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Immunological Evaluation of a Synthetic *Clostridium difficile* Oligosaccharide Conjugate Vaccine Candidate and Identification of a Minimal Epitope

Christopher E. Martin,[†,∥] Felix Broecker,[†,∥] Matthias A. Oberli,[†,§] Julia Komor,[‡] Jochen Mattner,[‡] Chakkumkal Anish,*[,†] and Peter H. Seeberger*[,†]

[†]Max Planck Institute of Colloids and Interfaces, 14424 Potsdam, Germany and Freie Universität Berlin, 14195 Berlin, Germany
[‡]Mikrobiologisches Institut − Klinische Mikrobiologie, Immunologie und Hygiene, Universitätsklinikum Erlangen and Friedrich-Alexander Universität Erlangen-Nürnberg, D-91054 Erlangen, Germany

Supporting Information

ABSTRACT: *Clostridium difficile* is the cause of emerging nosocomial infections that result in abundant morbidity and mortality worldwide. Thus, the development of a vaccine to kill the bacteria to prevent this disease is highly desirable. Several recently identified bacterial surface glycans, such as PS-I and PS-II, are promising vaccine candidates to preclude *C. difficile* infection. To circumvent difficulties with the generation of natural PS-I due to its low expression levels in bacterial cultures, improved chemical synthesis protocols for the pentasaccharide repeating unit of PS-I and oligosaccharide substructures were utilized to produce large quantities of well-defined PS-I related glycans. The analysis of stool and serum samples obtained from *C. difficile* patients using glycan microarrays of synthetic oligosaccharide epitopes revealed humoral immune responses to the PS-I related glycan epitopes. Two different vaccine candidates were evaluated in the mouse model. A synthetic PS-I repeating unit CRM_{197} conjugate was immunogenic in mice and induced immunoglobulin class switching as well as affinity maturation. Microarray screening employing PS-I repeating unit substructures revealed the disaccharide Rha-(1→3)-Glc as a minimal epitope. A CRM_{197}-Rha-(1→3)-Glc disaccharide conjugate was able to elicit antibodies recognizing the *C. difficile* PS-I pentasaccharide. We herein demonstrate that glycan microarrays exposing defined oligosaccharide epitopes help to determine the minimal immunogenic epitopes of complex oligosaccharide antigens. The synthetic PS-I pentasaccharide repeating unit as well as the Rha-(1→3)-Glc disaccharide are promising novel vaccine candidates against *C. difficile* that are currently in preclinical evaluation.

INTRODUCTION

Clostridium difficile is a Gram-positive, spore-forming bacterium and the most common cause of nosocomial diarrhea worldwide.[1,2] The disruption of the intestinal flora by antibiotics allows for the colonization with and/or the overgrowth by drug-resistant, toxin-producing *C. difficile* spores commonly found in healthcare facilities, causing *C. difficile* infection (CDI), which in its most severe forms is life-threatening.[3] In recent years, infection and death rates have been increasing drastically. In addition to the main risk group, the elderly, children, young adults, and pregnant women are now infected, thereby increasing the social and economic burden.[4−6] The emergence of new *C. difficile* strains, such as ribotype 027 (also designated BI, NAP1, or toxin type III), with increased virulence, toxin production, and antibiotics resistance is partially responsible for this development.[7,8] Ribotype 027 has quickly spread throughout North America, Europe, Asia, and Oceania[9] where it causes growing numbers of infections.[5] Vaccination as an alternative to antibiotic treatment is therefore highly desirable. Toxin-neutralizing immunization can protect against the lethal challenge with *C. difficile* in hamsters,[10−12] but toxin-based vaccines cannot inhibit bacterial colonization, which precedes toxin production. Furthermore, recurrent CDIs are serious clinical problems affecting up to 30% of patients after cessation of therapy, either due to recolonialization by the same or reinfection with a different *C. difficile* strain.[13−15] Thus, preventing colonialization by vaccination against surface antigens may more effectively limit recurrence than toxin-neutralizing approaches. Bacterial surface glycans are promising vaccine targets due to their well-documented role in adhesion to host tissues.[2,16−19] Several cell-surface glycans of *C. difficile* have been characterized,[20,21] and conjugate vaccines comprised of isolated or synthetic polysaccharide PS-II are in preclinical development.[22−25]

Cell-surface polysaccharide PS-I consisting of a pentasaccharide phosphate repeating unit [→4)-α-Rha*p*-(1→3)-β-Glc*p*-(1→4)-[α-Rha*p*-(1→3)]-α-Glc*p*-(1→2)-α-Glc*p*-(1→P] was originally described on ribotype 027[22] but has also been identified on other *C. difficile* strains.[25] Chemical synthesis is currently the only feasible approach to obtain large amounts of pure and well-defined PS-I related glycans due to low expression levels under culture conditions.[22,25] PS-I pentasaccharide repeating unit **1** equipped with an aminopentyl linker at the reducing end has been synthesized previously (Figure 1).[26]

Here, we describe the design and chemical synthesis of six PS-I related oligosaccharides **1**−**6** (Figure 1). Diglucoside **2** contains the reducing end moieties A and B, while trisaccharide **3** contains one additional glucose moiety C. Rha-(1→3)-Glc disaccharide **4** is found twice (BD and CD′) in **1**, whereas

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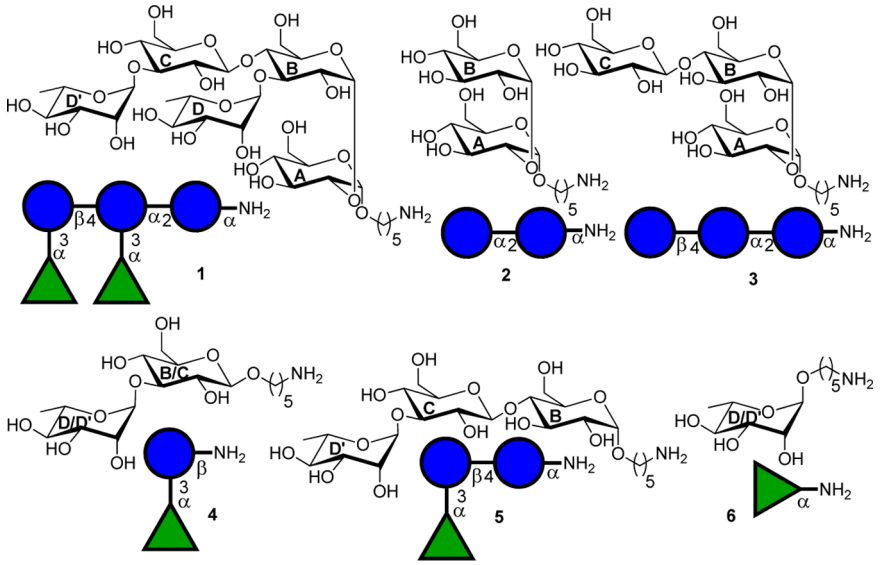


Figure 1. Synthetic PS-I pentasaccharide repeating unit **1** and substructures **2**–**6**.

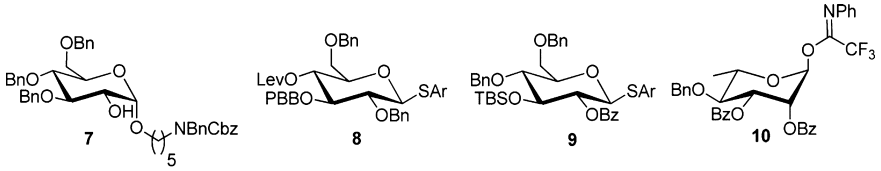
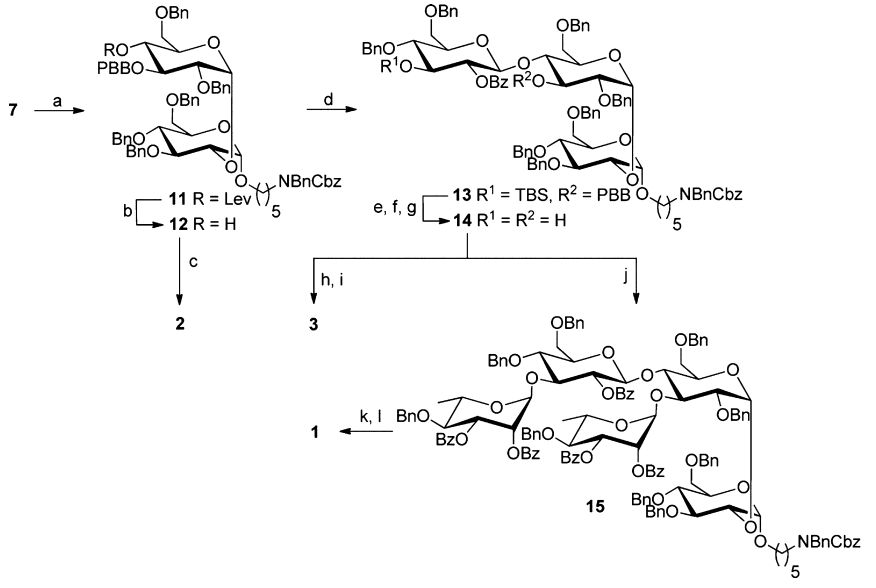


Figure 2. Monosaccharide building blocks **7**–**10** employed in the synthesis of oligosaccharides **1**–**6**. Ar = 2-methyl-5-*tert*-butylphenyl.

Scheme 1. Synthesis of Pentasaccharide 1 and Substructures 2 and 3[a]



[a]Reagents and conditions: (a) **8**, NIS/TfOH, Et_2O, −20 to −10 °C, 69%; (b) $N_2H_4{\cdot}H_2O$, AcOH/pyridine, DCM, 96%; (c) H_2, 10% Pd/C, MeOH, THF, H_2O, AcOH, 99%; (d) **9**, NIS/TfOH, DCM, −30 to −17 °C, 92%; (e) cat. $Pd(OAc)_2$, (3,4-dimethoxyphenyl)boronic acid, TBABr, K_3PO_4, EtOH; (f) DDQ, aq. $NaHCO_3$, H_2O, DCM; (g) TBAF·$3H_2O$, AcOH, DMF, 50 °C, 62% over 3 steps; (h) NaOMe, THF/MeOH; (i) H_2, 10% Pd/C, MeOH, THF, H_2O, AcOH, 49% over 2 steps; (j) **10**, TMSOTf, DCM, 4 Å MS, −40 to −20 °C, 88%; (k) NaOMe, THF/MeOH; (l) H_2, 10% Pd/C, MeOH, THF, H_2O, AcOH, 60% over 2 steps.

trisaccharide **5** covers the sequence BCD′ and **6** consists of a rhamnose residue (D/D′).

Glycan microarrays containing the *C. difficile* PS-I related glycans **1**–**6** (Figure 1) were used to screen patient samples for PS-I specific antibodies. The synthetic oligosaccharides represent natural epitopes that play a role in the immune response during CDI. Based on the insights gained from glycan microarray studies, a pentasaccharide **1**-CRM_{197} vaccine candidate was immunologically evaluated. The glycoconjugate proved immunogenic in mice, and immunoglobulin class-

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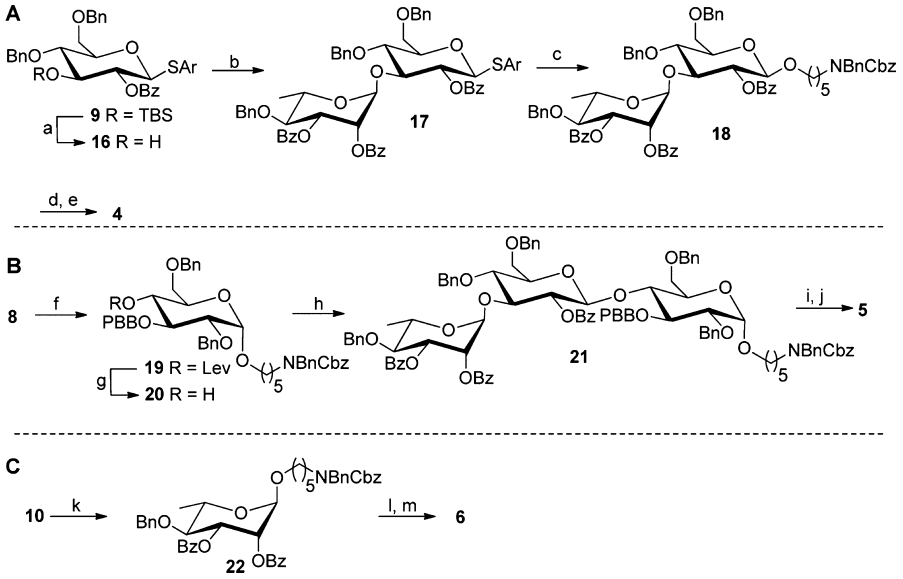
Scheme 2. Synthesis of Substructures 4−6[a]



[a]Reagents and conditions: Synthesis of **4** (A): (a) TBAF·3H_2O, AcOH, DMF, 35 °C, 92%; (b) **10**, TMSOTf, DCM, 4 Å MS, −40 to −20 °C, 86%; (c) HO$(CH_2)_5$NBnCbz, NIS/TfOH, DCM, −20 to 0 °C, 91%; (d) NaOMe, THF/MeOH; (e) H_2, 10% Pd/C, MeOH, THF, H_2O, AcOH, 76% over 2 steps. Synthesis of **5** (B): (f) HO$(CH_2)_5$NBnCbz, NIS/TfOH, Et_2O, −10 to 0 °C, 39%; (g) N_2H_4·H_2O, AcOH/pyridine, DCM, 81%; (h) **17**, NIS/TfOH, DCM, −20 to 0 °C, 95%; (i) NaOMe, THF/MeOH; (j) H_2, 10% Pd/C, MeOH, THF, H_2O, AcOH, 77% over 2 steps. Synthesis of **6** (C): (k) HO$(CH_2)_5$NBnCbz, TMSOTf, DCM, 4 Å MS, −30 to −20 °C, 94%; (l) NaOMe, THF/MeOH; (m) H_2, 10% Pd/C, MeOH, THF, H_2O, AcOH, 94% over 2 steps. Ar = 2-methyl-5-*tert*-butylphenyl.

switching and affinity maturation were observed. Glycan array-assisted epitope mapping using synthetic structures **1**−**6** revealed Rha-(1→3)-Glc disaccharide **4** as minimal epitope of pentasaccharide **1**. Mice immunized with a disaccharide **4**-CRM_{197} glycoconjugate produced antibodies that recognized the PS-I pentasaccharide. Pentasaccharide **1** as well as disaccharide **4** are promising vaccine candidates to protect from CDI.

RESULTS AND DISCUSSION

Synthesis of PS-I Pentasaccharide Repeating Unit 1 and Oligosaccharide Substructures. Pentasaccharide **1** and substructures **2**−**6** (Figure 1) were assembled from monosaccharide building blocks **7**−**10** (Figure 2). Two building blocks (**7**, **10**) had proven reliable previously, whereas the protecting group patterns of monosaccharides **8** and **9** were adjusted to overcome challenges encountered during previous synthetic efforts.[26] The nonparticipating benzyl group at C-2 of thioglucoside **8** allows for *α*-selective glycosylation to *α*-glucoside **7**, bearing the linker at the reducing end.[26] The two orthogonal temporary protecting groups *para*-bromobenzyl (PBB) ether[27,28] at C-3 and levulinic (Lev) ester at C-4 of **8** allow for installation of the branching point. Thioglucoside **9** was freed from the 4,6-*O*-benzylidene conformational restriction utilized previously to exhibit rather two benzyl ethers in the C-4 and C-6 positions. The *tert*-butyldimetylsilyl ether (TBS) at C-3 masks the hydroxyl group serving as attachment point for rhamnoside **10**.

Linear assembly of **1** proceeded from the reducing to the nonreducing end. Union of thioglucoside **8** and glucoside **7** furnished disaccharide **11** in 69% yield. After cleavage of the levulinic ester, coupling of thioglycoside **9** and disaccharide **12** proceeded in 92% yield to give trisaccharide **13**. A simple protecting group change more than doubled the yield when compared to our previous approach, where Fmoc was used as opposed to PBB.[26] Following conversion of **13** to diol **14**, bis-glycosylation with rhamnosyl-imidate **10** gave the fully protected pentasaccharide **15**. Global deprotection finally gave **1**. Disaccharide **2** and trisaccharide **3** were obtained by catalytic hydrogenation of protected **12** and **14**, respectively (Scheme 1).

Oligosaccharides **4** and **5**, containing terminal rhamnose residues, were synthesized relying on disaccharide **17** that in turn was obtained from building blocks **9** and **10** (Scheme 2). Rhamnoside **6** resulted from glycosylation of the linker with **10**, followed by deprotection.

Chemical synthesis of several PS-I related oligosaccharide structures **1**−**6** proved to be an efficient approach to obtain large amounts of pure and well-defined glycans for the generation of glycan microarrays and glycoconjugates.

Screening of Patient Samples for Antibodies to Oligosaccharides 1−6. Vaccine candidates and *C. difficile* diagnostics have to rely on glycan epitopes that are actually expressed by the bacterium. As the expression of PS-I is likely phase variable and thus not yet reliably established under culture conditions,[20,25] we chose the analysis of the native infection as an indirect way to investigate the antigenic properties by PS-I through the induction of antibody responses. We screened samples from *C. difficile* patients for antibodies to glycans **1**−**6** to determine whether these glycans represent true epitopes of the natural PS-I polysaccharide. Two CDI-negative control groups were included in this screening: one comprised of patients without diarrhea and one with patients suffering from diarrhea not caused by CDI. Initially, we aimed to detect secreted mucosal IgA antibodies to PS-I since IgA is the immunoglobulin critical for the control of mucosal infections. Accordingly, high levels of mucosal IgA to toxin A have been reported to correlate with protection from CDI.[29] While IgA antibodies to *C. difficile* toxins act by neutralizing toxicity,[30,31]

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IgA to surface antigens is proposed to inhibit colonialization of *C. difficile*.[32] Anti-*C. difficile* surface glycan PS-II IgA has been identified in human fecal samples.[23] In order to assess the importance of the mucosal immune response to the PS-I pentasaccharide **1** antigen during CDI, we first screened fecal samples of 36 hospitalized CDI patients that had tested positive for toxin A/B and the growth of *C. difficile* in bacterial cultures obtained from stool specimens by glycan array. Twenty-five of the patients had severe (persisting watery diarrhea, >5 times per day for >10 days and/or fever and or blood/pus in stool and/or abdominal cramping and pain) and 11 less severe (self-limiting disease, watery diarrhea of <3 times a day for <5 days associated with mild abdominal cramping and tenderness) clinical symptoms. Ten individuals not colonized by *C. difficile* served as controls. The known PS-II hexasaccharide epitope was also included in this analysis.[23] We observed that mucosal IgA antibody levels to both PS-I and PS-II antigens were higher in patients with less severe disease compared to the controls, while the patients with more severe clinical symptoms had lower IgA levels, with statistical significance ($p \leq 0.05$) compared to the patients with low disease severity (Figure 3A,B). This indicates that higher antibody levels to these antigens correlate with milder forms of the disease. To further follow the development of mucosal immune responses to PS-I, we screened fecal samples of 19 CDI patients suffering from acute CDI (tested positive for toxin A/B and the bacterial growth) for the presence of IgA to synthetic PS-I related epitopes using glycan arrays containing saccharides **1**–**6**. Twenty-three age- and sex-matched controls without CDI (tested negative for toxin A/B) were included. IgA antibodies specific to glycans **1**–**6** were present in most fecal samples of both patient and control groups, with highly variable antibody levels (Figure 3C). An increase in specific anti-PS-I IgA was in general observed around five days after the toxin ELISA became positive in patient samples that were followed as part of a longitudinal study. The CDI group showed a trend toward lower IgA levels to pentasaccharide **1** and rhamnose-containing substructures **4**–**6**, albeit without statistical significance likely due to small sample sizes (Figure 3C). These findings suggest that low levels of IgA antibodies to PS-I epitopes are associated with a risk of acquiring CDI. However, we cannot exclude the possibility that the lower IgA levels in patients' feces might be due to sequestration of antibodies through binding to a larger number of bacteria.

IgG antibodies to a variety of *C. difficile* antigens, including toxins[33−36] and surface antigens,[36−39] have been detected in sera of healthy and infected individuals. Elevated serum IgG to toxin and nontoxin antigens has been associated with protection from CDI,[40−42] suggesting that systemic IgG responses to *C. difficile* antigens correlate with clinical outcomes.[43] To assess whether patient blood contains IgG antibodies to PS-I epitopes, human sera were screened using glycan microarrays. Blood from 35 reconvalescent patients (diagnosed with CDI and recovered) and 24 age- and sex-matched controls without a history of CDI was screened for IgG recognizing glycans **1**–**6** using glycan microarrays. IgG specific to all glycan antigens was present in most sera of both reconvalescent patients and controls, with a high degree of variation in antibody levels (Figure 3D). In reconvalescent patients, statistically significantly higher IgG levels specific for pentasaccharide **1** as well as disaccharide **2** and trisaccharide **3** were detected (Figure 3D), correlating with results obtained with toxin A and B that had also been found to increase upon infection with *C. difficile*.[44]

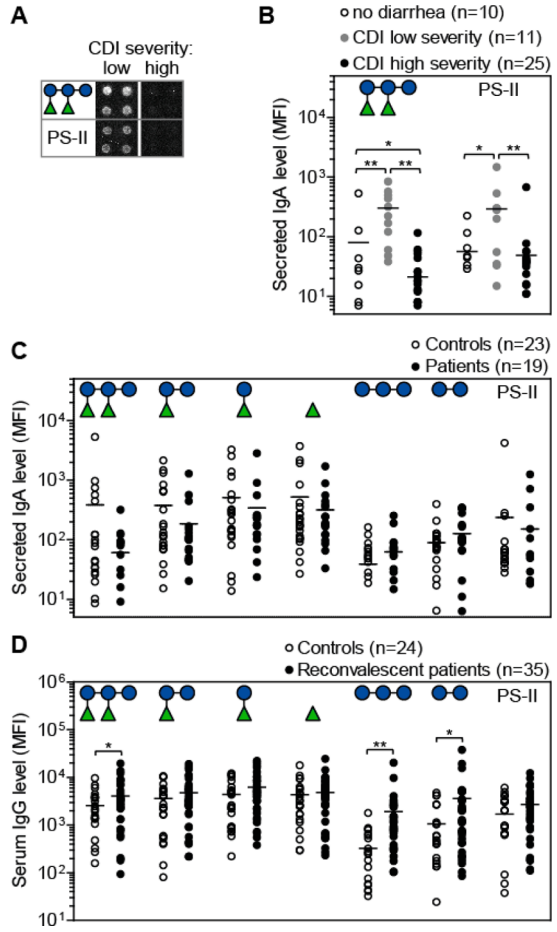
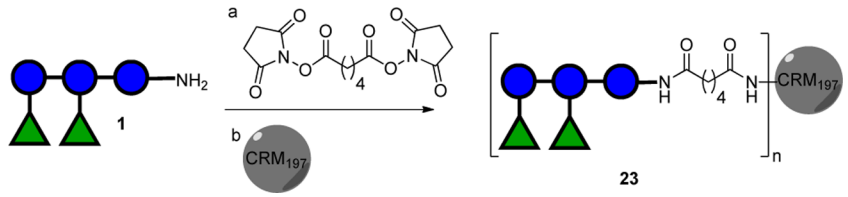


Figure 3. Detection of antibodies to pentasaccharide **1** and substructures **2**–**6** in human samples. (A) Exemplary microarray scans representing IgA antibodies to the PS-I pentasaccharide **1** and the PS-II hexasaccharide[23] epitopes found in fecal samples from two patients diagnosed with CDI; one with low disease severity and one with high disease severity. (B) Secreted IgA levels to both epitopes from fecal samples CDI patients with either low or high disease severity and control individuals without diarrhea, as indicated within the graph, expressed as MFI inferred by glycan array. Horizontal lines indicate mean values. (C) Secreted IgA levels in fecal samples of CDI patients and control patients with acute diarrhea due to other reasons than CDI. (D) Serum IgG levels of reconvalescent CDI patients and healthy controls. $*P \leq 0.1$, $**P \leq 0.05$, two-sided student's *t* test. Presence of IgG to rhamnose in human sera has been reported previously.[45,46] Correspondent microarray scans for (B–D) are depicted in Supporting Information, Figures 1–3. Note that data points for those patients with undetectable antibody levels are not shown in the graphs.

Elevated IgG to PS-I related antigens might be the result of exposure of the PS-I antigen to the immune system during CDI, raising the possibility that PS-I plays a role in immunity against *C. difficile*. Nonetheless, it needs to be defined whether serum IgG antibodies do not only correlate with the responsiveness of the immune system to the bacterial polysaccharides but also with the permeability and integrity of the intestinal epithelium and the immune status of the respective individual.

In summary, the presence of mucosal secreted IgA and serum IgG to the PS-I related pentasaccharide **1** and substructures in

Scheme 3. Conjugation of Pentasaccharide 1 to CRM_{197}[a]



[a]Reagents and conditions: (a) di-*N*-succinimidyl adipate, Et_3N DMSO; (b) CRM_{197}, 100 mM sodium phosphate, pH 7.4.

the majority of individuals indicates that these synthetic glycans represent biologically relevant epitopes that are recognized by the humoral immune system during CDI. Increased systemic IgG after reconvalescence from CDI suggests that an antibody response to PS-I epitopes is caused by an increased exposure of the bacterium to the immune system during the infection process. Furthermore, low mucosal IgA levels might be a risk factor for acquiring CDI. A part of the population appears to secrete insufficient amounts of antibodies to prevent the disease. A vaccine increasing the naturally existing antibody levels to *C. difficile* glycan antigens would especially benefit this group by leading to protection. Thus, the synthetic PS-I related glycans are promising target molecules for vaccination approaches against *C. difficile*.

Preparation and Characterization of a PS-I Pentasaccharide-CRM_{197} Conjugate. The observation that the synthetic PS-I related oligosaccharides **1**–**6** represent natural epitopes of *C. difficile* prompted the immunological evaluation of a pentasaccharide **1** protein conjugate. Conjugation to immunogenic carrier proteins improves the usually poor immunogenicity of oligosaccharide antigens and induces a T-cell dependent immune response.[47] The detoxified diphtheria toxin variant CRM_{197} served as carrier protein. This particular protein is a constituent of licensed conjugate vaccines against *Neisseria meningitidis*, *Haemophilus influenzae* type B, and *Streptococcus pneumoniae*.[48] To prepare the glycoconjugate, the primary amine present in the linker group of **1** was reacted with excess di-*N*-succinimidyl adipate in DMSO. The resulting monoester was reacted with the amine side chains of CRM_{197} lysine residues to obtain glycoconjugate **23** (Scheme 3).

Conjugation was confirmed by SDS-PAGE, showing a shift toward a higher mass of the glycoconjugate compared with unconjugated CRM_{197} and no evidence of unreacted protein (Supporting Information, Figure 4A). MALDI-TOF mass spectrometry analysis was used to determine the oligosaccharide-to-CRM_{197} molar ratio (Supporting Information, Figure 4B). Mass analysis of glycoconjugate **23** revealed that an average of 9.6 molecules **1** were loaded onto one molecule of CRM_{197}.

Immunological Evaluation of the PS-I Pentasaccharide-CRM_{197} Conjugate. To assess the ability of glycoconjugate **23** to elicit an antibody response to pentasaccharide **1**, three groups of C57BL/6 mice (each group, $n = 6$) were immunized, either without adjuvant, with Alum (Alhydrogel) adjuvant, or with Freund's adjuvant, in a prime-boost regimen (Figure 4A).

Alum was selected as adjuvant since it is approved for use in human vaccines.[49] Freund's adjuvant is an effective adjuvant in mice that has been successfully employed to raise antibodies to a synthetic oligosaccharide antigen.[23] Each injection comprised an amount of glycoconjugate corresponding to 3 μg of pentasaccharide **1**. The serum antibody response to **1** was monitored by glycan microarray analysis up to 18 weeks after the first injection (Figure 4A). Microarrays also included CRM_{197} and a BSA-GlcNAc dummy conjugate to assess antibody responses to the carrier protein and the generic spacer moiety (composed of pentyl and adipoyl groups), respectively. IgG, IgM, and IgA isotype antibodies to

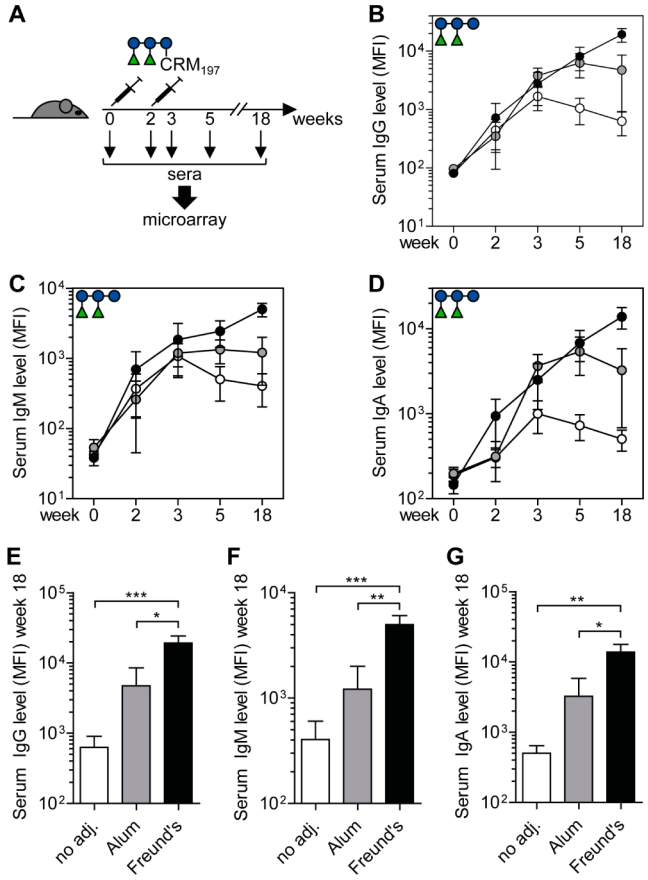


Figure 4. Serum antibody response to pentasaccharide **1** in mice immunized with glycoconjugate **23**. (A) C57BL/6 mice were immunized with **23** at weeks zero ('priming') and two ('boosting') using 3 μg of conjugated pentasaccharide **1** per injection. Sera obtained at the indicated time points were subjected to microarray analysis. Serum IgG, IgM, and IgA titers to pentasaccharide **1** at different time points expressed as MFI values of $n = 6 \pm$ SEM are shown in (B–D), respectively. White circles, no adjuvant; gray circles, Alum adjuvant; black circles, Freund's adjuvant. Serum IgG, IgM, and IgA titers to pentasaccharide **1** at week 18 are shown in (E–G), respectively. Bars represent mean values + SEM $^*P \leq 0.1$, $^{**}P \leq 0.05$, $^{***}P \leq 0.01$ (ANOVA with Bonferroni correction). Note that week 18, values of the Alum adjuvant group represent means of $n = 4$, as two mice of this group were sacrificed before for monoclonal antibody development. The complete microarray scans are depicted in Supporting Information, Figures 5–13.

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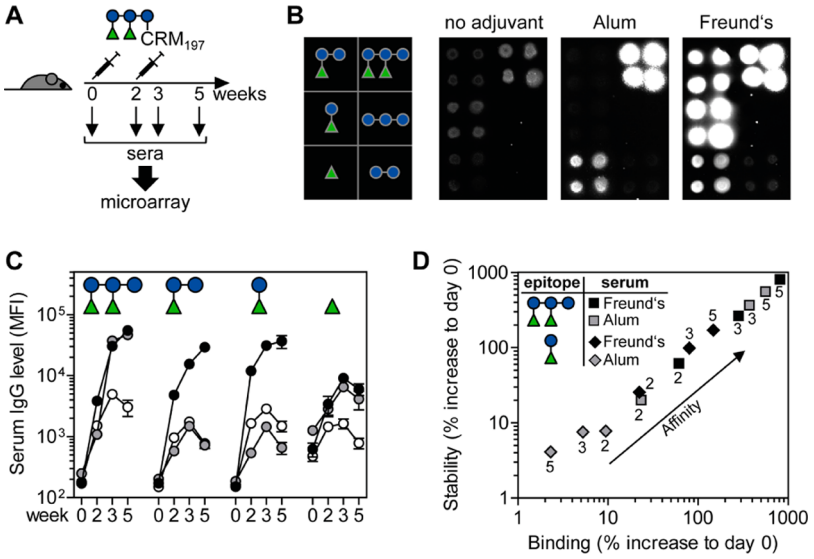

Figure 5. Serum IgG response to oligosaccharides **1**–**6** in mice immunized with glycoconjugate **23**. (A) C57BL/6 mice were immunized as described in the caption of Figure 4. Pooled sera (diluted 1:50 in PBS) were subjected to microarray analysis. (B) Representative microarray scans of week 5 indicating serum IgG to the PS-I derived synthetic epitopes. Note that Alum-adjuvanted **23** predominantly induced antibodies to pentasaccharide **1**, while Freund's-adjuvanted **23** also elicited high antibody titers to disaccharide **4** and trisaccharide **5**. The complete microarray scan is depicted in Supporting Information, Figure 14. (C) Antibody levels to PS-I derived antigens expressed as MFI values ± SD of four microarray spots. White circles, no adjuvant; gray circles, Alum adjuvant; black circles, Freund's adjuvant. Oligoglucoses **2** and **3** were omitted since responses to them were almost undetectable. (D) Binding and stability values of antibodies to **1** and **4** in pooled sera of the Freund's and Alum groups inferred by SPR are plotted, expressed as percent increase to day zero. Binding and stability correlate with antibody affinity. Weeks after initial immunization are indicated within the plot. Representative sensorgrams are shown in Supporting Information, Figure 15.

pentasaccharide **1** were detected in all three groups of mice (Figure 4B–D), demonstrating that immunoglobulin class switching was induced. Freund's adjuvant elicited higher antibody levels than Alum. Nonadjuvanted glycoconjugate was immunogenic as well but induced a weaker and shorter-lived antibody response. At week 18, serum IgG levels of the Freund's group (serum was diluted 1:100 in PBS) expressed as mean fluorescence intensity (MFI) were 30-fold higher than those of the nonadjuvanted group (Figure 4E) with statistical significance ($P \leq 0.01$) and 4-fold higher compared with the Alum-adjuvanted group ($P \leq 0.1$). Serum IgM levels were 12-fold higher in the Freund's group compared with the nonadjuvanted group ($P \leq 0.01$) and 4-fold higher compared with the Alum-adjuvanted group ($P \leq 0.05$) (Figure 4F). Serum IgA levels were 28-fold higher in the Freund's group compared with the nonadjuvanted group ($P \leq 0.05$) and 4-fold higher compared with the Alum-adjuvanted group ($P \leq 0.1$) (Figure 4G). The higher levels of serum IgG (8-fold), IgM (4-fold), and IgA (6-fold) in the Alum-adjuvanted group compared with the nonadjuvanted group were without statistical significance ($P > 0.1$), likely due to the small size of the Alum group ($n = 4$) in these comparisons, since two mice of the Alum group were sacrificed for monoclonal antibody development prior to week 18. Antibody levels to the CRM_{197} carrier protein and the adipoyl spacer moiety were also increased when the glycoconjugate was administered with adjuvants (Supporting Information, Figures 6–14). Immunization with glycoconjugate **23** was specific for pentasaccharide **1** and did not elicit antibodies against control oligosaccharides, *C. difficile* PS-II hexasaccharide[23] and *Leishmania* lipophosphoglycan capping tetrasaccharide[50,51] (Supporting Information, Figures 5–13).

Glycan Microarray-Assisted Epitope Mapping of PS-I Pentasaccharide 1. To identify the minimum size epitope of antigen **1**, we subjected pooled sera (diluted 1:50 in PBS) of mice immunized with glycoconjugate **23** to microarray analysis with slides containing oligosaccharides **1**–**6** (Figure 5A). Antibodies raised using glycoconjugate **23** and Freund's adjuvant not only recognized pentasaccharide **1** but also trisaccharide **5** and, more pronounced, disaccharide **4** (Figures 5B and C). In this group of mice, at week five after initial immunization, serum IgG levels in the pooled sera expressed as MFI were highest to pentasaccharide **1** (54 876 ± 5279, mean ± SD of four microarray spots), followed by disaccharide **4** (36 484 ± 8679), trisaccharide **5** (29 123 ± 2586), and monorhamnose **6** (5999 ± 1269). Interestingly, while the serum IgG levels to pentasaccharide **1**, disaccharide **4**, and trisaccharide **5** increased from weeks three to five, those to rhamnose **6** decreased, indicating that antibodies to monorhamnose are only transiently elicited. Antibodies recognizing the more complex rhamnose-containing glycan epitopes show a more robust induction. There was no significant induction of antibodies recognizing oligoglucose di- (**2**) and trisaccharide (**3**) (Supporting Information, Figure 14), even though both oligosaccharides appear to be immunodominant epitopes during natural infection (Figure 3B,C). Thus, contributions of other microbial infections that elevate responses to **2** and **3** cannot be ruled out. A similar epitope recognition pattern was observed in mice immunized with nonadjuvanted glycoconjugate **23** but with overall lower antibody levels and a shorter-lived immune response (Figure 5B,C). Here, serum IgG levels to pentasaccharide **1**, trisaccharide **5**, disaccharide **4**, and rhamnose **6** peaked at week three and decreased in week five. At week three, serum IgG levels were highest to pentasaccharide **1**, followed by disaccharide **4**, trisaccharide **5**, and rhamnose **6**. These observations indicate that immunizations with glycoconjugate **23** benefit from administration with an

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adjuvant. Again, serum IgG to the oligoglucoses **2** and **3** was not detectable.

The epitope recognition pattern in mice immunized with glycoconjugate **23** in the presence of Alum adjuvant differed from the other two groups. While antibody levels specific for **1** were comparable to mice immunized with Freund's adjuvant, relatively low levels of antibodies to epitopes **5** and **4** were observed. Interestingly, while the serum IgG level to pentasaccharide **1** increased from week three to five, at the same time the antibody response to trisaccharide **5**, disaccharide **4**, and monorhamnose **6** decreased (Figure 5C). This result indicates that a robust immune response was exclusively elicited to the complete pentasaccharide epitope **1**.

To gain further insights into the nature of the humoral immune response to the PS-I related epitopes and the differences observed in mice immunized with Freund's and Alum adjuvants, binding of serum antibodies to epitopes **1** and **4** was analyzed by surface plasmon resonance (SPR). Pooled sera of these two groups of mice (1:50 dilution in PBS) were flown over a CM5 sensor chip carrying immobilized epitopes **1** and **4**. Antibodies to both and **1** and **4** in the sera of the Freund's group showed increasing binding and stability values over time from week two over three to five, indicating affinity maturation to both epitopes during the course of the immune response (Figure 5D). Similarly, antibodies in the sera of the Alum group elicited increasing affinity to epitope **1** over time. In contrast, the affinity of serum antibodies to **4** was overall lower and decreased over time, with a minimum at week five. These results verify the epitope recognition to **1** and **4** observed in the glycan array studies (Figure 5B,C).

The differences in the recognition of oligosaccharides **1**–**6** between the Freund's and Alum-adjuvanted groups of mice might be attributed to the nature of the immune responses that are predominantly elicited by the adjuvants. Freund's adjuvant has been shown to evoke primarily a Th1-specific response profile including the generation of IgG2a, IgG2b, and IgG3 antibodies in mice.[52,53] Alum induces primarily a Th2-type response of IgG1 antibodies.[53−55] Th1-type responses have been linked to epitope spreading, the spread of the immune response to other areas of the antigen including substructures, or cryptic epitopes.[56] Our data indicate that disaccharide **4** is a cryptic epitope of pentasaccharide **1** that is accessible only via a Th1-type response induced by Freund's adjuvant but not by a Th2-type response elicited by Alum, as shown by lower antibody responses to substructure epitopes of **1** and decreasing affinity to epitope **4** during the course of the immune response (Figure 5B–D). Further investigations will need to address these questions and determine the IgG subclasses induced by glycoconjugates administered with Freund's or Alum adjuvant. While Alum as primarily Th2-directing adjuvant is most commonly used in human-approved vaccines,[49] the oil-in water emulsion adjuvant MF59, approved for seasonal flu vaccinations,[57] has been shown to elicit both Th1- and Th2-type responses[58] and has been successfully employed to mount an antibody response to oligosaccharide antigens formulated as CRM_{197} conjugates in mice.[24] MF59 is thus an interesting human-approved adjuvant for glycoconjugate **23** to elicit an antibody response to the cryptic epitope **4**.

The response to disaccharide **4** was highest compared to the other substructures when glycoconjugate **23** was administered; titers and robustness to **4** were similar to pentasaccharide **1** in the Freund's and nonadjuvanted groups of mice. In all three groups, IgG levels to rhamnose **6** were lower than to disaccharide **4**. Even in mice receiving Freund's adjuvant that elicited the highest IgG titers, titers against **6** increased only transiently, indicating that disaccharide **4**, and not rhamnose **6**, is the minimal epitope of **1**. This minimal epitope, however, was not accessible when glycoconjugate **23** was administered with the human-approved Alum adjuvant. Nevertheless disaccharide **4** is an interesting structure to be investigated as vaccine candidate as the data suggest that it could induce cross-reactive antibodies to the pentasaccharide **1**. Moreover, it can be procured with reduced synthetic efforts.

Preparation, Characterization and Immunological Evaluation of a PS-I Disaccharide 4-CRM_{197} Conjugate. Disaccharide **4** emerged as the major antigenic determinant of pentasaccharide **1**. Hence, we expected that immunization with **4** should not only induce antibodies recognizing disaccharide **4** but also pentasaccharide **1**. To test this hypothesis, we conjugated **4** to CRM_{197} for immunological evaluation. Conjugation was confirmed by SDS-PAGE and MALDI-TOF mass spectrometry indicated an average loading of 9.8 molecules disaccharide **4** per molecule CRM_{197} (Supporting Information, Figure 16). C57BL/6 mice were immunized with glycoconjugate **24** in the presence of Freund's adjuvant in a prime-boost regimen using 3 μg carbohydrate-based doses per injection (Figure 6A). We chose the more potent Freund's adjuvant rather than Alum since we expected lower immunogenicity of the smaller structure **4** compared with the pentasaccharide **1**. Indeed, glycoconjugate **24** proved less immunogenic than **23**, which is in accordance with the notion that simple carbohydrates, such as disaccharides, are usually less immunogenic than more complex ones.[61−64] Two boosting immunizations were required to evoke a detectable immune response against **4** in the serum of two of three mice (Figure

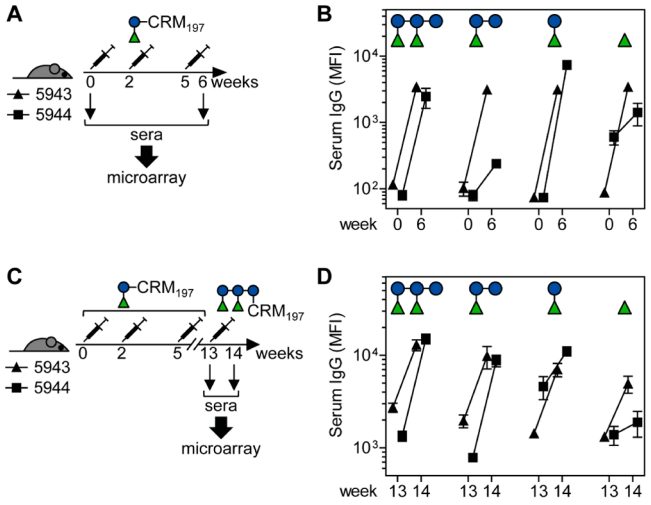

Figure 6. Serum IgG response to oligosaccharides **1**–**6** in mice immunized with glycoconjugate **24**. (A) Three C57BL/6 mice were immunized with **24** at weeks zero ('priming'), two ('first boosting') and five ('second boosting') using 3 μg of conjugated disaccharide **4** per injection and Freund's adjuvant. Two of these mice, 5943 and 5944, mounted an antibody response to disaccharide **4**. (B) Serum IgG levels of two mice to **1**–**6** expressed as MFI. Data points show mean ± SD of n = 4 microarray spots. (C) The mice were boosted with **23** at the indicated time point. (D) Serum IgG levels of the two mice expressed as MFI to **1**–**6**. Responses to **2** and **3** were omitted since they were undetectable. The microarray scans are depicted in Supporting Information, Figure 17.

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6B). The antibodies also recognized pentasaccharide **1** and trisaccharide **5** as well as monorhamnose **6**. Recognition of the rhamnose-containing epitopes cannot be explained by rhamnose-specific antibodies, since mouse 5944, prior to immunization, had antibodies to monorhamnose **6** that did neither recognize **1**, **4**, nor **5** (Figure 6B), and after the second boosting, IgG titers to **1** and **4** were both higher than those to **6**. This evidence lends further credence to the notion that disaccharide **4** is the minimal epitope of pentasaccharide **1**.

These findings underscore that disaccharide **4** is a minimal size epitope of pentasaccharide **1**. Furthermore, the presence of anti-disaccharide **4** antibodies in human sera and fecal samples indicates that this disaccharide is a natural epitope with a possible role during CDI (Figure 3). Thus, disaccharide **4** is a valid target structure for vaccination approaches against *C. difficile*. While oligosaccharide epitopes for conjugate vaccine development should ideally be larger,[59−63] immunogenic disaccharide epitopes have conferred protection against *Streptococcus pneumoniae* type 3[64] and *Candida albicans*[65] in preclinical settings. Thus, immunization with disaccharide **4** may protect against CDIs.

CONCLUSION

This study highlights the utility of synthetic oligosaccharides to identify and define glycan cell-surface epitopes as basis for antibody and vaccine development. The present focus is placed on *C. difficile* that causes serious infections and where a vaccine is urgently needed.[2] The chemical synthesis of PS-I pentasaccharide repeating unit **1** and related substructures **2**−**6** provided access to comparatively large amounts of pure glycans that were not available by isolation from natural sources. Glycan microarray analyses revealed that secreted IgA and serum IgG antibodies to pentasaccharide **1** and its substructures are found in the majority of the population, indicating that the synthetic glycans represent naturally occurring epitopes. Despite the observed interindividual variations in antibody responses, low IgA titers correlated with more severe acute disease among CDI patients. The variations among control individuals and CDI patients might reflect, for example, differences in the exposure status as children have been reported to be frequently asymptomatically colonized.[66,67]

It has originally been proposed that PS-I is a stochastically expressed antigen. However, the failure to reliably detect PS-I in different *C. difficile* isolates might be due to phase-variable expression rather than lack of biosynthetic machinery.[25] In contrast, our glycan array screens suggest that PS-I is a common antigen expressed *in vivo*. These findings prompted us to evaluate PS-I pentasaccharide **1** as vaccine candidate antigen.

Mice immunized with a pentasaccharide **1**-CRM_{197} glycoconjugate mounted a robust antibody response to pentasaccharide **1**, including immunoglobulin class switching and affinity maturation. Glycan microarray technology proved an elegant means to deduce the minimal antigenic determinant of complex oligosaccharide antigens. Here, we provide proof-of-concept for glycan microarray-assisted antigen definition by reducing the *C. difficile* PS-I pentasaccharide **1** antigen to disaccharide **4**. Our findings were supported by the observation that antibodies raised against disaccharide **4**-CRM_{197} glycoconjugate recognized pentasaccharide **1**. Thus, disaccharide **4**, in addition to pentasaccharide **1**, is a promising vaccine candidate to be taken into challenge trials in animal models. Recreating the native infection with *C. difficile* is currently the only feasible way to investigate whether antibodies raised with synthetic oligosaccharides **1** and **4** recognize the natural PS-I polysaccharide on the bacteria, since its expression has not yet been reliably established under culture conditions.[20,25] The presence of IgA antibodies in the stool specimens of CDI patients provides indirect evidence for the expression of PS-I and PS-II by *C. difficile* in the intestinal tract during infection. Cross-reactivity of naturally raised antibodies to the employed oligosaccharides indicates that the oligosaccharides represent natural epitopes of the PS-I polysaccharide. Challenge studies will show whether antibodies raised with the synthetic oligosaccharide epitopes recognize the natural PS-I polysaccharide. This will also be an opportunity to investigate immunization regimes that specifically promote intestinal IgA secretion like transcutaneous immunization with immunoadjuvantive proteins, such as *C. difficile* toxin A,[68] which would complement vaccination with PS-I related oligosaccharides.

In a broader sense the findings reported here are of relevance for the future design of synthetic carbohydrate antigens. Despite advances in chemical synthesis,[69−74] the assembly of complex oligosaccharides still poses challenges, particularly on large scale. Reducing carbohydrate antigens to less complex substructures by a systematic reductionist approach, delivering a molecule that still contains the desired antigenic properties is an important step toward cost-efficient synthetic carbohydrate-based vaccines. Monoclonal antibodies against pentasaccharide **1** and disaccharide **4** are being developed currently for passive immunization and to serve as diagnostic tools. Challenge studies with pentasaccharide **1** and disaccharide **4** antigens will be performed to evaluate their effectiveness in preventing CDI.

ASSOCIATED CONTENT

Supporting Information

Complete experimental procedures, NMR spectra of key compounds, Scheme S3, Figures 1−17, and methods. This information is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

Chakkumkal.Anish@mpikg.mpg.de; Peter.Seeberger@mpikg.mpg.de

Present Address

[§]Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, United States

Author Contributions

[‖]These authors contributed equally.

Notes

The authors declare no competing financial interest.

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