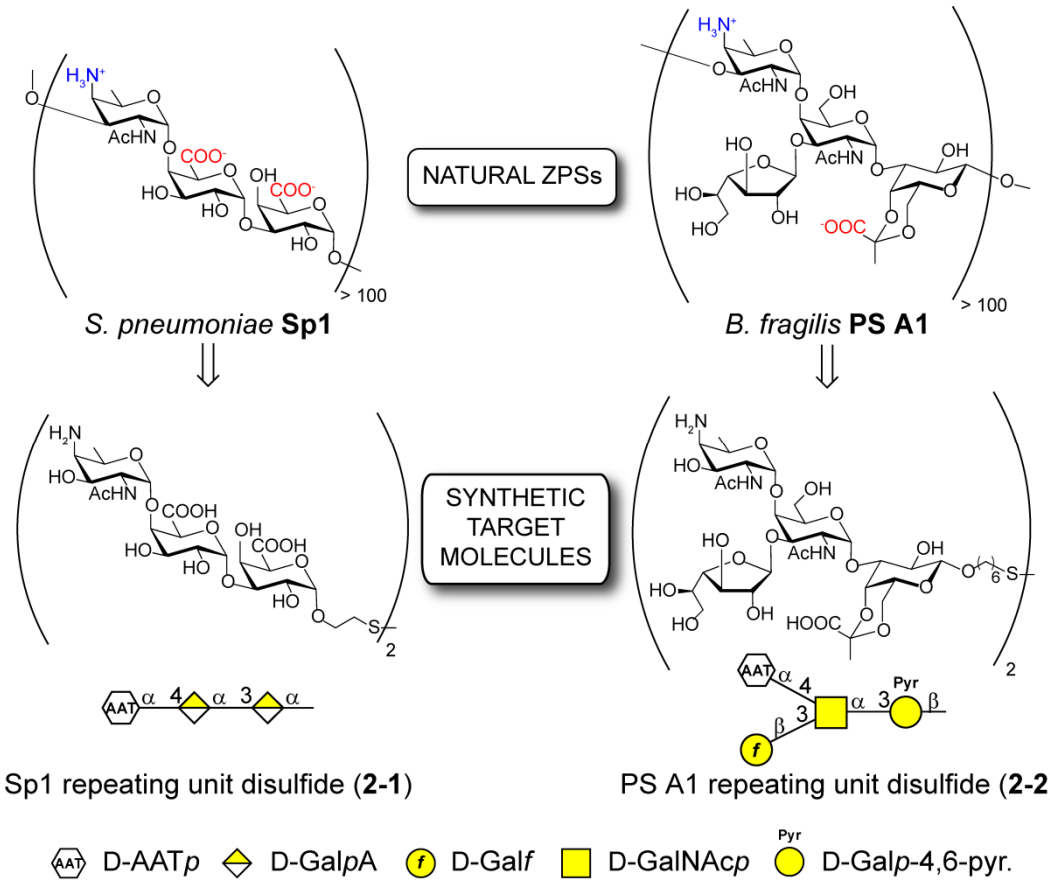
The immunomodulatory function of the ZPS representative Sp1 suggests a role of *S. pneumonaie* serotype 1 (ST1) as a facultative commensal bacterium.[125, 129, 216] Interestingly, bacterial lineages closely related to *S. pneumoniae* are purely commensal.[217] However, the incidence of pneumococcal infections is critically dependent upon environmental factors, such as nutrition and the availability of medical treatment.[218, 219] Developing countries are thus highly susceptible to IPD. Among the >90 serotypes described so far, ST1 is particularly aggressive and is the predominant serotype causing meningitis in sub-Saharan Africa.[220, 221] ST1 is the serotype with the highest incidence of IPD in children in Asia.[222]

Albeit ST1 has been included into 10- (Synflorix®) and 13-valent (Prevenar13®) PCV formulations, levels of functional anti-ST1 CPS antibodies raised by these vaccines have been low in clinical evaluations, with limited opsonophagocytic activity of immune sera from immunized humans.[80, 223-225] Additionally, contradictory results have been reported regarding the efficacy of ST1 conjugate vaccines in Africa.[226-228] Since the conjugation strategies of both marketed vaccines employ amine-reactive chemical modifications (1-cyano-4-dimethylaminopyridinium tetrafluoroborate activation and reductive amination, respectively),[80, 229] derivatization of the amino group found in D-AAT may mask crucial epitopes and compromise vaccine efficiency. Thus, a chemically

defined ST1 glycoconjugate vaccine is needed that does not modify the D-AAT moiety during conjugation.

2.1.4 Conjugation-Ready Zwitterionic Oligosaccharides

Both Sp1 and PS A1 polysaccharides are of high molecular weight and harbor a variety of highly unusual monosaccharides, even for bacterial glycans (Scheme 2.1). [126, 230, 231] The repeating unit of *S. pneumoniae* Sp1 is a trisaccharide consisting of two D-galacturonic acid moieties and D-AAT.[16, 17, 232, 233] The native *B. fragilis* PS A1 repeating unit is a branched tetrasaccharide, and the positive and negative charges are found on D-AAT and 4,6-*O*-pyruvalated D-galactose moieties, respectively.[234]



Scheme 2.1. Natural ZPSs and synthetic target molecules.

To date, several homogeneous ZPS fragments have been prepared by chemical synthesis, including a PS A1 tetrasaccharide and a Sp1 hexasaccharide.[132-134, 180] While these glycans are valuable tools to study the structural requirements of ZPS recognition, they do not bear a linker readily capable of chemoselective conjugation. In turn, forging an orthogonal linker at the reducing end of an oligosaccharide enables the conjugation to

reporter moieties, such as carrier proteins and microarray surfaces.[156, 235-239] In most cases, synthetic glycans are equipped with amine-containing linkers at the reducing end to form adducts with suitable electrophiles. Thiol linkers have been used in the conjugation of oligosaccharides to proteins, gold nanoparticles and surfaces.[169, 239-243] However, the thiol moiety is usually introduced at the very end of a synthetic route due to incompatibilities with oxidation reactions in oligosaccharide assembly, such as thioglycoside activation.[169, 238-245] Thus, thiol-linked glycans have seen limited use for oligosaccharide conjugation chemistry due to shortfalls associated with their synthesis.

2.1.5 Liposomal Display of Antigen-Adjuvant Systems as Promising Vaccine Formulations

The group of α-galactosylceramides that engage APC-associated CD1d presentation has recently gained attention due to their unique immunomodulatory properties. The glycolipid-CD1d complex is recognized by a T cell receptor bearing a genetically invariant α-chain (Vα14Jα18 in mice, Vα24Jα18 in humans) on a subset of natural killer T (NKT) cells called iNKT cells, thereby inducing a cytokine burst.[173] The predominant cytokines that play a role in iNKT-mediated immune responses are IL-4 and IFN-γ, leading to the recruitment of further immune cells and, eventually, the enhancement of immune responses.[173, 246]

The efficacy of vaccinations including KRN7000 or similar glycosphingolipids as adjuvants has been studied in pre-clinical and clinical trials.[173, 247, 248] Bendelac, Savage and coworkers co-formulated a KRN7000 derivative with a lipid-bearing tetrasaccharide derived from *S. pneumoniae* ST14 CPS in liposomes that elicited a long-lasting IgG response after immunization of mice.[249, 250] Care has to be taken with respect to the immunization regime: It is widely known that iNKT cells become anergic after systemic stimulation with α-galactosylceramides,[251-253] and a boost immunization must not be administered before these cells exit anergy. The optimal immunization regime is thus not known yet, and different schedules have been followed.[249, 250]

Here, the first total syntheses of conjugation-ready repeating units of the two most prominent ZPSs, *S. pneumoniae* Sp1 (**2-1**) and *B. fragilis* PS A1 (**2-2**), and their immunological characterization after conjugation to reporter moieties is disclosed

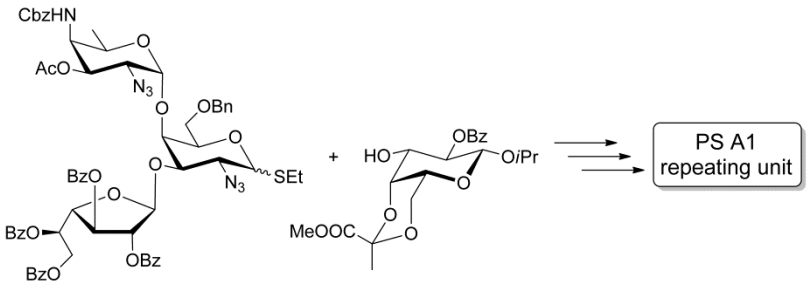
(Scheme 2.1). The introduction of a thioether-containing linker at an early stage of the synthesis called for establishing a method to chemoselectively activate thioglycosides in the presence of benzylthioethers. A collection of synthetic fragments of ZPS repeating units **2-1** and **2-2** were prepared and conjugated to glycan microarray slides and a carrier protein by virtue of the appended thiol groups. It was found that D-AAT plays a crucial role in the immune recognition of ZPS fragments, but a CRM197-D-AAT glycoconjugate did not raise an immune response against native ZPSs. Sp1 trisaccharide **2-1** was then studied as a vaccine hapten against *S. pneumoniae* ST1 both as a carbohydrate-protein conjugate and displayed on liposomes including the immunomodulatory adjuvant KRN7000.

2.2 Results

2.2.1 Development of a Chemoselective Thioglycoside Activation Strategy

Synthetic *S. pneumoniae* Sp1 and *B. fragilis* PS A1 disulfides (**2-1** and **2-2**, respectively) were targeted as homogeneous conjugation-ready ZPS fragments to study ZPS biology (Scheme 2.1). The use of an amine-functionalized linker toward this end was precluded by the presence of free primary amines in both ZPSs, which would complicate site-selective conjugation. Thiol groups can be chemoselectively coupled with suitable electrophiles in the presence of free amines.[254-256] Thus, oligosaccharides were targeted that were equipped with a thiol linker at the reducing end of the fragments as shown in **2-1** and **2-2** from the outset.[i] Introduction of the thiol linker at an early stage of the synthesis was proposed as this approach could be translated to solid-phase synthesis that originates from the reducing end, and also renders the synthesis more convergent. However, in an earlier synthesis of the PS A1 tetrasaccharide repeating unit,[132] the key [3+1] glycosylation could only be executed using thioglycoside chemistry (Scheme 2.2). Literature precedence did not provide any indication as to whether a protected thiol would survive thioglycoside activation conditions.[238, 244, 245]

[i] ZPS fragments **2-1** and **2-2** were furnished with different linkers in line with studies on the immunological implications of linker length.



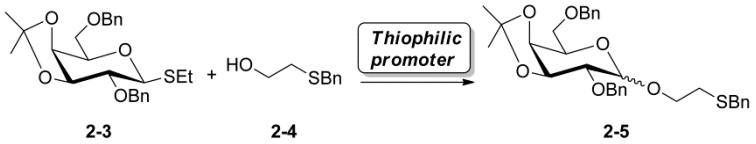
Scheme 2.2. Key step in a previous PS A1 synthesis.[132]

Thus, known thioglycoside **2-3**[257] and thioether-containing alcohol **2-4**[258] were used in a model glycosylation to evaluate the chemoselectivity of different thioglycoside activation methods (Table 2.1). The use of strong promoters, such as the well-known Ph_2SO/Tf_2O[259, 260] combination or the more recently reported Me_2S_2/Tf_2O system,[261] resulted in product formation in 43% and 51% yield, respectively (Table 2.1, entries 1 and 2). While in both cases a considerable amount of the hydrolyzed thioglycoside could be identified as a major side product, thioether **2-4** could not be recovered when Ph_2SO/Tf_2O was used as an activator system (entry 1). In the presence of NIS/TfOH[262] as a promoter mixture, the glycosylation reaction did not proceed to completion (Table 2.1, entry 3), resulting in 54% yield of **2-5** and recovery of unreacted thioglycoside **2-3** and alcohol **2-4**. Employing 3 Å acid-washed molecular sieves instead of unwashed 3 Å molecular sieves did not improve the outcome of the glycosylation (Table 2.1, entry 4). Incomplete turnover in these reactions was unexpected because of the highly reactive nature of thioglycoside **2-3**.[263] Indeed, a test glycosylation between **2-3** and monobenzyl ethylene glycol instead of thioether **2-4** with NIS/TfOH led to complete conversion (*see* Experimental Section). Thus, it is proposed in these glycosylation reactions (Table 2.1, entries 3 and 4) that the electrophilic iodonium species is in part sequestered by the alkyl benzylthioether moiety in **4**, resulting in incomplete turnover.[238]

When MeOTf was used as a promoter in presence of the acid scavenger 2,4,6-tri-*tert*-butylpyridine, only traces of product were obtained (Table 2.1, entry 5). Methylation of the benzyl thioether in **2-4** and **2-5** was observed instead, indicating that MeOTf does not discriminate between the thioglycoside and alkyl benzylthioether functional groups. Using mild activating agent dimethyl (methylthio) sulfonium trifluoromethanesulfonate (DMTST)[264] as a promoter provided glycoside **2-5** in 76% yield (Table 2.1, entry 6), with hydrolysis of the glycosylating agent being the only observable side reaction. Thus,

DMTST was found to be the best promoter for the chemoselective activation of thioglycoside **2-3** in presence of the benzyl thioether found in **2-4**. Of note, the diastereoselectivities of the depicted glycosylations between **2-3** and **2-4** were in the range of 2.1:1 to 2.4:1 (α:β). Neither additives, such as DMF[265] or thiophene,[190] nor the use of toluene/1,4-dioxane as a solvent mixture[266] altered this ratio significantly.

Table 2.1. Compatibility of thioglycoside activation methods with alkyl benzylthioether **2-4**.



2-3 **2-4** **2-5**

Entry[a]	Promoter (equiv.)[b,c]	Temperature	Yield, %
1	Ph_2SO/Tf_2O (1.1/1.1), TTBPy (1.5)[d,e]	-60 °C to -10 °C	43[g]
2	Me_2S_2/Tf_2O (1.5/1.5), TTBPy (1.5)	-40 °C	51
3	NIS/TfOH (1.5/0.2)	-40 °C to 5 °C	54[h]
4	NIS/TfOH (1.5/0.2)[f]	-40 °C to 5 °C	42[h]
5	MeOTf (1.2), TTBPy (2.0)	0 °C to r.t.	< 10[g]
6	DMTST (1.5), TTBPy (2.0)	0 °C	76

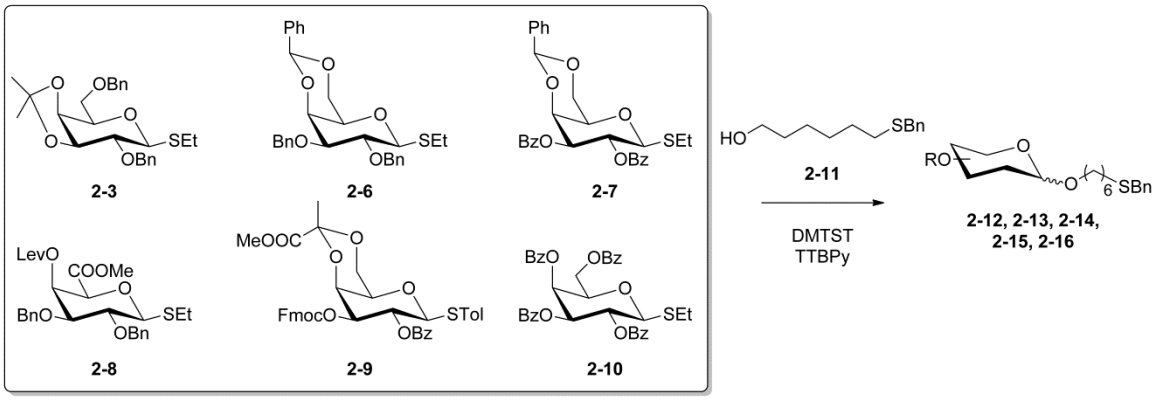
[a]1.0 equiv. glycosylating agent, 1.5 equiv. alcohol **2-4**. [b]Reactions performed in CH_2Cl_2/Et_2O 1:3 (v/v). [c]3 Å mol. sieves were used. [d]Reaction performed in CH_2Cl_2. [e]Pre-activation of glycosylating agent. [f]3 Å-AW mol. sieves were used. [g]Thioether decomposed. [h]Reaction incomplete. DMTST = dimethyl (methylthio) sulfonium trifluoromethanesulfonate. MeOTf = methyl trifluoromethanesulfonate. NIS = *N*-iodosuccinimide. Tf_2O = trifluoromethanesulfonic anhydride. TfOH = trifluoromethanesulfonic acid. TTBPy = 2,4,6-tri-*tert*-butylpyridine.

Next, the substrate scope of the DMTST-mediated thioglycoside activation in the presence of benzyl thioether **2-11** was evaluated (Table 2.2).[ii267] Nucleophile **2-11** was used in glycosylation reactions with reactive thioglycosides **2-3** and **2-6**[268] using DMTST activation at low temperature to provide glycosides **2-12** and **2-13** in 70% and 75% yield, respectively (Table 2.2, entries 1 and 2). Glycosylating agent **2-7**[269] (Table 2.2, entry 3) required reaction optimization due to the presence of the participating benzoyl ester protecting group at C2. Employing an excess of TTBPy (neutral conditions) led to the formation of high amounts of the respective orthoester as a side product, whereas benzylidene cleavage was observed when the scavenger was omitted (acidic conditions). It was found that using 1.0 to 1.2 equivalents of scavenger and two equivalents of

[ii] Alcohol **2-11** was chosen instead of alcohol **2-4** for further method evaluation because the respective products were easier to purify by flash chromatography.

DMTST produced a weakly acidic environment that yielded glycoside **2-14** without any major side reactions. Activation of galacturonic acid thioglycoside **2-8** (*see* Experimental Section) in the presence of alcohol **2-11** provided glycoside mixture **2-15** in 52% yield over two steps after removal of the C4 Lev ester (Table 2.2, entry 4). The moderate yield is consistent with previous reports on the low reactivity of galacturonic acid glycosylating agents.[134, 270-273] Nevertheless, complete chemoselectivity was observed in the activation of glycosylating agent **2-8**, leaving the alkyl benzylthioether intact.

Table 2.2. Scope and limitations of the chemoselective thioglycoside activation with DMTST/TTBPy.



Entry[a]	Thioglycoside[c]	DMTST/ TTBPy, equiv.	Time/temp.	Product (α:β)	Yield, %[e]
1	**2-3**	1.5/2.0	1 h/0 °C	**2-12** (1:1.6)	70
2	**2-6**	1.5/2.0	2 h/-10 °C	**2-13** (1:1.1)	75
3	**2-7**	2.0/1.2	1.5 h/r.t.	**2-14** (0:1)	70
4[b]	**2-8**	1.5/2.0	8 h/r.t.	**2-15** (4:3)	52[f]
5	**2-9**	1.8/1.1	16 h/r.t.	**2-16** (0:1)	58[f]
6	**2-10**	2.0/1.1	>100 h/r.t.	-	< 20
7	**2-3**[d]	1.5/2.0	2 h/0 °C	**2-12** (1:1.6)	91

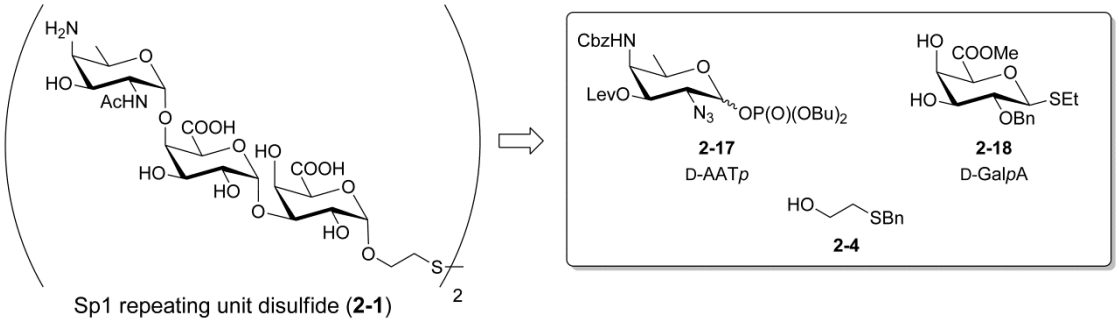
[a]Reactions performed in CH_2Cl_2. [b]Reaction performed in CH_2Cl_2/Et_2O 1:3 (v/v). [c]1.0 equiv. glycosylating agent, 1.5 equiv. alcohol **2-11**. [d]1.4 equiv. glycosylating agent, 1.0 equiv. alcohol **2-11**. [e]Isolated yields. [f]Isolated yield after consecutive step.

As a brief look at other anomeric thioether leaving groups, the chemoselective activation of *p*-tolyl thioglycoside **2-9**[iii][132] in the presence of the benzylthioether found in **2-11** was executed (Table 2.2, entry 5). It is known that aryl thioglycosides are less readily activated by DMTST than alkyl thioglycosides.[274] However, even tolyl thioglycoside **2-9** was chemoselectively activated by DMTST, giving pyruvalated

[iii] Building block **2-9** was synthesized by Dr. Rajan Pragani.

galactoside **2-16** in 58% yield after removal of the C3 Fmoc group. Finally, perbenzoylated galactose thioglycoside **2-10**[275] was employed as a highly electron-deficient glycosylating agent (Table 2.2, entry 6). Even after prolonged stirring at room temperature, only very little consumption of the starting material was observed. Increased amounts of DMTST or higher reaction temperatures did not result in the formation of the desired product, but led to decomposition of the thioether group in **2-11** (data not shown). It is speculated that the reactivities of both sulfur atoms in thioglycoside **2-10** and thioether **2-11** are comparable,[276] and thus, both compete for DMTST, leading to the decomposition of **2-11**.

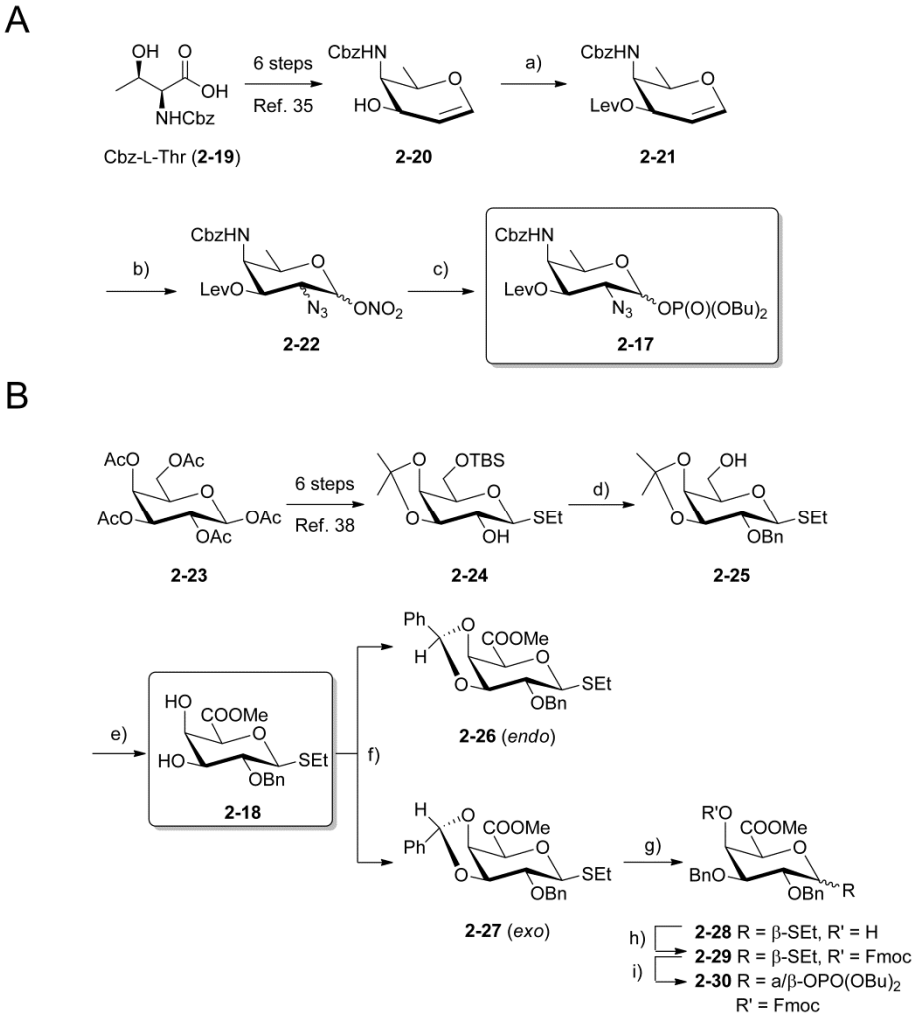
To fully confirm the chemoselectivity of DMTST-mediated thioglycoside activation, alcohol **2-11** was reacted with an excess of thioglycoside **2-3** and DMTST (Table 2.2, entry 7). Nearly full conversion of **2-11** was achieved, giving glycoside **2-12** in 91% yield without affecting the alkyl benzylthioether. Taken together, a wide range of thioglycosides of different reactivities can be efficiently activated by DMTST in presence of a benzyl thioether. However, highly deactivated thioglycosides, such as **2-10**, do not couple under these conditions.



Scheme 2.3. Retrosynthesis of Sp1 repeating unit disulfide **2-1**.

2.2.2 Total Synthesis of a Conjugation-ready Sp1 Repeating Unit Trisaccharide

With a method to conveniently introduce a benzyl thioether at an early stage in hand, attention was directed toward the synthesis of conjugation-ready zwitterionic oligosaccharides. It was envisaged that Sp1 trisaccharide dimer **2-1** could be assembled from D-AAT building block **2-17**, galacturonic acid diol **2-18**, and thioether **2-4** (Scheme 2.3).



Scheme 2.4. Synthesis of building blocks for the assembly of Sp1 repeating unit disulfide **2-1**. Reagents and conditions: a) LevOH, EDC, DMAP, pyr., CH_2Cl_2, r.t., 92%; b) CAN, NaN_3, CH_3CN, -20 °C; c) $CsOPO(OBu)_2$, DMF, r.t., 37% (two steps from 21); d) i. BnBr, NaH, DMF, 0 °C to r.t.; ii. TBAF, THF, 0 °C to r.t., 81% (two steps); e) i. $PhI(OAc)_2$, TEMPO, CH_2Cl_2, H_2O, 0 °C to r.t., 3 h; ii.AcCl, MeOH, 0 °C to r.t., 60% (two steps); f) $PhCH(OMe)_2$, TsOH, CH_3CN, r.t., 92% (1:1 *endo*:*exo*); g) TES, TFA, TFAA, 0 °C to r.t., 65%; h) FmocCl, pyr., 0 °C to r.t., 90%; i) NIS, $HOPO(OBu)_2$, CH_2Cl_2, r.t., 89%. CAN = ceric ammonium nitrate. DMAP = 4-(dimethylamino)pyridine. EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. TEMPO = 2,2,6,6-tetramethylpiperidine 1-oxyl. TES = triethylsilane. TFA = trifluoroacetic acid. TFAA = trifluoroacetic anhydride. TsOH = *p*-toluenesulfonic acid.

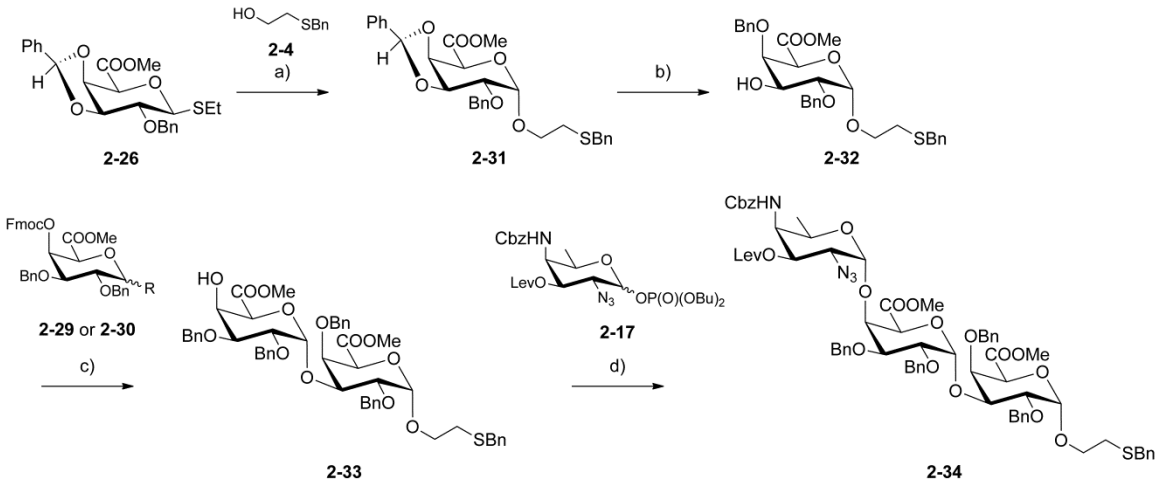
D-AAT building block **2-17** was prepared via a *de novo* synthetic route established recently, using Cbz-L-threonine **2-19** as a chiral, inexpensive precursor (Scheme 2.4A).[179] Alcohol **2-20** was condensed with levulinic acid to give ester **2-21** in 92% yield. Azidonitration provided glycosyl nitrate **2-22** as an inseparable 4:1 *galacto*:*talo* isomeric mixture.[179] Nucleophilic displacement of the anomeric nitrate with cesium dibutyl phosphate provided D-AAT phosphate **2-17** in 37% yield over two steps.[277, 278] Compared to already known D-AAT imidates,[134, 179, 279, 280] it was anticipated that a D-AAT

phosphate glycosylating agent would display enhanced stability towards decomposition while reducing the number of synthetic steps for preparation.

The native Sp1 repeating unit contains two galacturonic acid residues that are glycosylated at either C3 or C4 positions, respectively.[16, 17, 232, 233] For the generation of differentially C3-OH/C4-OH functionalized GalA building blocks, diol **2-18** was targeted as a common intermediate to minimize the total number of synthetic steps. Commercially available galactose pentaacetate **2-23** served as the starting point for the synthesis of diol **2-18**. Alcohol **2-24** was synthesized according to a literature procedure from **2-23** (Scheme 2.4B).[281] Benzyl protection of the C2-hydroxyl group followed by TBS deprotection at the C6 position afforded alcohol **2-25** in 81% yield over two steps. TEMPO-mediated oxidation and subsequent treatment with anhydrous hydrogen chloride in methanol then gave galacturonic acid diol **2-18** in 60% yield over two steps.

At this stage, attempts to introduce benzyl protecting groups under either basic (BnBr, NaH) or strongly acidic conditions (benzyl trichloroacetimidate, TfOH) resulted in the decomposition of compound **2-18** (not shown). Therefore, a two-step procedure was envisioned for the selective benzyl protection of either the C3- or C4-hydroxyl groups via intermediate 3,4-*O*-benzylidene acetals. In the first step, diol **2-18** was treated with benzaldehyde dimethyl acetal under weakly acidic conditions to furnish isomeric benzylidene acetals **2-26** and **2-27** in a 1:1 ratio in 92% overall yield. Since the regioselectivity of the second step, a reductive benzylidene acetal ring-opening, is dependent on the benzylidene configuration,[282-287] the generation of an equimolar mixture of both epimers was important for the synthesis of both galacturonic acids in Sp1 target trisaccharide **2-1**.[iv] The configurations of *endo*-acetal **2-26** and *exo*-acetal **2-27** were confirmed by HH-NOESY NMR spectroscopy (not shown).[288] As anticipated, exo-acetal **2-27** was ring-opened using TES and TFA to give dibenzyl ether **2-28** in 65% yield and complete regioselectivity. Fmoc protection of the free C4 hydroxyl group furnished carbonate **2-29** in 90% yield. To enable the screening of different glycosylating agents at a later stage, thioglycoside **2-29** was transformed into glycosyl phosphate **2-30** in 89% yield.

[iv] Dioxolane-type benzylidine protection may result in the preferential formation of one diastereomer for other monosaccharides.[284, 287]

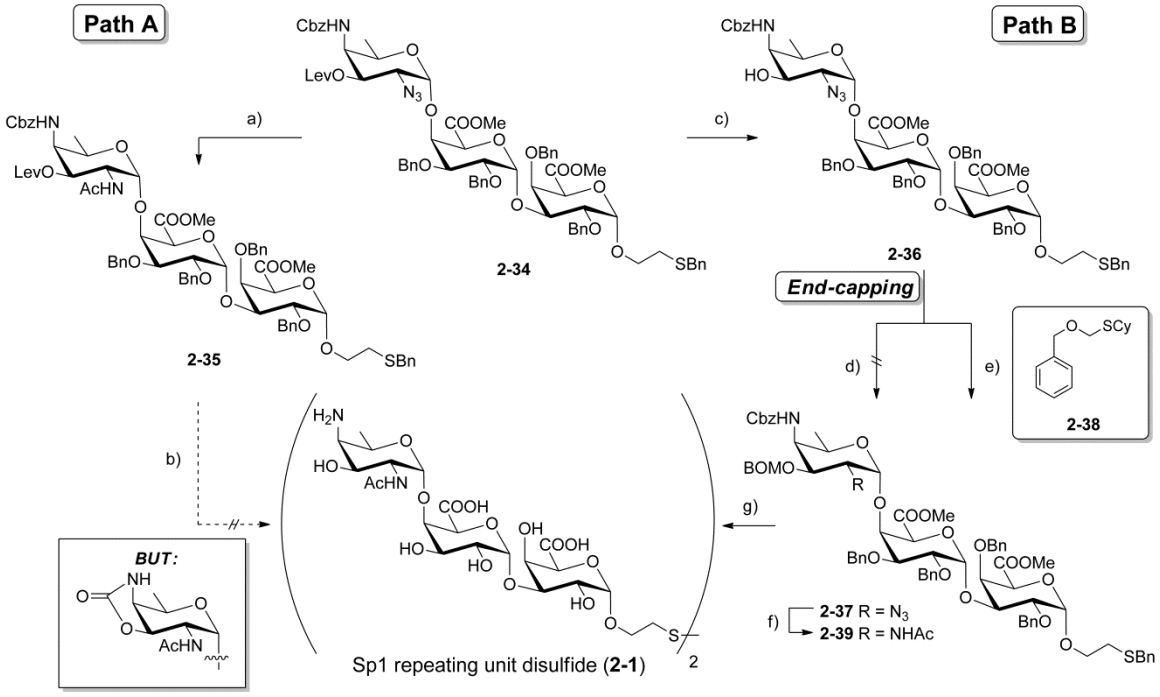


Scheme 2.5. Assembly of Sp1 trisaccharide **2-34**. Reagents and conditions: a) 1.5 equiv. **2-4**, DMTST, TTBPy, THF, 0 °C to r.t., 73% (1.7:1 α:β); b) BH_3•NMe_3, $AlCl_3$, THF, r.t., 70%. c) i. 1.5 equiv. **2-30**, TBSOTf, CH_2Cl_2, 80% (3:1 α:β) or 2.3 equiv. **2-29**, DMTST, TTBPy, Et_2O/CH_2Cl_2 3:1, r.t., 50% (3:1 α:β) ii. Et_3N, CH_2Cl_2, r.t., 68%; d) 1.5 equiv. **2-17**, TMSOTf, CH_2Cl_2, 0 °C, 85% (>19:1 α:β). TBSOTf = *tert*-butyldimethylsilyl trifluoromethanesulfonate. TMSOTf = trimethylsilyl trifluoromethanesulfonate.

With the building blocks in hand, the assembly of Sp1 trisaccharide disulfide **2-1** was undertaken (Scheme 2.5). DMTST-mediated glycosylation of thioglycoside **2-26** with thioether-containing alcohol **2-4** in THF gave glycoside **2-31** in 73% yield with modest α-selectivity. The low diastereoselectivity of this glycosylation is consistent with the results usually obtained with highly nucleophilic, primary alcohols.[193] A completely regioselective ring-opening of the *endo*-benzylidene in **2-31** using BH_3•NMe_3 and $AlCl_3$ gave alcohol **2-32** in 70% yield.[284, 289, 290] Glycosylation of alcohol **2-32** with glycosyl phosphate **2-30** (80% yield) with subsequent Fmoc removal (68% yield) provided digalacturonic acid **2-33** in a 3:1 α:β anomeric ratio. The use of ethereal solvents in the glycosylation reaction did not alter the diastereoselectivity. DMTST-mediated glycosylation of thioglycoside **2-29** with alcohol **2-32** was unable to undergo complete conversion, presumably due to the low reactivity of both alcohol and glycosylating agent.[273] Glycosylation of alcohol **2-33** with D-AAT phosphate **2-17** proceeded uneventfully using TMSOTf as the promoter. Thus, trisaccharide **2-34** was obtained in 85% yield and with complete α-selectivity, highlighting the suitability of AAT phosphate building block **2-17** in oligosaccharide synthesis.

With the fully protected Sp1 trisaccharide core (**2-34**) in hand, global deprotection strategies were assessed. Reductive acetylation of the azide followed by sequential

removal of all protecting groups present in **2-34** was attempted (Scheme 2.6, path A). Thus, acetamide **2-35** was obtained in 72% yield after treatment of **2-34** with thioacetic acid and pyridine.[132, 291] It was now important to hydrolyze the methyl esters prior to Birch reduction to prevent β-elimination of the galacturonic acid moieties under the harshly basic Birch conditions.[292] However, saponification of the methyl and Lev esters using either NaOH or Cs_2CO_3 in THF, water and methanol resulted in the concomitant cyclization of the D-AAT-NHCbz group to the corresponding cyclic carbamate. This side reaction has been associated with Cbz-protected AAT moieties previously.[132, 134]



Scheme 2.6. Global deprotection to yield Sp1 repeating unit disulfide **2-1**. Reagents and conditions: a) AcSH, pyr., r.t., 72%.; b) NaOH, H_2O, THF, 0 °C to r.t.; or Cs_2CO_3, H_2O, THF, 0 °C to r.t.; c) $H_4N_2{}^{\bullet}H_2O$, AcOH, pyr., CH_2Cl_2, r.t., quant.; d) BOMCl, *i*Pr_2NEt, CH_2Cl_2, reflux; e) **2-38**, DMTST, TTBPy, CH_2Cl_2, 0 °C to 15 °C, 84%; f) AcSH, pyr., 0 °C to r.t., 72%; g) i. NaOH, H_2O, THF, MeOH, 0 °C to r.t.; ii. Na, NH_3, *t*BuOH, THF, -78 °C, 93% (two steps).

A capping step of the D-AAT C3-OH was thus introduced to circumvent cyclization of the Cbz moiety (Scheme 2.6, path B). Capping of the D-AAT C3 hydroxyl group should ideally involve a stable, permanent protecting group. Thus, Lev deprotection of trisaccharide **2-34** gave C3 alcohol **2-36** in quantitative yield. Introduction of benzylic ethers under highly basic conditions was excluded due to the base-lability of alcohol **2-36**. Introduction of a *p*-methoxybenzyl (PMB) ether from the corresponding trichloroacetimidate under acidic conditions was also attempted, but was

not compatible with the thioether moiety.[v] Benzyloxymethyl (BOM) ethers can be introduced under mild, weakly basic conditions.[293, 294] Unfortunately, treatment of trisaccharide **2-36** with BOMCl and DIPEA in refluxing CH_2Cl_2 led to decomposition of the starting material. To enable the introduction of the BOM acetal moiety under milder conditions, a method described for the generation of formyl acetals by activation of *S*,*O*-acetal precursors was adapted.[295-297] Thereby, the established conditions on the chemoselective activation of thioglycosides in presence of thioethers were used to develop a procedure for introducing a BOM group under mild conditions. *S*,*O*-acetal **2-38**, readily prepared from commercially available reagents (*see* Experimental Section), was chosen as a suitable BOM precursor with a reactive *S*-cyclohexyl leaving group.[274] DMTST-mediated activation of *S*,*O*-acetal **2-38** under buffered reaction conditions and at low temperature converted alcohol **2-36** into the desired BOM-protected trisaccharide **2-37** in 85% yield. As anticipated, the alkyl benzylthioether remained unharmed despite the use of excess BOM precursor **2-38** as well as DMTST. This presents the mildest method so far to introduce a benzyloxymethyl ether into a complex substrate like **2-36**.

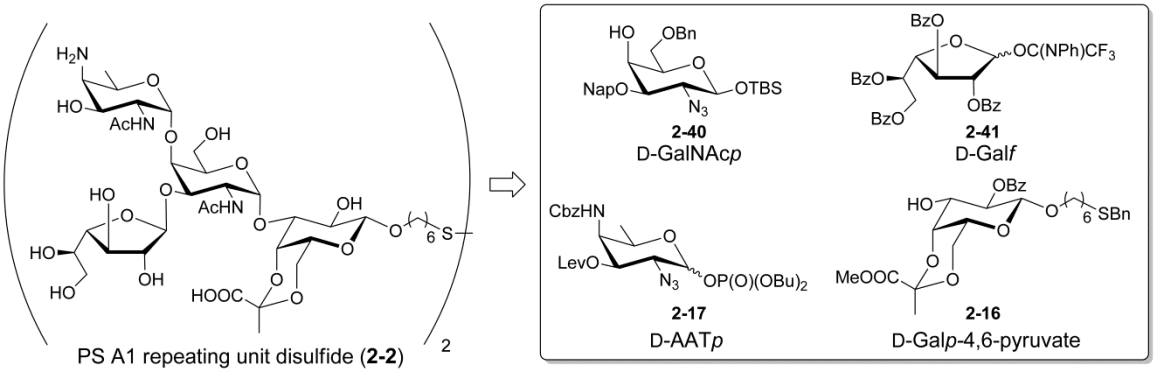
With capped trisaccharide **2-37** in hand, the stage was set for the completion of the synthesis. Reductive acetylation provided acetamide **2-39** in 72% yield. With the D-AAT C3 hydroxyl group capped, saponification of both methyl esters with NaOH in THF, methanol and water proceeded without any side reactions. Finally, Birch reduction was carried out to remove all benzyl ethers as well as the Cbz and BOM groups to afford disulfide **2-1** in 93% yield over two steps after size exclusion chromatography.[vi298] The identity of disulfide **2-1** was confirmed unambiguously by comparison of the analytical data with published results.[133, 134] Particularly, the presence of three α-anomeric linkages can be deduced from the small $^3J_{H,H}$ coupling constants of the anomeric protons (3.8 Hz each; Fig. 2.1A) in ^{1}H NMR.

[v] Treatment of thioether-containing alcohols, such as **2-36**, with PMB trichloroacetimidate under acidic conditions led to the *p*-methoxybenzylation of the thioether to form a sulfonium ion, identified by the formation of a highly polar product on TLC as well as the presence of a bis-PMB adduct by mass spectrometry.

[vi] Oligosaccharides were left at room temperature under air for 16 h after global deprotection to induce disulfide formation. Only traces of free thiol were observed.

2.2.3 Total Synthesis of a Conjugation-Ready PS A1 Repeating Unit Tetrasaccharide

Based on the lessons learned synthesizing the Sp1 repeating unit disulfide **2-1**, the preparation of PS A1 repeating unit disulfide **2-2** was undertaken. To assemble the tetrasaccharide backbone of **2-2**, the previously established [3+1] strategy was adapted in the retrosynthetic analysis based on building blocks **2-40**,[vii] **2-41**,[vii] **2-17** and **2-16** (Scheme 2.7).[132, 179]

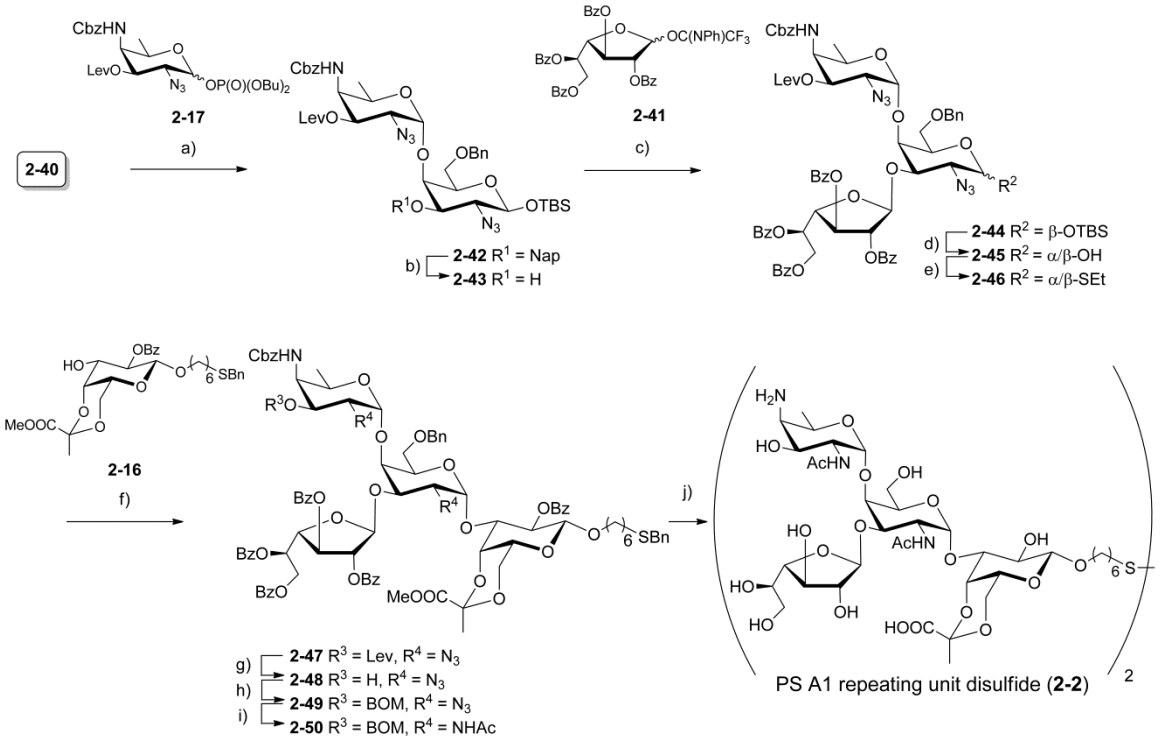


Scheme 2.7. Retrosynthesis of PS A1 repeating unit disulfide **2-2**.

Synthesis of PS A1 repeating unit disulfide **2-2** commenced with the glycosylation of D-AAT glycosylating agent **2-17** and D-galactosamine nucleophile **2-40** to give disaccharide **2-42** in 77% yield and 19:1 α:β selectivity (Scheme 2.8). Removal of the Nap group (84% yield) was followed by glycosylation of disaccharide **2-43** with galactofuranose imidate **2-41** to provide trisaccharide **2-44** in 90% yield. Modification of the trisaccharide reducing end included TBS deprotection to give lactol **2-45** (89% yield), formation of the corresponding trifluoroacetimidate and thioglycoside installation to obtain glycosylating agent **2-46** in 75% over two steps. Chemoselective glycosylation of thioglycoside **2-46** with thioether-containing alcohol **2-16** (*see* Table 2.2) was performed using DMTST to give tetrasaccharide **2-47** in 57% yield as the sole diastereomer without affecting the alkyl benzylthioether. Lev deprotection provided alcohol **2-48** in 96% yield, which was capped using novel reagent **2-38** and DMTST to give BOM protected tetrasaccharide **2-49** in 87% yield. Conversion of both azides to the corresponding acetamides with AcSH/pyridine produced diamide **2-50** in 60% yield.

[vii] Building blocks **2-40** and **2-41** were synthesized by Dr. Rajan Pragani.

Global deprotection of tetrasaccharide **2-50** was achieved in a straightforward two-step procedure. Saponification of all esters followed by Birch reduction provided fully-deprotected disulfide **2-2** in 88% over two steps after size exclusion chromatography. No major side reactions were observed during global deprotection, highlighting the feasibility of introducing a thioether at an early synthetic stage. The identity of disulfide **2-2** was confirmed by comparing the analytical data of **2-2** with published data on a similar synthetic PS A1 repeating unit (Fig. 2.1B).[132]



Scheme 2.8. Synthesis of PS A1 repeating unit disulfide **2-2**. Reagents and conditions: a) 1.5 equiv. **2-17**, TMSOTf, CH_2Cl_2, 0 °C, 77% (19:1 α:β); b) DDQ, MeOH, CH_2Cl_2, 0 °C to r.t., 84%; c) 1.4 equiv. **2-41**, TMSOTf, CH_2Cl_2, -30 °C, 90% (>19:1 β:α); d) TBAF, AcOH, THF, 0 °C to r.t., 89%; e) i. $F_3CC(NPh)Cl$, Cs_2CO_3, CH_2Cl_2, r.t.; ii. EtSH, TfOH, CH_2Cl_2, 0°C, 76% (two steps); f) 2.0 equiv. **2-16**, DMTST, TTBPy, CH_2Cl_2, r.t., 57% (>19:1 α:β); g) H_4N_2, pyridine, AcOH, CH_2Cl_2, r.t., 96%; h) **2-38**, DMTST, TTBPy, CH_2Cl_2, 0 °C to 10 °C, 87%; i) AcSH, pyr., r.t., 60%; j) i. NaOH, H_2O, THF, MeOH, 0 °C to r.t.; ii. Na, NH_3, *t*BuOH, THF, -78 °C, 88% (two steps).

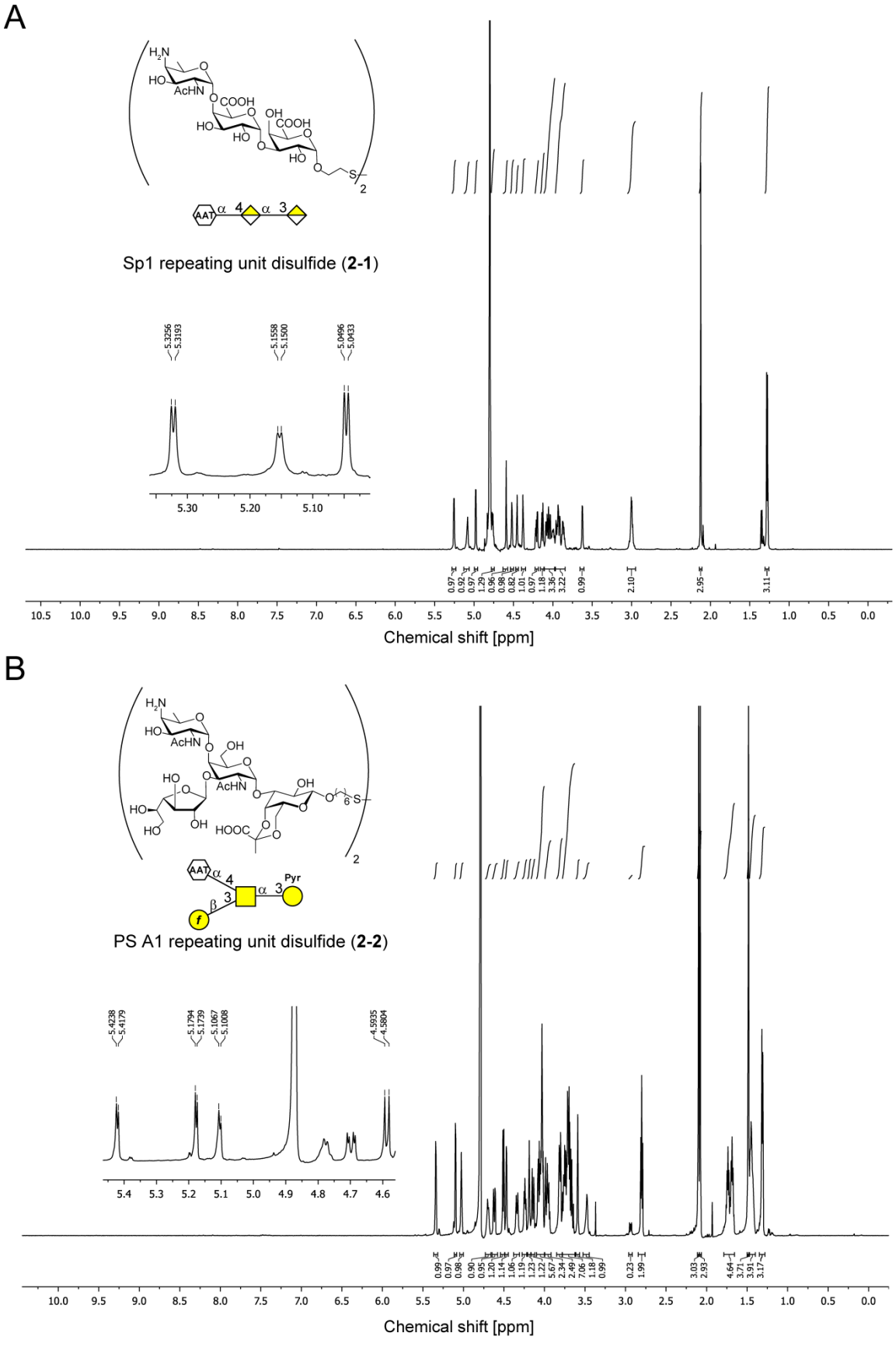
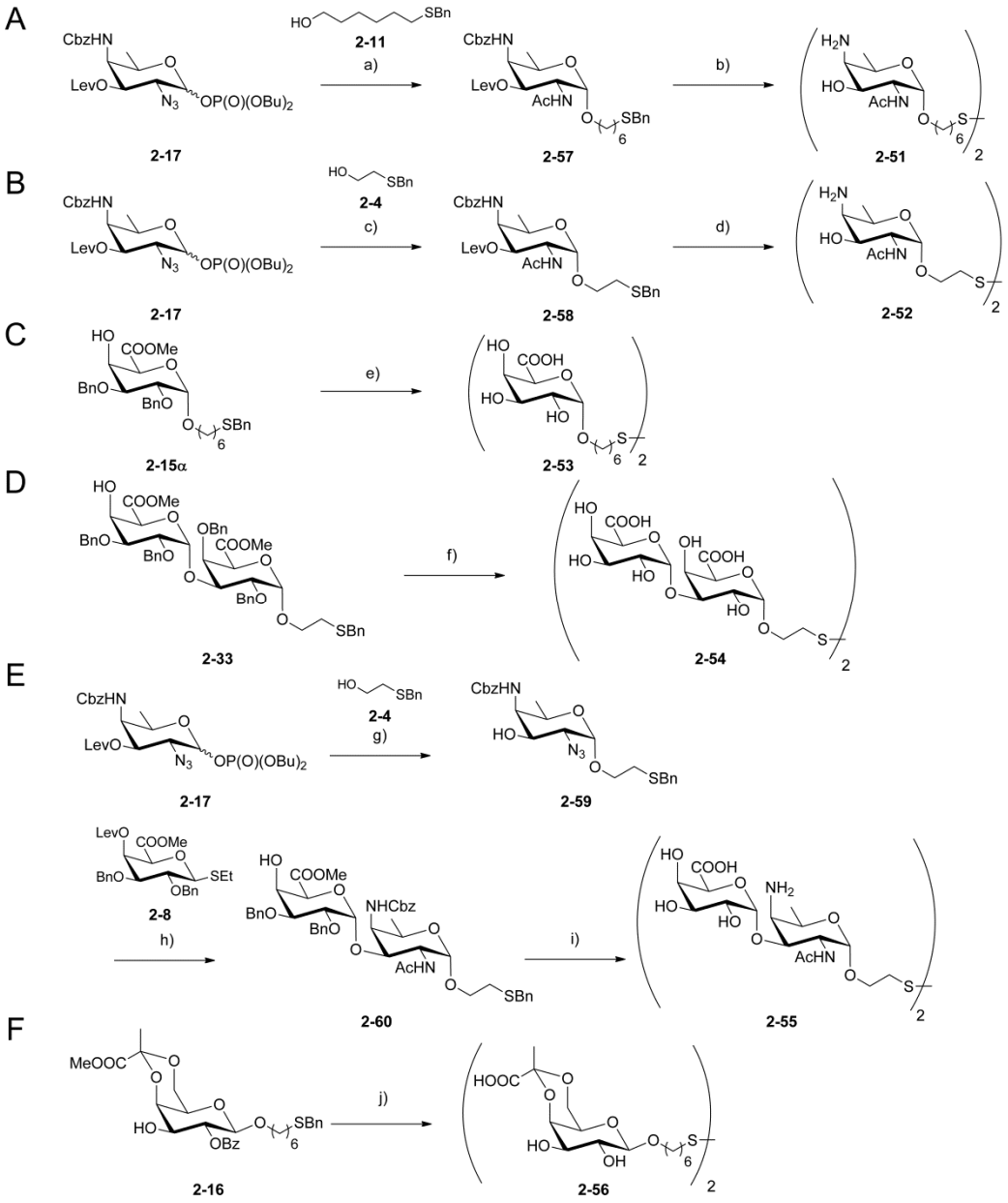


Figure 2.1. ^{1}H NMR spectra (600 MHz, D_2O, 25 °C) of Sp1 trisaccharide disulfide **2-1** (*A*) and PS A1 tetrasaccharide disulfide **2-2** (*B*).



Scheme 2.9. Synthesis of ZPS repeating unit substructures. *A-B*, common substructures of Sp1 and PS A1 **2-51** and **2-52**. *C-E*, Sp1 substructures **2-53**, **2-54** and **2-55**. *F*, PS A1 substructure **2-56**. Reagents and conditions: a) i. **2-11**, TMSOTf, CH_2Cl_2, 0 °C (1:1 α:β); ii.AcSH, pyr., r.t., 29% (two steps); b) i. $H_4N_2{\cdot}H_2O$, AcOH, pyr., CH_2Cl_2, r.t.; ii. Na, NH_3, *t*BuOH, THF, -78 °C, r.t, 34% (two steps); c) i. **2-4**, TMSOTf, Et_2O/CH_2Cl_2 5:2, 0 °C, 48% (5:2 α:β); ii. AcSH, pyr., r.t., 76%; d) i. $H_4N_2{\cdot}H_2O$, AcOH, pyr., ii. Na, NH_3, *t*BuOH, THF, -78 °C, r.t, 71% (two steps); e) i. NaOH, H_2O, THF, MeOH, 0 °C to r.t.; ii. Na, NH_3, *t*BuOH, THF, -78 °C, r.t, 60% (two steps); f) i. NaOH, H_2O, THF, MeOH, 0 °C to r.t.; ii. Na, NH_3, *t*BuOH, THF, -78 °C, r.t, 61% (two steps); g) i. **2-4**, TMSOTf, Et_2O/CH_2Cl_2 5:2, 0 °C, 48% (5:2 α:β); $H_4N_2{\cdot}H_2O$, AcOH, pyr., CH_2Cl_2, r.t., 92%; h) i. **2-8**, DMTST, TTBPy, THF, 0 °C to r.t.; ii. AcSH, pyr., r.t.; iii. $H_4N_2{\cdot}H_2O$, AcOH, pyr., CH_2Cl_2, r.t., 21% (three steps, based on recovered **2-59**); i) NaOH, H_2O, THF, MeOH, 0 °C to r.t.; ii. Na, NH_3, *t*BuOH, THF, -78 °C, 83% (two steps); j) NaOH, H_2O, THF, MeOH, 0 °C to r.t.; ii. Na, NH_3, *t*BuOH, THF, -78 °C, r.t, 56% (two steps).

2.2.4 Synthesis of Conjugation-ready ZPS Repeating Unit Fragments

To allow for a detailed view into the structural requirements of ZPS recognition by components of the immune system, a panel of oligosaccharides were prepared that represent Sp1 and PS A1 repeating unit substructures (Scheme 2.9 and Fig. 2.2A). Thiol groups were introduced at the reducing ends of all glycans to enable the conjugation of these probes to suitable electrophilic reporters.

Conjugation-ready D-AAT was equipped with both 6- and 2-carbon linkers (**2-51** and **2-52**, respectively, Scheme 2.9A and B). The longer aliphatic chain in **2-51** was chosen as a handle suitable for glycan microarray analysis, while the shorter linker in **2-52** was assumed to be less immunogenic during immunization experiments (*see* below). The thiol linkers were appended by glycosylation of phosphate **2-17** with alcohols **2-11** and **2-4**. Ensuing reductive acetylation gave acetamides **2-57** and **2-58** in 29% and 26% yield over two steps, respectively. Lev ester cleavage and Birch reduction of benzyl and Cbz groups afforded disulfides **2-51** and **2-52** in 34% and 71% yield, respectively.

Galacturonic acid **2-53** and digalacturonic acid **2-54** were prepared to assess the role of D-AAT in immune recognition of ZPS fragments (Scheme 2.9C and D). Both glycans were accessed by global deprotection of intermediates **2-15α** and **2-33** (*see* above), including ester saponification and Birch reduction to provide carboxylic acids **2-53** and **2-54** in 60% and 61% yield, respectively.

A D-AAT moiety was furnished at the reducing end of disaccharide **2-55** to probe the relevance of the position of this unusual monosaccharide within a glycan chain (Scheme 2.9E). Glycosylation of D-AAT precursor **2-17** with alcohol **2-4** furnished a 5:2 α:β anomeric mixture of the corresponding glycosides in 48% yield. Alcohol **2-59** was prepared by Lev deprotection in 92% yield. DMTST-mediated union of **2-59** with galacturonic acid thioglycoside **2-8**, reductive acetylation of the azide group and Lev deprotection gave disaccharide **2-60** in 21% over three steps, based on recovered **2-59**. The low yield of this synthetic sequence is mainly attributed to the low reactivity of

thioglycoside **2-8**. Global deprotection afforded disulfide **2-55** in 83% yield over two steps.

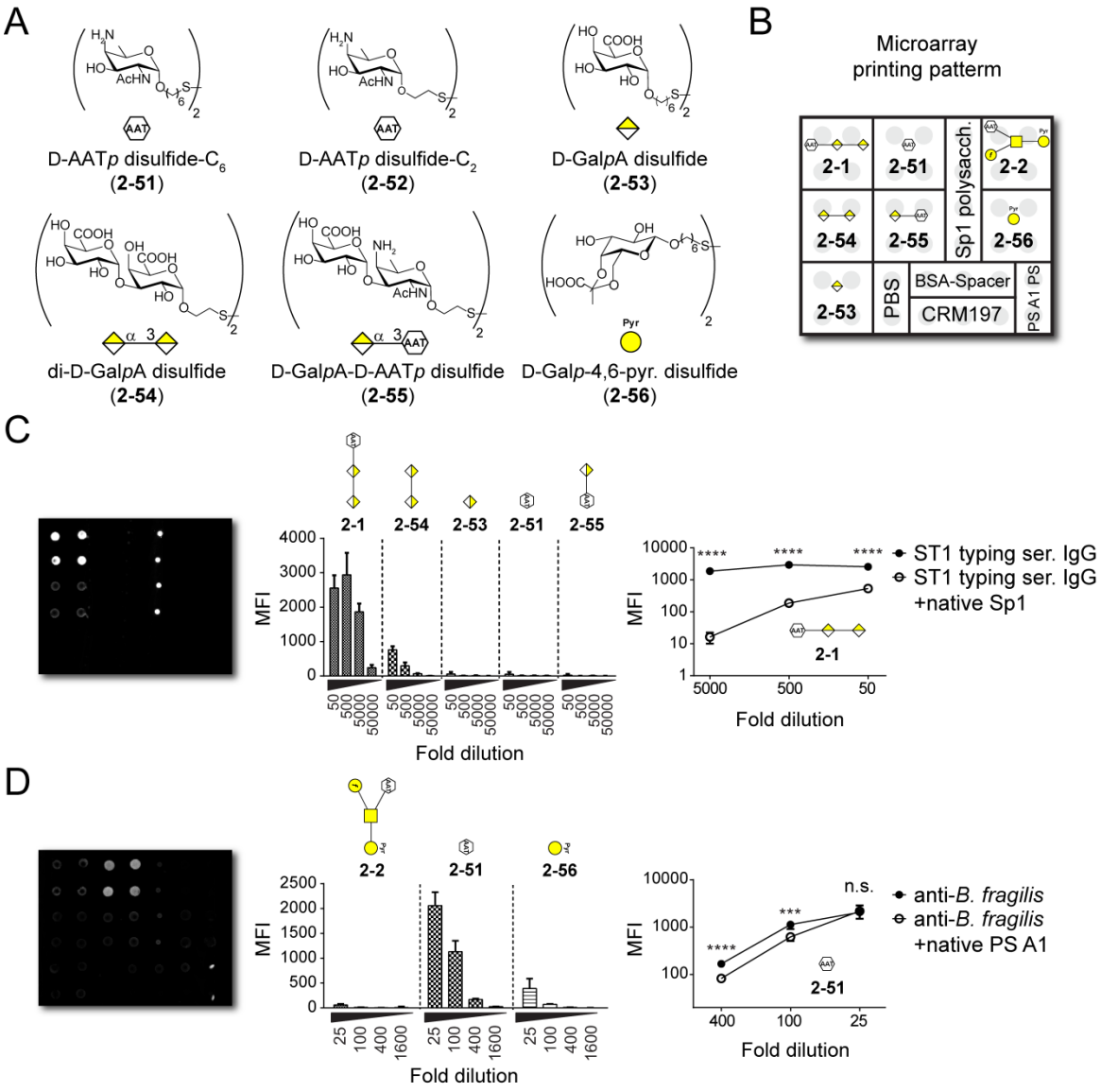


Figure 2.2. Immune recognition of synthetic ZPS substructures. *A*, synthetic fragments of ZPS repeating units **2-1** and **2-2**. *B*, glycanmicroarray printing pattern. *C*, recognition of synthetic Sp1 substructures by rabbit ST1 typing serum by glycan microarray at different concentrations (left and middle panel) and inhibition of antibody binding to Sp1 trisaccharide **2-1** by pre-adsorption with native Sp1 polysaccharide (10 µg/mL). Serum was pre-adsorbed with pneumococcal C-polysaccharide before application. Histograms show mean + SD of eight spots. Bars depict mean ± SD. *D*, recognition of synthetic PS A1 substructures by rabbit anti-*B. fragilis* antiserum by glycan microarray at different concentrations (left and middle panel) and inhibition of antibody binding to D-AAT by pre-adsorption with native PS A1 polysaccharide (10 µg/mL). Histograms show mean + SD of eight spots. Statistical analysis (One-tailed, unpaired t test with Welch's correction) of eight spots was performed of one out of at least two independent experiments. Asterisks indicate P values: n.s. not significant; *** $P < 0.001$; **** $P < 0.0001$. MFI = mean fluorescence intensity.

Finally, pyruvalated galactose **2-56** was synthesized to address the potentially immunodominant role of the pyruvate ketal, as seen for other polysaccharides (Scheme 2.9F).[299] Saponification and ensuing Birch reduction of intermediate **2-16** (*see* above) afforded disulfide **2-56** in 56% yield over two steps.

2.2.5 Recognition of Synthetic ZPS Fragments by Components of the Immune System

With a panel of synthetic, conjugation-ready ZPS fragments in hand, immune recognition of these oligosaccharides was examined. Disulfides **2-1**, **2-2**, **2-51**, **2-52**, **2-53**, **2-54**, **2-55** and **2-56** were reduced *in situ* with tris(2-carboxyethyl)phosphine (TCEP) and immobilized on maleimide-functionalized glass slides by virtue of the appended thiol functionalities (Fig. 2.2B).[149, 157, 300] Native Sp1 and PS A1 polysaccharides[viii] were included in the experiment as positive binding controls. Furthermore, proteins were immobilized to analyze the outcome of immunization experiments (*see* below). First, recognition of the synthetic ZPS substructures by recombinant human MHCII complexes[ix] and murine C-type lectins[x] was assessed, but no interaction was observed (data not shown).

Next, binding was examined of rabbit antisera against either Sp1 polysaccharide or whole *B. fragilis* bacteria (Fig. 2.2C and D).[viii] Both antisera bound to the respective immobilized native ZPSs, confirming the viability of these sera. A robust interaction was found between synthetic Sp1 trisaccharide **2-1** and antiserum against native Sp1 polysaccharide (Fig. 2.2C, left panel), indicating that the synthetic repeating unit efficiently presents epitopes found in the polysaccharide. Significant abrogation of binding by pre-adsorption of the antiserum to Sp1 polysaccharide confirmed this finding (Fig. 2.2C, right panel). Digalacturonic acid **2-54** was recognized with an at least threefold lower intensity than **2-1**, indicating that the D-AAT moiety plays a crucial role for immune recognition of Sp1. However, no binding was observed to D-AAT alone (**2-**

[viii] anti-*B. fragilis* antiserum and purified PS A1 polysaccharide were kindly provided by Prof. Dennis Kasper, Harvard Medical School, Boston, USA.

[ix] Recombinant MHCII complexes were kindly provided by Dr. Eddie James, Benaroya Research Institute, Seattle, USA.

[x] In collaboration with Prof. Bernd Lepenies, Max Planck Institute of Colloids and Interfaces, Potsdam, Germany.

51) or disaccharide **2-55** that harbors this rare monosaccharide at the reducing end. Thus, displaying the D-AAT moiety at the non-reducing end of an oligosaccharide is proposed to be important for antibody binding of Sp1 fragments. Negligible interaction was found between anti-*B. fragilis* antiserum and synthetic PS A1 repeating unit **2-2** (Fig. 2.2D, left panel). Recognition of pyruvalated galactose **2-56** was equally weak. In contrast, a robust interaction was observed towards D-AAT when presented as a linker-bearing monosaccharide (**2-51**), underlining the importance of this rare sugar for immune recognition of ZPSs. This interaction was physiologically relevant, as seen by the significant inhibition observed by antibody pre-adsorption to native PS A1 polysaccharide (Fig. 2.2D, right panel). Taken together, D-AAT plays an important role in the recognition of ZPS fragments by antisera against native ZPSs.

2.2.6 Evaluation of the Immune Response Against D-AAT

Unusual monosaccharides likely contribute to the "non-self" recognition of bacterial polysaccharides by the mammalian immune system.[301] To closer evaluate the role of the rare amino sugar D-AAT in immune recognition of ZPSs, thiol-containing D-AAT **2-52** was conjugated to the immunogenic carrier CRM197 (Fig. 2.3A). The carrier was first activated with thiophilic 2-bromoacetates on lysine residues by treatment with heterobifunctional spacer *N*-succinimidyl 3-(2-bromoacetamido)propionoate (SBAP). Approximately ten spacer molecules were introduced on average per protein molecule, as assessed by matrix-assisted laser desorption/ionization with time of flight detection (MALDI-TOF) mass spectrometry, and reacted with *in situ*-reduced D-AAT **2-52** in a second step to yield a glycoconjugate carrying on average five glycan moieties per protein molecule (Fig. 2.3B and C).

The immunogenicity of D-AAT as a hapten was evaluated in a mouse immunization model (Fig.2.4). C57BL/6 mice were immunized twice with the glycoconjugate formulated either with or without Freund's adjuvant (Fig. 2.4A). The immune response was monitored by glycan microarray (Fig. 2.4B). A consistent immune response against D-AAT was mounted by the CRM197-D-AAT (**2-52**) glycoconjugate in the presence of Freund's adjuvant, but not when the adjuvant was omitted (Fig. 2.4C and D). Cross-reactivity towards Sp1 trisaccharide **2-1** and PS A1 tetrasaccharide **2-2** was observed in one mouse (mouse 780, Fig. 2.4C), indicating that the corresponding

antibodies recognized D-AAT even when present on an oligosaccharide scaffold independent from a linker moiety.

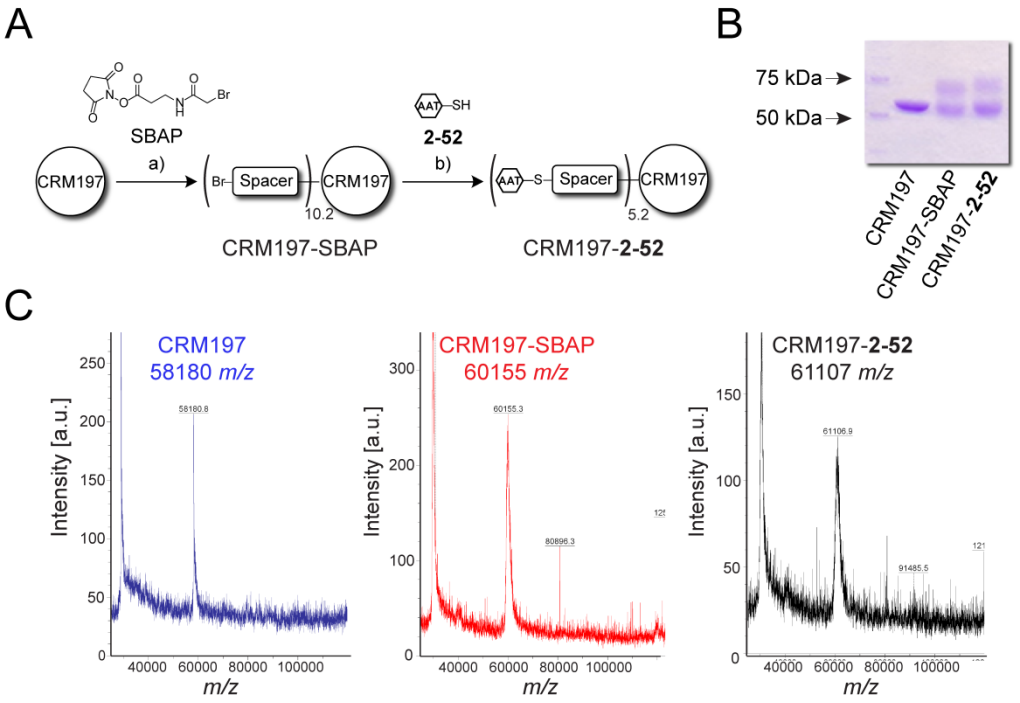


Figure 2.3. Preparation of a CRM197-D-AAT glycoconjugate. *A*, conjugation of D-AAT **2-52** to CRM197 using SBAP as a spacer. *B*, characterization by 10% SDS-PAGE. *C*, characterization by MALDI-TOF MS. Axes were re-drawn and labeled to improve visibility. Reagents and conditions: a) SBAP, 0.1 M NaPi pH 7.4, r.t.; b) i. **2-52**, TCEP, 0.1 M NaPi pH 7.4, r.t.; ii. L-cysteine, 0.1 M NaPi pH 7.4, r.t. a.u. = arbitrary units. NaPi = sodium phosphate buffer. SBAP = *N*-succinimidyl 3-(2-bromoacetamido)propanoate. TCEP = tris(2-carboxyethyl)phosphine.

No binding was found towards disaccharide **2-55** that contains D-AAT at the reducing end. Background binding events observed in glycan microarray experiments were attributed to the reactivity of antisera towards the reducing agent TCEP (background spots, Fig. 2.4C). Albeit commonly believed to be a disulfide-specific reducing agent, TCEP has been found to react with different electrophiles frequently (*see* below),[302] and may have reacted with α-bromoacetate and maleimide groups during glycoconjugate formation and glycan microarray fabrication, respectively.

Additionally, antisera were reactive towards the carrier protein CRM197 and a glycoconjugate containing non-related protein (bovine serum albumin, BSA) and glycan (D-galactose) components connected via the same linker/spacer chemistry (Fig. 2.4C).

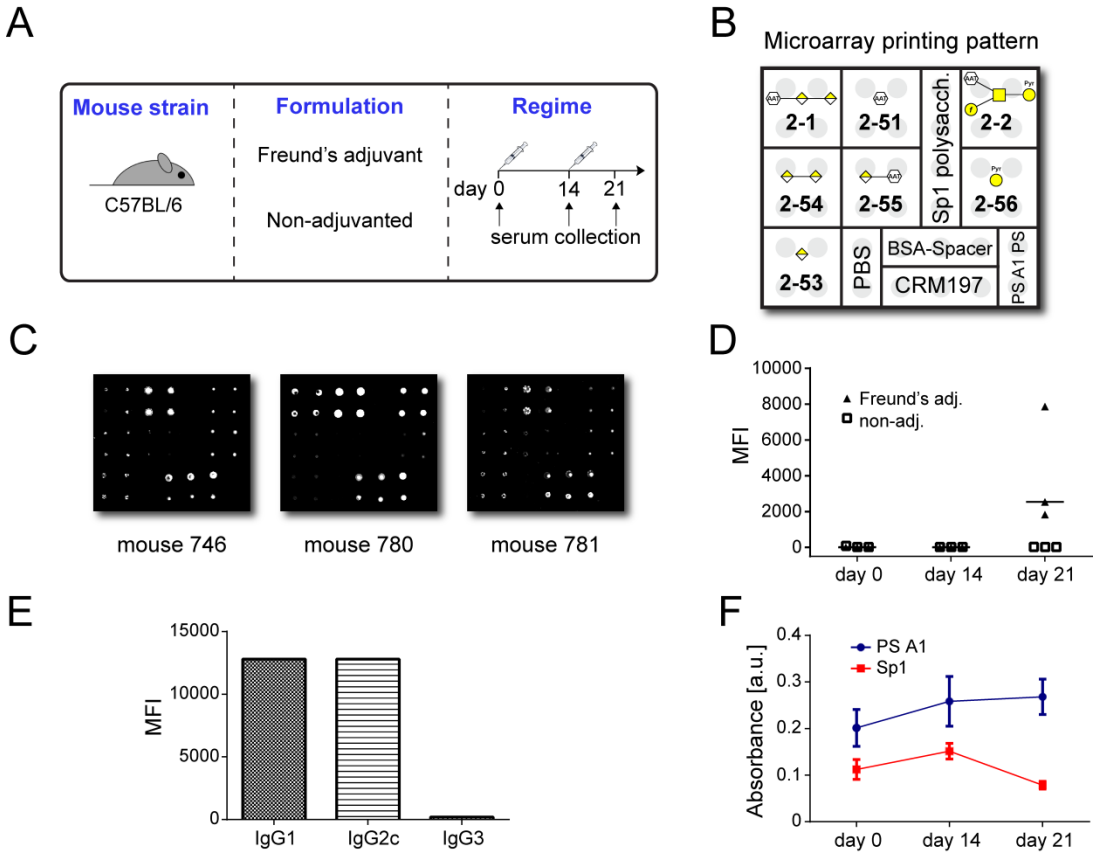


Figure 2.4. Evaluating a glycoconjugate of D-AAT in mice. *A*, immunization strategy. C57BL/6 mice ($n = 3$) were immunized two times with CRM197-D-AAT (**2-52**) with or without Freund's adjuvant. Sera were collected at days 0, 14 and 21. *B*, glycan microarray printing pattern. *C*, immune response at day 21 of mice immunized with CRM197-D-AAT (**2-52**) formulated with Freund's adjuvant, as assessed by glycan microarray. *D*, time course of the immune response against D-AAT in individual mice, as assessed by glycan microarray. Values show fluorescence of D-AAT binding after substracting the fluorescence intensity of a non-related glycan (D-GalA **2-53**) associated with TCEP-reactive antibodies. Values are given as individual data and median. *E*, IgG isotype distribution in antisera of mice immunized with CRM197-D-AAT formulated with Freund's adjuvant. Endpoint titers are shown as the reciprocal of the highest dilution of pooled sera ($n = 3$) with measurable fluorescence intensity on glycan microarray. *F*, Evaluation of the recognition of native ZPSs by antisera against D-AAT, as assessed by polysaccharide ELISA. Bars depict mean ± SD of triplicate measurements of one experiment.

Analysis of antibody subtypes revealed that the IgG response induced by the CRM197-D-AAT (**2-52**) glycoconjugate was mainly skewed towards IgG1 (endpoint titer 12800) and IgG2c (12800) rather than IgG3 (200) subtypes, consistent with a carrier protein-dependent Ig class switch (Fig. 2.4E).[45] Antisera raised against D-AAT did not recognize native Sp1 and PS A1 polysaccharides, as assessed by polysaccharide ELISA (Fig. 2.4F). Thus, despite the high immunogenicity of D-AAT as a hapten when conjugated to CRM197, antibodies raised by this glycoconjugate do not recognize D-AAT unless it is presented at the non-reducing end of a glycan chain.

2.2.7 Evaluation of an Sp1 Trisaccharide Repeating Unit as a Vaccine Hapten Against *Streptococcus pneumoniae* Serotype 1

The rare monosaccharide D-AAT is highly immunogenic as a hapten in mice, but did not induce an immune response against native Sp1. However, this monosaccharide was strongly recognized by *S. pneumoniae* ST1-reactive antiserum (that is directed against native Sp1) as a part of trisaccharide repeating unit **2-1**. This trisaccharide was thus deemed a suitable vaccine hapten against highly invasive ST1 (Fig. 2.5). CRM197 was functionalized with α-bromoacetate groups (*see* above) and reacted with *in situ*-reduced Sp1 trisaccharide **2-1** (Fig. 2.5A). On average, eight glycan chains were introduced per protein molecule (Fig. 2.5B and C).

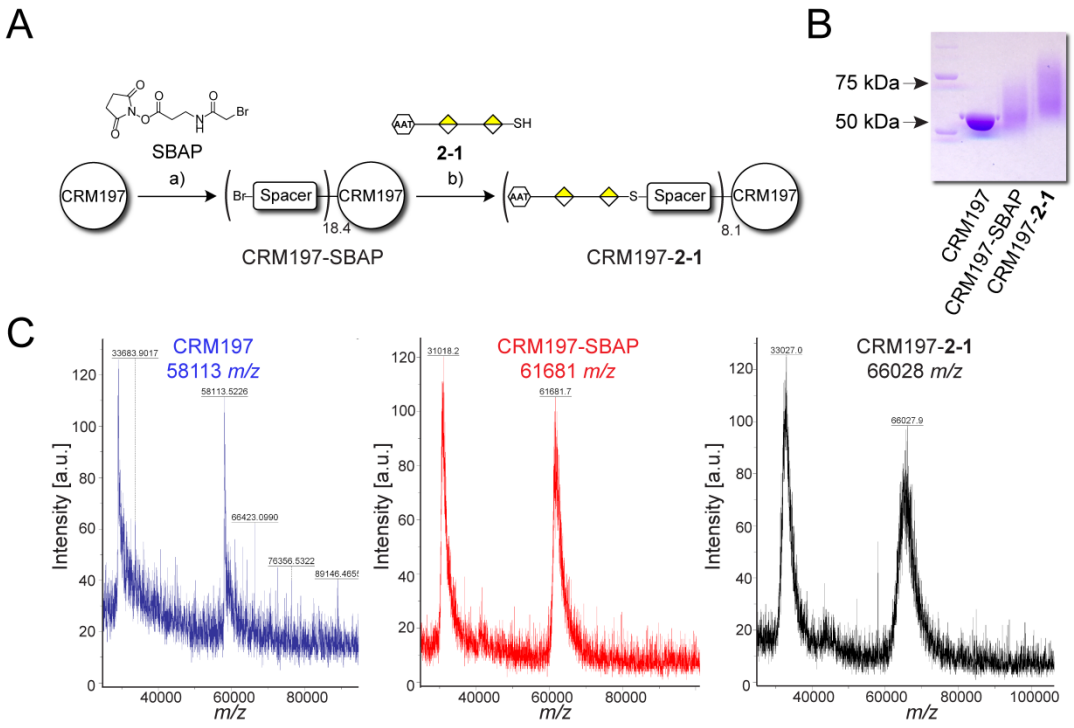


Figure 2.5. Preparation of a CRM197-Sp1 (**2-1**) glycoconjugate. *A*, conjugation of Sp1 trisaccharide **2-1** to CRM197 using SBAP as a spacer. *B*, characterization by 10% SDS-PAGE. *C*, characterization by MALDI-TOF MS. Axes were re-drawn and labeled to improve visibility. Reagents and conditions: a) SBAP, 0.1 M NaPi pH 7.4, r.t.; b) i. **2-1**, TCEP, 0.1 M NaPi pH 8.0, r.t.; ii. L-cysteine, 0.1 M NaPi pH 7.4, r.t.

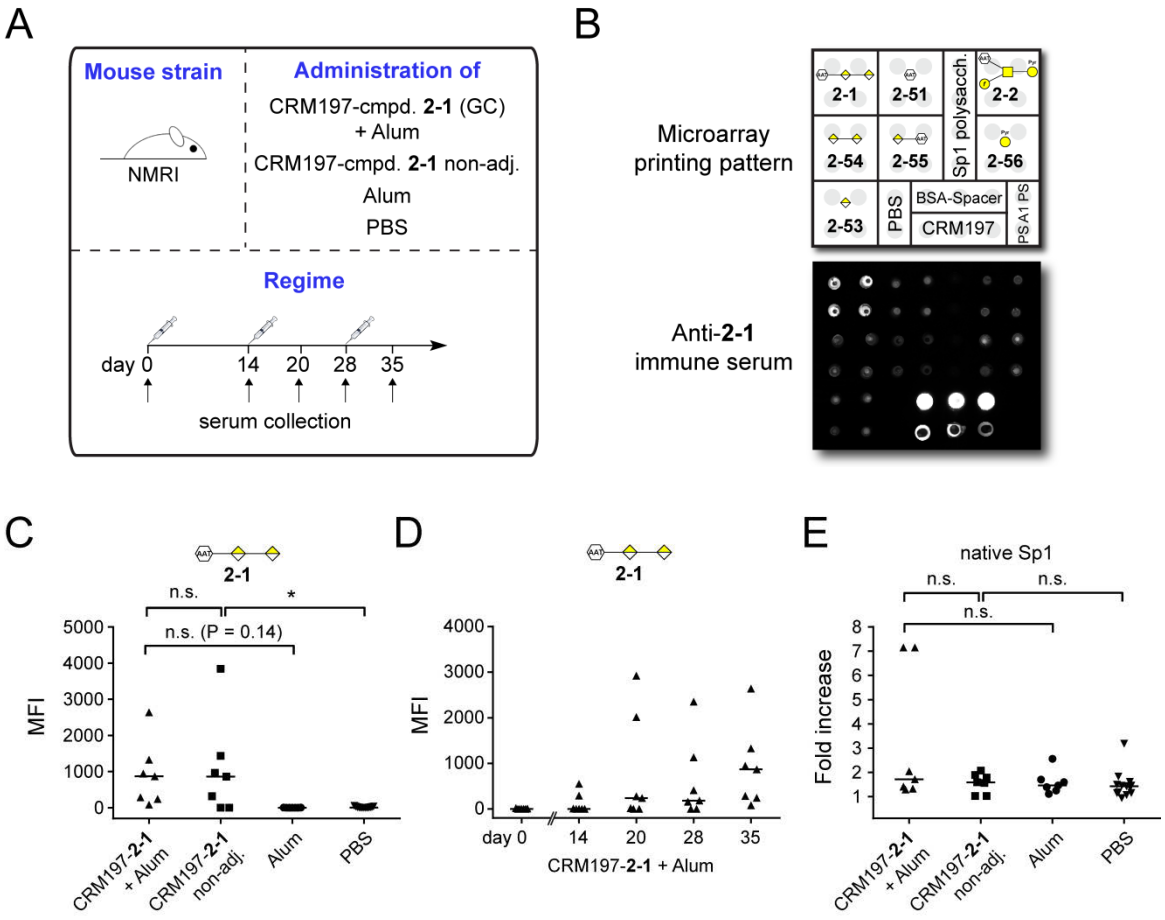


Figure 2.6. Evaluating a glycoconjugate of synthetic Sp1 trisaccharide **2-1** in mice. *A*, immunization strategy. NMRI mice (n = 7-13) were subcutaneously (s.c.) immunized three times with CRM197-Sp1 trisaccharide (**2-1**) formulated with or without Alum. Control immunizations contained Alum adjuvant alone or PBS. Sera were collected at several time points. *B*, glycan microarray printing pattern and glycan microarray exemplary of a detectable immune response against hapten **2-1**. *C*, immune responses of individual mice after immunization with CRM197-Sp1 trisaccharide (**2-1**) or controls, as assessed by glycan microarray. Values are given as individual data and median. *D*, time course of immune responses against Sp1 trissaccharide (**2-1**) of individual mice, as assessed by glycan microarray. Values are given as individual data and median. *E*, binding of antisera to native Sp1 polysaccharide, as assessed by polysaccharide ELISA. Values are given as individual data and median. Values in *C* and *D* show fluorescence of Sp1 trisaccharide (**2-1**) binding after substracting the fluorescence intensity of a non-related glycan (D-Gal-4,6-pyr. **2-56**) associated with TCEP-reactive antibodies. Statistical analysis was performed (one-way ANOVA with Bonferroni correction) and Asterisk indicates P value: n.s., non-significant; * $P < 0.05$. Reagents and conditions: a) SBAP, 0.1 M NaPi pH 7.4, r.t.; b) i. **2-1**, TCEP, 0.1 M NaPi pH 8.0, r.t.; ii. L-cysteine, 0.1 M NaPi pH 7.4, r.t.

To assess the immune response against Sp1 trisaccharide **2-1** *in vivo*, NMRI mice[xi] were immunized three times with the CRM197-Sp1 (**2-1**) glycoconjugate formulated either with or without the human-approved adjuvant Alum.[304] Control groups were treated with Alum or PBS, respectively, and the immune response was assessed by glycan microarray analysis (Fig. 2.6B). Mice immunized with CRM197-Sp1 (**2-1**) developed a pronounced immune response against the hapten **2-1** compared to control groups. The obtained immune response was independent from the use of adjuvant (Fig. 2.6C), highlighting the immunogenicity of trisaccharide **2-1**. An evaluation of the antibody time course revealed that immunizations at days 14 and 28 efficiently boosted the immune response, as increases in antibody levels were observed at days 20 and 35, respectively (Fig. 2.6D).

The primary goal of vaccination with glycoconjugates against encapsulated bacteria is the induction of an immune response directed against the respective native CPS. The capacity of antisera against CRM197-Sp1 trisaccharide **2-1** to bind native Sp1 was assessed by polysaccharide ELISA. A subset of mice ($n = 2$ out of 7) immunized with the glycoconjugate formulated with Alum, but neither with non-adjuvanted glycoconjugate nor with control formulations developed an anti-polysaccharide immune response (Fig. 2.6E). The high variability of antibody responses precludes the use of CRM197-Sp1 (**2-1**) as an antigen in the system studied.

The response to an immunogen may be dependent upon the animal system used. Despite the establishment of NMRI mice in pneumococcal challenge models, immune responses achieved in this outbred mouse strain are highly variable.[305] Therefore, inbred C57BL/6 mice were employed in further immunization experiments described below.

[xi] The NMRI mouse strain was employed here because a ST1 infection model has been described using this strain,[303] facilitating challenge experiments prospectively.

2.2.8 Liposomal Presentation of a Sp1 Trisaccharide as a Fully Synthetic Antigen Formulation

Multivalent presentation of glycans is a promising approach to induce anti-carbohydrate immune responses. Liposomal display of antigens is of special interest since this formulation offers the potential to develop fully synthetic vaccines devoid of protein components and the need to maintain a cold chain.[165, 167]

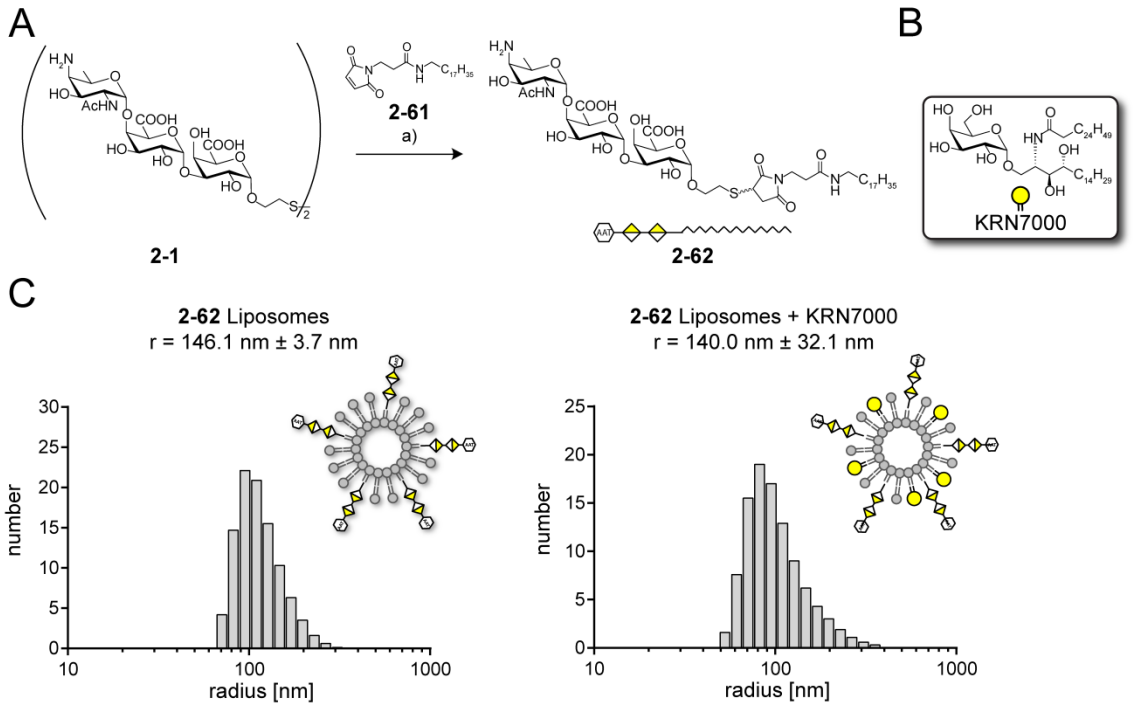


Figure 2.7. Preparation of liposomes displaying Sp1 trisaccharide **2-1**. *A*, synthesis of alkyl-derivatized Sp1 trisaccharide **2-62**. *B*, Structure of KRN7000. *C*, preparation of Sp1-displaying liposomes without (left panel) or with KRN7000 (right panel). Sizes depict average ± SD of three independent liposome preparations. The size distribution of a representative liposome preparation is shown, as determined by dynamic light scattering. Reagents and conditions: a) DTT, 0.1 M NaPi pH 7.4, r.t., then **2-61**, $CHCl_3$, MeOH, H_2O, r.t., 51%.

To enable the construction of a Sp1 trisaccharide-containing liposome system, disulfide **2-1** was first conjugated to maleimide-functionalized octadecylamine **2-61** (Fig. 2.7A). The disulfide in **2-1** was first reduced in aqueous phosphate buffer and directly reacted with **2-61** in a ternary $CHCl_3$/methanol/water solvent system. 1,4-Dithiothreitol (DTT) was used as a disulfide reducing agent as the water-soluble phosphine reagent TCEP was found to react with the maleimide moiety.[302] Thus, alkyl-containing, amphiphilic Sp1 trisaccharide derivative **2-62** was obtained in 51% yield after purification by solid-phase extraction and size exclusion chromatography.

Next, liposomes were fabricated by incorporating alkyl-derivatized trisaccharide **2-62** and, optionally, the lipophilic immunomodulator KRN7000 (Fig. 2.7B) into a mixture of distearyl phosphatidyl choline and cholesterol (Fig. 2.7C).[306] Liposomes were found to be of reproducible size, with a radius of 146 nm ± 3.7 nm (292 nm diameter, Sp1-alkyl **2-62** liposomes) and 140.0 nm ± 32.1 nm (280 nm diameter, Sp1-alkyl **2-62** liposomes + KRN7000), respectively, as determined by dynamic light scattering (Fig. 2.7C).

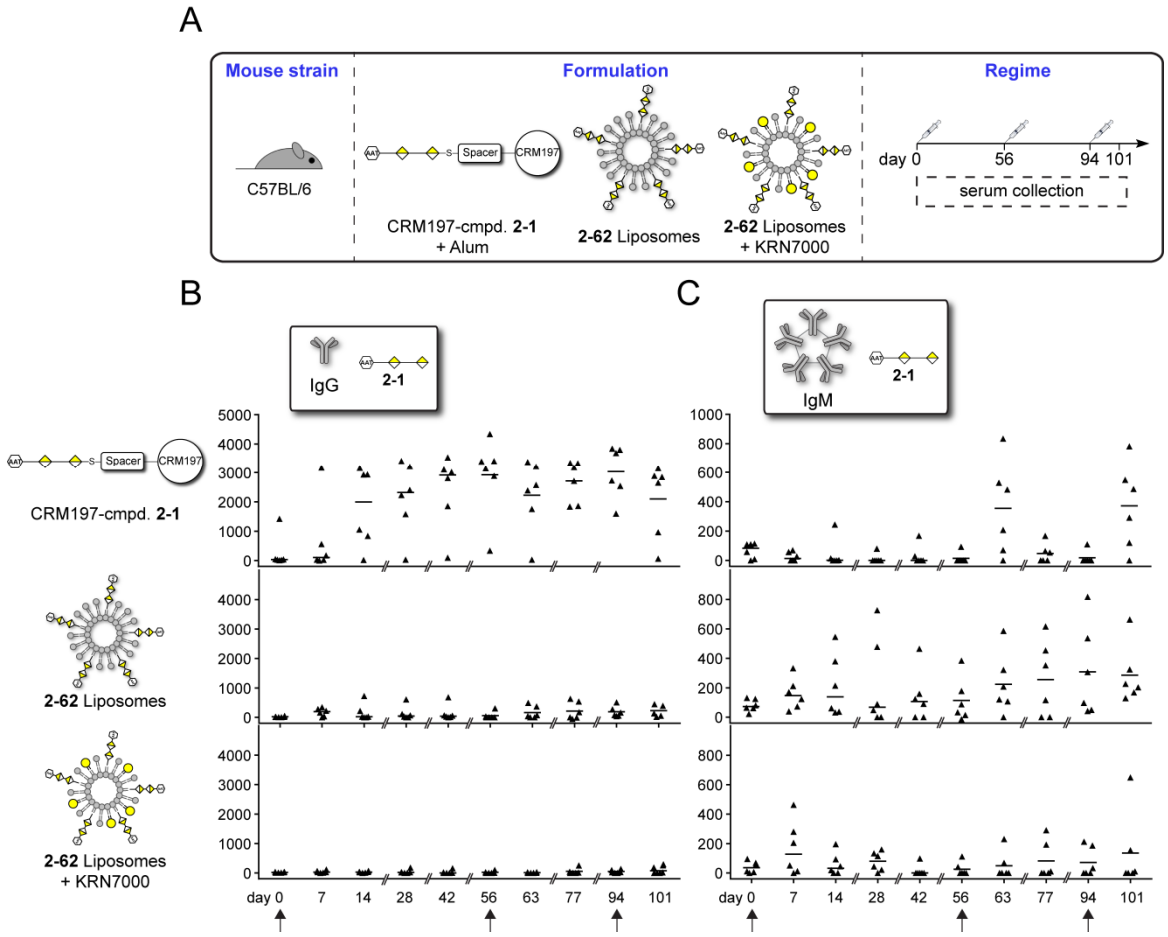


Figure 2.8. Evaluating different antigen formulations on the immune response against Sp1 trisaccharide **2-1**. *A*, immunization regime. C57BL/6 mice (n = 6) were intraperitoneally (i.p.) immunized three times either with CRM197-Sp1 trisaccharide (**2-1**) formulated with Alum, liposomes containing Sp1 derivative **2-62** or liposomes containing **2-62** and KRN7000. Sera were collected at several time points. *B*, time course of the IgG response of individual mice against Sp1 trisaccharide **2-1**, as assessed by glycan microarray. Values are given as individual data and median. *C*, time course of the IgM response of individual mice against Sp1 trisaccharide **2-1**, as assessed by glycan microarray. Values are given as individual data and median. Values of CRM197-Sp1 trisaccharide **2-1** immune sera in *C* and *D* show fluorescence of Sp1 trisaccharide (**2-1**) binding after substracting the fluorescence intensity of a non-related glycan (D-Gal-4,6-pyr. **2-56**) associated with TCEP-reactive antibodies. Arrows depict time points of immunization.

The potency of liposomal formulations of Sp1 trisaccharide **2-1** to elicit anti-carbohydrate antibody responses was elucidated *in vivo* (Fig. 2.8). C57BL/6 mice were immunized three times with liposomes containing either alkyl-linked Sp1 trisaccharide **2-62** alone or in conjunction with KRN7000. A CRM197-Sp1 trisaccharide (**2-1**) conjugate (*see* above) was utilized as a control antigen. The immunization regime was adjusted to the physiological prerequisites of KRN7000 immunomodulation: As KRN7000-sensitive iNKT cells become anergic for several weeks after stimulation,[249, 251-253] the first boost immunization was administered eight weeks after the prime immunization. A recall of the immune response was performed by administration of a second boost immunization after 94 days, and sera were collected at regular intervals. Antibody responses against Sp1 trisaccharide **2-1** were monitored by glycan microarray. As expected, the CRM197-Sp1 (**2-1**) glycoconjugate induced a robust immune response that was associated with a long-term antibody response with measurable IgG levels (Fig. 2.8B). However, antisera of CRM197-Sp1 (**2-1**)-immunized mice did not cross-react with native Sp1 polysaccharide in an ELISA assay (data not shown). In contrast to the protein-based antigen, neither liposome formulation invoked an IgG response against Sp1 trisaccharide **2-1** (Fig. 2.8B). IgM responses were observed in mice immunized with a CRM197-Sp1 (**2-1**) conjugate one week after each boost immunization (days 63 and 101, respectively, Fig. 2.8C). Liposomes containing the alkyl-Sp1 (**2-62**) construct without KRN7000 induced a pronounced IgM response, as expected after administration of a T cell-independent, multivalent antigen presentation. IgM levels were lower when KRN7000 was included in the formulation. The lack of antibody responses after administration of a co-formulation of carbohydrate antigen and iNKT cell agonist is in stark contrast to literature reports, and it is concluded that KRN7000 is not suitable as an adjuvant to increase the immune response against trisaccharide **2-1**.[249, 250]

2.3 Conclusion and Outlook

Despite the progress in studying the molecular determinants of ZPS-based immunomodulation, the precise mechanisms remain ill-defined. ZPSs have been depolymerized to study the effect of polysaccharide size on immunomodulation.[230] Thereby, it has been found that ZPS fragments as small as 5 kDa (six repeating units)

do not efficiently activate T cells. Due to their complexity, no synthetic ZPS fragments larger than two repeating units have been prepared, and these synthetic glycans did not result in T cell activation *in vitro* (Wu et al.[133] and own observations). However, immune recognition of oligosaccharides may be dependent upon factors other than size. The abundance of unusual monosaccharides, glycosidic linkages and modifications called for the generation of defined, synthetic ZPS fragments to study the role of these structural attributes.

Amine-containing linkers, commonly used to couple synthetic oligosaccharides to reporter molecules or carrier proteins, are not compatible with oligosaccharides derived from ZPSs Sp1 and PS A1. These molecules already contain free primary amines leading to a chemoselectivity problem during conjugation reactions. The synthetic approach to generating conjugation-ready homogeneous Sp1 (**2-1**) and PS A1 (**2-2**) oligosaccharides presented here relied on known orthogonal conjugation conditions that chemoselectively couple thiols and electrophiles in the presence of a free amine.[254, 255, 307] It was demonstrated that benzyl thioethers can be introduced early in the synthesis of oligosaccharides **2-1** and **2-2** without considerably decreasing the variability of chemical transformations. This approach depended on the finding that thioglycosides can generally be activated by DMTST without affecting thioethers found elsewhere in the molecule. This strategy will influence the generation of other disulfide conjugation-ready oligosaccharides by solution and solid phase syntheses. The approach was illustrated by the total syntheses of Sp1 (**2-1**) and PS A1 (**2-2**) conjugation-ready oligosaccharides, along with a panel of synthetic substructures.

Immune recognition of these glycans was studied after immobilization on functionalized glass slides. While recombinant MHCII molecules and C-type lectins did not bind to zwitterionic oligosaccharides on glycan microarray slides (data not shown), incubation with antisera against native ZPSs revealed a distinct role of the amino sugar D-AAT for antibody binding. The positive charge found on D-AAT under physiological conditions is essential for immunomodulation *in vitro* and *in vivo*, and abrogation of that charge by *N*-acylation leads to loss of function.[118, 308] In line with these findings, the charge motifs in every ZPS repeating are important to maintain the three-dimensional structure of these polysaccharides.[131, 309] The importance of D-AAT for antibody

recognition even in short oligosaccharides points towards a function during carbohydrate-protein interaction. Immunization with a D-AAT-containing glycoconjugate induced a robust antibody response against this monosaccharide *in vivo*, and antiserum even recognized D-AAT when present on an oligosaccharide scaffold. Antibodies recognizing exposed monosaccharides have been reported, albeit rarely.[146] Despite the high structural variety of antigen binding sites of antibodies,[310] carbohydrate-reactive antibodies are thought to require oligosaccharides of a certain size.[311] Concomitantly, precedence on the immunization with monosaccharide-based glycoconjugates is scarce.[312] The finding that antibodies can be raised against D-AAT irrespective of the scaffold this monosaccharide is connected to may require a reconsideration of the minimal size of carbohydrate antigens. It is important to note that mAbs generated from splenocytes of mice immunized with the CRM197-D-AAT glycoconjugate did not bind that monosaccharide with sufficiently high affinity to allow for detailed binding characterization.

Glycoconjugate vaccines against *S. pneumoniae* are highly successful in preventing IPD caused by the most common serotypes.[224, 313] Clinical assessment of current 10- or 13-valent vaccines has revealed shortcomings in the induction of functional immune responses against certain serotypes, including ST1, ST3 and ST5.[80, 223] The capsules synthesized by these serotypes are unique with respect to either structure, stability or biosynthesis,[15, 17] and ST1 and ST5 are particularly resistant towards antibody-dependent complement deposition and opsonization.[314] Due to the high invasiveness of these serotypes, it is imperative to develop novel vaccines with optimized efficiency.

Synthetic oligosaccharide haptens have to be carefully designed with respect to the position of unusual functionalities that will be recognized as non-self by the mammalian immune system. Sp1 trisaccharide **2-1** harbors the immunogenic monosaccharide D-AAT at the non-reducing end. A hapten-specific immune response was induced in mice upon immunization with a CRM197-Sp1 trisaccharide (**2-1**) glycoconjugate even when Alum adjuvant was omitted, confirming the high immunogenicity of this trisaccharide. Cross-reactivity to native Sp1 polysaccharide was found in a small subset of mice, and changing the mouse model from outbred NMRI to inbred C57BL/6 mice did not improve this outcome. Despite its immunogenicity, it is likely that Sp1 trisaccharide **2-1** is too short to efficiently display protective glycotopes of native Sp1 and is thus not sufficiently

recognized by appropriate B cells. Alternatively, furnishing D-AAT, presumably the most immunogenic monosaccharide within native Sp1, at the outermost part of a hapten may divert induced antibody responses away from protective glycotopes. It has been proposed that certain immunogenic glycotopes function as decoys to induce non-protective immune responses,[116] and highly immunogenic D-AAT may play that role in Sp1 polysaccharide. In that respect, it is noteworthy that D-AAT is found at the reducing end of the biological repeating unit of Sp1,[16] and hence unlikely to be displayed at the outermost end of each polysaccharide chain. However, it is unclear why disaccharide **2-55**, containing D-AAT at the reducing end, is not recognized by antiserum against the native polysaccharide.

Fully synthetic formulations carry great potential as novel carbohydrate-based vaccines. α-Galactosylceramides have been employed as adjuvants in various vaccination settings to stimulate either antibody-dependent or cellular immune responses.[85, 249, 250] In contrast to recent reports,[249, 250] immunization of liposomes containing an amphiphilic glycan derivative co-formulated with an iNKT cell agonist did not result in an IgG response against the synthetic hapten. Insufficient glycan presentation on the liposomes was excluded by the induction of a robust IgM response, especially in the absence of KRN7000. iNKT cell anergy is induced after stimulation with CD1d ligands.[251-253] Anergy was excluded here by carefully adjusting the immunization regime according to literature precedence.[249] Thus, the lack of induced antibody responses in the KRN7000-**2-62** co-formulation cannot be explained by suboptimal iNKT cell help, and further efforts need to be invested into optimizing either antigen presentation or nature of the adjuvant.

Taken together, a panel of oligosaccharide fragments derived from ZPSs Sp1 and PS A1 were prepared using a newly developed chemoselective thioglycoside activation protocol. Immunological evaluation of these glycans gave insight into the role of the rare monosaccharide D-AAT for immune recognition of ZPSs. A Sp1 repeating unit trisaccharide was evaluated as a vaccine hapten against *S. pneumoniae* ST1 *in vivo* using both carbohydrate-protein conjugate and liposomal antigen formulations. Further efforts will target the generation of alternative oligosaccharide haptens and the optimization of vaccine formulations.

2.5 Experimental Section

2.5.1 Methods of Synthetic Chemistry

General Experimental Details

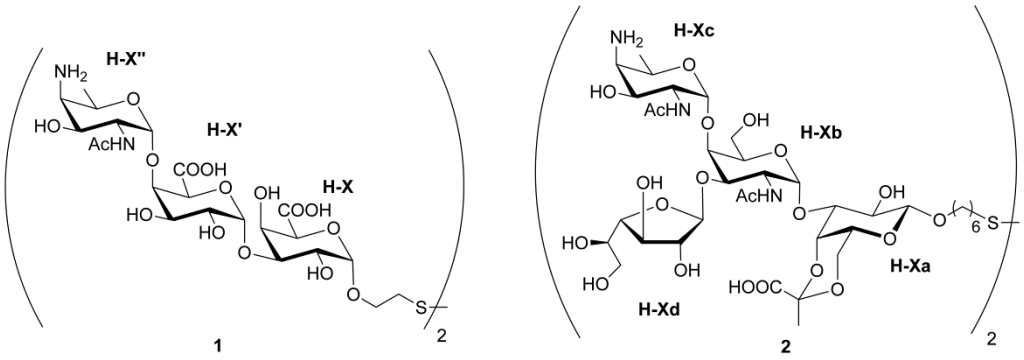
Commercial grade solvents and reagents were used unless stated otherwise. Anhydrous solvents were obtained from a Dry Solvent System (Waters, Milford, USA). Solvents for chromatography were of technical grade and distilled under reduced pressure prior to use. Sensitive reactions were carried out in oven-dried glassware and under an argon atmosphere. Molecular sieves were activated by heating under high vacuum prior to use. Analytical thin layer chromatography (t.l.c.) was performed on Kieselgel 60 F254 glass plates pre-coated with silica gel of 0.25 mm thickness (Macherey-Nagel, Düren, Germany). Spots were visualized with sugar stain (0.1% (v/v) 3-methoxyphenol, 2.5% (v/v) sulfuric acid in EtOH) or CAM stain (5% (w/v) ammonium molybdate, 1% (w/v) cerium(II) sulfate and 10% (v/v) sulfuric acid in water) dipping solutions. Flash chromatography was performed on Kieselgel 60 with 230-400 mesh (Sigma-Aldrich, St. Louis, USA). Automated flash chromatography was carried out with a Biotage flash purification system using high-purity Kieselgel 60 with 230-400 mesh (Sigma-Aldrich). Solvents were removed under reduced pressure using a rotary evaporator and high vacuum (<1 mbar). Freeze-drying of aqueous solutions was performed using an Alpha 2-4 LD Lyophilizer (Christ, Osterode am Harz, Germany).

^{1}H, ^{13}C and two-dimensional NMR spectra were measured with a Varian 400-MR spectrometer or a Varian 600 spectrometer (both Agilent, Santa Clara, USA) at 298 K. 1D HH-NOESY measurements were performed at Freie Universität Berlin, NMR Core Facility, with a AMX500 spectrometer (Bruker, Billerica, USA), at 298 K. Chemical shifts (δ) are reported in parts per million (ppm) relative to the respective residual solvent peaks ($CDCl_3$: δ 7.26 in ^{1}H and 77.16 in ^{13}C NMR; acetone-D_6: δ 2.05 in ^{1}H and 29.84 in ^{13}C NMR; CD_3OD: δ 3.31 in ^{1}H and 49.00 in ^{13}C NMR; D_2O: δ 4.79 in ^{1}H NMR). Two-dimensional NMR experiments (HH-COSY, CH-HSQC, CH-HMBC) were performed to assign peaks in ^{1}H and ^{13}C spectra. The following abbreviations are used to indicate peak multiplicities: *s* singlet; *d* doublet; *dd* doublet of doublets; *t* triplet; *dt* doublet of triplets; *m* multiplet; *br s* broad singlet. Coupling constants (*J*) are reported

in Hertz (Hz). NMR spectra were evaluated using MestreNova 6.2 (MestreLab Research SSL, Santiago de Compostella, Spain). Optical rotation (OR) measurements were carried out with a UniPol L1000 polarimeter (Schmidt&Haensch, Berlin Germany) at $\lambda = 589$ nm and a concentration (c) expressed in g/100 mL in the solvent noted in parentheses. High resolution mass spectrometry by electrospray ionization (ESI-HRMS) was performed at Freie Universität Berlin, Mass Spectrometry Core Facility, with a 6210 ESI-TOF mass spectrometer (Agilent). Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) high resolution mass spectra were recorded on a Daltonics Autoflex Speed spectrometer (Bruker, Billerica, USA) using 2,5-dihydroxyacetophenone and 2,4,6-trihydroxyacetophenone matrices for proteins and organic compounds, respectively. Infrared (IR) spectra were measured with a 100 FTIR spectrometer (Perkin Elmer, Waltham, USA). Schemes were prepared using ChemBioDraw Ultra 12.0.2 (Cambridgesoft, Waltham, USA).

When handling small quantities (<5 mg) of thioether-containing compounds for analytical measurements, a drop of dimethyl sulfide (0.1% v/v) was added to the solution to avoid sulfoxide formation under the influence of air.

Monosaccharide residues are labeled in ^{1}H NMR spectra according to the following scheme:

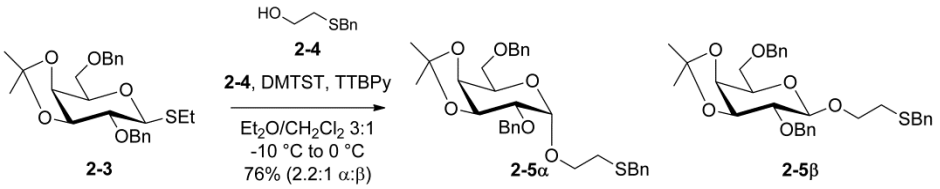


Chemoselective Thioglycoside Activation using DMTST

Typically, thioglycoside (0.20 mmol) and thioether-containing alcohol (0.3 mmol) were co-evaporated twice with anhydrous toluene and kept for 1 h under high vacuum. The mixture was dissolved in anhydrous CH_2Cl_2 or a mixture of anhydrous CH_2Cl_2 and Et_2O (4 mL), and 2,4,6-tri-*tert*-butylpyridine (TTBPy) and activated molecular sieves (3 Å)

were added. The solution was stirred for 30 min at room temperature and cooled to the indicated temperature. The mixture was treated with DMTST[264, 315] and stirred until t.l.c. indicated complete consumption of the thioglycoside. The reaction was then diluted with CH_2Cl_2 (10 mL) and quenched by addition of 10% aq. $Na_2S_2O_3$ (10 mL) and sat. aq. $NaHCO_3$ (10 mL). After separation, the aqueous fraction was extracted with CH_2Cl_2 (3x10 mL). The organic fractions were pooled, dried over Na_2SO_4 and concentrated. The product was purified by flash chromatography using the indicated solvents.

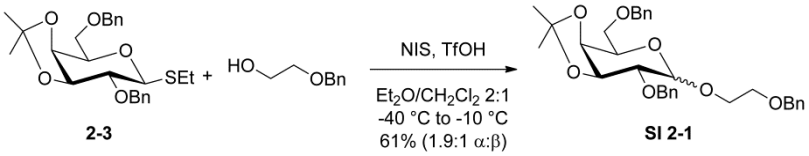
2,6-Di-*O*-benzyl-3,4-*O*-isopropylidene-α-D-galactopyranosyl-(1→1)-2-(benzylthio)ethanol (2-5α) and 2,6-di-*O*-benzyl-3,4-*O*-isopropylidene-β-D-galactopyranosyl-(1→1)-2-(benzylthio)ethanol (2-5β)



Thioglycoside **2-3**[257] (98 mg, 0.221 mmol) was glycosylated with alcohol **2-4**[258] (56 mg, 0.331 mmol) using DMTST (85 mg, 0.331 mmol) and TTBPy (109 mg; 0.441 mmol) in Et_2O (3.3 mL) and CH_2Cl_2 (1.1 mL) from -10 °C to 0 °C for 1 h. Flash chromatography was performed (EtOAc/hexanes 0:1 to 1:3) to give thioethers **2-5α** (63 mg, 0.114 mmol, 52%) and **2-5β** (29 mg, 0.052 mmol, 24%). Analytical data for **2-5α**: Clear oil; R_f (EtOAc/hexanes 1:2) = 0.77; $[\alpha]_D^{20}$ = +75.4° (c = 2.0, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$,) δ 7.39 – 7.19 (m, 15H, arom.), 4.80 (m, 2H, H-1, A of AB, $PhCH_2$), 4.70 (d, J = 12.5 Hz, 1H, B of AB, $PhCH_2$), 4.65 (d, J = 12.1 Hz, 1H, A of AB, $PhCH_2$), 4.55 (d, J = 12.1 Hz, 1H, B of AB, $PhCH_2$), 4.35 (dd, J = 7.8, 5.5 Hz, 1H, H-3), 4.29 (dt, J = 5.1, 2.5 Hz, 1H, H-5), 4.21 (dd, J = 5.5, 2.5 Hz, 1H, H-4), 3.81 (dt, J = 10.2, 6.9 Hz, 1H, A of AB, O-C*H*$_2$-CH_2), 3.78 – 3.68 (m, 4H, $PhCH_2$, H-6), 3.57 (dt, J = 10.2, 6.9 Hz, 1H, B of AB, O-C*H*$_2$-CH_2), 3.53 (dd, J = 7.9, 3.5 Hz, 1H, H-2), 2.71 – 2.58 (m, 2H, CH_2-C*H*$_2$-S), 1.40 (s, 3H, *i*Pr-CH_3), 1.34 (s, 3H, *i*Pr-CH_3); ^{13}C NMR (150 MHz, $CDCl_3$) δ 138.4, 138.3, 129.1, 128.6, 128.42, 128.41, 128.0, 127.8, 127.67, 127.66, 127.1, 109.2, 97.3, 76.6, 76.0, 73.8, 73.5, 72.4, 69.6, 67.9, 66.9, 36.5, 30.3, 28.2, 26.5; IR (thin film) 3063, 3029, 2985, 2916, 2869, 2340, 1953, 1812, 1576, 1495, 1453, 1380, 1370, 1313, 1243, 1218, 1165, 1099, 1074, 1044, 1028, 906, 873, 845, 791, 737, 698 cm^{-1}; HRMS (ESI) calcd. for $C_{32}H_{38}O_6S$ $(M+Na)^+$ 573.2287 found 573.2288 *m/z*. Analytical data for **2-5β**: Clear oil; R_f

(EtOAc/hexanes 1:2) = 0.63. $[\alpha]_D^{20}$ = +16.4° (c = 1.0, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.44 – 7.20 (m, 15H, arom.), 4.84 (d, J = 11.7 Hz, A of AB, 1H, $PhCH_2$), 4.79 (d, J = 11.7 Hz, B of AB, 1H, $PhCH_2$), 4.64 (d, J = 11.9 Hz, A of AB, 1H, $PhCH_2$), 4.57 (d, J = 11.9 Hz, B of AB, 1H, $PhCH_2$), 4.30 (d, J = 8.0 Hz, 1H, H-1), 4.15 (m, 2H, H-3, H-4), 4.01 (m, 1H, A of AB, O-CH_2-CH_2), 3.90 (ddd, J = 5.3, 1.5, 6.9 Hz, 1H, H-5), 3.83 – 3.72 (m, 4H, H-6, $PhCH_2$), 3.66 (m, 1H, B of AB, O-CH_2-CH_2), 3.42 – 3.36 (m, 1H, H-2), 2.79 – 2.65 (m, 2H, CH_2-CH_2-S), 1.37 (s, 3H, *i*PrCH_3), 1.34 (s, 3H, *i*Pr-CH_3); ^{13}C NMR (150 MHz, $CDCl_3$) δ 138.5, 138.4, 138.3, 129.0, 128.6, 128.5, 128.3, 128.3, 127.82, 127.78, 127.7, 127.2, 110.12, 110.05, 103.1, 79.6, 79.1, 73.9, 73.8, 73.7, 72.4, 69.6, 69.1, 36.6, 30.9, 27.9, 26.5. IR (thin film) 3063, 3030, 2986, 2924, 2870, 1602, 1496, 1454, 1397, 1242, 1219, 1161, 1097, 1079, 1045, 872, 801, 738, 698 cm^{-1}; HRMS (ESI) calcd. for $C_{32}H_{38}O_6S$ $(M+Na)^+$ 573.2287 found 573.2286 m/z.

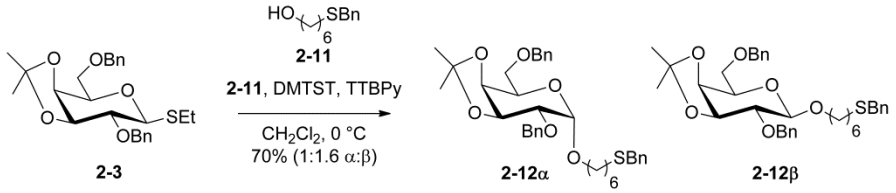
Control Reaction: Activation of Thioglycoside 2-3 with NIS/TfOH



Thioglycoside **2-3** (65 mg, 0.146 mmol) and monobenzyl ethylene glycol (33 mg, 0.219 mmol) were co-evaporated with anhydrous toluene (2x10 mL) and kept for 1 h under high vacuum. The mixture was dissolved in anhydrous Et_2O (2.1 mL) and anhydrous CH_2Cl_2 (0.7 mL) and activated molecular sieves (3 Å-AW) were added. The solution was stirred for 30 min at room temperature and cooled to -40 °C. The mixture was treated with NIS (49 mg, 0.219 mmol) and triflic acid (1.7 µL, 0.019 mmol) and slowly warmed to -10 °C over a period of 2 h, when t.l.c. indicated complete conversion of thioglycoside **2-3**. The reaction was diluted with CH_2Cl_2 (10 mL), quenched by adddition of Et_3N (0.2 mL), filtered and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 1:3) to give glycosides **SI 2-1** as an inseparable 1.9:1 (α:β) mixture of both isomers (48 mg, 0.090 mmol, 61%) as a clear oil. R_f (EtOAc/hexanes 1:3) = 0.47; $[\alpha]_D^{20}$ = +51.8° (c = 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42 – 7.21 (m, 15H), 4.89 (dd, J = 9.5, 7.7 Hz, 1.00H), 4.84 – 4.76 (m, 1H), 4.75 – 4.68 (m, 0.65H), 4.64 (d, J = 7.0 Hz, 0.35H), 4.61 (d, J = 7.2 Hz, 0.65H), 4.58 – 4.50 (m, 2H), 4.42 – 4.34 (m, 1H), 4.32 (m, 0.65H), 4.18 (dd, J = 5.6, 2.5 Hz, 0.65H), 4.15 (dd, J = 3.4, 1.2 Hz, 0.65H), 4.12 – 4.04 (m, 0.35H), 3.94 – 3.89 (m, 0.35H), 3.89 – 3.84 (m, 0.35H), 3.81 – 3.77 (m, 0.65H),

3.75 – 3.65 (m, 3H), 3.53 (dd, J = 7.8, 3.5 Hz, 0.65H), 3.45 – 3.39 (m, 0.35H), 1.39 (s, 1.95H), 1.37 (s, 1.05H), 1.34 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 138.6, 138.49, 138.48, 138.47, 138.43, 138.37, 133.1, 129.83, 129.80, 128.52, 128.47, 128.44, 128.43, 128.37, 128.34, 128.28, 128.0, 127.9, 127.79, 127.78, 127.76, 127.71, 127.69, 127.67, 127.65, 127.64, 127.57, 110.14, 110.13, 110.0, 109.2, 103.3, 97.4, 79.7, 79.1, 76.6, 76.0, 73.96, 73.94, 73.73, 73.70, 73.5, 73.4, 73.2, 72.4, 72.3, 69.7, 69.5, 69.4, 69.0, 67.5, 66.7, 28.3, 28.0, 26.5, 26.5; IR (thin film) 3031, 2986, 2931, 2968, 1721, 1497, 1454, 1380, 1370, 1243, 1219, 1166, 1100, 1074, 1044, 873, 737, 698 cm^{-1}; HRMS (ESI) calcd. for $C_{32}H_{38}O_7$ $(M+Na)^+$ 557.2515 found 557.2489 m/z.

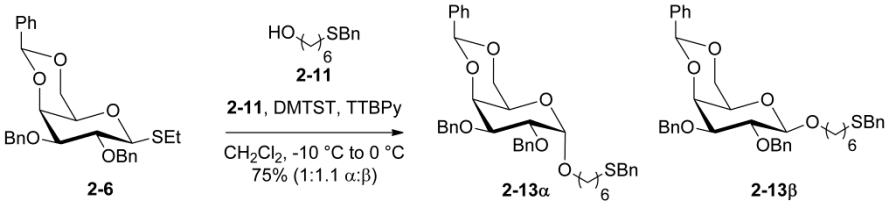
2,6-Di-*O*-benzyl-3,4-*O*-isopropylidene-α-D-galactopyranosyl-(1→1)-6-(benzylthio)hexanol (2-12α) and 2,6-di-*O*-benzyl-3,4-*O*-isopropylidene-β-D-galactopyranosyl-(1→1)-6-(benzylthio)hexanol (2-12β)



Thioglycoside **2-3** (95 mg, 0.214 mmol) was glycosylated with alcohol **2-11**[267] (72 mg, 0.321 mmol) using DMTST (83 mg, 0.321 mmol) and TTBPy (79 mg; 0.321 mmol) in CH_2Cl_2 (4.3 mL) at 0 °C for 1.5 h. Flash chromatography was performed (EtOAc/hexanes 0:1 to 1:4) to give thioethers **2-12α** (35 mg, 0.058 mmol, 27%, clear oil) and **2-12β** (56 mg, 0.092 mmol, 43%, clear oil). Analytical data for **2-12α**: R_f (EtOAc/hexanes 1:4) = 0.60; $[\alpha]_D^{20}$ = +54.6° (c = 0.2, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 – 7.20 (m, 15H, arom.), 4.78 (m, 2H, H-1, A of AB, $PhCH_2$), 4.69 (d, J = 12.6 Hz, 1H, B of AB, $PhCH_2$), 4.64 (d, J = 12.1 Hz, 1H, A of AB, $PhCH_2$), 4.53 (d, J = 12.1 Hz, 1H, B of AB, $PhCH_2$), 4.33 (dd, J = 7.8, 5.4 Hz, 1H, H-3), 4.23 – 4.11 (m, 2H, H-4, H-5), 3.77 – 3.61 (m, 5H, H-6, $PhCH_2$, A of AB, O-CH_2-CH_2), 3.51 (dd, J = 7.8, 3.5 Hz, 1H, H-2), 3.38 (dt, J = 9.8, 6.6 Hz, 1H, B of AB, O-CH_2-CH_2), 2.43 – 2.36 (m, 2H, S-CH_2-CH_2), 1.65 – 1.50 (m, 4H, aliph.), 1.44 – 1.28 (m, 10H, aliph, iPr-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 138.8, 138.6, 138.5, 129.0, 128.6, 128.48, 128.45, 128.0, 127.8, 127.71, 127.67, 127.0, 109.2, 97.2, 76.1, 74.0, 73.6, 72.4, 69.8, 68.4, 66.8, 36.5, 31.5, 29.4, 29.3, 28.8, 28.3, 26.6, 25.9; IR (thin film) 3029, 2929, 1496, 1454, 1380, 1243, 1219, 1167, 1102, 1073, 1044, 874, 735, 698 cm^{-1}; HRMS (ESI) calcd. for $C_{36}H_{46}O_6S$ $(M+Na)^+$ 629.2913

found 629.2889 *m/z*. Analytical data for **2-12β:** R_f (EtOAc/hexanes 1:4) = 0.27; $[\alpha]_D^{20}$ = +12.6° (c = 0.33, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41 – 7.18 (m, 15H, arom.), 4.84 (d, *J* = 11.7 Hz, 1H, A of AB, $PhCH_2$), 4.78 (d, *J* = 11.7 Hz, 1H, B of AB, $PhCH_2$), 4.64 (d, *J* = 11.9 Hz, 1H, A of AB, $PhCH_2$), 4.56 (d, *J* = 11.9 Hz, 1H, B of AB, $PhCH_2$), 4.29 (d, *J* = 8.1 Hz, 1H, H-1), 4.16 – 4.11 (m, 2H, H-3, H-4 or H-5), 3.97 – 3.87 (m, 2H, H-4 or H-5, A of AB, H-6), 3.82 – 3.76 (m, 2H, B of AB, H-6, A of AB, O-C*H*$_2$-CH_2), 3.69 (s, 2H, $PhCH_2$), 3.55 – 3.45 (m, 1H, B of AB, O-C*H*$_2$-CH_2), 3.43 – 3.34 (m, 1H, H-2), 2.40 (t, *J* = 7.2 Hz, 2H, S-C*H*$_2$-CH_2), 1.69 – 1.49 (m, 4H, aliph.), 1.41 – 1.31 (m, 10H, aliph., *i*Pr-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 138.8, 138.5, 138.4, 128.9, 128.54, 128.49, 128.3, 128.2, 127.8, 127.7, 127.6, 127.0, 110.0, 103.1, 79.8, 79.2, 74.0, 73.73, 73.71, 72.4, 69.9, 69.7, 36.5, 31.5, 29.7, 29.3, 28.8, 27.9, 26.5, 25.9; IR (thin film) 2934, 1454, 1370, 1219, 1076, 1045, 872, 737, 697 cm^{-1}; HRMS (ESI) calcd. for $C_{36}H_{46}O_6S$ $(M+Na)^+$ 629.2913 found 629.2990 *m/z*.

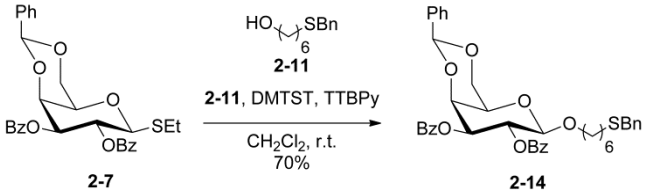
2,3-Di-O-benzyl-4,6-O-benzylidene-α-D-galactopyranosyl-(1→1)-6-(benzylthio)hexanol (2-13α) and 2,3-di-O-benzyl-4,6-O-benzylidene-β-D-galactopyranosyl-(1→1)-6-(benzylthio)hexanol (2-13β)



Thioglycoside **2-6**[268] (110 mg, 0.223 mmol) was glycosylated with alcohol **2-11** (75 mg, 0.335 mmol) using DMTST (87 mg, 0.335 mmol) and TTBPy (110 mg; 0.447 mmol) in CH_2Cl_2 (4.5 mL) from -10 °C to 0 °C for 1.5 h. Flash chromatography was performed twice (EtOAc/hexanes 1:6, then EtOAc/toluene 1:10) to give thioethers **2-13α** (52 mg, 0.080 mmol, 35%, white foam) and **2-13β** (58 mg, 0.089 mmol, 40%, clear oil). Analytical data for **2-13α**: R_f (EtOAc/hexanes 1:4) = 0.5; $[\alpha]_D^{20}$ = +84.3° (c = 0.5, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.55 – 7.50 (m, 2H, arom.), 7.44 – 7.22 (m, 18H, arom.), 5.47 (s, 1H, PhCH), 4.89 (d, *J* = 3.5 Hz, 1H, H-1), 4.88 – 4.78 (m, 2H, $PhCH_2$), 4.74 (d, *J* = 12.2 Hz, 1H, B of AB, $PhCH_2$), 4.66 (d, *J* = 12.0 Hz, 1H, B of AB, $PhCH_2$), 4.22 – 4.17 (m, 2H, H-4, A of AB, H-6), 4.06 (dd, *J* = 10.1, 3.5 Hz, 1H, H-2), 4.03 – 3.96 (m, 2H, H-3, B of AB, H-6), 3.70 (s, 2H, $PhCH_2$), 3.65 – 3.57 (m, 2H, H-5, A of AB, O-C*H*$_2$-CH_2), 3.49 – 3.40 (m, 1H, B of AB, O-C*H*$_2$-CH_2), 2.41 (t, *J* = 5.6 Hz, 2H, S-C*H*$_2$-

CH_2), 1.62 – 1.51 (m, 4H, aliph.), 1.40 – 1.26 (m, 4H, aliph.); ^{13}C NMR (100 MHz, $CDCl_3$) δ 139.1, 139.0, 138.8, 138.1, 129.0, 128.6, 128.43, 128.42, 128.2, 128.0, 127.8, 127.7, 127.6, 127.0, 126.5, 101.2, 98.3, 76.3, 75.9, 75.0, 73.7, 72.3, 69.7, 68.5, 62.8, 36.54, 31.53, 29.5, 29.3, 28. 8, 26.0; IR (thin film) 3030, 2922, 2858, 1496, 1454, 1400, 1364, 1340, 1247, 1100, 1049, 1028, 796, 739, 697 cm^{-1}; HRMS (ESI) calcd. for $C_{40}H_{46}O_6S$ $(M+Na)^+$ 677.2912 found 677.2905 *m/z*. Analytical data for **2-13β**: R_f (EtOAc/hexanes 1:4) = 0.29; $[\alpha]_D^{20}$ = +41.1° (c = 0.33, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.61 – 7.52 (m, 2H, arom.), 7.42 – 7.21 (m, 18H, arom.), 5.50 (s, 1H, PhCH), 4.92 (d, *J* = 10.9 Hz, 1H, A of AB, $PhCH_2$), 4.81 – 4.74 (m, 3H, $PhCH_2$), 4.37 (d, *J* = 7.8 Hz, 1H, H-1), 4.30 (dd, *J* = 12.3, 1.5 Hz, 1H, A of AB, H-6), 4.11 (dd, *J* = 3.7, 0.8 Hz, 1H, H-4), 4.01 (dd, *J* = 12.3, 1.8 Hz, 1H, B of AB, H-6), 3.96 (dt, *J* = 9.4, 6.4 Hz, 1H, A of AB, O-C*H*$_2$-CH_2), 3.83 (dd, *J* = 9.7, 7.7 Hz, 1H, H-2), 3.68 (s, 2H, $PhCH_2$), 3.55 (dd, *J* = 9.7, 3.7 Hz, 1H, H-3), 3.49 (dt, *J* = 9.4, 6.8 Hz, 1H, B of AB, O-C*H*$_2$-CH_2), 3.31 (d, *J* = 1.0 Hz, 1H, H-5), 2.43 – 2.35 (t, *J* = 5.2 Hz, 2H, S-C*H*$_2$-CH_2), 1.71 – 1.59 (m, 2H, aliph.), 1.58 – 1.50 (m, 2H, aliph.), 1.44 – 1.30 (m, 4H, aliph.); ^{13}C NMR (100 MHz, $CDCl_3$) δ 139.1, 138.8, 138.7, 138.1, 129.02, 128.97, 128.6, 128.5, 128.41, 128.25, 128.1, 127.9, 127.8, 127.7, 127.0, 126.7, 103.9, 101.5, 79.5, 78.6, 75.4, 74.23, 72.17, 70.0, 69.5, 66.6, 36.5, 31.5, 29.8, 29.3, 28.9, 25.9; IR (thin film) 3031, 2925, 2858, 1496, 1454, 1365, 1178, 1101, 1057, 1028, 734, 698 cm^{-1}; HRMS (ESI) calcd. for $C_{40}H_{46}O_6S$ $(M+Na)^+$ 677.2912 found 677.2905 *m/z*.

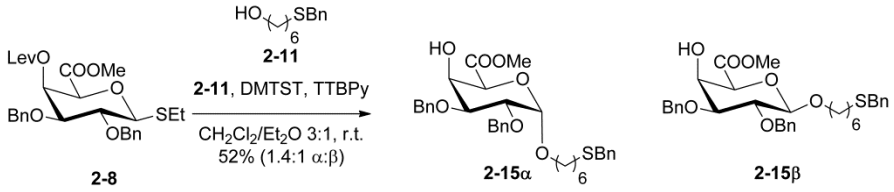
2,3-Di-*O*-benzoyl-4,6-*O*-benzylidene-β-D-galactopyranosyl-(1→1)-6-(benzylthio)hexanol (2-14)



Thioglycoside **2-7**[269] (100 mg, 0.192 mmol) was glycosylated with alcohol **2-11** (65 mg, 0.288 mmol) using DMTST (99 mg, 0.384 mmol) and TTBPy (57 mg; 0.231 mmol) in CH_2Cl_2 (3.2 mL) at room temperature for 3 h. Flash chromatography was performed (EtOAc/CH_2Cl_2/hexanes 1:1:3 to 1:1:2) to give thioether **2-14** (92 mg, 0.134 mmol, 70%) as a clear oil, which solidified upon standing. R_f (EtOAc/CH_2Cl_2/hexanes 1:1:2) = 0.66; $[\alpha]_D^{20}$ = +100.2° (c = 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.15 – 7.89 (m, 4H, arom.), 7.58 – 7.43 (m, 4H, arom.), 7.43 – 7.19 (m, 12H, arom.), 5.87 (dd, *J* = 10.4, 8.0

Hz, 1H, H-2), 5.55 (s, 1H, PhCH), 5.38 (dd, J = 10.4, 3.6 Hz, 1H, H-3), 4.73 (d, J = 8.0 Hz, 1H, H-1), 4.62 – 4.56 (m, 1H, H-4), 4.41 (dd, J = 12.4, 1.4 Hz, 1H, A of AB, H-6), 4.14 (dd, J = 12.4, 1.7 Hz, 1H, B of AB, H-6), 3.96 (dt, J = 9.6, 6.1 Hz, 1H, A of AB, O-CH_2-CH_2), 3.69 – 3.62 (m, 3H, H-5, S-CH_2-Ph), 3.51 (dt, J = 9.6, 6.7 Hz, 1H, B of AB, O-CH_2-CH_2), 2.26 (t, J = 7.4 Hz, 2H, CH_2-SBn), 1.59 – 1.41 (m, 2H, aliph.), 1.31 (m, 2H, aliph.), 1.17 (m, 4H, aliph.); ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.4, 165.3, 138.8, 137.7, 133.5, 133.1, 130.1, 129.9, 129.8, 129.3, 129.0, 128.9, 128.5, 128.5, 128.4, 128.2, 127.0, 126.4, 101.4, 101.0, 73.8, 72.9, 69.6, 69.3, 69.1, 66.6, 36.4, 31.4, 29.4, 29.1, 28.6, 25.6; IR (thin film) 2933, 2857, 1723, 1602, 1451, 1368, 1315, 1275, 1179, 1110, 1097, 1000, 737, 709 cm^{-1}; HRMS (ESI) calcd. for $C_{40}H_{42}O_8S$ $(M+Na)^+$ 705.2498 found 705.2476 m/z.

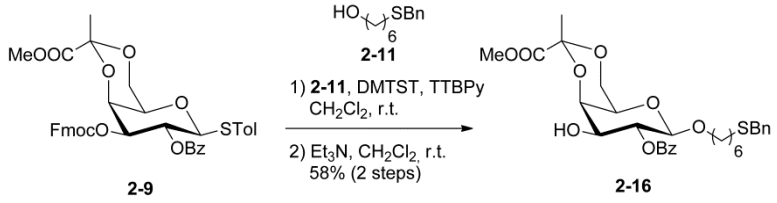
Methyl (2,3-di-*O*-benzyl-4,6-*O*-benzylidene-α-D-galactopyranosid)uronate-(1→1)-6-(benzylthio)hexanol (15α) and methyl (2,3-di-*O*-benzyl-4,6-*O*-benzylidene-β-D-galactopyranosid)uronate-(1→1)-6-(benzylthio)hexanol (2-15β)



Thioglycoside **2-8** (87 mg, 0.164 mmol; *see* below) was glycosylated with alcohol **2-11** (85 mg, 0.379 mmol) using DMTST (63.5 mg, 0.246 mmol) and TTBPy (97 mg; 0.392 mmol) in Et_2O (2.4 mL) and CH_2Cl_2 (0.8 mL) at room temperature for 8 h. Flash chromatography was performed (EtOAc/CH_2Cl_2/hexanes 0:0:1 to 2:1:1) to give the corresponding glycosides as an inseparable 1.4:1 (α:β) mixture of both isomers as a clear oil (60 mg). The compound mixture in CH_2Cl_2 (2.2 mL) was then treated with a mixture of glacial acetic acid (0.14 mL, 2.393 mmol) and pyridine (0.2 mL, 2.411 mmol), and subsequently with hydrazine hydrate (5.9 µL, 0.121 mmol) at room temperature. The reaction was stirred for 2 h at that temperature, quenched by addition of acetone (0.2 mL) and concentrated. The residue was dissolved in CH_2Cl_2 (20 mL) and washed with 1 M aq. HCl (10 mL) and sat. aq. $NaHCO_3$ (10 mL). The combined aqueous fractions were extracted with CH_2Cl_2 (2x10 mL), the combined organic fractions were dried over Na_2SO_4 and concentrated. Flash chromatography was performed (EtOAc/hexanes 1:2) to give alcohols **2-15α** (29 mg, 0.049 mmol, 30% over two steps, clear oil) and **2-15β** (22

mg, 0.037 mmol, 22% over two steps, clear oil). Analytical data for **2-15α**: R_f (EtOAc/hexanes 1:1) = 0.57; $[\alpha]_D^{20}$ = +47.1° (c = 1.0, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 7.42 – 7.18 (m, 15H, arom.), 4.87 (d, *J* = 3.5 Hz, 1H, H-1), 4.83 – 4.77 (m, 2H, $PhCH_2$), 4.72 (d, *J* = 11.4 Hz, 1H, B of AB, $PhCH_2$), 4.62 (d, *J* = 12.1 Hz, 1H, B of AB, $PhCH_2$), 4.39 (m, 2H, H-4, H-5), 3.95 (dd, *J* = 9.8, 3.0 Hz, 1H, H-3), 3.86 (dd, *J* = 9.8, 3.5 Hz, 1H, H-2), 3.81 (s, 3H, $COOCH_3$), 3.70 (s, 2H, $PhCH_2$), 3.63 (dt, *J* = 9.7, 6.9 Hz, 1H, A of AB, O-C*H*$_2$-CH_2), 3.42 (dt, *J* = 9.7, 6.9 Hz, 1H, B of AB, O-C*H*$_2$-CH_2), 2.52 (br s, 1H, OH), 2.40 (t, *J* = 7.3 Hz, 2H, CH_2-C*H*$_2$-S), 1.57 (m 4H, aliph.), 1.45 – 1.22 (m, 4H, aliph.); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.4, 138.7, 138.4, 138.0, 128.9, 128.7, 128.58, 128.56, 128.18, 128.11, 128.07, 128.0, 127.9, 127.0, 97.7, 77.0, 75.3, 73.6, 73.0, 69.9, 68.9, 52.7, 36.5, 31.4, 29.3, 29.2, 28.7, 25.8; IR (thin film) 2929, 1764, 1454, 1208, 1101, 1028, 738, 699 cm^{-1}; HRMS (ESI) calcd. for $C_{34}H_{42}O_7S$ $(M+Na)^+$ 617.2548 found 617.2542 *m/z*. Analytical data for **2-15β**: R_f (EtOAc/hexanes 1:1) = 0.63; $[\alpha]_D^{20}$ = -7.1° (c = 1.0, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 7.38 – 7.19 (m, 15H, arom.), 4.90 (d, *J* = 11.1 Hz, 1H, A of AB, $PhCH_2$), 4.77 – 4.67 (m, 3H, B of AB, $PhCH_2$), 4.34 (d, *J* = 7.7 Hz, 1H, H-1), 4.31 (dd, *J* = 3.5, 1.4 Hz, 1H, H-4), 4.04 (d, *J* = 1.4 Hz, 1H, H-5), 4.00 (dt, *J* = 9.4, 6.4 Hz, 1H, A of AB, O-C*H*$_2$-CH_2), 3.82 (s, 3H, $COOCH_3$), 3.69 – 3.63 (m, 3H, $PhCH_2$, H-2), 3.55 (dd, *J* = 9.3, 3.4 Hz, 1H, H-3), 3.49 (dt, *J* = 9.4, 6.8 Hz, 1H, B of AB, O-C*H*$_2$-CH_2), 2.47 – 2.34 (t, *J* = 7.6 Hz, 2H, CH_2-SBn), 1.62 (d, *J* = 6.2 Hz, 2H, aliph.), 1.52 (dd, *J* = 14.1, 6.8 Hz, 2H, aliph.), 1.45 – 1.28 (m, 4H, aliph.); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.7, 138.8, 138.6, 137.7, 129.0, 128.7, 128.6, 128.5, 128.20, 128.17, 128.0, 127.8, 127.0, 103.5, 79.8, 78.4, 75.3, 73.8, 72.7, 70.2, 68.1, 52.7, 36.5, 31.5, 29.7, 29.3, 28.8, 25.9; IR (thin film) 2930, 1765, 1454, 1211, 1099, 1040, 738, 698 cm^{-1}; HRMS (ESI) calcd. for $C_{34}H_{42}O_7S$ $(M+Na)^+$ found 617.2548 found 617.2547 *m/z*.

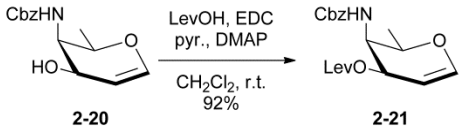
2-*O*-Benzoyl-4,6-[1-(*R*)-(methoxycarbonyl)-ethylidene]-β-D-galactopyranosyl-(1→1)-6-(benzylthio)hexanol (2-16)



Thioglycoside **2-9**[132] (200 mg, 0.287 mmol) was glycosylated with alcohol **2-11** (129 mg, 0.574 mmol) using DMTST (148 mg, 0.574 mmol) and TTBPy (74.7 mg, 0.301 mmol) in

CH_2Cl_2 (5.7 mL) at room temperature for 20 h. Flash chromatography was performed (EtOAc/hexanes 1:3 to 1:1) to give the crude glycoside, which contained excess alcohol **2-11**. The material was then dissolved in CH_2Cl_2 (5.2 mL) and treated with Et_3N (0.3 mL, 2.15 mmol) at room temperature. The mixture was stirred for 1.5 h at that temperature, diluted with toluene (10 mL) and concentrated. The crude product was purified by flash chromatography (EtOAc/hexanes 1:2 to 1:1) to give alcohol **2-16** (96 mg, 0.167 mmol, 58% over two steps) as a clear oil. R_f (EtOAc/hexanes 1:2) = 0.18; $[\alpha]_D^{20}$ = -8.7° (c = 1.0, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.12 – 7.89 (m, 2H, arom.), 7.73 – 7.12 (m, 8H, arom.), 5.32 (dd, *J* = 10.0, 8.0 Hz, 1H, H-2), 4.50 (d, *J* = 8.0 Hz, 1H, H-1), 4.18 (dd, *J* = 3.8, 0.9 Hz, 1H, H-4), 4.08 (ddd, *J* = 32.6, 12.8, 1.7 Hz, 2H, H-6), 3.90 (dt, *J* = 9.7, 6.1 Hz, 1H, A of AB, O-C*H*$_2$-CH_2), 3.84 – 3.78 (m, 4H, $COOCH_3$, H-3), 3.62 (s, 2H, $PhCH_2$), 3.42 (m, 2H, B of AB, O-C*H*$_2$-CH_2, H-5) 2.43 (br s, 1H, OH), 2.23 (t, *J* = 7.3 Hz, 2H, CH_2-SBn), 1.63 (s, 3H, CH_3), 1.53 – 1.38 (m, 2H, aliph.), 1.38 – 1.07 (m, 6H, aliph.); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.3, 166.2, 138.7, 133.2, 130.1, 129.9, 128.9, 128.5, 128.44, 128.42, 126.9, 101.0, 98.9, 72.7, 71.6, 71.4, 69.9, 65.8, 65.2, 52.9, 36.3, 31.3, 29.3, 29.0, 28.6, 25.9, 25.6; IR (thin film) 2935, 1726, 1452, 1373, 1270, 1206, 1178, 1121, 1087, 983, 711 cm^{-1}; HRMS (ESI) calcd. for $C_{30}H_{38}O_9S$ $(M+Na)^+$ 597.2134 found 597.2119 *m/z*.

4-*O*-(Benzyloxycarbonyl)amino-3-*O*-levulinoyl-4,6-dideoxy-D-galactal (2-21)

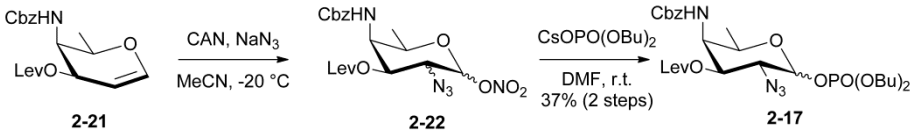


2-20 **2-21**

To a stirred solution of alcohol **2-20**[179] (1.64 g, 6.21 mmol) in CH_2Cl_2 (40 mL) were added at 0 °C pyridine (0.5 mL, 6.23 mmol), levulinic acid (0.96 mL, 9.32 mmol), DMAP (0.15 g, 1.24 mmol) and EDC (1.2 mL, 6.83 mmol). The mixture was warmed to room temperature and stirred at that temperature. After 3 h, 0.5 equiv. levulinic acid and 0.5 equiv. EDC were added to drive the reaction to completion. After 5 h, the mixture was diluted with 100 mL CH_2Cl_2 and washed with water (50 mL), sat. aq. NH_4Cl (50 mL), sat. aq. $NaHCO_3$ (50 mL) and brine (50 mL). The organic fraction was dried over Na_2SO_4 and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 1:1) to give ester **2-21** (2.07 g, 5.73 mmol, 92%) as a clear oil. R_f (EtOAc/hexanes 3:7) = 0.40; $[\alpha]_D^{20}$ = +1.2° (c = 1.0, $CHCl_3$); ^{1}H NMR (400 MHz,

$CDCl_3$) δ 7.44 – 7.28 (m, 5H, arom.), 6.41 (dd, J = 6.3, 1.8 Hz, 1H, H-1), 5.50 (d, J = 5.5 Hz, 1H, H-3), 5.17 (d, J = 12.2 Hz, 1H, A of AB, $PhCH_2$), 5.07 (d, J = 12.3 Hz, 1H, B of AB, $PhCH_2$), 4.99 (d, J = 10.0 Hz, 1H, NH), 4.65 (dt, J = 6.3, 1.7 Hz, 1H, H-2), 4.20 (m, 2H, H-4, H-5), 2.87 – 2.69 (m, 1H, Lev-CH_2), 2.68 – 2.47 (m, 2H, Lev-CH_2), 2.35 (dt, J = 17.1, 6.2 Hz, 1H, Lev-CH_2), 2.15 (s, 3H, Lev-CH_3), 1.29 (d, J = 6.5 Hz, 3H, H-6); ^{13}C NMR (100 MHz, $CDCl_3$) δ 206.7, 172.3, 157.0, 146.3, 136.6, 128.6, 128.3, 128.1, 99.6, 76.8, 72.9, 67.0, 66.5, 48.5, 38.0, 29.9, 28.0, 16.7; HRMS (ESI) calcd. for $C_{19}H_{23}NO_6$ $(M+Na)^+$ 384.1423 found 384.1415 m/z.

Dibutyl [2-azido-4-(benzyloxycarbonyl)amino-3-*O*-levulinoyl-αβ-2,4,6-trideoxy-D-galactopyranosyl] phosphate (2-17)

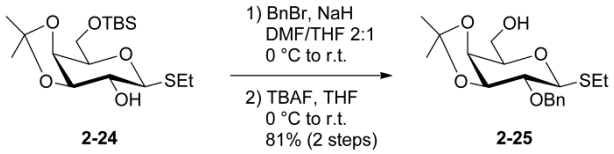


To a stirred solution of galactal **2-21** (3.17 g, 8.77 mmol) in anhydrous MeCN (44 mL) were added at -25°C ceric ammonium nitrate (14.42 g, 26.3 mmol) and sodium azide (0.86 g, 13.15 mmol). The reaction was stirred vigorously between -25 °C and -20 °C for 6 h. The mixture was diluted with cold Et_2O (50 mL), washed with cold water (3x30 mL), dried over Na_2SO_4 and concentrated. The residue was filtered through a plug of silica gel (EtOAc/hexanes/Et_3N 1:1:0.01) to give crude glycosyl nitrate **2-22** as a 4:1 *galacto*:*talo* mixture (2.0 g) as a slightly yellow oil.

To crude glycosyl nitrate **2-22** (2.0 g) was added at room temperature a solution of cesium dibutyl phosphate (2.21 g, 6.45 mmol) in anhydrous DMF (28 mL). The mixture was stirred at that temperature for 4.5 h, diluted with EtOAc (100 mL) and poured into water (100 mL). The organic phase was washed with water (5x50 mL) and the combined aqueous fractions were extracted with EtOAc (50 mL). The combined organic fractions were dried over Na_2SO_4 and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 45:55 to 50:50) to give glycosyl phosphate **2-17** (1.84 g, 3.00 mmol, 37%, 1:10 α:β) as a clear oil. R_f (EtOAc/hexanes 4:1) = 0.50 (β-anomer) & 0.72 (α-anomer). Analytical data for the β-anomer: $[\alpha]_D^{20}$ = +10.8° (c = 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 – 7.28 (m, 5H, arom.), 5.15 (d, J = 12.1 Hz, 2H, NH, A of AB, $PhCH_2$), 5.10 – 5.03 (d, J = 12.3 Hz, 1H, B of AB, $PhCH_2$), 4.98 (t, J

= 8.6 Hz, 1H, H-1), 4.74 (dd, J = 10.7, 3.9 Hz, 1H, H-3), 4.22 – 4.00 (m, 5H, H-4, PO-CH_2-CH$_2$), 3.91 – 3.79 (m, 1H, H-5), 3.61 (dd, J = 10.7, 8.2 Hz, 1H, H-2), 2.87 – 2.72 (m, 1H, Lev-CH$_2$), 2.72 – 2.51 (m, 2H, Lev-CH$_2$), 2.41 (ddd, J = 13.1, 9.4, 5.5 Hz, 1H, Lev-CH$_2$), 2.21 – 2.11 (s, 3H, Lev-CH$_3$), 1.79 – 1.56 (m, 4H, aliph.), 1.53 – 1.31 (m, 4H, aliph.), 1.30 – 1.19 (s, 3H, H-6), 0.92 (t, J = 7.4 Hz, 6H, aliph.); ^{13}C NMR (100 MHz, CDCl$_3$) δ 206.4, 172.0, 156.6, 136.4, 128.7, 128.4, 128.1, 97.83, 97.77, 73.3, 70.6, 68.3, 68.22, 68.16, 68.1, 67.3, 61.5, 61.4, 51.8, 38.0, 32.3, 32.24, 32.21, 32.17, 29.89, 27.91, 18.71, 18.70, 16.5, 13.67, 13.65; IR (thin film) 3658, 2981, 2115, 1720, 1462, 1382, 1252, 1153, 1073, 1030, 957, 820, 755 cm^{-1}; HRMS (ESI) calcd. for $C_{27}H_{41}N_4O_{10}P$ $(M+Na)^+$ 635.2458 found 635.2422 m/z.

Ethyl 2-*O*-benzyl-3,4-isopropylidene-1-thio-β-D-galactopyranoside (2-25)



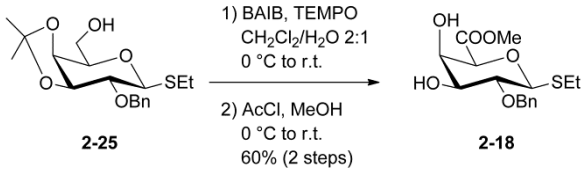
2-24 **2-25**

To a stirred solution of alcohol **2-24**[281] (45.7 g, 121 mmol) in DMF (150 mL) and THF (75 mL) were added at 0 °C first portionwise sodium hydride (60% (w/w), 7.24 g, 181 mmol) and then benzyl bromide (17.2 mL, 145 mmol). The mixture was stirred for 1 h at 0 °C, slowly warmed to room temperature and stirred for 16 h at that temperature. The reaction was quenched at 0 °C with sat. aq. NH$_4$Cl (20 mL), diluted with water (200 mL) and EtOAc (150 mL) and stirred for 15 min at 0 °C. After separation, the organic phase was washed with water (5x100 mL) and the combined aqueous fractions were extracted with EtOAc (2x100 mL). The combined organic extracts were dried over Na$_2$SO$_4$ and concentrated to give the crude benzyl ether (61 g) as a yellow oil.

To a stirred solution of the crude benzyl ether (61 g) in THF (370 mL) was added at 0 °C tetrabutylammonium fluoride (1 M in THF, 166 mL, 166 mmol). The mixture was warmed to room temperature and stirred for 1 h at that temperature. The reaction was diluted with sat. aq. NaHCO$_3$ (200 mL) and EtOAc (100 mL). After separation, the aqueous phase was extracted with EtOAc (3x100 mL), the combined organic fractions were dried over MgSO$_4$ and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 0:1 to 1:3 to 1:1) to give alcohol **2-25** (35 g, 98 mmol, 81% over two steps) as a clear oil. R$_f$ (EtOAc/hexanes 1:2) = 0.25; $[\alpha]_D^{20}$ = +1.1° (c =

2.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45 – 7.38 (m, 2H, arom.), 7.37 – 7.23 (m, 3H, arom.), 4.84 (d, J = 11.4 Hz, 1H, A of AB, $PhCH_2$), 4.76 (d, J = 11.4 Hz, 1H, B of AB, $PhCH_2$), 4.43 (d, J = 9.6 Hz, 1H, H-1), 4.25 (dd, J = 13.1, 7.2 Hz, 1H, H-3), 4.19 (dd, J = 5.7, 2.0 Hz, 1H, H-4), 3.94 (dd, J = 11.1, 6.9 Hz, 1H, A of AB, H-6), 3.84 – 3.73 (m, 2H, H-5, B of AB, H-6), 3.46 (dd, J = 9.6, 5.3 Hz, 1H, H-2), 2.72 (m, 2H, S-CH_2-CH_3), 2.22 (s, 1H, OH), 1.43 (s, 3H, *i*Pr-CH_3), 1.35 (s, 3H, *i*Pr-CH_3), 1.33 – 1.26 (m, 3H, S-CH_2-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 137.9, 128.41, 128.37, 127.9, 110.3, 83.9, 79.8, 79.1, 76.8, 74.1, 73.6, 62.7, 27.9, 26.5, 24.8, 15.1; IR (thin film) 3234, 2980, 2934, 1455, 1381, 1368, 1245, 1217, 1079, 1037, 875, 743, 697 cm^{-1}; HRMS (ESI) calcd. for $C_{18}H_{26}O_5S$ $(M+Na)^+$ 377.1398 found 377.1416 m/z.

Methyl (ethyl 1-thio-β-D-galactopyranosid)uronate (2-18)

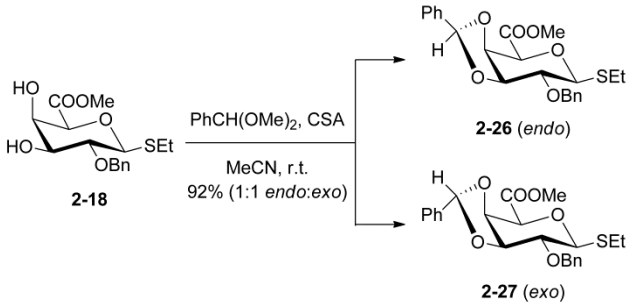


To a vigorously stirred solution of alcohol **2-25** (6.0 g, 16.9 mmol) in CH_2Cl_2 (50 mL) and water (25 mL) were added at 0 °C TEMPO (0.53 g, 3.4 mmol) and BAIB (10.9 g, 33.9 mmol). The mixture was warmed to room temperature and stirred for 1 h at that temperature. The reaction was quenched with 10% aq. $Na_2S_2O_3$ (10 mL) and diluted with EtOAc (30 mL). After separation, the organic phase was washed with 10% $Na_2S_2O_3$ (4x20 mL). The aqueous phase was extracted with EtOAc (2x20 mL), the combined organic fractions were dried over Na_2SO_4 and concentrated to give the crude acid (7.92 g) as a yellow oil.

To a stirred solution of acetyl chloride (6.04 mL, 85 mmol) in MeOH (300 mL) was added dropwise at 0 °C a solution of the crude acid (7.92 g) in MeOH (40 mL). The mixture was warmed to room temperature and stirred for 2 h at that temperature. The reaction was quenched at 0 °C with sat. aq. $NaHCO_3$ (30 mL) and neutralized to pH 7 with solid $NaHCO_3$. The volatiles were evaporated and the mixture was diluted with EtOAc (70 mL). After separation, the aqueous phase was extracted with EtOAc (5x50 mL). The combined organic fractions were dried over Na_2SO_4 and concentrated. Flash chromatography was performed (EtOAc/hexanes 2:3 to 1:1 to 1:0) to give the crude product, which was crystallized in methanol at -20 °C (5 mL/g crude product) to give

diol **2-18** (3.47 g, 10.1 mmol, 60% over two steps) as a white solid. R_f (EtOAc) = 0.51; $[\alpha]_D^{20}$ = -32.0° (c = 0.50, MeOH); ^{1}H NMR (400 MHz, CD_3OD) δ 7.46 – 7.40 (m, 2H, arom.), 7.34 – 7.23 (m, 3H, arom.), 4.84 – 4.77 (m, 2H, $PhCH_2$), 4.49 (d, J = 9.7 Hz, 1H, H-1), 4.26 (s, 1H, H-5), 4.18 (dd, J = 3.4, 1.3 Hz, 1H, H-4), 3.77 (s, 3H, $COOCH_3$), 3.70 (dd, J = 9.2, 3.5 Hz, 1H, H-3), 3.52 (t, J = 9.4 Hz, 1H, H-2), 2.88 – 2.66 (m, 2H, S-C*H*$_2$-CH_3), 1.30 (t, J = 7.4 Hz, 3H, S-CH_2-C*H*$_3$); ^{13}C NMR (100 MHz, CD_3OD) δ 170.5, 139.9, 129.3, 129.1, 128.6, 86.1, 79.6, 78.7, 76.3, 75.7, 71.9, 52.6, 25.6, 15.5. IR (thin film) 3461, 2929, 1744, 1440, 1350, 1267, 1216, 1101, 1029, 836, 741, 699 cm^{-1}; HRMS (ESI) calcd. for $C_{16}H_{22}O_6S$ $(M+Na)^+$ 365.1034 found 365.1058 *m/z*.

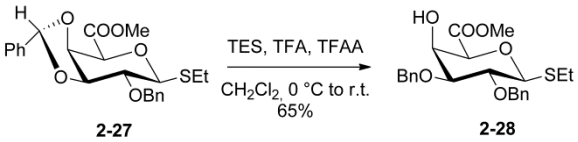
Methyl (ethyl 2-*O*-benzyl-3,4-*O*-*endo*-benzylidene-1-thio-β-D-galactopyranosid)uronate (26) and methyl (ethyl 2-*O*-benzyl-3,4-*O*-*exo*-benzylidene-1-thio-β-D-galactopyranosid)uronate (2-27)



To a stirred solution of diol **2-18** (2.99 g, 8.73 mmol) in anhydrous MeCN (29 mL) were added at room temperature benzaldehyde dimethyl acetal (6.57 mL, 43.6 mmol) and DL-camphorsulfonic acid (0.51 g, 2.18 mmol). The mixture was stirred at room temperature for 5 h and the reaction was quenched by addition of Et_3N (0.35 mL). The mixture was concentrated and the residue was filtered through a short plug of silica gel (EtOAc/hexanes/Et_3N 1:8:0.02 to 1:1:0.02) to give benzylidene acetals **2-26** (*endo*) and **2-27** (*exo*) (3.46 g, 8.03 mmol, 92%) as a 1:1 mixture. The isomers were separated by selective crystallization of *exo*-isomer **2-27** from EtOAc/hexanes and chromatographic separation of the mother liquor (Biotage, flat gradient of 10% to 40% (v/v) EtOAc in hexanes + 0.5% Et_3N). Analytical data for **2-26**: Clear oil; R_f (EtOAc/hexanes 1:2) = 0.40; $[\alpha]_D^{20}$ = -59.3° (c = 2.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$,) δ 7.37 (m, 10H, arom.), 5.93 (s, 1H, PhCH), 4.69 (dd, J = 11.5 Hz, 2H, $PhCH_2$), 4.64 – 4.61 (dd, J = 6.4 Hz, 2.5 Hz, 1H, H-4), 4.58 (d, J = 8.6 Hz, 1H, H-1), 4.46 (m, 2H, H-3, H-5), 3.81 (s, 3H, $COOCH_3$) 3.61 (dd, J = 8.6, 5.7 Hz, 1H, H-2), 2.88 – 2.60 (m, 2H, S-C*H*$_2$-CH_3), 1.30 (t, J

= 7.4 Hz, 3H, S-CH_2-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.5, 137.5, 136.8, 129.5, 128.4, 128.39, 128.35, 127.9, 127.1, 104.9, 84.1, 78.6, 78.1, 75.7, 75.1, 73.3, 52.6, 24.8, 14.9; IR (thin film) 2928, 2874, 1767, 1737, 1455, 1438, 1267, 1216, 1150, 1091, 1076, 1028, 756, 698 cm^{-1}; HRMS (ESI) calcd. for $C_{23}H_{26}O_6S$ $(M+Na)^+$ 453.1348 found 453.1352 *m/z*. Analytical data for **2-27**: White foam; R_f (EtOAc/hexanes 1:2) = 0.50; $[\alpha]_D^{20}$ = -42.0° (c = 2.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46 – 7.22 (m, 10H, arom.), 6.06 (s, 1H, PhCH), 4.91 – 4.76 (dd, 2H, *J* = 15.2, 11.5 Hz, $PhCH_2$), 4.71 – 4.55 (m, 3H, H-1, H-3, H-4), 4.37 (d, *J* = 1.8 Hz, 1H, H-5), 3.79 (s, 3H, $COOCH_3$), 3.69 (dd, *J* = 8.4, 5.1 Hz, 1H, H-2), 2.93 – 2.59 (m, 2H, S-CH_2-CH_3), 1.33 (t, *J* = 7.4 Hz, 3H, S-CH_2-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.6, 138.0, 137.3, 129.4, 128.5, 128.5, 128.1, 126.4, 104.0, 83.7, 79.3, 75.6, 75.5, 74.3, 73.4, 52.6, 25.1, 15.0; IR (thin film) 2903, 2873, 1765, 1738, 1497, 1455, 1438, 1344, 1267, 1213, 1140, 1098, 1075, 1028, 1002, 969, 920, 758, 737, 697 cm^{-1}; HRMS (ESI) calcd. for $C_{23}H_{26}O_6S$ $(M+Na)^+$ 453.1348 found 453.1338 *m/z*.

Methyl (ethyl 2,3-*O*-benzyl-1-thio-β-D-galactopyranosyl)uronate (2-28)

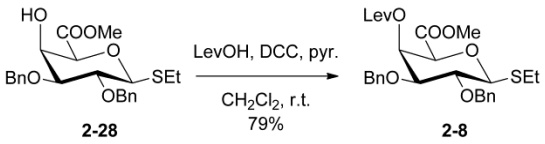


2-27 **2-28**

To a stirred solution of acetal **2-27** (206 mg, 0.48 mmol) and triethylsilane (2.29 mL, 14.35 mmol) in CH_2Cl_2 (7 mL) over activated molecular sieves (3 Å-AW) were added at 0 °C trifluoroacetic anhydride (20 µL, 0.14 mmol) and then trifluoroacetic acid (0.22 mL, 2.87 mmol). The reaction was warmed to room temperature and stirred for 5 h at that temperature. The mixture was diluted with EtOAc (10 mL), quenched with Et_3N (0.4 mL) at 0 °C and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 1:1) to give alcohol **2-28** (134 mg, 0.31 mmol, 65%) as a white foam. R_f (EtOAc/hexanes 2:3) = 0.47; $[\alpha]_D^{20}$ = -14.0° (c = 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44 – 7.27 (m, 10H, arom.), 4.89 (d, *J* = 10.3 Hz, 1H, A of AB, $PhCH_2$), 4.81 – 4.67 (m, 3H, $PhCH_2$), 4.43 (d, *J* = 9.6 Hz, 1H, H-1), 4.39 (d, *J* = 0.9 Hz, 1H, H-4), 4.06 (s, 1H, H-5), 3.82 (s, 3H, $COOCH_3$), 3.71 (t, *J* = 9.3 Hz, 1H, H-2), 3.61 (dd, *J* = 8.9, 3.3 Hz, 1H, H-3) 2.96 – 2.67 (m, 2H, S-CH_2-CH_3), 2.52 (d, *J* = 1.8 Hz, 1H, OH), 1.32 (t, *J* = 7.4 Hz, 3H, S-CH_2-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.5, 138.1, 137.5, 128.7, 128.51, 128.50, 128.3, 128.1, 128.0, 85.2, 81.7, 77.4, 77.1, 76.0, 72.4, 68.1, 52.8, 25.0, 15.1; IR (thin film) 3492, 2927, 2870, 1764, 1736, 1497, 1454, 1351, 1266, 1210, 1128, 1098,

1029, 903, 844, 741, 698 cm^{-1}; HRMS (ESI) calcd. for $C_{23}H_{28}O_6S$ $(M+Na)^+$ 455.1504 found 455.1511 *m/z*.

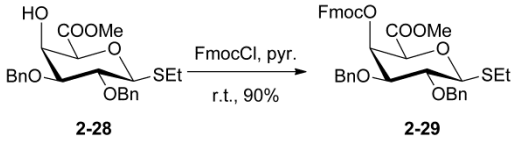
Methyl (ethyl 2,3-*O*-benzyl-4-*O*-levulinoyl-1-thio-β-D-galactopyranosyl)uronate (2-8)



2-28 **2-8**

To a stirred solution of alcohol **2-28** (94 mg, 0.217 mmol) in CH_2Cl_2 (1.9 mL) were added at room temperature levulinic acid (386 mg, 3.26 mmol), DCC (673 mg, 3.26 mmol) and pyridine (0.26 mL, 3.26 mmol). The mixture was stirred at that temperature for 35 h, diluted with CH_2Cl_2 (5 mL) and filtered through Celite. The mixture was concentrated, the residue was dissolved in a minimal volume of CH_2Cl_2 (1-3 mL) and filtered through cotton wool. The same procedure was repeated 3 times. The residue was purified by flash chromatography (EtOAc/toluene 1:1) to give ester **2-8** (91 mg, 0.171 mmol, 79%) as a slightly yellow oil. R_f (EtOAc/toluene 2:3) = 0.57; $[\alpha]_D^{20}$ = +24.2° (c = 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45 – 7.28 (m, 10H, arom.), 5.84 (dd, *J* = 3.3, 1.3 Hz, 1H, H-4), 4.83 (d, *J* = 10.2 Hz, 1H, A of AB, $PhCH_2$), 4.79 – 4.74 (m, 2H, B of AB, $PhCH_2$, A of AB, $PhCH_2$), 4.53 (d, *J* = 11.3 Hz, 1H, B of AB, $PhCH_2$), 4.48 (d, *J* = 9.4 Hz, 1H, H-1), 4.16 (d, *J* = 1.3 Hz, 1H, H-5), 3.79 (s, 3H, $COOCH_3$), 3.64 (m, 2H, H-2, H-3), 2.87 – 2.59 (m, 6H, Lev-CH_2, S-CH_2-CH_3), 2.16 (s, 3H, Lev-CH_3), 1.33 (t, *J* = 7.4 Hz, 3H, S-CH_2-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 206.1, 171.8, 167.2, 138.1, 137.6, 128.50, 128.46, 128.4, 128.3, 128.0, 127.9, 85.4, 80.4, 77.3, 76.0, 75.9, 72.0, 68.1, 52.8, 38.1, 29.9, 28.1, 25.1, 15.1; IR (thin film) 2968, 2868, 1746, 1720, 1497, 1454, 1363, 1264, 1213, 1156, 1124, 1104, 1028, 988, 736, 699 cm^{-1}; HRMS (ESI) calcd. for $C_{28}H_{34}O_8S$ $(M+Na)^+$ 553.1872 found 553.1872 *m/z*.

Methyl (ethyl 2,3-*O*-benzyl-4-*O*-fluorenylmethoxycarbonyl-1-thio-β-D-galactopyranosyl)uronate (2-29)

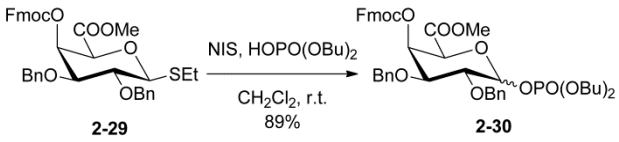


2-28 **2-29**

To a stirred solution of alcohol **2-28** (160 mg, 0.370 mmol) in pyridine (1.2 mL) was added at 0 °C FmocCl (383 mg, 1.48 mmol). The mixture was warmed to room

temperature and stirred for 3 h at that temperature. The mixture was diluted with EtOAc (50 mL) and washed with 1 M aq. HCl (2x30 mL) and sat. aq. $NaHCO_3$ (30 mL). The organic phase was dried over Na_2SO_4 and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 1:2) to give carbonate **2-29** (217 mg, 0.331 mmol, 90%) as a white foam. R_f (EtOAc/hexanes 1:4) = 0.35; $[\alpha]_D^{20}$ = -0.3° (c = 3.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (dd, *J* = 7.6, 0.8 Hz, 2H, arom.), 7.66 – 7.58 (m, 2H, arom.), 7.44 – 7.27 (m, 11H, arom.), 7.24 – 7.19 (m, 3H, arom.), 5.72 (dd, *J* = 3.1, 1.3 Hz, 1H, H-4), 4.85 (dt, *J* = 10.2, 8.7 Hz, 3H, $PhCH_2$), 4.61 (d, *J* = 11.4 Hz, 1H, $PhCH_2$), 4.51 (d, *J* = 9.2 Hz, 1H, H-1), 4.42 – 4.32 (m, 2H, Fmoc), 4.28 – 4.18 (m, 2H, H-5, Fmoc), 3.81 – 3.69 (m, 5H, $COOCH_3$, H-2, H-3), 2.92 – 2.72 (m, 2H, S-C*H*$_2$-CH_3), 1.36 (t, *J* = 7.4 Hz, 3H, S-CH_2-C*H*$_3$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.2, 154.8, 143.6, 143.3, 141.4, 138.0, 137.5, 128.44, 128.42, 128.39, 128.0, 127.93, 127.87, 127.2, 125.5, 125.3, 120.1, 120.0, 99.5, 85.5, 80.5, 76.1, 75.8, 72.2, 72.0, 70.4, 52.8, 46.6, 25.1, 15.1; IR (thin film) 2953, 1752, 1451, 1386, 1257, 1108, 1028, 741, 699 cm^{-1}; HRMS (ESI) calcd. for $C_{38}H_{38}O_8S$ $(M+Na)^+$ 677.2185 found 677.2167 *m/z*.

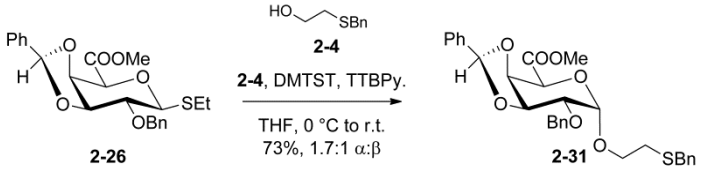
Dibutyl [methyl (2,3-*O*-benzyl-4-*O*-fluorenylmethoxycarbonyl-αβ-D-galactopyranosyl)uronate] phosphate (2-30)



Thioglycoside **2-29** (200 mg, 0.31 mmol) was co-evaporated with anhydrous toluene (2x30 mL), kept under high vacuum for 1 h and dissolved in anhydrous CH_2Cl_2 (3 mL). Activated molecular sieves (3 Å-AW) were added and the solution was stirred for 15 min at room temperature. The solution was then cooled to 0 °C, treated with dibutyl phosphoric acid (128 mg, 0.61 mmol) and stirred for another 15 min. The mixture was then treated with NIS (89 mg, 0.40 mmol), warmed to room temperature and stirred for 3 h at that temperature. The reaction was diluted with CH_2Cl_2 (20 mL) and quenched with a 1:1 (v/v) mixture of 10% aq. $Na_2S_2O_3$ and sat. aq. $NaHCO_3$ (20 mL). After separation, the aqueous phase was extracted with CH_2Cl_2 (3x30 mL), the combined organic fractions were dried over Na_2SO_4 and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 1:1 to 2:1) to give glycosyl phosphate **2-30** (218 mg, 0.27 mmol, 89%, 10:1 α:β) as a clear oil. Analytical data for **2-30α**: R_f

(EtOAc/hexanes 1:2) = 0.44; $[\alpha]_D^{20}$ = +61.8° (c = 3.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, *J* = 7.5 Hz, 2H, arom.), 7.59 (ddd, *J* = 8.3, 7.5, 0.8 Hz, 2H, arom.), 7.47 – 7.19 (m, 14H, arom.), 5.99 (dd, *J* = 6.9, 2.9 Hz, 1H, H-1), 5.77 (dd, *J* = 2.7, 1.7 Hz, 1H, H-4), 4.81 (m, 4H, H-5, $PhCH_2$), 4.67 (d, *J* = 11.6 Hz, 1H, $PhCH_2$), 4.37 (m, 2H, Fmoc), 4.21 (t, *J* = 7.6 Hz, 1H, Fmoc), 4.12 – 3.97 (m, 6H, P-O-CH_2, H-2, H-3), 3.76 (s, 3H, $COOCH_3$), 1.68 – 1.52 (m, 4H, aliph.), 1.45 – 1.24 (m, 4H, aliph.), 0.91 (dt, *J* = 11.5, 7.4 Hz, 6H, aliph.); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.1, 154.6, 143.5, 143.2, 141.33, 141.25, 137.9, 137.6, 128.4, 128.3, 128.0, 127.94, 127.89, 127.85, 127.8, 127.24, 127.23, 125.4, 125.2, 120.1, 120.0, 95.7, 95.6, 77.4, 74.7, 74.4, 74.3, 73.8, 72.5, 72.3, 70.4, 70.3, 68.1, 68.0, 67.84, 67.78, 52.8, 46.6, 32.3, 32.21, 32.19, 32.1, 18.7, 18.6, 13.63, 13.61; IR (thin film) 3065, 3033, 2960, 2934, 2874, 1755, 1452, 1385, 1351, 1259, 1113, 1028, 997, 956, 910, 858, 781, 758, 741, 699 cm^{-1}; HRMS (ESI) calcd. for $C_{44}H_{51}O_{12}P$ $(M+Na)^+$ 825.3015 found 825.3020 *m/z*.

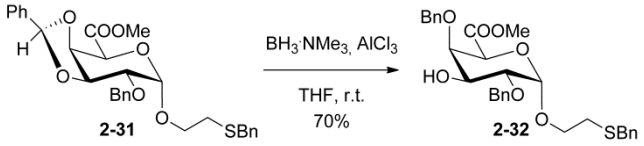
Methyl (2-*O*-benzyl-3,4-*O*-*endo*-benzylidene-α-D-galactopyranosyl)uronate-(1→1)-2-(benzylthio)ethanol (2-31)



Thioglycoside **2-26** (102 mg, 0.237 mmol), alcohol **2-4** (60 mg, 0.355 mmol) and TTBPy (117 mg, 0.474 mmol) were co-evaporated with anhydrous toluene (3x10 mL) and kept under high vacuum for 30 min. The mixture was dissolved in THF (4.8 mL) and stirred over activated molecular sieves (3 Å) for 30 min at room temperature. The solution was cooled to 0 °C and treated with DMTST (92 mg, 0.355 mmol in 0.2 mL anhydrous CH_2Cl_2). The reaction was warmed to room temperature and stirred for 2 h at that temperature. The reaction was quenched with a 1:1 (v/v) mixture of MeOH and Et_3N (0.1 mL) and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes/Et_3N 0:1:0.01 to 30:70:0.01 to 45:55:0.01) to give thioether **2-31** (59 mg, 0.110 mmol, 46%), along with the corresponding β-isomer (35 mg, 0.065 mmol, 27%). Analytical data for **2-31**: Clear oil; R_f (EtOAc/hexanes 2:3) = 0.56; $[\alpha]_D^{20}$ = +25.0° (c = 2.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.52 – 7.14 (m, 15H, arom.), 5.96 (s, 1H, PhCH), 4.88 (d, *J* = 3.0 Hz, 1H, H-5), 4.86 (d, *J* = 3.6 Hz, 1H, H-1), 4.71 (d, *J* = 12.3

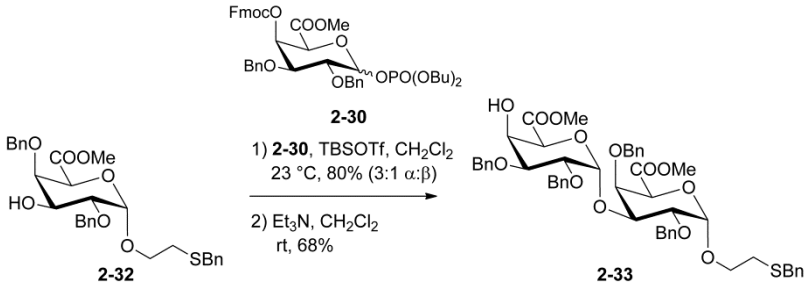
Hz, 1H, A of AB, $PhCH_2$), 4.64 (dd, J = 6.1, 3.0 Hz, 1H, H-4), 4.58 – 4.50 (m, 2H, H-3, B of AB, $PhCH_2$), 3.84 (s, 3H, $COOCH_3$), 3.82 – 3.73 (m, 3H, $PhCH_2$, A of AB, O-CH_2-CH_2), 3.67 – 3.57 (m, 2H, H-2, B of AB, O-CH_2-CH_2), 2.75 – 2.59 (m, 2H, CH_2-CH_2-S); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.5, 138.2, 138.0, 137.4, 129.4, 129.0, 128.7, 128.49, 128.47, 128.1, 127.9, 127.3, 126.8, 104.2, 98.0, 76.1, 75.8, 75.6, 72.9, 68.5, 67.9, 52.7, 36.8, 30.6; IR (thin film) 2918, 1767, 1737, 1495, 1454, 1215, 1166, 1094, 1045, 923, 759, 698 cm^{-1}; HRMS (ESI) calcd. for $C_{30}H_{32}O_7S$ $(M+Na)^+$ 559.1766 found 559.1731 *m/z*.

Methyl (2,4-di-*O*-benzyl-α-D-galactopyranosid)uronate-(1→1)-2-(benzylthio)ethanol (2-32)



To a stirred solution of acetal **2-31** (100 mg, 0.186 mmol) in anhydrous THF (5.3 mL) were added at room temperature borane trimethylamine complex (57 mg, 0.745 mmol) and then aluminium chloride (149 mg, 1.12 mmol). The reaction was stirred at that temperature for 4.5 h and quenched by addition of water (10 mL) and 1 M aq. HCl (5 mL). The mixture was extracted with EtOAc (3x10 mL), the combined organic fractions were dried over Na_2SO_4 and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 2:5 to 1:1) to give alcohol **2-32** (70.0 mg, 0.130 mmol, 70%) as a clear oil. R_f (EtOAc/hexanes 1:1) = 0.71; $[\alpha]_D^{20}$ = +60.8° (c = 3.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 – 7.17 (m, 15H, arom.), 4.93 (d, J = 3.5 Hz, 1H, H-1), 4.84 (d, J = 11.7 Hz, 1H, A of AB, $PhCH_2$), 4.70 (d, J = 11.9 Hz, 1H, A of AB, $PhCH_2$), 4.66 – 4.59 (m, 2H, B of AB, $PhCH_2$), 4.53 (d, J = 1.6 Hz, 1H, H-5), 4.28 (dd, J = 3.2, 1.7 Hz, 1H, H-4), 4.22 – 4.08 (m, 1H, H-3), 3.88 (dd, J = 10.1, 3.5 Hz, 1H, H-2), 3.78 – 3.68 (m, 3H, $PhCH_2$, A of AB, O-CH_2-CH_2), 3.66 (s, 3H, $COOCH_3$), 3.49 (m, 1H, B of AB, O-CH_2-CH_2), 2.63 – 2.54 (m, 2H, 2H, CH_2-CH_2-S), 2.43 – 2.35 (m, 1H, OH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.2, 138.3, 138.2, 138.0, 129.0, 128.62, 128.58, 128.4, 128.23, 128.17, 128.0, 127.8, 127.1, 97.3, 77.9, 76.7, 75.2, 73.1, 70.7, 69.8, 68.1, 52.3, 50.3, 36.6, 30.6; IR (thin film) 3506, 3029, 2918, 1762, 1496, 1454, 1344, 1211, 1152, 1106, 1060, 1026, 917, 739, 698 cm^{-1}; HRMS (ESI) calcd. for $C_{30}H_{34}O_7S$ $(M+Na)^+$ 561.1923 found 561.1879 *m/z*.

Methyl (2,3-di-*O*-benzyl-4-*O*-fluorenylmethoxycarbonyl-α-D-galactopyranosyl)uronate-(1→3)-methyl (2,4-di-*O*-benzyl-α-D-galactopyranosyl)uronate-(1→1)-(2-(benzylthio)ethanol (2-33)

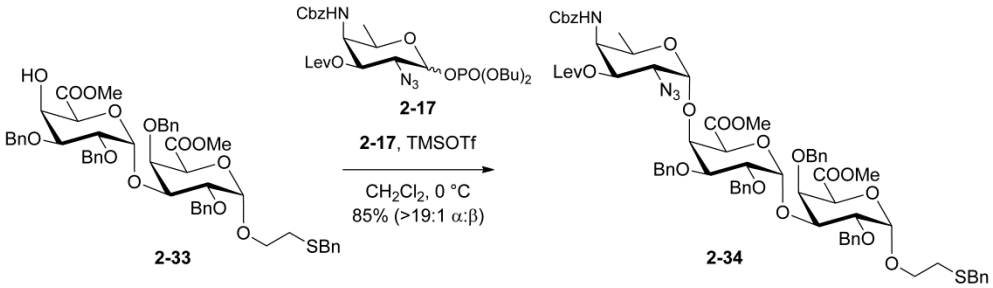


Alcohol **2-32** (90 mg, 0.166 mmol) and glycosyl phosphate **2-30** (208 mg, 0.259 mmol) were co-evaporated with anhydrous toluene (3x10 mL) and kept under high vacuum for 1 h. The mixture was dissolved in anhydrous CH_2Cl_2 (3.3 mL) and stirred over activated molecular sieves (3 Å-AW) for 30 min at room temperature. The solution was cooled to 0 °C and treated dropwise with TBSOTf (57 µL, 0.133 mmol, in 0.2 mL anhydrous CH_2Cl_2). The solution was warmed to room temperature and stirred for 20 h at that temperature. The reaction was diluted with CH_2Cl_2 (10 mL) and quenched with a 1:1 (v/v) mixture of MeOH and pyridine (0.2 mL). The solution was filtered through Celite and concentrated. The crude product was filtered through a short plug of silica gel (EtOAc/hexanes 1:1) to give the intermediate disaccharide mixture (150 mg, 0.133 mmol, 80%, 3:1 α:β) as an inseparable mixture as a clear oil.

To a stirred solution of the disaccharide mixture (150 mg) in CH_2Cl_2 (2.6 mL) was added at room temperature Et_3N (1.1 mL, 7.96 mmol). The reaction was stirred for 3 h at that temperature and co-evaporated with toluene (2x10 mL). The residue was purified by flash chromatography (EtOAc/hexanes 1:6 to 2:3 to 1:1) to give alcohol **2-33** (62 mg, 0.068 mmol, 51%) along with the corresponding β-anomer (20 mg, 0.022 mmol, 17%). Analytical data for **2-33**: Clear oil; R_f (EtOAc/hexanes 1:1) = 0.36; $[\alpha]_D^{20}$ = +86.3° (c = 0.50, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 – 7.15 (m, 23H, arom.), 7.04 (dd, J = 6.6, 2.6 Hz, 2H, arom.), 5.28 (d, J = 2.4 Hz, 1H, H-1'), 5.03 (A of AB, d, J = 11.6 Hz, 1H, $PhCH_2$), 4.96 (d, J = 3.5 Hz, 1H, H-1), 4.84 (dd, J = 11.6, 6.4 Hz, 2H, A of AB, $PhCH_2$, H-5'), 4.75 – 4.63 (m, 3H, $PhCH_2$), 4.61 – 4.52 (m, 2H, $PhCH_2$), 4.45 (s, 1H, H-5), 4.37 (B of AB, d, J = 11.5 Hz, 1H, $PhCH_2$), 4.30 (s, 1H, H-4), 4.26 (dd, J = 10.2, 2.6 Hz, 1H, H-3), 4.19 (m, 1H, H-4'), 4.05 (dd, J = 10.2, 3.5 Hz, 1H, H-2), 3.98 (m,

2H, H-2', H-3'), 3.82 – 3.68 (m, 3H, A of AB, O-CH_2-CH_2, $PhCH_2$), 3.63 (s, 3H, $COOCH_3$), 3.56 – 3.47 (m, 4H, B of AB, O-CH_2-CH_2, $COOCH_3$), 2.54 (m, 2H, CH_2-CH_2-S), 2.44 (br s, 1H, OH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.2, 169.1, 138.7, 138.4, 138.2, 137.9, 137.7, 129.2, 128.7, 128.62, 128.56, 128.55, 128.3, 128.2, 128.1, 128.02, 127.98, 127.9, 127.7, 127.3, 127.1, 97.6, 95.8, 76.0, 75.2, 75.1, 74.6, 74.1, 72.9, 72.4, 71.0, 70.2, 68.8, 68.6, 52.4, 52.2, 36.7, 30.3; IR (thin film) 3517, 3062, 3030, 2933, 1763, 1735, 1603, 1496, 1454, 1344, 1273, 1212, 1148, 1105, 1061, 1028, 916, 805, 740, 698 cm^{-1}; HRMS (ESI) calcd. for $C_{51}H_{56}O_{13}S$ $(M+Na)^+$ 931.3339 found 931.3340 *m/z*.

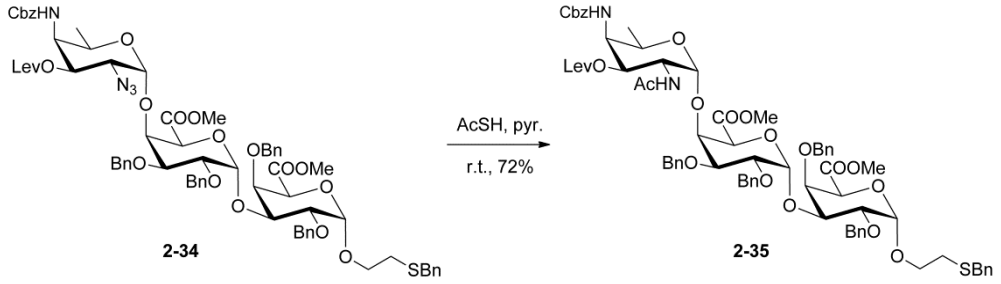
2-Azido-4-(benzyloxycarbonyl)amino-3-*O*-levulinoyl-2,4,6-trideoxy-α-D-galactopyranosyl-(1→4)-methyl (2,3-di-*O*-benzyl-α-D-galactopyranosyl)uronate-(1→3)-methyl (2,4-di-*O*-benzyl-α-D-galactopyranosyl)uronate-(1→1)-2-(benzylthio)ethanol (2-34)



Alcohol **2-33** (65 mg, 0.062 mmol) and glycosyl phosphate **2-17** (61 mg, 0.100 mmol) were co-evaproated with anhydrous toluene (3x10 mL) and kept under high vacuum for 30 min. The mixture was dissolved in CH_2Cl_2 (2.1 mL) and stirred over activated molecular sieves (3 Å-AW) for 1 h at room temperature. The solution was cooled to 0 °C and treated with TMSOTf (17 µL, 0.093 mmol in 0.2 mL anhydrous CH_2Cl_2). The reaction was stirred for 3 h at that temperature and quenched with a 1:1 (v/v) mixture of MeOH and Et_3N (0.5 mL). The mixture was diluted with CH_2Cl_2 (20 mL), filtered through Celite and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 1:2 to 1:1) to give trisaccharide **2-34** (69 mg, 0.053 mmol, 85%) as a clear oil. R_f (EtOAc/hexanes 2:3) = 0.43; $[\alpha]_D^{20}$ = +116.6° (c = 0.50, acetone); ^{1}H NMR (400 MHz, acetone-D_6) δ 7.51 – 7.10 (m, 30H, arom.), 6.28 (d, *J* = 10.1 Hz, 1H, NH), 5.45 (d, *J* = 3.1 Hz, 1H, H-1'), 5.22 – 5.11 (m, 3H, H-3'', 2x A of AB, $PhCH_2$), 5.05 – 4.97 (m, 2H, H-1, A of AB, $PhCH_2$), 4.93 – 4.81 (m, 5H, H-1'', H-5', $PhCH_2$), 4.76 (d, *J* = 12.8 Hz, 1H, B of AB, $PhCH_2$), 4.61 (d, *J* = 11.7 Hz, 1H, A of AB, $PhCH_2$), 4.54 (m,

2H, H-5, B of AB, $PhCH_2$), 4.49 (m, 2H, H-4, H-5''), 4.43 (d, J = 1.4 Hz, 1H, H-4'), 4.39 (d, J = 11.2 Hz, 1H, B of AB, $PhCH_2$), 4.29 (dd, J = 10.4, 2.8 Hz, 1H, H-3), 4.16 – 4.01 (m, 3H, H-2', H-3', H-4''), 3.91 (dd, J = 10.4, 3.5 Hz, 1H, H-2), 3.83 – 3.73 (m, 3H, $PhCH_2$, A of AB, O-CH_2-CH_2), 3.70 – 3.63 (m, 4H, H-2'', $COOCH_3$), 3.61 – 3.52 (m, 1H, B of AB, O-CH_2-CH_2), 3.44 (s, 3H, $COOCH_3$), 2.80 – 2.63 (m, 2H, Lev-CH_2), 2.63 – 2.45 (m, 3H, Lev-CH_2, CH_2-CH_2-S), 2.38 (m, 1H, Lev-CH_2), 2.13 (s, 3H, Lev-CH_3), 0.92 – 0.81 (m, 3H, H-6''); ^{13}C NMR (100 MHz, acetone-D_6) δ 206.0, 172.3, 169.5, 169.3, 157.8, 140.3, 139.7, 139.4, 139.0, 138.3, 129.4, 129.14, 129.11, 129.08, 129.06, 129.05, 128.7, 128.6, 128.6, 128.5, 128.4, 128.31, 128.26, 128.17, 128.15, 127.7, 127.5, 99.9, 97.9, 96.3, 77.6, 77.3, 76.2, 75.8, 75.7, 75.1, 74.6, 73.0, 72.8, 71.4, 71.0, 70.2, 69.4, 66.7, 65.8, 58.3, 53.8, 52.0, 51.8, 38.1, 36.9, 17.8, 16.6; IR (thin film) 2925, 2210, 1719, 1497, 1454, 1347, 1215, 1147, 1108, 1028, 742, 699 cm^{-1}; HRMS (ESI) calcd.. for $C_{70}H_{78}N_4O_{19}S$ $(M+Na)^+$ 1333.4879 found 1333.4911 *m/z*.

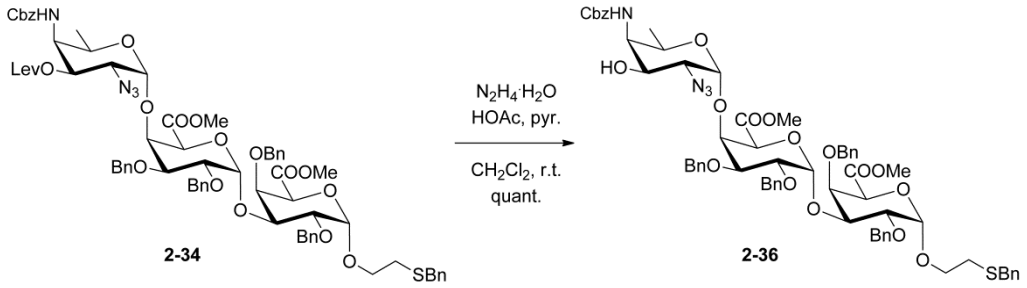
2-Acetamido-4-(benzyloxycarbonyl)amino-3-*O*-levulinoyl-2,4,6-trideoxy-α-D-galactopyranosyl-(1→4)-methyl (2,3-di-*O*-benzyl-α-D-galactopyranosyl)uronate-(1→3)-methyl (2,4-di-*O*-benzyl-α-D-galactopyranosyl)uronate-(1→1)-(2-(benzylthio)ethanol (2-35)



To a stirred solution of azide **2-34** (20.0 mg, 0.015 mmol) in anhydrous pyridine (0.4 mL) was added at room temperature thioacetic acid (0.4 mL). The reaction was stirred for 24 h at that temperature. The mixture was co-evaporated with toluene (2x5 mL) and the residue was purified by flash chromatography (EtOAc/hexanes 1:8 to 5:1) to give acetamide **2-35** (15 mg, 0.011 mmol, 72%) as a white foam. R_f (EtOAc/hexanes 4:1) = 0.25; $[\alpha]_D^{20}$ = +99.5° (c = 1.0, MeOH); ^{1}H NMR (400 MHz, CD_3OD) δ 7.47 – 7.13 (m, 30H, arom.), 5.43 (d, J = 3.3 Hz, 1H, H-1'), 5.17 (d, J = 12.6 Hz, 1H, A of AB, $PhCH_2$), 5.11 (d, J = 11.0 Hz, 1H, A of AB, $PhCH_2$), 5.05 – 4.99 (m, 2H, H-1, B of AB, $PhCH_2$), 4.93 (dd, J = 6.6, 2.5 Hz, 1H, H-3''), 4.80 (m, 3H, $PhCH_2$), 4.73 – 4.65 (m, 3H, H-5,

$PhCH_2$), 4.60 (d, J = 3.9 Hz, 1H, H-1''), 4.57 (d, J = 1.2 Hz, 1H, H-5'), 4.51 (d, J = 11.1 Hz, 1H, A of AB, $PhCH_2$), 4.48 (d, J = 2.4 Hz, 1H, H-4'), 4.42 – 4.33 (m, 3H, H-5'', B of AB, $PhCH_2$), 4.25 – 4.19 (m, 2H, H-2'', H-3'), 4.16 (d, J = 1.9 Hz, 1H, H-4), 4.07 (dd, J = 10.4, 3.3 Hz, 1H, H-2'), 3.99 (dd, J = 3.9, 1.9 Hz, 1H, H-4''), 3.96 – 3.90 (m, 2H, H-2, H-3), 3.78 – 3.67 (m, 6H, A of AB, O-CH_2-CH_2, $COOCH_3$, $PhCH_2$), 3.59 – 3.52 (m, 1H, B of AB, O-CH_2-CH_2), 3.25 (s, 3H, $COOCH_3$), 2.75 – 2.47 (m, 4H, Lev-CH_2, CH_2-CH_2-S), 2.41 – 2.31 (m, 1H, A of AB, Lev-CH_2), 2.31 – 2.18 (m, 1H, B of AB, Lev-CH_2), 2.10 (s, 3H, Ac-CH_3), 1.94 (s, 3H, Lev-CH_3), 0.89 (d, J = 6.3 Hz, 3H, H-6''); ^{13}C NMR (100 MHz, CD_3OD) δ 209.2, 173.9, 173.7, 171.0, 160.0, 159.5, 140.2, 140.1, 139.8, 139.3, 139.1, 138.6, 130.3, 130.1, 129.6, 129.53, 129.49, 129.41, 129.38, 129.1, 129.0, 128.9, 128.81, 128.77, 128.4, 127.9, 100.3, 98.4, 96.8, 78.2, 77.4, 76.4, 76.3, 75.9, 75.5, 75.0, 73.8, 73.7, 71.9, 71.6, 71.1, 70.0, 67.5, 66.4, 54.1, 52.7, 52.4, 38.4, 37.5, 31.2, 29.7, 29.1, 23.1, 16.9; IR (thin film) 3444, 2927, 2112, 1763, 1724, 1660, 1497, 1455, 1349, 1263, 1111, 1028, 913, 743, 700 cm^{-1}; HRMS (ESI) calcd. for $C_{72}H_{82}N_2O_{20}S$ $(M+Na)^+$ 1349.5079 found 1349.5029 *m/z*.

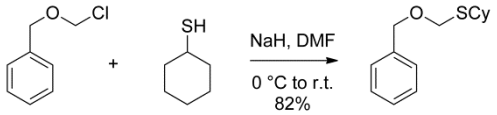
2-Azido-4-(benzyloxycarbonyl)amino-2,4,6-trideoxy-α-D-galactopyranosyl-(1→4)-methyl (2,3-di-*O*-benzyl-α-D-galactopyranosyl)uronate-(1→3)-methyl (2,4-di-*O*-benzyl-α-D-galactopyranosyl)uronate-(1→1)-2-(benzylthio)ethanol (2-36)



To a stirred solution of Lev ester **2-34** (30 mg, 0.023 mmol) in CH_2Cl_2 (1.0 mL) was added at room temperature first a mixture of pyridine (56 µL, 0.692 mmol) and acetic acid (37 µL, 0.646 mmol), and then hydrazine hydrate (2 µL, 0.041 mmol). The mixture was stirred for 4 h at room temperature, diluted with EtOAc (2 mL), quenched with acetone (0.1 mL) and poured into water (15 mL). The aqueous phase was extracted with EtOAc (4x10 mL), the combined organic extracts were dried over Na_2SO_4 and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 0:1 to 1:2 to 2:3) to give alcohol **2-36** (28 mg, 0.023 mmol, quant.) as a clear oil. R_f (EtOAc/hexanes 1:1) = 0.34; $[\alpha]_D^{20}$ = +122.0° (c = 0.36, acetone); ^{1}H NMR (400 MHz,

acetone-D_6) δ 7.52 – 7.08 (m, 30H, arom.), 6.14 (d, J = 10.1 Hz, 1H, NH), 5.48 (d, J = 2.8 Hz, 1H, H-1'), 5.15 (d, J = 11.2 Hz, 1H, A of AB, $PhCH_2$), 5.10 (d, J = 12.6 Hz, 1H, A of AB, $PhCH_2$), 5.05 – 4.99 (m, 2H, H-1, B of AB, $PhCH_2$), 4.91 (d, J = 11.7 Hz, 3H, $PhCH_2$), 4.82 – 4.73 (m, 3H, H-1'', H-5', B of AB, $PhCH_2$), 4.60 (d, J = 11.7 Hz, 1H, A of AB, $PhCH_2$), 4.54 (m, 2H, H-5, B of AB, $PhCH_2$), 4.49 (s, 1H, H-4), 4.44-4.35 (m, 4H, H-4', H-5'', $PhCH_2$), 4.29 (dd, J = 10.4, 2.8 Hz, 1H, H-3), 4.16 – 4.09 (m, 1H, H-3'), 4.08 – 4.02 (dd, J = 2.6, 5.8 Hz, 1H, H-2'), 3.96 (dd, J = 10.1, 2.7 Hz, 1H, H-4''), 3.91 (dd, J = 10.4, 3.5 Hz, 1H, H-2), 3.82 – 3.74 (m, 3H, $PhCH_2$, A of AB, O-CH_2-CH_2), 3.68 (s, 3H, $COOCH_3$), 3.56 (m, 1H, B of AB, O-CH_2-CH_2), 3.43 (s, 3H, $COOCH_3$), 3.38 (dd, J = 11.3, 3.9 Hz, 1H, H-2''), 2.59 (dd, J = 7.4, 5.3 Hz, 2H, CH_2-CH_2-S), 0.86 (d, J = 6.4 Hz, 3H, H-6''); ^{13}C NMR (100 MHz, acetone-D_6) δ 169.6, 169.4, 158.3, 140.4, 139.9, 139.8, 139.5, 139.3, 138.3, 130.0, 129.3, 129.2, 129.13, 129.12, 129.08, 128.8, 128.7, 128.6, 128.53, 128.45, 128.4, 128.3, 128.2, 127.8, 127.6, 100.2, 97.9, 96.3, 77.4, 77.1, 76.4, 75.8, 75.2, 74.5, 72.8, 71.5, 71.2, 69.5, 67.6, 66.70, 66.66, 61.2, 57.2, 52.1, 51.8, 37.0, 30.8, 16.9; IR (thin film) 3363, 3031, 2929, 2111, 1764, 1728, 1522, 1497, 1455, 1347, 1263, 1107, 1028, 915, 741, 699 cm^{-1}; HRMS (ESI) calcd. for $C_{65}H_{72}N_4O_{17}S$ $(M+Na)^+$ 1235.4511 found 1235.4539 *m/z*.

Benzyloxymethyl cyclohexyl sulfide (2-38)

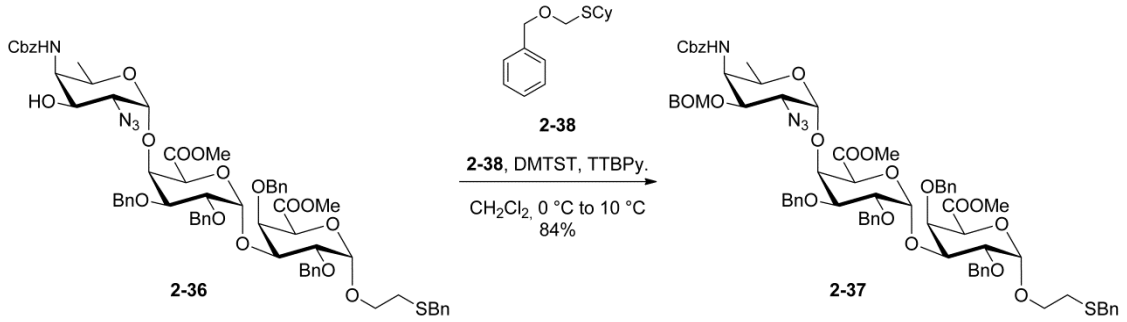


2-38

To a stirred solution of cyclohexanethiol (1.46 mL, 11.9 mmol) in DMF (36 mL) was added at 0 °C sodium hydride (0.338 g, 14.1 mmol). The mixture was treated with benzyloxymethyl chloride (2.0 mL, 75% (w/w), 10.8 mmol) and warmed to room temperature. The reaction was stirred at that temperature for 16 h, quenched at 0 °C with 1 M aq. NaOH (20 mL) and diluted with water (100 mL) and hexanes (70 mL). After separation, the aqueous phase was extracted with hexanes (3x70 mL), the combined organic fractions were dried over Na_2SO_4 and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 0:1 to 1:50) to give *S,O*-acetal **2-38** (2.1 g, 8.9 mmol, 82%) as a slightly yellow oil. R_f (EtOAc/hexanes 1:10) = 0.80; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 – 7.27 (m, 5H, arom.), 4.77 (s, 2H, O-CH_2-S), 4.63 (s, 2H, O-CH_2Ph), 2.93 – 2.80 (m, 1H, S-CH), 2.07 – 1.98 (m, 2H, aliph.), 1.83 – 1.70 (m,

2H, aliph.), 1.61 (dd, J = 10.1, 4.0 Hz, 1H, aliph.), 1.47 – 1.20 (m, 5H, aliph.); ^{13}C NMR (100 MHz, $CDCl_3$) δ 137.8, 128.6, 128.3, 127.9, 71.8, 69.6, 43.3, 34.2, 26.3, 25.9; IR (thin film) 2928, 2852, 1497, 1449, 1310, 1265, 1061, 1028, 739, 697 cm^{-1}; HRMS (ESI) calcd for $C_{14}H_{20}OS$ $(M+Na)^+$ 259.1133 found 259.1132 *m/z*.

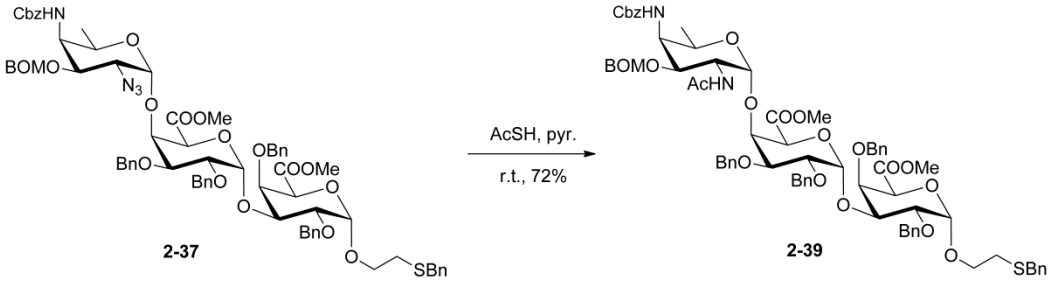
2-Azido-4-(benzyloxycarbonyl)amino-3-*O*-benzyloxymethyl-2,4,6-trideoxy-α-D-galactopyranosyl-(1→4)-methyl (2,3-di-*O*-benzyl-α-D-galactopyranosyl)uronate-(1→3)-methyl (2,4-di-*O*-benzyl-α-D-galactopyranosyl)uronate-(1→1)-2-(benzylthio)ethanol (2-37)



Alcohol **2-36** (49 mg, 0.040 mmol), *S,O*-acetal **2-38** (180 mg, 0.81 mmol) and TTBPy (400 mg, 1.62 mmol) were co-evaporated with anhydrous toluene (3x10 mL). The mixture was dissolved in anhydrous CH_2Cl_2 (2.0 mL) and stirred over activated molecular sieves (3 Å) for 30 min at room temperature. The mixture was cooled to 0 °C and DMTST (24 mg, 0.598 mmol in 0.3 mL CH_2Cl_2) was added dropwise over a period of 1.5 h, while the reaction temperature was kept below 10 °C. The reaction was stirred for another 45 min, quenched by addition of a 10:1 (v/v) mixture of MeOH and Et_3N (0.5 mL) and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 1:10 to 1:2) to give acetal **2-37** (46 mg, 0.034 mmol, 84%) as a clear oil. R_f (EtOAc/hexanes 2:3) = 0.58; $[\alpha]_D^{20}$ = +127.0° (c = 0.27, acetone); ^{1}H NMR (400 MHz, acetone-D_6) δ 7.54 – 7.03 (m, 35H, arom.), 6.24 (d, J = 9.5 Hz, 1H, NH), 5.48 (d, J = 2.8 Hz, 1H, H-1'), 5.15 (d, J = 11.3 Hz, 1H, A of AB, $PhCH_2$), 5.08 (s, 2H, $PhCH_2$), 5.03 (d, J = 3.5 Hz, 1H, H-1), 4.98 (d, J = 7.2 Hz, 1H, A of AB, BnO-CH_2-O), 4.87 – 4.80 (m, 5H, H-1'', $PhCH_2$, H-5'), 4.78 – 4.71 (m, 3H, B of AB, BnO-CH_2-O, $PhCH_2$), 4.66 (d, J = 11.7 Hz, 1H, B of AB, $PhCH_2$), 4.61 (d, J = 11.7 Hz, 1H, A of AB, $PhCH_2$), 4.54 (m, 2H, H-5, B of AB, $PhCH_2$), 4.49 (m, 1H, H-4), 4.42 (s, 1H, H-4'), 4.40 – 4.34 (m, 2H, H-5'', B of AB, $PhCH_2$), 4.30 (dd, J = 10.4, 2.8 Hz, 1H, H-3), 4.15 (d, J = 9.4

Hz, 2H, H-3'', H-4''), 4.05 (m, 2H, H-2',H-3'), 3.91 (dd, J = 10.4, 3.5 Hz, 1H, H-2), 3.84 – 3.73 (m, 3H, $PhCH_2$, A of AB, O-CH_2-CH_2), 3.68 (s, 3H, $COOCH_3$), 3.60 – 3.54 (m, 2H, H-2'', B of AB, O-CH_2-CH_2), 3.44 (s, 3H, $COOCH_3$), 2.59 (dd, J = 7.5, 5.2 Hz, 2H, CH_2-CH_2-S), 0.88 (d, J = 6.3 Hz, 3H, H-6''); ^{13}C NMR (100 MHz, acetone-D_6) δ 169.6, 169.4, 158.0, 140.4, 139.9, 139.8, 139.5, 139.20, 139.16, 138.4, 130.0, 129.4, 129.2, 129.14, 129.11, 129.0, 128.8, 128.7, 128.6, 128.5, 128.38, 128.36, 128.3, 128.2, 127.8, 127.6, 100.0, 98.0, 96.3, 93.1, 77.53, 77.48, 77.0, 76.3, 75.9, 75.8, 75.3, 74.5, 73.0, 72.9, 72.1, 71.5, 71.2, 70.4, 69.5, 66.7, 66.4, 60.2, 53.7, 52.1, 51.9, 37.0, 30.9, 16.9; IR (thin film) 2925, 2110, 1765, 1727, 1497, 1455, 1345, 1238, 1106, 1039, 915, 739, 698 cm^{-1}; HRMS (ESI) calcd. for $C_{73}H_{80}N_4O_{18}S$ $(M+Na)^+$ 1355.5086 found 1355.5071 *m/z*.

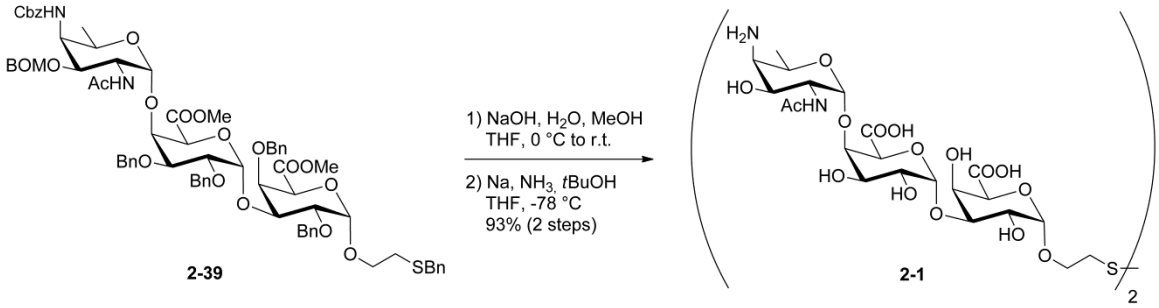
2-Acetamido-4-(benzyloxycarbonyl)amino-3-*O*-benzyloxymethyl-2,4,6-trideoxy-α-D-galactopyranosyl-(1→4)-methyl (2,3-di-*O*-benzyl-α-D-galactopyranosyl)uronate-(1→3)-methyl (2,4-di-*O*-benzyl-α-D-galactopyranosyl)uronate-(1→1)-2-(benzylthio)ethanol (2-39)



To a stirred solution of azide **2-37** (29 mg, 0.022 mmol) in anhydrous pyridine (0.35 mL) was added at 0 °C thioacetic acid (0.35 mL). The mixture was warmed to room temperature and stirred for 24 h at that temperature. The solution was co-evaporated with toluene (2x5 mL) and the residue was purified by flash chromatography (EtOAc/hexanes 1:10 to acetone/hexanes 1:7 to 1:5 to 1:3) to give acetamide **2-39** (21 mg, 0.016 mmol, 72%) as a white foam. R_f (acetone/hexanes 2:3) = 0.25; $[\alpha]_D^{20}$ = +104.7° (c = 0.36, acetone); ^{1}H NMR (600 MHz, acetone-D_6) δ 7.48 (d, J = 7.0 Hz, 2H, arom.), 7.42 – 7.18 (m, 31H, arom.), 7.16 – 7.08 (m, 2H, arom.), 6.49 (d, J = 10.3 Hz, 1H, NH), 5.93 (d, J = 9.5 Hz, 1H, NH), 5.52 (d, J = 2.9 Hz, 1H, H-1'), 5.15 (d, J = 11.2 Hz, 1H, A of AB, $PhCH_2$), 5.09 (s, 2H, $PhCH_2$), 5.05 (d, J = 3.5 Hz, 1H, H-1), 4.92 – 4.80 (m, 4H, $PhCH_2$, A of AB, BnO-CH_2-O), 4.77 (d, J = 12.8 Hz, 2H, $PhCH_2$, H-5'), 4.71 – 4.56 (m, 5H, H-1'', $PhCH_2$, B of AB, Bn-O-CH_2-O), 4.51 (d, J = 11.4 Hz, 2H, H-5,

B of AB, $PhCH_2$), 4.48 (m, 1H, H-4), 4.43 – 4.33 (m, 2H, H-4', H-5'', B of AB, $PhCH_2$), 4.29 (dd, J = 10.4, 2.8 Hz, 1H, H-3), 4.24 – 4.15 (m, 1H, H-2''), 4.14 – 4.01 (m, 3H, H-2', H-3', H-4''), 3.91 (dd, J = 10.3, 3.5 Hz, 1H, H-2), 3.86 (dd, J = 11.4, 4.2 Hz, 1H, H-3''), 3.83 – 3.72 (m, 3H, $PhCH_2$, A of AB, O-CH_2-CH_2), 3.69 (s, 3H, $COOCH_3$), 3.62 – 3.54 (m, 1H, B of AB, O-CH_2-CH_2), 3.33 (s, 3H, $COOCH_3$), 2.60 (dd, J = 8.1, 4.7 Hz, 2H, CH_2-CH_2-S), 1.81 (s, 3H, Ac-CH_3), 0.92 (d, J = 6.3 Hz, 3H, H-6''); ^{13}C NMR (100 MHz, acetone-D_6) δ 170.0, 169.6, 169.2, 158.1, 140.4, 140.0, 139.8, 139.6, 139.4, 139.2, 130.0, 129.3, 129.18, 129.15, 128.9, 128.7, 128.6, 128.5, 128.4, 128.32, 128.28, 127.9, 127.6, 99.9, 97.9, 96.7, 93.6, 77.6, 77.1, 76.0, 75.8, 75.6, 74.4, 73.4, 73.1, 72.9, 71.6, 71.1, 69.8, 69.49, 66.47, 54.0, 52.1, 51.9, 49.2, 37.0, 30.9, 23.5, 17.2; IR (thin film) 3030, 2933, 1764, 1718, 1670, 1520, 1455, 1368, 1248, 1107, 1043, 916, 740, 699 cm^{-1}; HRMS (ESI) calcd. for $C_{75}H_{84}N_2O_{19}S$ $(M+Na)^+$ 1371.5281 found 1371.5314 *m/z*.

2,2'-Dithiobis[2-acetamido-4-amino-2,4,6-trideoxy-α-D-galactopyranosyl-(1→4)-α-D-galactopyranosyluronate-(1→3)-α-D-galactopyranosyluronate-(1→1)-1-ethanol] (2-1)

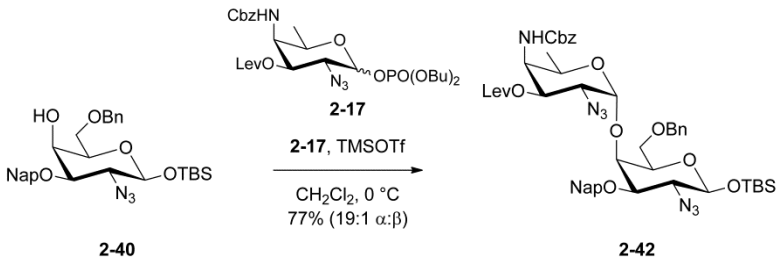


To a stirred solution of ester **2-39** (18 mg, 13.3 µmol) in THF (2.0 mL) and MeOH (0.75 mL) was added at 0 °C a 1 M aq. solution of NaOH (0.8 mL, 0.800 mmol). The reaction was slowly warmed to room temperature and stirred for 16 h. The reaction was diluted with water (5 mL), acidified to pH 4 with 0.5 M aq. $NaHSO_4$, and poured into EtOAc (5 mL). After separation, the aqueous fraction was extracted with EtOAc (8x10 mL), the combined organic fractions were dried over Na_2SO_4 and concentrated to give the intermediate diacid as a white foam.

To a stirred solution of liquid ammonia (10 mL) was added at -78 °C a solution of the crude diacid in THF (2.0 mL). The mixture was treated with *t*BuOH (0.8 mL) and lumps of freshly cut sodium (90 mg) were added until a deeply blue color persisted. The reaction was stirred at -78 °C for 45 min and quenched by addition of solid NH_4OAc (300

mg). The solution was warmed to room temperature under a stream of argon and co-evaporated with MeOH (2x10 mL) and water (2x5 mL). The residue was left exposed to air for 16 h, purified by size exclusion chromatography (MeOH/5 mM aq. NH_4OAc 1:10, Sephadex® G-25, GE Healthcare, Little Chalfont, UK) and lyophilized repeatedly to give disulfide **2-1** (7.6 mg, 6.2 µmol, 93% over two steps) as a white solid. $[\alpha]_D^{20} = +80.3°$ (c = 0.10, H_2O); ^1H NMR (600 MHz, D_2O) δ 5.32 (d, J = 3.8 Hz, 1H, H-1'), 5.12 (d, J = 3.8 Hz, 1H, H-1), 5.05 (d, J = 3.8 Hz, 1H, H-1''), 4.83 (d, J = 5.3 Hz, 1H, H-5''), 4.66 (s, 1H, H-5'), 4.59 (s, 1H, H-4), 4.48 (s, 1H, H-4'), 4.45 (d, J = 2.5 Hz, 1H, H-5), 4.26 (d, J = 11.1 Hz, 1H, H-3''), 4.21 (dd, J = 10.6, 3.0 Hz, 1H, H-3'), 4.11 (m, 3H, H-2'', H-3, A of AB, O-CH_2-CH_2), 4.03 – 3.96 (m, 2H, H-2, H-2'), 3.96 – 3.89 (m, 1H, B of AB, O-CH_2-CH_2), 3.67 (s, 1H, H-4''), 3.11 – 3.01 (m, 2H, CH_2-CH_2-S-S), 2.20 (s, 3H, Ac-CH_3), 1.35 (d, J = 6.6 Hz, 3H, H-6''); HRMS (MALDI) calcd. for $C_{44}H_{70}N_4O_{32}S_2$ $(M-H)^-$ 1229.3330 found 1229.3342 m/z.

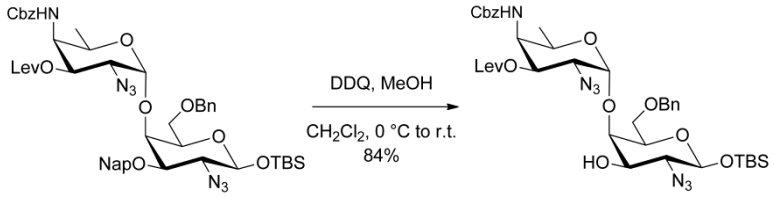
tert-Butyldimethylsilyl 2-azido-4-(benzyloxycarbonyl)amino-3-*O*-levulinoyl-2,4,6-trideoxy-α-D-galactopyranosyl-(1→4)-2-azido-6-*O*-benzyl-2-deoxy-3-*O*-naphthyl-β-D-galactopyranoside (2-42)



Alcohol **2-40**[132] (40 mg, 0.073 mmol) and glycosyl phosphate **2-17** (67 mg, 0.109 mmol) were co-evaporated with anhydrous toluene (3x5 mL) and kept under high vacuum for 48 h. The mixture was dissolved in anhydrous CH_2Cl_2 (3.7 mL) and stirred over activated molecular sieves (3 Å-AW) for 30 min at room temperature. The solution was cooled to 0 °C and treated dropwise with TBSOTf (25 µL, 0.109 mmol). The reaction was stirred for 1.5 h at that temperature and quenched by addition of a 1:1 (v/v) mixture of MeOH and Et_3N. The mixture was diluted with CH_2Cl_2 (20 mL), filtered through Celite and concentrated. The crude product was purified by flash chromatography (EtOAc/hexanes 1:4 to 1:2) to give disaccharide **2-42** (53 mg, 0.056 mmol, 77%) as a clear oil. R_f (EtOAc/hexanes 1:2) = 0.63; $[\alpha]_D^{20} = +66.1°$ (c = 5.0, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 7.84 (dd, J = 7.6, 2.9 Hz, 4H, arom.), 7.63 – 7.46 (m, 3H, arom.), 7.43 – 7.28

(m, 10H, arom.), 5.30 (dd, J = 11.3, 3.8 Hz, 1H, H-3c), 5.14 (d, J = 12.2 Hz, 1H, A of AB, $PhCH_2$), 5.03 (d, J = 12.3 Hz, 1H, B of AB, $PhCH_2$), 4.96 – 4.89 (m, 3H, H-1c, A of AB, CH_2Ph, $NapCH_2$), 4.84 (d, J = 12.5 Hz, 1H, B of AB, $NapCH_2$), 4.73 – 4.64 (m, 1H, H-5c), 4.51 (d, J = 1.4 Hz, 2H, $PhCH_2$), 4.42 (d, J = 7.5 Hz, 1H, H-1b), 4.17 (dd, J = 9.5, 2.1 Hz, 1H, H-4c), 4.11 (d, J = 2.9 Hz, 1H, H-4b), 3.92 (t, J = 9.1 Hz, 1H, H-6b), 3.67 (dd, J = 10.7, 7.5 Hz, 1H, H-2b), 3.55 (dd, J = 9.1, 5.6 Hz, 1H, H-6b), 3.44 (dd, J = 8.9, 5.7 Hz, 1H, H-5b), 3.31 (dd, J = 11.3, 3.9 Hz, 1H, H-2c), 3.18 (dd, J = 10.7, 3.0 Hz, 1H, H-3b), 2.91 – 2.56 (m, 3H, Lev-CH_2), 2.46 (m, 1H, Lev-CH_2), 2.18 (d, J = 4.5 Hz, 3H, Lev-CH_3), 0.92 (s, 9H, TBS), 0.84 (d, J = 6.4 Hz, 3H, H-6c), 0.12 (d, J = 0.4 Hz, 6H, TBS); ^{13}C NMR (100 MHz, $CDCl_3$) δ 206.6, 172.2, 156.7, 137.6, 136.5, 135.1, 133.3, 133.2, 128.7, 128.6, 128.42, 128.38, 128.11, 128.09, 128.07, 128.0, 127.8, 126.6, 126.3, 126.1, 125.7, 98.9, 97.8, 78.1, 77.4, 73.6, 72.9, 72.8, 70.2, 67.1, 65.5, 64.9, 58.0, 52.9, 38.1, 29.9, 28.1, 25.8, 25.7, 18.1, 16.4, -4.1, -5.0; IR (thin film) 3346, 2931, 2858, 2109, 1718, 1603, 1510, 1408, 1349, 1253, 1146, 1076, 1040, 895, 839, 784, 752, 698 cm^{-1}; HRMS (ESI) calcd. for $C_{49}H_{61}N_7O_{11}Si$ $(M+Na)^+$ 974.4096 found 974.4051 m/z.

tert-Butyldimethylsilyl 2-azido-4-(benzyloxycarbonyl)amino-3-*O*-levulinoyl-2,4,6-trideoxy-α-D-galactopyranosyl-(1→4)-2-azido-6-*O*-benzyl-2-deoxy-β-D-galactopyranoside (2-43)

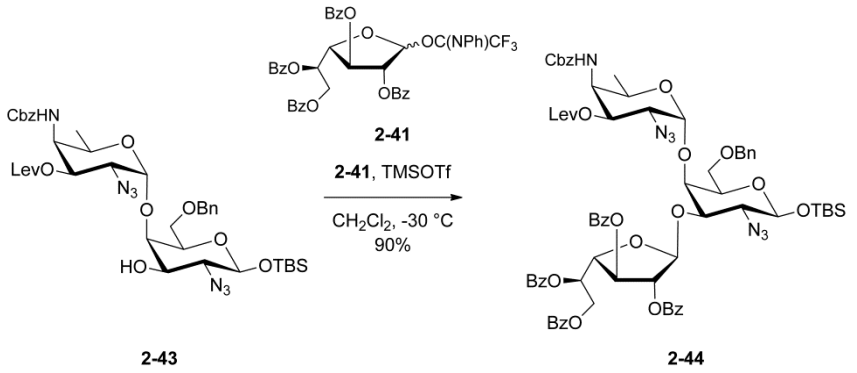


2-42 **2-43**

To a stirred solution of naphthyl ether **2-42** (270 mg, 0.28 mmol) in a CH_2Cl_2 (5.1 ml) and MeOH (0.6 mL) was added at 0 °C DDQ (193 mg, 0.85 mmol). The mixture was slowly warmed to room temperature and stirred for 12 h at that temperature. The reaction was diluted with Et_2O (20 mL), quenched by addition of a 1:1 (v/v) mixture of sat. aq. $NaHCO_3$ and 10% aq. $Na_2S_2O_3$ (10 mL) and stirred vigorously for 15 min. After separation, the aqueous layer was extracted with Et_2O (3x20 mL). The combined organic fractions were dried over Na_2SO_4 and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 1:8 to 1:1) to give alcohol **2-43** (230 mg, 0.24 mmol, 84%) as a clear oil. R_f (EtOAc/hexanes 1:2) = 0.31; $[\alpha]_D^{20}$ = +66.4° (c = 1.0, $CHCl_3$); ^{1}H

NMR (400 MHz, $CDCl_3$) δ 7.43 – 7.28 (m, 10H, arom.), 5.24 (dd, J = 11.2, 3.7 Hz, 1H, H-3c), 5.16 (d, J = 12.2 Hz, 1H, A of AB, $PhCH_2$), 5.06 (d, J = 12.3 Hz, 1H, B of AB, $PhCH_2$), 5.00 (d, J = 9.4 Hz, 1H, NH), 4.90 (d, J = 4.0 Hz, 1H, H-1c), 4.65 (dd, J = 13.0, 6.5 Hz, 1H, H-5c), 4.53 (s, 2H, $PhCH_2$), 4.48 (d, J = 7.1 Hz, 1H, H-1b), 4.26 (dd, J = 9.5, 2.2 Hz, 1H, H-4c), 3.97 (d, J = 1.7 Hz, 1H, H-4b), 3.92 (t, J = 10.4 Hz, 1H, A of AB, H-6b), 3.59 (m, 2H, H-5b, B of AB, H-6b), 3.51 (dd, J = 11.2, 4.0 Hz, 1H, H-2c), 3.42 – 3.33 (m, 2H, H-2b, H-3b), 3.00 – 2.53 (m, 4H, Lev-CH_2, OH), 2.51 – 2.39 (m, 1H, Lev-CH_2), 2.18 (s, 3H, Lev-CH_3), 1.16 (d, J = 6.5 Hz, 3H, H-6c), 0.93 (s, 9H, TBS), 0.14 (s, 6H, TBS); ^{13}C NMR (100 MHz, $CDCl_3$) δ 206.5, 172.2, 156.7, 137.9, 136.4, 129.72, 128.71, 128.6, 128.4, 128.1, 127.9, 127.8, 99.3, 97.7, 77.3, 73.5, 73.3, 71.7, 70.8, 67.6, 67.2, 67.0, 65.6, 58.3, 52.7, 38.0, 29.9, 28.1, 25.8, 18.1, 16.4, -4.1, -5.0; IR (thin film) 3355, 2930, 2858, 2111, 1719, 1719, 1524, 1456, 1363, 1253, 1179, 1253, 1145, 1116, 1076, 840, 784, 751, 699, 676 cm^{-1}; HRMS (ESI) calcd. for $C_{38}H_{53}N_7O_{11}Si$ $(M+Na)^+$ 834.3470 found 834.3439 m/z.

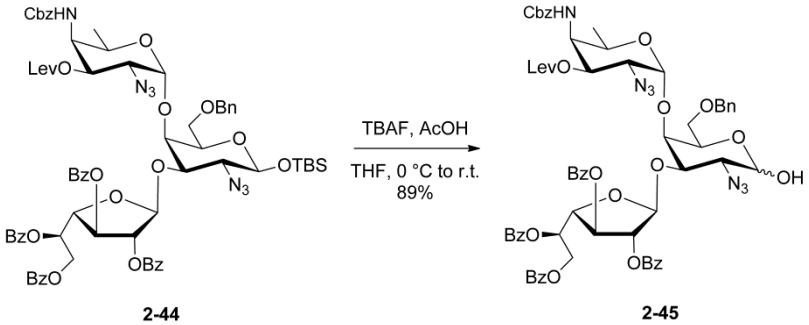
tert-Butyldimethylsilyl 2-azido-4-(benzyloxycarbonyl)amino-3-*O*-levulinoyl-2,4,6-trideoxy-α-D-galactopyranosyl-(1→4)-[2,3,5,6-tetra-*O*-benzoyl-β-D-galactofuranosyl-(1→3)]-2-azido-6-*O*-benzyl-2-deoxy-β-D-galactopyranoside (2-44)



Alcohol **2-43** (181 mg, 0.22 mmol) and imidate **2-41**[132] (250 mg, 0.33 mmol) were co-evaporated with anhydrous toluene (3x20 mL) and kept under high vacuum for 16 h. The mixture was dissolved in anhydrous CH_2Cl_2 (11 mL) and stirred over activated molecular sieves (3 Å-AW) for 1 h at room temperature. The solution was cooled to -30 °C and treated dropwise with TMSOTf (10 µL, 0.055 mmol in 0.2 mL anhydrous CH_2Cl_2). The reaction was stirred for 1.5 h at that temperature, quenched with a 1:1 (v/v) mixture of EtOH and Et_3N (0.5 mL) and diluted with CH_2Cl_2 (20 mL). The mixture was filtered through Celite and concentrated. The residue was purified by flash

chromatography (EtOAc/hexanes 1:3 to 2:3) to give trisaccharide **2-44** (280 mg, 0.20 mmol, 90%) as a clear oil; R_f (EtOAc/hexanes 2:3) = 0.59; $[\alpha]_D^{20}$ = +46.5° (c = 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08 (d, *J* = 7.2 Hz, 2H, arom.), 8.00 (dd, *J* = 13.7, 7.2 Hz, 4H, arom.), 7.89 – 7.82 (m, 2H, arom.), 7.58 – 7.45 (m, 5H, arom.), 7.42 – 7.27 (m, 17H, arom.), 6.02 (dt, *J* = 6.4, 4.5 Hz, 1H, H-5d), 5.63 (m, 3H, H-1d, H-2d, H-3d), 5.29 – 5.13 (m, 2H, H-3c, A of AB, $PhCH_2$), 5.06 (d, *J* = 12.3 Hz, 1H, B of AB, $PhCH_2$), 4.94 (d, *J* = 3.6 Hz, 1H, H-1c), 4.87 – 4.69 (m, 4H, H-4d, NH, H-6d), 4.63 – 4.44 (m, 4H, H-5c, $PhCH_2$, H-1b), 4.18 (d, *J* = 7.1 Hz, 1H, H-4c), 4.03 (d, *J* = 2.1 Hz, 1H, H-4b), 3.85 (dd, *J* = 10.4, 10.4 Hz, 1H, H-6b), 3.71 – 3.51 (m, 4H, H-2b, H-3b, H-5b, H-6b), 3.10 (dd, *J* = 11.2, 3.6 Hz, 1H, H-2c), 2.92 – 2.52 (m, 3H, Lev-CH_2), 2.50 – 2.37 (m, 1H, Lev-CH_2), 2.18 (s, 3H, Lev-CH_3), 1.18 (d, *J* = 6.4 Hz, 3H, H-6c), 0.91 (s, 9H, TBS), 0.13 (s, 6H, TBS); ^{13}C NMR (100 MHz, $CDCl_3$) δ 206.6, 172.2, 166.3, 165.9, 165.8, 156.7, 137.9, 136.6, 133.8, 133.5, 133.3, 130.1, 130.0, 129.94, 129.89, 129.7, 129.5, 129.1, 129.0, 128.8, 128.7, 128.62, 128.58, 128.5, 128.4, 128.2, 128.0, 127.9, 106.3, 98.6, 98.0, 82.1, 81.7, 78.2, 77.4, 76.2, 75.0, 73.6, 70.7, 70.6, 68.1, 67.2, 65.3, 65.1, 63.5, 58.6, 52.9, 38.1, 30.0, 29.9, 28.1, 25.8, 18.1, 16.7, -4.0, -5.0; IR (thin film) 3419, 2930, 2858, 2113, 1725, 1602, 1585, 1507, 1492, 1316, 1264, 1178, 1109, 1096, 1070, 1027, 840, 785, 711 cm^{-1}; HRMS (ESI) calcd. for $C_{72}H_{79}N_7O_{20}Si$ $(M+Na)^+$ 1412.5047 found 1412.5050 *m/z*.

2-Azido-4-(benzyloxycarbonyl)amino-3-*O*-levulinoyl-2,4,6-trideoxy-α-D-galactopyranosyl-(1→4)-[2,3,5,6-tetra-*O*-benzoyl-β-D-galactofuranosyl-(1→3)]-2-azido-6-*O*-benzyl-2-deoxy-αβ-D-galactopyranose (2-45)

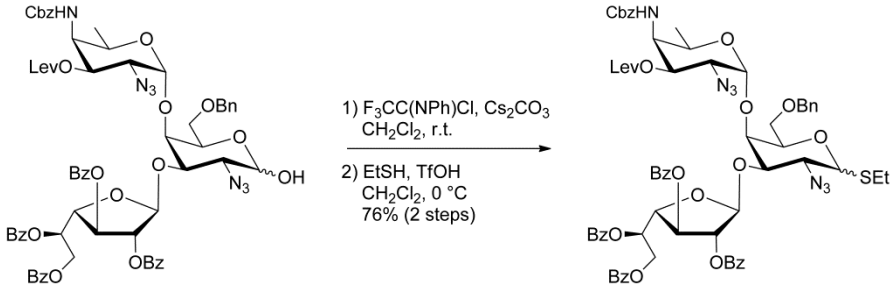


2-44 **2-45**

To a stirred solution of silyl ether **2-44** (280 mg, 0.20 mmol) in THF (10 mL) were added at 0 °C acetic acid (115 µL, 2.014 mmol) and tetrabutylammonium fluoride (1 M in THF, 2.0 mL, 2.0 mmol). The reaction was slowly warmed to room temperature and stirred for 2 h at that temperature. The mixture was diluted with Et_2O (50 mL) and

washed with water (3x30 mL). The combined aqueous fractions were extracted with Et_2O (2x20 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated. The residue was filtered through a short plug of silica gel (EtOAc/hexanes 1:2 to 1:1) to give lactol **2-45** (229 mg, 0.18 mmol, 89%, 3:2 α:β) as a clear oil. R_f (EtOAc/hexanes 2:3) = 0.20; $[\alpha]_D^{20}$ = +69.2° (c = 1.0, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.06 (dd, *J* = 11.0, 4.0 Hz, 2H), 8.03 – 7.93 (m, 5H), 7.90 – 7.84 (m, 2H), 7.55 – 7.42 (m, 5H), 7.42 – 7.27 (m, 22H), 6.08 – 5.83 (m, 1.5H), 5.74 – 5.57 (m, 3H), 5.37 (t, *J* = 3.0 Hz, 0.6H), 5.26 – 5.02 (m, 4H), 4.98 – 4.65 (m, 6H), 4.65 – 4.43 (m, 4.4H), 4.26 (dd, *J* = 12.2, 4.9 Hz, 1.4H), 4.20 – 4.10 (m, 2H), 4.04 (d, *J* = 2.2 Hz, 0.4H), 4.00 (d, *J* = 7.1 Hz, 0.4H), 3.84 – 3.57 (m, 5H), 3.22 (dd, *J* = 11.2, 3.7 Hz, 0.6H), 3.13 (dd, *J* = 11.2, 3.7 Hz, 0.4H), 2.89 – 2.74 (m, 1H), 2.72 – 2.51 (m, 2H), 2.49 – 2.36 (m, 1H), 2.21 – 2.13 (m, 3H), 1.18 (d, *J* = 6.4 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 206.7, 172.1, 166.3, 166.2, 165.9, 165.79, 165.78, 165.6, 165.0, 156.7, 138.0, 137.7, 137.6, 136.5, 133.83, 133.76, 133.6, 133.52, 133.49, 133.4, 130.1, 130.04, 129.98, 129.92, 129.90, 129.85, 129.8, 129.59, 129.57, 129.38, 129.35, 129.1, 128.99, 128.95, 128.9, 128.8, 128.72, 128.68, 128.6, 128.5, 128.4, 128.33, 128.30, 128.25, 128.14, 128.07, 128.0, 125.4, 107.0, 106.3, 98.8, 98.4, 97.0, 92.1, 82.09, 82.08, 81.99, 81.95, 81.9, 81.8, 81.4, 78.1, 77.7, 77.4, 76.6, 75.2, 74.6, 73.62, 73.55, 70.6, 70.5, 69.8, 67.2, 65.3, 63.3, 60.5, 58.9, 52.8, 38.0, 29.9, 28.1, 21.6, 16.7; IR (thin film) 3426, 2935, 2111, 1722, 1602, 1505, 1452, 1316, 1264, 1778, 1109, 1070, 1027, 711 cm^{-1}; HRMS (ESI) calcd. for $C_{66}H_{65}N_7O_{20}$ $(M+Na)^+$ 1298.4182 found 1298.4198 *m/z*.

Ethyl 2-azido-4-(benzyloxycarbonyl)amino-3-*O*-levulinoyl-2,4,6-trideoxy-α-D-galactopyranosyl-(1→4)-[2,3,5,6-tetra-*O*-benzoyl-β-D-galactofuranosyl-(1→3)]-2-azido-6-*O*-benzyl-2-deoxy-1-thio-αβ-D-galactopyranoside (2-46)



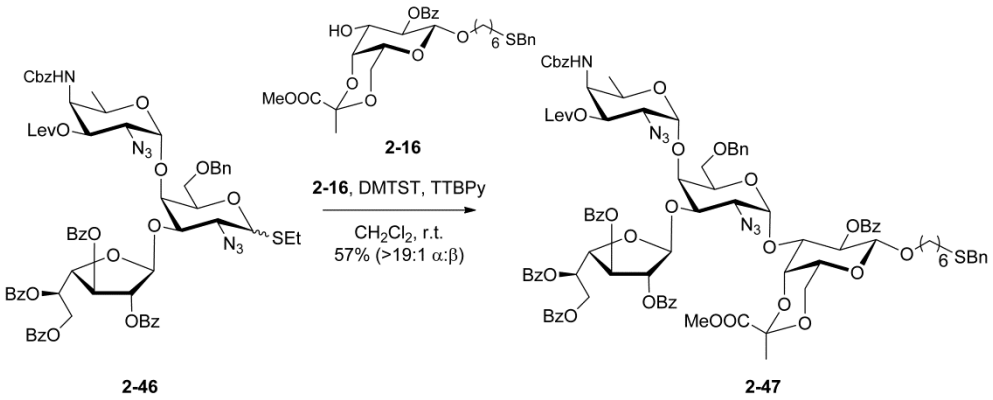
2-45 **2-46**

To a stirred solution of alcohol **2-45** (115 mg, 0.090 mmol) in CH_2Cl_2 (4.5 mL) were added at room temperature 2,2,2-trifluoro-*N*-phenylacetimidoyl chloride (47 mg, 0.225 mmol) and cesium carbonate (73 mg, 0.225 mmol). The reaction was stirred for 1.5 h, diluted with hexanes/0.5% Et_3N (20 mL) and filtered through basic Celite. The mixture was concentrated and the residue was filtered through a short plug of silica gel (EtOAc/hexanes/Et_3N 1:10:0.05 to 1:2:0.15) to give the crude imidate (125 mg) as a clear oil.

To a stirred solution of the crude imidate (125 mg) in CH_2Cl_2 (4.3 mL) over activated molecular sieves (3 Å-AW) was added ethanethiol (7 µL, 0.095 mmol) and the mixture was stirred for 30 min at room temperature. The solution was cooled to 0 °C and treated with trifluoromethanesulfonic acid (1.5 µL, 0.017 mmol). The reaction was stirred for 1.5 h at that temperature, quenched with Et_3N (0.05 mL) and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 0:1 to 1:8 to 1:5) to give thioglycoside **2-46** (90 mg, 0.068 mmol, 76% over two steps, 1:1 α:β) as a clear oil. R_f (EtOAc/hexanes 1:2) = 0.44-0.59; $[\alpha]_D^{20}$ = +74.3° (c = 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, acetone-D_6) δ 8.18 – 8.03 (m, 4H), 8.01 – 7.96 (m, 2H), 7.95 – 7.89 (m, 2H), 7.65 – 7.56 (m, 4H), 7.52 – 7.23 (m, 18H), 6.24 – 6.16 (m, 1H), 6.09 – 6.01 (m, 1H), 5.91 (d, *J* = 1.5 Hz, 0.5H, H-1d), 5.85 (d, *J* = 1.3 Hz, 0.5H, H-1d), 5.83 – 5.73 (m, 2H), 5.65 (d, *J* = 5.5 Hz, 1H), 5.32 – 5.24 (m, 1H), 5.22 – 5.17 (m, 1H, H-1c), 5.16 – 5.09 (m, 2H), 5.05 (d, *J* = 12.5 Hz, 0.5H), 4.89 – 4.69 (m, 3H), 4.60 – 4.48 (m, 3H), 4.43 – 4.31 (m, 2H), 4.23 (ddd, *J* = 10.1, 4.1, 2.1 Hz, 1H), 4.16 (dd, *J* = 10.7, 2.5 Hz, 0.5H), 4.05 (dd, *J* = 10.2, 2.6 Hz, 0.5H), 3.98 – 3.83 (m, 3H), 3.74 – 3.64 (m, 1H), 2.81 – 2.47 (m, 5H), 2.44 – 2.29 (m, 1H), 2.13 (s, 3H), 1.40 – 1.18 (m, 6H); ^{13}C NMR (100 MHz, acetone-D_6) δ 206.1, 172.40,

172.35, 166.5, 166.31, 166.29, 166.2, 166.00, 165.96, 157.8, 139.3, 139.2, 138.3, 134.48, 134.45, 134.35, 134.32, 134.29, 134.13, 134.10, 130.7, 130.58, 130.56, 130.54, 130.49, 130.47, 130.3, 130.18, 130.16, 130.1, 129.54, 129.52, 129.47, 129.43, 129.41, 129.32, 129.29, 129.14, 129.12, 128.8, 128.72, 128.65, 128.5, 128.42, 128.36, 108.1 (C-1d), 107.8 (C-1d), 99.74 (C-1c), 99.65 (C-1c), 85.1, 83.2, 82.9, 82.7, 82.2, 82.0, 80.4, 78.9, 78.8, 77.9, 77.4, 77.3, 76.4, 73.7, 71.7, 71.6, 71.3, 70.9, 69.1, 68.7, 66.9, 66.1, 64.3, 63.9, 61.1, 59.6, 53.9, 38.2, 28.8, 28.7, 24.41, 24.37, 17.24, 17.19, 15.7, 15.1; IR (thin film) 3365, 2927, 2111, 1724, 1585, 1504, 1452, 1316, 1264, 1178, 1109, 1097, 1027, 975, 740, 711 cm^{-1}; HRMS (ESI) calcd. for $C_{68}H_{69}N_7O_{19}S$ $(M+Na)^+$ 1342.4267 found 1342.4253 m/z.

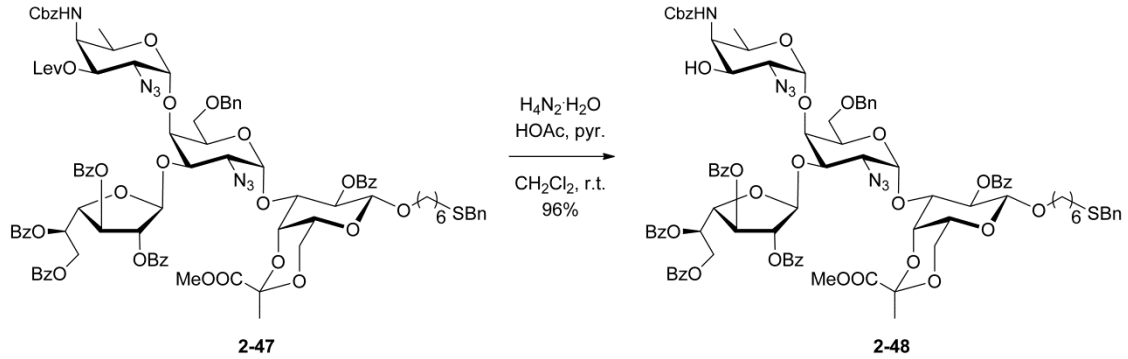
2-Azido-4-(benzyloxycarbonyl)amino-3-*O*-levulinoyl-2,4,6-trideoxy-α-D-galactopyranosyl-(1→4)-[2,3,5,6-tetra-*O*-benzoyl-β-D-galactofuranosyl-(1→3)]-2-azido-6-*O*-benzyl-2-deoxy-α-D-galactopyranosyl-(1→3)-2-*O*-benzoyl-4,6-*O*-[1-(*R*)-(methoxycarbonyl)-ethylidene]-β-D-galactopyranosyl-(1→1)-6-(benzylthio)-1-hexanol (2-47)



Thioglycoside **2-46** (88 mg, 0.067 mmol) and alcohol **2-16** (77 mg, 0.133 mmol) were glycosylated using DMTST (34 mg, 0.167 mmol) and TTBPy (116 mg, 0.467 mmol) in CH_2Cl_2 (3.3 mL) at room temperature for 14 h. Then, 0.5 equiv. of DMTST were added to drive the reaction to completion and stirring was continued for 6 h. The reaction was quenched with a 1:1 (v/v) mixture of sat. aq. $NaHCO_3$ and 10% aq. $Na_2S_2O_3$ (10 mL), and diluted with CH_2Cl_2 (10 mL). After separation, the aqueous layer was extracted with CH_2Cl_2 (5x10 mL), the combined organic fractions were dried over Na_2SO_4 and concentrated. The residue was purified by flash chromatography (EtOAc/toluene 0:1 to 1:4 to 1:3) to obtain a mixture of tetrasaccharide **2-47** and excess acceptor **2-16**. The residue was subjected to size exclusion chromatography (MeOH/CH_2Cl_2 1:1, Sephadex®

LH-20, GE Healthcare) to give tetrasaccharide **2-47** (70 mg, 57%) as a white foam. R_f (EtOAc/toluene 1:2) = 0.46; $[\alpha]_D^{20}$ = +40.0° (c = 0.74, acetone); ^{1}H NMR (600 MHz, acetone-D_6) δ 8.13 (d, *J* = 7.9 Hz, 2H, arom.), 8.09 (d, *J* = 7.1 Hz, 2H, arom.), 8.05 (d, *J* = 7.7 Hz, 2H, arom.), 7.98 (d, *J* = 7.3 Hz, 2H, arom.), 7.86 (d, *J* = 7.7 Hz, 2H, arom.), 7.62 (m, 5H, arom.), 7.53 – 7.25 (m, 25H, arom.), 6.19 (d, *J* = 10.0 Hz, 1H, NH), 6.00 (dd, *J* = 4.1, 3.2 Hz, 1H, H-5d), 5.76 (s, 1H, H-1d), 5.74 (s, 1H, H-2d), 5.71 – 5.68 (m, 1H, H-3d), 5.53 (dd, *J* = 5.2 Hz, 6.4 Hz, 1H, H-2a), 5.45 (s, 1H, H-1b), 5.25 (dd, *J* = 11.4, 3.9 Hz, 1H, H-3c), 5.15 (d, *J* = 12.5 Hz, 1H, A of AB, $PhCH_2$), 5.06 – 5.02 (m, 2H, H-1c, B of AB, $PhCH_2$), 4.95 (s, 1H, H-4d), 4.80 – 4.74 (m, 1H, A of AB, H-6d), 4.72 (m, 1H, H-5c), 4.63 (m, 2H, H-1a, B of AB, H-6d), 4.50 (d, *J* = 3.6 Hz, 1H, H-4a), 4.42 (dd, *J* = 8.4 Hz, 2H, $PhCH_2$), 4.24 – 4.17 (m, 2H, H-3a, H-4c), 4.14 (d, *J* = 10.8 Hz, 1H, H-3b), 4.10 (s, 3H, H-4b, H-5b, A of AB, H-6a), 3.99 (d, *J* = 12.3 Hz, 1H, B of AB, H-6a), 3.88 – 3.80 (m, 2H, A of AB, O-C*H*$_2$-CH_2, H-2b), 3.80 – 3.73 (m, 5H, $COOCH_3$, A of AB, H-6b, H-2c), 3.63 (m, 3H, S-C*H*$_2$-Ph, B of AB, H-6b), 3.58 (s, 1H, H-5a), 3.46 (dt, *J* = 10.0, 6.4 Hz, 1H, B of AB, O-C*H*$_2$-CH_2), 2.74 (m, 1H, Lev-CH_2), 2.66 (m, 1H, Lev-CH_2), 2.50 (dt, *J* = 17.0, 7.4 Hz, 1H, Lev-CH_2), 2.43 – 2.33 (m, 1H, Lev-CH_2), 2.22 (t, *J* = 7.3 Hz, 2H, CH_2-C*H*$_2$-S), 2.11 (s, 3H, Lev-CH_3), 1.51 (s, 3H, pyruv.-CH_3), 1.47 – 1.37 (m, 3H, aliph.), 1.25 (m, 5H, aliph., H-6c), 1.19 – 1.11 (m, 3H, aliph.); ^{13}C NMR (150 MHz, acetone-D_6) δ 206.1, 172.4, 171.1, 166.5, 166.3, 166.2, 165.8, 165.7, 157.8, 140.0, 138.3, 134.4, 134.33, 134.25, 134.1, 134.00, 130.8, 130.7, 130.6, 130.5, 130.4, 130.3, 130.2, 130.1, 129.7, 129.6, 129.5, 129.42, 129.37, 129.3, 129.2, 129.1, 128.74, 128.69, 128.4, 128.3, 127.5, 108.5 (C-1d), 101.8 (C-1a), 99.6, 99.5 (C-1c), 94.4 (C-1b), 82.6, 82.0, 78.6, 75.9, 73.73, 73.71, 73.6, 71.7, 71.3, 70.9, 69.9, 67.4, 66.9, 66.5, 66.0, 64.3, 60.0, 59.3, 53.9, 52.8, 38.2, 36.5, 31.7, 26.3, 26.2, 26.1, 17.2; IR (thin film) 2111, 1726, 1452, 1266, 1110, 711 cm^{-1}; HRMS (ESI) calcd. for $C_{96}H_{101}N_7O_{28}S$ $(M+Na)^+$ 1854.6307 found 1854.6344 *m/z*.

2-Azido-4-(benzyloxycarbonyl)amino-2,4,6-trideoxy-α-D-galactopyranosyl-(1→4)-[2,3,5,6-tetra-O-benzoyl-β-D-galactofuranosyl-(1→3)]-2-azido-6-O-benzyl-2-deoxy-α-D-galactopyranosyl-(1→3)-2-O-benzoyl-4,6-O-[1-(R)-(methoxycarbonyl)-ethylidene]-β-D-galactopyranosyl-(1→1)-6-(benzylthio)-1-hexanol (2-48)

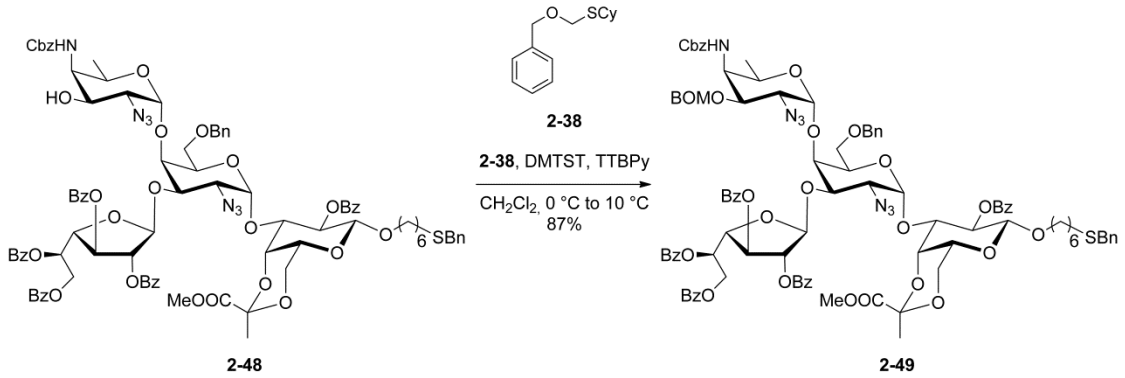


2-47 **2-48**

To a stirred solution of Lev ester **2-47** (49 mg, 0.027 mmol) in CH_2Cl_2 (2.7 mL) was added at room temperature first a mixture of pyridine (65 µL, 0.802 mmol) and acetic acid (43 µL, 0.749 mmol), and then hydrazine hydrate (2.3 µL, 0.045 mmol). The mixture was stirred for 1.5 h at room temperature, quenched with acetone (0.1 mL) and subjected to size exclusion chromatography (MeOH/CH_2Cl_2 1:1, Sephadex® LH-20, GE Healthcare) to give alcohol **2-48** (45 mg, 0.026 mmol, 96%) as a white foam. R_f (EtOAc/toluene 2:3) = 0.31; $[\alpha]_D^{20}$ = +98.9° (c = 0.27, acetone); ^{1}H NMR (600 MHz, acetone-D_6) δ 8.13 (d, J = 7.7 Hz, 2H, arom.), 8.09 (d, J = 7.2 Hz, 2H, arom.), 8.03 (d, J = 7.7 Hz, 2H, arom.), 7.98 (d, J = 7.6 Hz, 2H, arom.), 7.87 (d, J = 7.7 Hz, 2H, arom.), 7.62 (m, 4H, arom.), 7.54 – 7.25 (m, 26H, arom.), 6.05 (d, J = 9.9 Hz, 1H, NH), 5.98 (dd, J = 9.3, 5.3 Hz, 1H, H-5d), 5.76 – 5.71 (m, 2H, H-1d, H-2d), 5.70 (dd, J = 5.9, 3.2 Hz, 1H, H-3d), 5.57 – 5.49 (dd, J = 6.8, 5.2 Hz, 1H, H-2a), 5.45 (s, 1H, H-1b), 5.12 (d, J = 12.6 Hz, 1H, A of AB, $PhCH_2$), 5.04 (d, J = 12.6 Hz, 1H, B of AB, $PhCH_2$), 4.94 (m, 2H, H-1c, H-4d), 4.74 (dd, J = 11.9, 3.9 Hz, 1H, A of AB, H-6d), 4.66 – 4.56 (m, 3H, H-1a, H-5c, B of AB, H-6d), 4.51 (d, J = 3.7 Hz, 1H, H-4a), 4.47 – 4.39 (m, 2H, $PhCH_2$), 4.34 – 4.26 (m, 1H, H-3c), 4.19 (dd, J = 10.0, 3.7 Hz, 1H, H-3a), 4.14 – 4.03 (m, 5H, H-3b, H-4b, H-5b, A of AB, H-6a, H-4c), 3.99 (d, J = 12.8 Hz, 1H, B of AB, H-6a), 3.85 – 3.74 (m, 6H, A of AB, O-CH_2-CH_2, H-2b, A of AB, H-6b, $COOCH_3$), 3.70 – 3.62 (m, 3H, S-CH_2-Ph, B of AB, H-6b), 3.58 (s, 1H, H-5a), 3.52 – 3.43 (m, 2H, H-2c, B of AB, O-CH_2-CH_2), 2.22 (t, J = 7.4 Hz, 2H, CH_2-CH_2-S), 1.51 (s, 3H, pyruv.-CH_3), 1.44 (m, 2H, aliph.), 1.34 – 1.19 (m, 6H, aliph., H-6c), 1.15 (m, 3H, aliph.); ^{13}C NMR (150 MHz, acetone-D_6) δ 171.1, 166.4, 166.3, 165.8, 165.7, 158.3, 140.0, 138.3, 134.4, 134.3, 134.2,

134.1, 130.73, 130.65, 130.6, 130.49, 130.46, 130.4, 130.3, 130.2, 130.1, 129.7, 129.6, 129.5, 129.37, 129.35, 129.23, 129.16, 129.14, 128.70, 128.67, 128.4, 128.2, 127.5, 108.2 (C-1d), 101.8 (C-1a), 99.5 (C-1c), 94.1 (C-1b), 82.5, 81.7, 78.5, 76.0, 73.6, 71.6, 71.3, 69.9, 67.4, 66.8, 66.5, 66.1, 64.2, 62.0, 60.1, 57.3, 52.8, 36.5, 31.7, 30.4, 29.8, 29.1, 26.2, 26.1, 17.4; IR (thin film) 2936, 2326, 2163, 2111, 1728, 1602, 1452, 1266, 1109, 1071, 1029, 985, 711 cm^{-1}; HRMS (ESI) calcd. for $C_{91}H_{95}N_7O_{26}S$ $(M+Na)^+$ 1756.5940 found 1756.5895 m/z.

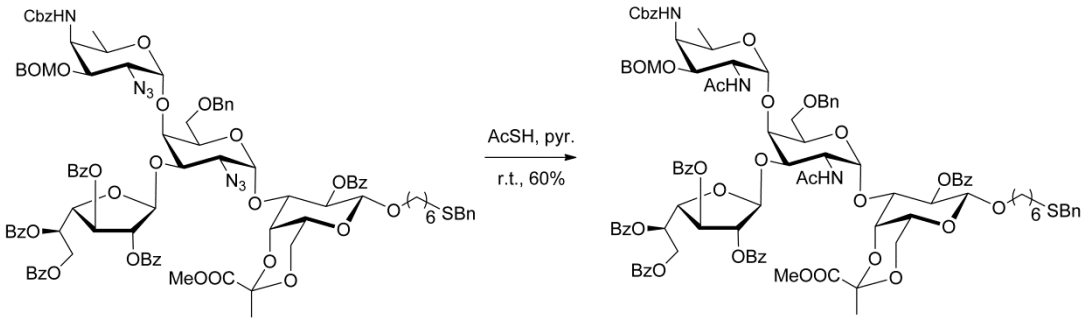
2-Azido-4-(benzyloxycarbonyl)amino-3-*O*-benzyloxymethyl-2,4,6-trideoxy-α-D-galactopyranosyl-(1→4)-[2,3,5,6-tetra-*O*-benzoyl-β-D-galactofuranosyl-(1→3)]-2-azido-6-*O*-benzyl-2-deoxy-α-D-galactopyranosyl-(1→3)-2-*O*-benzoyl-4,6-*O*-[1-(R)-(methoxycarbonyl)-ethylidene]-β-D-galactopyranosyl-(1→1)-6-(benzylthio)-1-hexanol (2-49)



Alcohol **2-48** (23 mg, 0.013 mmol), *S*,*O*-acetal **2-38** (95 mg, 0.401 mmol) and TTBPy (132 mg, 0.535 mmol) were co-evaproated with anhydrous toluene (3x10 mL). The mixture was dissolved in CH_2Cl_2 (2.0 mL) and stirred over activated molecular sieves (3 Å) for 30 min at room temperature. The mixture was cooled to 0 °C and DMTST (16 mg, 0.401 mmol in 0.3 mL CH_2Cl_2) was added dropwise over a period of 1.5 h, while the reaction temperature was kept below 10 °C. The reaction was stirred for another 45 min, quenched by addition of a 10:1 (v/v) mixture of MeOH and Et_3N (0.5 mL) and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 0:1 to 1:1) to give acetal **2-49** (22 mg, 0.012 mmol, 87%) as a white foam. R_f (EtOAc/hexanes 2:3) = 0.46; $[\alpha]_D^{20}$ = +76.5° (c = 0.53, acetone); ^{1}H NMR (400 MHz, acetone-D_6) δ 8.16 – 8.07 (m, 4H, arom.), 8.04 (d, J = 7.3 Hz, 2H, arom.), 8.01 – 7.96 (d, J = 7.6 Hz, 2H, arom.), 7.86 (d, J = 8.3 Hz, 2H, arom.), 7.70 – 7.55 (m, 5H, arom.), 7.52 – 7.24 (m, 30H, arom.), 6.09 (d, J = 10.1 Hz, 1H, NH), 5.98 (dt, J = 7.4, 3.8 Hz, 1H, H-5d), 5.76 – 5.72 (m, 2H, H-1d, H-2d), 5.70 (dd, J = 5.8, 3.2 Hz, 1H, H-3d), 5.54 (dd, J = 10.0, 8.1 Hz,

1H, H-2a), 5.46 (s, 1H, H-1b), 5.13 – 5.06 (m, 2H, $PhCH_2$), 5.01 (dd, J = 8.1, 5.5 Hz, 2H, H-1c, A of AB, BnO-CH_2-O), 4.95 (dd, J = 5.8, 3.0 Hz, 1H, H-4d), 4.75 (m, 3H, A of AB, H-6d, A of AB, $PhCH_2$, B of AB, BnO-CH_2-O), 4.69 – 4.54 (m, 4H, H-1a, H-5c, B of AB, H-6d, B of AB, $PhCH_2$), 4.52 (d, J = 3.6 Hz, 1H, H-4a), 4.43 (s, 2H, $PhCH_2$), 4.35 (dd, J = 11.0, 4.2 Hz, 1H, H-3c), 4.30 (d, J = 10.0 Hz, 1H, H-4c), 4.20 (dd, J = 10.1, 3.8 Hz, 1H, H-3a), 4.15 – 4.03 (m, 4H, H-3b, H-4b, H-5b, A of AB, H-6a), 3.99 (d, J = 12.6 Hz, 1H, B of AB, H-6a), 3.88 – 3.76 (m, 3H, A of AB, O-CH_2-CH_2, H-2b, A of AB, H-6b), 3.74 (s, 3H, $COOCH_3$), 3.72 – 3.65 (m, 2H, H-2c, B of AB, H-6b), 3.64 (s, 2H, CH_2-Ph), 3.57 (s, 1H, H-5a), 3.46 (dt, J = 10.0, 6.5 Hz, 1H, B of AB, O-CH_2-CH_2), 2.23 (t, J = 7.3 Hz, 2H, CH_2-CH_2-S), 1.51 (s, 3H, pyruv-CH_3), 1.49 – 1.37 (m, 2H, aliph.), 1.35 – 1.20 (m, 6H, H-6c, aliph.), 1.19 – 1.12 (m, 3H, aliph.); ^{13}C NMR (100 MHz, acetone-D_6) δ 171.2, 166.5, 166.3, 165.9, 165.7, 157.9, 140.0, 139.4, 139.2, 138.4, 134.4, 134.34, 134.27, 131.1, 130.8, 130.7, 130.64, 130.57, 130.5, 130.4, 130.3, 130.2, 130.1, 129.72, 129.65, 129.5, 129.43, 129.39, 129.24, 129.18, 129.16, 128.9, 128.7, 128.41, 128.37, 128.3, 127.5, 108.6 (C-1d), 101.9 (C-1a), 99.7, 99.5 (C-1c), 94.2 (C-1b), 92.9, 82.6, 81.8, 78.6, 76.1, 73.67, 73.65, 73.62, 72.5, 71.7, 71.4, 71.1, 70.3, 69.9, 69.2, 67.4, 66.8, 66.6, 66.5, 66.1, 64.2, 61.1, 60.1, 53.6, 52.8, 36.5, 31.8, 30.1, 29.9, 29.7, 26.2, 26.1, 17.4; IR (thin film) 2940, 2111, 1728, 1452, 1264, 1110, 1042, 711 cm^{-1}; HRMS (ESI) calcd. for $C_{99}H_{103}N_7O_{27}S$ $(M+Na)^+$ 1876.6520 found 1876.6628 m/z.

2-Acetamido-4-(benzyloxycarbonyl)amino-3-*O*-benzyloxymethyl-2,4,6-trideoxy-α-D-galactopyranosyl-(1→4)-[2,3,5,6-tetra-*O*-benzoyl-β-D-galactofuranosyl-(1→3)]-2-acetamido-6-*O*-benzyl-2-deoxy-α-D-galactopyranosyl-(1→3)-2-*O*-benzoyl-4,6-*O*-[1-(*R*)-(methoxycarbonyl)-ethylidene]-β-D-galactopyranosyl-(1→1)-6-(benzylthio)-1-hexanol (2-50)

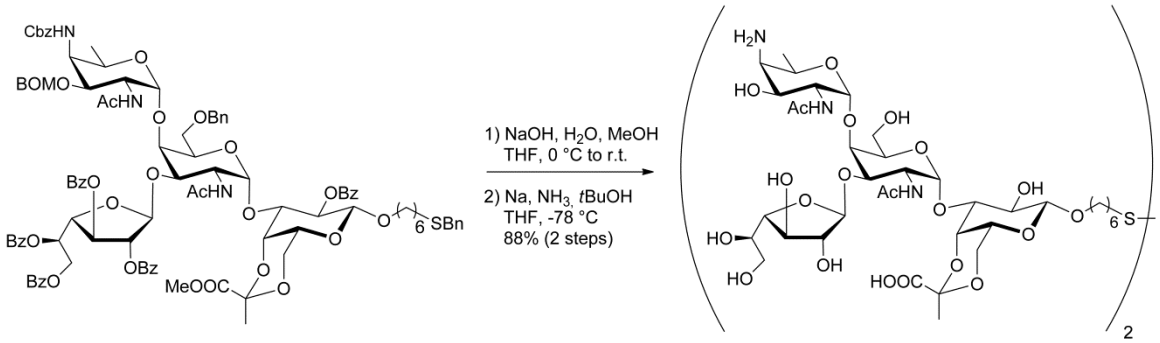


2-49 **2-50**

To a stirred solution of diazide **2-49** (21 mg, 0.011 mmol) in anhydrous pyridine (0.22 mL) was added at 0 °C thioacetic acid (0.2 mL). The mixture was warmed to room temperature and stirred for 48 h at that temperature. The solution was co-evaporated with toluene (2x5 mL) and the residue was purified by flash chromatography (EtOAc/hexanes 1:2 to acetone/hexanes 1:3 to 1:2 to 3:4) to give diacetamide **2-50** (13 mg, 0.007 mmol, 60%) as a white foam. R_f (acetone/hexanes 1:2) = 0.29; $[\alpha]_D^{20}$ = +56.4° (c = 0.25, acetone); ^{1}H NMR (400 MHz, acetone-D_6) δ 8.11 – 8.08 (m, 2H, arom.), 8.06 – 7.92 (m, 8H, arom.) 7.68 – 7.54 (m, 4H, arom.), 7.52 – 7.27 (m, 31H, arom.), 6.86 (d, J = 9.8 Hz, 1H, NH), 6.71 (d, J = 7.2 Hz, 1H, NH), 5.92 (m, 2H, NH, H-5d), 5.71 – 5.64 (m, 3H, H-1d, H-2d, H-3d), 5.41 (dd, J = 10.2, 8.1 Hz, 1H, H-2a), 5.10 (d, J = 3.9 Hz, 3H, H-1b, $PhCH_2$), 5.06 – 5.01 (m, 1H, H-1c), 4.98 (dd, J = 7.1, 3.4 Hz, 1H, H-4d), 4.89 (d, J = 6.9 Hz, 1H, A of AB, BnO-CH_2-O), 4.79 – 4.49 (m, 8H, H-1a, H-2b, H-5c, H-6d, B of AB, BnO-CH_2-O, $PhCH_2$), 4.46 – 4.35 (m, 3H, H-4a, $PhCH_2$), 4.07 (m, 7H, H-2c, H-3a, H-4b, H-4c, H-5b, H-6a), 3.90 – 3.78 (m, 3H, H-3c, H-3b, A of AB, O-C*H*$_2$-CH_2), 3.77 (s, 3H, $COOCH_3$), 3.70 – 3.58 (m, 3H, $PhCH_2$, A of AB, H-6b), 3.54 (s, 1H, H-5a), 3.51 – 3.39 (m, 2H, B of AB, O-C*H*$_2$-CH_2, B of AB, H-6b), 2.21 (t, J = 6.4 Hz, 2H, CH_2-C*H*$_2$-S), 1.83 (s, 3H, Ac-CH_3), 1.80 (s, 3H, Ac-CH_3), 1.49 (s, 3H, pyruv.-CH_3), 1.47 – 1.37 (m, 2H, aliph.), 1.31 – 1.09 (m, 9H, H-6c, aliph.); ^{13}C NMR (100 MHz, acetone-D_6) δ 171.2, 170.2, 170.1, 166.5, 166.2, 166.0, 165.6, 140.0, 139.6, 139.54, 138.51, 134.46, 134.3, 134.2, 134.1, 130.82, 130.81, 130.78, 130.7, 130.6, 130.5, 130.4, 130.1, 129.7, 129.6, 129.47, 129.45,

129.4, 129.22, 129.19, 129.15, 128.7, 128.64, 128.58, 128.4, 128.3, 128.21, 128.18, 127.6, 109.1 (C-1d), 101.9 (C-1a), 99.6, 99.5 (C-1b), 94.5 (C-1c), 93.8, 82.9, 80.9, 78.6, 76.6, 73.6, 73.2, 71.6, 71.5, 71.1, 70.03, 69.98, 67.7, 66.7, 66.5, 66.3, 66.0, 64.2, 54.1, 53.0, 50.6, 49.1, 36.5, 32.3, 31.8, 30.6, 30.3, 30.1, 29.9, 29.1, 26.4, 26.2, 23.3, 23.2, 17.7; IR (thin film) 2928, 1726, 1683, 1509, 1452, 1267, 1111, 1071, 1047, 711 cm^{-1}; HRMS (ESI) calcd. for $C_{103}H_{111}N_3O_{29}S$ $(M+Na)^+$ 1908.6921 found 1908.6906 *m/z*.

6,6'-Dithiobis[2-*N*-acetyl-4-amino-2,4,6-trideoxy-α-D-galactopyranosyl-(1→4)-[β-D-galactofuranosyl-(1→3)]-2-*N*-acetyl-2-deoxy-α-D-galactopyranosyl-(1→3)-4,6-*O*-[1-(*R*)-(carboxy)-ethylidene]-β-D-galactopyranoside-(1→1)-1-hexanol] (2-2)



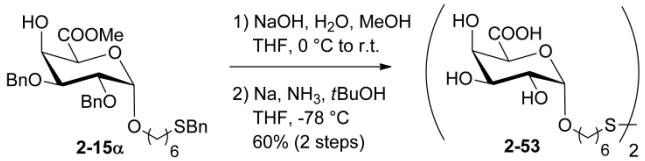
2-50 **2-2**

To a stirred solution of ester **2-50** (12 mg, 6.4 µmol) in THF (1.5 mL) and MeOH (0.75 mL) was added at 0 °C a 1 M aq. solution of NaOH (0.8 mL). The reaction was slowly warmed to room temperature and stirred for 16 h at that temperature. The mixture was diluted with water (5 mL), acidified to pH 4 with 0.5 M aq. $NaHSO_4$, and poured into EtOAc (5 mL). After separation, the aqueous fraction was extracted with EtOAc (8x10 mL), the combined organic fractions were dried over Na_2SO_4 and concentrated to give the intermediate acid as a white foam.

To a stirred solution of liquid ammonia (8 mL) was added at -78 °C a solution of the crude acid in THF (2.0 mL). The mixture was treated with *t*BuOH (0.8 mL) and lumps of freshly cut sodium (65 mg) were added until a deeply blue color persisted. The reaction was stirred at -78 °C for 45 min and quenched by addition of solid NH_4OAc (200 mg). The solution was warmed to room temperature under a stream of argon and co-evaporated with MeOH (2x10 mL) and water (2x5 mL). The residue was left exposed to air for 16 h, purified by size exclusion chromatography (MeOH/5 mM aq. NH_4OAc 4:6, Sephadex® G-25, GE Healthcare) and lyophilized repeatedly to give disulfide **2** (5 mg,

2.8 µmol, 88%) as a white solid, containing approx. 10% of the corresponding thiol. $[\alpha]_D^{20}$ = +60.6° (c = 0.14, H_2O); ^1H NMR (600 MHz, D_2O) δ 5.42 (d, *J* = 3.7 Hz, 1H, H-1b), 5.18 (d, *J* = 3.4 Hz, 1H, H-1d), 5.10 (d, *J* = 3.8 Hz, 1H, H-1c), 4.77 (d, *J* = 8.1 Hz, 1H, H-5c), 4.70 (dd, *J* = 11.2, 3.5 Hz, 1H, H-2b), 4.59 (d, *J* = 7.9 Hz, 1H, H-1a), 4.55 (d, *J* = 4.0 Hz, 1H, H-4a), 4.41 (dd, *J* = 11.4, 4.3 Hz, 1H, H-3c), 4.32 (dd, *J* = 7.5, 5.5 Hz, 1H, H-5b), 4.26 (d, *J* = 2.2 Hz, 1H, H-4d or H-3d), 4.22 (dd, *J* = 11.1, 2.4 Hz, 1H, H-3b), 4.18 – 4.08 (m, 5H, H-2c, H-2d, H-4d or H-3d, H-4b, A of AB, H-6a), 4.04 (m, 2H, B of AB, H-6a, A of AB, O-C*H*$_2$-CH$_2$), 3.92 – 3.86 (m, 2H, H-3a, H-5d), 3.87 – 3.72 (m, 6H, H-2a, H-6b, H-6d, B of AB, O-C*H*$_2$-CH$_2$), 3.67 (s, 1H, H-5a), 3.55 (s, 1H, H-4c), 3.02 (dd, *J* = 9.1, 6.8 Hz, 0.2H, CH$_2$-C*H*$_2$-SH), 2.88 (t, *J* = 7.2 Hz, 1.9H, CH$_2$-C*H*$_2$-S-S), 2.18 (s, 3H, Ac-CH$_3$), 2.16 (s, 3H, Ac-CH$_3$), 1.85 – 1.73 (m, 4H, aliph.), 1.56 (s, 3H, pyruv.-CH$_3$), 1.55 – 1.50 (m, 4H, aliph.), 1.39 (d, *J* = 6.7 Hz, 3H, H-6c); HRMS (MALDI) calcd. for $C_{74}H_{124}N_6O_{42}S_2$ $(M+Na)^+$ 1855.7085 found 1855.7010 *m/z*.

6,6'-Dithiobis[α-D-galactopyranosyluronate-(1→1)-1-hexanol] (2-53)

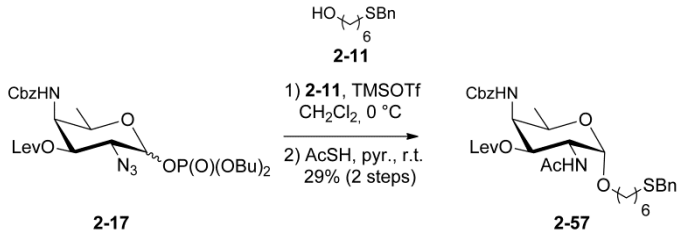


To a stirred solution of ester **2-15α** (10 mg, 0.017 mmol) in THF (1.0 mL) and MeOH (0.5 mL) was added at 0 °C 1 M aq. NaOH (0.8 mL). The reaction was slowly warmed to room temperature and stirred for 16 h at that temperature. The reaction was diluted with EtOAc (5 mL) and water (5 mL) and acidified to pH 4 with 0.5 M aq. $NaHSO_4$. After separation, the aqueous fraction was extracted with EtOAc (8x5 mL), the combined organic fractions were dried over Na_2SO_4 and concentrated to give the intermediate acid as a white foam.

To a stirred solution of liquid ammonia (8 mL) was added at -78 °C a solution of the crude acid in THF (2 mL). The mixture was treated with *t*BuOH (0.4 mL) and lumps of freshly cut sodium (45 mg) were added until a deeply blue color persisted. The reaction was stirred at -78 °C for 45 min and quenched by addition of solid NH_4OAc (100 mg). The solution was warmed to room temperature under a stream of argon and co-evaporated with MeOH (2x10 mL) and water (2x5 mL). The residue was left exposed to air for 16 h, purified by size exclusion chromatography (9:1 MeOH/5 mM aq. NH_4OAc,

Sephadex® G-25, GE Healthcare) and lyophilized repeatedly to give disulfide **2-53** (3.1 mg, 5.1 µmol, 60% over two steps) as a white solid. $[\alpha]_D^{20}$ = +29.8° (c = 0.29, H_2O); ^1H NMR (400 MHz, D_2O) δ 5.09 (d, *J* = 3.7 Hz, 1H, H-1), 4.47 – 4.33 (m, 2H, H-4, H-5), 4.04 (dd, *J* = 10.1, 3.1 Hz, 1H, H-3), 3.97 (dd, *J* = 10.1, 3.7 Hz, 1H, H-2), 3.90 – 3.76 (m, 1H, A of AB, O-C*H*$_2$-CH_2), 3.74 – 3.63 (m, 1H, B of AB, O-C*H*$_2$-CH_2), 2.91 (t, *J* = 7.2 Hz, 2H, CH_2-C*H*$_2$-S), 1.91 – 1.68 (m, 4H, aliph.), 1.67 – 1.46 (m, 4H, aliph.); HRMS (MALDI) calcd. for $C_{24}H_{42}O_{14}S_2$ $(M-H)^-$ 617.1938 found 617.1954 *m/z*.

2-Acetamido-4-(benzyloxycarbonyl)amino-3-*O*-levulinoyl-2,4,6-trideoxy-α-D-galactopyranosyl-(1→1)-6-(benzylthio)hexanol (2-57)



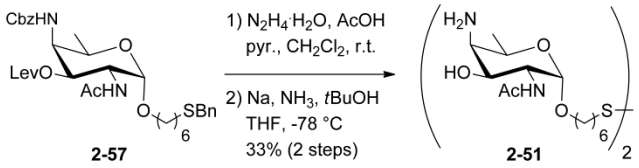
2-17 2-57

Alcohol **2-11** (29 mg, 0.171 mmol) and glycosyl phosphate **2-17** (70 mg, 0.114 mmol) were co-evaproated with anhydrous toluene (3x10 mL) and kept under high vacuum for 30 min. The mixture was dissolved in CH_2Cl_2 (1.8 mL) and stirred over activated molecular sieves (4 Å-AW) for 1 h at room temperature. The solution was cooled to 0 °C and treated with TMSOTf (31 µL, 0.171 mmol in 0.2 mL anhydrous CH_2Cl_2). The mixture was stirred for 3 h at that temperature, quenched with a 1:1 (v/v) mixture of MeOH and Et_3N (0.5 mL), diluted with CH_2Cl_2 (20 mL) and filtered through Celite. The residue was purified by flash chromatography (EtOAc/hexanes 2:3 to 3:2) to give the corresponding glycosides (57 mg) as an inseparable α/β mixture.

To a stirred solution of the glycoside mixture in anhydrous pyridine (0.9 mL) was added at 0 °C thioacetic acid (0.9 mL). The mixture was warmed to room temperature and stirred for 24 h at that temperature. The solution was co-evaporated with toluene (2x5 mL) and the residue was purified by flash chromatography (EtOAc/hexanes 1:2 to 2:1 to 6:1) to give acetamide **2-57** (22 mg, 0.034 mmol, 29% over two steps) as a white solid, along with the corresponding β-isomer (21.6 mg, 0.034 mmol, 29%). R_f (EtOAc/hexanes 4:1) = 0.36; $[\alpha]_D^{20}$ = +24.7° (c = 0.50, CH_2Cl_2); ^1H NMR (400 MHz, $CDCl_3$) δ 7.45 – 7.28 (m, 8H, arom.), 7.28 – 7.20 (m, 2H), 5.57 (d, *J* = 9.3 Hz, 1H, NH),

5.09 (m, 4H, $PhCH_2$, H-3, NH), 4.74 (d, J = 3.9 Hz, 1H, H-1), 4.34 – 4.22 (m, 1H, H-2), 4.11 (dt, J = 13.0, 5.3 Hz, 2H, H-4, H-5), 3.71 (s, 2H, $PhCH_2$), 3.61 (m, 1H, A of AB, O-CH_2-CH_2), 3.35 (m, 1H, B of AB, O-CH_2-CH_2), 2.68 (m, 1H, Lev-CH_2), 2.61 – 2.30 (m, 5H, Lev-CH_2, CH_2-CH_2-S), 2.12 (s, 3H, Ac-CH_3 or Lev-CH_3), 1.98 (s, 3H, Ac-CH_3 or Lev-CH_3), 1.56 (dd, J = 15.6, 8.3 Hz, 4H, aliph.), 1.43 – 1.23 (m, 4H, aliph.), 1.15 (d, J = 6.4 Hz, 3H, H-6); ^{13}C NMR (100 MHz, $CDCl_3$) δ 206.7, 172.6, 170.2, 156.7, 138.6, 136.4, 128.8, 128.5, 128.4, 128.1, 128.0, 126.9, 97.4, 77.2, 70.2, 68.2, 66.9, 64.4, 52.8, 47.7, 37.7, 36.3, 31.3, 29.7, 29.2, 29.1, 28.5, 28.1, 25.8, 23.3, 16.5; IR (thin film) 2927, 1719, 1665, 1533, 1242, 1122, 1045, 699 cm^{-1}; HRMS (ESI) calcd. for $C_{34}H_{46}N_2O_8S$ $(M+Na)^+$ 665.2872 found 665.2865 *m/z*.

6,6'-Dithiobis[2-acetamido-4-amino-2,4,6-trideoxy-α-D-galactopyranosyl-(1→1)-1-hexanol] (2-51)

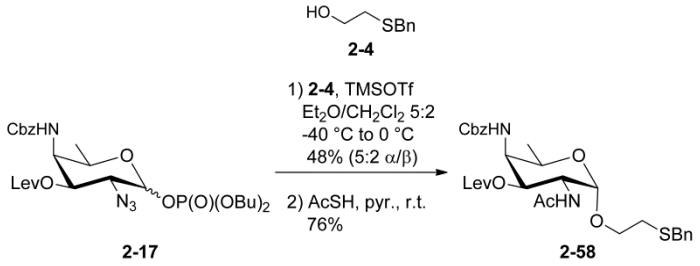


To a stirred solution of ester **2-57** (10 mg, 0.016 mmol) in anhydrous CH_2Cl_2 (1.0 mL) were added at room temperature first a mixture of pyridine (38 µL, 0.467 mmol) and acetic acid (24.9 µL, 0.436 mmol), and then hydrazine hydrate (1.0 µL, 0.020 mmol). The mixture was stirred for 2 h at that temperature, quenched with acetone (0.1 mL) and purified by size exclusion chromatography (CH_2Cl_2/MeOH 2:1, Sephadex® LH-20, GE Healthcare) to give the corresponding alcohol as a clear oil.

To a stirred solution of liquid ammonia (5 mL) was added at -78 °C a solution of the intermediate alcohol in THF (1.2 mL). The mixture was treated with *t*BuOH (0.5 mL) and lumps of freshly cut sodium (80 mg) were added until a deeply blue color persisted. The reaction was stirred at -78 °C for 45 min and quenched by addition of solid ammonium acetate (100 mg). The solution was warmed to room temperature under a stream of argon and co-evaporated with MeOH (2x10 mL) and water (2x5 mL). The residue was left under air for 16 h, purified by size exclusion chromatography (9:1 MeOH/5 mM aq. NH_4OAc, Sephadex® G-25, GE Healthcare) and lyophilized repeatedly to give thiol-linked monosaccharide **2-51** (acetate salt, 1.7 mg, 2.7 µmol, 33% over two steps) as a mixture of thiol and disulfide as a white solid. $[\alpha]_D^{20}$ = +19.9° (c = 0.02,

H_2O); ^1H NMR (400 MHz, D_2O) δ 4.76 (d, J = 3.1 Hz, 1H), 4.11 (d, J = 6.9 Hz, 1H), 3.99 – 3.91 (m, 2H), 3.65 – 3.55 (m, 1H), 3.43 – 3.34 (m, 1H), 3.19 – 3.04 (m, 1H), 2.88 – 2.76 (m, 0.5H), 2.68 (t, J = 7.1 Hz, 1H), 1.95 (s, 3H), 1.82 (s, 3H), 1.71 – 1.57 (m, 2H), 1.56 – 1.42 (m, 2H), 1.39 – 1.27 (m, 4H), 1.19 – 1.13 (m, 3H). HRMS (ESI) calcd. for $C_{28}H_{54}N_4O_8S_2$ $(M+Na)^+$ 661.3281 found 661.3306 *m/z*.

2-Acetamido-4-(benzyloxycarbonyl)amino-3-*O*-levulinoyl-2,4,6-trideoxy-α-D-galactopyranosyl-(1→1)-6-(benzylthio)hexanol (2-58)

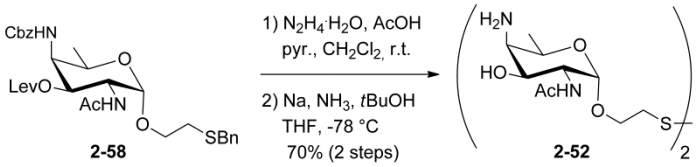


Alcohol **2-4** (71 mg, 0.421 mmol) and glycosyl phosphate **2-17** (171 mg, 0.281 mmol) were co-evaproated with anhydrous toluene (3x10 mL) and kept under high vacuum for 30 min. The mixture was dissolved in CH_2Cl_2 (1.8 mL) and stirred over activated molecular sieves (4 Å-AW) for 1 h at room temperature. The solution was then cooled to -40 °C and treated with TMSOTf (56 µL, 0.309 mmol in 0.2 mL anhydrous CH_2Cl_2). The mixture was slowly warmed to 0 °C (2 h), quenched with a 1:1 (v/v) mixture of MeOH and Et_3N (0.5 mL), diluted with CH_2Cl_2 (20 mL), filtered through Celite and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 1:3 to 1:1) to give the corresponding α-glycoside (55 mg, 0.096 mmol, 34%) along with the corresponding β-glycoside (22 mg, 0.039 mmol, 14%).

To a stirred solution of the intermediate α-glycoside (40 mg, 0.070 mmol) in anhydrous pyridine (0.4 mL) was added at 0 °C thioacetic acid (0.4 mL). The mixture was warmed to room temperature and stirred for 24 h at that temperature. The solution was co-evaporated with toluene (2x5 mL) and the residue was purified by flash chromatography (EtOAc/hexanes 1:3 to acetone/hexanes 1:2 to 2:3) to give acetamide **2-58** (31 mg, 0.053 mmol, 76%) as a white solid. R_f (acetone/hexanes 2:3) = 0.43; $[\alpha]_D^{20}$ = +109.5° (c = 0.50, acetone); ^1H NMR (400 MHz, CD_3OD) δ 7.46 – 7.14 (m, 10H, arom.), 5.18 (d, J = 12.6 Hz, 1H, A of AB, $PhCH_2$), 5.07 – 4.99 (m, 2H, H-2, B of AB, $PhCH_2$), 4.75 (d, J = 3.8 Hz, 1H, H-1), 4.30 (dd, J = 11.7, 3.8 Hz, 1H, H-3), 4.23 – 4.16 (m, 1H,

H-5), 4.11 – 4.04 (m, 1H, H-4), 3.77 (s, 2H, $PhCH_2$), 3.75 – 3.67 (m, 1H, A of AB, O-C*H*$_2$-CH_2), 3.53 (m, 1H, B of AB, O-C*H*$_2$-CH_2), 2.71 – 2.50 (m, 4H, Lev-CH_2, CH_2-C*H*$_2$-S), 2.42 – 2.18 (m, 2H, Lev-CH_2), 2.09 (s, 3H, Lev-CH_3 or Ac-CH_3), 1.93 (s, 3H, Lev-CH_3 or Ac-CH_3), 1.11 (d, J = 6.5 Hz, 3H, H-6); ^{13}C NMR (100 MHz, CD_3OD) δ 209.3, 173.8, 173.5, 159.5, 140.0, 138.6, 1230.0, 129.52, 129.48, 129.0, 128.8, 128.1, 99.0, 71.5, 68.4, 67.5, 65.8, 54.0, 38.5, 37.2, 31.9, 29.7, 29.1, 22.7, 16.9; IR (thin film) 3322, 2930, 1718, 1667, 1533, 1423, 1244, 1158, 1122, 1050, 699 cm^{-1}; HRMS (ESI) calcd. for $C_{30}H_{38}N_2O_8S$ $(M+Na)^+$ 609.2246 found 609.2256 *m/z*.

2,2'-Dithiobis[2-acetamido-4-amino-2,4,6-trideoxy-α-D-galactopyranosyl-(1→1)-1-hexanol] (2-52)

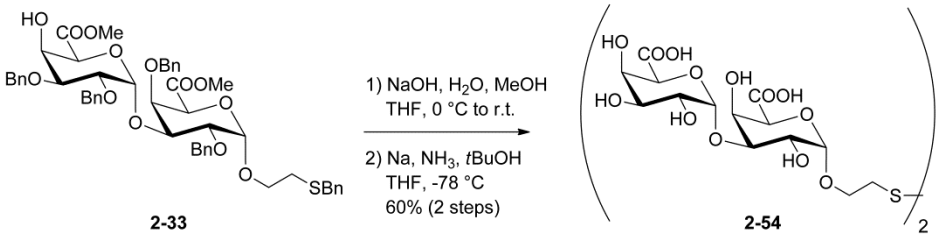


To a stirred solution of ester **2-58** (20.7 mg, 0.035 mmol) in anhydrous CH_2Cl_2 (3.0 mL) were added at room temperature first a mixture of pyridine (86 µL, 1.058 mmol) and acetic acid (57 µL, 0.988 mmol), and then hydrazine hydrate (3.4 µL, 0.071 mmol). The mixture was stirred for 5 h at that temperature, diluted with EtOAc (2 mL), quenched with acetone (0.1 mL) and poured into water (10 mL). The aqueous phase was extracted with EtOAc (4x5 mL), the combined organic fractions were dried over Na_2SO_4 and concentrated. The residue was purified by flash chromatography (acetone/hexanes 1:1) to give the intermediate alcohol as a white solid.

To a stirred solution of liquid ammonia (6 mL) was added at -78 °C a solution of the intermediate alcohol in THF (1.5 mL). The mixture was treated with *t*BuOH (0.5 mL) and lumps of freshly cut sodium (45 mg) were added until a deeply blue color persisted. The reaction was stirred at -78 °C for 45 min and quenched by addition of solid ammonium acetate (100 mg). The solution was warmed to room temperature under a stream of argon and co-evaporated with MeOH (2x10 mL) and water (2x5 mL). The residue was left under air for 16 h, purified by size exclusion chromatography (1:10 MeOH/5 mM aq. NH_4OAc, Sephadex® G-25, GE Healthcare) and lyophilized repeatedly to give disulfide **2-52** as the corresponding diacetate salt (7.91 mg, 12.3 µmol, 70% over two steps) as a white solid. $[\alpha]_D^{20}$ = +130.9° (c = 0.11, H_2O); ^{1}H NMR (400 MHz, D_2O) δ

5.09 (d, J = 3.9 Hz, 1H, H-1), 4.56 (q, J = 6.6 Hz, 1H, H-5), 4.36 (dd, J = 11.2, 4.4 Hz, 1H, H-3), 4.13 (m, 2H, H-2, A of AB, O-CH_2-CH_2), 3.95 (m, 1H, B of AB, O-CH_2-CH_2), 3.75 (d, J = 4.1 Hz, 1H, H-4), 3.11 (t, J = 5.7 Hz, 2H, CH_2-CH_2-S), 2.19 (s, 3H, Ac-CH_3), 1.45 (d, J = 6.7 Hz, 3H, H-6); HRMS (ESI) calcd. for $C_{20}H_{38}N_4O_8S_2$ $(M+Na)^+$ 549.2029 found 549.2086 *m/z*.

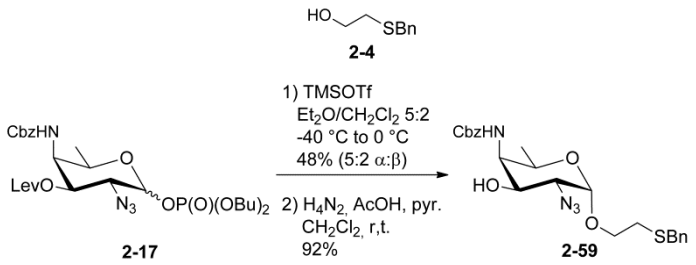
2,2'-Dithiobis[α-D-galactopyranosyluronate-(1→3)-α-D-galactopyranosyluronate-(1→1)-1-ethanol] (2-54)



To a stirred solution of ester **2-33** (8.6 mg, 9.5 µmol) in THF (0.6 mL) and MeOH (0.3 mL) was added at 0 °C a 1 M solution of NaOH in water (0.5 mL). The reaction was slowly warmed to room temperature and stirred for 16 h at that temperature. The reaction was diluted with EtOAc (5 mL) and water (5 mL) and acidified to pH 4 with 0.5 M aq. $NaHSO_4$. After separation, the aqueous fraction was extracted with EtOAc (8x5 mL), the combined organic fractions were dried over Na_2SO_4 and concentrated to give the intermediate diacid as a white solid.

To a stirred solution of liquid ammonia (6 mL) was added at -78 °C a solution of the crude diacid in THF (1.5 mL). The mixture was treated with *t*BuOH (0.4 mL) and lumps of freshly cut sodium (75 mg) were added until a deeply blue color persisted. The reaction was stirred at -78 °C for 45 min and quenched by addition of solid ammonium acetate (100 mg). The solution was warmed to room temperature under a stream of argon and co-evaporated with MeOH (2x10 mL) and water (2x5 mL). The residue was left under air for 16 h, purified by size exclusion chromatography (1:9 MeOH/5 mM aq. NH_4OAc, Sephadex® G-25, GE Healthcare) and lyophilized repeatedly to give disulfide **2-54** (2.5 mg, 2.9 µmol, 61% over two steps) as a white solid. $[\alpha]_D^{20}$ = +21.4° (c = 0.10, H_2O); ^1H NMR (600 MHz, D_2O) δ 5.32 (s, 1H), 5.13 (s, 1H), 4.71 (s, 1H), 4.61 (s, 1H), 4.55 (s, 1H), 4.41 (s, 1H), 4.21 – 3.88 (m, 6H), 3.13 – 3.00 (m, 2H); HRMS (MALDI) calcd. for $C_{28}H_{42}O_{26}S_2$ ($M+2Na^+-3H^+$) 901.0966 found 901.0981 *m/z*.

2-Azido-4-(benzyloxycarbonyl)amino-2,4,6-trideoxy-α-D-galactopyranosyl-(1→1)-6-(benzylthio)ethanol (2-59)

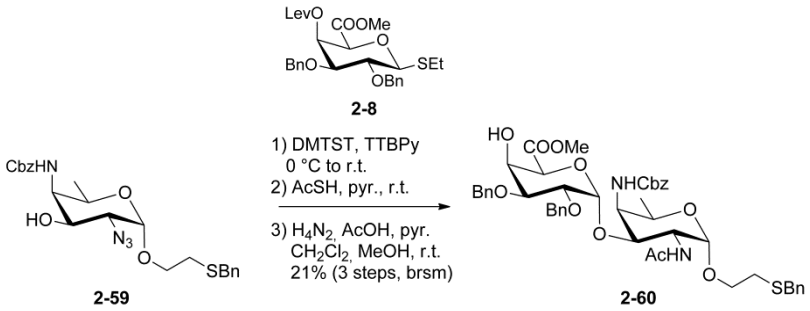


Alcohol **2-4** (71 mg, 0.421 mmol) and glycosyl phosphate **2-17** (171 mg, 0.281 mmol) were co-evaproated with anhydrous toluene (3x10 ml) and kept under high vacuum for 30 min. The mixture was dissolved in CH_2Cl_2 (1.8 ml) and stirred over activated molecular sieves (4 Å-AW) for 1 h at room temperature. The solution was then cooled to -40 °C and treated with TMSOTf (56 µl, 0.309 mmol in 0.2 ml anhydrous CH_2Cl_2). The mixture was slowly warmed to 0 °C (2 h), quenched with a 1:1 (v/v) mixture of MeOH and triethylamine (0.5 ml), diluted with CH_2Cl_2 (20 ml), filtered through Celite and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 1:3 to 1:1) to give the corresponding α-glycoside (55 mg, 0.096 mmol, 34%) along with the corresponding β-glycoside (22 mg, 0.039 mmol, 14%).

To a stirred solution of the intermediate Lev ester (17 mg, 0.03 mmol) in anhydrous CH_2Cl_2 (1 mL) were added at room temperature first a mixture of pyridine (72 µl, 0.894 mmol) and acetic acid (48 µl, 0.834 mmol), and then hydrazine hydrate (3 µl, 0.062 mmol). The mixture was stirred for 5 h at that temperature, diluted with EtOAc (2 ml), quenched with acetone (0.1 mL) and poured into water (10 mL). The aqueous phase was extracted with CH_2Cl_2 (4x5 ml), the combined organic fractions were dried over Na_2SO_4 and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 3:1) to give alcohol **2-58** (13 mg, 0.028 mmol, 92%) as a clear oil. $[\alpha]_D^{20}$ = +105.3° (c = 0.57, acetone); ^{1}H NMR (400 MHz, acetone-D_6) δ 7.41 – 7.16 (m, 10H), 6.24 (d, J = 10.0 Hz, 1H), 5.12 – 4.96 (m, 2H), 4.82 (d, J = 3.7 Hz, 1H), 4.46 (d, J = 6.0 Hz, 1H), 4.33 – 3.97 (m, 4H), 3.80 – 3.42 (m, 4H), 2.65 (t, J = 6.6 Hz, 2H), 1.08 (d, J = 6.4 Hz, 3H); ^{13}C NMR (100 MHz, acetone-D_6) δ 129.9, 129.23, 129.17, 128.58, 128.55, 127.7, 99.2, 68.7, 68.1, 68.0, 66.7, 66.3, 61.2, 57.1, 36.9, 31.3, 16.9; IR (thin film) 3416,

2924, 2109, 1701, 1522, 1454, 1347, 1240, 1106, 1074, 1032, 828, 741, 699 cm^{-1}; HRMS (ESI) calcd. for $C_{23}H_{28}N_4O_5S$ $(M+Na)^+$ 495.1678 found 495.1679 *m/z*.

Methyl (2,3-di-*O*-benzyl-α-D-galactopyranosyl)uronate-(1→3)-2-azido-4-(benzyloxycarbonyl)amino-2,4,6-trideoxy-α-D-galactopyranosyl-(1→1)-2-(benzylthio)ethanol (2-60)



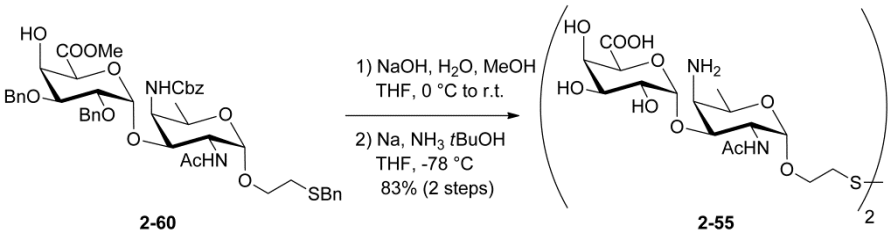
Alcohol **2-59** (13 mg, 0.028 mmol), TTBPy (45, 0.138 mmol) and thioglycoside **2-8** (37 mg, 0.069 mmol) were co-evaporated with anhydrous toluene (3x10 mL) and kept under high vacuum for 1 h. The mixture was dissolved in anhydrous THF (1.5 mL) and stirred over activated molecular sieves (3 Å) for 30 min at room temperature. The solution was cooled to 0 °C and treated dropwise with DMTST (17 mg, 0.069 mmol). The mixture was warmed to room temperature and treated with an additional 2 equiv. of DMTST after 2 h. The reaction was stirred for 16 h and quenched with 1:1 (v/v) mixture of 10% aq. $Na_2S_2O_3$ and sat. aq. $NaHCO_3$ (5 mL). The mixture was extracted with CH_2Cl_2 (3x10 mL), dried over Na_2SO_4 and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 1:2) to give the intermediate disaccharide as a clear oil.

To a stirred solution of the intermediate disaccharide in anhydrous pyridine (0.2 ml) was added at 0 °C thioacetic acid (0.2 ml). The mixture was warmed to room temperature and stirred for 24 h at that temperature. The solution was co-evaporated with toluene (2x5 ml) and the residue was purified by flash chromatography (EtOAc/hexanes 1:3 to acetone/hexanes 1:2) to give the intermediate acetamide as a white foam.

To a stirred solution of the intermediate acetamide in anhydrous CH_2Cl_2 (0.6 mL) and MeOH (60 µL) were added at room temperature first a mixture of pyridine (12 µl, 0.16 mmol) and acetic acid (8 µl, 0.15 mmol), and then hydrazine hydrate (1 µl, 0.021

mmol). The mixture was stirred for 3 h at that temperature, diluted with CH_2Cl_2 (2 ml), quenched with acetone (0.1 mL) and poured into water (5 mL). The aqueous phase was extracted with CH_2Cl_2 (4x5 ml), the combined organic fractions were dried over Na_2SO_4 and concentrated. The residue was purified by flash chromatography (acetone/hexanes 0:1 to 1:1) to give acetamide **2-60** (2.7 mg, 3.14 µmol, 21% over 3 steps based on recovered **2-69**) as a white foam. R_f (acetone/hexanes 1:1) = 0.46; $[\alpha]_D^{20}$ = +90.9° (c = 0.14, acetone); ^{1}H NMR (400 MHz, acetone-D_6) δ 7.43 – 7.17 (m, 20H), 6.98 (d, J = 9.3 Hz, 1H), 6.71 (d, J = 10.1 Hz, 1H), 5.28 (d, J = 2.8 Hz, 1H), 5.07 (d, J = 12.8 Hz, 1H), 4.77 (t, J = 13.5 Hz, 2H), 4.71 – 4.64 (m, 2H), 4.62 – 4.50 (m, 2H), 4.45 – 4.29 (m, 3H), 4.13 – 4.07 (m, 2H), 4.04 – 3.89 (m, 2H), 3.88 – 3.80 (m, 1H), 3.78 (s, 2H), 3.75 – 3.68 (m, 2H), 3.64 (s, 3H), 3.59 – 3.35 (m, 2H), 2.61 (dd, J = 12.1, 5.3 Hz, 3H), 1.11 (d, J = 7.0 Hz, 3H); IR (thin film) 3459, 2931, 1723, 1652, 1424, 1347, 1246, 1122, 1036, 823, 742, 699 cm^{-1}; HRMS (ESI) calcd. for $C_{46}H_{54}N_2O_{12}S$ $(M+Na)^+$ 881.3295 found 881.3286 *m/z*.

2,2'-Dithiobis[α-D-galactopyranosyluronate-(1→3)-2-acetamido-4-amino-2,4,6-trideoxy-α-D-galactopyranosyl-(1→1)-1-ethanol] (2-55)

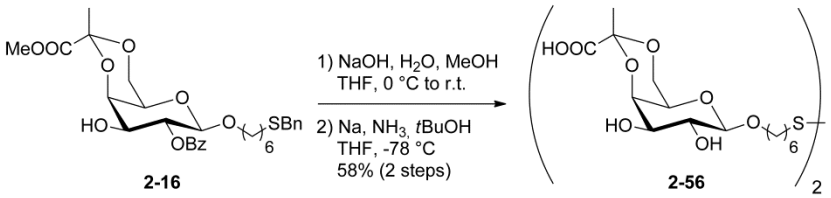


To a stirred solution of ester **2-60** (2.7 mg, 3.14 µmol) in THF (1 mL) and MeOH (0.25 mL) was added at 0 °C a 1 M solution of NaOH in water (0.4 mL). The reaction was slowly warmed to room temperature and stirred for 16 h. The reaction was diluted with EtOAc (5 ml) and water (5 ml) and acidified to pH 4 with 0.5 M aq. $NaHSO_4$. After separation, the aqueous fraction was extracted with EtOAc (8x5 ml), the combined organic fractions were dried over Na_2SO_4 and concentrated to give the intermediate diacid as a white solid.

To a stirred solution of liquid ammonia (10 ml) was added at -78 °C a solution of the crude diacid in THF (1.5 ml). The mixture was treated with *t*BuOH (0.4 ml) and lumps of freshly cut sodium (80 mg) were added until a deeply blue color persisted. The reaction was stirred at -78 °C for 45 min and quenched by addition of solid ammonium

acetate (100 mg). The solution was warmed to room temperature under a stream of argon and co-evaporated with MeOH (2x10 ml) and water (2x5 ml). The residue was left under air for 16 h, purified by size exclusion chromatography (1:3 MeOH/5 mM aq. NH_4OAc Sephadex® G-25, GE Healthcare) and lyophilized repeatedly to give disulfide **2-55** (1.15 mg, 2.61 µmol, 83% over two steps) as a white solid. ^{1}H NMR (600 MHz, D_2O) δ 5.06 (d, J = 2.2 Hz, 1H), 4.88 (d, J = 3.8 Hz, 1H), 4.30 – 4.23 (m, 3H), 4.15 (s, 1H), 4.04 (dd, J = 11.0, 3.5 Hz, 1H), 4.01 – 3.94 (m, 1H), 3.85 (s, 2H), 3.83 – 3.74 (m, 2H), 3.32 (s, 1H), 3.30 (d, J = 26.8 Hz, 1H), 2.98 (t, J = 5.7 Hz, 2H), 2.02 (s, 3H), 1.28 (d, J = 6.6 Hz, 3H); HRMS (ESI) calcd. for $C_{32}H_{54}N_4O_{20}S_2$ $(M+Na)^+$ 901.2670 found 901.2681 *m/z*.

6,6'-Dithiobis[4,6-*O*-[1-(*R*)-(carboxy)-ethylidene]-β-D-galactopyranoside-(1→1)-1-hexanol] (2-56)

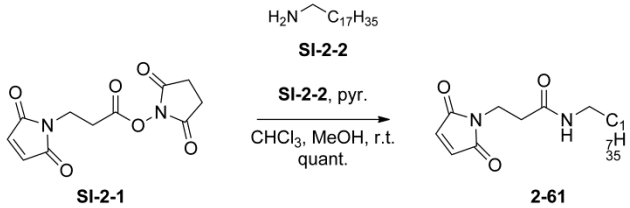


To a stirred solution of ester **2-16** (30 mg, 0.052 mmol) in THF (0.6 mL) and MeOH (0.3 mL) was added at 0 °C a 1 M solution of NaOH in water (0.6 mL). The reaction was slowly warmed to room temperature and stirred for 16 h at that temperature. The reaction was diluted with MeOH (2 mL), neutralized with Amberlite 120 (H^+), filtered and concentrated to give the intermediate acid as a white foam.

To a stirred solution of liquid ammonia (8 mL) was added at -78 °C a solution of the crude acid in THF (2 mL). The mixture was treated with *t*BuOH (0.6 mL) and lumps of freshly cut sodium (80 mg) were added until a deeply blue color persisted. The reaction was stirred at -78 °C for 45 min and quenched by addition MeOH (2 mL) and solid NH_4OAc (100 mg). The solution was warmed to room temperature under a stream of argon and co-evaporated with MeOH (2x5 mL) and water (2x5 mL). The residue was left exposed to air for 16 h, purified by size exclusion chromatography (4:1 MeOH/5 mM aq. NH_4OAc, Sephadex® G-25, GE Healthcare) and solid phase extraction (Chromafix® C18 cartridge, Macherey-Nagel, Düren, Germany) and lyophilized repeatedly to give disulfide **2-56** (11 mg, 0.030 mmol, 58% over two steps) as a white solid, containing

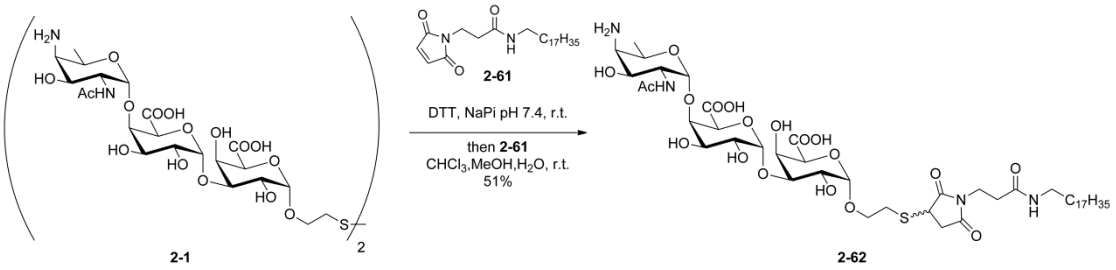
approx. 10% of the corresponding thiol. $[\alpha]_D^{20}$ = -22.3° (c = 0.44, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 4.53 (d, *J* = 7.8 Hz, 1H, H-1), 4.26 (d, *J* = 3.7 Hz, 1H, H-4), 4.13 (d, *J* = 12.0 Hz, 1H, A of AB, H-6), 4.08 – 3.96 (m, 2H, B of AB, H-6, A of AB, O-C*H*$_2$-CH_2), 3.83 – 3.63 (m, 4H, H-2, H-3, H-5, B of AB, O-C*H*$_2$-CH_2), 2.88 (t, *J* = 7.2 Hz, 1.65H, CH_2-C*H*$_2$-S-S), 2.66 (t, *J* = 7.3 Hz, 0.35H, CH_2-C*H*$_2$-SH), 1.92 – 1.69 (m, 4H, aliph.), 1.60 – 1.46 (m, 7H, pyruv.-CH_3, aliph.); HRMS (MALDI) calcd. for $C_{30}H_{50}O_{16}S_2$ $(M-H)^-$ 729.2462 found 729.2480 *m/z*.

3-Maleimido-(*N*-octadecyl)propionamide (2-61)



To a stirred solution of 1-octadecylamine **SI-2-2** (10 mg, 37 µmol) in $CHCl_3$ (600 µL) and MeOH (200 µL) were added at room temperature 3-(maleimido)propionic acid *N*-hydroxysuccinimide **SI-2-1** (12.8 mg, 48 µmol) and pyridine (10 µL). The reaction was stirred for 1 h at that temperature. The solvents were evaporated, the residue was suspended in ice-cold MeOH (5 mL) and filtered. The solid residue was washed repeatedly with cold MeOH and dried to give maleimide **2-61** (15.6 mg, 37 µmol, quant.) as a white solid. R_f (EtOAc/hexanes 3:1) = 0.88; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.70 (s, 2H), 5.57 (s, 1H), 3.83 (t, *J* = 7.2 Hz, 2H), 3.20 (dd, *J* = 13.3, 6.9 Hz, 2H), 2.51 (t, *J* = 7.2 Hz, 2H), 1.51 – 1.37 (m, 2H), 1.25 (m, 32H), 0.87 (t, *J* = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.7, 169.4, 134.3, 39.8, 34.9, 34.4, 32.1, 29.9, 29.82, 29.80, 29.74, 29.69, 29.7, 29.5, 29.4, 27.1, 22.8, 14.3; HRMS (ESI) calcd. for $C_{25}H_{44}N_2O_3$ $(M+Na)^+$ 443.3249 found 443.3256 *m/z*.

1-[2-(*N*-Octadecyl)ethanecarboxamidyl]-3-[2-acetamido-4-amino-2,4,6-trideoxy-α-D-galactopyranosyl-(1→4)-α-D-galactopyranosyluronate-(1→3)-α-D-galactopyranosyluronate-(1→2)-2-hydroxyethylthio]-2,5-dipyrrolidone (2-62)



To a stirred solution of disulfide **2-1** (1.68 mg, 1.36 µmol) in water (54 µL) were added at room temperature 0.1 M sodium phosphate buffer (NaPi) pH 7.4 (20 µL) and 1,4-dithiothreitol (DTT, 209 µg, 1.36 µmol) in water (14 µL). The mixture was stirred under argon for 1 h at that temperature and added to a solution of maleimide **2-61** (1.49 mg, 3.54 µmol) in a 2:3 (v/v) mixture of $CHCl_3$ and MeOH (300 µL). Water (approx. 50 µL) and a 1:1 (v/v) mixture of $CHCl_3$ and MeOH (approx. 150 µL) were added until all components were dissolved, and the reaction was stirred at room temperature for 96 h under argon. The reaction had proceeded to completion when a white precipitate appeared, as indicated by mass spectrometry. The solvents were evaporated under a stream of nitrogen and the aqueous residue was directly loaded on a pre-conditioned solid-phase extraction cartridge (Chromabond C18ec 730015G, Macherey-Nagel). Thioether **2-62** was eluted using 30-40 % (v/v) MeCN in water, and concentrated. The residue was further purified by size exclusion chromatography (MeCN/water 1:1, Sephadex® LH-20, GE Healthcare) and concentrated. The residue was purified again by solid phase extraction (*see* above) to give thioether **2-62** (1.44 mg, 1.39 µmol, 51% with respect to free thiol from **2-1**) as a white solid. ^{1}H NMR [600 MHz, $D_2O/CD_3CN/CD_3OD$ 10:10:1 (v/v)] δ 5.43 (s, 1H), 5.28 – 5.17 (m, 1H), 5.16 – 5.06 (m, 1H), 4.71 – 4.69 (m, 1H), 4.59 – 4.47 (m, 2H), 4.38 – 3.88 (m, 10H), 3.89 – 3.59 (m, 5H), 3.46 – 3.27 (m, 3H), 3.23 – 2.80 (m, 2H), 2.75 – 2.58 (m, 2H), 2.32 (s, 3H), 1.78 – 1.64 (m, 2H), 1.58 – 1.52 (m, 30H), 1.44 (d, J = 6.6 Hz, 3H), 1.15 (t, J = 6.8 Hz, 3H); HRMS (MALDI) calcd. for $C_{47}H_{80}N_4O_{19}S$ $(M+Na)^+$ 1059.5035 found 1059.5071 m/z.

2.5.2 Methods of Biochemistry

Figures were prepared using Illustrator CS5 (Adobe Systems, San Jose, USA).

Antisera, Polysaccharides and Carrier Protein

Rabbit ST1 typing serum (Type 1 Neufeld antiserum, cat. no. 16744) and ST1 capsular polysaccharide (cat. no. 76851) was purchased from SSI Diagnostica (Hillerød, Denmark). Rabbit anti-*B. fragilis* serum and purified PS A1 polysaccharide were gifts from Prof. Dennis Kasper, Harvard Medical School, Boston, USA. CRM197 was purchased from Pfenex (San Diego, USA).

Preparation of Glycan Microarray Slides

Maleimide-functionalized glycan array slides were prepared as reported previously.[10] Disulfide-containing glycans [1 mM or 0.5 mM in phosphate-buffered saline (PBS, 10 mM Na_2HPO_4, 1.8 mM K_2HPO_4, 137 mM NaCl, 2.7 mM KCl)] were reduced using 1.0 equivalent of tris(2-carboxyethyl)phosphine (TCEP) and spotted onto the functionalized microarray slides using an automatic piezoelectric arraying robot (Scienion, Berlin, Germany) at 0.2 nL per spot. PS A1 polysaccharide was spotted onto the same slides at a concentration of 0.05 mg/mL in PBS. Slides were then incubated in a humid chamber for 24 h at room temperature and quenched in a 0.2% (v/v) solution of 2-mercaptoethanol in PBS for 1 h at room temperature. The slides were washed with water (3x) and MeOH (3x), dried and stored under argon until use.

Glycan Microarray Binding Experiments

Glycan-functionalized slides were blocked using blocking solution [1% (w/v) BSA in PBS] for 1 h at room temperature. Slides were washed with water (3x) and MeOH (3x) and dried. A 64-well gasket (FlexWell 64, Grace Bio-Labs, Bend, US) was appended and antisera were applied in the depicted dilutions. The slides were incubated for 16 h at 4 °C, washed with washing buffer [0.1% (v/v) Tween 20 in PBS, 3x] and incubated with secondary antibody [goat anti-rabbit-FITC conjugate (abcam, Cambridge, UK), 1:200 in blocking solution] for 2 h at room temperature. The slides were washed with washing buffer (3x) and water (3x) and dried by centrifugation in a 50 mL tube. Fluorescence readout was performed using an Axon GenePix 4300A microarray scanner and GenePix

Pro 7 software (both MDS, Sunnyvale, US). Negative fluorescence intensities were arbitrarily set to 0. All statistical analyses were perfomed using Prism 6 (Graphpad Software Inc., La Jolla, USA). Brightness and contrast of images were adjusted equally using Photoshop CS5 (Adobe Systems).

Conjugation of D-AAT (2-52) to CRM197

To a stirred solution of CRM197 (2 mg, 34.5 nmol) in 0.1 M sodium phosphate buffer (NaPi) pH 7.4 (2 mL) was added at room temperature a solution of *N*-Succinimidyl-3-(bromoacetamido)propionate (SBAP) (530 µg, 1.7 µmol) in DMF (40 µL). The mixture was stirred for 1 h at that temperature, and dialyzed using a centrifugal filter (10 kDa molecular weight cut-off, Millipore, Darmstadt, Germany). The protein solution was diluted to 4 mL with sterile water and concentrated again. This process was repeated three times and the solution was diluted to 0.5 mL using sterile water. 20 µL were taken for analysis, and the protein solution was re-buffered to 0.1 M NaPi pH 7.4 (2 mL) using membrane filtration. Disulfide-containing D-AAT **2-52** (336 µg acetate salt, 1.035 µmol resp. to the monomer) in 0.1 M NaPi pH 7.4 (15 µL) was treated at room temperature with tris(2-carboxyethyl)phosphine (TCEP, 25 µL of a 100 mM stock solution), left for 1 h at that temperature under an argon atmosphere and added to the solution of the activated protein. The mixture was stirred at 4 °C for 16 h, and washed with sterile water using membrane filtration (see above). Another analytical sample was taken, and the solution was re-buffered to 0.1 M NaPi pH 7.4 (0.5 mL). The glycoconjugate was then treated at room temperature with L-cysteine (420 µg, 3.47 µmol) in 100 µL sterile water. The mixture was left for 1 h at that temperature and purified by membrane filtration. Incorporation of glycan into the glycoconjugate was assessed by MALDI-TOF-MS.

Conjugation of Sp1 Trisaccharide (2-1) to CRM197

To a stirred solution of CRM197 (2 mg, 34.5 nmol) in 0.1 M NaPi pH 7.4 (1.33 mL) was added at room temperature a solution of *N*-succinimidyl-3-(bromoacetamido)propionate (SBAP) (1.05 mg, 3.4 µmol) in DMF (40 µL). The mixture was stirred for 1 h at that temperature, and dialyzed using a centrifugal filter. The protein solution was diluted to 4 mL with sterile water and concentrated again. This process was repeated three times and the solution was diluted to 0.5 mL using sterile water. 20 µL were taken for analysis, and

the protein solution was re-buffered to 0.1 M NaPi pH 8.0 (0.5 mL) using membrane filtration. Disulfide-containing Sp1 trisaccharide **2-1** (1.44 mg, 2.33 µmol resp. to the monomer) in 0.1 M NaPi pH 8.0 (0.2 mL) was treated at room temperature with TCEP (25 µL of a 100 mM stock solution, pH 7.4), left for 1 h at that temperature under an argon atmosphere and added to the solution of the activated protein. The mixture was stirred at room temperature for 16 h, and washed with sterile water using membrane filtration (see above). Another analytical sample was taken, and the solution was re-buffered to 0.1 M NaPi pH 7.4 (0.5 mL). The glycoconjugate was then treated at room temperature with L-cysteine (625 µg, 5.1 µmol) in 100 µl sterile water. The mixture was left for 2 h at that temperature and purified by membrane filtration. Incorporation of glycan into the glycoconjugate was assessed by MALDI-TOF MS.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Discontinuous SDS-PAGE was performed according to Lämmli's protocol,[316] using a MiniProtean system (Bio-Rad, Hercules, USA). An alkaline separating gel (375 mM Tris/HCl pH 8.8, 10 to 12% (w/v) of a 29:1 acrylamide/*N*,*N*'-methylenebisacrylamide mixture) and an acidic stacking gel (100 mM Tris/HCl pH 6.8, 4.5% (w/v) of a 29:1 acrylamide/*N*,*N*'-methylenebisacrylamide mixture), polymerized by the addition of TEMED and 10% (w/v) ammonium peroxodisulfate, were used. Proteins were visualized using Coomassie G-250. Brightness and contrast of images were adjusted using Photoshop CS5 (Adobe Systems).

Liposome Fabrication

Liposomes were generated from lipid mixtures containing a 35:40:20:5 molar ration of distearyl phosphatidyl choline, cholesterol, antigen **2-62** and KRN7000, respectively. Control liposomes were fabricated without KRN7000. Lipids were dissolved in $CHCl_3$/MeOH, mixed in the appropriate ratio and dried under a stream of nitrogen. Lipid mixtures were hydrated in sterile PBS (4 µg of glycan per 100 µL solution) and subjected to repeated freeze (-20 °C)-thaw (60 °C) cycles. Liposomes of defined sizes were then prepared by extrusion through a 400 nm membrane using a Mini-Extruder (both Avanti Polar Lipids, Inc., Alabaster, USA) and stored at 4 °C until use (not longer than two days). Liposome size was assessed by dynamic light scattering using a Zetasizer µV (Malvern, Worcestershire, UK).

Ethics Statement

All animal experiments were approved by local institutional (Charité - Universitätsmedizin Berlin) and governmental authorities (Landesamt für Gesundheit und Soziales Berlin, approval ID G0128/12, A 0305/12 and G104/13). Animal housing and experiments were in strict accordance with the regulations of the Federation of European Laboratory Animal Science Associations (FELASA) and recommendations for the care and use of laboratory animals. All mice were housed under specific pathogen-free conditions.

Immunization Experiments

Mice (6-8 week old female NMRI or C57BL/6J mice, Charles River, Sulzfeld, Germany) were immunized subcutaneously with the respective glycoconjugates corresponding to 1.7 µg (D-AAT **2-52**) or 4 µg (Sp1 trisaccharide **2-1**) synthetic glycan and formulated either as a 1:1 (v/v) emulsion with Complete Freund's Adjuvant (CFA, Sigma-Aldrich), a 1:1 (v/v) suspension with Alum (Alhydrogel, Brenntag, Mülheim, Germany) or without adjuvant at a total volume of 100 µL. Booster doses were given at days 14 and 28 using the same strategy (mice primed with CFA received booster doses with Incomplete Freund's Adjuvant (Sigma-Aldrich). Blood (50 µL) was withdrawn once a week from the tail vein or the facial vein and centrifuged (5000 g, 10 min, room temperature) to retrieve serum.

For liposome immunizations, liposome suspensions or CRM197-Sp1 trisaccharide **2-1** were formulated in sterile PBS (liposomes) or with Alum (*see* above) and doses corresponding to 4 µg synthetic glycan were administered intraperitoneally (i.p.) at days 0, 56 and 94. Serum was collected weekly.

Enzyme-linked Immunosorbent Assay (ELISA)

ELISA was performed using Costar™ high-binding polystyrene 96-well plates (cat. no. 3361, Corning, Corning, USA). Plates were coated using native ST1 capsular polysaccharide at a concentration of 10 µg/mL in PBS for 20 h at 4 °C. Plates were blocked with 10% (v/v) fetal calve serum in PBS for 2 h at 37 °C and washed once with PBS-T. After applying cell culture supernatants or mAb dilutions (30-50 µL), Plates were incubated for 1 h at 37 °C, washed with PBS-T three times and treated with a

horseradish peroxidase (HRP)-labeled secondary antibody [Goat anti-Mouse IgG HRP conjugate (cat. no. 115-035-062**,** dianova, Hamburg, Germany)]. Plates were washed with PBS-T three times and HRP activity was measured with TMB substrate (BD Biosciences, San Jose, USA) according to the manufacturer's instructions.

3 Reverse Engineering of Antibodies Against *Streptococcus pneumoniae* Serotype 8 Capsular Polysaccharide Using Synthetic Oligosaccharides

3.1 Introduction

3.1.1 *Streptococcus pneumoniae* Serotype 8

Initially described in 1928 as a particularly virulent strain that is "related to, but not identical with typical strains of [serotype 3]",[317, 318] *Streptococcus pneumoniae* ST8 was soon categorized as a distinct serotype.[319] To date, ST8 ranks amongst the most common serotypes causing IPD worldwide, and disease incidence seems to be highest amongst adults.[20, 76, 320, 321] Outbreaks of invasive disease caused by ST8 are frequently reported in cohorts with comorbidities, such as human immunodeficiency virus, or low health standards, and isolates often display broad antibiotic resistance.[19, 20, 322-324] In correlation to the high virulence of this serotype, low asymptomatic carriage rates are observed.[19] The severity of ST8-mediated infections underscores the need for highly efficient vaccines. While ST8 is a part of the polysaccharide vaccine Pneumovax23®, this serotype is not included in modern glycoconjugate vaccines, and thus poses a risk to replace serotypes that are eliminated by vaccine administration.[325]

3.1.2 Serotype 8 Capsular Polysaccharide

The significant, albeit not complete, serological cross-reactivity of ST8 pneumococci with anti-ST3 immune sera has been the subject of multiple studies,[317, 319, 326] and was ultimately attributed to the presence of the disaccharide cellobiuronic acid (β-D-Glc*p*A-(1→4)-β-D-Glc*p*) in CPSs of both strains.[327, 328] While this disaccharide constitutes the full repeating unit of ST3 CPS, cellobiuronic acid is embedded in a tetrasaccharide repeating unit in ST8 CPS with the glycan sequence [→4)α-D-Glc*p*-(1→4)-α-D-Gal*p*-(1→4)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→] (Fig. 3.1A).[16, 17, 327] In spite of the immunological overlap of both CPSs, cross-reactivity of antisera is incomplete, and vaccines containing either polysaccharide are unlikely to confer full protection against each other.[326, 329-331]

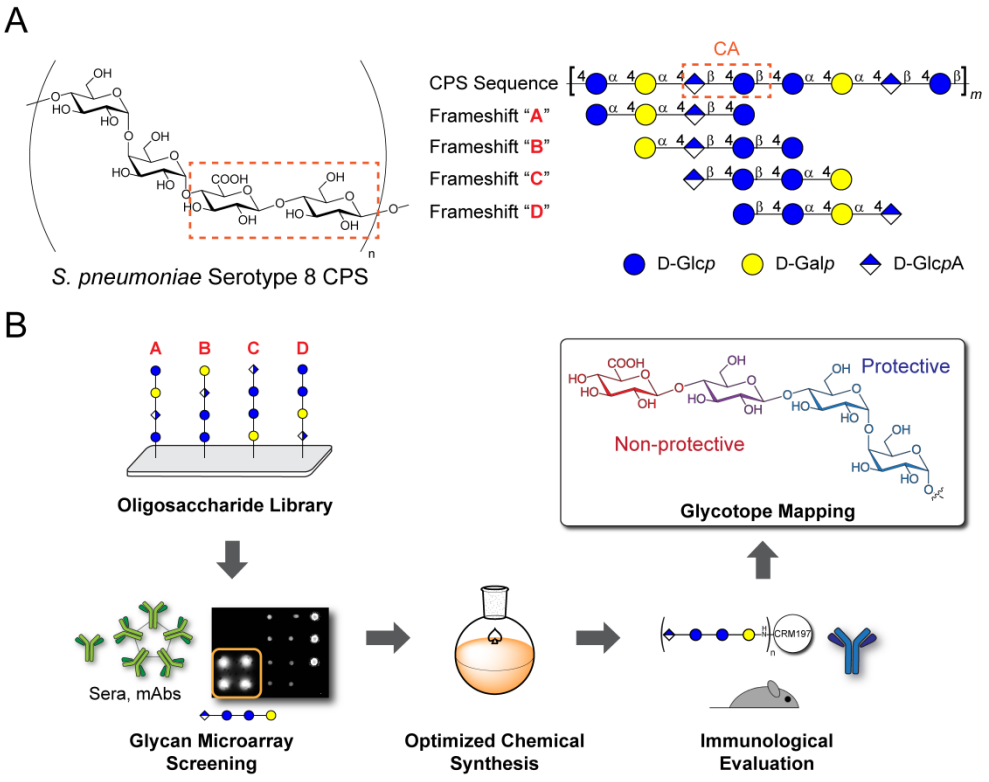


Figure 3.1. Discovery of a protectice glycotope of *S. pneumonie* serotype 8 CPS. *A*, ST8 CPS structure and annotation of frameshifts. The cellobiuronic acid moiety (CA) is highlighted. *B*, discovery of both protective and non-protective glycotopes based on the reverse engineering of available antibody samples.

The biosynthetic mechanism of ST8 CPS differs markedly from ST3 CPS: While the former is generated *in vivo* from tetrasasccharide precursors that are assembled by the action of distinct glycosyltransferases and added to the growing polysaccharide chain,

the latter is produced by a single processive polymerase.[15] Thus, the structural similarity between CPSs of both strains is not caused by an evolutionary relationship, but probably associated with a functional advantage of cellobiuronic acid within CPS structures. The strict organization of ST8 CPS based on repeating units distinguishes four tetrasaccharide frameshifts (Fig. 3.1A).

3.1.3 The Relevance of Monoclonal Antibodies Against Serotype 8

Anticapsular antibodies are crucial mediators of protection against infections with *Streptococcus pneumoniae*. A multitude of mAbs have been raised against ST8 CPS and mechanisms of protection have been studied.[86, 87, 91-93] For instance, immunization of mice with a glycoconjugate containing ST8 CPS and tetanus toxoid led to the generation of mAbs either recognizing the CPS or C-polysaccharide, a contaminant present in abundance in ST8 CPS preparations (*see* below).[92] MAbs directed against C-polysaccharide mainly target the immunogenic component phosphocholine, but do not protect from ST8 infections *in vivo*.[92]

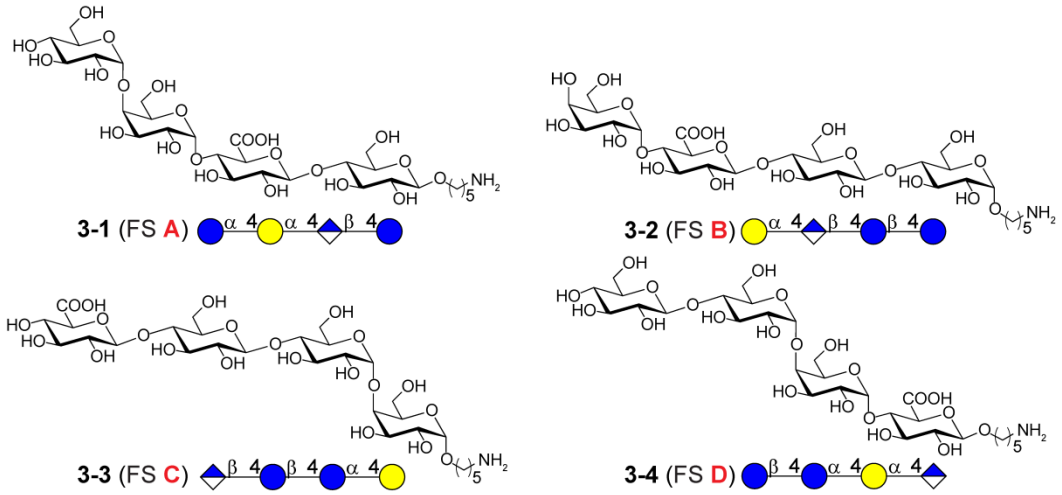
The importance of antibodies to orchestrate host immune responses against *S. pneumoniae* is indisputable.[14] While opsonization of bacteria with subsequent phagocyte-dependent clearance is the most important mechanism to defend the host from pneumococcal infections, it has been found that opsonization may not be essential for protection. Instead, certain protective mAbs directed against either ST8 or ST3 CPSs agglutinate pneumococci, but do not promote opsonophagocytosis *in vitro*.[89, 91, 332] Quorum sensing of bacterial populations is enhanced upon treatment with these mAbs, and the induction of bacterial fratricide has been discussed as a potential strategy of protection.[91] Alternatively, simpler mechanisms, such as the deposition of complement component C3, could be at play.[332] The molecular determinants that govern the different mechanisms of action have not been elucidated yet

3.1.4 Reverse Engineering of Antibodies as a Concept of Vaccine Design

Structural information on protective and non-protective glycotopes is key to the design of novel carbohydrate-based vaccine antigens. Chemically defined, synthetic

oligosaccharides are essential for this process since isolated structures are too heterogeneous to provide information on precise glycotopes. In addition to conventional iterative approaches of antigen synthesis and glycoconjugate evaluation, the reverse engineering of antigen binding sites of protective mAbs has greatly advanced the field of carbohydrate-based vaccines.[1, 109, 111, 112, 144-146, 333-337] A shortfall of these conventional glycotope mapping strategies is the plethora of putative glycotopes within a polysaccharide that are laborious to obtain by traditional chemical synthesis. Since protective glycotopes can vary with respect to length, frameshift and covalent modifications, the number of putative immunogens is enormous. Therefore, methods that allow for educated antigen design by providing insight into structural determinants are key to the rational design of carbohydrate-based vaccines.

Here, glycan microarray screening of a collection of synthetic oligosaccharides was combined with the reverse engineering of a protective mAb by chemical synthesis and immunological evaluation to elucidate a protective glycotope of CPS from *S. pneumoniae* ST8 (Fig. 3.1B). Furthermore, glycan-binding characteristics of a set of opsonic and non-opsonic mAbs against ST8 CPS were mapped to give insight into the molecular principles of *in vitro* ST8 recognition phenotypes.



Scheme 3.1. Synthetic ST8 CPS-derived oligosaccharides used for glycotope mapping of antibody samples.[xii]

[xii] Tetrasaccharides **3-1**, **3-2**, **3-3** and **3-4** were synthesized by Heung Sik Hahm.

3.2 Results

3.2.1 Glycotope Mapping of ST8-recognizing Antibody Samples

The search for protective ST8 CPS glycotopes was prompted by the rather well-established immunological implications of ST8 infections and the availability of protective mAbs against native CPS.[86, 91-93] Due to a lack of synthetic, conjugation-ready glycan probes, no information regarding the contribution of distinct glycotopes to polysaccharide immunogenicity is available.

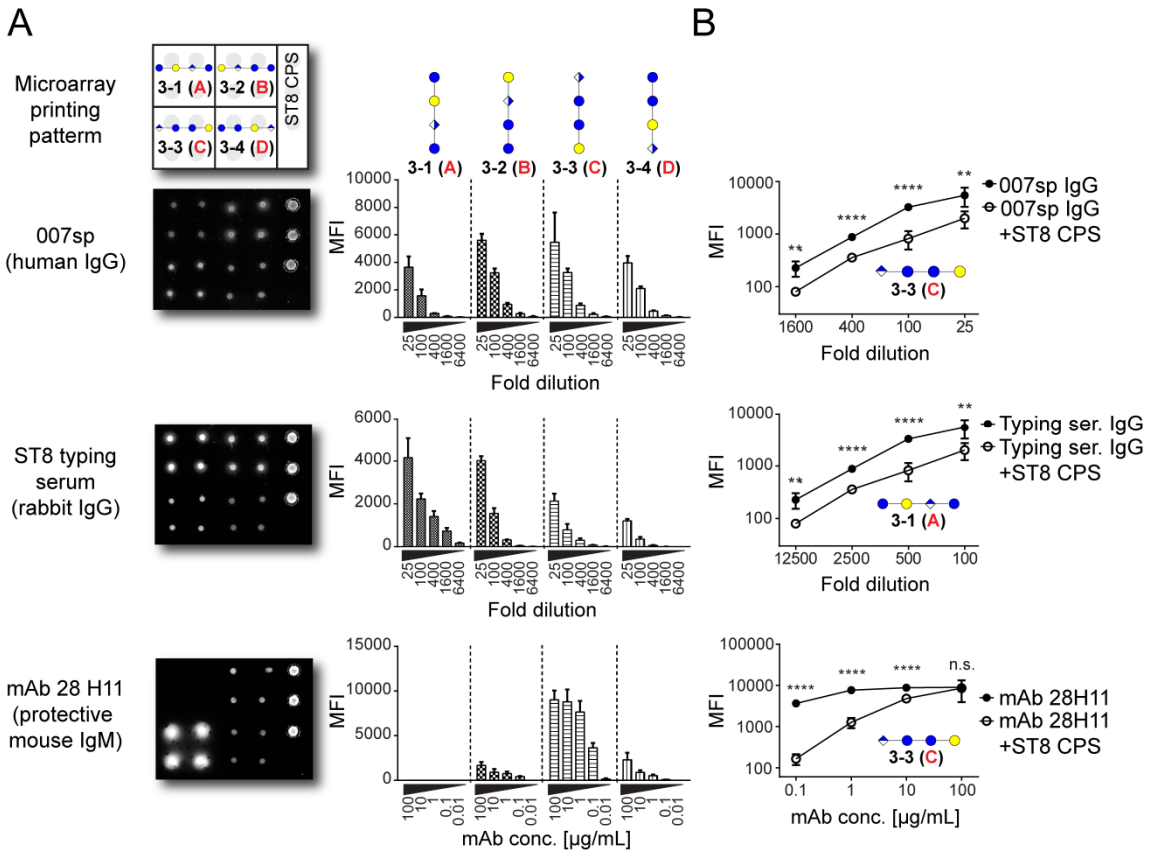


Figure 3.2. Differential immune recognition of synthetic ST8 CPS frameshifts. *A*, glycan microarray analysis of pooled sera from Pneumovax23®-vaccinated humans ("007sp"), rabbit ST8 typing serum and a protective murine mAb 28H11 at different concentrations. Sera were pre-adsorbed with pneumococcal C-polysaccharide before application. Histograms show mean + SD of eight spots. *B*, inhibition of antibody binding by pre-adsorption with native ST8 CPS (10 µg/mL). Statistical analysis (One-tailed, unpaired t test with Welch's correction) of eight spots was performed of one out of at least two independent experiments. Asterisks indicate P values: n.s. not significant; ** $P < 0.005$; *** $P < 0.001$; **** $P < 0.0001$. Bars depict mean ± SD.MFI, mean fluorescence intensity.

In contrast to the long oligosaccharide haptens that are necessary to confer protection against bacteria that display structural CPS glycotopes,[113, 335, 338] haptens as small as tri- or tetrasaccharides have been used to induce protective immunity against several *S. pneumoniae* serotypes.[109, 334] Thus, to gain insight into the nature of glycotopes that confer protective immunity against ST8, all four tetrasaccharide frameshifts prepared by automated glycan assembly[xiii] were used to map the binding specificities of several antibody samples in a glycan microarray experiment. Arrays were fabricated by covalent immobilization of amine-equipped oligosaccharides **3-1**,[xiii] **3-2**,[xiii] **3-3**[xiii] and **3-4**[xiii] (Scheme 3.1) or native ST8 CPS[28] on *N*-hydroxysuccinimide (NHS) ester-functionalized glass slides.[149] Fluorescently labeled secondary antibodies were employed to visualize binding of antibodies to the immobilized structures (Fig. 3.2).

Initially, polyclonal antibody mixtures in commercially available sera raised against native ST8 CPS were screened. All four synthetic glycans were recognized along with the native polysaccharide by a human reference serum mixture (007sp[339]) as well as rabbit-borne ST8 typing serum (Fig. 3.2A and B). Thereby, species-specific preferences for certain FSs were observed: The human serum bound preferentially to FSs B and C (**3-2** and **3-3**, respectively, Fig. 3.2A), but only the latter interaction was inhibited by antibody pre-adsorption with native ST8 CPS (Fig. 3.2B). Non-ST8-dependent recognition of FS B may reflect the presence of naturally occurring antibodies recognizing certain glycotopes such as the terminal α-galactoside of that oligosaccharide in human sera.[340] The rabbit-derived typing serum preferentially bound to FS A and, to a significantly ($P < 0.0001$) lesser extent, FS B (**3-1** and **3-2**, respectively, Fig. 3.2A). Binding was abrogated by ST8 CPS pre-adsorption (Fig. 3.2B). Thus, all synthetic ST8 frameshifts are recognized by antisera against ST8 CPS with species-specific preferences mainly towards FS C (in humans) and FS A (in rabbits).[54]

Binding analyses of complex, polyclonal antisera are unlikely to reveal the identity of protective glycotopes of a CPS due to the presence of non-protective antibodies that target immunogenic substructures (*see* above). We envisaged that a protective mAb would shed light onto the nature of the optimal frameshift representing ST8 CPS. MAb

[xiii] *See* dissertation of Heung Sik Hahm for the synthesis of tetrasaccharides **3-1**, **3-2**, **3-2** and **3-4**.

28H11, a murine IgM raised against native ST8 CPS, is well-characterized and protects mice from infection with live ST8 pneumococci in various *in vivo* challenge models.[xiv92] Glycan microarray analysis revealed a robust interaction of mAb 28H11 with ST8 FS C (**3-3**, Fig. 3.2A), with at least five times higher binding intensities than FSs B and D (**3-2** and **3-4**, respectively). In turn, no interaction was observed with FS A (**3-1**). Recognition of FS C was specific to ST8, as shown by the ablation of binding by native ST8 CPS at mAb concentrations of up to 10 µg/mL (Fig. 3.2B). Thus, FS C displays a protective glycotope of ST8 CPS.

3.2.2 An Optimized Chemical Synthesis of ST8 Frameshift C

To enable a detailed immunological evaluation of ST8 CPS-derived oligosaccharides, a convenient and scalable route to synthesize ST8 FSs A and C was targeted. Particularly, stereoselective installation of the 1,2-*cis*-glycosidic linkage at the reducing end of FS C had been found cumbersome during automated glycan assembly[xv] and was a central goal in the synthetic strategy.

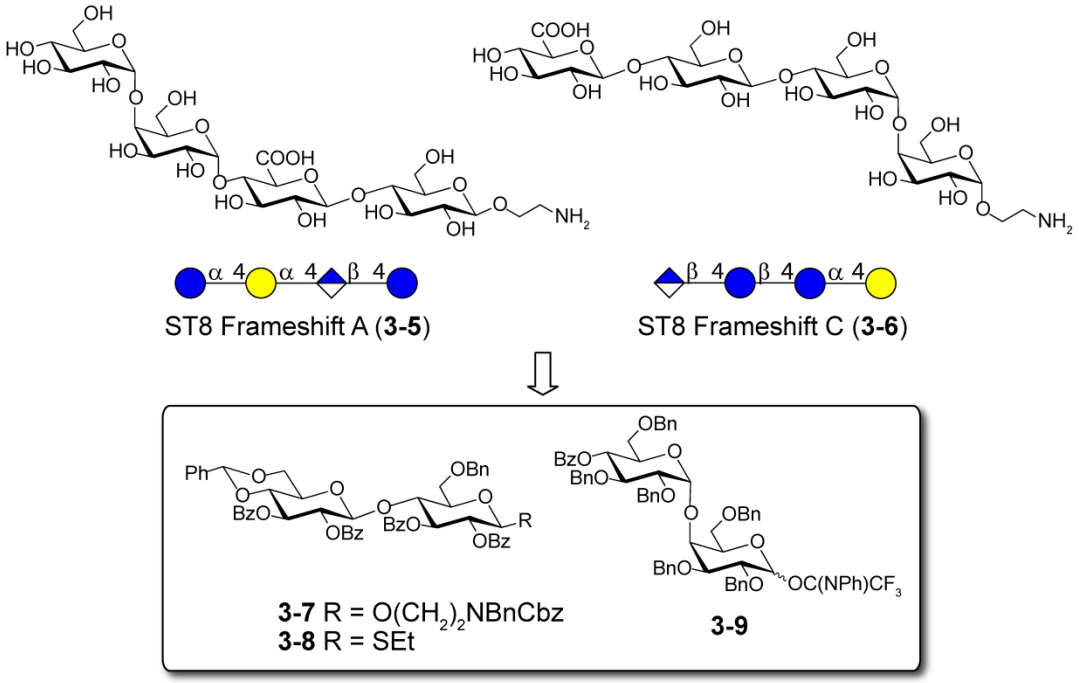
A divergent route was designed to synthesize tetrasaccharides **3-5** and **3-6** based on disaccharide building blocks **3-7**,[xvi] **3-8**[xvi] and **3-9** (Scheme 3.2). In an attempt to produce an alternative disaccharide precursor to **3-9** in a smaller number of synthetic transformations, glycosyl phosphate **3-10** was reacted with alcohol **3-11**, but no formation of the desired disaccharide **3-12** was obtained (Scheme 3.3A). Intermolecular aglycon transfer was observed instead, with thioglycoside **3-13** as the major product.[341] Thus, galactose derivative **3-14**,[342] bearing an anomeric silyl protecting group instead of a reactive leaving group, was employed. The benzylidene moiety in **3-14** was subjected to regioselective ring-opening under acidic conditions to give alcohol **3-15** in 87% yield (Scheme 3.3B). Union of **3-15** with glycosylating agent **3-13β**[343] furnished disaccharide **3-16** in 75% yield and 2.4:1 α:β-selectivity. A two-step procedure of silyl ether cleavage and installation of an anomeric imidate leaving group was appended to give glycosylating

[xiv] MAb 28H11 was kindly provided by Prof. Liise-anne Pirofski, Albert Einstein College of Medicine, New York, USA.

[xv] *See* dissertation of Heung Sik Hahm.

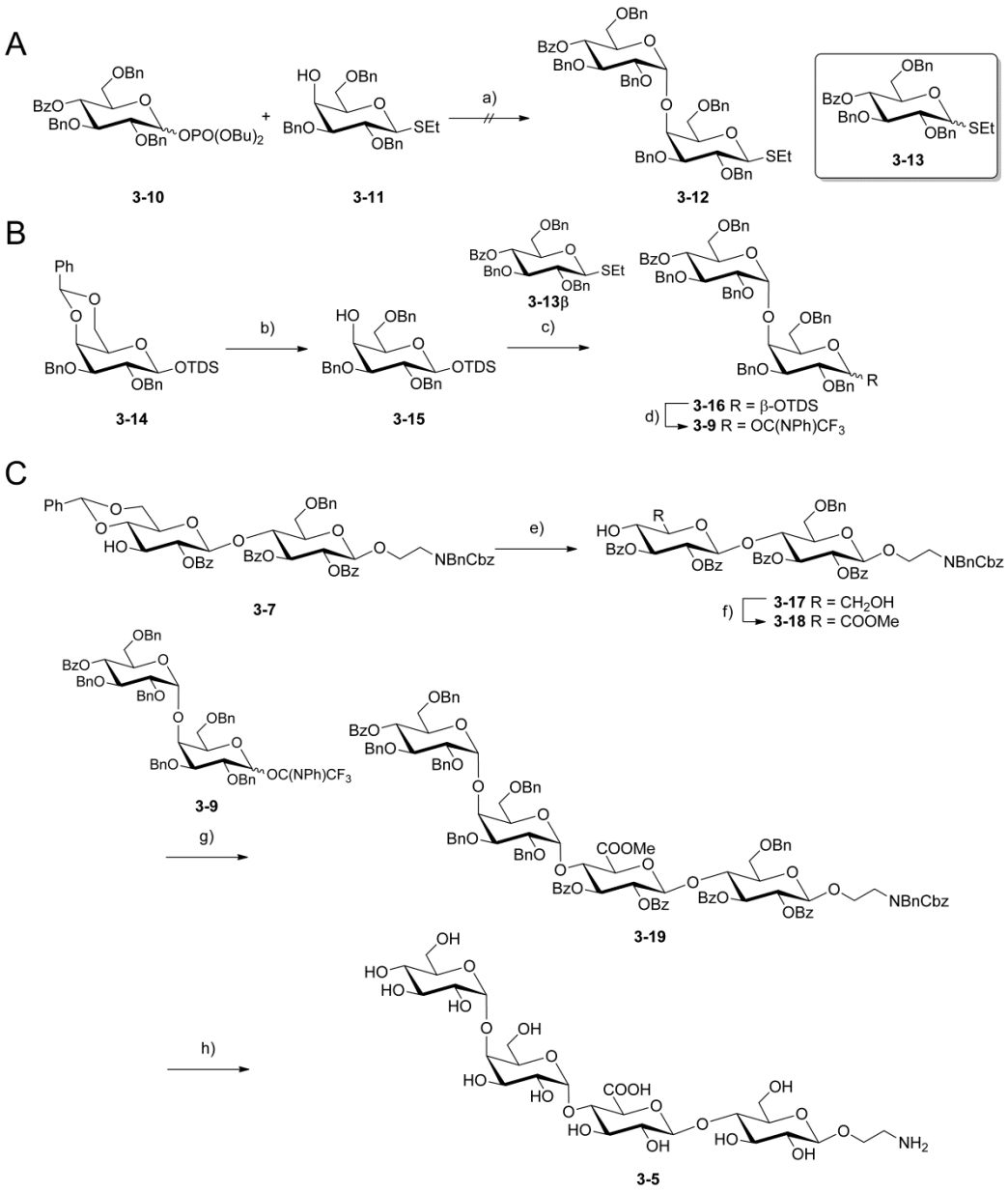
[xvi] Disaccharides **3-7** and **3-8** were synthesized by Dr. Sharavathi G. Parameswarappa and Dr. Subramanian Govindan.

agent **3-9** in 84% yield (from **3-16**) that was key to the divergent synthetic strategy *en route* toward tetrasaccharides **3-5** and **3-6** (Scheme 3.3B).



Scheme 3.2. Retrosynthesis of ST8 CPS frameshifts A (**3-5**) and C (**3-6**) from disacccharides **3-7**, **3-8** and **3-9**.

The reducing-end cellobiuronic acid moiety of FS A was generated by benzoylation of alcohol **3-7** with subsequent removal of the benzylidene acetal in 97% over two steps (Scheme 3.3C). The primary alcohol found in diol **3-17** was then oxidized to the corresponding uronic acid using TEMPO with $PhI(OAc)_2$ as a co-oxidant, and treated with TMS-diazomethane to yield methyl ester **3-18** in 79% over two steps. Assembly of the FS A tetrasaccharide backbone proceeded smoothly under Lewis-acidic conditions with disaccharide precursors **3-9** and **3-18** as glycosylating agent and nucleophile, respectively. Thus, protected tetrasaccharide **3-19** was obtained in 71% yield and with complete 1,2-*cis*-stereoselectivity. Global deprotection commenced by mild hydrolysis of the methyl ester in **3-19** with aqueous lithium peroxide. All benzoate esters were then removed by the sequential use of aqueous sodium hydroxide and methanolic sodium methoxide. The resulting pentaol was finally subjected to hydrogenation conditions with Pd/C as a catalyst in a mixture of methanol, water and acetic acid to isolate tetrasaccharide **3-5** in 69% yield over three steps.



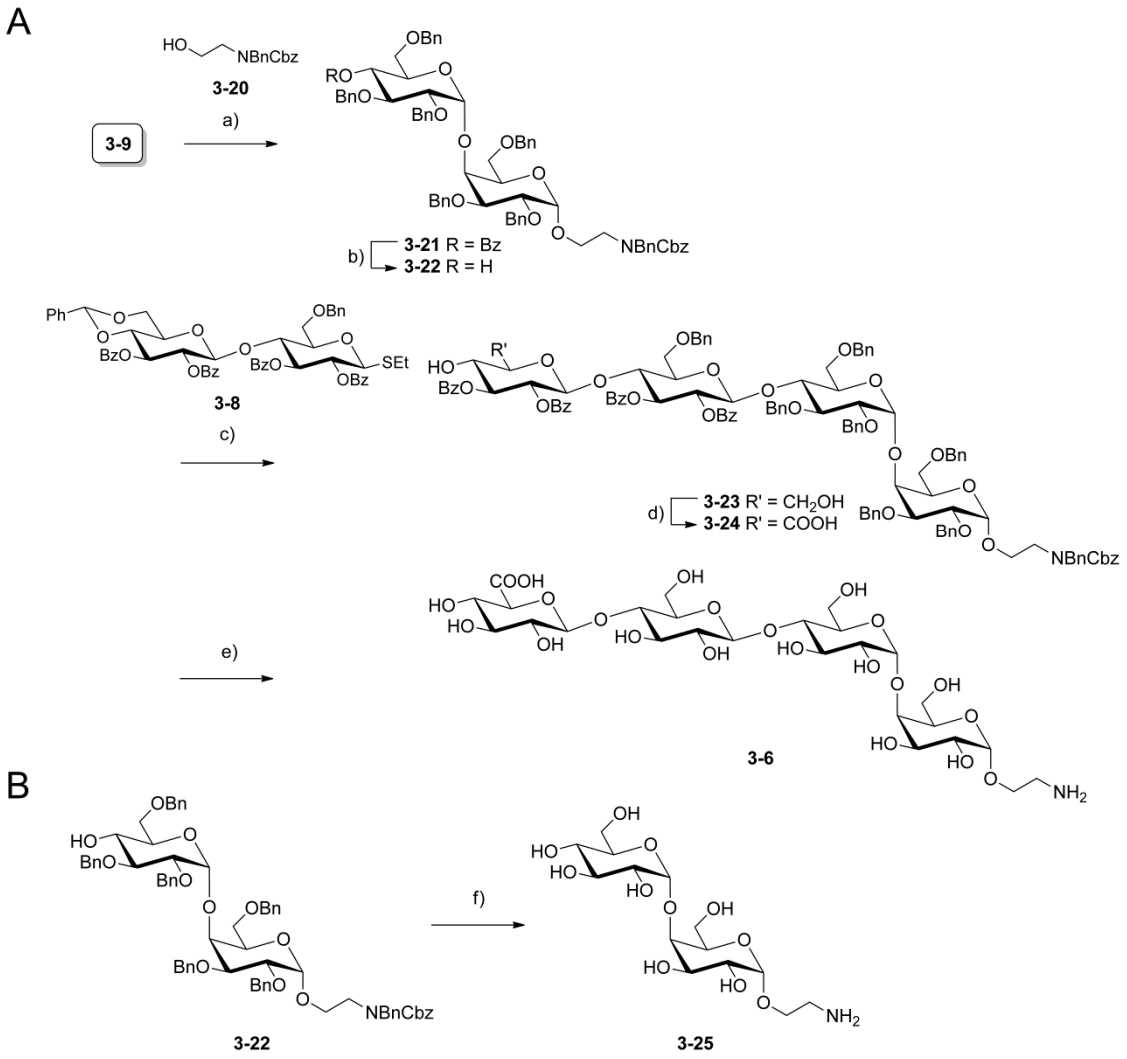
Scheme 3.3. Total synthesis of ST8 tetrasaccharide **3-5**. Reagents and conditions: a) TBSOTf, , Et_2O/CH_2Cl_2 3:1, -10 °C, 0%; b) TES, TFA, CH_2Cl_2, 0 °C to r.t., 87%; c) **3-13β**, NIS, TfOH, Et_2O/CH_2Cl_2 3:1, -20 °C to -10 °C, 75% (2.4:1 α:β); d) i. TBAF, AcOH, THF, 0 °C to r.t.; ii. $F_3CC(NPh)Cl$, Cs_2CO_3, CH_2Cl_2, r.t., 84% (two steps); e) i. BzCl, pyr., 0 °C to r.t., ii. EtSH, *p*TsOH, CH_2Cl_2, r.t., 97% (two steps); f) i. $PhI(OAc)_2$, TEMPO, CH_2Cl_2/H_2O 5:2, 0 °C to r.t., ii. TMS-CHN_2, tol./MeOH 2:1, r.t., 79% (two steps); g) TMSOTf, Et_2O/CH_2Cl_2 3:1, -20 °C to 0 °C, 71% (>19:1 α:β); h) H_2O_2, aq. LiOH, THF, MeOH, H_2O, 0 °C to r.t., then aq. NaOH, 0 °C to r.t., ii. NaOMe, MeOH, r.t.; iii. H_2, Pd/C, MeOH, H_2O, AcOH, r.t., 69% (three steps). NIS = *N*-iodosuccinimide; TBAF = tetra-n-butylammonium fluoride; TBSOTf = *tert*-butyldimethylsilyl trifluoromethanesulfonate; TEMPO = 2,2,6,6-tetramethylpiperidine 1-oxyl; TES = triethylsilane; TFA = trifluoroacetic acid = TMSOTf, trimethylsilyl trifluoromethanesulfonate = *p*TsOH, *p*-toluenesulfonic acid = TfOH, trifluoromethanesulfonic acid.

The lessons learned during the assembly of ST8 FS A (**3-5**) were then applied to the generation of ST8 FS C tetrasaccharide **3-6** (Scheme 3.4). The synthetic route was

initiated by reacting protected linker moiety **3-20** with imidate **3-9** in a glycosylation reaction (Scheme 3.4A). A gratifying 5:1 *cis*:*trans*-stereoselectivity was obtained in this reaction despite the highly nucleophilic nature of alcohol **3-20**, likely because the glucoside residue in **3-9** sterically shields the nucleophilic approach from the β-face of the electrophile. Thus, linker-containing disaccharide **3-21** was isolated in 79% yield. sodium methoxide-mediated removal of the benzoate ester then furnished alcohol **3-22** in 85% yield. Thioglycoside **3-8** served as a suitable glycosylating agent to effect the installation of the FS C tetrasaccharide backbone with **3-22** as a nucleophile and complete 1,2-*trans*-stereoselectivity. The benzylidene moiety was then removed from the terminal glucose moiety to furnish tetrasaccharide **3-23** in 95% yield over two steps. Completion of the synthesis included selective oxidation of the primary alcohol in **3-23** using TEMPO/$PhI(OAc)_2$ to give uronic acid **3-24** (74% yield), saponification of all esters and hydrogenation of benzyl and Cbz groups under acidic conditions to produce tetrasaccharide **3-6** in 70% yield over two steps. Of note, the use of Pearlman's catalyst ($Pd(OH)_2$) in dichloromethane, *tert*-butanol and water gave comparable results in the hydrogenation step (not shown).

To enable initial screening of immune recognition of ST8 CPS substructures, disaccharide **3-22** was subjected to hydrogenation conditions to give deprotected ST8 CPS fragment **3-25** in 87% yield (Scheme 3.4B).

The anomeric configurations of saccharides **3-5**, **3-6** and **3-25** were unambiguously confirmed through the corresponding $^3J_{H,H}$ coupling constants (J = 3.7 - 3.9 Hz for 1,2-*cis*-linkages and 7.9 - 8.0 Hz for 1,2-*trans*-linkages) in ^1H NMR and the spectral comparison with commercial ST8 CPS (*see* below).



Scheme 3.4. Total syntheses of ST8 tetrasaccharide **3-6** and disaccharide **3-25**. Reagents and conditions: a) **3-20**, TMSOTf, Et_2O/CH_2Cl_2 4:1, -40 °C to -10 °C, 79% (5:1 α:β); b) NaOMe, MeOH, THF, 37 °C, 85%; c) i. **3-8**, NIS, TfOH, CH_2Cl_2, -10 °C (>19:1 α:β), ii. EtSH, *p*TsOH, CH_2Cl_2, r.t., 95% (two steps); d) $PhI(OAc)_2$, TEMPO, CH_2Cl_2/H_2O 5:2, r.t., 74%; e) i. NaOH, THF, MeOH, H_2O, 0 °C to r.t.; ii. H_2, Pd/C, MeOH, H_2O, AcOH, r.t., 70% (two steps); f) H_2, Pd/C, EtOAc, MeOH, H_2O, AcOH, r.t., 87%.

3.2.3 Evaluation of the Immune Response Against Synthetic ST8 Frameshifts A and C

To recapitulate the glycan binding patterns of the screened antibody samples (rabbit typing serum and mAb 28H11, *see* above) by immunization, tetrasaccharides **3-5** and **3-6** were conjugated to the immunogenic carrier protein CRM197 using bifunctional spacer di-*N*-succinimidyl adipate (DSAP, Fig. 3.3A). The resulting glycoconjugates were found to contain an average loading of 10.7 and 9.6 chains of oligosaccharides **3-5** and **3-6** on each protein, respectively, by MALDI-TOF MS (Fig. 3.3B) and SDS-PAGE (Fig. 3.3C,

top panel). Western blot analysis using either ST8 typing serum or mAb 28H11 as antibody sources confirmed the incorporation of ST8 oligosaccharide chains into the glycoconjugates (Fig. 3.3C, bottom panel). The typing serum exhibited a preference towards CRM197-FS A over CRM197-FS C, whereas mAb 28H11 displayed the reversed recognition pattern, in line with the binding profiles of the same antibody samples in glycan microarray experiments (*see* Fig. 3.2).

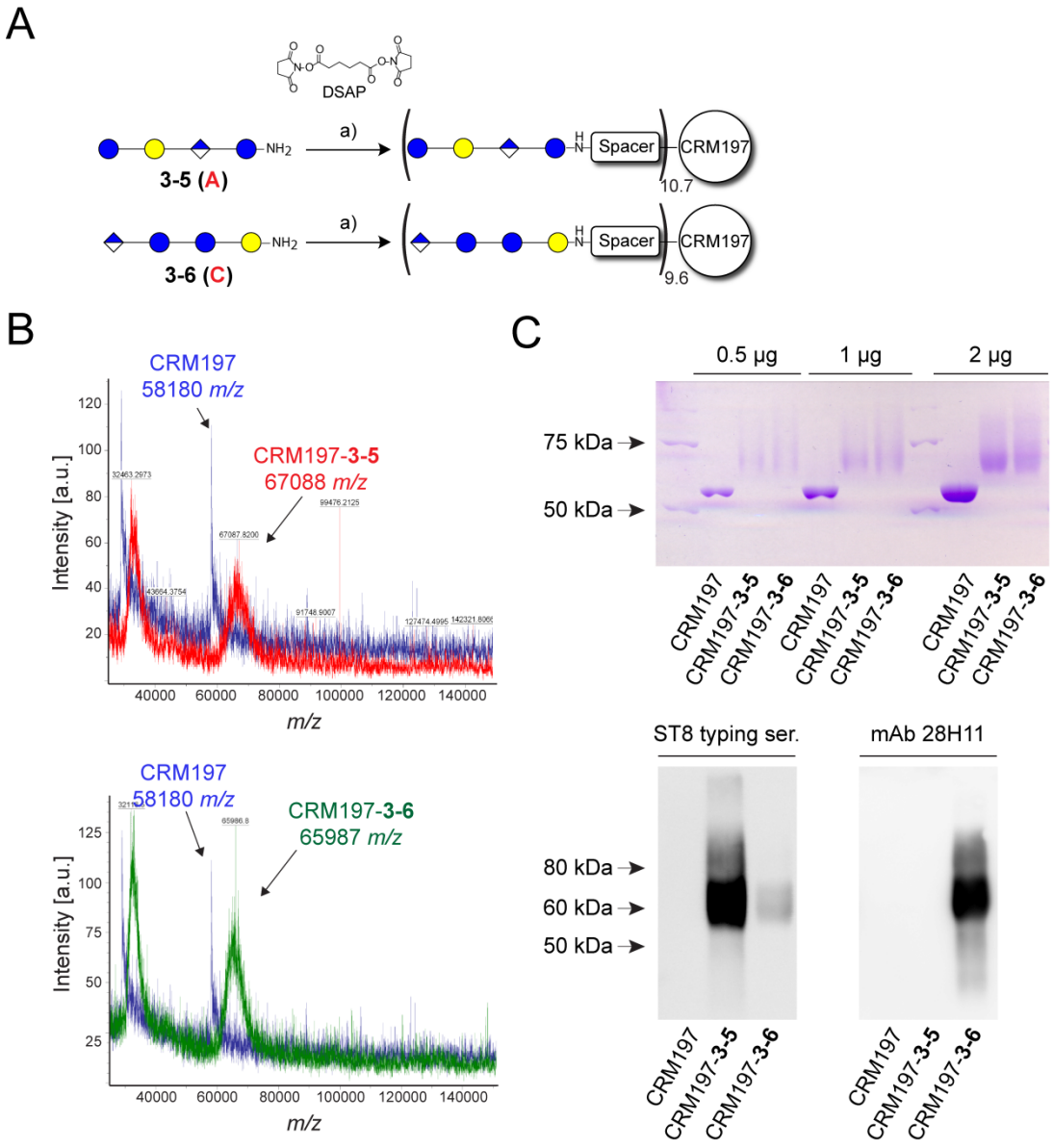


Figure 3.3. Conjugation of ST8 FS A (**3-5**) and FS C (**3-6**) to CRM197. *A*, conjugation reaction using the bifunctional spacer DSAP. *B*, characterization by MALDI-TOF MS. Axes were re-drawn and labeled to improve visibility. *C*, characterization by 10% SDS-PAGE (top panel) and immunoblot using either ST8 rabbit typing serum or mAb 28H11 as antibody sources (bottom panel). Reagents and conditions: a) i. DSAP, DMSO, Et_3N, r.t.; ii. CRM197, 0.1 M sodium phosphate buffer pH 7.4, r.t. a.u.= arbitrary units. DSAP = di-*N*-succinimidyl adipate.

CRM197-ST8 oligosaccharide conjugates were then employed in an immunogenicity screening experiment with multiple variable parameters (Fig. 3.4). Mice (BALB/c or C57BL/6; $n = 3$ per group) were immunized subcutaneously with the CRM197-FS A (**3-5**) and CRM197-FS C (**3-6**) conjugates formulated either with Freund's adjuvant, Alum or without adjuvant in a prime-boost regime and a total of three immunization doses (Fig. 3.4A).[34] The immune responses mounted by the glycoconjugates were monitored by glycan microarray analysis (Fig. 3.4B). To enable further evaluation of antibody specificities, synthetic saccharides **3-25** and **3-26**[xvii] were included in the experiment.[60] Tetrasaccharide **3-26**, a synthetic dimer of cellobiuronic acid, is under evaluation as a vaccine candidate against *S. pneumoniae* ST3[109] and was chosen to analyze antibody cross-reactivity to ST3 CPS. A non-related glycoconjugate (BSA-GlcNAc) was used to assess the immunogenicity of the linker/spacer construct.[159]

No anti-glycan antibodies were invoked upon immunization with FS A (**3-5**) as a hapten in any immunization setting used, indicating that FS A is not immunogenic in mice (Fig. 3.4C). Robust antibody responses against CRM197 and the linker/spacer construct were observed instead. Immunization with the CRM197-FS C glycoconjugate resulted in two opposing types of anti-glycan immune responses (Fig. 3.4D, left panel), depending on the cross-reactivity of antibodies either toward native ST8 CPS (as intended) or ST3 tetrasaccharide **3-26**. The latter binding pattern was observed more frequently, indicating that cross-reactivity towards tetrasaccharide **3-26** was induced by a more immunogenic glycotope (Fig. 3.4D, right panel). Both types of immune response were mutually exclusive since no serum sample bound to both ST3 tetrasaccharide **3-26** and ST8 CPS by glycan microarray (Fig. 3.4D, right panel). Immune responses were enhanced by boost immunizations, and no anti-glycan-antibodies were seen in naïve mice (Fig. 3.4E). Antibodies reactive towards both CRM197 and the linker/spacer construct were observed in all mice.

Taken together, ST8 CPS-binding antibodies can be raised by immunization with a glycoconjugate containing synthetic FS C, but not FS A. The induction of antibodies

[xvii] Tetrasaccharide **3-26** was synthesized by Dr. Sharavathi G. Parameswarappa and Dr. Subramanian Govindan.

reactive against FS C and native ST8 CPS is overshadowed by the immune response against a glycotope that is included in an ST3 CPS tetrasaccharide.

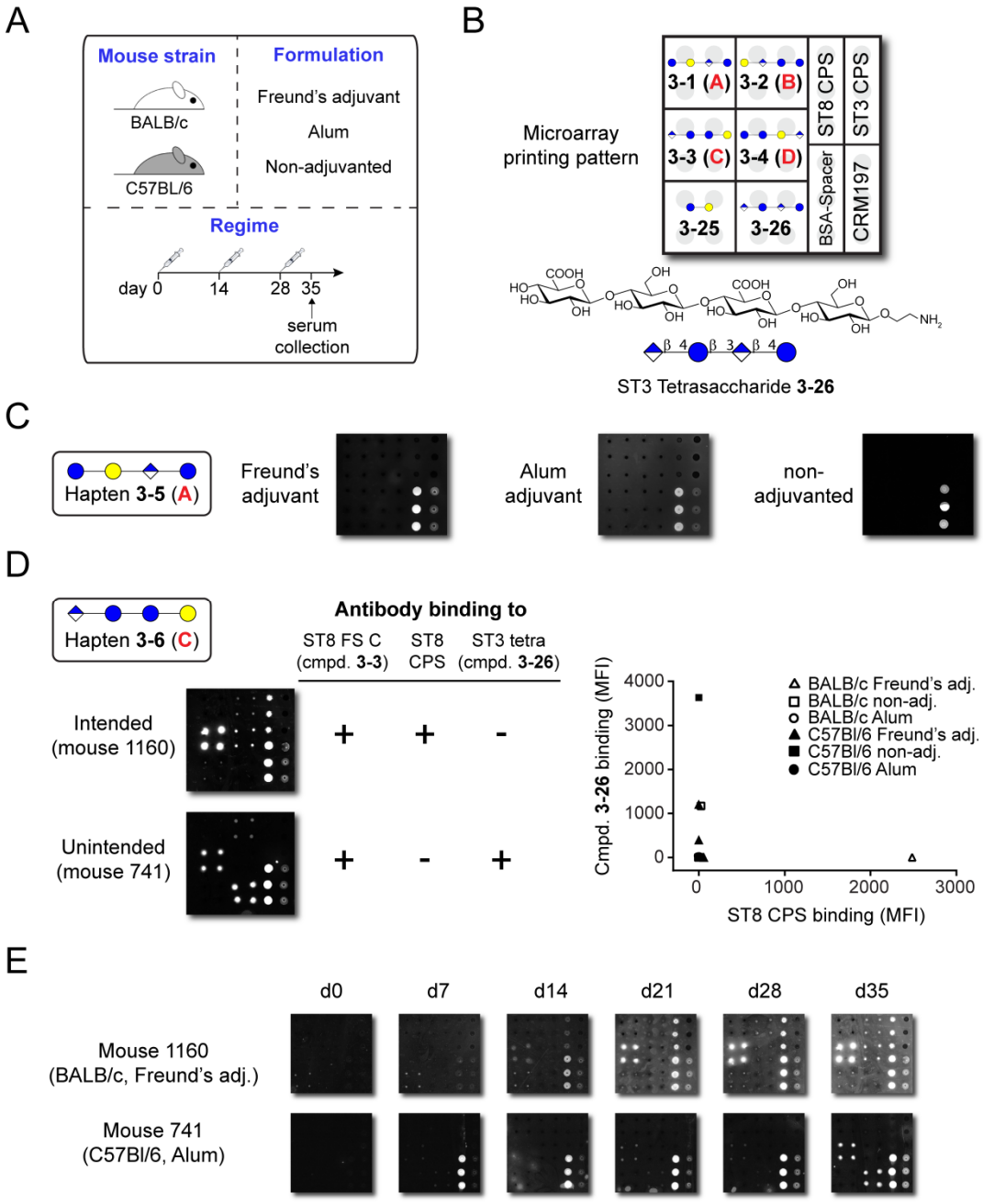


Figure 3.4. Evaluating glycoconjugates of synthetic ST8 tetrasaccharides in mice. *A*, immunization strategy. Mice were subcutaneously (s.c.) immunized three times with CRM197-FS A (**3-5**) or CRM197-FS C (**3-6**). Different mouse strains and formulations were screened. *B*, glycan microarray printing pattern and structure of tetrasaccharide **3-26**. *C*, immune response of C57BL/6 mice immunized with ST8 FS A (**3-5**) as a hapten, as assessed by glycan microarray of pooled sera (1:100 dilution). *D*, immune response of mice immunized with ST8 FS C (**3-6**) as a hapten, as assessed by glycan microarray (1:100 dilution). Antibody binding of sera from mice reacting in intended (cross-reactivity towards ST8 CPS) and unintended (cross-reactivity towards ST3 tetrasaccharide **3-26**) manners is shown. *E*, time course of the immune response against ST8 FS C.

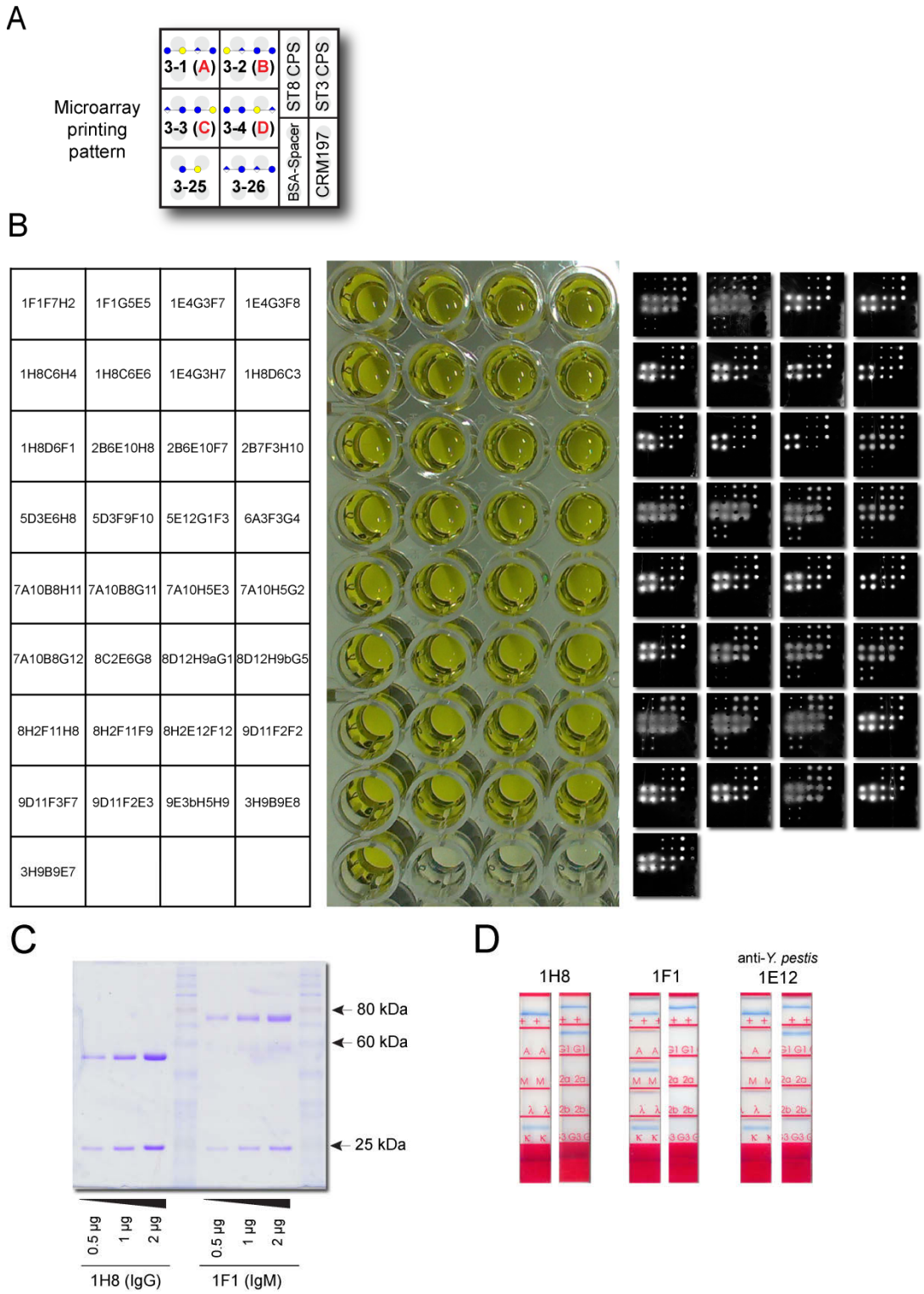


Figure 3.5. Characterization of monoclonal antibodies raised against ST8 CPS frameshift C (**3-6**). *A*, glycan microarray printing pattern. *B*, clone names (left) and glycan binding of cell culture supernatants by ST8 polysaccharide ELISA (middle) and glycan microarray (right). Since mAb isotypes were not determined for all clones, anti-mouse IgG secondary antibodies were used for ELISA and microarray analyses that display cross-reactivity towards IgM (our own observations). *C*, analysis of purified mAbs 1H8 and 1F1 by 12% SDS-PAGE. *D*, determination of mAb isotypes of 1H8 and 1F1 and anti-*Y. pestis* LPS core mAb 1E12[83] that was used as an IgG1 isotype control in subsequent experiments.

3.2.4 Monoclonal Antibodies Against ST8 Frameshift C Bind to Internal Glycotopes of Native ST8 Polysaccharide

A closer investigation of the different types of immune response invoked by the CRM197-FS C glycoconjugate followed (*see* Fig. 3.4D). MAbs were generated using splenocytes of a mouse that had shown an immune response against both synthetic FS C and ST8 CPS (no. 1160, *see* Fig. 3.4D). Two mAbs, 1H8 (IgG1[κ]) and 1F1 (IgM[κ]), were isolated out of a pool of more than 30 ST8 glycan-specific clones (Fig. 3.5) to enable both structural and functional characterization of saccharide binding. Both mAbs bound to immobilized ST8 CPS, as assessed by polysaccharide ELISA (Fig. 3.6A). Thereby, mAb 1H8 exhibited a similar endpoint concentration as mAb 28H11, with detectable binding at antibody concentrations as low as 50 ng/mL.

Binding to synthetic ST8 CPS frameshifts of mAbs 1H8 and 1F1 was then investigated by glycan microarray analysis and compared to mAb 28H11. Upon incubation with the array of synthetic ST8-derived oligosaccharides, mAb 1H8 closely resembled the binding pattern of mAb 28H11 (*see* Fig. 3.2), with specificity towards frameshift C and less robust binding to FSs B and D (Fig. 3.6A). These results confirm the finding that immunization with a FS C-containing glycoconjugate can induce an antibody response that recapitulates the binding of mAb 28H11. In contrast, mAb 1F1 is more promiscuous towards other synthetic glycans, especially FSs B and D (Fig. 3.6F). All interactions were blocked by mAb pre-adsorption to ST8 CPS (Fig. 3.6D and G), indicating that the observed antibody-glycan interactions are ST8-specific.

Polysaccharide-binding antibodies can be classified into two categories depending on their specificity towards either internal glycotopes found in each repeating unit or terminal sugars at the non-reducing end of the glycan chain.[95, 146, 344] Surface plasmon resonance (SPR) measurements can distinguish between the two modes of interaction. The recognition of terminal glycotopes by an immobilized antibody results in a 1:1 binding pattern with detectable dissociation rates of a soluble polysaccharide analyte.

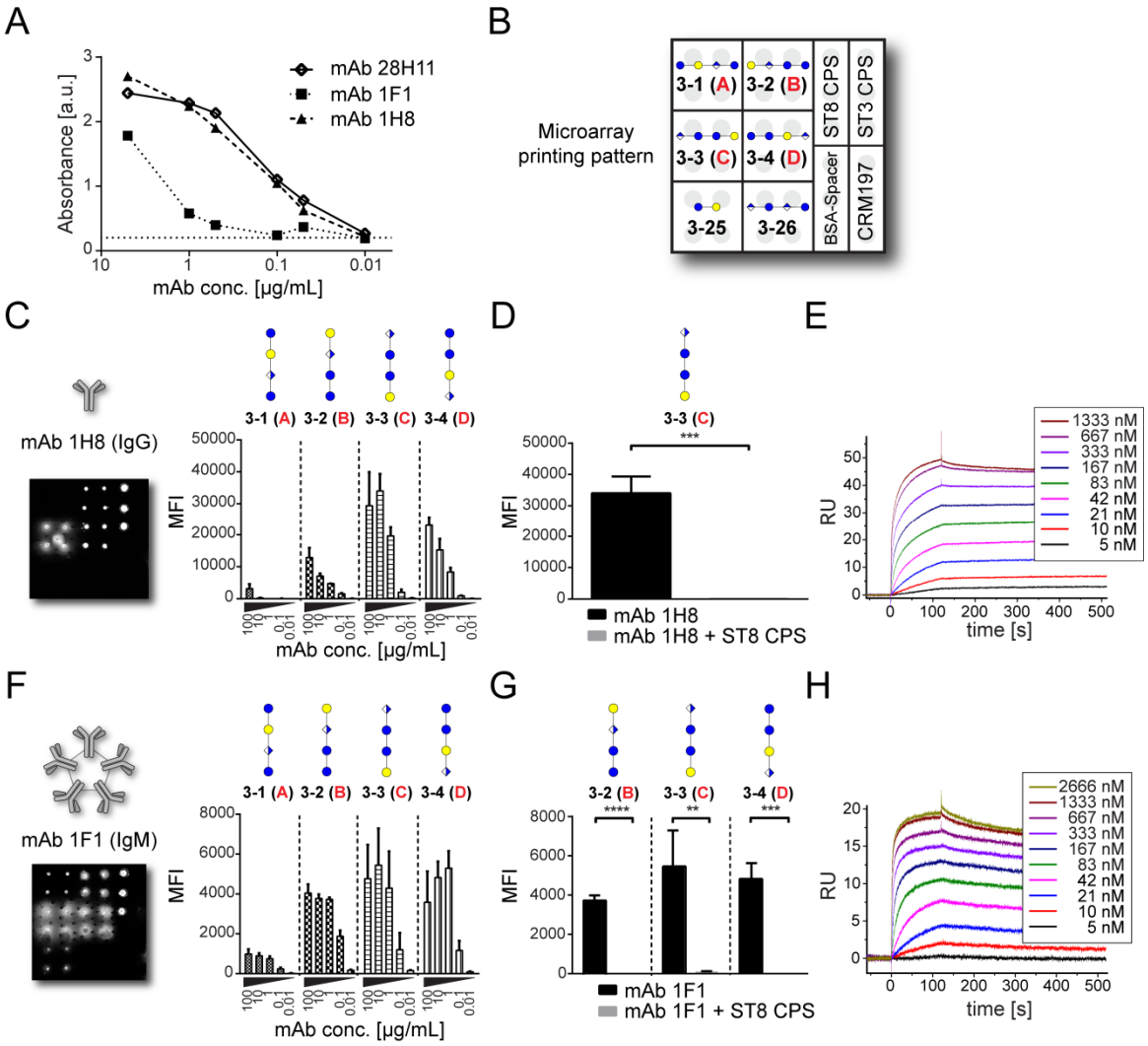


Figure 3.6. Binding characterization of ST8 CPS-related glycans by mAbs 1H8 and 1F1. *A*, comparison of ST8 CPS binding of mAbs 1H8 and 1F1 with mAb 28H11 by polysaccharide ELISA. A dotted line indicates signal background. *B*, glycan microarray printing pattern. *C* and *F*, glycan microarray analysis of mAbs 1H8 (*C*) and 1F1 (*F*). Histograms show mean + SD of fluorescence intensities normalized to background of eight spots. *D* and *G*, inhibition of oligosaccharide binding of mAbs 1H8 (*D*) and 1F1 (*G*) by pre-adsorption to ST8 CPS. Histpgrams show mean + SD of fluorescence intensities normalized to background of at least four spots. Statistical analysis (one-tailed, unpaired t test with Welch's correction) of at least four spots was performed of one out of at least two independent experiments. Asterisks indicate P values: ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. *E* and *H*, SPR analysis of immobilized mAbs 1H8 (*E*) and 1F1 (*H*) using ST8 CPS as an analyte in the indicated concentrations.

When internal glycotopes are recognized by the immobilized antibody, extensive re-binding occurs, and little dissociation of the polysaccharide analyte is observed.[95] Both mAbs 1H8 and 1F1 exhibited interaction patterns by SPR that indicate that these antibodies recognize an internal glycotope (Fig. 3.6E and H). To test this notion, ST8 CPS fragments of intermediate chain lengths (one to eight repeating units) were generated by acid-mediated partial depolymerization.[345] Dissociation of partially

depolymerized ST8 CPS towards mAb 1H8 was markedly accelerated compared to full-length polysaccharide (Fig. 3.7A and B). Finally, synthetic FS C tetrasaccharide **3-6** that spans a single repeating unit exhibited a 1:1 binding pattern to immobilized mAb 1H8 (Fig. 3.7C). These results confirm that dissociation is slow when the analyte contains multiple CPS repeating units, but fast when CPS fragments are used. Thus, mAbs raised against ST8 FS C recognize internal rather than terminal glycotopes of the native polysaccharide.

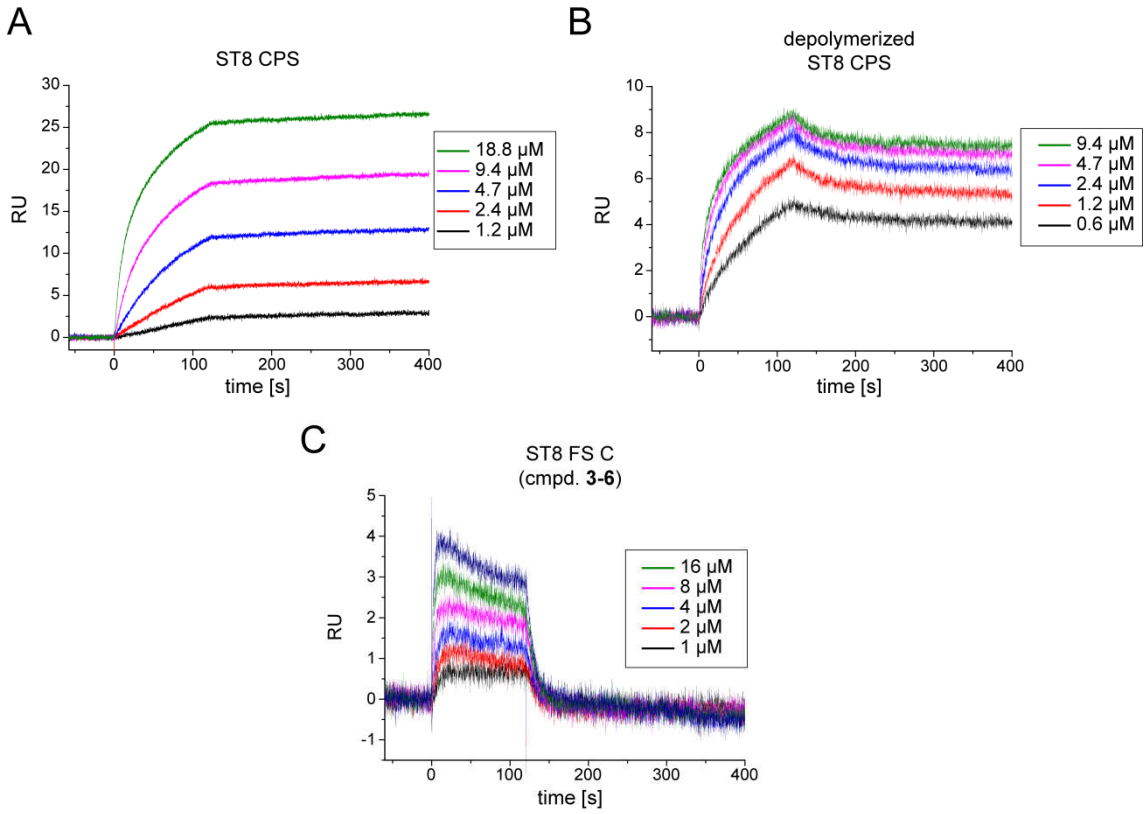


Figure 3.7. Qualitative comparison of binding kinetics of mAb 1H8 towards ST8 CPS-derived glycans of different chain lengths. MAb 1H8 was immobilized (500-1000 RU) and subjected to a flow of native ST8 CPS (*A*), depolymerized ST8 CPS containing one to eight repeating units (*B*) or synthetic tetrasaccharide **3-6** (*C*). Sensorgrams (30 µL/min flow rate; 120 s association phase; 280 s dissociation phase) were double-referenced to buffer and a flow cell containing a non-related, immobilized murine IgG. Analyte concentrations are calculated based on repeating units present (M_r = 664 for polysaccharide preparations and M_r = 723 for tetrasaccharide **3-6**). Kinetic constants could not be determined due to the heterogeneous nature of polysaccharide analytes.

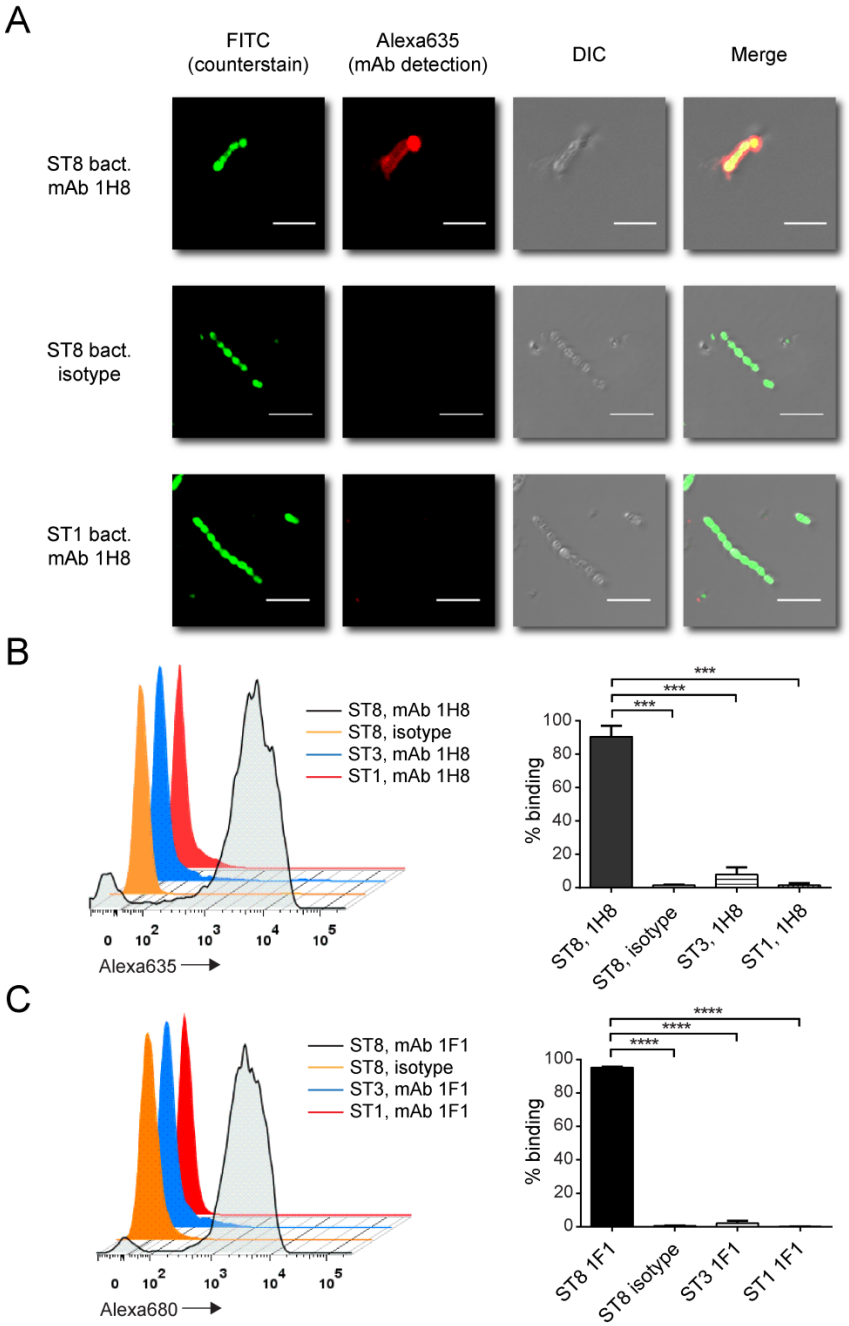


Figure 3.8. MAbs 1H8 and 1F1 specifically bind ST8 pneumococci. *A*, immunofluorescence of inactivated, FITC-labeled ST8 or ST1 pneumococci by 1H8 or an IgG1 isotype. Scale bar: 5 µm. *B*, flow cytometry of pneumococci after 1H8-mediated fluoredcent labeling. Left panel: Representative flow cytometry result after incubation with mAb 1H8 or IgG1 isotype (10 µg/mL) and Alexa Fluor® 635-labeled secondary antibody. Histograms show mean + SD of positive binding. Right panel: Cumulated results of at least three independent labeling experiments using mAb 1H8. *C*, flow cytometry of pneumococci after 1F1-mediated fluoredcent labeling. Left panel: Representative flow cytometry result after incubation with mAb 1F1 or IgM isotype (10 µg/mL) and Alexa Fluor® 680-labeled secondary antibody. Right panel: Cumulated results of at least three independent labeling experiments using mAb 1F1 Histograms show mean + SD of positive binding. Statistical analysis was performed (paired, one-tailed t test) and Asterisks indicate P values: *** $P < 0.001$; **** $P < 0.0001$. DIC = differential interference contrast. FITC = fluorescein isothiocyanate. RU = response units.

3.2.5 Monoclonal Antibodies Against ST8 Frameshift C Bind Serotype 8 Pneumococci and Protect from Lethal Pneumococcal Infection

3.2.5.1 Binding of Serotype 8 Pneumococci

MAbs 1H8 and 1F1 that are directed towards synthetic tetrasaccharide **3-6** specifically recognized native ST8 CPS. To assess whether capsule binding led to the recognition of bacteria, immunofluorescence labeling was performed using inactivated, fluorescein isothiocyanate-labeled *S. pneumoniae*. Both mAbs bound to the capsule of ST8, as assessed after incubation with secondary antibodies carrying compatible fluorescent labels by fluorescence microscopy (Fig. 3.8A) and flow cytometry (Fig. 3.8B and C). Interactions between mAbs and bacteria were ST8-specific, since no binding was observed by antibody isotype controls or towards *S. pneumoniae* ST1 or ST3.

3.2.5.2 Opsonophagocytic Killing

Next, the viability of mAbs raised against synthetic tetrasaccharide **3-6** to orchestrate anti-pneumococcal defense mechanisms *in vitro* was evaluated. MAb 1H8 was chosen over 1F1 due to the higher binding capacity towards ST8 CPS and the higher relevance of IgG over IgM in vaccinations.[80]

Antibody-dependent opsonization of bacteria is the most important defense mechanism against *S. pneumoniae* infections.[8] An opsonophagocytic killing assay was performed employing widely-used, differentiated HL-60 cells as a phagocyte source to elucidate the capability of antibodies against FS C (**3-6**) to opsonize ST8 bacteria.[79] MAb 1H8, but not an isotype-matched control mAb, efficiently triggered opsonophagocytic killing in the same order of magnitude as high concentrations of reference sera (Fig. 3.9A).

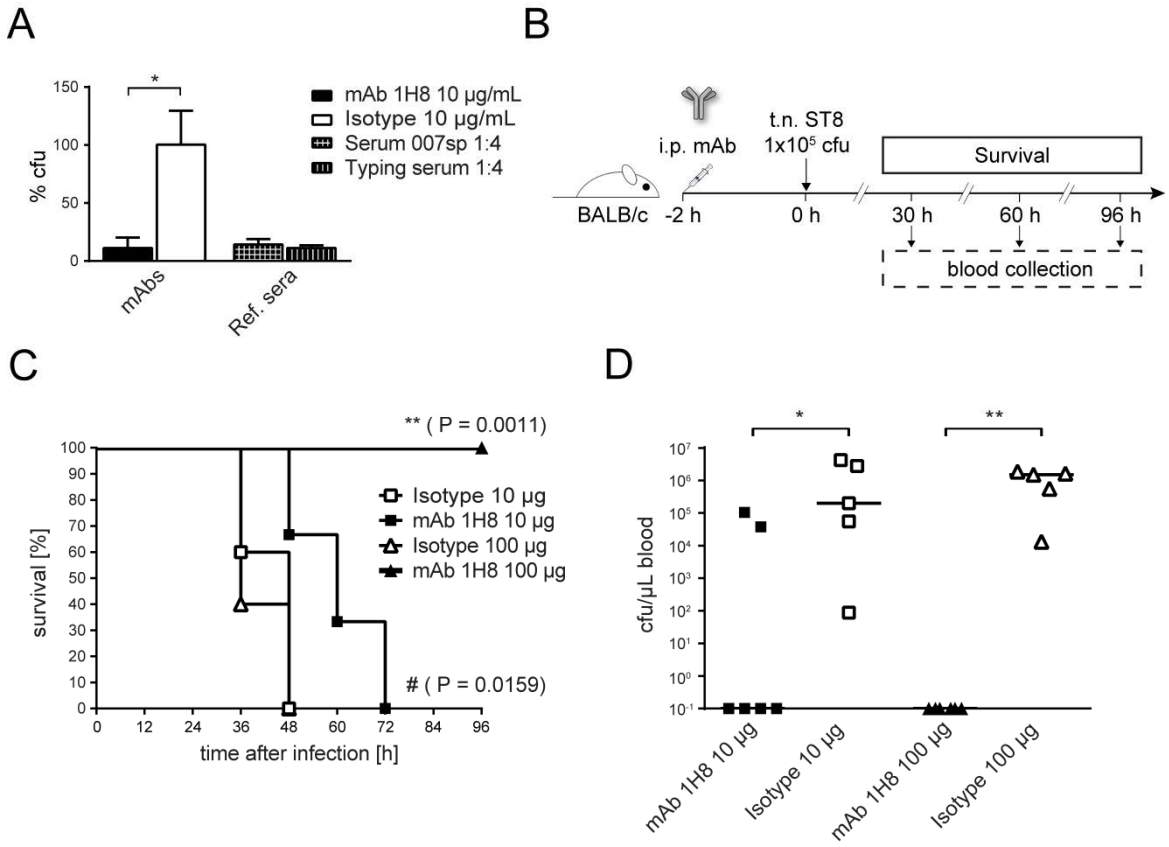


Figure 3.9. MAb 1H8 opsonizes bacteria and protects from lethal pneumococcal challenge. *A*, opsonophagocytic killing assay using differentiated HL-60 cells as phagocyte, rabbit serum as complement and mAbs as antibody sources, respectively. Reduction of cfu is shown relative to control wells lacking an antibody source. Histograms show mean + SD of triplicate wells. Statistical analysis (One-tailed, unpaired t test with Welch's correction) was performed of one out of two independent experiments. Asterisk indicates P value: * P $<$ 0.05. *B-D*, passive immunization and lethal challenge with *S. pneumoniae* ST8. *B*, passive immunization regime. Mice (n = 5-6 per group) were treated i.p. with 10 µg or 100 µg of mAb 1H8 or isotype control 2 h prior to lethal t.n. challenge with ST8 bacteria. Blood was withdrawn at regular intervals and survival was monitored for 96 h. *C*, survival of antibody-treated mice after ST8 infection. Statistical analysis (Mantel-Cox log-rank test) was performed between groups of equal antibody doses: ** 100 µg mAb; # 10 µg mAb. *D*, bacterial burden in the blood of mice 30 h p.i. Values are given as individual data and median. Statistical analysis (Mann-Whitney U test) was performed between groups of equal antibody doses. Asterisks indicate P values: * P $<$ 0.05; ** P $<$ 0.005.

3.2.5.3 Passive Immunization

To determine whether polysaccharide binding of mAbs raised against FS C is functional in a disease setting, protection of mice from pneumococcal infection by mAb 1H8 was studied.[xviii] MAb 1H8 or an isotype-matched control mAb were administered at either 10

[xviii] Passive immunization was performed together with Dr. Katrin Reppe, Denise Barthel and Prof. Dr. Martin Witzenrath at Charité Universitätsmedizin, Berlin, Germany.

µg or 100 µg doses two hours prior to transnasal infection with a lethal dose of *S. pneumoniae* serotype 8 (Fig. 3.9B). [57] The distinct glycan array binding pattern of mAb 1H8 allowed for the monitoring of antibody levels during the course of infection by glycan microarray analysis. Both survival and bacteremia were monitored and correlated to bloodstream levels of mAb 1H8.

A 10 µg dose of mAb 1H8 induced significantly prolonged survival of infected mice when compared to the isotype-matched control mAb (Fig. 3.9C), whereas a 100 µg dose of mAb 1H8 resulted in complete survival. Clinical parameters of bacterial sepsis were absent in mice treated with the 100 µg dose of mAb 1H8 (not shown). As expected, survival was inversely correlated to bacterial burden, and mice with measurable amounts of bacteria in the bloodstream died within 12 hours after detection of bacteremia (Fig. 3.9D and Fig. 3.10A and B).

Bacteremia of ST8-infected mice inversely correlated with bloodstream levels of mAb 1H8. Antibody levels in the same order of magnitude as the administered amounts (mAb concentrations of 6 to 13.5 µg/mL in blood for 10 µg and 15.5 to 44.1 µg/mL in blood for 100 µg groups, respectively) were observed in mice without bacteremia (Fig. 3.10C and D). When no mAb 1H8 was present in the bloodstream, bacterial burden was observed to indicate that the presence of anti-capsular antibody excludes bloodstream invasion of pneumococci. Analysis of the time course of both bacteremia and mAb levels confirmed that the presence of antibody was essential for survival, as mice receiving the 10 µg dose of mAb 1H8 survived until mAb levels disappeared and, simultaneously, bloodstream bacterial burden increased (Fig. 3.10C).

3.2.6 Synthesis of Oligosaccharides Related to ST8 Frameshift C

Immunization with FS C as a hapten can induce protective antibodies against ST8, but more frequently induces antibodies against a non-protective glycotope (*see* above) To foster the generation of optimized vaccine antigens, it was now important to uncover the nature of the protective glycotope within FS C. A panel of synthetic, FS C-related oligosaccharides was synthesized to this end (Scheme 3.5).

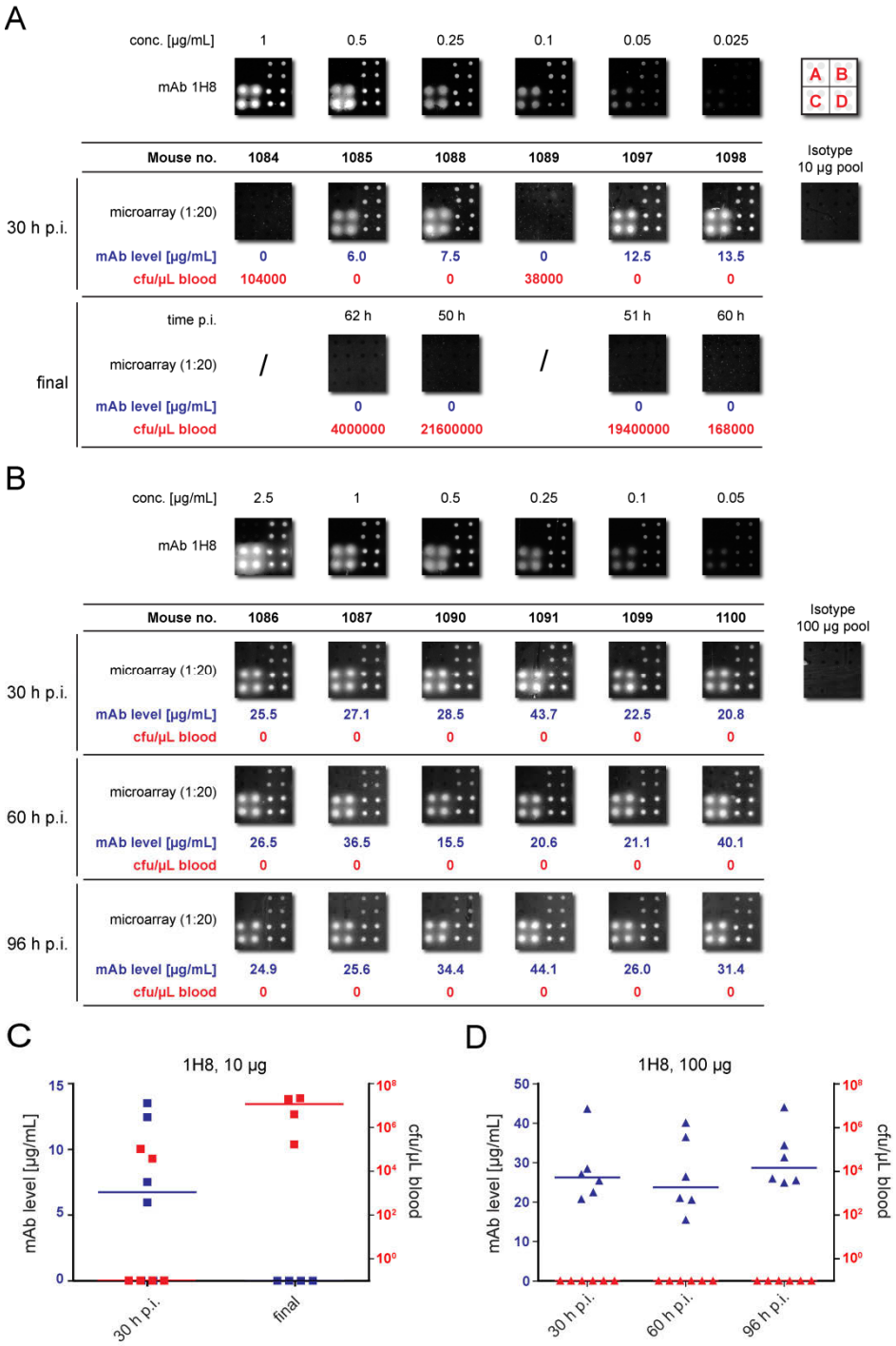
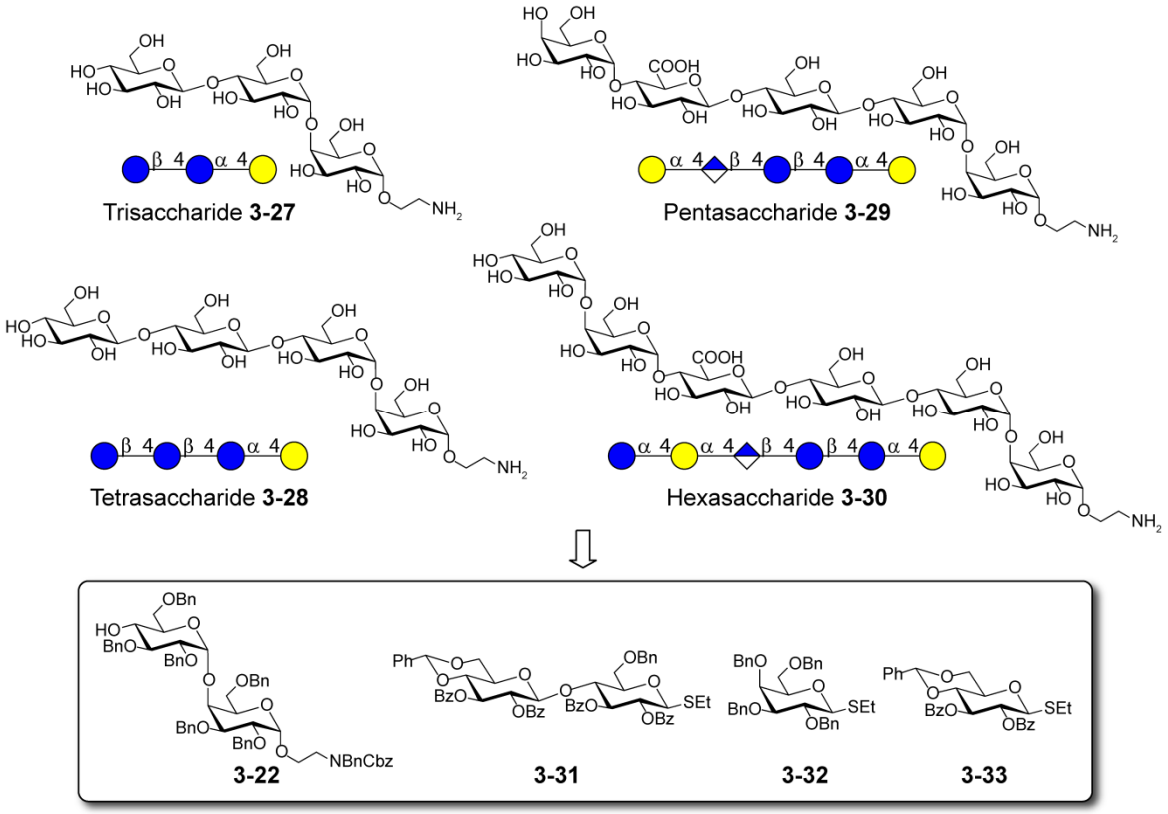


Figure 3.10. Correlation between mAb 1H8 levels and bacterial burden in blood after passive immunization and lethal pneumococcal challenge. Mice were passively immunized intraperitoneally (i.p.) with mAb 1H8 or IgG1 isotype and infected transnasally (t.n.) with $1x10^5$ cfu *S. pneumoniae* ST8 two hours later. Blood was withdrawn at regular intervals and upon euthanizing mice and analyzed for mAb 1H8 levels (glycan microarray) and bacterial burden. MAb concentrations were assessed by comparing fluorescent to standard curves of mAb 1H8. Mean values after fluorescence read-out of at least 8 spots are shown. Pooled sera of isotype-treated mice 30 h p.i. are shown for comparison. *A*, 10 µg mAb. Blood was analyzed after 30 h and at indicated time points upon euthanizing ("final"). *B*, 100 µg mAb doses. Blood was analyzed after 30 h, 60 h and 96 h. *C* and *D*, correlation of mAb levels and bacterial burden in blood of mice receiving a 10 µg (*C*) or a 100 µg dose of mAb 1H8 (*D*). Values are given as individual data and median.

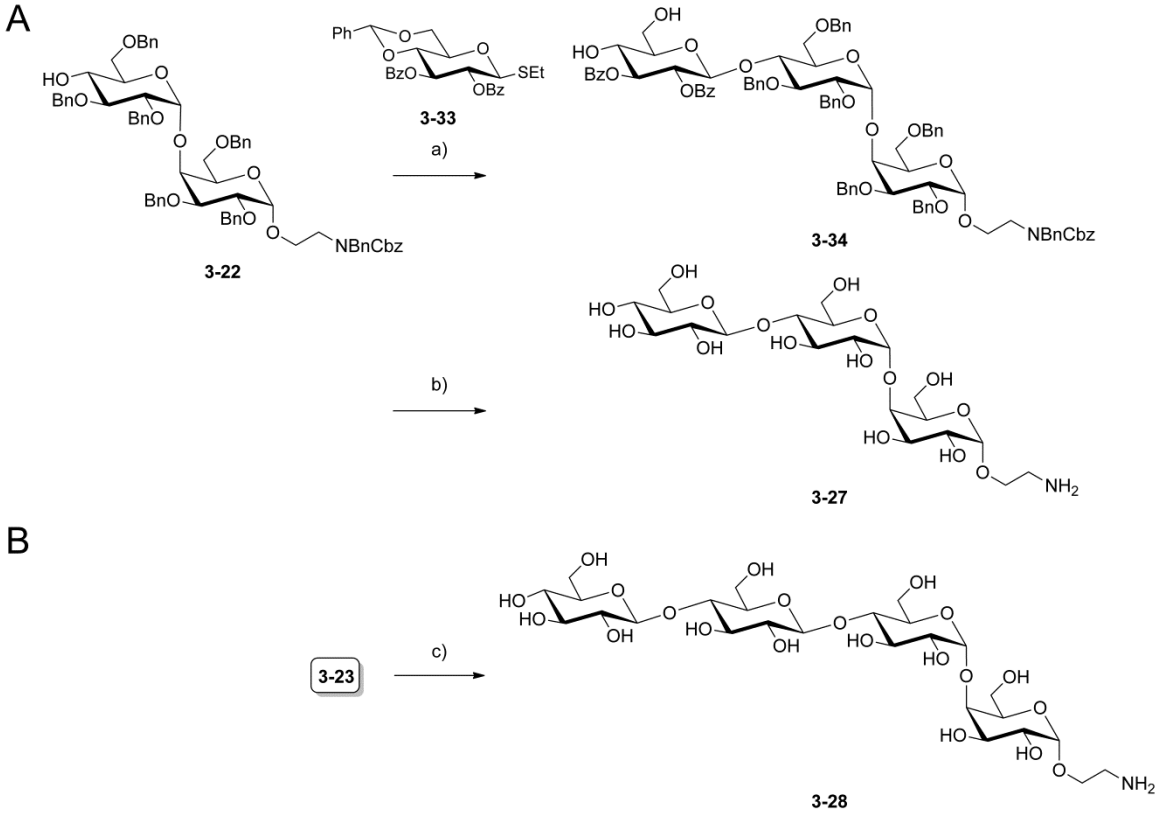


Scheme 3.5. Retrosynthesis of ST8 substructures **3-27**, **3-28**, **3-29** and **3-30** based on building blocks **3-22**, **3-31**, **3-32** and **3-33**.

Saccharides **3-27** and **3-28** were of interest due to the absence of the terminal glucuronic acid moiety that is found on ST3 tetrasaccharide **3-26**. Particularly, tetrasaccharide **3-28** was chosen as a "reduced" congener of FS C containing a terminal glucose unit instead of glucuronic acid to evaluate the role of the negative charge present under physiological conditions. Penta- and hexasaccharides **3-29** and **3-30** served to study the effects of saccharide extensions at the non-reducing end of FS C. Disaccharide **3-22** was used along with diglucoside **3-31** and protected monosaccharides **3-32** and **3-33** to generate conjugation-ready glycans **3-27**, **3-28**, **3-29** and **3-30**.

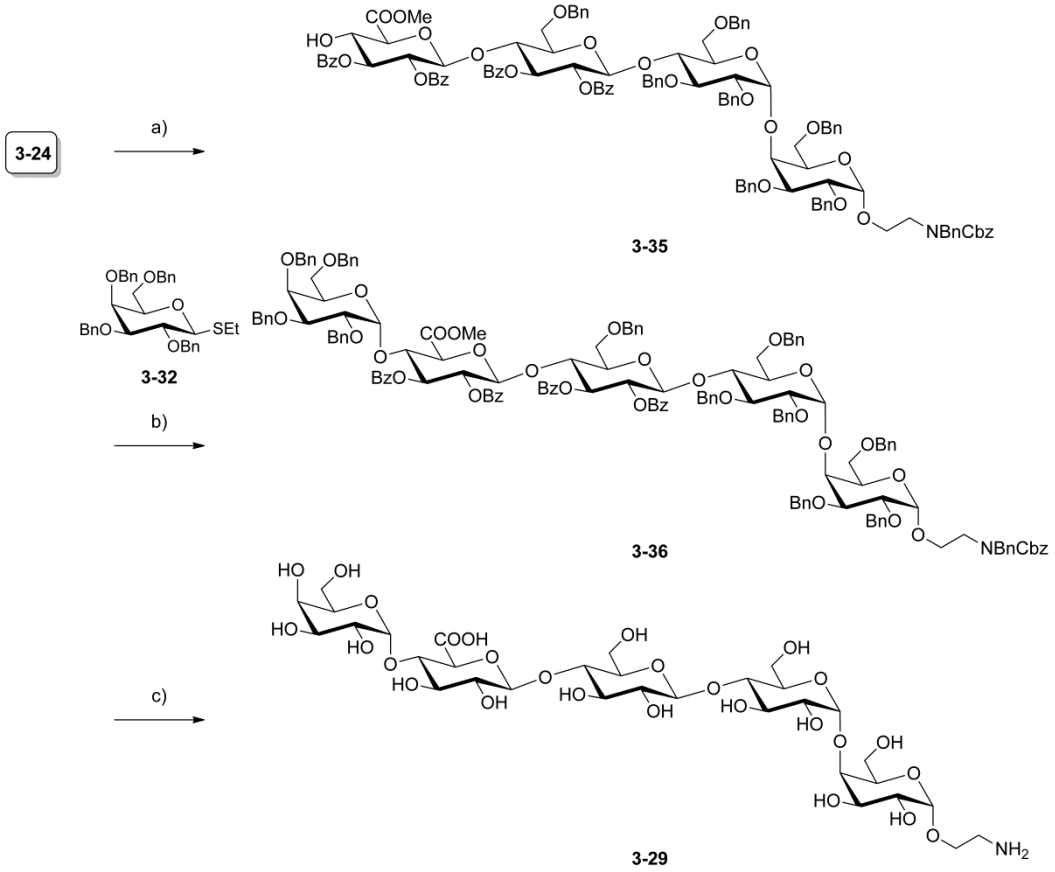
The synthesis of trisaccharide **3-27** commenced by appending a terminal glycose moiety to alcohol **3-22** through thioglycoside **3-33**[333] (Scheme 3.6A). Subsequent removal of the benzylidene moiety from the trisaccharide intermediate under acidic conditions furnished diol **3-34** in 75% yield over two steps. Removal of all permanent protecting groups was effected by transesterification with methanolic sodium methoxide and hydrogenation of the benzyl and Cbz groups using Pearlman's catalyst in a ternary mixture of dichloromethane, *tert*-butanol and water to give trisaccharide **3-27** in 79%

yield over two steps. Following tetrasaccharide assembly (*see* Scheme 3.4A), global deprotection of diol **3-23** produced FS C tetrasaccharide congener **3-28** in 89% yield over two steps (Scheme 3.6B).



Scheme 3.6. Total syntheses of ST8 trisaccharide **3-27** and tetrasaccharide **3-28**. Reagents and conditions: a) **3-33**, NIS, TfOH, CH_2Cl_2, -20 °C, ii. EtSH, *p*TsOH, CH_2Cl_2, r.t., 75% (two steps); b) i. NaOMe, MeOH, CH_2Cl_2, r.t.; ii. H_2, $Pd(OH)_2$, CH_2Cl_2, *t*BuOH, H_2O, r.t., 79% (two steps); i. NaOMe, THF, MeOH, 40 °C; ii. H_2, $Pd(OH)_2$, CH_2Cl_2, *t*BuOH, H_2O, r.t., 89% (two steps).

Further elongation of the FS C oligosaccharide backbone toward the generation of penta- and hexasaccharides commenced by treatment of carboxylate **3-24** with methyl iodide under basic conditions to afford ester **3-35** in 80% yield (Scheme 3.7). The use of trimethylsilyl diazomethane instead resulted in the formation of multiple products. The terminal galactose moiety was introduced through thioglycoside **3-32**[346] using NIS/TfOH as a promoter system to give pentasaccharide **3-36** in 89% yield and 4.7:1 α:β stereoselectivity. Global deprotection featured the initial mild hydrolysis of the methyl esters in **3-36** using lithium peroxide, transesterification of benzoate esters and hydrogenolysis using Pearlman's catalyst (*see* above). Pentasaccharide **3-29** was obtained in 88% yield over three steps.



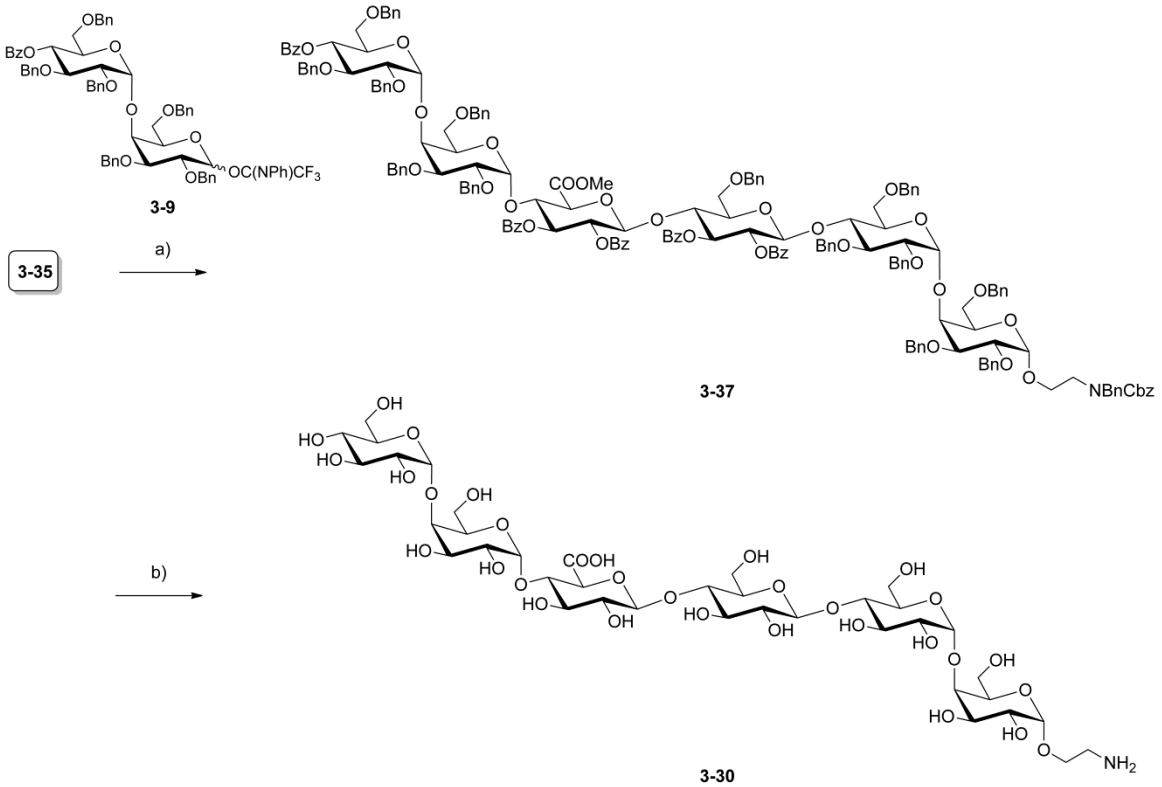
Scheme 3.7. Total synthesis of ST8 pentasaccharide **3-27**. Reagents and conditions: a) MeI, Cs_2CO_3, DMF, r.t., 80%; b) **3-30**, NIS, TMSOTf, Et_2O/CH_2Cl_2 3:1, -20 °C to 0 °C, 89% (4.7:1 α:β); c) i. H_2O_2, aq. LiOH, THF, MeOH, H_2O, r.t., then aq. NaOH, r.t., ii. NaOMe, MeOH, r.t.; iii. H_2, $Pd(OH)_2$, CH_2Cl_2, *t*BuOH, H_2O, r.t., 88% (three steps).

Next, the total synthesis of hexasaccharide **3-30** was undertaken (Scheme 3.8). Methyl ester **3-35** and glycosylating agent **3-9** served as suitable precursors to give protected hexasaccharide **3-37** in a TMSOTf-catalyzed glycosylation reaction in 68% yield and complete α-selectivity. Careful analysis of anomeric $^1J_{C\text{-}H}$ coupling constants in **3-37** confirmed the configurations of all anomeric linkages on that stage. Global deprotection of **3-37** proceeded uneventfully, using the reaction sequence established for the deprotection of pentassaccharide **3-36** (*see* Scheme 3.7) to give deprotected hexasaccharide **3-30** in 86% yield over three steps.

Assessment of $^3J_{H,H}$ coupling constants in ^{1}H NMR spectra of saccharides **3-27**, **3-28**, **3-29** and **3-30** confirmed the configurations of all anomeric linkages. Furthermore, comparison of ^{1}H NMR spectra of all synthetic ST8 CPS-derived oligosaccharides permitted the assignment of anomeric peaks and the confirmation of the ST8 CPS structure that had so far only been determined by wet chemical and biosynthetic

analyses (Fig. 3.10).[16, 327] Thereby, commercial ST8 CPS turned out to be heavily contaminated with pneumococcal C-polysaccharide, in line with observations that antibodies against C-polysaccharide are generated in abundance after immunization with a ST8 CPS glycoconjugate.[92] This finding further underscores the power of chemical synthesis to generate glycans devoid of cellular contaminants.

Taken together, a collection of seven ST8 FS C-related oligosaccharides were synthesized from a small set of building blocks in a highly divergent manner, enabling the mapping of glycotopes recognized by FS C-directed antibody samples (*see* below).



Scheme 3.8. Total synthesis of ST8 hexasaccharide **3-30**. Reagents and conditions: k) **3-9**, TMSOTf, Et_2O/CH_2Cl_2 3:1, -20 °C to 0 °C, 68% (>19:1 α:β); l) i. H_2O_2, aq. LiOH, THF, MeOH, H_2O, r.t., then aq. NaOH, r.t., ii. NaOMe, MeOH, r.t.; iii. H_2, $Pd(OH)_2$, CH_2Cl_2, *t*BuOH, H_2O, r.t., 86% (three steps).

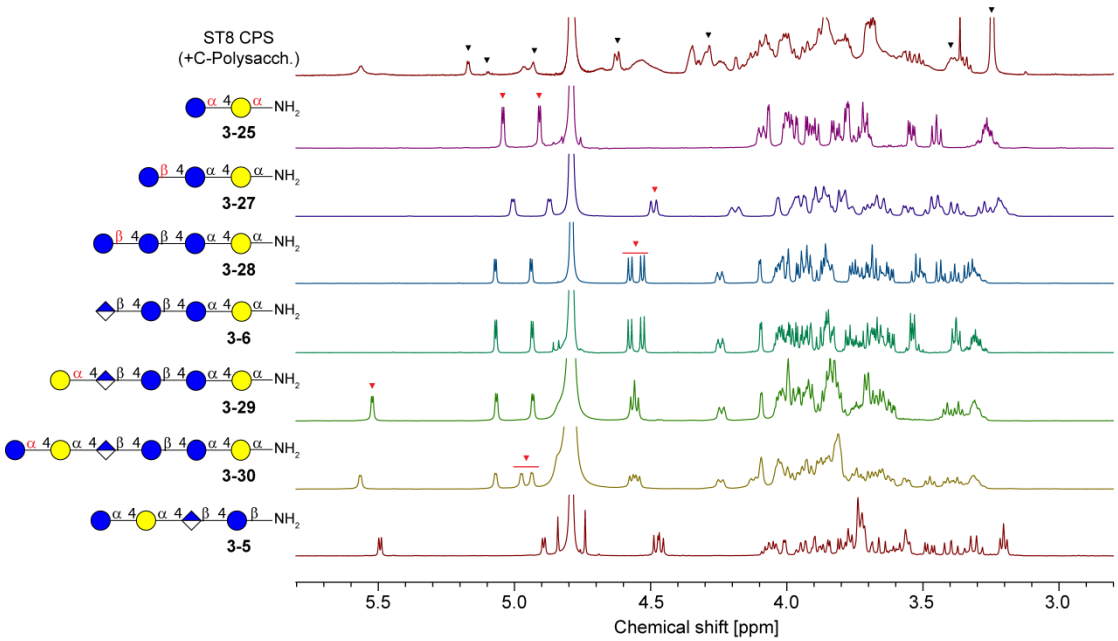


Figure 3.10. Comparison of ^{1}H NMR spectra of native ST8 CPS (600 MHz) with ST8-derived oligosaccharides **3-25** (600 MHz), **3-27** (400 MHz), **3-28** (600 MHz), **3-6** (600 MHz), **3-29** (600 MHz), **3-30** (600 MHz) and **3-5** (400 MHz). All spectra were recorded at 25 °C. C-polysaccharide impurities in commercial ST8 CPS are highlighted with black arrowheads according to Xu et al.[100] Signals of anomeric protons different from spectra of smaller glycans are highlighted with red arrowheads. A red line depicts signals that are too close to differentiate.

3.2.7 Mapping of Protective and Non-protective Glycotopes Within ST8 Frameshift C

3.7.2.1 Mapping a Non-protective Glycotope Within ST8 Frameshift C

Immunization with a CRM197-FS C glycoconjugate yielded immune responses either recognizing native ST8 CPS or cellobiuronic acid-containing tetrasaccharide **3-24** (*see* Fig. 3.4D). ST8 CPS recognition was key to protection from pneumococcal infection. In order to uncover the nature of non-protective glycotopes found in ST8 FS C, a collection of synthetic, ST3 CPS-derived oligosaccharides was first employed in a glycan microarray experiment (Fig. 3.11A).[59] Antisera raised against ST8 FS C and binding to ST3 tetrasaccharide **3-26** exhibited an even more robust interaction with cellobiuronic acid **3-38**, whereas mAb 1H8 did not recognize that disaccharide (Fig. 3.11B). Little or no binding was found to ST3 CPS-derived trisaccharides (**3-40**[xix] and **3-41**[xix]), a

[xix] Oligosaccharides **3-39**, **3-40** and **3-41** were synthesized by Dr. Sharavathi G. Parameswarappa and Dr. Subramanian Govindan.

frameshifted ST3 disaccharide (**3-39**[xix]) or native ST3 CPS (Fig. 3.11B), confirming that cellobiuronic acid alone is highly immunogenic.

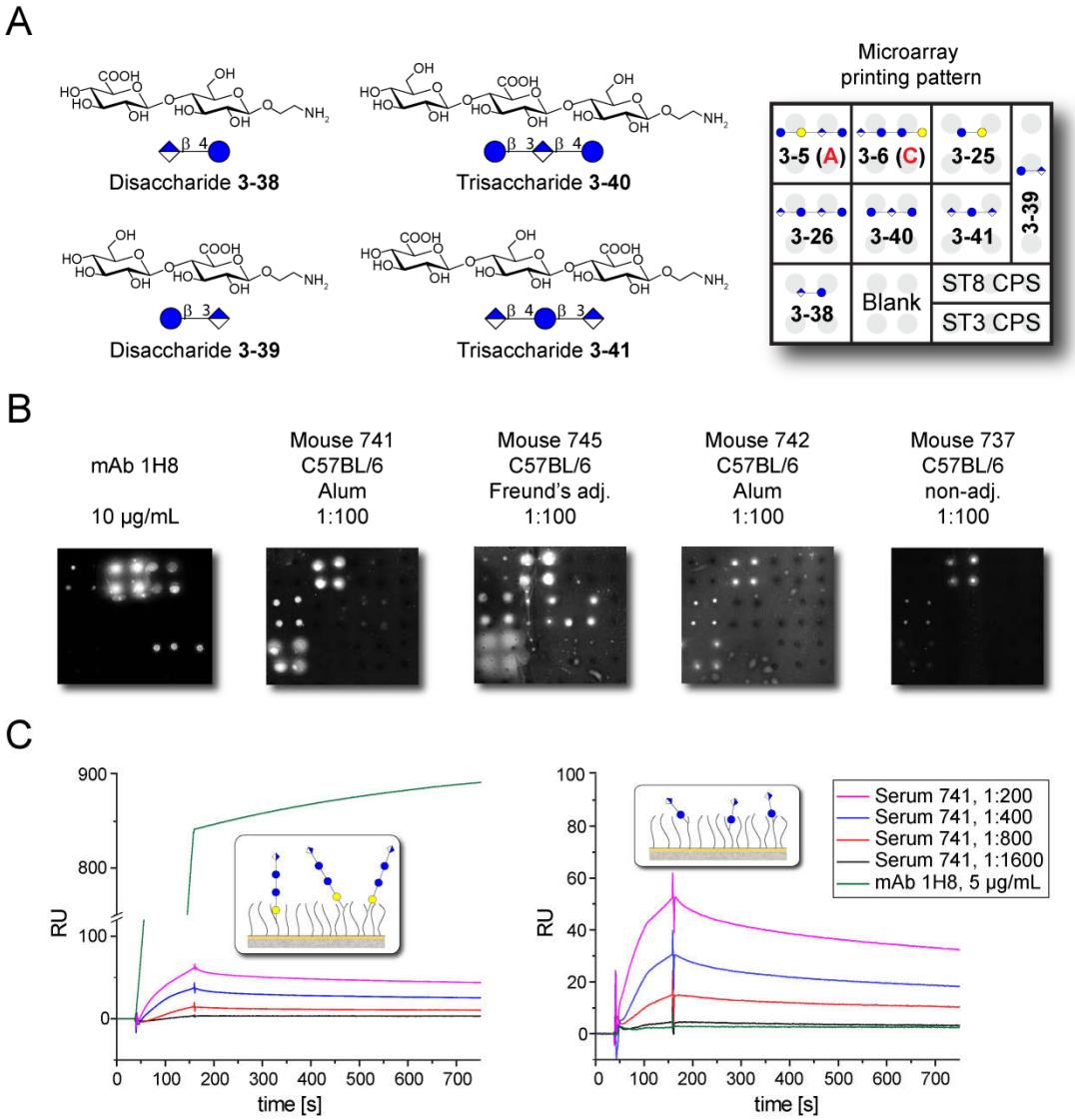


Figure 3.11. Mapping the non-protective glycotope of ST8 FS C. *A*, synthetic ST3-related oligosaccharides **3-38**, **3-39**, **3-40** and **3-41**,[xix] and glycan microarray printing pattern. Disaccharide **3-38** represents conjugation-ready cellobiuronic acid. *B*, glycan microarray analysis comparing ST8 CPS-recognizing mAb 1H8 and antiserum of mouse 741 recognizing a non-protective glycotope of FS C (*see* Fig. 3.4) by using synthetic ST8 and ST3 CPS-derived oligosaccharides. *C*, SPR analysis of mAb 1H8 and antiserum 781 with immobilized glycans FS C (**3-6**, left panel) and disaccharide **3-38** (right panel).

The binding preferences of serum 741 in contrast to mAb 1H8 were confirmed by SPR: While both serum and mAb interacted with immobilized ST8 FS C (**3-6**), only serum 741 bound cellobiuronic acid disaccharide **3-38** (Fig. 3.11C). Taken together, these findings indicate that the immunodominant glycotope within ST8 FS C is the terminal cellobiuronic acid moiety. The immune response induced by that glycotope does not

confer cross-reactivity towards ST8 or ST3 CPSs. Thus, the information conferring protective immunity against ST8 CPS is likely located elsewhere in the FS C tetrasaccharide.

3.7.2.2 The Protective Glycotope Within ST8 Frameshift C Does not Include the Terminal Glucuronic Acid

A detailed investigation of the protective glycotope of ST8 CPS that is recognized by mAbs 1H8 and 1F1 was then carried out making use of oligosaccharides **3-27**, **3-28**, **3-29**, **3-30** and **3-42**[xx] in a glycan microarray experiment (Fig. 3.12). Omission of the terminal glucuronic acid moiety of FS C did not abrogate binding by either mAb as long as the reducing-end sequence β-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Gal*p* was intact (Fig. 3.12B and D): Both trisaccharide **3-27** and tetrasaccharide **3-28** were recognized with comparable intensities to FS C. In contrast, truncation of trisaccharide **3-27** by a single monosaccharide residue at either reducing or non-reducing end (**3-25** and **3-42**, respectively) led to almost complete ablation of binding. Longer oligosaccharides (**3-29** and **3-30**) were recognized with intensities comparable to FS C, indicating that saccharide extensions at the non-reducing end neither ablate nor facilitate antibody interaction. All interactions of mAbs 1H8 and 1F1 with synthetic, ST8 FS C-related oligosaccharides were inhibited by mAb pre-adsorption to native ST8 CPS (Fig. 3.12C and E) to confirm that the observed oligosaccharide binding events are relevant for the recognition of ST8 pneumococci.

To determine whether the binding preferences found for anti-ST8 mAbs correlate with affinities, the dissociation constants (K_D) of mAb 1H8 and synthetic ST8 CPS-derived oligosaccharides were measured by SPR (Fig. 3.13). Similar affinities were determined for mAb 1H8 binding of ST8 FS C **3-6** (K_D = 5.8 ± 0.7 µM), trisaccharide **3-27** (K_D = 4.5 ± 1.5 µM) and tetrasaccharide congener **3-28** (K_D = 2.0 ± 2.3 µM), while binding of disaccharide **3-25** was approximately fivefold weaker (K_D =24.7 ± 2.7 µM). These results are in good agreement with the microarray data on the respective mAb-oligosaccharide interactions (*see* Fig. 3.12B and D) and confirm the role of the trisaccharide found at the reducing end of FS C as a protective glycotope of ST8 CPS.

[xx] Disaccharide **3-42** was synthesized by Dr. Sharavathi G. Parameswarappa.

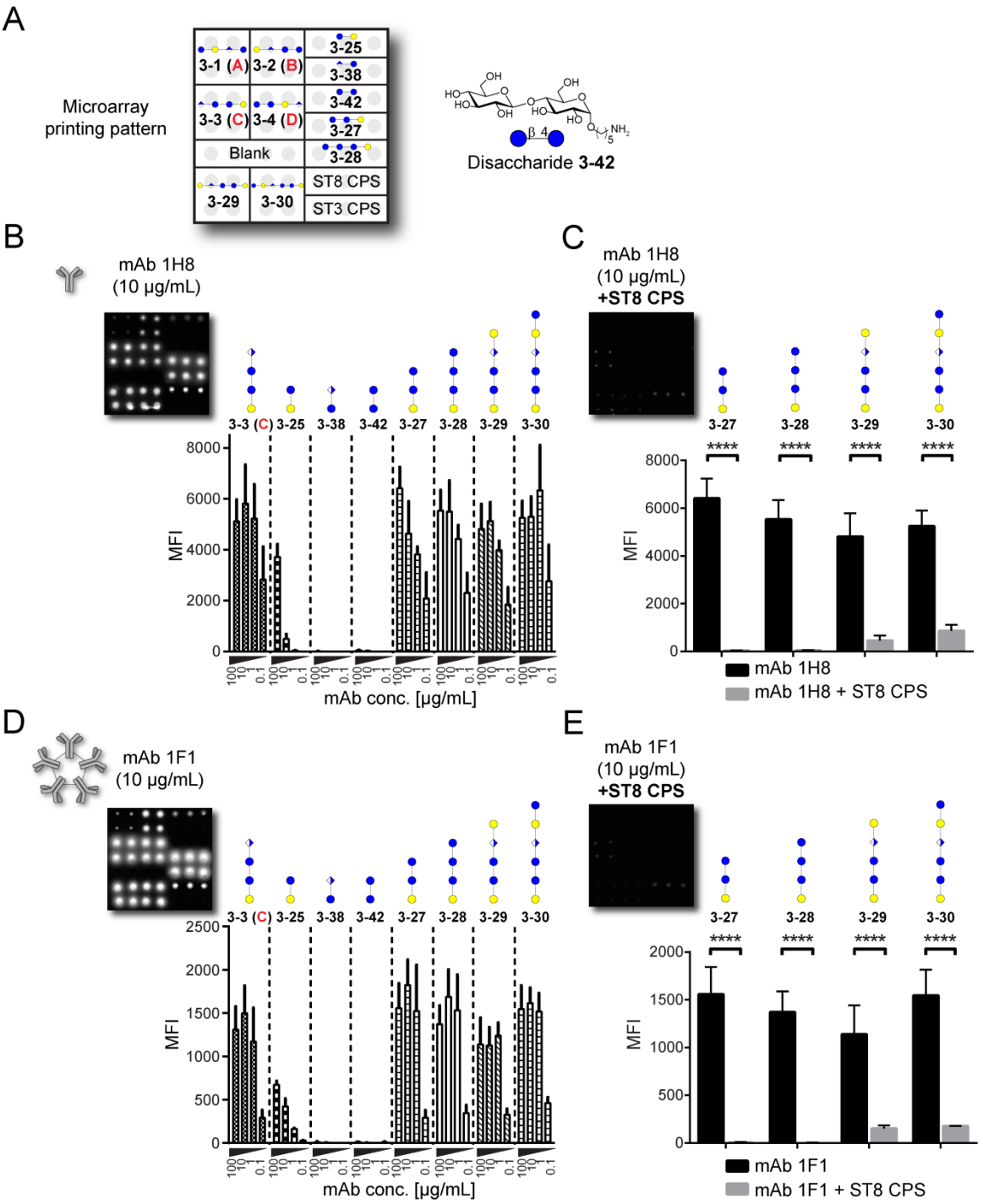


Figure 3.12. Mapping the protective glycotope of ST8 FS C. *A*, glycan microarray printing pattern. *B* and *D*, glycan microarray analysis of mAb 1H8 (*B*) and 1F1 (*D*) using a collection of synthetic ST8 CPS-related oligosaccharides. Histograms show mean + SD of fluorescence intensities normalized to background of at least six spots. *C* and *E*, glycan microarray analysis of mAb 1H8 (*C*) and 1F1 (*E*) with pre-adsorption to ST8 CPS and quantitation of binding inhibition to oligosaccharides **3-27** - **3-30** by native ST8 CPS. Histograms show mean + SD. Statistical analysis (one-tailed, unpaired t test with Welch's correction) of at least six spots was performed. Asterisks indicate P value: **** $P < 0.0001$.

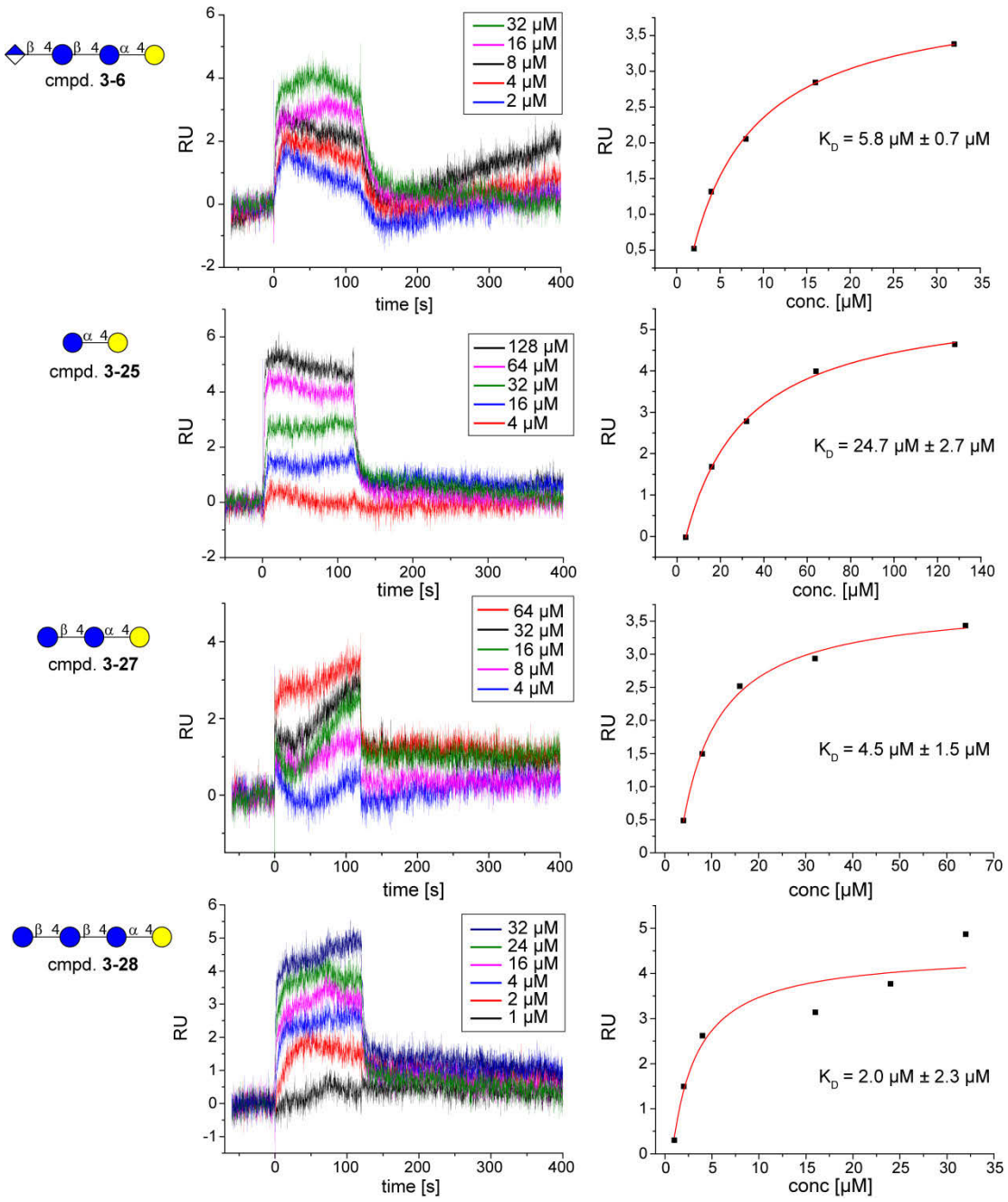


Figure 3.13. Determination of affinities of mAb 1H8 towards synthetic, ST8 CPS-related oligosaccharides. MAb 1H8 was immobilized (500-1000 RU) and subjected to a flow of synthetic oligosaccharides (50 µL/min flow rate; 120 s association phase; 280 s dissociation phase). Sensorgrams were double-referenced to buffer and a flow cell containing a non-related, immobilized murine IgG.

Thus, the trisaccharide β-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Gal*p*, represented by synthetic glycan **3-27**, is identified as the smallest structure containing a protective glycotope of ST8 FS C, and further glycan extension does not lead to improved recognition by polysaccharide-binding mAbs (Fig. 3.14). Particularly, the charge present

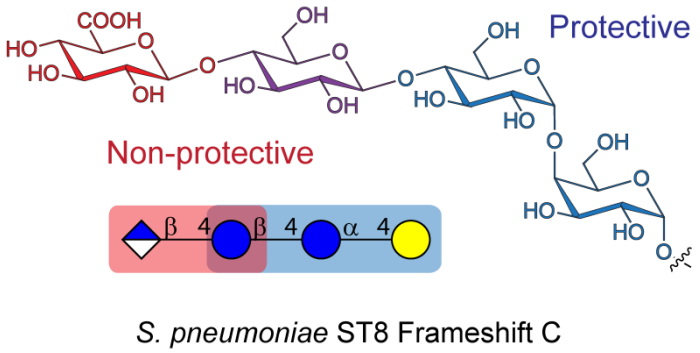


Figure 3.14. Protective (blue) and non-protective (red) glycotopes of ST8 FS C.

on the terminal GlcA moiety is dispensable for mAb recognition and a part of an immunodominant, non-protective glycotope. In turn, truncation of the protective trisaccharide by one monosaccharide at either end abrogates recognition by these mAbs.

3.2.8 Mapping the Glycan Binding Characteristics of Opsonizing and Agglutinating mAbs Against ST8 CPS

Contrasting the classical notion of opsonophagocytosis as the major mechanism of protection, recently-described, protective mAbs agglutinate pneumococci, but lack opsonophagocytic activity *in vitro*.[91] MAbs that display either *in vitro* phenotype have been raised after immunization with a ST8 CPS-TT glycoconjugate.[92] To shed light onto the molecular mechanisms governing these phenotypes, the glycan binding characteristics of three protective, ST8 CPS-directed murine mAbs were determined: 28H11,[xxi] an agglutinating IgM (*see* above), 31B12,[xxi] an opsonizing IgG and 30H9,[xxi] an IgG that has not been evaluated for opsonophagocytic potential.[91, 92] Furthermore, opsonizing mAb 1H8 raised against synthetic FS C (*see* above) was included in the evaluation.

Glycan microarray analysis revealed differential recognition of synthetic ST8 FSs by all three ST8 CPS-directed mAbs (Fig. 3.15A): While 28H11 robustly bound to FS C (*see* above), mAbs 30H9 and 31B12 displayed a significant preference towards FSs D and A, respectively. All interactions were inhibited by antibody pre-adsorption to ST8 CPS (Fig. 3.15B), and all mAbs bound to native ST8 CPS. It is thus concluded that, in addition to ST8 FS C, FSs A and D display protective glycotopes of the native

[xxi] MAbs 28H11, 30H9 and 31B12 were kindly provided by Prof. Liise-anne Pirofski, Albert Einstein College of Medicine, New York, USA.

polysaccharide. Since agglutinating 28H11 and opsonizing 1H8 target the same ST8 frameshift, it is unlikely that glycotope specificity determines the *in vitro* phenotype.

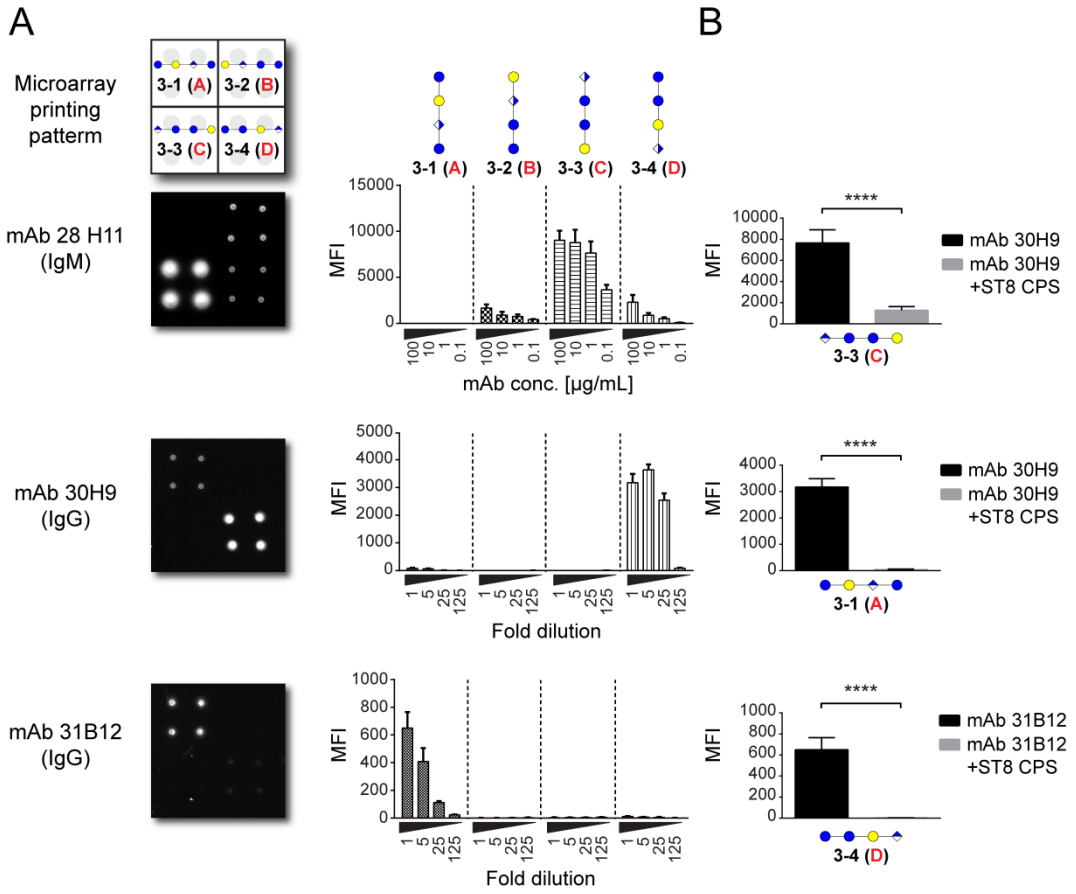


Figure 3.15. Glycotope mapping of protective, ST8 CPS-directed mAbs. *A*, glycan array analysis of mAbs 28H11 (absolute concentrations), 30H9 and 31B12 (dilutions of cell culture supernatants for both). Histograms show mean + SD of eight spots. *B*, inhibition of antibody binding of indicated FSs by pre-adsorption with native ST8 CPS (10 µg/mL). Statistical analysis (One-tailed, unpaired t test with Welch's correction) of eight spots was performed. Asterisks indicate P value: **** $P < 0.0001$. Bars depict mean + SD.

The *in vitro* ST8 recognition phenotype of anti-capsular mAbs may be influenced by glycan binding characteristics other than targeted frameshifts. For instance, polysasccharide binding affinity or the specificity for internal versus terminal glycotopes may influence mAb behaviour (*see* above).[95] Using SPR, the characteristics of ST8 CPS binding by mAbs 28H11, 30H9, 31B12 and 1H8 were studied (Fig. 3.16).

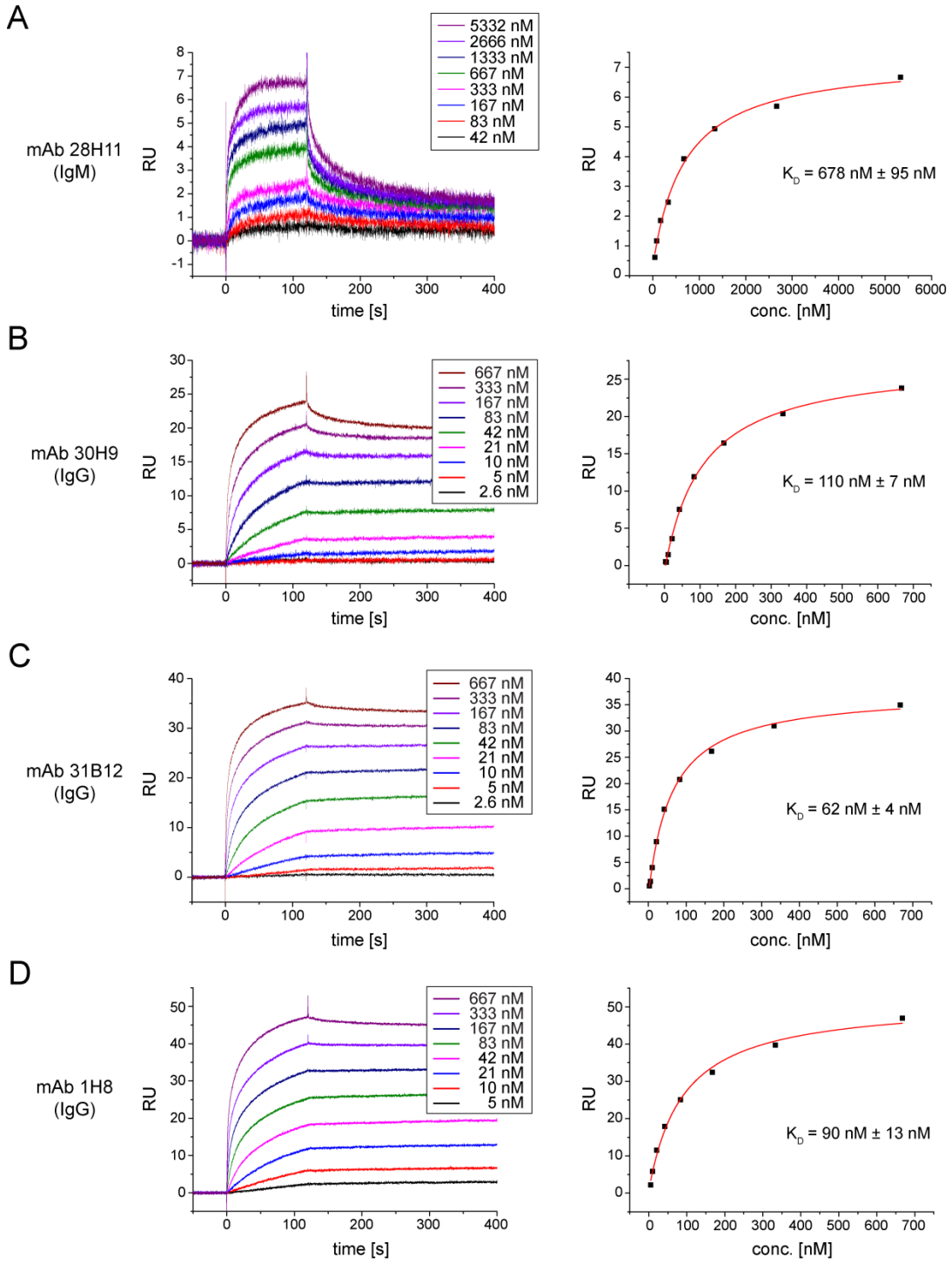
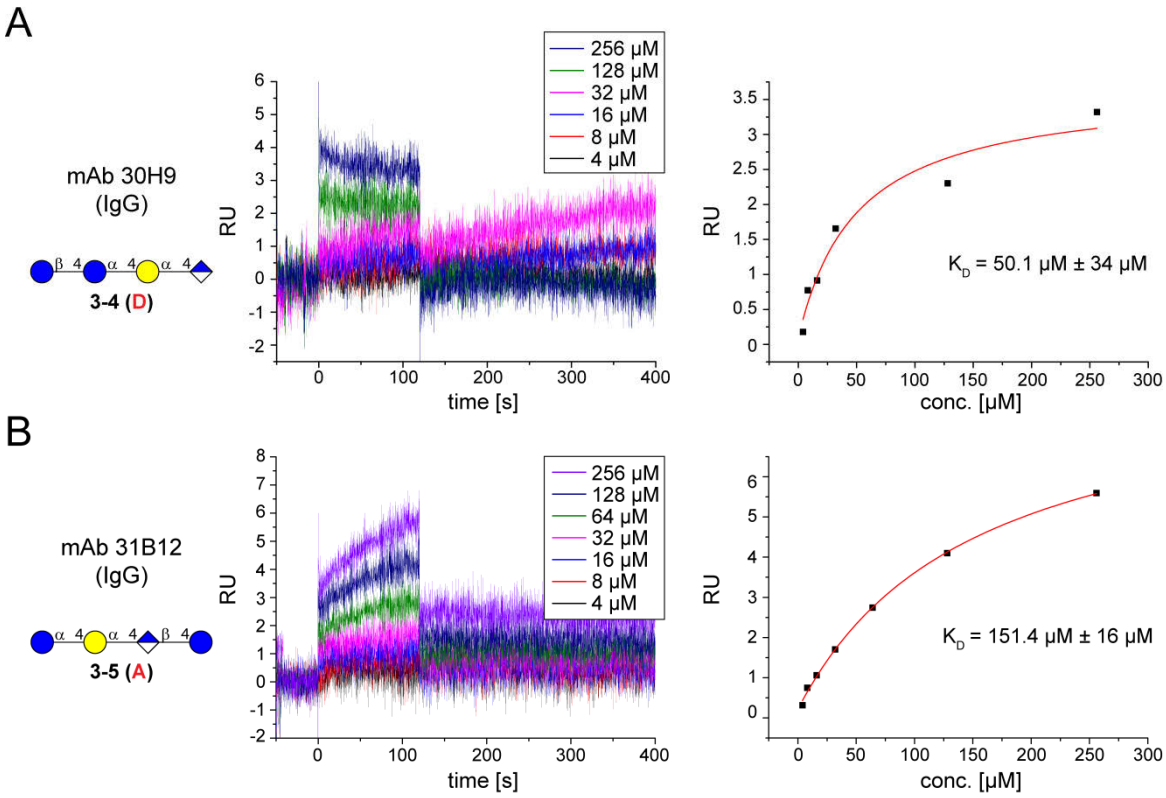


Figure 3.16. Characterization of ST8 CPS binding of protective anti-capsular mAbs by SPR. MAbs 28H11 (*A*), 30H9 (*B*) , 31B12 (*C*) or 1H8 (*D*) were immobilized (500-1000 RU) and subjected to a flow of ST8 CPS (30 µL/min flow rate; 120 s association phase; 280 s dissociation phase). Sensorgrams were double-referenced to buffer and a flow cell containing a non-related, immobilized murine IgG.

Striking binding differences were observed, with 28H11 (K_D = 678 nM ± 95 nM, Fig. 3.16A) displaying a five- to tenfold lower affinity towards ST8 CPS than 30H9 (K_D = 110 nM ± 7 nM, Fig. 3.16B), 31B12 (K_D = 62 nM ± 4 nM, Fig. 3.16C) and 1H8 (K_D

= 90 nM ± nM, Fig. 3.16D). Affinities towards the preferred ST8 FSs (*see* Fig. 3.8) of mAbs 30H9 (K_D = 50.1 µM ± 34 µM towards FS D (**3-4**), Fig. 3.17A) and 31B12 (K_D = 151.4 µM ± 15 µM towards FS A (**3-5**), Fig. 3.17B) were found to be in the micromolar range and one order of magnitude lower than the affinity of mAb 1H8 towards FS C (**3-6**, 5.8 µM, *see* Supporting Fig. 3.11). The affinity of mAb 28H11 towards FS C (**3-6**) was not measurable by SPR (not shown).



Supporting Figure 3.17. Characterization of synthetic ST8 tetrasaccharide binding of protective anti-capsular mAbs by SPR. MAbs 30H9 (*A*) or 31B12 (*B*) were immobilized (500-1000 RU) and subjected to a flow of ST8 CPS (30 µL/min flow rate; 120 s association phase; 280 s dissociation phase). Sensorgrams were double-referenced to buffer and a flow cell containing a non-related, immobilized murine IgG.

When assessing the ST8 CPS binding behavior by SPR, pronounced CPS dissociation was observed from immobilized mAb 28H11, but not from immobilized mAbs 30H9, 31B12 and 1H8 (Fig. 3.16). Glycan dissociation from the latter group of mAbs was enhanced when using synthetic tetrasaccharides as analytes (Fig. 3.17). These findings suggest that agglutinating mAb 28H11 may specifically bind terminal glycotopes at the non-reducing end of the polysaccharide chain with low affinity, while opsonizing mAbs 1H8 and 31B12 as well as mAb 30H9 display high affinities towards internal glycotopes (Table 3.1).

Table 3.1. ST8 CPS-binding mAbs used in this study.

mAb	Raised against	Specificity (FS)	*In vitro* phenotype	Glycotope location	Affinity to ST8 CPS [nM]
28H11	TT-ST8 CPS[92]	C	agglutination	terminal (?)	678 ± 95
30H9	TT-ST8 CPS[92]	D	unknown	internal	110 ± 7
31B12	TT-ST8 CPS[92]	A	opsonization	internal	62 ± 4
1H8	CRM197-FS C (**3-6**)	C	opsonization	internal	90 ± 13

3.3 Conclusion and Outlook

3.3.1 Elucidation of a Protective Glycotope of ST8 CPS

The design of synthetic immunogens from first principles is key to the development of novel glycoconjugate vaccines but cannot yet be addressed satisfactorily. Some evidence into the role of distinct glycotopes for immunogenicity has been obtained during studies aimed at developing glycoconjugate vaccines based on synthetic oligosaccharides.[1, 2] Glycan-based antigens that induce a protective antibody response have been identified for *S. pneumoniae* serotypes 3 and 14 via laborious total synthetic efforts paired with immunization trials.[109, 334] Bundle used structural analysis to reverse-engineer a carbohydrate-recognizing mAb and develop a vaccine hapten against *Candida albicans*.[144, 145] This approach works well when structural information is available, but crystal structures are often difficult to obtain.

Here, an approach is described to learn about glycan immunogenicity and protective glycotopes of *S. pneumoniae* serotype 8 CPS. This serotype was chosen to illustrate the principle since it is an important non-PCV target and had been studied only marginally by chemical synthesis.[347, 348] No information was available on the immunogenicity of ST8 CPS fragments. The strategy employed here featured glycotope mapping using all four tetrasaccharide frameshifts of ST8 CPS. Depending on the geometry of antigen binding sites and the structure of glycan antigens,[310] carbohydrate-binding antibodies have been found to accommodate between one and six monosaccharide residues.[146, 147, 311] In spite of the possibility to neglect longer glycotopes, screening all ST8 tetrasaccharide frameshifts was rationalized by the relatively small glycans associated with protective immunity in other *S. pneumoniae* serotypes.[109, 111, 334]

Furthermore, utilizing longer oligosaccharides from the outset may impair glycotope mapping due to significant structural overlap between glycan probes.

Antisera raised against polysaccharides can provide valuable information about the glycotopes that are predominantly recognized by the immune system. However, polyclonal sera often contain non-protective antibodies that may be induced by immunodominant glycotopes in native glycans, co-isolated impurities or artifacts arising during isolation and antigen preparation.[92, 99, 116, 349, 350] Additionally, non-related antibodies in sera may cross-react with synthetic structures (*see* Fig. 3.2). The latter interaction can be uncovered by inhibition of binding with the native polysaccharide, ruling out potential false-positive screening results. Since mAbs recognize glycotopes of restricted lengths in a defined manner, information on a protective glycotope can be obtained easier by studying mAbs than by studying polyclonal antisera. Protective mAb 28H11 exhibited a clear preference for FS C in glycan microarray screening experiments, and this binding event was chosen for mAb reverse engineering.

The design of a scalable synthetic route for the generation of multiple, CPS-derived glycans can be challenging, especially if multiple 1,2-*cis*-linkages have to be introduced. An efficient strategy was developed for the synthesis of ST8 CPS-derived oligosaccharides that alleviates the difficulties in stereoselectivities seen during automated glycan assembly.[xxii] Remarkable 1,2-*cis*-stereoselectivities were achieved using disaccharide **3-9** as a glycosylating agent, even when a primary alcohol found in a linker precursor was employed as the nucleophile. This finding is in stark contrast to the low stereoselectivities often obtained using this and similar, highly nucleophilic alcohols in glycosylation reactions.[193] The herein developed route served to access ST8 CPS FSs A (18% over 8 steps) and C (11% over 11 steps) in high-yielding linear syntheses from precursor **3-14** and was extended to the generation of a family of FS C-related glycans.

Upon immunization, a CPS frameshift hapten that harbors a protective glycotope may still display glycotopes that induce non-protective immunity. The striking preference of mAb 28H11 for FS C was contrasted by the predominant induction of an antibody response against the terminal cellobiuronic acid moiety following immunization with a FS C glycoconjugate. Cellobiuronic acid represents the repeating unit of *S.*

[xxii] *See* dissertation of Heung Sik Hahm.

pneumoniae serotype 3 CPS, and ST3-derived oligosaccharides are among the most immunogenic synthetic oligosaccharides described to date.[109] It is thus not surprising that ST8 FS C primarily induced an immune response directed against the terminal cellobiuronic acid moiety, but not against native ST3 or ST8 polysaccharides. In spite of the immunodominance of cellobiuronic acid, murine FS C-directed mAbs were generated that specifically bound ST8 pneumococci and retained the frameshift binding pattern of anti-ST8 CPS mAb 28H11. Bacterial binding was associated with the recognition of internal glycotopes in the native ST8 polysaccharide, thus confirming the presence of a protective, albeit less immunogenic glycotope within that tetrasaccharide frameshift.

S. pneumoniae ST8 is a particularly virulent serotype in both humans and mice, with rapid bloodstream invasion as a frequent clinical outcome of infections.[19, 20, 92, 322, 323] In contrast, clinical symptoms of pneumonia are mild compared to other serotypes such as ST3 (our own observations). Similar to mAbs raised against ST8 CPS,[89, 91] mAb 1H8 raised against synthetic FS C mediated opsonophagocytic killing of pneumococci and protected from infection by preventing bloodstream invasion in a dose-dependent fashion. A clear-cut correlation between systemic antibody levels and bacterial burden was observed, whereby bacteremia was not observed in the presence of mAb 1H8. A lower dose of mAb 1H8 was associated with delayed bacteremia and prolonged survival, connected to a steep decline of mAb levels that was probably caused by antibody consumption in the process of phagocyte-dependent bacterial killing.

A detailed investigation of mAb 1H8 using an array of synthetic, ST8 CPS-derived oligosaccharides suggests that the minimal protective glycotope is the trisaccharide β-D-Gl*p*c-(1→4)-α-D-Glc*p*-(1→4)-α-D-Gal*p*. Interestingly, a FS C-derived tetrasaccharide harboring a terminal glucose unit instead of glucuronic acid (**3-28**) was recognized by anti-FS C mAb 1H8 with a similar affinity as FS C, indicating that glucuronic acid is not part of the protective glycotope of ST8 FS C. Instead, the glucuronic acid-containing cellobiuronic acid moiety is a crucial determinant for the induction of a non-protective immune response upon immunization with FS C as a hapten.

In preliminary experiments, immunization of mice with a glycoconjugate containing FS C tetrasaccharide congener **3-28** induced antibodies that were reactive towards ST8 CPS and FS C (data not shown). However, these results could not be translated to a

statistically significant number of animals, underscoring the low immunogenicity of the protective glycotope. Presentation of the synthetic antigens used on a carrier protein may not be ideal to induce antibodies that are directed against an internal glycotope such as the protective trisaccharide identified herein. Thus, turning oligosaccharides that contain protective glycotopes of ST8 CPS, such as tetrasaccharide **3-28**, into productive vaccine haptens may likely involve optimization of antigen presentation on the carrier protein.

The immunogenicity of synthetic ST8-related glycans tested as haptens may be associated with the animal model used for immunizations. Antibody repertoires displayed by mice differ from other mammals, including humans,[351, 352] and species-specific variations of antigen immunogenicity have been observed for certain carbohydrate haptens (observations by members of the Seeberger group). Thus, ST8 CPS-based oligosaccharides may be efficient immunogens in animal models other than mice, and the respective immunization experiments are underway.

In summary, a protective trisaccharide glycotope of *S. pneumoniae* ST8 has been identified by combining automated glycan assembly, glycan microarray analysis and immunological evaluation. A plethora of synthetic oligosaccharides helped identifying both protective and non-protective glycotopes within a single synthetic frameshift.

3.3.2 Characterization of Glycan Binding Properties of Opsonizing and Agglutinating Monoclonal Antibodies Against ST8

Anticapsular antibodies are thought to protect from pneumococcal infection by mediating opsonophagocytic killing. This model has been extended by the recent discovery of mAbs that protect from *S. pneumoniae* infection, but do not trigger opsonophagocytosis.[89, 91, 92, 332] These mAbs induce agglutination of bacteria and enhance the transformation efficiency of pneumococci,[91] and fratricide as a result of bacterial quorum sensing has been suggested as a possible means of protection.[353] Multiple factors may influence the function of these antibodies, including Ig isotype, structure of antigen binding sites and glycan binding characteristics. The results presented herein suggest that target glycotope does not correlate with *in vitro* phenotype. First, distinct glycotope specificities were found for all mAbs targeting ST8 CPS, including the two opsonizing

mAbs 31B12 (FS A) and 1H8 (FS C) as well as mAb 30H9 (FS D).[91] Second, agglutinating mAb 28H11 displayed the same overall glycotope specificity to FS C as opsonizing mAb 1H8 (*see* above), incidating that specificity towards FS C alone does dictate an agglutinating phenotype. Marked differences in both affinity and location of target glycotopes were found for mAbs that either agglutinate or opsonize bacteria. Only agglutinating mAb 28H11 bound to terminal glycotopes at the non-reducing end of the polysaccharide chain. The affinity of IgM 28H11 towards ST8 CPS was at least fivefold weaker than the affinity of IgGs 30H9, 31B12 and 1H8, as expected considering affinity maturation during the generation of IgG isotypes. Of note, the Ig isotype is unlikely to be a major determinant for the *in vitro* phenotype, since an agglutinating IgG has been described against ST3 CPS[89, 91] and antibodies of the IgM isotype are known to efficiently deposit C3 complement, priming bacteria to opsonophagocytosis by a range of effector cells.[332, 354]

The three-dimensional structures of antigen binding sites that define antibody specificities are mainly determined by CDR sequences. Assigning these peptide sequences to target glycotopes is crucial to understand carbohydrate-protein interactions. Interestingly, the CDR3 peptide sequences of ST8 CPS-binding mAbs 28H11 and 30H9 are nearly identical despite their differences of target glycotopes.[92] The structural interpretation of antigen binding sites based on CDR sequences is hampered by the variability of these sites.[310] More detailed structural analysis will be required to relate CDR sequences to glycotope specificity.

Taken together, the results presented herein point towards a role of either glycotope location or affinity towards native CPS in determining the *in vitro* phenotype. MAb 30H9 has not been studied for opsonizing capacity. This mAb displays a strong preference for FS A and a binding behavior towards ST8 CPS similar to all other IgGs tested herein. It will be interesting to study the capacity of 30H9 to mediate opsonophagocytosis and correlate that data to glycan binding charcteristics. Additionally, the investigation of other ST8 CPS-targeting antibodies will provide further insight into the molecular mechanisms of protection from pneumococcal infection.

3.5 Experimental Section

3.5.1 Methods of Synthetic Chemistry

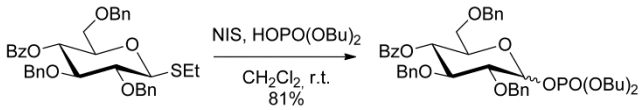
General Experimental Details

Commercial grade solvents and reagents were used unless stated otherwise. Anhydrous solvents were obtained from a Dry Solvent System (Waters, Milford, USA). Solvents for chromatography were of technical grade and distilled under reduced pressure prior to use. Sensitive reactions were carried out in oven-dried glassware and under an argon atmosphere. Molecular sieves were activated by heating under high vacuum prior to use. Analytical thin layer chromatography (t.l.c.) was performed on Kieselgel 60 F254 glass plates pre-coated with silica gel of 0.25 mm thickness (Macherey-Nagel, Düren, Germany). Spots were visualized with sugar stain (0.1% (v/v) 3-methoxyphenol, 2.5% (v/v) sulfuric acid in EtOH) or CAM stain (5% (w/v) ammonium molybdate, 1% (w/v) cerium(II) sulfate and 10% (v/v) sulfuric acid in water) dipping solutions. Flash chromatography was performed on Kieselgel 60 with 230-400 mesh (Sigma-Aldrich, St. Louis, USA). Solvents were removed under reduced pressure using a rotary evaporator and high vacuum (<1 mbar). Freeze-drying of aqueous solutions was performed using an Alpha 2-4 LD Lyophilizer (Christ, Osterode am Harz, Germany).

^{1}H, ^{13}C and two-dimensional NMR spectra were measured with a Varian 400-MR spectrometer or a Varian 600 spectrometer (both Agilent, Santa Clara, USA) at 298 K. Chemical shifts (δ) are reported in parts per million (ppm) relative to the respective residual solvent peaks ($CDCl_3$: δ 7.26 in ^{1}H and 77.16 in ^{13}C NMR; acetone-D_6: δ 2.05 in ^{1}H and 29.84 in ^{13}C NMR; D_2O: δ 4.79 in ^{1}H NMR). Two-dimensional NMR experiments (HH-COSY, CH-HSQC, CH-HMBC) were performed to confirm newly-formed linkages. The following abbreviations are used to indicate peak multiplicities: *s* singlet; *d* doublet; *dd* doublet of doublets; *t* triplet; *dt* doublet of triplets; *m* multiplet. Coupling constants (*J*) are reported in Hertz (Hz). NMR spectra were evaluated using MestreNova 6.2 (MestreLab Research SSL, Santiago de Compostella, Spain). Optical rotation (OR) measurements were carried out with a UniPol L1000 polarimeter (Schmidt+Haensch, Berlin, Germany) at λ = 589 nm and a concentration (c) expressed in g/100 mL in the solvent noted in parentheses. High resolution mass spectrometry by electrospray

ionization (ESI-HRMS) was performed at Freie Universität Berlin, Mass Spectrometry Core Facility, with a 6210 ESI-TOF mass spectrometer (Agilent). Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) high resolution mass spectra were recorded on a Daltonics Autoflex Speed spectrometer (Bruker, Billerica, USA) using 2,5-dihydroxyacetophenone and 2,4,6-trihydroxyacetophenone matrices for proteins and organic compounds, respectively. Infrared (IR) spectra were measured with a Spectrum 100 FTIR spectrometer (Perkin-Elmer, Waltham, USA). Schemes were prepared using ChemBioDraw Ultra 12.0.2 (Cambridgesoft, Waltham, USA).

Dibutyl (4-*O*-benzoyl-2,3,6-tri-*O*-benzyl-D-galactopyranosyl) phosphate (3-10)

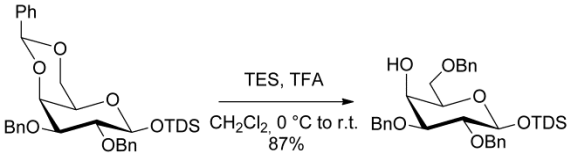


3-13β **3-10**

Thioglycoside **3-13β**[343] (100 mg, 167 µmol) was co-evaproated with anhydrous toluene (2x5 mL), kept under high vacuum for 15 min and dissolved in anhydrous CH_2Cl_2 (2 mL). The solution, over activated molecular sieves (3 Å-AW), was treated at room temperature with dibutyl phosphoric acid (66 µL, 334 µmol) and stirred for another 15 min. The mixture was then treated with NIS (56 mg, 251 µmol) and stirred for 30 min. The reaction was diluted with CH_2Cl_2 (5 mL) and quenched with a 1:1 (v/v) mixture of 10% aq. $Na_2S_2O_3$ and sat. aq. $NaHCO_3$ (5 mL). After separation, the aqueous phase was extracted with CH_2Cl_2 (3x10 mL), the combined organic fractions were dried over Na_2SO_4 and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 1:10 to 1:5 to 1:1) to give glycosyl phosphate mixture **3-10** (101 mg, 135 µmol, 81%, 1:2 α:β) as a clear oil. R_f (EtOAc/hexanes 1:4) = 0.36; $[\alpha]_D^{20}$ = +26.8° (c = 1.00, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.06 – 7.91 (m, 2H), 7.63 – 7.54 (m, 1H), 7.51 – 7.02 (m, 17H), 5.90 (dd, *J* = 7.1, 3.1 Hz, 1H), 5.44 – 5.34 (m, 1H), 5.29 (t, *J* = 7.4 Hz, 0.5H), 4.92 (dd, *J* = 13.0, 7.1 Hz, 0.5H), 4.84 – 4.69 (m, 3H), 4.62 (dd, *J* = 14.2, 11.5 Hz, 1.5H), 4.54 – 4.41 (m, 2H), 4.31 – 4.15 (m, 1H), 4.15 – 3.95 (m, 5.5H), 3.87 – 3.70 (m, 2H), 3.70 – 3.45 (m, 3H), 1.69 – 1.53 (m, 4H), 1.45 – 1.25 (m, 4H), 1.01 – 0.80 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.4, 165.3, 137.99, 137.97, 137.74, 137.70, 137.68, 137.65, 133.4, 133.3, 129.92, 129.89, 129.7, 129.6, 128.51, 128.45, 128.32, 128.29, 128.27, 128.2, 128.1, 128.0, 127.89, 127.87, 127.85, 127.79, 127.75, 127.73, 127.66, 127.65, 127.6, 98.8, 98.7, 95.13, 95.07, 82.0, 81.9, 81.4, 79.2, 79.1, 78.0, 77.4, 75.3, 75.2, 75.1, 74.2, 73.7,

73.6, 73.2, 71.2, 70.9, 70.2, 69.3, 68.7, 68.00, 67.95, 67.8, 67.7, 32.34, 32.29, 32.3, 32.22, 32.19, 18.72, 18.69, 18.66, 13.73, 13.69; IR (thin film) 2962, 2875, 1729, 1603, 1455, 1364, 1267, 1104, 1028, 954, 737, 712, 698 cm^{-1}; HRMS (ESI) calcd. for $C_{42}H_{51}O_{10}P$ $(M+Na)^+$ 769.3118 found 769.3140 *m/z*.

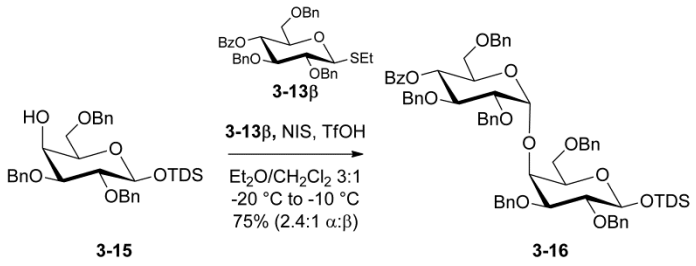
Thexyl 2,3,6-tri-*O*-benzyl-β-D-galactopyranoside (3-15)



3-14 **3-15**

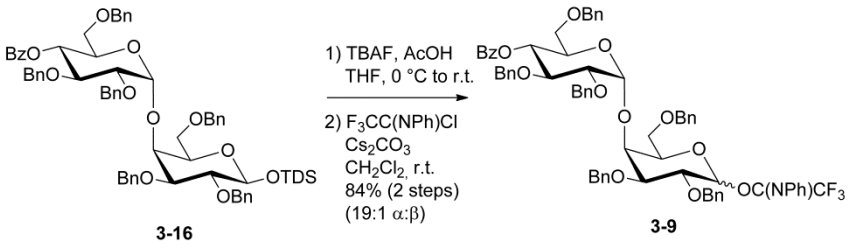
To a stirred solution of benzylidene acetal **3-14**[342] (1.68 g, 2.84 mmol) in CH_2Cl_2 (60 mL) over activated molecular sieves (3 Å-AW) were added at 0 °C TES (2.72 mL, 17.06 mmol) and trifluoroacetic acid (1.81 mL, 17.06 mmol). The mixture was slowly warmed to room temperature and stirred for 16 h at that temperature. The reaction was quenched with Et_3N (2 mL), filtered through Celite and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 1:20 to 1:7) to give alcohol **3-15** (1.46 g, 2.46 mmol, 87%) as a clear oil. R_f (EtOAc/toluene/hexanes 1:1:3) = 0.68; $[\alpha]_D^{20}$ = +12.5° (c = 2.20, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42 – 7.27 (m, 15H), 4.93 (d, *J* = 11.0 Hz, 1H), 4.75 (d, *J* = 11.0 Hz, 1H), 4.70 (s, 2H), 4.62 – 4.56 (m, 3H), 4.01 (s, 1H), 3.80 (dd, *J* = 9.8, 5.9 Hz, 1H), 3.70 (dd, *J* = 9.8, 6.0 Hz, 1H), 3.57 (m, 2H), 3.49 (dd, *J* = 9.4, 3.4 Hz, 1H), 2.51 (d, *J* = 1.6 Hz, 1H), 1.69 (dt, *J* = 13.7, 6.8 Hz, 1H), 0.92 – 0.85 (m, 12H), 0.20 (d, *J* = 10.5 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 138.8, 138.2, 138.1, 128.6, 128.5, 128.4, 128.1, 127.99, 127.98, 127.8, 127.7, 98.4, 81.0, 80.9, 75.4, 73.8, 73.3, 72.6, 69.5, 67.1, 33.9, 25.0, 20.3, 20.1, 18.8, 18.6, -1.6, -3.0; IR (thin film) 2866, 1497, 1454, 1365, 1252, 1181, 1072, 1029, 876, 832, 781, 735, 696 cm^{-1}; HRMS (ESI) calcd. for $C_{35}H_{48}O_6Si$ $(M+Na)^+$ 615.3117 found 615.3104 *m/z*.

Thexyl 4-*O*-benzoyl-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-β-D-galactopyranoside (3-16)



Alcohol **3-15** (550 mg, 0.93 mmol) and thioglycoside **3-13β**[343] (667 mg, 1.11 mmol) were co-evaporated with anhydrous toluene (3x10 mL) and kept under high vacuum for 30 min. The mixture was dissolved in Et_2O (14 mL) and CH_2Cl_2 (2.8 mL) and stirred over activated molecular sieves (3 Å-AW) for 30 min at room temperature. The solution was cooled to -20 °C and treated with NIS (250 mg, 1.11 mmol) and TfOH (16 µL, 0.19 mmol). The mixture was stirred for 1 h and slowly warmed to -10 °C. The reaction was quenched with Et_3N (0.05 mL), diluted with CH_2Cl_2 (20 mL), filtered through Celite and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 0:1 to 1:8 to 1:6) to give disaccharide **3-16** (553 mg, 0,490 mmol, 53%) along with the corresponding β-anomer (231 mg, 0.205 mmol, 22%). Analytical data for **3-16**: Clear oil. R_f (EtOAc/hexanes 1:1) = 0.28; $[\alpha]_D^{20}$ = +70.6° (c = 1.46, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.95 (dd, *J* = 8.3, 1.3 Hz, 2H), 7.64 – 7.52 (m, 1H), 7.49 – 7.02 (m, 32H), 5.52 (dd, *J* = 10.1, 9.5 Hz, 1H), 5.12 (d, *J* = 3.4 Hz, 1H), 4.99 (d, *J* = 11.1 Hz, 1H), 4.88 (t, *J* = 12.0 Hz, 2H), 4.84 – 4.59 (m, 6H), 4.49 – 4.33 (m, 2H), 4.33 – 4.20 (m, 4H), 4.14 (d, *J* = 2.9 Hz, 1H), 4.01 (m, 1H), 3.83 – 3.63 (m, 2H), 3.62 – 3.48 (m, 2H), 3.44 (dd, *J* = 10.0, 2.9 Hz, 1H), 3.05 (m, 2H), 1.80 – 1.64 (m, 1H), 1.04 – 0.84 (m, 12H), 0.29 (s, 3H), 0.23 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.2, 138.9, 138.61, 138.58, 138.2, 138.1, 137.9, 133.0, 130.3, 129.8, 128.5, 128.43, 128.39, 128.37, 128.3, 128.2, 128.14, 128.12, 128.0, 127.8, 127.7, 127.6, 127.5, 127.50, 127.45, 127.4, 99.5, 98.7, 81.3, 80.8, 80.3, 79.0, 75.1, 75.0, 74.9, 73.7, 73.6, 73.4, 73.3, 72.7, 70.8, 69.5, 68.1, 67.6, 34.2, 25.0, 20.4, 18.8, 18.7, -1.6, -2.3; IR (thin film) 2866, 1727, 1497, 1454, 1364, 1267, 1096, 834, 782, 734, 697 cm^{-1}; HRMS (ESI) calcd. for $C_{69}H_{80}O_{12}Si$ $(M+Na)^+$ 1151.5316 found 1151.5293 *m/z*.

4-*O*-Benzoyl-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-αβ-D-galactopyranosyl trifluoro-(*N*-phenyl)acetimidate (3-9)

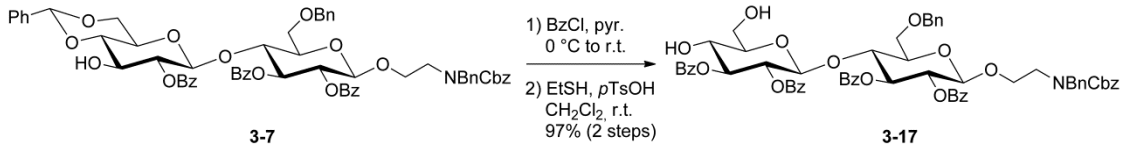


To a stirred solution of silyl ether **3-16** (470 mg, 0.416 mmol) in THF (8.3 mL) were added at 0 °C acetic acid (0.24 mL, 4.19 mmol) and tetrabutylammonium fluoride(1.0 M solution in THF, 4.2 mL, 4.20 mmol). The reaction was slowly warmed to room temperature and stirred for 2 h at that temperature. Acetic acid (0.24 mL, 4.19 mmol) and tetrabutylammonium fluoride (1.0 M solution in THF, 4.2 mL, 4.20 mmol) were added to drive the reaction to completion and the mixture was stirred for 16 h at room temperature. The reaction was diluted with Et_2O (50 mL), washed with water (3x30 mL) and the aqueous phase was re-extracted with Et_2O (2x20 mL). The combined organic fractions were dried over Na_2SO_4 and concentrated. The residue was filtered through a short plug of silica gel (EtOAc/hexanes 1:3 to 1:1) to give the intermediate lactol mixture as a clear oil.

To a stirred solution of the intermediate lactol mixture in CH_2Cl_2 (7.8 mL) were added at room temperature cesium carbonate (318 mg, 0.975 mmol) and $F_3CC(NPh)Cl$ (202 mg, 0.975 mmol). The mixture was stirred for 2.5 h at that temperature, diluted with hexanes containing 0.5% (v/v) Et_3N (10 mL) and filtered through Celite. The residue was purified by flash chromatography (EtOAc/hexanes 0:1 + 0.5% (v/v) Et_3N to 1:3 + 0.5% (v/v) Et_3N) to give imidate mixture **3-9** (404 mg, 0.349 mmol, 84% over two steps, predominantly α-isomer) as a clear oil. R_f (EtOAc/hexanes 1:3) = 0.63 – 0.74; $[\alpha]_D^{20}$ = +65.4° (c = 1.00, acetone); ^{1}H NMR (400 MHz, acetone-D_6) δ 8.03 – 7.95 (m, 2H), 7.76 – 7.61 (m, 1H), 7.56 – 7.00 (m, 35H), 6.93 – 6.72 (m, 2H), 5.44 (t, *J* = 9.8 Hz, 1H), 5.25 (d, *J* = 3.3 Hz, 1H), 4.96 – 4.58 (m, 9H), 4.55 – 4.46 (m, 1H), 4.45 – 3.96 (m, 10H), 3.77 (m, 1H), 3.64 (m, 1H), 3.30 – 3.13 (m, 2H); ^{13}C NMR (100 MHz, acetone-D_6) δ 165.7, 139.6, 139.4, 133.9, 131.3, 130.4, 129.9, 129.7, 129.4, 129.2, 129.1, 128.8, 128.8, 128.6, 128.5, 128.41, 128.37, 128.3, 128.2, 128.1, 128.0, 126.6, 125.1, 121.6, 120.1, 99.8, 81.6, 81.3, 79.7, 78.5, 75.5, 75.4, 74.1, 73.8, 73.5, 73.1, 72.2, 70.3, 69.5; IR (thin film)

2868, 1724, 1454, 1315, 1268, 1209, 1102, 1028, 737, 697 cm^{-1}; HRMS (ESI) calcd. for $C_{69}H_{66}F_3NO_{12}$ $(M+Na)^+$ 1180.4434 found 1180.4458 *m/z*.

2,3-Di-*O*-benzoyl-β-D-glucopyranosyl-(1→4)-2,3-di-*O*-benzoyl-6-*O*-benzyl-β-D-glucopyranosyl-(1→1)-(2-*N*-benzyl-*N*-benzyloxycarbonylamino)ethanol (3-17)



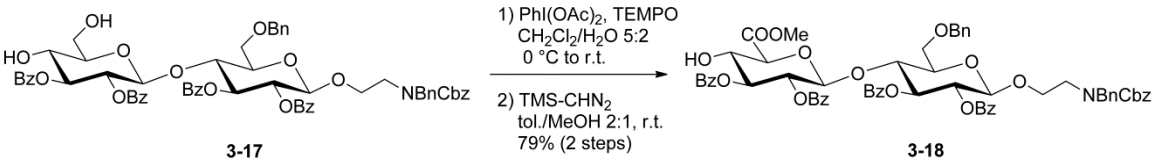
To a stirred solution of alcohol **3-7**[xxiii] (400 mg, 0.36 mmol) in pyridine (5.0 mL) was added at 0 °C benzoyl chloride (63 µL, 0.55 mmol). The reaction was slowly warmed to room temperature and stirred for 16 h at that temperature. An additional 0.5 equiv. BzCl was added to drive the reaction to completion. The mixture was stirred for 2 h at room temperature, quenched with water (30 ml) and diluted with EtOAc (50 mL). After separation, the organic fraction was washed with 0.1 M HCl (20 mL) and the aqueous fraction was re-extracted with EtOAc (30 mL). The combined organic fractions were washed with sat. aq. $NaHCO_3$ (20 mL) and brine (10 mL), dried over Na_2SO_4 and concentrated to give the intermediate tetrabenzoate as a yellow oil.

To a stirred solution of the intermediate tetrabenzoate in CH_2Cl_2 (6.5 mL) were added at room temperature ethanethiol (0.36 mL, 4.9 mmol) and *p*-toluenesulfonic acid (12 mg, 0.06 mmol). The mixture was stirred for 2 h at that temperature, quenched with Et_3N (50 µL) and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 0:1 to 1:10 to 1:5) to give diol **3-17** (389 mg, 0.349 mmol, 97% over two steps) as a white foam. R_f (EtOAc/hexanes 1:1) = 0.34; $[\alpha]_D^{20}$ = +9.1° (c = 1.0, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.02 (d, *J* = 8.0 Hz, 2H), 7.98 – 7.79 (m, 6H), 7.62 – 7.08 (m, 27H), 7.04 – 6.86 (m, 1H), 5.68 – 5.52 (m, 1H), 5.51 – 5.34 (m, 1H), 5.30 – 5.24 (m, 1H), 5.19 (m, 1H), 5.15 – 4.95 (m, 2H), 4.69 (t, *J* = 10.8 Hz, 2H), 4.53 (d, *J* = 7.9 Hz, 1H), 4.48 – 4.29 (m, 3H), 4.28 – 4.14 (m, 1H), 4.04 – 3.93 (m, 1H), 3.84 (dd, *J* = 10.3, 5.3 Hz, 1H), 3.64 (dd, *J* = 21.4, 10.1 Hz, 2H), 3.52 (d, *J* = 10.4 Hz, 1H), 3.48 – 3.13 (m, 7H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.3, 165.4, 165.2, 164.9, 156.3, 156.2, 137.92,

[xxiii] Disaccharide **3-7** was synthesized by Dr. Sharavathi G. Parameswarappa and Dr. Subramanian Govindan.

137.85, 137.79, 136.7, 136.6, 133.60, 133.55, 133.47, 133.3, 130.0, 129.8, 129.5, 129.4, 129.2, 129.0, 128.9, 128.8, 128.6, 128.53, 128.48, 128.4, 128.3, 128.2, 128.1, 128.0, 127.8, 127.3, 127.2, 101.2, 100.3, 77.4, 76.9, 75.8, 75.3, 74.6, 73.8, 73.5, 71.9, 71.8, 71.6, 69.3, 68.9, 67.4, 67.2, 67.1, 61.6, 51.7, 46.8, 45.8; IR (thin film) 3448, 2945, 1729, 1602, 1452, 1418, 1365, 1315, 1264, 1093, 1069, 1027, 989, 854, 709 cm^{-1}; HRMS (ESI) calcd. for $C_{64}H_{61}NO_{17}$ $(M+Na)^+$ 1138.3837 found 1138.3850 *m/z*.

Methyl (2,3-di-*O*-benzoyl-β-D-glucopyranosyl)uronate-(1→4)-2,3-di-*O*-benzoyl-6-*O*-benzyl-β-D-glucopyranosyl-(1→1)-(2-*N*-benzyl-*N*-benzyloxycarbonylamino)ethanol (3-18)

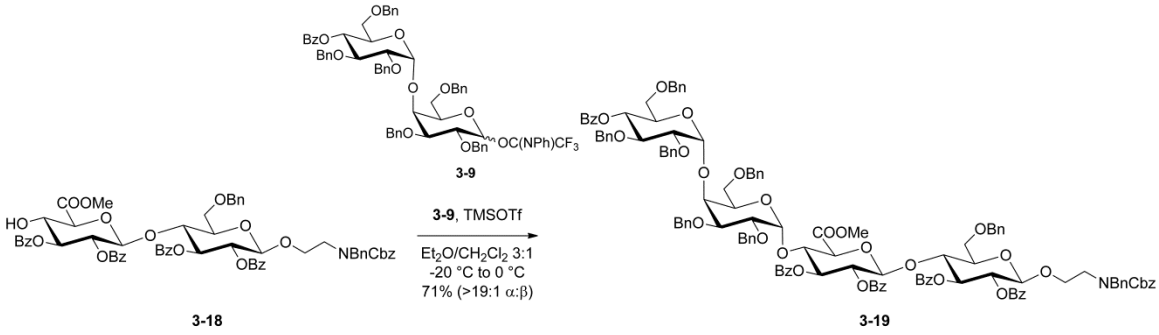


To a stirred solution of alcohol **3-17** (90 mg, 0.081 mmol) in CH_2Cl_2 (2.0 mL) and water (0.8 mL) were added at 0 °C TEMPO (2.5 mg, 0.016 mmol) and $PhI(OAc)_2$ (55 mg, 0.170 mmol). The reaction was stirred for 20 min at that temperature and warmed to room temperature. The mixture was stirred for 2 h at that temperature and diluted with EtOAc (20 mL) and water (10 mL). After separation, the aqueous fraction was extracted with EtOAc (2x10 mL), the combined organic fractions were dried over Na_2SO_4 and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 1:2 to 1:1, then 1:1 + 5% AcOH) to give the intermediate carboxylic acid as a white foam.

To a stirred solution of the intermediate carboxylic acid in toluene (1.6 mL) and MeOH (0.8 mL) was added at room temperature TMS-diazomethane (40 µL, 0.081 mmol). The reaction was stirred for 2 h at that temperature. An additional 0.25 equiv. TMS-diazomethane was added to drive the reaction to completion. The mixture was stirred for 1 h, quenched with AcOH (0.1 mL) and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 0:1 to 1:1) to give methyl ester **3-18** (73 mg, 0.064 mmol, 79% over two steps) as a clear oil. R_f (EtOAc/hexanes 1:1) = 0.47; $[\alpha]_D^{20}$ = +16.7° (c = 1.47, CH_2Cl_2); ^1H NMR (400 MHz, $CDCl_3$) δ 7.90 (m, 8H), 7.58 – 7.29 (m, 20H), 7.24 – 6.93 (m, 7H), 5.71 – 5.49 (m, 1H), 5.50 – 5.22 (m, 3H), 5.14 – 4.94 (m, 2H), 4.77 – 4.62 (m, 2H), 4.55 – 4.17 (m, 5H), 4.09 – 3.78 (m, 2H), 3.65 (m, 3H), 3.59 – 3.38 (m, 5H), 3.34 – 3.19 (m, 2H), 3.12 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ

168.4, 166.6, 165.6, 165.4, 164.8, 156.4, 156.2, 138.0, 137.9, 137.8, 136.6, 133.5, 133.31, 133.26, 133.0, 132.9, 130.06, 130.05, 130.0, 129.9, 129.7, 129.42, 129.38, 129.37, 129.11, 129.10, 129.05, 128.9, 128.6, 128.53, 128.49, 128.3, 128.2, 128.10, 128.06, 128.0, 127.8, 127.3, 127.2, 101.3, 100.4, 77.4, 75.1, 74.8, 74.6, 74.4, 73.8, 73.7, 73.4, 72.2, 72.1, 71.4, 70.4, 68.9, 67.4, 67.2, 52.7, 51.7, 46.8, 45.8; IR (thin film) 3442, 2951, 1731, 1602, 1452, 1366, 1270, 1094, 1069, 1028, 992, 752, 709 cm^{-1}; HRMS (ESI) calcd. for $C_{65}H_{61}NO_{18}$ $(M+Na)^+$ 1166.3786 found 1166.3762 *m/z*.

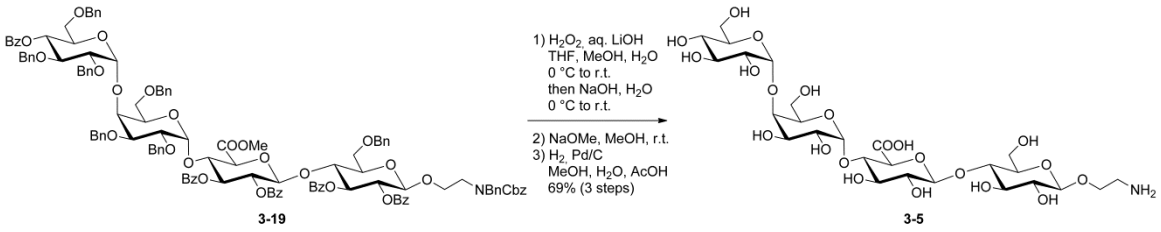
4-*O*-Benzoyl-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-galactopyranosyl-(1→4)-methyl (2,3-Di-*O*-benzoyl-β-D-glucopyranosyl)uronate-(1→4)-2,3-di-*O*-benzoyl-6-*O*-benzyl-β-D-glucoyranosyl-(1→1)-(2-*N*-benzyl-*N*-benzyloxycarbonylamino)ethanol (3-19)



Alcohol **3-18** (100 mg, 87 µmol) and imidate **3-9** (121 mg, 105 µmol) were co-evaproated with anhydrous toluene (3x10 mL) and kept under high vacuum for 30 min. The mixture was dissolved in Et_2O (3.3 mL) and CH_2Cl_2 (1.1 mL) and stirred over activated molecular sieves (3 Å-AW) for 30 min at room temperature. The solution was cooled to -20 °C and treated with TMSOTf (3.2 µL, 17 µmol). The mixture was stirred for 1 h at that temperature and slowly warmed to 0 °C. The reaction was quenched with sat. aq. $NaHCO_3$ (10 mL), extracted with CH_2Cl_2 (3x20 mL) and the combined organic fractions were dried over Na_2SO_4 and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes/toluene 1:3:3 to 1:2:2) to give tetrasaccharide **3-19** (130 mg, 62 µmol, 71%) as a clear oil. R_f (EtOAc/hexanes/toluene 1:1:2) = 0.66; $[\alpha]_D^{20}$ = +35.8° (c = 1.03, CH_2Cl_2); ^1H NMR (400 MHz,$CDCl_3$) δ 7.92 (m, 10H), 7.64 – 6.97 (m, 60H), 5.54 (m, 3H), 5.37 (m, 2H), 5.16 – 4.97 (m, 3H), 4.86 – 4.58 (m, 8H), 4.56 – 4.45 (m, 1H), 4.46 – 4.17 (m, 10H), 4.19 – 4.07 (m, 3H), 4.03 – 3.60 (m, 10H), 3.57 – 3.37 (m, 3H), 3.32 – 3.18 (m, 3H), 3.13 (s, 3H), 3.07 – 2.92 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$)

δ 167.0, 165.6, 165.5, 165.4, 165.1, 164.8, 156.3, 156.2, 138.8, 138.5, 138.4, 138.3, 138.1, 138.0, 137.9, 137.8, 136.74, 136.66, 133.5, 133.3, 133.0, 132.7, 130.3, 129.9, 129.9, 129.7, 129.42, 129.38, 129.2, 129.0, 128.6, 128.54, 128.49, 128.3, 128.2, 128.1, 127.9, 127.84, 127.79, 127.7, 127.63, 127.58, 127.5, 127.4, 127.3, 127.2, 101.2, 100.6, 100.0, 99.3, 80.1, 79.7, 77.4, 77.0, 75.8, 75.2, 75.2, 75.1, 74.7, 74.2, 73.9, 73.8, 73.5, 73.4, 73.2, 73.0, 72.4, 72.3, 71.6, 71.0, 70.0, 69.1, 68.9, 67.4, 67.2, 67.1, 66.3, 52.3, 51.7; IR (thin film) 2870, 1732, 1602, 1496, 1453, 1363, 1269, 1094, 1070, 1051, 1027, 738, 709 cm^{-1}; HRMS (ESI) calcd. for $C_{126}H_{121}NO_{29}$ $(M+Na)^+$ 2134.7921 found 2134.7879 *m/z*.

α-D-Glucopyranosyl-(1→4)-α-D-galactopyranosyl-(1→4)-β-D-glucopyranosyluronate-(1→4)-β-D-glucoyranosyl-(1→1)-(2-amino)ethanol (3-5)

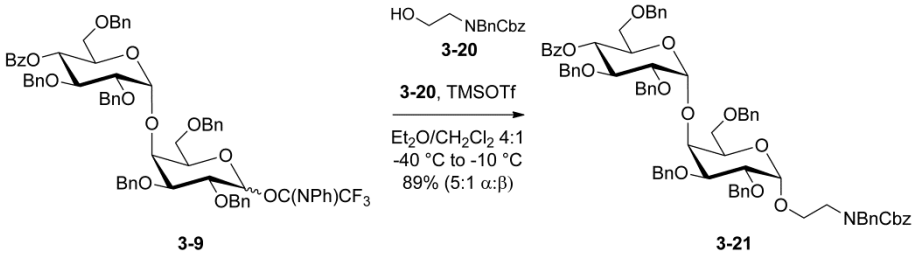


To a stirred solution of ester **3-19** (56 mg, 26 µmol) in THF (5 mL) and MeOH (1 mL) were added at 0 °C H_2O_2 (6% aq. solution, 265 µL, 530 µmol) and LiOH (1 M aq. solution, 265 µL, 132 mol). The reaction was stirred for 1 h and warmed to room temperature. The reaction was kept at that temperature and treated after 2 h with H_2O_2 (6% aq. solution, 265 µL, 530 µmol) and LiOH (1 M aq. solution, 265 µL, 132 mol). After 2 h, NaOH (15% (w/v) aq. solution, 1 mL) was added and the mixture was stirred for 72 h at room temperature. The solvents were evaporated under reduced pressure, the residue was co-evaporated with toluene (2x5 mL) and dissolved in MeOH (5 mL). The solution was treated at room temperature with NaOMe (143 mg, 2.65 mmol) and stirred for 96 h at that temperature. The solvent was evaporated and the residue was dissolved in water (5 mL). The solution was acidified at 0 °C with 0.5 M aq. $NaHSO_4$ to approx. pH 4 and extracted with EtOAc (5x5 mL). The combined organic fractions were dried over Na_2SO_4 and concentrated to give the intermediate acid as a white foam.

The intermediate acid in MeOH (2 mL) was added at room temperature to a suspension of Pd/C (50 mg) in MeOH (1 mL), water (0.1 mL) and AcOH (5 drops). The mixture was purged with hydrogen, stirred under hydrogen atmosphere for 48 h at room temperature, filtered and concentrated. The residue was purified by solid phase

extraction (Chromabond C18, Macherey-Nagel) and lyophilized to give tetrasaccharide **3-5** (acetate salt, 13.6 mg, 18 µmol, 69% over three steps) as a white solid. ^{1}H NMR (400 MHz, D_2O) δ 5.49 (d, J = 3.8 Hz, 1H), 4.89 (d, J = 3.8 Hz, 1H), 4.51 – 4.43 (2xd, J = 7.9 and 7.9 Hz, 2H), 4.11 – 4.01 (m, 3H), 3.97 – 3.83 (m, 4H), 3.82 – 3.53 (m, 13H), 3.48 (dd, J = 10.0, 3.8 Hz, 1H), 3.44 – 3.27 (m, 3H), 3.20 (t, J = 5.0 Hz, 2H); ^{13}C NMR (150 MHz, D_2O) δ 177.7, 104.7, 104.5, 102.8, 100.9, 81.01, 80.97, 78.8, 78.7, 78.6, 77.4, 76.6, 75.7, 75.4, 75.3, 74.5, 74.4, 73.5, 71.9, 71.3, 71.1, 68.4, 62.7, 62.5, 62.4, 42.0; HRMS (MALDI) calcd. for $C_{26}H_{45}NO_{22}$ $(M+Na)^+$ 746.2330 found 746.2323 *m/z*.

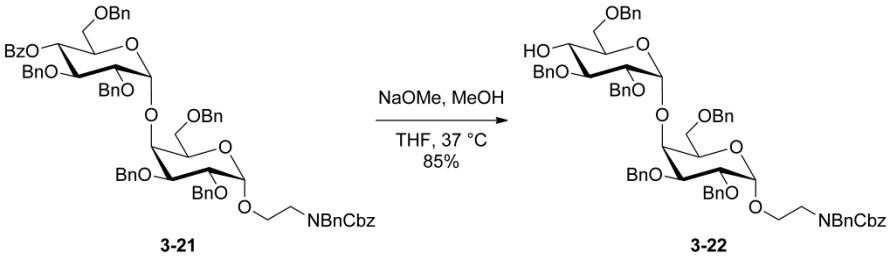
4-*O*-Benzoyl-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-galactopyranosyl-(1→1)-(2-*N*-benzyl-*N*-benzyloxycarbonylamino)ethanol (31)



Imidate **3-9** (200 mg, 0.173 mmol) and alcohol **3-20**[355] (74 mg, 0.259 mmol) were co-evaproated with anhydrous toluene (2x5 mL) and kept under high vacuum for 30 min. The mixture was dissolved in Et_2O (2.8 mL) and CH_2Cl_2 (0.7 mL) and stirred over activated molecular sieves (3 Å-AW) for 30 min at room temperature. The solution was cooled to -40 °C and treated with TMSOTf (6.2 µL, 35 µmol). The mixture was stirred for 10 min at that temperature and then slowly warmed to -10 °C. The reaction was quenched with sat. aq. $NaHCO_3$ (5 mL), extracted with CH_2Cl_2 (3x20 mL) and the combined organic fractions were dried over Na_2SO_4 and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 0:1 to 1:6 to 1:4) to give disaccharide **3-21** (160 mg, 0.128 mmol, 74%) along with the corresponding β-anomer (32 mg, 0.026 mmol, 15%). Analytical data for **3-21**: Clear oil. R_f (EtOAc/hexanes 1:3) = 0.58; $[\alpha]_D^{20}$ = +68.2° (c = 1.48, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.03 – 7.86 (m, 2H), 7.58 (t, J = 7.4 Hz, 1H), 7.48 – 7.04 (m, 42H), 5.52 (t, J = 9.8 Hz, 1H), 5.22 – 5.12 (m, 2H), 5.08 (s, 1H), 4.94 – 4.65 (m, 8H), 4.65 – 4.52 (m, 3H), 4.45 (d, J = 8.8 Hz, 1H), 4.38 (d, J = 12.1 Hz, 1H), 4.17 (m 5H), 4.06 – 3.96 (m, 1H), 3.96 – 3.79 (m, 3H), 3.76 – 3.60 (m, 2H), 3.58 – 3.34 (m, 4H), 3.12 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.2, 156.6, 156.3, 138.7, 138.33, 138.30, 138.2, 138.0, 137.9, 136.8, 133.1, 130.3, 129.8, 128.6, 128.53, 128.51,

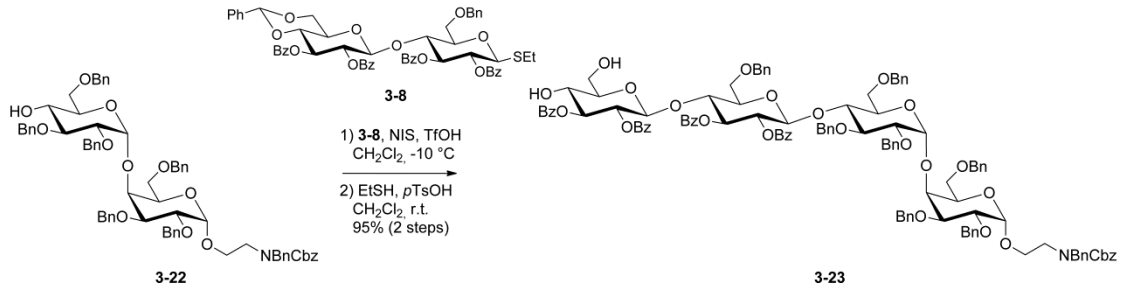
128.4, 128.3, 128.2, 128.1, 128.01, 127.96, 127.9, 127.82, 127.77, 127.61, 127.57, 127.5, 127.4, 127.3, 99.6, 98.1, 98.0, 80.1, 79.7, 77.4, 76.2, 76.1, 75.7, 75.6, 75.3, 74.1, 73.6, 73.3, 73.2, 73.1, 72.7, 71.1, 69.7, 69.6, 69.4, 68.1, 68.1, 67.7, 67.43, 67.37, 66.9, 51.4, 46.5, 45.6; IR (thin film) 3031, 2922, 1727, 1699, 1497, 1453, 1417, 1363, 1267, 1097, 1041, 1028, 736, 697 cm^{-1}; HRMS (ESI) calcd. for $C_{78}H_{79}NO_{14}$ $(M+Na)^+$ 1276.5398 found 1276.5405 *m/z*.

4-*O*-Benzoyl-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-galactopyranosyl-(1→1)-(2-*N*-benzyl-*N*-benzyloxycarbonylamino)ethanol (3-22)



To a stirred solution of ester **3-21** (126 mg, 0.100 mmol) in THF (5 mL) and MeOH (5 mL) was added at 0 °C sodium methoxide (0.5 M in MeOH, 1 mL, 0.500 mmol). The reaction was slowly warmed to room temperature and stirred for 24 h. Sodium methoxide (0.5 M in MeOH, 1 mL, 0.500 mmol) was added and the reaction was warmed to 37 °C. The mixture was stirred for 7 h at that temperature, neutralized with Amberlite IR120 (H^+ form), filtered and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 0:1 to 1:6 to 1:4) to give alcohol **3-22** (98 mg, 85 µmol, 85%) as a clear oil. R_f (EtOAc/hexanes 1:3) = 0.51; $[\alpha]_D^{20}$ = +65.3° (c = 0.5, CH_2Cl_2); ^1H NMR (400 MHz, $CDCl_3$) δ 7.44 – 7.07 (m, 40H), 5.19 – 5.10 (m, 2H), 5.01 (s, 1H), 4.94 (d, *J* = 11.3 Hz, 1H), 4.89 – 4.72 (m, 5H), 4.72 – 4.61 (m, 3H), 4.55 (q, *J* = 15.5 Hz, 2H), 4.40 (d, *J* = 12.2 Hz, 1H), 4.22 (dd, *J* = 15.8, 11.1 Hz, 4H), 4.09 (d, *J* = 6.0 Hz, 1H), 4.01 – 3.67 (m, 7H), 3.65 – 3.27 (m, 6H), 3.19 (dd, *J* = 10.2, 3.9 Hz, 1H), 2.44 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 156.6, 156.3, 139.1, 138.82, 138.77, 138.63, 138.55, 138.4, 138.2, 138.14, 138.05, 137.9, 137.7, 136.8, 136.7, 128.6, 128.52, 128.50, 128.45, 128.4, 128.3, 128.1, 128.0, 127.78, 127.75, 127.7, 127.6, 99.7, 98.2, 81.8, 80.1, 77.4, 76.4, 76.3, 75.5, 75.4, 73.9, 73.5, 73.4, 73.2, 72.7, 71.8, 70.4, 69.7, 69.6, 69.2, 68.1, 68.0, 67.4, 66.9, 66.8, 51.4, 46.5, 45.5; IR (thin film) 3031, 2925, 1699, 1497, 1454, 1364, 1234, 1096, 1059, 736, 698 cm^{-1}; HRMS (ESI) calcd. for $C_{71}H_{75}NO_{13}$ $(M+Na)^+$ 1172.5136 found 1172.5103 *m/z*.

2,3-Di-*O*-benzoyl-β-D-glucopyranosyl-(1→4)-2,3-di-*O*-benzoyl-6-*O*-benzyl-β-D-glucoyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-galactopyranosyl-(1→1)-(2-*N*-benzyl-*N*-benzyloxycarbonylamino)ethanol (3-23)



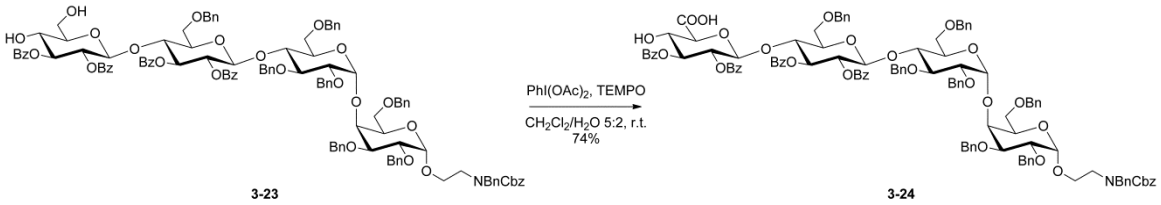
Alcohol **3-22** (47 mg, 41 µmol) and thioglycoside **3-8**[xxiv] (60 mg, 61 µmol) were co-evaporated with anhydrous toluene (2x5 mL) and kept under high vacuum for 30 min. The mixture was dissolved in CH_2Cl_2 (2 mL) and stirred over activated molecular sieves (3 Å-AW) for 30 min at room temperature. The solution was cooled to -10 °C and treated with NIS (13.8 mg, 61 µmol) and TfOH (1 µL, 11 µmol). The mixture was kept for 1 h at that temperature and slowly warmed to 0 °C. The reaction was quenched with Et_3N (50 µL), filtered and concentrated to give the intermediate benzylidene acetal as a yellow oil.

To a stirred solution of the intermediate benzylidene acetal in CH_2Cl_2 (2 mL) were added at room temperature ethanethiol (0.3 mL, 4.06 mmol) and *p*-toluenesulfonic acid (10 mg, 0.053 mmol). The mixture was stirred for 1 h at that temperature, quenched with Et_3N (20 µL) and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 0:1 to 1:3 to 1:2) to give diol **3-23** (78 mg, 39 µmol, 95% over two steps) as a clear oil. R_f (EtOAc/hexanes 1:1) = 0.67; $[\alpha]_D^{20}$ = +46.1° (c = 0.5, CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 (d, J = 7.8 Hz, 2H), 7.95 (d, J = 7.9 Hz, 2H), 7.89 (d, J = 7.8 Hz, 2H), 7.70 (d, J = 7.8 Hz, 2H), 7.59 (t, J = 7.4 Hz, 1H), 7.55 – 7.10 (m, 56H), 5.44 – 5.26 (m, 3H), 5.26 – 5.05 (m, 4H), 4.96 (s, 1H), 4.75 (t, J = 13.1 Hz, 2H), 4.69 – 4.56 (m, 4H), 4.55 – 4.42 (m, 4H), 4.35 – 4.05 (m, 8H), 4.03 – 3.84 (m, 5H), 3.79 – 3.60 (m, 5H), 3.59 – 3.25 (m, 10H), 3.23 – 3.08 (m, 2H), 3.02 (d, J = 10.0 Hz, 2H), 2.00 (s, 1H), 0.98 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.5, 165.0, 164.9, 164.7, 156.5, 156.2, 140.0, 138.8, 138.7, 138.5, 138.3, 138.2, 138.0, 137.9, 137.5, 136.8, 133.7,

[xxiv] Disaccharide **3-8** was synthesized by Dr. Sharavathi G. Parameswarappa and Dr. Subramanian Govindan.

133.6, 133.4, 133.2, 130.1, 130.0, 129.8, 129.7, 129.4, 129.2, 129.1, 129.0, 128.9, 128.84, 128.80, 128.73, 128.65, 128.6, 128.43, 128.37, 128.3, 128.13, 128.10, 128.0, 127.9, 127.6, 127.5, 127.4, 127.2, 127.0, 100.2, 99.9, 99.8, 98.5, 98.4, 80.7, 79.0, 77.4, 76.6, 76.5, 75.7, 75.2, 74.9, 74.7, 74.5, 74.3, 74.1, 73.8, 73.7, 73.6, 73.4, 73.0, 72.1, 72.0, 71.5, 70.4, 69.5, 67.74, 67.68, 67.3, 67.2, 66.9, 66.7, 61.7, 51.3, 46.4, 45.5, 29.8; IR (thin film) 2926, 1733, 1602, 1453, 1364, 1315, 1272, 1094, 1070, 1028, 737, 710 cm^{-1}; HRMS (ESI) calcd. for $C_{118}H_{117}NO_{27}$ $(M+Na)^+$ 2002.7710 found 2002.7731 *m/z*.

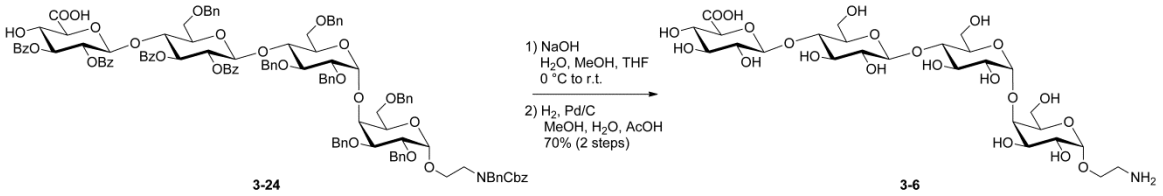
2,3-Di-*O*-benzoyl-β-D-glucopyranosyluronate-(1→4)-2,3-di-*O*-benzoyl-6-*O*-benzyl-β-D-glucoyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-galactopyranosyl-(1→1)-(2-*N*-benzyl-*N*-benzyloxycarbonylamino)ethanol (3-24)



To a vigorously stirred solution of alcohol **3-23** (45 mg, 23 µmol) in CH_2Cl_2 (2 mL) and water (0.8 mL) were added at 0 °C TEMPO (3 crystals) and $PhI(OAc)_2$ (15.4 mg, 48 µmol). The reaction was stirred for 20 min at that temperature and slowly warmed to room temperature. After 1 h, TEMPO (2 crystals) and $PhI(OAc)_2$ (10 mg, 31 µmol) were added and the mixture was stirred for 2 h at room temperature. The reaction was diluted with CH_2Cl_2 (5 mL) and quenched with 10% (w/v) aq. $Na_2S_2O_3$ (5 mL). The aqueous phase was extracted with EtOAc (2x10 mL), the combined organic fractions were dried over Na_2SO_4 and concentrated. The residue was purified by flash chromatography twice (EtOAc/hexanes 0:1 to 1:2 to 8:1, then EtOAc/hexanes 1:1 + 1% (v/v) AcOH) and co-evaporated with heptane repeatedly to give acid **3-24** (33 mg, 17 µmol, 74%) as a clear oil. R_f (EtOAc/hexanes 1:1 + 0.5% (v/v) AcOH) = 0.63; $[\alpha]_D^{20}$ = +33.7° (c = 0.5, CH_2Cl_2); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.88 (dd, *J* = 22.7, 7.7 Hz, 6H), 7.65 (d, *J* = 7.5 Hz, 2H), 7.56 (t, *J* = 7.4 Hz, 1H), 7.52 – 7.27 (m, 26H), 7.24 – 7.06 (m, 30H), 5.36 – 5.26 (m, 3H), 5.23 (d, *J* = 9.3 Hz, 2H), 5.17 – 5.03 (m, 2H), 4.92 (s, 1H), 4.75 – 4.66 (m, 3H), 4.62 – 4.52 (m, 4H), 4.51 – 4.40 (m, 4H), 4.35 (t, *J* = 10.1 Hz, 2H), 4.22 (mz, 3H), 4.13 (d, *J* = 9.9 Hz, 2H), 4.04 (d, *J* = 11.9 Hz, 2H), 3.96 (d, *J* = 11.9 Hz, 2H), 3.91 – 3.79 (m, 4H), 3.76 – 3.56 (m, 5H), 3.46 (m, 6H), 3.30 (m, 3H), 3.05 (d, *J* = 9.3 Hz, 1H), 2.97 (d, *J* = 9.6 Hz, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 166.2, 166.1, 164.9, 156.6, 156.3, 140.0,

138.8, 138.5, 138.4, 138.3, 138.0, 137.9, 137.6, 136.8, 136.7, 133.6, 133.4, 133.2, 130.0, 129.9, 129.7, 129.3, 129.2, 129.0, 128.9, 128.72, 128.69, 128.64, 128.56, 128.5, 128.4, 128.3, 128.1, 128.0, 127.9, 127.7, 127.6, 127.5, 127.4, 127.2, 127.0, 100.1, 100.0, 99.8, 98.4, 98.3, 80.6, 79.3, 76.7, 75.2, 75.0, 74.4, 74.2, 74.1, 73.6, 73.4, 73.1, 72.4, 72.2, 71.3, 70.5, 70.2, 69.6, 69.5, 67.9, 67.8, 67.5, 67.4, 67.0, 66.8, 66.8, 51.4, 46.5, 45.5; IR (thin film) 3031, 2924, 1735, 1602, 1496, 1453, 1363, 1316, 1272, 1094, 1070, 1028, 737, 710 cm^{-1}; HRMS (ESI) calcd. for $C_{118}H_{115}NO_{28}$ $(M+Na)^+$ 2016.7503 found 2016.7558 *m/z*.

β-D-Glucopyranosyluronate-(1→4)-β-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-α-D-galactopyranosyl-(1→1)-(2-amino)ethanol (3-6)

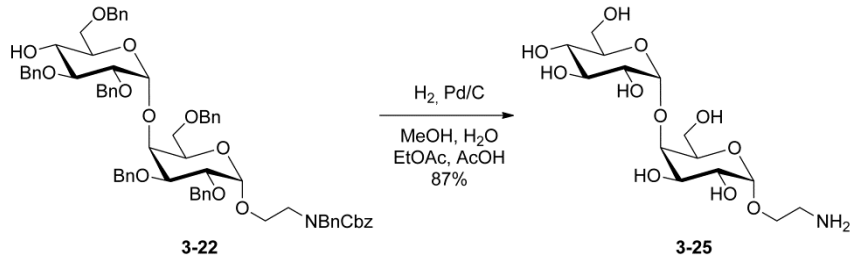


To a stirred solution of ester **3-24** (45 mg, 23 µmol) in THF (4 mL) and MeOH (0.5 mL) was added at 0 °C NaOH (1 M aq. solution, 1 mL). The reaction was slowly warmed to room temperature and stirred for 16 h at that temperature. The solution was neutralized at 0 °C with 0.5 M aq. $NaHSO_4$ and extracted with EtOAc (5x5 mL). The combined organic fractions were dried over Na_2SO_4 and concentrated to give the intermediate alcohol as a white foam.

The intermediate alcohol in MeOH (3 mL) was added at room temperature to a suspension of Pd/C (20 mg) in MeOH (6 mL), water (6 drops) and AcOH (3 drops). The suspension was purged with hydrogen, stirred under hydrogen atmosphere for 96 h, filtered and concentrated. Since the reaction had not proceeded to completion, the residue was subjected to the same conditions again and stirred for 72 h at room temperature. The mixture was filtered and concentrated, the residue was purified by solid phase extraction (Chromabond C18, Macherey-Nagel) and lyophilized to give tetrasaccharide **3-6** (11.3 mg, 16 µmol, 70% over two steps) as a white solid. ^{1}H NMR (600 MHz, D_2O) δ 5.07 (d, J = 3.7 Hz, 1H), 4.93 (d, J = 3.9 Hz, 1H), 4.58 (d, J = 8.0 Hz, 1H), 4.53 (d, J = 7.9 Hz, 1H), 4.28 – 4.21 (m, 1H), 4.10 (d, J = 2.7 Hz, 1H), 4.06 – 3.97 (m, 4H), 3.97 – 3.90 (m, 3H), 3.90 – 3.82 (m, 4H), 3.80 – 3.72 (m, 2H), 3.72 – 3.64 (m, 4H), 3.62 (dd, J = 10.1, 3.9 Hz, 1H), 3.56 – 3.51 (m, 2H), 3.40 – 3.36 (m, 2H), 3.35 – 3.26 (m, 2H); ^{13}C NMR (150 MHz, D_2O) δ 178.1, 104.9, 104.8, 102.5, 101.2, 81.23, 81.17,

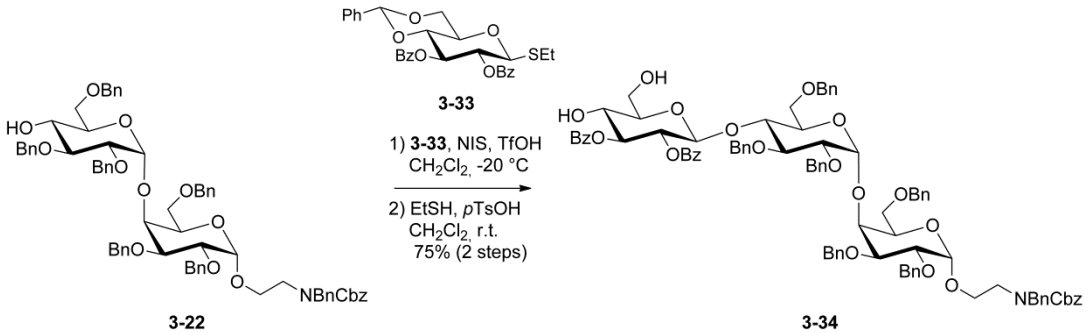
80.9, 78.4, 77.9, 77.4, 76.7, 75.53, 75.52, 74.3, 74.2, 74.1, 73.8, 73.3, 71.4, 70.8, 66.6, 63.1, 62.6, 62.1, 41.9; HRMS (MALDI) calcd. for $C_{26}H_{45}NO_{22}$ $(M+Na)^+$ 746.2330 found 746.2416 *m/z*.

α-D-Glucopyranosyl-(1→4)-α-D-galactopyranosyl-(1→1)-(2-amino)ethanol (3-25)



Benzyl ether **3-22** (10 mg, 8.69 µmol) in EtOAc (1 mL) was added at room temperature to a suspension of Pd/C (30 mg) in MeOH (3 mL), water (0.5 mL) and AcOH (3 drops). The reaction was purged with hydrogen, stirred under hydrogen atmosphere for 24 h, filtered and concentrated to give disaccharide **3-25** (acetate salt, 2.9 mg, 6.55 µmol, 76%) as a white solid. ^{1}H NMR (600 MHz, D_2O) δ 5.04 (d, J = 3.7 Hz, 1H), 4.91 (d, J = 3.8 H z, 1H), 4.16 – 4.05 (m, 2H), 3.99 (m, 3H), 3.91 (m, 2H), 3.86 – 3.69 (m, 6H), 3.54 (dd, J = 10.1, 3.8 Hz, 1H), 3.45 (t, J = 9.7 Hz, 1H), 3.32 – 3.21 (m, 2H); ^{13}C NMR (150 MHz, D_2O) δ 102.8, 101.2, 81.2, 75.3, 74.6, 74.3, 74.2, 71.8, 71.4, 70.9, 66.7, 63.1, 62.7, 41.9, 25.8. HRMS (ESI) calcd. for $C_{14}H_{27}NO_{11}$ $(M+Na)^+$ 408.1481 found 408.1499 *m/z*.

2,3-Di-*O*-benzoyl-β-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-galactopyranosyl-(1→1)-(2-*N*-benzyl-*N*-benzyloxycarbonylamino)ethanol (3-34)

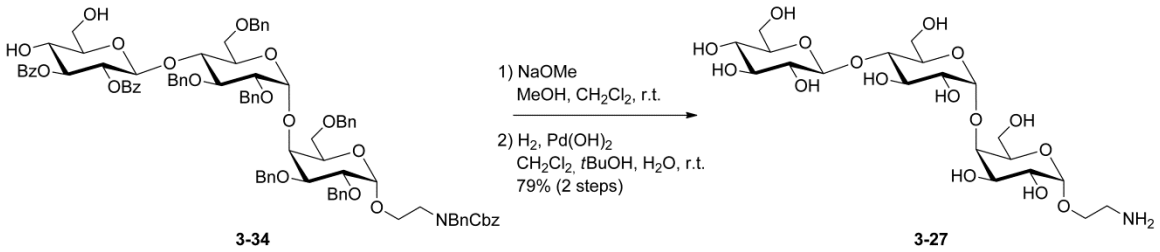


Alcohol **3-22** (15 mg, 13 µmol) and thioglycoside **3-33**[333] (20.4 mg, 39 µmol) were co-evaporated with anhydrous toluene (2x5 mL) and kept under high vacuum for 10 min. The mixture was dissolved in CH_2Cl_2 (1.3 mL) and stirred over activated molecular sieves (3 Å-AW) for 30 min at room temperature. The solution was cooled to -20 °C and

treated with NIS (8.8 mg, 39 µmol) and TfOH (1 µL, 11 µmol). The mixture was stirred for 1 h at that temperature and slowly warmed to 0 °C. The reaction was quenched with a 1:1 (v/v) mixture of sat. aq. $NaHCO_3$ (10 mL) and 10% (w/v) Na_2SO_3 (5 mL) and extracted with CH_2Cl_2 (4x10 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 1:5 to 1:4 to 1:3) to give the intermediate benzylidene acetal as a yellow oil.

To a stirred solution of the intermediate benzylidene acetal in CH_2Cl_2 (2 mL) were added at room temperature ethanethiol (0.2 mL, 2.8 mmol) and *p*-toluenesulfonic acid (6 mg, 32 µmol). The mixture was stirred for 1 h at that temperature, quenched with Et_3N (100 µL) and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 0:1 to 1:3 to 2:3) to give diol **3-34** (14.7 mg, 9.7 µmol, 75% over two steps) as a clear oil. R_f (EtOAc/hexanes 1:1) = 0.37; $[\alpha]_D^{20}$ = +26.4° (c = 0.1, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.91 (d, *J* = 7.3 Hz, 2H), 7.61 (d, *J* = 7.4 Hz, 2H), 7.52 – 7.26 (m, 27H), 7.24 – 6.97 (m, 19H), 5.38 – 5.26 (m, 1H), 5.14 – 5.07 (m, 2H), 5.05 (d, *J* = 11.0 Hz, 1H), 5.01 – 4.94 (m, 2H), 4.84 – 4.80 (m, 2H), 4.70 (dd, *J* = 14.1, 12.4 Hz, 2H), 4.62 – 4.39 (m, 6H), 4.33 – 4.25 (m, 1H), 4.18 (m, 3H), 4.10 – 3.91 (m, 4H), 3.86 (m, 2H), 3.69 (m, 6H), 3.55 – 3.19 (m, 8H), 3.02 (s, 1H), 2.95 (d, *J* = 9.5 Hz, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 167.8, 164.9, 139.54, 138.47, 138.3, 137.8, 133.7, 133.3, 130.1, 129.7, 129.1, 129.0, 128.9, 128.8, 128.6, 128.5, 128.4, 128.1, 128.0, 127.8, 127.7, 127.6, 127.4, 127.2, 99.89, 99.87, 98.5, 80.1, 79.3, 77.8, 76.0, 75.2, 74.2, 73.8, 73.1, 72.3, 71.8, 70.51, 70.48, 69.6, 67.9, 67.4, 66.9, 66.8, 62.1; IR (thin film) 3453, 2928, 1733, 1701, 1454, 1273, 1094, 1029, 739, 699 cm^{-1}; HRMS (MALDI) calcd. for $C_{91}H_{93}NO_{20}$ $(M+Na)^+$ 1542.6188 found 1542.6145 *m/z*.

β-D-Glucoyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-α-D-galactopyranosyl-(1→1)-(2-amino)ethanol (3-27)

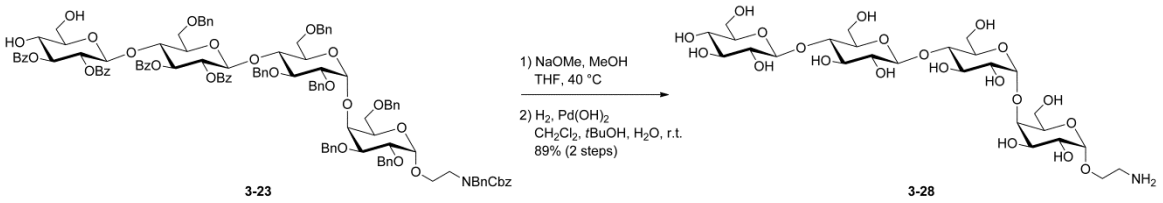


3-34 **3-27**

To a stirred solution of ester **3-34** (26 mg, 17 µmol) in CH_2Cl_2 (1 mL) and MeOH (1 mL) was added at room temperature NaOMe (0.5 M solution in MeOH, 0.5 mL). The reaction was stirred for 2 h at that temperature, neutralized at 0 °C with Amberlite IR-120 (H^+ form), filtered and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 1:3 to 2:1) to give the intermediate tetraol as a white foam.

The intermediate tetraol in CH_2Cl_2/*t*BuOH/water (1:6:2, 5 mL) was purged with argon and treated at 0 °C with a suspension of $Pd(OH)_2$ on carbon (20% (w/w) loading, 30 mg) in the same solvent mixture (1 mL). The suspension was purged with hydrogen, stirred under hydrogen atmosphere for 24 h, filtered and concentrated. Since the reaction had not proceeded to completion, the residue was subjected to the same conditions again and stirred for 48 h at room temperature. The mixture was filtered and concentrated, the residue was purified by solid phase extraction (Chromabond C18, Macherey-Nagel) and lyophilized to give trisaccharide **3-27** (7.3 mg, 13 µmol, 79% over two steps) as a white solid. ^{1}H NMR (400 MHz, D_2O) δ 5.01 (d, J = 3.3 Hz, 1H), 4.87 (d, J = 3.4 Hz, 1H), 4.49 (d, J = 7.9 Hz, 1H), 4.19 (d, J = 10.1 Hz, 1H), 4.03 (s, 1H), 4.00 – 3.74 (m, 10H), 3.67 (m, 3H), 3.55 (dd, J = 10.0, 3.5 Hz, 1H), 3.52 – 3.34 (m, 3H), 3.24 (m, 3H); ^{13}C NMR (100 MHz, D_2O) δ 102.4, 99.8, 98.4, 78.4, 78.2, 75.8, 75.4, 73.0, 71.5, 71.3, 71.1, 70.5, 69.3, 68.7, 68.1, 63.8, 60.4, 60.3, 59.3, 39.1; HRMS (ESI) calcd. for $C_{20}H_{37}NO_{16}$ $(M+Na)^+$ 570.2010 found 570.2000 *m/z*.

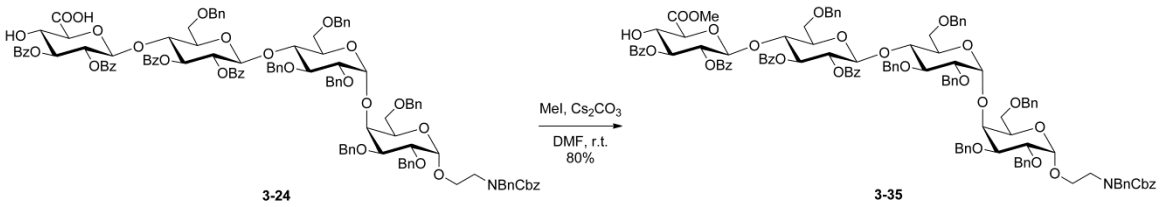
β-D-Glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-α-D-galactopyranosyl-(1→1)-(2-amino)ethanol (3-28)



To a stirred solution of ester **3-23** (20 mg, 10.1 µmol) in THF (1 mL) and MeOH (0.33 mL) was added at room temperature NaOMe (0.5 M solution in MeOH, 0.5 mL). The reaction was warmed to 40 °C and stirred for 5 h at that temperature. The mixture was cooled to room temperature and stirred for 16 h at that temperature. The reaction was neutralized with Amberlite IR-120 (H^+ form), filtered and concentrated. The residue was purified by size exclusion chromatography (CH_2Cl_2/MeOH 2:1, Sephadex® LH-20, GE Healthcare, Little Chalfont, UK) to give the intermediate hexaol as a white foam.

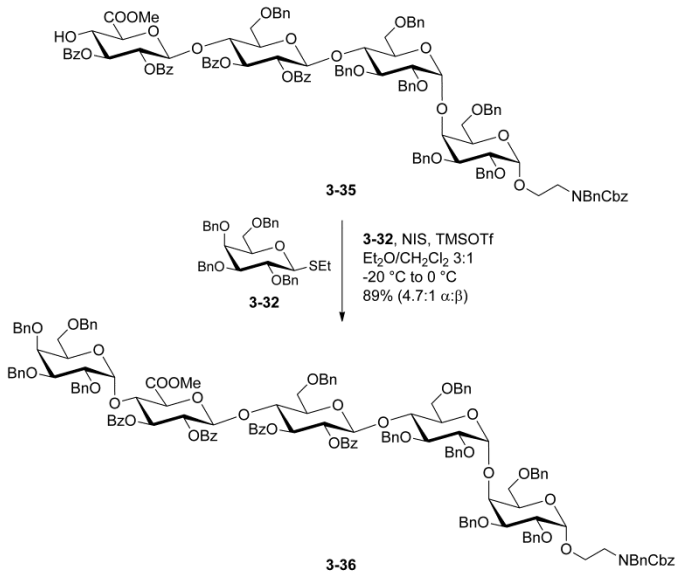
The intermediate hexaol in CH_2Cl_2/*t*BuOH/water (1:16:8, 1 mL) was purged with argon and treated at 0 °C with a suspension of $Pd(OH)_2$ on carbon (20% (w/w) loading, 20 mg) in the same solvent mixture (1 mL). The suspension was purged with hydrogen, stirred under hydrogen atmosphere for 18 h, filtered and concentrated. The residue was purified by solid phase extraction (Chromabond C18, Macherey-Nagel) and lyophilized to give tetrasaccharide **3-28** (6.8 mg, 9.0 µmol, 89% over two steps) as a white solid. ^{1}H NMR (600 MHz, D_2O) δ 5.15 (d, J = 3.5 Hz, 1H), 5.02 (d, J = 3.7 Hz, 1H), 4.66 (d, J = 7.9 Hz, 1H), 4.61 (d, J = 7.9 Hz, 1H), 4.33 (d, J = 10.1 Hz, 1H), 4.18 (d, J = 2.2 Hz, 1H), 4.14 – 4.06 (m, 4H), 4.05 – 3.97 (m, 5H), 3.96 – 3.89 (m, 4H), 3.87 – 3.67 (m, 8H), 3.64 – 3.56 (m, 2H), 3.55 – 3.49 (m, 1H), 3.46 (t, J = 8.4 Hz, 1H), 3.45 – 3.34 (m, 3H); ^{13}C NMR (150 MHz, D_2O) δ 105.2, 104.9, 102.5, 101.2, 81.2, 81.0, 80.9, 78.6, 78.1, 77.4, 76.7, 75.7, 75.6, 74.2, 74.1, 73.8, 73.3, 72.1, 71.4, 70.9, 66.6, 63.2, 63.1, 62.5, 62.1, 41.9; HRMS (ESI) calcd. for $C_{26}H_{47}NO_{21}$ $(M+Na)^+$ 732.2538 found 732.2504 *m/z*.

Methyl (2,3-di-*O*-benzoyl-β-D-glucopyranosyl)uronate-(1→4)-2,3-di-*O*-benzoyl-6-*O*-benzyl-β-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-galactopyranosyl-(1→1)-(2-*N*-benzyl-*N*-benzyloxycarbonylamino)ethanol (3-35)



To a stirred solution of carboxylic acid **3-24** (100 mg, 50 µmol) in DMF (2.5 mL) were added at room temperature Cs_2CO_3 (24.5 mg, 75 µmol) and methyl iodide (10.7 mg, 75 µmol) and the reaction was stirred at that temperature. After 2 h, methyl iodide (10.7 mg, 75 µmol) was added and the mixture was stirred for another 2 h at room temperature. The reaction was quenched with sat. aq. NH_4Cl (5 mL), extracted with EtOAc (4x10 mL), the combined organic extracts were dried over Na_2SO_4 and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 0:1 to 2:3) to give methyl ester **3-35** (81 mg, 40 µmol, 80%) as a white foam. R_f (EtOAc/hexanes 1:1) = 0.83; $[\alpha]_D^{20}$ = +33.1° (c = 0.25, CH_2Cl_2); ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.07 – 7.91 (m, 6H), 7.76 (d, *J* = 7.4 Hz, 2H), 7.66 (t, *J* = 7.4 Hz, 1H), 7.62 – 7.03 (m, 56H), 5.39 (m, 3H), 5.30 (t, *J* = 9.5 Hz, 1H), 5.25 (t, *J* = 9.5 Hz, 1H), 5.21 – 5.14 (m, 2H), 5.00 (s, 1H), 4.81 (d, *J* = 11.9 Hz, 1H), 4.77 (d, *J* = 11.4 Hz, 1H), 4.71 (d, *J* = 8.0 Hz, 1H), 4.68 – 4.62 (m, 3H), 4.60 – 4.47 (m, 5H), 4.32 (m, 4H), 4.22 (d, *J* = 9.2 Hz, 2H), 4.12 (d, *J* = 12.1 Hz, 2H), 4.08 – 3.87 (m, 6H), 3.83 – 3.64 (m, 5H), 3.62 – 3.47 (m, 6H), 3.45 (s, 3H), 3.42 – 3.29 (m, 2H), 3.17 (d, *J* = 2.6 Hz, 1H), 3.05 (d, *J* = 9.4 Hz, 2H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 168.4, 166.7, 165.6, 164.83, 164.79, 156.6, 156.3, 140.1, 138.80, 138.76, 138.6, 138.4, 138.34, 138.29, 138.1, 138.0, 137.9, 137.6, 136.83, 136.76, 133.6, 133.5, 133.1, 132.6, 130.4, 130.0, 129.84, 129.80, 129.7, 129.3, 129.18, 129.15, 129.1, 129.0, 128.9, 128.8, 128.7, 128.6, 128.51, 128.46, 128.44, 128.35, 128.3, 128.2, 128.1, 128.0, 127.94, 127.87, 127.7, 127.6, 127.5, 127.43, 127.40, 127.0, 100.3, 100.2, 99.8, 98.5, 80.7, 79.2, 76.7, 76.6, 75.2, 75.12, 75.07, 75.0, 74.8, 74.6, 74.4, 74.4, 74.1, 73.7, 73.62, 73.59, 73.4, 73.1, 72.5, 72.1, 71.4, 70.5, 69.6, 69.5, 67.8, 67.3, 67.0, 66.8, 52.6, 51.4, 46.5, 45.5; IR (thin film) 2928, 1734, 1602, 1453, 1364, 1272, 1094, 1070, 740, 710 cm^{-1}; HRMS (MALDI) calcd. for $C_{119}H_{117}NO_{28}$ $(M+Na)^+$ 2030.7659 found 2030.7660 *m/z*.

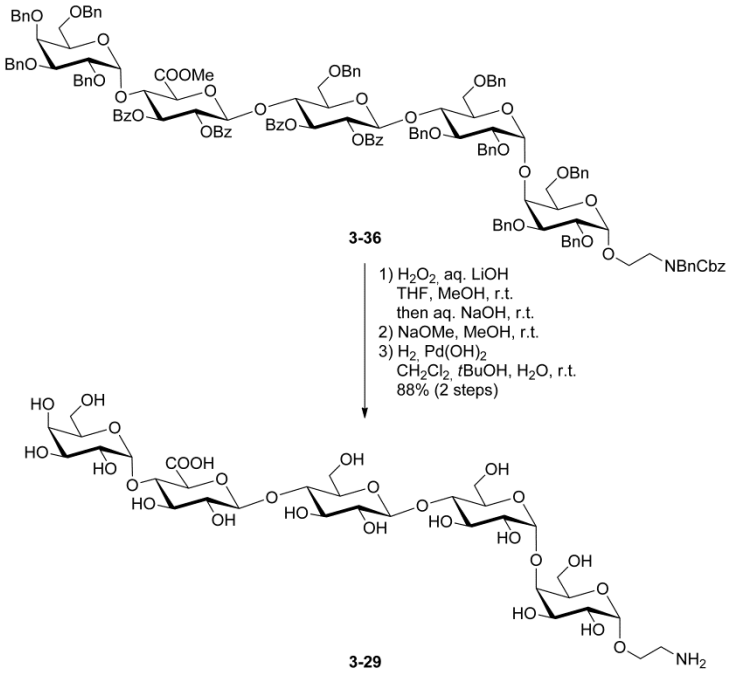
2,3,4,6-Tetra-*O*-benzyl-α-D-galactopyranosyl-(1→4)-methyl (2,3-di-*O*-benzoyl-β-D-glucopyranosyl)uronate-(1→4)-2,3-di-*O*-benzoyl-6-*O*-benzyl-β-D-glucoyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-galactopyranosyl-(1→1)-(2-*N*-benzyl-*N*-benzyloxycarbonylamino)ethanol (3-36)



Alcohol **3-35** (14 mg, 7 µmol) and thioglycoside **3-32**[346] (16 mg, 28 µmol) were co-evaproated with anhydrous toluene (3x10 mL) and kept under high vacuum for 30 min. The mixture was dissolved in Et_2O (1.05 mL) and CH_2Cl_2 (0.35 mL) and stirred over activated molecular sieves (3 Å-AW) for 30 min at room temperature. The solution was cooled to -20 °C and treated with NIS (6.3 mg, 28 µmol) and TMSOTf (1 µL, 5.5 µmol). The mixture was stirred for 1 h at that temperature and slowly warmed to 0 °C. The reaction was quenched with a 1:1 (v/v) mixture of sat. aq. $NaHCO_3$ (10 mL) and 10% (w/v) Na_2SO_3 (5 mL) and extracted with CH_2Cl_2 (4x10 mL). The combined organic fractions were dried over Na_2SO_4 and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 0:1 to 1:4 to 1:3) to give pentasaccharide **3-36** (12.5 mg, 4.9 µmol, 70%) along with the corresponding β-anomer (3.4 mg, 1.3 µmol, 19%). Analytical data for **3-36**: Clear oil. R_f (EtOAc/hexanes 2:3) = 0.63; $[\alpha]_D^{20}$ = +20.4° (c = 0.33, CH_2Cl_2); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.97 – 7.82 (m, 5H), 7.67 (d, J = 7.3 Hz, 2H), 7.57 (t, J = 7.4 Hz, 1H), 7.48 – 6.98 (m, 77H), 5.52 (t, J = 9.6 Hz, 1H), 5.37 (dd, J = 9.9, 8.2 Hz, 1H), 5.27 (t, J = 9.1 Hz, 2H), 5.17 (t, J = 9.5 Hz, 1H), 5.11 (t, J = 9.9 Hz, 2H), 4.92 (s, 1H), 4.82 (d, J = 11.3 Hz, 1H), 4.77 – 4.53 (m, 9H), 4.52 – 4.37 (m, 7H), 4.32 – 4.07 (m, 9H), 4.03 (d, J = 6.9 Hz, 1H), 3.96 (d, J = 12.0 Hz, 2H), 3.91 – 3.78

(m, 6H), 3.76 – 3.56 (m, 7H), 3.54 – 3.39 (m, 7H), 3.38 – 3.21 (m, 4H), 3.07 (s, 3H), 3.05 – 2.91 (m, 2H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 166.8, 165.49, 165.46, 164.84, 164.79, 156.6, 156.3, 140.0, 139.1, 138.9, 138.8, 138.6, 138.5, 138.4, 138.3, 138.2, 138.1, 138.0, 137.5, 136.8, 133.5, 133.0, 132.5, 130.2, 129.9, 129.8, 129.7, 129.4, 129.2, 129.1, 129.03, 128.95, 128.8, 128.7, 128.6, 128.5, 128.43, 128.41, 128.35, 128.28, 128.26, 128.22, 128.15, 128.03, 127.98, 127.93, 127.91, 127.8, 127.6, 127.52, 127.49, 127.45, 127.2, 127.0, 100.5, 100.2, 99.8, 99.47, 98.46, 80.7, 79.2, 78.4, 76.8, 76.61, 76.55, 75.34, 75.30, 75.26, 75.1, 75.0, 74.9, 74.8, 74.5, 74.2, 73.7, 73.6, 73.5, 73.4, 73.3, 73.1, 72.6, 72.1, 71.6, 70.5, 70.0, 69.6, 67.9, 67.5, 67.3, 67.0, 66.9, 52.2, 51.4, 46.5, 45.5; IR (thin film) 2928, 1737, 1498, 1454, 1271, 1094, 1047, 1028, 738, 699 cm^{-1}; HRMS (MALDI) calcd. for $C_{153}H_{151}NO_{33}$ $(M+Na)^+$ 2553.0066 found 2553.0066 *m/z*.

α-D-Galactopyranosyl-(1→4)-β-D-glucopyranosyluronate-(1→4)-β-D-glucoyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-α-D-galactopyranosyl-(1→1)-(2-amino)ethanol (3-29)

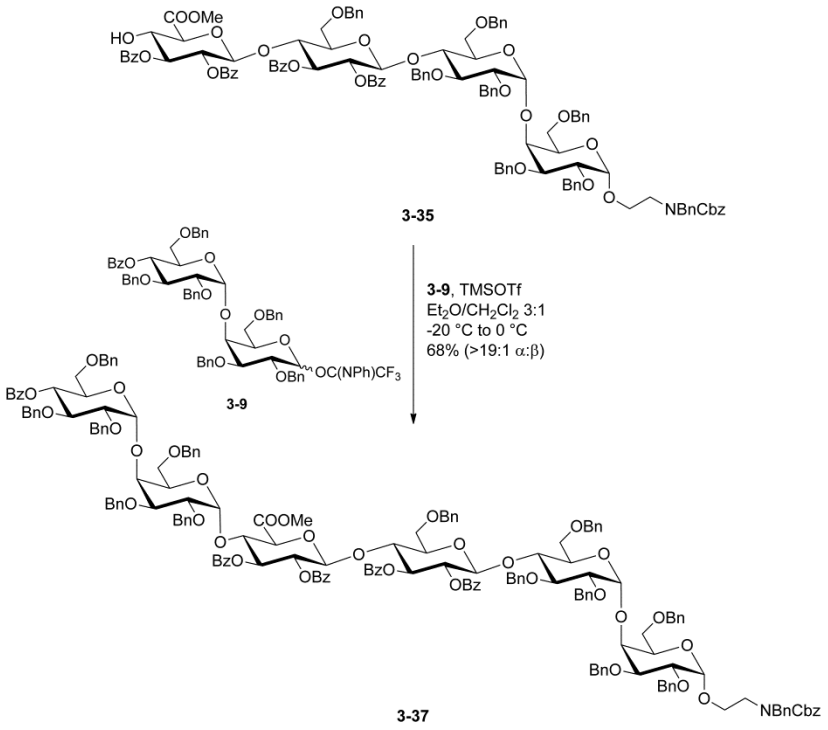


To a stirred solution of ester **3-36** (26 mg, 10.3 µmol) in THF (1 mL) and MeOH (1 mL) was added at 0 °C a 1:1 (v/v) mixture (450 µL) of H_2O_2 (6% (v/v) aq. solution, 397 µmol) and LiOH (0.5 M aq. solution, 113 µmol). The reaction was warmed to room temperature and stirred for 1 h at that temperature. The reaction was treated with NaOH (0.5 M aq. solution, 1 mL) and stirred for 16 h at room temperature. The solvents

were evaporated under reduced pressure, the residue was co-evaporated with toluene (2x5 mL) and dissolved in MeOH (1 mL). The solution was treated at room temperature with NaOMe (0.5 M in MeOH, 1 mL) and stirred for 16 h at that temperature. The reaction was diluted with water (0.5 mL) and CH_2Cl_2 (0.5 mL), neutralized at 0 °C with Amberlite IR-120 (H^+ form), filtered and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 0:1 to 1:4 to 1:2 to 1:2 + 1% (v/v) AcOH to 1:1 + 1% (v/v) AcOH) to give the intermediate carboxylic acid as a clear oil.

The intermediate carboxylic acid in CH_2Cl_2/*t*BuOH/water (1:16:8, 1 mL) was purged with argon and treated at 0 °C with a suspension of $Pd(OH)_2$ on carbon (20% (w/w) loading, 20 mg) in the same solvent mixture (0.5 mL). The suspension was purged with hydrogen, stirred under hydrogen atmosphere for 16 h, filtered and concentrated. Since the reaction had not proceeded to completion, the residue was subjected to the same conditions again and stirred for 24 h at room temperature. The mixture was filtered and concentrated, the residue was purified by solid phase extraction (Chromabond C18, Macherey-Nagel) and lyophilized to give pentasaccharide **3-29** (8.1 mg, 9.1 µmol, 88% over two steps) as a white solid. ^{1}H NMR (600 MHz, D_2O) δ 5.52 (d, J = 3.5 Hz, 1H), 5.07 (d, J = 3.7 Hz, 1H), 4.93 (d, J = 3.8 Hz, 1H), 4.56 (2xd, J = 8.4 and 8.4 Hz, 2H), 4.24 (d, J = 10.0 Hz, 1H), 4.09 (d, J = 2.6 Hz, 1H), 4.07 – 3.78 (m, 18H), 3.77 – 3.58 (m, 8H), 3.39 (dt, J = 24.4, 8.5 Hz, 2H), 3.34 – 3.26 (m, 2H); ^{13}C NMR (150 MHz, D_2O) δ 104.86, 104.85, 102.5, 101.2, 101.0, 81.2, 81.0, 80.9, 78.7, 78.6, 77.4, 76.6, 75.7, 75.5, 74.2, 74.1, 73.8, 73.31, 73.29, 71.8, 71.6, 71.5, 71.0, 70.9, 66.5, 63.3, 63.1, 62.5, 62.1, 41.9; HRMS (MALDI) calcd. for $C_{32}H_{55}NO_{27}$ $(M+Na)^+$ 884.2883 found 884.2942 *m/z*.

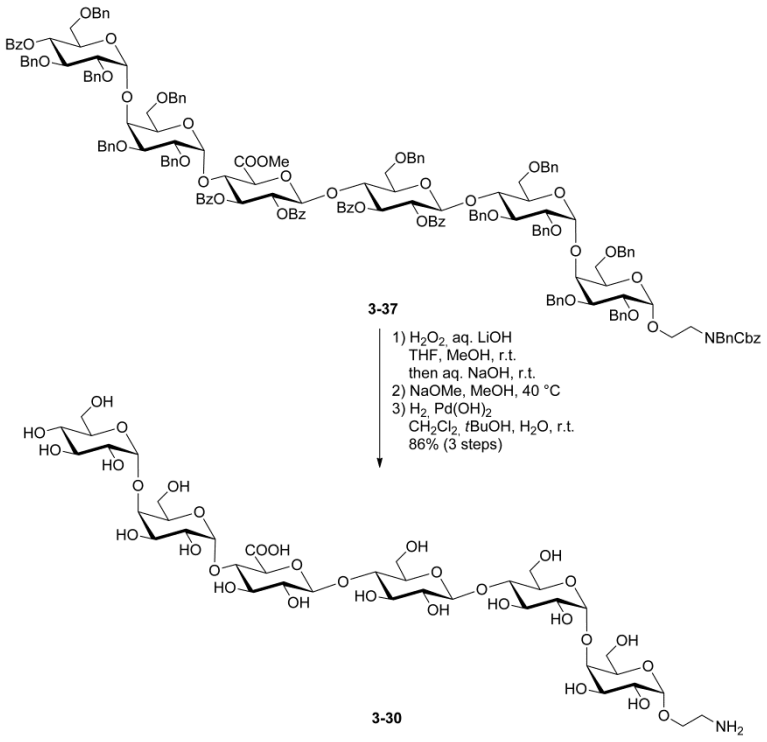
4-*O*-Benzoyl-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-galactopyranosyl-(1→4)-methyl (2,3-di-*O*-benzoyl-β-D-glucopyranosyl)uronate-(1→4)-2,3-di-*O*-benzoyl-6-*O*-benzyl-β-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-galactopyranosyl-(1→1)-(2-*N*-benzyl-*N*-benzyloxycarbonylamino)ethanol (3-37)



Alcohol **3-35** (50 mg, 25 µmol) and imidate **3-9** (72.1 mg, 62 µmol) were co-evaproated with anhydrous toluene (3x10 mL) and kept under high vacuum for 30 min. The mixture was dissolved in Et_2O (2 mL) and CH_2Cl_2 (0.67 mL) and stirred over activated molecular sieves (3 Å-AW) for 30 min at room temperature. The solution was cooled to -20 °C and treated with TMSOTf (2 µL, 11 µmol). The mixture was stirred for 1 h at that temperature and slowly warmed to 0 °C. The reaction was quenched with sat. aq. $NaHCO_3$ (10 mL) and extracted with CH_2Cl_2 (4x10 mL). The combined organic fractions were dried over Na_2SO_4 and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 0:1 to 1:3 to 3:7 to 1:2) to give hexasaccharide **3-37** (51 mg, 17 µmol, 68%) as a clear oil. R_f (EtOAc/hexanes 2:3) = 0.63; $[\alpha]_D^{20}$ = +36.6° (c = 0.21, CH_2Cl_2); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.95 (d, J = 7.3 Hz, 2H), 7.89 (d, J = 7.7 Hz, 4H), 7.84 (d, J = 7.3 Hz, 2H), 7.67 (d, J = 7.4 Hz, 2H), 7.55 (dt, J = 26.6, 7.4 Hz, 2H), 7.48 – 6.98 (m, 88H), 5.49 (dt, J = 19.5, 9.8 Hz, 2H), 5.39 (dd, J = 9.9, 8.2 Hz, 1H), 5.28 (m, 2H), 5.16 (t, J = 9.5 Hz, 1H), 5.09 (m, 3H), 4.92 (s, 1H), 4.74 (dd, J = 11.7, 9.1

Hz, 2H), 4.71 – 4.66 (m, 3H), 4.60 (m, 7H), 4.53 – 4.41 (m, 6H), 4.35 – 4.28 (m, 3H), 4.28 – 4.20 (m, 5H), 4.18 – 4.01 (m, 8H), 4.00 – 3.78 (m, 9H), 3.77 – 3.59 (m, 8H), 3.55 (d, J = 9.6 Hz, 1H), 3.53 – 3.20 (m, 8H), 3.17 (dd, J = 8.8, 4.9 Hz, 1H), 3.06 (s, 3H), 2.97 (m, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 167.0, 165.54, 165.50, 165.2, 164.8, 156.6, 156.3, 140.1, 138.9, 138.8, 138.8, 138.6, 138.41, 138.36, 138.3, 138.2, 138.1, 138.0, 137.6, 136.84, 136.77, 133.5, 133.1, 133.0, 132.5, 130.4, 130.2, 130.1, 129.9, 129.8, 129.7, 129.3, 129.2, 129.1, 129.04, 128.95, 128.8, 128.7, 128.6, 128.44, 128.41, 128.37, 128.35, 128.33, 128.30, 128.25, 128.18, 128.15, 128.1, 128.03, 127.99, 127.96, 127.94, 127.85, 127.8, 127.7, 127.64, 127.61, 127.55, 127.4, 127.3, 127.2, 127.0, 100.4 ($^1J_{C\text{-}H}$ = 165.6 Hz; β), 100.2 ($^1J_{C\text{-}H}$ = 165.6 Hz; β), 100.0 ($^1J_{C\text{-}H}$ = 171.3 Hz; α), 99.8 ($^1J_{C\text{-}H}$ = 171.0 Hz; α), 99.3 ($^1J_{C\text{-}H}$ = 172.8 Hz; α), 98.5 ($^1J_{C\text{-}H}$ = 174.0 Hz; α), 80.7, 80.1, 79.7, 79.2, 77.6, 76.64, 76.56, 75.9, 75.3, 75.2, 74.9, 74.4, 74.4, 74.2, 73.9, 73.6, 73.6, 73.4, 73.3, 73.2, 73.1, 73.0, 72.6, 72.4, 72.1, 71.6, 71.1, 70.5, 70.1, 69.6, 69.5, 69.2, 67.9, 67.8, 67.6, 67.3, 67.0, 66.9, 66.8, 66.3, 52.2, 51.40, 51.37, 46.5, 45.5; IR (thin film) 2926, 1736, 1454, 1271, 1095, 1045, 737, 699 cm^{-1}; HRMS (MALDI) calcd. for $C_{180}H_{177}NO_{39}$ $(M+2Na)^{2+}$ 1511.0847 found 1511.0576 *m/z*.

α-D-Glucopyranosyl-(1→4)-α-D-galactopyranosyl-(1→4)-β-D-glucopyranosyluronate-(1→4)-β-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-α-D-galactopyranosyl-(1→1)-(2-amino)ethanol (3-30)



To a stirred solution of ester **3-37** (22 mg, 7.4 µmol) in THF (1 mL) and MeOH (1 mL) was added at 0 °C a 1:1 (v/v) mixture (296 µL) of H_2O_2 (6% (v/v) aq. solution, 295 µmol) and LiOH (0.5 M aq. solution, 74 µmol). The reaction was warmed to room temperature and treated after 2 h and 4 h with another 294 µL of the same lithium peroxide solution, respectively. The mixture was stirred for 16 h at room temperature and treated with NaOH (1 M aq. solution, 0.5 mL) and MeOH (0.5 mL). The reaction was stirred for 20 h at that temperature, quenched with 10% aq. Na_2SO_3 (0.8 mL) and concentrated under reduced pressure. The residue was dissolved in water (4 mL), acidified at 0 °C with 0.5 M aq. $NaHSO_4$ to approx. pH 4 and extracted with EtOAc (4x10 mL). The combined organic fractions were dried over Na_2SO_4 and concentrated. The residue was treated with NaOMe (0.5 M solution in MeOH, 1 mL), warmed to 40 °C and stirred for 5 h at that temperature. The reaction was cooled to room temperature, stirred for another 16 h at that temperature and treated with water (0.5 mL). The mixture was neutralized with Amberlite IR-120 (H^+ form), filtered and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 0:1:0 to 1:4 + 2% (v/v) AcOH to 1:1 + 2% (v/v) AcOH) to give the intermediate carboxylic acid as a clear oil.

The intermediate carboxylic acid in CH_2Cl_2/*t*BuOH/water (1.5:16:8, 3 mL) was purged with argon and treated at 0 °C with a suspension of $Pd(OH)_2$ on carbon (20% (w/w) loading, 30 mg) in the same solvent mixture (1 mL). The suspension was purged with hydrogen, stirred under hydrogen atmosphere for 18 h, filtered and concentrated. The residue was purified by solid phase extraction (Chromabond C18, Macherey-Nagel) and lyophilized to give hexasaccharide **3-30** (7 mg, 6.7 µmol, 86% over three steps) as a white solid. ^{1}H NMR (600 MHz, D_2O) δ 5.57 (d, J = 3.2 Hz, 1H), 5.07 (d, J = 2.9 Hz, 1H), 4.97 (d, J = 3.0 Hz, 1H), 4.94 (d, J = 3.0 Hz, 1H), 4.56 (2xd, J = 8.0 and 7.9 Hz, 2H), 4.24 (d, J = 9.8 Hz, 1H), 4.15 – 4.07 (m, 3H), 4.01 (m, 4H), 3.89 (m, 16H), 3.77 – 3.60 (m, 7H), 3.56 (dd, J = 9.9, 3.1 Hz, 1H), 3.47 (t, J = 9.6 Hz, 1H), 3.43 – 3.28 (m, 4H); ^{13}C NMR (150 MHz, D_2O) δ 104.9, 104.82, 102.80, 102.5, 101.2, 101.0, 81.2, 81.01, 80.98, 80.9, 78.7, 78.7, 77.4, 76.6, 75.7, 75.5, 75.4, 74.5, 74.4, 74.2, 74.1, 73.8, 73.6, 73.3, 71.9, 71.5, 71.3, 71.1, 70.9, 66.5, 63.1, 62.7, 62.5, 62.4, 62.1, 41.9; HRMS (ESI) calcd. for $C_{38}H_{65}NO_{32}$ $(M+Na)^+$ 1070.3387 found 1070.3391 *m/z*.

3.4.2 Methods of Biochemistry

Figures were prepared using Illustrator CS5 (Adobe Systems, San Jose, USA).

Antisera, Polysaccharides and Carrier Protein

Human pooled pneumococcal antiserum (WHO 1st International Standard for Human Anti-pneumococcal capsule Reference Serum, prod. no. 007sp[339] was obtained from NIBSC (South Mimms, UK). Rabbit ST8 typing serum (Type 8 Neufeld antiserum, cat. no. 16751), ST8 capsular polysaccharide (cat. no. 76875), ST3 capsular polysaccharide (cat. no. 76853) and pneumococcal cell wall polysaccharide (cat. no. 3459) were purchased from SSI Diagnostica (Hillerød, Denmark). CRM197 was purchased from Pfenex (San Diego, USA).

ST8 CPS fragments were prepared according to a literature protocol.[345] Briefly, ST8 CPS (200 µg) in 0.5 M aqueous trifluoroacetic acid (200 µL) was warmed to 100 °C in a sealed tube. The solution was directly shock-frozen and lyophilized to remove excess acid.

Glycan Microarray Analysis

Microarray slides were fabricated as described recently.[155] Briefly, synthetic oligosaccharides [0.2 mM solutions in printing buffer (50 mM sodium phosphate buffer, NaPi, pH 8.5)], polysaccharides (0.02 or 0.04 µg/mL in printing buffer) and proteins (0.5 µM in printing buffer) were spotted onto CodeLink *N*-hydroxysuccinimide-activated glass slides (SurModics Inc., Eden Prairie, USA) using an automated piezoelectric arraying robot (Scienion, Berlin, Germany) at 0.4 nL per spot and incubated for 24 h at room temperature in a humified chamber. Slides were quenched for 1 h at room temperature using 100 mM ethanolamine in 0.1 M NaPi pH 9, washed with water and stored in a anhydrous chamber until use.

Slides were blocked for 1 h at room temperature with 1% (w/v) bovine serum albumin (BSA) in phosphate buffered saline (PBS, 10 mM Na_2HPO_4, 1.8 mM K_2HPO_4, 137 mM NaCl, 2.7 mM KCl) and antibody dilutions were applied using a 64 well gasket (FlexWell 64, Grace Bio-Labs, Bend, US). The slides were incubated for 16 h at 4 °C in a humified chamber, washed three times with 0.1 % (v/v) Tween® 20 in PBS (PBS-T)

and treated with the appropriate secondary antibody solutions (see below). The slides were incubated for 1.5 h at room temperature in a dark, humified chamber, washed three times with washing buffer and with water. Fluorescence read-out was performed using an Axon GenePix 4300A microarray scanner and GenePix Pro 7 software (both MDS, Sunnyvale, US). Negative fluorescence intensities were arbitrarily set to 0. All statistical analyses were perfomed using Prism 6 (Graphpad Software Inc., La Jolla, USA). Brightness and contrast of related images (e.g. all sera of the same mouse) were adjusted equally using Photoshop CS5 (Adobe Systems).

Conjugation of Tetrasaccharides 3-5 and 3-6 to CRM197

To a stirred solution of di-*N*-succinimidyl adipate (DSAP, 10 mg, 29 µmol) and triethylamine (10 µL, 72 µmol) in anhydrous DMSO (150 µL) was added at room temperature dropwise a solution of tetrasaccharide **3-5** or **3-6** (approx. 2 mg, 2.8 µmol) in anhydrous DMSO (150 µL). The reaction was stirred for 2 h at that temperature under an Argon atmosphere and treated with conjugation buffer (100 mM NaPi pH 7.4, 200 µL). The mixture was extracted with chloroform (10 mL) and the phases separated by centrifugation (2 min, 1800 g). The organic phase was discarded and the extraction step was repeated two times. The aqueous layer was clarified by centrifugation in a 1.5 mL reaction tube (1 min, 14500 g, room temperature) and added to a stirred solution of CRM197 (1 mg, 17.3 nmol) in conjugation buffer (1 mL). The mixture was stirred for 16 h at room temperature and dialyzed using a centrifugal filter (10 kDa molecular weight cut-off, Millipore, Darmstadt, Germany). The glycoconjugates were characterized by MALDI-TOF MS, SDS-PAGE and western blot.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Discontinuous SDS-PAGE was performed according to Lämmli's protocol,[316] using a MiniProtean system (Bio-Rad, Hercules, USA). An alkaline separating gel (375 mM Tris/HCl pH 8.8, 10 to 12% (w/v) of a 29:1 acrylamide/*N*,*N*'-methylenebisacrylamide mixture) and an acidic stacking gel (100 mM Tris/HCl pH 6.8, 4.5% (w/v) of a 29:1 acrylamide/*N*,*N*'-methylenebisacrylamide mixture), polymerized by the addition of TEMED and 10% (w/v) ammonium peroxodisulfate, were used. Proteins were visualized using Coomassie G-250. Brightness and contrast of images were adjusted using Photoshop CS5 (Adobe Systems).

Western Blotting and Immunodetection of Proteins

Tank western blotting of proteins was performed using a Mini Trans-Blot Cell (Bio-Rad). After SDS-PAGE, proteins in the separating gel were transferred to a nitrocellulose membrane (GE Healthcare) using transfer buffer (25 mM Tris, 192 mM glycine, 15% (v/v) MeOH) at 100 V for 1 h. Transfer was confirmed by Ponceau S staining (0.1% (w/v) Ponceau S in 5% (v/v) aq. acetic acid). The membrane was washed briefly with Tris buffered saline (TrBS, 20 mM Tris/HCl pH 7.5, 154 mM NaCl) and blocked using blocking solution (5% (w/v) skimmed milk powder in TrBS) for 1 h at room temperature. Primary antibody (1:1500 rabbit ST8 typing serum or 0.4 µg/mL anti-ST8 mAb 28H11) in blocking solution was applied and the membrane was incubated for 1 h at room temperature or for 16 h at 4 °C. The membrane was washed with TrBS and TrBS supplemented with 0.1% (v/v) Tween® 20 (TrBS-T). Secondary antibody [goat anti-rabbit IgG HRP conjugate (ab6721, abcam) or goat anti-mouse IgM HRP conjugate (62-6820, Thermo Fisher Scientific, Waltham, USA)] was applied in the dilution specified by the manufacturer in blocking buffer. The membrane was incubated for 1 h at room temperature, washed with TrBS and TrBS-T and analyzed by chemiluminescence detection using Pierce™ ECL Western Blotting Substrate (Thermo Fisher). Brightness and contrast of images were adjusted using Photoshop CS5 (Adobe Systems).

Ethics Statement

All animal experiments were approved by local institutional (Charité - Universitätsmedizin Berlin) and governmental authorities (Landesamt für Gesundheit und Soziales Berlin, approval ID G0128/12 and A 0305/12). Animal housing and experiments were in strict accordance with the regulations of the Federation of European Laboratory Animal Science Associations (FELASA) and recommendations for the care and use of laboratory animals. All mice were housed under specific pathogen-free conditions.

Glycoconjugate Immunization

Mice (6-8 week old female BALB/c or C57BL/6N mice, Charles River, Sulzfeld, Germany) were immunized subcutaneously with the CRM197-FS A (**3-5**) CRM197-FS C

(**3-6**) glycoconjugates (corresponding to 4 µg synthetic glycan) formulated either as a 1:1 (v/v) emulsion with Complete Freund's Adjuvant (CFA, Sigma-Aldrich), a 1:1 (v/v) suspension with Alum (Alhydrogel, Brenntag, Mülheim, Germany) or without adjuvant at a total volume of 100 µL. Booster doses were given at days 14 and 28 using the same strategy (mice primed with CFA received booster doses with Incomplete Freund's Adjuvant (Sigma-Aldrich). Blood (50 µL) was withdrawn once a week from the tail vein or the facial vein and centrifuged (5000 g, 10 min, room temperature) to retrieve serum.

Enzyme-linked Immunosorbent Assay (ELISA)

ELISA was performed using Costar™ high-binding polystyrene 96-well plates (cat. no. 3361, Corning, Corning, US). Plates were coated using native ST8 polysaccharide (SSI Diagnostica, Kopenhagen) at a concentration of 10 µg/mL in PBS for 20 h at 4 °C. Plates were blocked with 10% (v/v) fetal calve serum in PBS for 2 h at 37 °C and washed once with PBS-T. After applying cell culture supernatants or mAb dilutions (30-50 µL), Plates were incubated for 1 h at 37 °C, washed with PBS-T three times and treated with a horseradish peroxidase (HRP)-labeled secondary antibody (see below). Plates were washed with PBS-T three times and HRP activity was measured with TMB substrate (BD Biosciences, San Jose, US) according to the manufacturer's instructions.

Antibodies

Monoclonal antibodies were prepared using BM-Condimed H1 (Roche, Penzberg, Germany) according to the manufacturer's instructions. After fusion of plasma cells with P3X63Ag8.653 (ATCC CRL-1580™) cells, single clones were generated using limited dilution and two subsequent rounds of subcloning. Antibody production was monitored by glycan array and enzyme-linked immunosorbent assay (ELISA). 33 clones were eventually isolated that produced mAbs recognizing both ST8 FS C (cmpd. **3**) and ST8 CPS. Clones 1H8C6H4 (termed "1H8") and 1F1F7H2 (termed "1F1") were expanded in ISF-1 serum-free medium (Biochrom, Berlin, Germany) supplemented with Penicillin and Streptomycin (life Technologies, Carlsbad, US). MAb 1H8 was purified using a Protein G Antibody Purification kit (Pro-Chem, Littleton, USA). MAb 1F1 was purified by gel filtration chromatography using a Superdex HiLoad 16/600 200 prep grade column mounted on an ÄKTA 900 system (GE Healthcare). MAb isotypes were determined

using a Mouse Isotyping Test Kit (AbD Serotec, Kidlington, UK) and purity was confirmed by SDS-PAGE. Antibodies were stored in PBS supplemented with 0.02% (w/v) sodium azide at 4 °C or in 50% (v/v) glycerol/PBS at -20 °C. MAbs were dialyzed against azide-free PBS before application in animal or cell-based experiments.

Table 3.2. Fluorescently labeled antibodies used in this study.

Antibody	Source	cat. no.
Goat anti-Rabbit IgG H+L FITC conjugate	abcam (Camrbidge, UK)	ab6717
Goat anti-Human IgG H+L Alexa Fluor® 647 conjugate	life Technologies	A-21445
Goat anti-Mouse IgG H+L FITC conjugate	Sigma-Aldrich	F9137
Goat anti-Mouse IgG H+L Alexa Fluor® 635 conjugate	life Technologies	A-31574
Goat anti-Mouse IgM H chain Alexa Fluor® 680 conjugate	life Technologies	A-21048
Donkey anti-Mouse IgM H chain Alexa Fluor® 594 conjugate	dianova (Hamburg, Germany)	715-585-020

Antibody isotype controls employed were an anti-*Y. pestis* lipopolysaccharide core trisaccharide mAb IgG1 (clone 1E12)[83] and a purified mouse myeloma IgM (cat. no. 02-6800, Invitrogen).

Fluorescent antibodies were used from commercial sources (Table 3.2). Secondary antibodies used for ELISA were horseradish peroxidase (HRP)-labeled: Goat anti-Mouse IgG HRP conjugate (cat. no. 115-035-062**,** dianova, Hamburg, Germany) or Goat anti-mouse IgM H chain HRP conjugate (cat. no. 62-6820, Life Technologies).

Surface Plasmon Resonance

Surface plasmon resonance was performed on a Biacore® T100 instrument (GE Healthcare). Murine antibodies were immobilized using the Mouse Antibody Capture Kit and Amine Coupling Kit (GE Healthcare). Approx. 10000 response units (RU) of capture antibody were immobilized. A commercial mouse IgG (cat. no. 026502, Invitrogen, Carlsbad, US) was immobilized as a dummy in the reference cell (approx. 10000 RU). Approx. 1000 RU of mAb 1H8 or 500 RU of mAb 1F1 were captured prior to every run. Runs were performed using PBS (for ST8 CPS analyte) or PBS supplemented with 0.001% (v/v) Tween® 20 (for synthetic oligosaccharide analytes) as running buffers at a flow rate of 30-50 µL/min with 120 s association and 280-600 s dissociation periods. Flow

cells were regenerated using 10 mM glycine-HCl pH 1.7 and 100 mM glycine-NaOH pH 12 with 0.3% (v/v) Triton™ X100. To calculate the concentration of ST8 polysaccharide, a molecular weight of 150 kDa (assessed by gel filtration, data not shown) was assumed.[327] Affinities and standard errors were calculated using Biacore® T100 Evaluation Software (GE Healthcare).

Immobilization of oligosaccharides was performed using the Amine coupling Kit (GE Healthcare) according to the manufacturer's recommendations with 0.66 mM glycan solutions in printing buffer. Approx. 200 RU of each glycan were immobilized.

Bacteria

S. pneumoniae serotype 8 (ATCC 6308), serotype 1 (ATCC 6301) or serotype 3 (PN36, NCTC7978) bacteria (a gift from Prof. Sven Hammerschmidt, Universität Greifswald, Germany) were plated from frozen stocks on Columbia Agar plates with 5% (v/v) sheep blood, grown for approx. 9 h at 37 °C/5% CO_2 and inoculated as single colonies in Todd Hewitt Broth with 0.5% (w/v) yeast extract (growth medium). Cultured were grown at 37 °C/5% CO_2 to log phase (OD_{600} approx. 0.3) and harvested by centrifugation.

For UV-inactivation, bacteria were washed with PBS once, harvested, suspended in PBS to approx. $4x10^8$ colony-forming units (cfu)/mL and inactivated by irradiation at λ = 254 nm for 10 min at room temperature. Cells were harvested, washed once with PBS and frozen at approx. $8x10^8$ cfu/mL in growth medium supplemented with with 20% (v/v) glycerol (freezing medium) at -20 °C.

Immunofluorescence of UV-inactivated *S. pneumoniae*

Bacteria were thawed, harvested by centrifugation (16800 g, 15 min, r.t.) and washed once in 50 mM $NaHCO_3$, 100 mM NaCl, pH 7.5 (buffer A). Cells were resuspended in Buffer A (1 mL) and treated with fluorescein isothiocyanate (FITC, Sigma-Aldrich) solution (10 mg/mL in DMSO) to a final FITC concentration of 0.1 mg/mL. Bacteria were labeled in the dark for 1 h at 37 °C, harvested by centrifugation and washed twice with 0.25% (w/v) BSA in PBS (1 mL). Labeling was monitored by fluorescence microscopy using an Axio Imager.M2 system equipped with a LSM 700 confocal laser scanning microscope (Carl Zeiss Microscopy, Jena, Germany). Cells were suspended in 1% (w/v) BSA in PBS (1 mL for ST8, 0.5 mL for ST1) and the suspension was

distributed into two aliquots. The suspensions were treated with mAb 1H8 or mAb 1E12 as an isotype control to a final mAb concentration of 10 µg/mL. Bacteria were incubated in the dark for 16 h at 4 °C under agitation and washed with 1% (w/v) BSA in PBS (0.5 mL). The cells were suspended in a solution of goat anti-mouse IgG-Alexa635 conjugate (1:100 dilution in 200 µL 1% (w/v) BSA in PBS, Invitrogen), incubated in the dark for 1.5 h at room temperature and washed with 1% (w/v) BSA in PBS and PBS (0.5 mL, respectively). Fluorescently labeled bacteria were visualized by fluorescence microscopy and images were processed with using Zen 2011 software (Carl Zeiss Microscopy).

Flow Cytometry

S. pneumoniae serotype 8 (ATCC 6308), serotype 1 (ATCC 6301) or serotype 3 (PN36, NCTC7978) were UV-inactivated, FITC-labeled and treated with a fluorescent secondary antibody (anti-mouse IgG-Alexa635 conjugate or anti-mouse IgM-Alexa680 conjugate, see above) as described above. Flow cytometry was performed by counting 10000 bacteria using a FACSCanto II flow cytometer (BD Pharmingen, Heidelberg, Germany) and analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

Opsonophagocytic Killing Assay

An opsonophagocytic killing (OPKA) assay was peformed as described by Romero-Steiner et al.[79] Briefly, HL-60 cells were differentiated for one week with *N*,*N*-dimethylformamide as reported (Romero-Steiner et al., 1997), washed twice with Hanks' buffer supplemented with 0.1% (w/v) gelatin (OPKA buffer) and diluted to a density of 10^7 cells/mL in the same buffer directly before use. Bacteria were grown in growth medium at 37 °C/5% CO_2 to log phase (OD_{600} approx. 0.2-0.3), diluted in freezing medium to a density of 10^6 cfu/mL and frozen in 0.5 mL aliquots at -80 °C. Bacteria were diluted with OPKA buffer and aliquoted (1000 cfu in 20 µL each) in a 96 well-plate. Bacterial suspensions were treated with 10 µg/mL mAb solutions or control antisera (1:4) dilutions and incubated for 15 min at 37 °C. Complement source (10 µL,baby rabbit complement, CedarLane, Ontario, Canada) and differentiated HL-60 cell suspension (40 µL, phagocyte/bacteria ratio 400:1) were added and the suspensions were incubated for 45 min at 37 °C with shaking (220 rpm). Opsonophagocytosis was performed in triplicates. 10% of the contents of each well were plated on Columbia Agar

plates with 5% (v/v) sheep blood, and cfu were counted after incubation at 37 °C/5% CO_2 overnight. Control wells lacked either antibody or complement sources.

Passive immunization and lethal pneumococcal challenge

Streptococcus pneumoniae serotype 8 was cultured as described above and suspended in sterile PBS. Female BALB/c mice (12 weeks, 20-22 g, Charles River, Sulzfeld, Germany) were treated intraperitoneally (i.p.) with monoclonal antibodies 1H8 or anti-*Y. pestis* 1E12 at doses of either 10 or 100 µg in 100 µL sterile PBS 2 h prior to infection. Mice were anaesthetized by i.p administration of ketamine (80 mg/kg, Ketavet®, Pfizer, Berlin, Germany) and xylazine (25 mg/kg, Rompun®, Bayer, Leverkusen, Germany) and transnasally inoculated with $1x10^5$ cfu *S. pneumoniae* in 20 µL PBS. Disease severity was evaluated at 12 h intervals (more often if severely ill) for 96 h after bacterial infection to assess appearance, behavior, grooming, respiration, body weight and rectal temperature (BAT-12 Microprobe Thermometer, Physitemp Instruments, Clifton, USA). Blood samples (max. 20 µL) were removed from tail veins of surviving mice 30 h and 60 h post infection.

Mice were humanely sacrificed when they reached at least one of the predefined criteria [(i) body temperature <30°C, (ii) body weight loss >20%, (iii) cumbersome breathing, (iv) accelerated breathing in combination with staggering, pain or paleness] by exsanguination via the caudal *Vena cava* after i.p. injection of ketamine (160 mg/kg body weight) and xylazine (75 mg/kg). 96 h after infection, surviving mice were anaesthetized with i.p. ketamine (160 mg/kg) and xylazine (75 mg/kg). After heparinization, blood was drawn from the *Vena cava caudalis* and lungs were removed.

After each bleeding, serial dilutions of blood were plated on Columbia agar plates with 5% (v/v) sheep blood and incubated at 37°C under 5% CO_2 overnight to count cfu. Blood antibody levels were monitored by glycan microarray analysis after 0.2 µm sterile filtration of 1:20 dilutions of blood samples and comparison of the FS C (**3-6**) binding signal with a standard curve of different concentrations of mAb 1H8.

To assess the residual bacterial burden of mice 96 h after infection, lungs were homogenized by passage through a cell strainer (100 µm, BD Bioscience). Serial dilutions of lung homogenates were assessed for cfu growth as described above.

4 Introduction of a Linker System with Low Nucleophilicity and High α-Selectivity in Glycosylations

4.1 Introduction

Manufacturing novel synthetic oligosaccharide-based vaccines requires reliable methods that enable the construction of a variety of glycosidic linkages.[2, 184] However, unusual glycosidic linkages especially found in bacterial polysaccharides pose a synthetic challenge to generating these glycans. Numerous strategies have been devised to address the difficulties associated with the installation of 1,2-*cis*-glycosidic bonds.[184] In the past years, mechanistic studies have tried to rationalize experimental observations to develop kinetic principles that may help to increase 1,2-*cis*-selectivities.[196, 270, 356, 357] However, kinetic pathways are often difficult to discern, and deducing general trends is cumbersome. A particular challenge is the 1,2-*cis*-stereoselective introduction of protected linker alcohols at the reducing end of oligosaccharide chains. Typically, *N*-protected, aliphatic amino alcohols are used for that purpose. These alcohols are conformationally flexible, comparably strong nucleophiles, and common concepts to enhance 1,2-*cis*-selectivities in the corresponding glycosylation reactions have seen limited use (*see* Chapters 2 and 3).[155, 156, 201, 238, 358-360] Due to the low 1,2-*cis*-stereoselectivities typically observed in the introduction of reducing-end linkers, these molecules are usually appended in early steps of oligosaccharide synthesis.[156] An efficient strategy to introduce linker moieties at late synthetic stages would significantly boost synthetic flexibility.

Strategies to increase 1,2-*cis*-selectivities ("α" in the D-*galacto* series studied here) in glycosylations comprise the use of certain solvent combinations, leaving groups and intermediates.[184] Few studies, however, have focused on altering the properties of the

nucleophile. Woerpel found that nucleophile strength significantly alters the outcome of glycosylations.[194, 195] Thereby, the reactivity of *C*-nucleophiles inversely correlated with α-selectivity in the reaction with a model glycosylating agent.[194] Highly reactive *C*-nucleophiles were associated with an erosion of stereochemistry.[194, 195] The same trends were later confirmed using *O*-nucleophiles of different reactivities,[193] and increasing the number of electronegative fluorine substituents in ethanol-derived nucleophiles led to increased α-selectivities. It was thus proposed that weak nucleophiles react via an energetically favored kinetic pathway, while strong nucleophiles exhibit reaction rates at the diffusion limit and thus erode stereoselectivities.[193] These mechanistic studies did not provide conjugation-ready oligosaccharides, and it is not known whether the observed effects can be universally translated to other glycosylations to furnish biologically relevant glycosidic bonds.

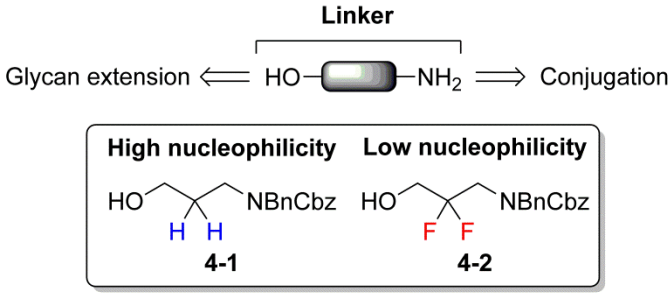
Fluorination is a well-practiced strategy to alter the physical and biochemical properties of organic compounds, with up to 25% of all drugs in development containing fluorine substituents.[361] As a substituent, fluorine is closest to hydrogen in size, with van der Waals-radii of 1.47 Å and 1.10 Å, respectively.[362] In contrast to their size, the physicochemical properties of both substituents differ substantially. The high electronegativity of fluorine affects pK values of neighboring acidic and basic functional groups, often leading to differences in drug bioavailability.[361] Other physicochemical effects of fluorine substitution include conformational preferences and altered lipophilicity of the respective compounds.[361] Biochemically, fluorine substitution can provide a protective effect towards biodegradation of organic compounds, leading to higher metabolic stability.[361] Although there is little evidence for biodegradative pathways of organofluorine compounds,[363] the effects of fluorine substitutions in a medicinal context are well-studied and widely employed.

The lack of reliable methods to α-selectively introduce linker alcohols into synthetic glycans inspired the development of a linker with low *O*-nucleophilicity. In particular, the question whether the introduction of two fluorine substituents would decrease *O*-nucleophilicity enough to increase α-selectivity was of interest.

4.2 Results

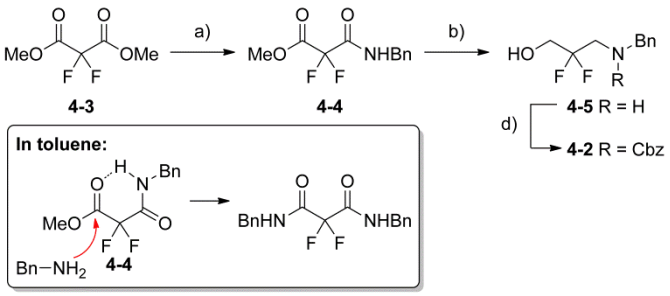
4.2.1 Design of Linker Alcohols with Distinct *O*-Nucleophilicities

Classically, *N*-protected aliphatic amino alcohols, such as **4-1**, are used as linkers to attach glycans to carrier proteins, glycan array surfaces and reporter probes by virtue of a free amino group (Scheme 4.1).[2] To enable the α-selective introduction of a linker at the reducing end of glycans, it was reasoned that the formal substitution of the methylene group in **4-1** by a difluoromethylene group (in **4-2**) would reduce *O*-nucleophilicity enough to increase the stereoselectivities of glycosylation reactions without abrogating the bifunctional nature of the linker.



Scheme 4.1. Linker alcohols used in this study. An amine-containing linker is introduced into a synthetic glycan chain to enable the chemoselective conjugation to surfaces, reporter moieties or carrier proteins using appropriate electrophiles (see Chapter 3). Conventional, *N*-protected amino alcohol **4-1** bears a highly nucleophilic hydroxyl function, whereas difluorinated derivative **4-2** exhibits reduced *O*-nucleophilicity.

A strategy was thus envisioned to synthesize alcohol **4-2** from commercially available dimethyl difluoromalonate **4-3** as a precursor (Scheme 4.2). Treatment of **4-3** with substoichiometric benzylamine in methanol provided amide **4-4** in 59% yield. The respective diamide was obtained as a side product but became the major product when toluene was used as a solvent. It is reasoned that the reactivity of the ester group in monoamide **4-4** towards a second amidation reaction is increased due to an intramolecular hydrogen bond in non-protic solvents (Scheme 4.2, insert). A two-step reduction of the ester and amide groups in **4-4** followed, employing sodium borohydride and, subsequently, borane dimethylsulfide at 50 °C, to give amino alcohol **4-5** in 73% yield over two steps. The amine moiety in **4-5** was then reacted with *N*-benzylchloroformate to provide difluorinated linker **4-2** in 95% yield.



Scheme 4.2. Synthesis of difluorinated linker **4-2**. Insert: proposed mechanism for the formation of the corresponding diamide using toluene as a solvent. Reagents and conditions: a) $BnNH_2$, MeOH, 0 °C to r.t., 59%; b) i. NaBH4, MeOH, 0 °C; ii. BH_3•Me_2S, THF, reflux, 73% (2 steps); c) CbzCl, $NaHCO_3$, EtOAc, H_2O, r.t., 95%.

4.2.2 Impact of *O*-Nucleophilicity on the Stereoselectivity of Glycosylation Reactions

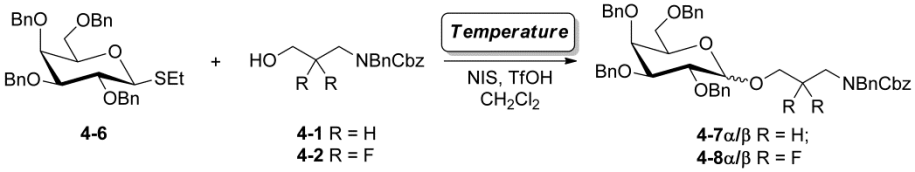
Attention was then turned towards assessing the effect of difluorination on the stereoselectivity of glycosylation reactions. Tetrabenzylated galactose thioglycoside **4-6** served as a glycosylating agent to assess the effects of various determinants on the stereochemical outcome of glycosylations with nucleophiles **4-1** and **4-2** (Table 4.1).

Reaction temperature was selected as the first variable, and both activating agent (NIS/TfOH) and solvent (dichloromethane) were kept constant. Glycosylations using non-fluorinated alcohol **4-1** were generally β-selective with profound temperature dependence: increasing β-selectivities of up to 1:26 α:β were obtained when reactions were conducted at -40 °C or -78 °C (Table 4.1, entries 1 and 2, left column), while higher temperatures led to erosion of stereoselectivity (entries 3 to 5, left column). In contrast, difluorinated linker alcohol **4-2** produced an excess of the corresponding α-glycoside of at least 5.5:1 α:β at nearly all reaction temperatures tested (entries 2 to 5, right column). No conversion was observed at -78 °C (entry 1, right column), providing evidence for the limited reactivity of nucleophile **4-2**. Nucleophiles **4-1** and **4-2** produced opposite stereoselectivities in glycosylations, confirming that the outcome of a glycosylation reaction is critically dependent upon *O*-nucleophilicity

It was next assessed whether the influence of *O*-nucleophilicity on stereoselectivity is sensitive towards the choice of glycosylating agent and activation method (Table 4.2). A plethora of strategies are available to activate thioglycosides. Similar to the most

widely employed NIS/TfOH system (Table 4.2, entry 1), mild thiophilic promoter DMTST[264] led to opposing selectivities of 1:4 vs. 10:1 α:β using alcohols **4-1** and **4-2** as nucleophiles, respectively (Table 4.2, entry 2).

Table 4.1. Impact of *O*-nucleophilicity on the stereoselectivities of glycosylation reactions at different temperatures.



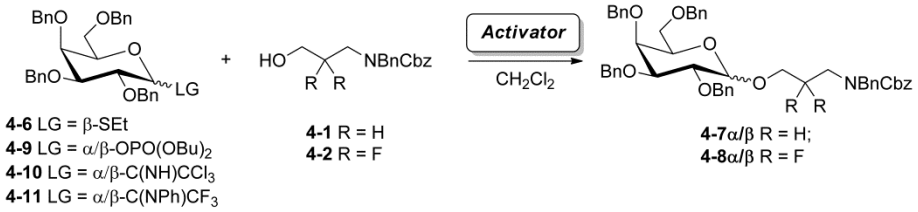
		Selectivity, α:β (yield, %)[e,f]	
Entry[a,b,c,d]	**Temperature**	**Nucleophile 4-1 (R = H)**	**Nucleophile 4-2 (R = F)**
1	-78 °C	1:26 (67)	n.r.
2	**-40 °C**	**1:26 (83)**	**5.5:1 (88)**
3	-20 °C	1:5.4 (67)	8:1 (83)
4	0 °C	1:2.6 (76)	9:1 (66)
5	r.t.	1:1.9 (74)	6:1 (62)

[a]1.5 equiv. glycosylating agent **4-6**, 1.0 equiv. nucleophile. [b]Reactions performed in CH_2Cl_2. [c]3 Å-AW mol. sieves were used. [d]NIS/TfOH was used as an activator system. [e]Selectivity determined by HPLC. [f]Isolated yield.

Pre-activation of thioglycosides using the Ph_2SO/Tf_2O[259] activator system can lead to stereoselectivities that are inaccessible otherwise, presumably due to the emergence of covalent anomeric triflates.[364] Pre-activation did not significantly alter the stereoselectivities obtained in glycosylation reactions of thioglycoside **4-6** with alcohols **4-1** and **4-2**, with α:β-ratios of 1:6 and 11.5:1, respectively (entry 3). Glycosyl phosphate **4-9**[365] as well as glycosyl imidates **4-10**[366] and **4-11**[367] were included in the experiment to assess alternative anomeric leaving groups. Preferential formation of the β-anomer was observed in all reactions using non-fluorinated alcohol **4-1**, whereas the α-anomer was preferred in reactions using fluorinated linker **4-2** (entries 4 to 6). Thereby, glycosyl phosphate displayed a remarkable selectivity difference (1:10 vs. 10:1 α:β for nucleophiles **4-1** and **4-2**, respectively), while trifluoroacetimidate **4-11** was associated with the smallest difference in stereoselectitvity (1:3 vs. 1.3:1 α:β for **4-1** and **4-2**, respectively). Thus, *O*-nucleophilicity impacts stereoselectivity in glycosylations using a range of

different activator and leaving group systems. Pre-activation of thioglycosyides does not override this effect.

Table 4.2. Impact of *O*-nucleophilicity on the stereoselectivities of glycosylation reactions using different leaving groups and activators.



4-6 LG = β-SEt
4-9 LG = α/β-OPO(OBu)$_2$
4-10 LG = α/β-C(NH)CCl$_3$
4-11 LG = α/β-C(NPh)CF$_3$

4-1 R = H
4-2 R = F

4-7α/β R = H;
4-8α/β R = F

			Selectivity, α:β[e,f]	
Entry[a,b,c,d]	**LG (compound)**	**Activator**	**Nucleophile 4-1 (R = H)**	**Nucleophile 4-2 (R = F)**
1	β-SEt, (**4-6**)	NIS, TfOH	1:26	5.5:1
2	β-SEt, (**4-6**)	DMTST[h,i]	1:4	10:1
3	β-SEt, (**4-6**)	Ph_2SO, Tf_2O, TTBPy[g,h,i]	1:6	11.5:1
4	α/β-OPO(OBu)$_2$ (**4-9**)	TMSOTf	1:10	10:1
5	α/β-C(NH)CCl$_3$ (**4-10**)	TMSOTf	1:9.6	2:1
6	α/β-C(NPh)CCl$_3$ (**4-11**)	TMSOTf	1:3	1.3:1

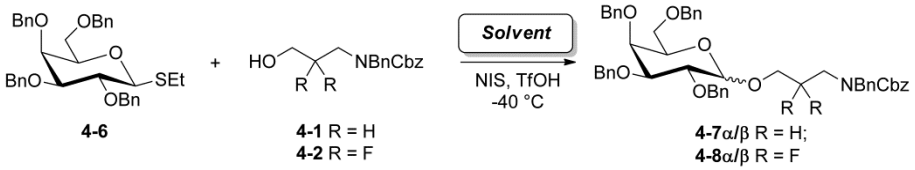
[a]1.5 equiv. glycosylating agent, 1.0 equiv. nucleophile. [b]Reactions performed in CH_2Cl_2. [c]3 Å-AW mol. sieves were used. [d]Reaction performed at -40 °C. [e]Selectivity determined by HPLC. [f]Isolated yields were generally between 60% and 90%. [g]Pre-activation. [h]Reaction performed at -40 °C to r.t. [i]4 Å mol. sieves were used. LG = leaving group. TTBPy = tri-*tert*-butylpyridine.

The choice of the solvent can have a pronounced effect on the stereoselectivity of a glycosylation reaction. In D-*galacto* series such as building block **4-6**, ethereal solvents are known to promote α-selectivity, while nitriles efficiently induce β-selectivity ("nitrile effect").[177, 368] It was thus interesting to assess to what extent the solvent affects stereoselectivities of glycosylation reactions between thioglycoside **4-6** and alcohols with different *O*-nucleophilicities (Table 4.3). In comparison to dichloromethane as a solvent, addition of diethyl ether markedly increased the α-selectivity of glycosylations using both **4-1** and **4-2** as nucleophiles in the NIS/TfOH-promoted reaction at -40 °C (Table 4.3, entries 1 and 2). Nevertheless, stereoselectivities were still opposed, with preferential formation of the β-glycoside of alcohol **4-1** (1:5.9 α:β) and the α-glycoside of fluorinated alcohol **4-2** (15.7:1 α:β). When a mixture of toluene and 1,4-dioxane[266] was used as solvent, both nucleophiles displayed α-selectivity (entry 3), yet of a significantly higher extent when using difluorinated alcohol **4-2** (3:1 vs. 28:1 α:β for **4-1** and **4-2**,

respectively). Thus, a synergistic effect is found to yield increased α-selectivities when an alcohol with low nucleophilicity is employed in ethereal solvents instead of a non-participating solvent. In turn, this solvent effect reduces, but does not nullify the β-stereoselectivity associated with highly nucleophilic alcohols in the system studied.

As expected, glycosylations were β-selectivite when conducted in acetonitrile due to the nitrile effect (entry 4).[177, 368] Thereby, non-fluorinated alcohol **4-1** was associated with a notable 1:31 α:β-stereoselectivity, while fluorinated alcohol **4-2** produced a lower β-stereoselectivity of 1:3.5 α:β. These results complement the synergism observed for ethereal solvents (*see* above), and the use of a highly nucleophilic alcohol in combination with a nitrile solvent enhances the inherent β-stereoselectivity of this reaction even further. In contrast, low nucleophilicity and nitrile effect seem to somewhat counteract each other, leading to low β-stereoselectivity.

Table 4.3. Impact of *O*-nucleophilicity on the stereoselectivities of glycosylation reactions using different solvents.



Entry[a,b,c,d]	Solvent	Selectivity, α:β (yield, %) [e,f] Nucleophile 4-1 (R = H)	Nucleophile 4-2 (R = F)
1	CH_2Cl_2	1:26	5.5:1
2	CH_2Cl_2/Et_2O[g]	1:5.9	15.7:1
3	toluene/1,4-dioxane[h]	3:1	28:1
4	MeCN	1:31	1:3.5
5	Trichloroethylene	1:6	9:1

[a]1.5 equiv. glycosylating agent **4-6**, 1.0 equiv. nucleophile. [b]3 Å-AW mol. sieves were used. [c]NIS/TfOH was used as an activator system. [d]Reaction performed at -40 °C. [e]Selectivity determined by HPLC. [f] Isolated yields were generally between 60% and 90%. [g]A 1:4 (v/v) CH_2Cl_2:Et_2O mixture was used. [h]A 1:3 (v/v) toluene:1,4-dioxane mixture was used.

In addition to direct participation by oxacarbenium ion coordination, the polarity of a solvent exerts a crucial effect on the stereochemical outcome of a glycosylation.[369] The solvent trichloroethylene is less polar than dichloromethane and has been found to alter stereoselectivity by favoring less polar reaction intermediates.[369] Using trichloroethylene as a solvent in the reactions between thioglycoside **4-6** and alcohols **4-1**

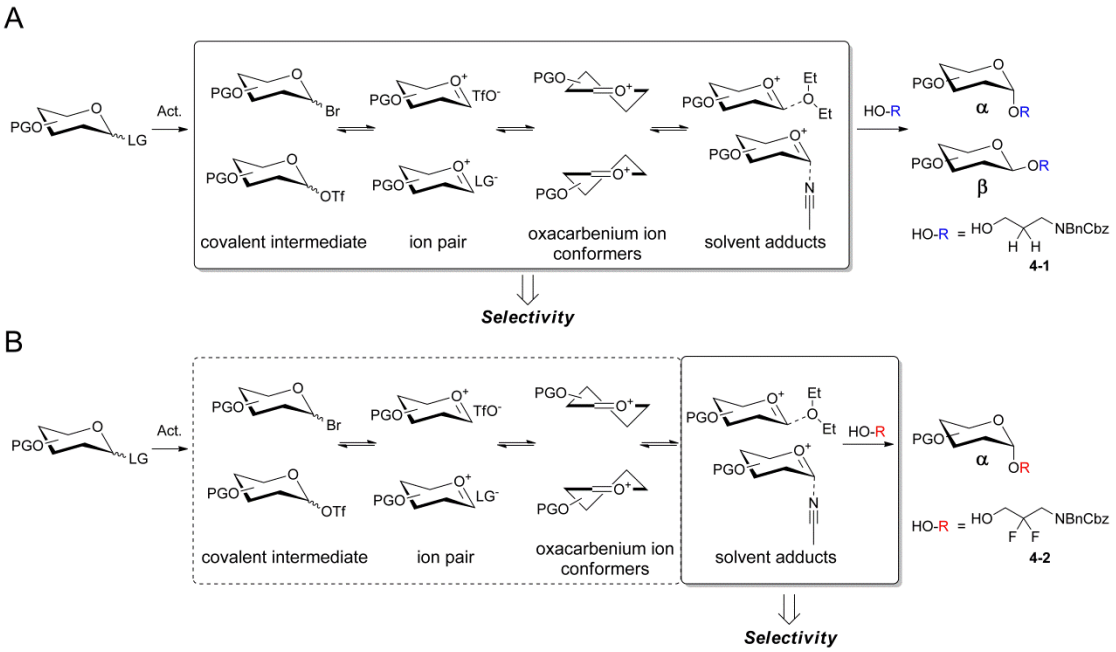
and **4-2** led to β-selective (1:6 α:β) introduction of the former, non-fluorinated alcohol, while the latter, fluorinated alcohol displayed good α-selectivity (9:1 α:β) that was even higher than in the presence of dichloromethane as a solvent (Table 4.2, entry 5). Thus, *O*-nucleophilicity can be used to robustly tune the stereoselectivity of glycosylation reactions, especially in combination with solvents that confer synergism towards a particular anomeric configuration.

4.3 Conclusion and Outlook

Glycosidic bond formation remains one of the least predictable transformations in organic chemistry. A multitude of effects influence the outcome of a glycosylation, and just as many variables exist to manipulate glycosylations. With the exception of thermodynamically controlled Fischer glycosylations (*see* Chapter 1), glycosylation reactions are usually under kinetic control, and the selectivity-determining step is likely dependent on reaction intermediates (Scheme 4.3). The use of simplified model systems has provided important insight into the kinetic processes that govern stereoselectivity.[186, 193-196, 270, 357, 370, 371] However, the translation of that data into a comprehensive Curtin-Hammett-type model to predict stereoselectivity has been precluded by the sheer number of stereodirecting parameters. A continuum of transient intermediates have been proposed ranging from covalent adducts via ion pairs to "naked" oxacarbenium ions.[194] Additionally, the relevance of these intermediates critically depends upon steric and stereoelectronic effects contributed by functional groups elsewhere in the molecule, rendering the prediction of stereoselectivity often impossible.[196, 371]

Considering the efforts that have been invested into studying the intermediates of glycosylation reactions, it is surprising how little is known about the role of the nucleophile. Although nucleophilicity is irrelevant for the rate of a classical S_N1 reaction, the structure and reactivity of a nucleophile can tremendously influence the outcome of the reaction.[372] This notion is underlined by the cumbersome 1,2-*cis*-selective introduction of primary aliphatic alcohols as part of linker molecules through glycosylation. A method to reliably achieve this transformation would immensely facilitate oligosaccharide synthesis, and enable a scenario in which linker molecules could be stereoselectively introduced at a late stage of a synthetic route.

The development of a linker that displays low *O*-nucleophilicity was instigated by a recent finding that correlated nucleophilicity to stereoselectivity in model glycosylations.[193] An appropriate linker was designed by formal substitution of a methylene group by a difluoromethylene group of a known linker alcohol. In proof-of-principle experiments, a non-fluorinated control alcohol displayed excellent β(1,2-*trans*)-selectivities in the glycosylation reaction studied, in contrast to recent findings reporting an erosion of stereoselectivity when highly reactive nucleophiles are used. Interestingly, difluorination led to a complete reversal of stereoselectivity to α(1,2-*cis*). Preliminary quantum mechanical modelling of both linker alcohols revealed very similar ground state conformations of both molecules, but a tremendous decrease of electron density at the alcohol oxygen due to the presence of the two fluoride substituents (data not shown). These findings rule out an influence of conformational or steric differences and highlight the importance of nucleophilicity for stereoselectivity.



Scheme 4.3. Selectivity-determining factors of glycosylation reactions. *A*, activation of a glycosylating agent leads to the generation of intermediates that can be in equilibrium. Reaction conditions, steric and stereoelectronic effects enable the predominance of certain intermediates. Stereoselectivity is determined based on the reactivity and stability of these intermediates (Curtin-Hammett principle) when a highly reactive alcohol is used as a nucleophile. *B*, The stereoselectivity induced by the use of an alcohol with low nucleophilicity overrides many of the stereodirecting parameters of glycosylation reactions to predominantly yield the corresponding α-glycoside (1,2-*cis* for *galacto* and *gluco* series, for instance). The use of participating solvents can, however, enhance or (to a certain extent) revert this stereoinduction. Act. = activation.

The stereochemical outcome of glycosylations is often temperature-dependent due to the interplay of multiple factors such as the stability of certain intermediates[370] or the interconversion of distinct conformations.[371] The temperature dependence of β(1,2-*trans*)-selectivities obtained with the non-fluorinated control nucleophile is a testimony to that fact. Remarkably, α(1,2-*cis*)-selectivities were constantly above 5.5:1 α:β using the difluorinated linker irrespective of the reaction temperature. Furthermore, low *O*-nucleophilicity led to the preferential formation of α(1,2-*cis*)-glycosides when using a range of different leaving groups and activators. These results suggest that low *O*-nucleophilicity could be a general determinant that overrides many of the stereodirecting parameters of glycosylation reactions, such as the occurrence of covalent or ion pair intermediates and the conformational preferences of oxacarbenium ions (Scheme 4.3B). Although imidate-based glycosylating agents displayed lower α(1,2-*cis*)-selectivities than glycosyl phosphates and thioglycosides, the evaluation of different solvents suggests that a synergism between low *O*-nucleophilicity and the directing effect of ethereal solvents can be exploited to further improve selectivities. Importantly, combining the effects of alcohol nucleophilicity with solvent effects yielded stereoselectivities between 1:31 and 28:1 α:β. These values correspond to diastereomeric ratios (d.r.) of 97:3 and are unparalleled by most other methods that mediate the 1,2-*cis*-selective introduction of glycosidic bonds, especially using aliphatic linker alcohols as nucleophiles. Thus, judicious choice of nucleophile and reaction conditions provides access to linker-bearing glycosides of either anomeric configuration with essentially complete stereoselectivity.

Taken together, a method was devised to 1,2-*cis*-stereoselectively introduce an amine-functionalized linker alcohol at the reducing end of synthetic glycans. The method features the use of an alcohol with low nucleophilicity, and ought to be applicable to a range of different glycosylating agents. Furthermore, it will be interesting to study the introduction of that linker at a late synthetic stage of oligosaccharide synthesis. These studies are currently underway.

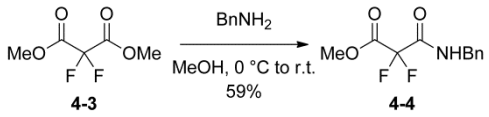
4.4 Experimental Section

General Experimental Details

Commercial grade solvents and reagents were used unless stated otherwise. Anhydrous solvents were obtained from a Dry Solvent System (Waters, Milford, USA). Solvents for chromatography were of technical grade and distilled under reduced pressure prior to use. Sensitive reactions were carried out in heat-dried glassware and under an argon atmosphere. Analytical thin layer chromatography (t.l.c.) was performed on Kieselgel 60 F254 glass plates pre-coated with silica gel of 0.25 mm thickness (Macherey-Nagel, Düren, Germany). Spots were visualized with sugar stain (0.1% (v/v) 3-methoxyphenol, 2.5% (v/v) sulfuric acid in EtOH) or CAM stain (5% (w/v) ammonium molybdate, 1% (w/v) cerium(II) sulfate and 10% (v/v) sulfuric acid in water) dipping solutions. Flash chromatography was performed on Kieselgel 60 with 230-400 mesh (Sigma-Aldrich, St. Louis, USA). Solvents were removed under reduced pressure using a rotary evaporator and high vacuum (<1 mbar).

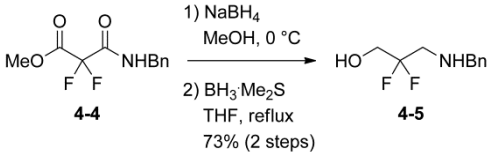
^{1}H, ^{13}C and two-dimensional NMR spectra were measured with a Varian 400-MR spectrometer or a Varian 600 spectrometer (both Agilent, Santa Clara, USA) at 298 K. Chemical shifts (δ) are reported in parts per million (ppm) relative to the respective residual solvent peaks ($CDCl_3$: δ 7.26 in ^{1}H and 77.16 in ^{13}C NMR). Two-dimensional NMR experiments (HH-COSY, CH-HSQC, CH-HMBC) were performed to assign peaks in ^{1}H and ^{13}C spectra. The following abbreviations are used to indicate peak multiplicities: *s* singlet; *d* doublet; *dd* doublet of doublets; *t* triplet; *m* multiplet. Coupling constants (*J*) are reported in Hertz (Hz). NMR spectra were evaluated using MestreNova 6.2 (MestreLab Research SSL, Santiago de Compostella, Spain). High resolution mass spectrometry by electrospray ionization (ESI-HRMS) was performed at Freie Universität Berlin, Mass Spectrometry Core Facility, with a 6210 ESI-TOF mass spectrometer (Agilent). High performance liquid chromatography (HPLC) was carried out with a 1200 HPLC system equipped with an Evaporating Light Scattering Detector (both Agilent). Schemes were prepared using ChemBioDraw Ultra 12.0.2 (Cambridgesoft, Waltham, USA).

Methyl *N*-benzyl-2,2-difluoromalonate monoamide (4-4)



To a stirred solution of dimethyl 2,2-difluoromalonate **4-3** (3.0 g, 17.85 mmol) in MeOH (100 mL) was added dropwise at 0 °C a solution of benzylamine (1.56 mL, 14.28 mmol) in MeOH (10 mL). The mixture was warmed to room temperature, stirred for 18 h at that temperature, filtered and concentrated. The residue was purified by flash chromatography (EtOAc/toluene 1:50 to 1:10) to give monoamide **4-4** (2.06 g, 8.47 mmol, 59%) as a white foam. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42 – 7.27 (m, 5H), 6.71 (s, 1H), 4.54 (d, J = 5.8 Hz, 2H), 3.94 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 136.3, 129.1, 128.3, 128.0, 54.3, 44.0; HRMS (ESI) calcd. for $C_{11}H_{11}F_2NO_3$ $(M+Na)^+$ 266.0604 found 266.0597 m/z.

N-Benzyl-2,2-difluoro-3-amino-1-propanol (4-5)

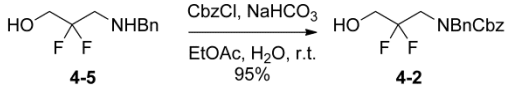


To a stirred solution of ester **4-4** (2.0 g, 8.22 mmol) in MeOH (50 mL) was added at 0 °C sodium borohydride (1.56 g, 41.1 mmol). The mixture was stirred for 2 h at that temperature, quenched with water (1 mL) and concentrated. The residue was dissolved in EtOAc (25 mL) and water (50 mL). After separation, the aqueous phase was extracted with EtOAc (2x25 mL), the combined organic fractions were dried over Na_2SO_4 and concentrated. The residue was purified by flash chromatography (EtOAc/toluene 1:50 to 1:10) to give the intermediate monoamide (1.4 g) as a white foam.

To a stirred solution of the intermediate monoamide in THF (25 mL) was added dropwise at room temperature borane dimethylsulfide (2 M solution in THF, 16.3 mL, 32.5 mmol). The reaction was refluxed for 3 h, slowly quenched with MeOH (5 mL) and cooled to room temperature. The mixture was stirred for 18 h at that temperature and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 1:10 to 1:1) to give amine **4-5** (1.2 g, 5.96 mmol, 73% over two steps) as a clear oil. ^{1}H NMR

(400 MHz, $CDCl_3$) δ 7.38 – 7.27 (m, 5H), 3.93 – 3.82 (m, 4H), 3.11 (t, J = 13.1 Hz, 2H), 2.67 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 138.9, 128.8, 128.3, 127.7, 123.5, 121.1, 118.7, 77.36, 64.7, 64.4, 64.1, 53.8, 51.8, 51.5, 51.2; HRMS (ESI) calcd. for $C_{10}H_{13}F_2NO$ $(M+H)^+$ 202.1043 found 202.1043 m/z.

N-Benzyl-*N*-benzyloxycarbonyl-2,2-difluoro-3-amino-1-propanol (4-2)



To a stirred solution of amine **4-5** (1.2 g, 5.96 mmol) in EtOAc (50 mL) and sat. aq. $NaHCO_3$ (50 mL) was added at room temperature benzyl chloroformate (1.02 mL, 7.16 mmol). The mixture was stirred for 2 h at that temperature and the layers were separated. The aqueous layer was extracted with EtOAc (3x20 mL), the combined organic fractions were washed with brine, dried over Na_2SO_4 and concentrated. The residue was purified by flash chromatography (EtOAc/hexanes 1:20 to 1:5) to give carbamate **4-2** (1.89 g, 5.64 mmol, 95%) as a clear oil. ^1H NMR (400 MHz, $CDCl_3$) δ 7.44 – 7.28 (m, 8H), 7.23 – 7.06 (m, 2H), 5.23 (s, 2H), 4.60 (s, 2H), 4.23 (t, J = 7.9 Hz, 1H), 3.77 – 3.54 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.3, 136.2, 135.7, 129.0, 128.8, 128.6, 128.2, 128.0, 127.7, 127.1, 124.9, 122.4, 120.0, 77.4, 68.7, 61.7, 61.4, 61.1, 51.8, 47.4, 47.0, 46.7; HRMS (ESI) calcd. for $C_{18}H_{19}F_2NO_3$ $(M+H)^+$ 358.1230 found 358.1221 m/z.

General Glycosylation Procedures Using Alcohols 4-1 and 4-2 as Nucleophiles

Thioglycoside activation using NIS/TfOH

Typically, linker alcohol **4-1** or **4-2** (0.075 mmol) and thioglycoside (0.112 mmol) were co-evaporated twice with anhydrous toluene and kept for 30 min under high vacuum. The mixture was dissolved in the indicated solvent (2 mL), and activated molecular sieves (3 Å-AW) were added. The solution was stirred for 15 min at room temperature and cooled to the indicated temperature. The mixture was treated with NIS (25 mg, 0.112 mmol) and TfOH (1.3 µL, 0.015 mmol) and stirred until t.l.c. indicated consumption of the thioglycoside (at least 1.5 h). The reaction was then quenched with Et_3N (1 mL) and stirred for another 10 min. The mixture was diluted with CH_2Cl_2 (9 mL), filtered and concentrated. The residue was filtered through a pad of silica gel (EtOAc/hexanes 0:1 to 1:4).

Thioglycoside Activation Using DMTST

Linker alcohol **4-1** or **4-2** (0.075 mmol) and thioglycoside **4-6**[346] (0.112 mmol) were co-evaporated twice with anhydrous toluene and kept for 30 min under high vacuum. The mixture was dissolved in CH_2Cl_2 (2 mL), and TTBPy (41 mg, 0.164 mmol) and activated molecular sieves (4 Å) were added. The solution was stirred for 15 min at room temperature and cooled to -40 °C. The mixture was treated with a solution of DMTST (38.5 mg, 0.149 mmol) in CH_2Cl_2 (1 mL) and kept at that temperature for 15 min. The reaction was slowly warmed to room temperature over 1.5 h, quenched with sat. aq. $NaHCO_3$ (1 mL) and stirred for another 10 min. The mixture was diluted with CH_2Cl_2 (9 mL), filtered and concentrated. The residue was filtered through a pad of silica gel (EtOAc/hexanes 0:1 to 1:4).

Thioglycoside Activation Using Ph_2SO/Tf_2O

Thioglycoside **4-6** (0.112 mmol) was co-evaporated twice with anhydrous toluene and kept for 30 min under high vacuum. Ph_2SO (45 mg, 0.224 mmol) and TTBPy (37 mg, 0.15 mmol) were added, the mixture was dissolved in CH_2Cl_2 (2 mL) and activated molecular sieves (4 Å) were added. The solution was stirred for 15 min at room temperature and cooled to -40 °C. The mixture was treated with Tf_2O (25 µL, 0.15 mmol) and kept at that temperature for 10 min. Linker alcohol **4-1** or **4-2** (0.075 mmol) was added in CH_2Cl_2 (1 mL), the reaction was kept at that temperature for 15 min and slowly warmed to room temperature over 1.5 h. The reaction was then quenched with sat. aq. $NaHCO_3$ (1 mL) and stirred for another 10 min. The mixture was diluted with CH_2Cl_2 (9 mL), filtered and concentrated. The residue was filtered through a pad of silica gel (EtOAc/hexanes 0:1 to 1:4).

Glycosyl Phosphate Activation

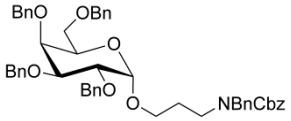
Linker alcohol **4-1** or **4-2** (0.075 mmol) and glycosyl phosphate **4-9**[365] (0.112 mmol) were co-evaporated twice with anhydrous toluene and kept for 30 min under high vacuum. The mixture was dissolved in the indicated solvent (2 mL), and activated molecular sieves (3 Å-AW) were added. The solution was stirred for 15 min at room temperature and cooled to -40 °C. The mixture was treated with TMSOTf (20 µL, 0.112 mmol) and stirred until t.l.c. indicated consumption of the glycosylating agent (at least 1.5 h). The reaction was then quenched with Et_3N (1 mL) and stirred for another 10 min. The

mixture was diluted with CH_2Cl_2 (9 mL), filtered and concentrated. The residue was filtered through a pad of silica gel (EtOAc/hexanes 0:1 to 1:4).

Glycosyl Imidate Activation

Linker alcohol **4-1** or **4-2** (0.075 mmol) and glycosyl imidate **4-10**[366] or **4-11**[367] (0.112 mmol) were co-evaporated twice with anhydrous toluene and kept for 30 min under high vacuum. The mixture was dissolved in the indicated solvent (2 mL), and activated molecular sieves (3 Å-AW) were added. The solution was stirred for 15 min at room temperature and cooled to -40 °C. The mixture was treated with TMSOTf (2.7 µL, 0.015 mmol) and stirred until t.l.c. indicated consumption of the glycosylating agent (at least 1.5 h). The reaction was then quenched with Et_3N (1 mL) and stirred for another 10 min. The mixture was diluted with CH_2Cl_2 (9 mL), filtered and concentrated. The residue was filtered through a pad of silica gel (EtOAc/hexanes 0:1 to 1:4).

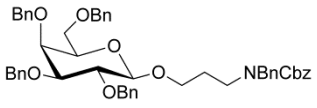
2,3,4,6-Tetra-*O*-benzyl-α-D-galactopyranosyl-(1→1)-(3-*N*-benzyl-*N*-benzyloxycarbonylamino)propanol (4-8α)



4-8α

Clear oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46 – 7.11 (m, 30H), 5.18 (s, 2H), 4.96 (d, J = 11.5 Hz, 1H), 4.90 – 4.69 (m, 4H), 4.67 – 4.50 (m, 3H), 4.50 – 4.33 (m, 3H), 4.08 – 3.79 (m, 4H), 3.68 – 3.26 (m, 6H), 1.97 – 1.79 (m, 2H); HRMS (ESI) calcd. for $C_{52}H_{55}NO_8$ $(M+H)^+$ 844.3825 found 844.3773 m/z.

2,3,4,6-Tetra-*O*-benzyl-β-D-galactopyranosyl-(1→1)-(3-*N*-benzyl-*N*-benzyloxycarbonylamino)propanol (4-8β)

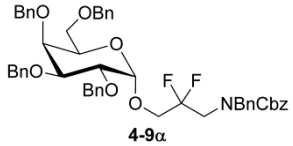


4-8β

Clear oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.51 – 7.09 (m, 30H), 5.18 (s, 2H), 4.96 (d, J = 11.6 Hz, 1H), 4.88 – 4.68 (m, 4H), 4.64 (d, J = 11.7 Hz, 1H), 4.59 – 4.37 (m, 4H), 4.37 – 4.24 (m, 1H), 4.00 – 3.87 (m, 2H), 3.83 – 3.75 (m, 1H), 3.61 – 3.56 (m, 2H), 3.56 – 3.47

(m, 2H), 3.46 – 3.31 (m, 2H), 1.99 – 1.79 (m, 2H); HRMS (ESI) calcd. for $C_{52}H_{55}NO_8$ $(M+H)^+$ 844.3825 found 844.3773 m/z.

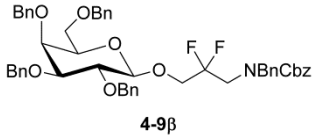
2,3,4,6-Tetra-*O*-benzyl-α-D-galactopyranosyl-(1→1)-(3-*N*-benzyl-*N*-benzyloxycarbonylamino)-2,2-difluoropropanol (4-9α)



4-9α

Clear oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 – 7.18 (m, 30H), 5.17 (s, 2H), 4.98 – 4.90 (m, 2H), 4.82 – 4.71 (m, 2H), 4.71 – 4.53 (m, 5H), 4.51 – 4.30 (m, 3H), 4.10 – 3.73 (m, 7H), 3.56 – 3.45 (m, 2H); HRMS (ESI) calcd. for $C_{52}H_{53}F_2NO_8$ $(M+H)^+$ 880.3636 found 880.3606 m/z.

2,3,4,6-Tetra-*O*-benzyl-β-D-galactopyranosyl-(1→1)-(3-*N*-benzyl-*N*-benzyloxycarbonylamino)-2,2-difluoropropanol (4-9β)



4-9β

Clear oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45 – 7.14 (m, 30H), 5.20 (d, J = 29.3 Hz, 2H), 4.94 (dd, J = 11.5, 2.7 Hz, 1H), 4.79 – 4.53 (m, 7H), 4.48 – 4.29 (m, 3H), 4.13 – 3.86 (m, 3H), 3.84 – 3.64 (m, 4H), 3.59 – 3.44 (m, 3H); HRMS (ESI) calcd. for $C_{52}H_{53}F_2NO_8$ $(M+H)^+$ 880.3636 found 880.3606 m/z.

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