

Synthetic Oligosaccharide Bacterial Antigens to Produce Monoclonal Antibodies for Diagnosis and Treatment of Disease Using *Bacillus anthracis* as a Case Study

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2.1 Introduction

Bacillus anthracis is a Gram-positive, spore-forming soil bacterium that is closely related to *Bacillus cereus* and *Bacillus thuringiensis*. Infections with *Bacillus anthracis* result in a disease called anthrax (Mock and Fouet 2001; Sylvestre et al. 2002). Anthrax is primarily an infection of grazing cattle. Ingested spores germinate within the host to the vegetative form. Vegetative cells multiply, disseminate in the host organism, and kill the host by their virulence factors. Upon contact with air and depending on other environmental factors, the vegetative cells start to sporulate to form the dormant, durable spores again. *B. anthracis* spores are remarkably resistant to physical stress such as extreme temperatures, radiation, harsh chemicals, desiccation, and physical damage. These properties allow them to persist in the soil for decades (Nicholson et al. 2000). Human anthrax infections are very rare and only occur when humans are closely exposed to infected animals, tissue from infected animals or when they are directly exposed to *B. anthracis* spores (Quinn and Turnbull 1998). Depending on the route of infection, anthrax can occur in three forms: cutaneous, gastrointestinal or inhalation anthrax.

More than 95% of all naturally occurring *B. anthracis* infections are cutaneous. This form of anthrax is associated with handling infected animals or products

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thereof such as meat, wool, or leather (Lucez 2005). The majority of cutaneous anthrax lesions proceed locally in the area of the exposed tissue. The anthrax lesion begins as a small papule that quickly enlarges and develops a central vesicle, which ruptures, leaving an underlying necrotic ulcer. Historically, case-fatality rates for cutaneous anthrax have been as high as 20% but are now less than 1% with appropriate antimicrobial treatments (Quinn and Turnbull 1998).

Gastrointestinal anthrax typically occurs after eating raw or undercooked contaminated meat. This form is very rare and the case-fatality ratio is unknown, but estimated to range from 25% to 60% (Beatty et al. 2003).

Inhalation anthrax is a systemic infection that is caused by inhalation of *B. anthracis* spores and is associated with very high fatality rates. The highly resistant spores of *B. anthracis* have been a focus of offensive and defensive biological warfare research programs worldwide (Rotz et al. 2002). The high danger of *B. anthracis* spores used as biological weapons was demonstrated by the accidental release of spores from a biological weapons factory in the Soviet Union. This accident killed more than 100 people. The intentional release of anthrax spores through contaminated letters caused the death of five people out of 11 infected with inhalation anthrax and widespread panic among the American population in the autumn of 2001. Following this terrorist attack, *B. anthracis* research programs with the aim to detect, prevent and cure *B. anthracis* were strongly intensified.

Most symptoms of anthrax infections are caused by three *B. anthracis* protein toxins (Moayeri and Leppla 2004). These three toxins work in concert to result in pathogenesis: protective antigen (PA) forms a membrane channel that mediates the entry of two other toxins, the lethal factor (LF) and the edema factor (EF), into host cells. EF, an adenyl cyclase, catalyzes the conversion of ATP to cAMP. LF, a protease, cleaves members of the MEK family and other targets (Moayeri and Leppla 2004). In this way, the toxins interfere with host cell homeostasis and facilitate pathogen survival. The toxins cause the shock-induced death of the host when they are systemically released at the later stages of infection.

The first effective anthrax vaccines using live, attenuated cultures of *B. anthracis* were developed in 1880 by Greenfield (Tigertt 1980) and Pasteur in 1881 (Pasteur 1881). Although effective, the virulence of these heat-attenuated vaccines varied. In 1939, Sterne developed a live, attenuated vaccine from an avirulent, non-encapsulated variant of *B. anthracis* (Sterne 1939). The Sterne-type vaccine replaced the Pasteur heat-attenuated formulation and is still used today as the veterinary vaccine of choice. Due to strong side effects, improved vaccines were developed for human use. While attenuated bacteria are still used in Russia for vaccination, cell free vaccines were developed in the USA and the UK. The only licensed human anthrax vaccine in the United States today is Anthrax Vaccine Adsorbed (AVA), sold as BioThrax (FDA 2005). AVA is a sterile suspension prepared from cell-free filtrates of the non-encapsulated strain *B. anthracis* V770-NP1-R. While effective, considerable adverse effects are observed after AVA vaccination, mild local reactions, including erythema, edema, and indurations, occur in 20% of the recipients and severe local reactions are observed in 1% of those receiving the vaccine. Systemic reactions such

as fever, chills, body aches, or nausea occurred in up to 0.2% of AVA immunizations (Brachman et al. 1962). Thus, a safer vaccine is highly desirable.

Like several other bacteria, *B. anthracis* also expresses unique oligosaccharides on the surface. Such strain-specific oligosaccharides create excellent opportunities to generate synthetic anti-bacterial vaccines (Seeberger and Werz 2007). By combination of degradation reactions, extensive NMR spectroscopy and mass spectrometry, two interesting carbohydrate structures of *B. anthracis* were elucidated in recent years. One oligosaccharide antigen, tetrasaccharide **1**, that is probably part of a larger oligosaccharide, was structurally assigned in 2004. This tetrasaccharide is found on the surface of the exosporium glycoprotein BclA of *B. anthracis* spores (Daubenspeck et al. 2004). The BclA tetrasaccharide **1** contains three rhamnose residues and an unusual, non-reducing terminal sugar, 2-*O*-methyl-4-(3-hydroxy-3-methylbutanamido)-4,6-dideoxy-D-glucopyranose, that was named anthrose. This sugar had not been observed previously in other organisms. Tetrasaccharide **1** as well as analogues and truncated sequences thereof have become very attractive targets for the development of a synthetic vaccine and the induction of a highly specific immune response against *Bacillus anthracis*. The second interesting carbohydrate structure, which has been elucidated in 2004, is hexasaccharide **2**. This portion is the repeating unit of the major cell wall polysaccharide from *B. anthracis* vegetative cells (Choudhury et al. 2006). Composition analyses have shown that the structure of this hexasaccharide is different from that of even closely related *B. cereus* strains. Thus, this saccharide may have a function in determining the virulence of *B. anthracis* strains and may become also a component for the development of a multi-subunit vaccine against anthrax (Fig. 2.1).

2.2 The Anthrax Tetrasaccharide

Several groups started synthetic efforts towards the so-called anthrax tetrasaccharide right after its structural characterization. The first complete syntheses were published by Seeberger (Werz and Seeberger 2005) and later Kováč (Saksena et al. 2005, 2006, 2007; Adamo et al. 2005). Other routes leading to the same target structure or the

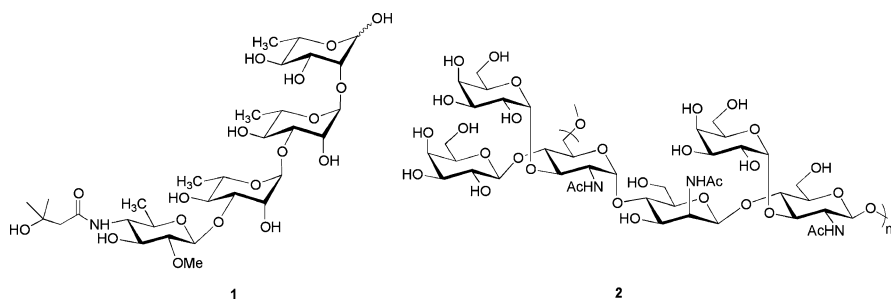
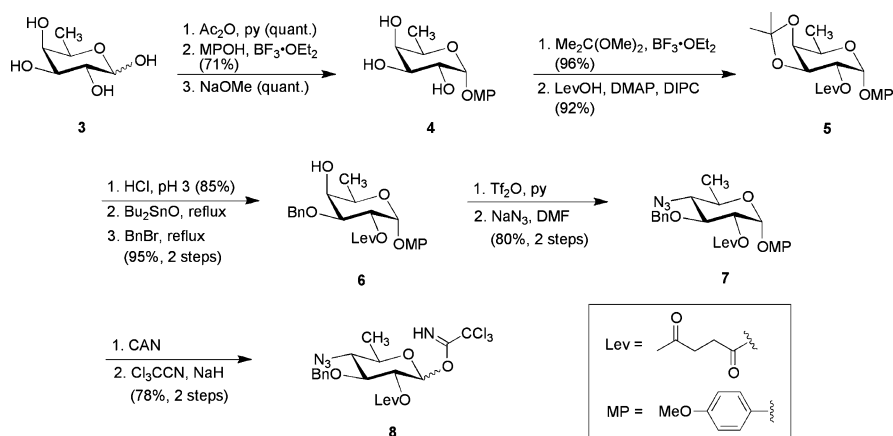


Fig. 2.1 Anthrax tetrasaccharide **1** released from *B. anthracis* spores and hexasaccharide **2**, a repeating unit of major cell wall polysaccharide from *B. anthracis* vegetative cells

shorter trisaccharide portion have been reported by Boons and Crich (Mehta et al. 2006; Crich and Vinogradova 2007). All these protocols start from the chiral pool and utilize readily available carbohydrates such as D-fucose, D-galactose and L-rhamnose as precursors. A completely different route was developed by O'Doherty who used a series of Pd-catalyzed glycosylations as key reactions for the preparation of the oligosaccharide chain (Guo and O'Doherty 2007a,b). A number of enantioselective manipulations are necessary to create the correct stereochemistry starting from 2-acetylfuran. In the following section one synthetic route to the tetrasaccharide is summarized.

2.2.1 Synthesis of Anthrax Tetrasaccharide 1

The synthesis of the unique monosaccharide anthrose is a key step in all syntheses. Seeberger's synthesis of the terminal anthrose started from commercially available D-fucose (**3**) (Scheme 2.1). Acetylation of **3**, followed by immediate protection of the anomeric position with *p*-methoxyphenol (MPOH) and subsequent cleavage of acetate protecting groups furnished **4**. A levulinoyl group proved to be the best choice to protect the C2 hydroxyl group during installation of the $\beta(1\rightarrow3)$ glycosidic linkage in anticipation of its selective removal prior to subsequent *O*-2 methylation. Hence, reaction of **4** with 2,2-dimethoxypropane and introduction of the C2-*O*-levulinic ester furnished **5**. Removal of the isopropylidene and tin-mediated, selective benzylation of the C3 hydroxyl group afforded **6**. Inversion of the stereocenter at C4 was achieved by reaction of the hydroxyl group with triflic anhydride to install a triflate that is displaced by sodium azide in an SN2-type fashion to give **7** (Golik et al. 1991). Removal of the anomeric *p*-methoxyphenyl group using wet cerium ammonium nitrate was followed by the formation of the anthrose trichloroacetimidate **8** using conditions reported by Schmidt.

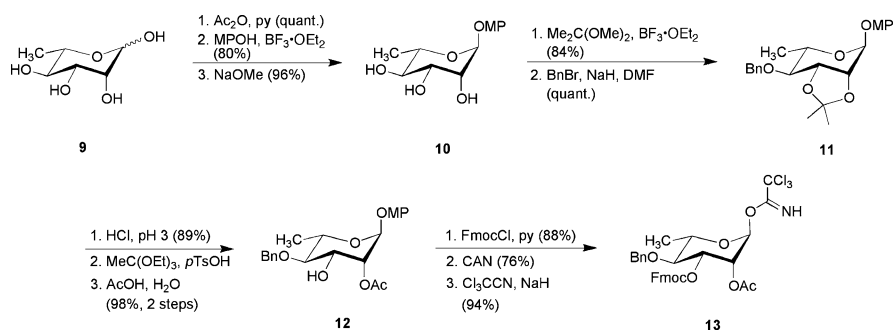


Scheme 2.1 Synthesis of anthrose building block **8** starting from D-fucose **3**

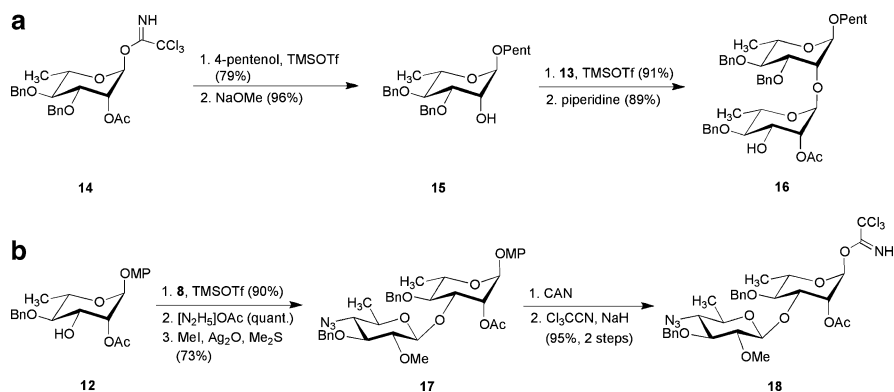
The rhamnose building block **13**, equipped with a robust C2 participating group to ensure α -selectivity and a readily removable temporary protecting group in the C3 position, was synthesized as shown in Scheme 2.2. Placement of an anomeric *p*-methoxyphenol moiety gave **10** (Sarkar et al. 2003). Formation of the *cis*-fused acetal and subsequent benzylation afforded **11**. The transformation of the acetal to the corresponding orthoester and ring-opening resulted in the kinetically preferred axial acetate in **12**. Placement of Fmoc, cleavage of the *p*-methoxyphenyl glycoside and reaction with trichloroacetonitrile in the presence of traces of sodium hydride afforded the trichloroacetimidate building block **13**.

The assembly of the anthrax tetrasaccharide *n*-pentenyl glycoside analogue *via* a (2 + 2) approach commenced with the reaction of building block **14** (Fürstner and Müller 1999) with 4-pentenol (Scheme 2.3a). At a later stage, the *n*-pentenyl moiety can serve as a handle for conjugation to proteins or to glass surfaces for the preparation of microarrays. Removal of acetate, further glycosylation with **13** and subsequent removal of the Fmoc protecting group yielded disaccharide **16**.

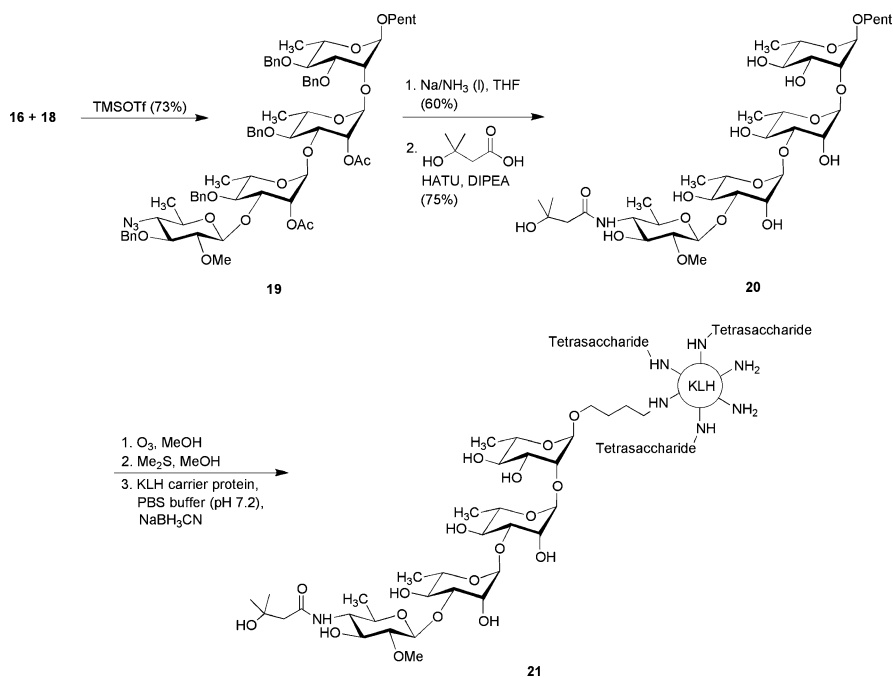
The second disaccharide (Scheme 2.3b) was assembled by glycosylation of **12**, an intermediate in the synthesis of building block **13**, with anthrose building block **8**.



Scheme 2.2 Synthesis of rhamnose building block **13**



Scheme 2.3 Syntheses of disaccharide building blocks **16** (a) and **18** (b)



Scheme 2.4 Completion of the total synthesis and conjugation to a carrier protein

The levulinoyl (Lev) protecting group that ensured β -selectivity was replaced by the final C2 methoxy substituent. Methylation in the presence of acetate was achieved by the action of MeI/Ag₂O in the presence of catalytic amounts of dimethyl sulfide. The commonly used maneuver to convert the methoxyphenyl glycoside into the corresponding trichloroacetimidate furnished disaccharide unit **18**.

To complete the total synthesis, the two disaccharide units **16** and **18** were unified to afford tetrasaccharide **19**. Sodium in liquid ammonia removed all permanent protecting groups and reduced the azide moiety into an amine, thus achieving global deprotection. The formation of the amide with 3-hydroxy-3-methylbutyric acid (Carpino and El-Faham 1995) led to tetrasaccharide **20** including the *n*-pentenyl handle. The double bond in the handle on the reducing terminus was utilized to install a reactive terminal aldehyde moiety *via* ozonolysis. Covalent attachment (Ragupathi et al. 1998; Wang et al. 2000) of tetrasaccharide **20** to keyhole limpet hemocyanine (KLH) carrier protein by reductive amination yielded conjugate **21** that was successfully used as immunogen to produce monoclonal antibodies (mABs) in mice (Tamborini et al. 2006) (Scheme 2.4).

2.2.2 Immunology

The tetrasaccharide–KLH conjugate was formulated in ImmunEasyTM adjuvant (QIAGEN) and this mixture was injected into mice. Following the second booster immunization, antibodies against tetrasaccharide **1** were detected in the blood of

immunized mice as determined by using carbohydrate microarrays bearing the tetrasaccharide **1** structure. Spleen cells of immunized mice were fused with immortalized cell lines to generate hybridoma cell lines. These hybridoma cells were screened for the production of antibodies against tetrasaccharide **1** and cell lines eliciting tetrasaccharide **1** binding IgG antibodies were isolated. Finally, three B cell hybridoma lines producing tetrasaccharide specific monoclonal IgG antibodies (MTA1-3) were generated. All three mAbs bound native *B. anthracis* endospores as determined by an indirect immunofluorescence assay (Tamborrini et al. 2006). By contrast, endospores of close relatives of *B. anthracis* including *B. cereus*, *B. subtilis*, and *B. thuringiensis* strains were not bound by the mAbs MTA1-3.

Independently, other research groups generated anti-anthrax-tetrasaccharide antibodies using neoglycoconjugates comprised of synthetic tetrasaccharide **1** and carrier proteins. Kováč et al. as well as Boons et al. produced antibodies in rabbits with a high specificity for *B. anthracis* (Kuehn et al. 2009; Mehta et al. 2006).

Such antibodies that are highly specific for *B. anthracis* spores are excellent tools to detect *B. anthracis*. Efforts to exploit these antibodies to generate *B. anthracis* spore tests have met with success. Such tests once fully developed will ensure that spores will be detected more readily in order to allow for faster, life-saving treatment and to take the necessary measures when *B. anthracis* spores are used as biological weapons.

2.2.3 Analysis of Anthrax Carbohydrate–Antibody Interactions

Interactions of antibodies with the anthrax oligosaccharide antigen were biochemically analyzed in great detail due to the enormous importance and medical potential of this antigen. A combination of synthetic glycan microarray screening, surface plasmon resonance (SPR), and Saturation Transfer Difference (STD) NMR were used to identify crucial antibody-binding positions on the sugar antigen (Oberli et al. 2010). First, mAbs against a truncated structure, the non reducing terminal disaccharide **36**, (designated MTD1-6) were generated as described above in order to investigate and compare the importance of the anthrose moiety for immunogenicity in the following biochemical experiments.

A collection of synthetic oligosaccharides related to the BclA tetrasaccharide were chemically synthesized. The synthetic oligosaccharide analogues and fragments ranged from mono- to tetrasaccharides related to the original anthrax tetrasaccharide **1**. These synthetic glycans were analyzed for their ability to bind the anti-disaccharide mAbs (MTD1-6) and the anti-tetrasaccharide mAbs (MTA1-3) (Fig. 2.2).

To uncover which structural elements of the anthrax carbohydrate influence the selectivity of antibody–carbohydrate interactions, microarray screening was performed using the generated mAbs (MTA1-3 and MTD1-6) and microarrays bearing the collection of synthetic anthrax oligosaccharides. The anti-tetrasaccharide and the anti-disaccharide mAbs exhibited profoundly different binding patterns. The anti-disaccharide mAbs recognized all synthetic structures with an intact anthrose moiety (structures **22–27** and **33–37**), including anthrose monosaccharide

	anti-disach. mAbs MTD1-6	anti-tetrasach. mAbs MTA1-3	anti-disach. mAbs MTD1-6	anti-tetrasach. mAbs MTA1-3	anti-disach. mAbs MTD1-6	anti-tetrasach. mAbs MTA1-3
						
	+	+	-	-	-	-
	+	+	-	-	+	+
	+	(+)	-	-	+	+
	+	(+)	-	-	-	-
	+	-	-	-	+	+
	+	(+)	-	-	+	+
	-	-	-	-	-	-
						
						
						
						
						
						
						
						

Fig. 2.2 Synthetic glycans related to the *B. anthracis* cell surface tetrasaccharide BcIA. The synthetic glycans were used for antibody mapping by microarray screening, SPR and STD NMR analysis. Microarray analysis demonstrates the cross-reactivity of monoclonal antibodies generated against anthrose-rhamnose disaccharide **36** (MTD1–MTD6) and tetrasaccharide **22** (MTA1–MTA3) (Reprinted with permission from Oberli et al. (2010). Copyright © 2010, American Chemical Society)

34. Similarly, the anti-tetrasaccharide mAbs strongly bound tetrasaccharide analogues **22** and **23**, and the trisaccharide **37**. However, the anti-tetrasaccharide antibodies bound tetrasaccharide analogues **24**, **25** and **27** only weakly, and tetrasaccharide analogues **26** and **28** were not bound at all. Notably, each of these structures contained a modified terminal anthrose. No antibody, neither anti-disaccharide nor anti-tetrasaccharide mAbs, recognized mono-, di-, or trirhamnose structures (**29–32**). Altogether, these results demonstrate that anthrose is the minimal unit required for binding anti-disaccharide mAbs. Interestingly, while a terminal anthrose is absolutely required for oligosaccharide recognition by the anti-tetrasaccharide mAbs, these mAbs failed to bind the anthrose containing truncated mono- and disaccharide structures (**32–36**). Therefore, the anti-tetrasaccharide mAbs require at least two rhamnose units as well as the terminal anthrose for tight oligosaccharide binding.

Anthrose, other than most glycans in mammalian systems contains a side chain appendage other than *N*-acetylation. Since the anthrose unit is essential for antibody binding, its distinctive side chain was investigated in greater detail. A drastic truncation of the chain, produced by reducing 3-hydroxy-3-methylbutyrate to acetate (Fig. 2.2, **28**), resulted in a structure that was not recognized by any of the mAbs that were tested. However, deleting a methyl group within the side chain, by replacing 3-hydroxy-3-methylbutyrate with 3-hydroxybutyrate (Fig. 2.2, carbohydrates **24** and **25**), reduced binding of the anti-tetrasaccharide mAbs dramatically, but had little effect on binding anti-disaccharide mAbs. Similarly, placement of a trimethylacetyl moiety (Fig. 2.2, **26**), or deletion of a 3-hydroxyl group (Fig. 2.2, **27**) only affected anti-tetrasaccharide mAb binding significantly. Therefore, while the anthrose side chain must be present on the glycan to bind both classes of mAbs, only the anti-tetrasaccharide mAbs are affected by altering the specific chemical composition of the side chain, such as removing the C3 methyl group for instance.

A more detailed analysis of the anthrax carbohydrate–antibody interactions relied on SPR experiments (Fig. 2.3). These measurements confirmed and underscored the tight interaction ($K_D = 9.1 \mu\text{M}$) between the anti-tetrasaccharide mAb MTA1 and the tetrasaccharide **1** antigen used to elicit the immune response. Consistent with the microarray results, MTA1 did not bind any other synthetic glycan tested with strong affinity. SPR analysis further demonstrated that the kinetics of the tetrasaccharide **1**–MTA1 interaction are fast and are indeed much faster than the binding kinetics of anti-disaccharide antibodies with any ligand. Notably, the anti-disaccharide mAb MTD6 showed unusually high affinity ($K_D = 0.51 \mu\text{M}$) to its original disaccharide antigen (**36**). Few carbohydrate–antibody interactions with K_D s below $1 \mu\text{M}$ have been reported, thus making this discovery particularly significant. Interestingly, K_D values were comparable for interactions between MTD6 and two structurally diverse oligosaccharides, the tetrasaccharide **1** ($K_D = 3.7 \mu\text{M}$) and the anthrose monosaccharide ($K_D = 7.2 \mu\text{M}$). Given this small difference in K_D , we can conclude that the rhamnose units in the tetrasaccharide contribute little to MTD6 binding.

The molecular details of anthrax carbohydrate–antibody interactions were analyzed using STD NMR to determine individual groups and atoms that are important for binding. STD NMR is particularly suited to establish differences in binding to different

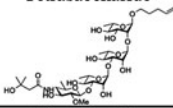
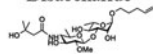
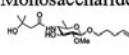

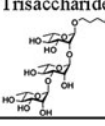
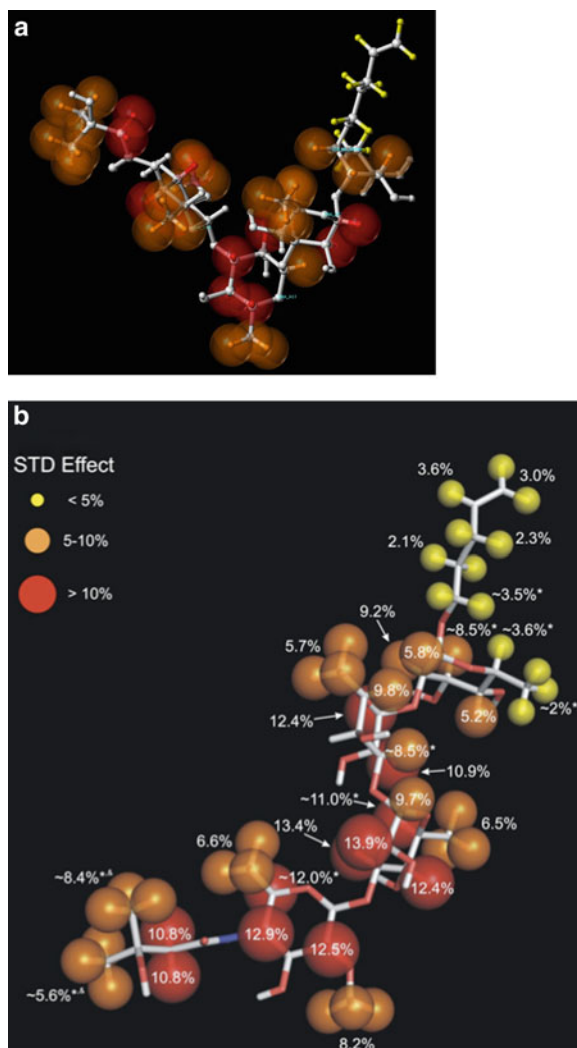
Oligosaccharide	anti-tetrasaccharide mAb MTA1		anti-disaccharide mAb MTD6	
	Affinity K_D [μ M]	Kinetics (on-/off-Rate)	Affinity K_D [μ M]	Kinetics (on-/off Rate)
Tetrasaccharide 	9.1	fast*	3.7	slow ($k_a = 19540 \text{ M}^{-1}\text{s}^{-1}$ $k_d = 0.0596 \text{ s}^{-1}$ $k_d/k_a = 3.05 \text{ }\mu\text{M}$)
Disaccharide 	> 1.000	n.a.	0.51	slow ($k_a = 46300 \text{ M}^{-1}\text{s}^{-1}$ $k_d = 0.0049 \text{ s}^{-1}$ $k_d/k_a = 0.11 \text{ }\mu\text{M}$)
Monosaccharide 	> 1.000	n.a.	7.2	slow ($k_a = 8786 \text{ M}^{-1}\text{s}^{-1}$ $k_d = 0.05312 \text{ s}^{-1}$ $k_d/k_a = 6.0 \text{ }\mu\text{M}$)
Rhamnose-Disaccharide 	> 1.000	n.a.	> 1.000	n.a.
Rhamnose-Trisaccharide 	> 1.000	n.a.	> 1.000	n.a.

Fig. 2.3 Determination of affinity and interaction kinetics by surface plasmon resonance. Dissociation constants as determined by steady-state measurements (dilution series), and interaction kinetics as determined by fitting individual binding curves (*The sensorgrams of the tetrasaccharide/MTA1 interaction indicated mass transport-limited association. Kinetic constants could therefore not be determined reliably by SPR)

ligands (discriminating tightly-bound domains from weakly-bound domains) without having to assign the resonance of the macromolecular receptor. Unfortunately, extremely slow dissociation of the MTD6–disaccharide complex prevented further analysis of this antibody–oligosaccharide pair by STD NMR (Meyer and Meyer 1999; Meyer and Peters 2003). Such slow kinetics result in very limited transfer of ligands from the antibody-bound state to the free state, and greatly affect the signal-to-noise ratio of STD NMR experiments. In addition, the increasing antibody–ligand complex lifetime in such cases results in intra-ligand spin diffusion that decreases the discrimination between individual positions of the ligand and prevents detailed mapping.

The complex of MTA1 and tetrasaccharide **1**, however, was a good candidate for further analysis by STD NMR. By assessing antibody binding at a 30:1 ratio of carbohydrate ligand to protein, it was confirmed that MTA1 tightly binds all four sugars of the tetrasaccharide (Fig. 2.4), but had little effect on the non-natural linker at the reducing end of rhamnose D. Strong STD effects indicate that tight-binding sites were located throughout the entire tetrasaccharide on all four sugars, with a cluster of tight-binding sites found within the β -anthrose-(1 \rightarrow 3)-rhamnose sub-structure. Binding was relatively weaker at the opposite end of the molecule, but STD effects of 12.4% (rhamnose C–H1) and 9.2% (rhamnose D–H2), showed that

Fig. 2.4 Epitope mapping of the BclA tetrasaccharide **1**/MTA1 interaction by STD NMR spectroscopy. Percent STD effects are shown for individual protons of tetrasaccharide **1**. In addition, strong (>10%), medium (5–10%) and weak (<5%) STD effects are indicated by *red, orange, and yellow* spheres of decreasing size. Positions marked with an *asterisk* could not be determined to high accuracy due to resonance overlap. The methyl groups marked with *asterisk* were not assigned stereospecifically. Microarray data support the assignment of stronger STD effects to the pro-*S* methyl group, as shown here. The MTA1 antibody recognizes a complex epitope comprising of all four monosaccharide units. Protons of the anthrose moiety and the adjacent rhamnose sugar receive the strongest saturation transfer and are thus most tightly bound by the antibody (Reprinted with permission from Oberli et al. (2010). Copyright © 2010, American Chemical Society)



binding in this region is still significant. Looking at STD effects throughout the structure, it was observed that one face of the triirhamnose chain, (protons H1–H2–H3), was bound more tightly by the antibody than the opposite side (protons H4–H5–H6). Indeed, for rhamnose B, the combined average STD value for protons H1–H2–H3 was 12.3% and 10% for H4–H5–H6, for rhamnose C the average STD values were 10.6% (H1–H2–H3) and 7.8% (H4–H5–H6), and for rhamnose D the average STD values were 7.8% (H1–H2–H3) and 3.6% (H4–H5–H6). The H1–H2–H3 face of the triirhamnose chain is apparently oriented closer to the antibody within the tetrasaccharide–antibody complex. STD NMR analysis indicated that there is a cluster of sites that are tightly bound by MTA1 within the anthrose unit. Specifically, on the

anthrose sugar ring, three protons showed strong STD effects. However, the 2-*O*-methyl group is bound less strongly, with an STD effect of 8.2%. This observation agrees with the microarray data that indicated that this side chain appendage is of minor importance for recognition by MTA1. The microarray data also indicated that the anthrose C4 chain, a methylene group, as well as two methyl groups appear to have significant STD values. Interestingly, the two methyl groups have different STD values (8.4% and 5.6%), indicating that one methyl group is oriented closer to MTA1 and this group is bound more tightly (8.4%). It is remarkable that this difference in binding affinity was also detected by microarray screening, where tetrasaccharide **25**, containing a 4-((3*S*)-3-hydroxy-3-methylbutyrate) side chain, showed stronger affinity towards anti-tetrasaccharide mAb MTA2 compared to compound **24** which is decorated with a 4-((3*R*)-3-hydroxy-3-methylbutyrate) side chain. We therefore conclude that the methyl group presented in the (*S*)-configured isomer **25** is proximal to the antibody and thus makes a greater contribution to binding.

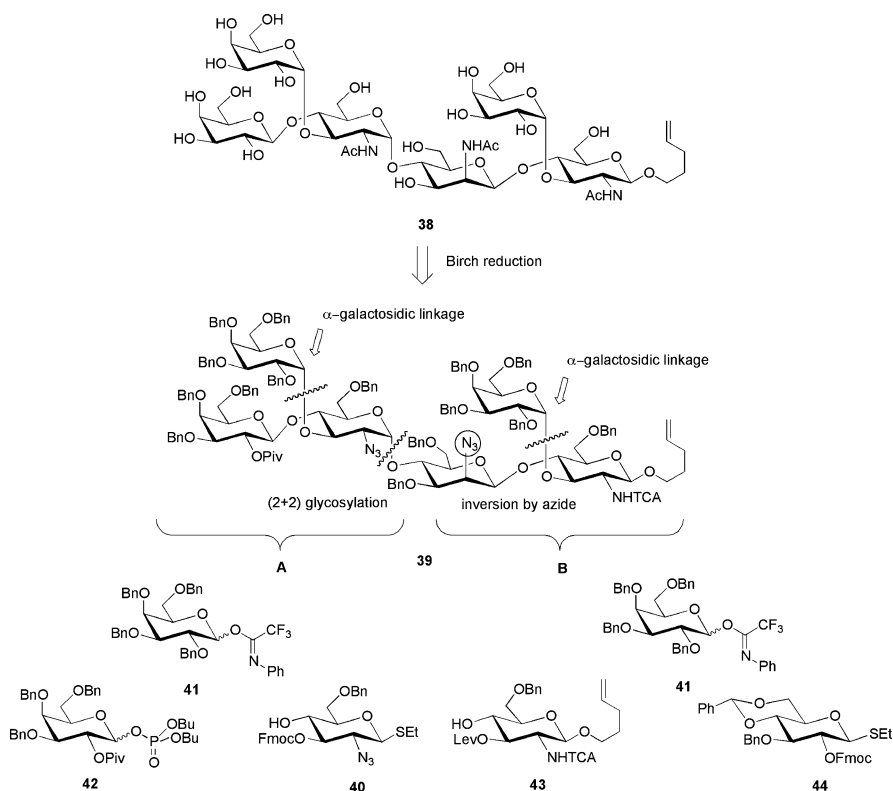
The power of combining microarray profiling, SPR, and STD NMR was demonstrated in this study to precisely map the molecular elements of the BcIA tetrasaccharide that participate in tight antibody binding. Understanding which structural features of the oligosaccharide are most important for this interaction will enable the design of better carbohydrate-based anthrax vaccines. Furthermore, this approach ultimately aids elucidating general principles of carbohydrate–antibody interactions, enabling guided structure-based design of a broad spectrum of carbohydrate-based antigens and therapeutics thereof.

2.3 The Anthrax Hexasaccharide

In addition to the anthrax carbohydrate present on the surface of spores described above, the structure of the secondary cell wall polysaccharide of *B. anthracis* vegetative cells was elucidated (Choudhury et al. 2006). This capsular polysaccharide of *B. anthracis* vegetative cells mainly consists of hexasaccharide repeating units (hexasaccharide **2**) and is tethered to the *B. anthracis* cell surface S-layer proteins. This structure represents another promising carbohydrate antigen for the development of vaccines and diagnostics, because the β -linked *N*-acetylmannosamine is a pattern that does not occur in mammalian carbohydrates and is therefore presumably a highly immunogenic structure. Following the disclosure of the structure elucidation data, two research groups reported on efforts towards the chemical synthesis of the anthrax hexasaccharide. While the Boons group reported on the synthesis of two trisaccharide fragments (Vasan et al. 2008), we accomplished the synthesis of the complete hexasaccharide repeating unit (Oberli et al. 2008).

2.3.1 Synthesis of the Hexasaccharide Repeating Unit

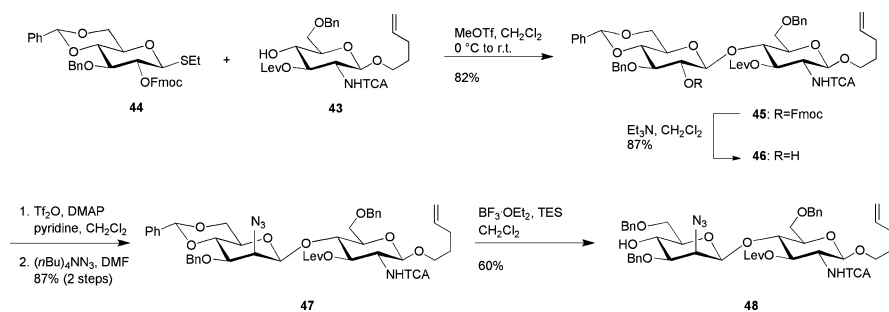
The retrosynthetic analysis of the fully protected hexasaccharide **39** (Scheme 2.5) dissected the target molecule into two terminal α -galactose building blocks **41**, **42**,



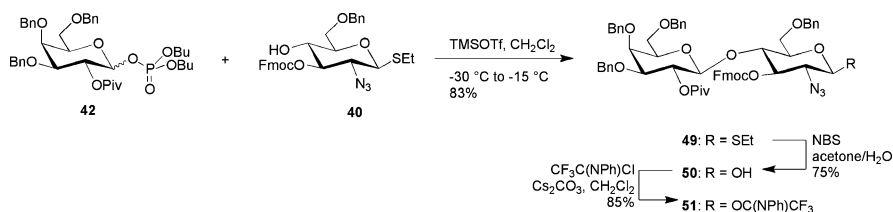
Scheme 2.5 Retrosynthetic analysis of a hexasaccharide repeating unit of the capsular polysaccharide of *B. anthracis*

and two disaccharide parts **A** and **B**. For part **A**, two building blocks **40** and **42** were identified. The *N*-acetyl group of the glucosamine of part **A** was masked as an azide to ensure α -selectivity in the (2 + 2) glycosylation. For part **B**, the trichloroacetyl-protected glucosamine **43** was to be used already attached to the pentenyl handle in order to allow for a facile transformation either into an aldehyde or a thiol. The β -mannosaminic linkage is introduced by inverting the C2-hydroxyl of the glucose moiety after construction of the β -glucosidic bond with **44**.

The synthesis commenced with the union of glucose thioglycoside **44** and glucosamine *n*-pentenyl glucoside **43** to form a $\beta(1\rightarrow4)$ glycosidic linkage. The challenging *cis*- β -mannosamine linkage was installed by creating the *trans*-glucosidic bond, readily prepared with the help of the participating fluorenylmethoxycarbonate (Fmoc) group in C2. Subsequently, the Fmoc group was cleaved and the C2 stereocenter was inverted by displacement of the triflate *via* the action of tetrabutylammonium azide to afford the disaccharide **47** that contains the mannosamine motif. Benzylidene protection of the C4 and C6 hydroxyls of the glucose unit proved to be essential for the successful inversion. Selective benzylidene ring opening yielded disaccharide acceptor **48** ready for glycosylation (Scheme 2.6).



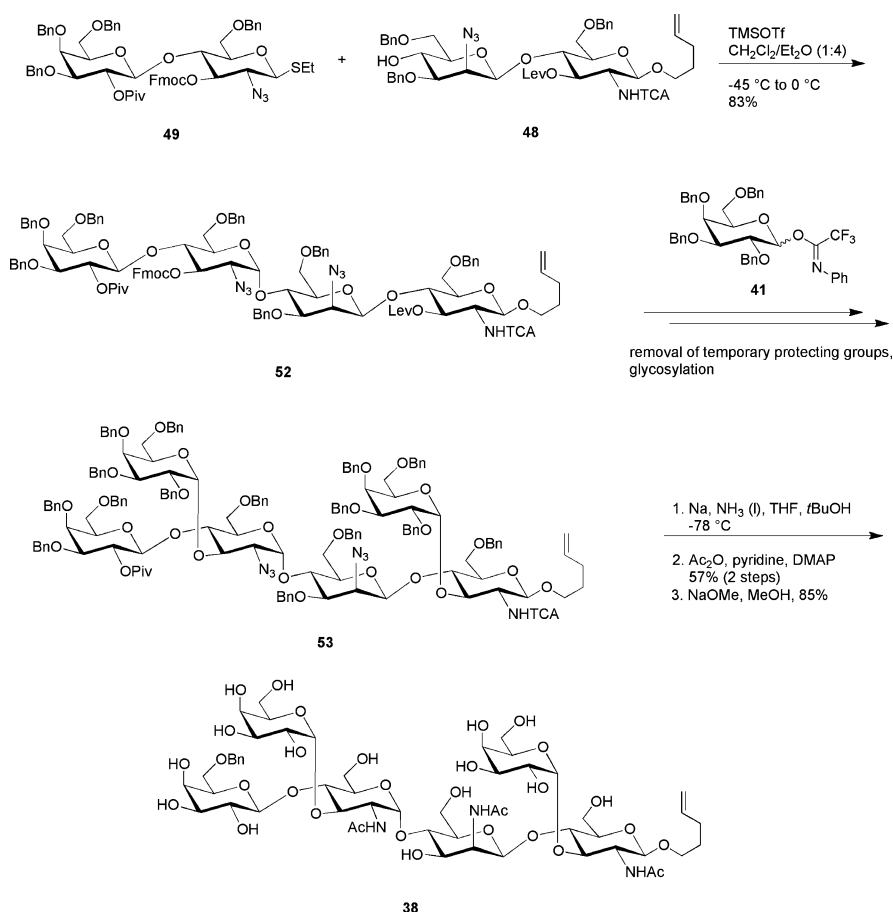
Scheme 2.6 Synthesis of disaccharide **48**



Scheme 2.7 Synthesis of disaccharide building block **51**

The differentially protected lactosamine building block **49** was prepared from galactose phosphate **42** and thioglycoside **40** (Scheme 2.7). The pivaloyl group (Piv) as neighboring-participating group in C2 ensured the selective creation of the β -linkage. In a subsequent step, the C–S bond was cleaved by the action of *N*-bromosuccinimide and water to afford hemiacetal **50**. This hemiacetal was converted into the *N*-phenyltrifluoroacetimidate **51**.

Glycosylation of disaccharide **48** with *N*-phenyltrifluoroacetimidate **51** in a mixture of dichloromethane and diethyl ether selectively afforded tetrasaccharide **52** with the corresponding α -glucosaminic linkage (Scheme 2.8). To complete the synthesis, tetrasaccharide **52** was treated with triethylamine to remove the Fmoc group. A first glycosylation with perbenzylated *N*-phenyltrifluoroacetimidate **41** yielded a pentasaccharide (not shown). Further removal of the levulinoyl ester protecting group by action of hydrazine monohydrate and subsequent glycosylation with the same building block **41** afforded the hexasaccharide **53**. This step-by-step procedure proved superior to a double glycosidation of the tetrasaccharide for the installation of α -galactosidic linkages. Complete deprotection and the transformation of azide moieties into amines were achieved by the action of sodium in liquid ammonia. To reveal NHAc groups – and also to allow for a more facile purification – the completely deprotected carbohydrate was acetylated with a mixture of acetic anhydride, pyridine and DMAP. Final saponification of the completely acetylated structure removed all ester protecting groups leading to target compound **38**. Further functionalization of the *n*-pentenyl moiety allowed for attachment to a carrier protein to yield a neoglycoconjugate that can be readily used in immunization studies.



Scheme 2.8 Assembly of the pentenyl hexasaccharide **38**

2.3.2 Immunology

The immunogenicity of the *B. anthracis* cell wall polysaccharide and synthetic carbohydrate antigens were investigated (Vasan et al. 2008). Isolated *B. anthracis* cell wall polysaccharide was coupled to KLH as carrier protein. Rabbits were immunized with this polysaccharide–KLH conjugate or live- or irradiated *B. anthracis* spores. As determined by ELISA, all immunized rabbits elicited IgG antibodies that recognized the isolated polysaccharide, thereby indicating that the polysaccharide structure is also present on the spores. Furthermore, sera from all types of immunized rabbits recognized the synthetic trisaccharide fragments, albeit to a different extent. Whereas both trisaccharides were bound equally well by sera from rabbit immunized with polysaccharide–KLH and irradiated spores, one of the two trisaccharides was bound much better than the other by sera from rabbits that

were immunized with live spores. The different recognition of the trisaccharides presumably results from different presentation of the antigens on the polysaccharide chains. Most interesting is the observation that spores can elicit anti-polysaccharide antibodies that also recognize the small synthetic trisaccharides. This observation suggests that not only vegetative cells but also *B. anthracis* spores express the polysaccharide. This finding implies that a subunit vaccine based on the polysaccharide may provide immunity towards vegetative cells as well as spores.

2.4 Conclusion and Outlook

Two carbohydrate structures that were identified on the surface of *Bacillus anthracis* are the focus of this account. The first one, tetrasaccharide **1**, is attached to the major glycoprotein BclA that is present on the exosporium of *B. anthracis* spores. Several groups have reported the chemical synthesis of this target structure and related tri- and pentasaccharide. The second structure, hexasaccharide **2**, is a hexasaccharide repeating unit of the major cell wall capsular polysaccharide of vegetative cells. Again, chemical syntheses of the hexasaccharide and its substructures served as targets of challenging total syntheses, but also provided synthetic antigens to investigate potential vaccine candidates and can be exploited as tools to create diagnostic antibodies.

The history of these two *B. anthracis* antigens illustrates how fast and efficient today's glycoscientists can work. After the terror attack in the US in 2001 where several people died after the exposure to *B. anthracis* spores, research efforts with respect to the underlying mechanisms of this horrible disease and with respect to structure elucidation of relevant target molecules were intensified. Immediately, after disclosure of the oligosaccharide structure, several groups started their synthetic approaches. As soon as the target was assembled by chemical means, conjugation to carrier proteins and immunization studies were performed yielding mAbs able to detect spores. In the meantime, it has been shown that the anti-tetrasaccharide antibodies are not completely selective (Tamborrini et al. 2009), a view that has been confirmed by an analysis of the corresponding anthrose gene cluster that has also been found in a few *B. cereus* strains (Dong et al. 2008). Even though few *B. cereus* strains show cross-reactivity a detection system based on these antibodies is currently under development in Switzerland. The potential of these oligosaccharides as part of a multi-component vaccine is still valid and challenge studies with anthrax tetra- and hexasaccharide structures are ongoing.

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