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Phosphorylation of the Synthetic Hexasaccharide Repeating Unit Is Essential for the Induction of Antibodies to *Clostridium difficile* PSII Cell Wall Polysaccharide

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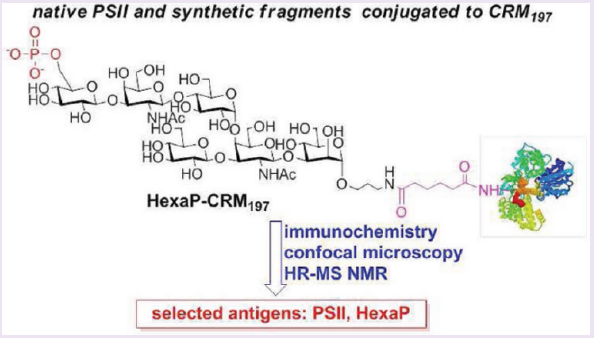
Supporting Information

ABSTRACT: *Clostridium difficile* is emerging worldwide as a major cause of nosocomial infections. The negatively charged PSII polysaccharide has been found in different strains of *C. difficile* and, thereby, represents an important target molecule for a possible carbohydrate-based vaccine. In order to identify a synthetic fragment that after conjugation to a protein carrier could be able to induce anti-PSII antibodies, we exploited a combination of chemical synthesis with immunochemistry, confocal immunofluorescence microscopy, and solid state NMR. We demonstrate that the phosphate group is crucial in synthetic glycans to mimic the native PSII polysaccharide; both native PSII and a phosphorylated synthetic hexasaccharide repeating unit conjugated to CRM_{197} elicit comparable immunogenic responses in mice. This finding can aid design and selection of carbohydrate antigens to be explored as vaccine candidates.



Clostridium difficile is the most important identifiable cause of healthcare-acquired diarrhea.[1,2] The incidence and mortality associated with this Gram-positive spore-forming anaerobic pathogen have dramatically increased in the recent years, with a reported attack rate of 22.5 cases per 1000 hospital admissions in Canada and a significantly high mortality rate of 6.9%.[3] Outbreaks of severe and recurrent *C. difficile* infections in the U.S. and Canada are associated with the spread of the hypervirulent ribotype 027 strain or North American pulsotype 1 (NAP1).[4] This strain has subsequently been registered also in many European countries,[5] although a prevalence of ribotypes other than 027 has been reported.[6]

The incidence of this microorganism is increasing in hospitals worldwide as a consequence of the widespread use of broad-spectrum antibiotics.[7] Treatment failures and recurrences with antibiotics such as vancomycin and metronidazole are emphasizing the need for the discovery of new therapeutic and preventative agents.[8] The major virulence factors of *C. difficile* are the two large exotoxins A (TcdA) and B (TcdB).[9] In addition, a limited number of isolates also produce a binary toxin (CDT), whose patho-physiological role in *C. difficile* infection (CDI) remains poorly understood.[10] Infusion with human mAbs against toxins A and B appears to be protective against secondary CDI episodes.[11] However, the anticipated high costs associated with this passive immunotherapy render a vaccine more attractive in terms of efficacy, availability, and affordability.[1]

Recently the structural analysis of the cell wall polysaccharides of *C. difficile* ribotype 027 and two additional strains, MOH900 and MOH718, identified two different structures, named PSI and PSII. Only PSII was found to be expressed by all the three analyzed strains.[12] On the basis of the hypothesis that this polysaccharide might be a conserved surface antigen, in the perspective of a vaccine that could be effective in inducing bacterial clearance following infection, we considered the use of PSII for the development of a possible carbohydrate-based anti-*C. difficile* vaccine. The immunogenicity of weak T-independent antigens, such as carbohydrates, can be increased in quantity and quality by conjugation to protein carriers, which induce T-cell help.[13] This strategy has already found application in the development of a number of carbohydrate-based vaccines against *Haemophilus influenzae*

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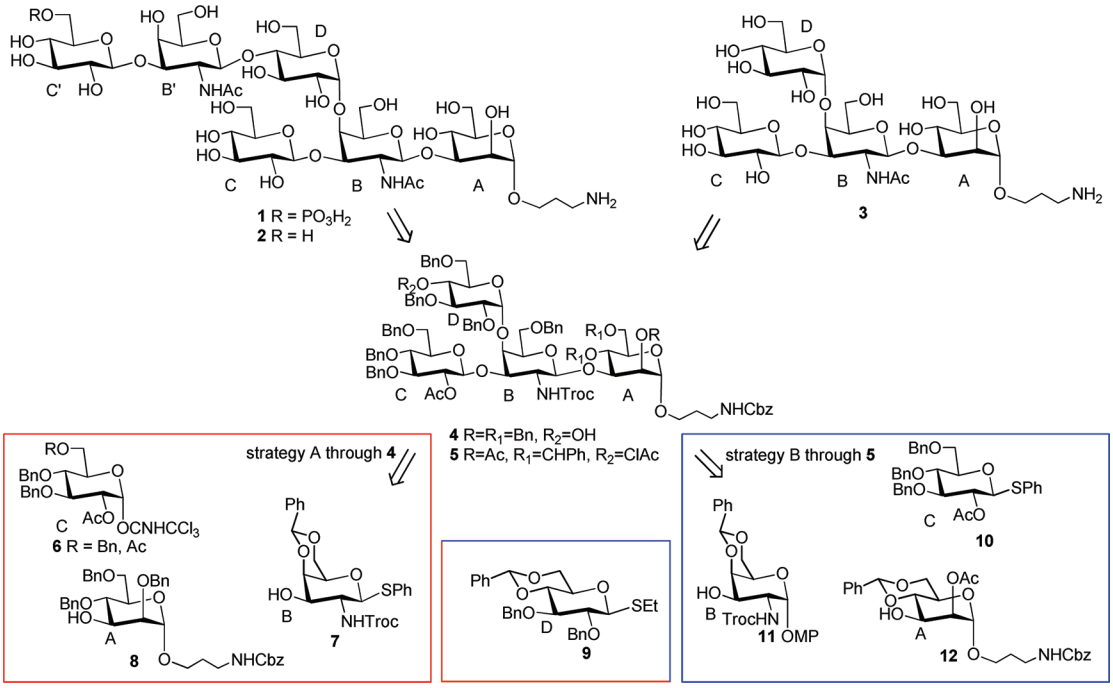

Figure 1. PSII repeating unit and retrosynthetic strategies A[20] and B for the preparation of its synthetic fragments.

type b, *Neisseria meningitidis*, *Streptococcus pneumoniae*, and Group B *Streptococcus*.[14]

Glycoconjugate vaccines are among the safest and most efficacious vaccines developed so far. A breakthrough for this type of vaccine was made in 2004 by a Cuban and Canadian team[15] who reported the large-scale synthesis and the introduction of a synthetic oligosaccharide vaccine against *H. influenzae* type b in humans. Advantages of synthetic carbohydrate-based vaccines include their well-defined chemical structures and lack of the impurities present in polysaccharides obtained from bacterial isolation. In the past decade, a variety of oligosaccharides resembling the surface carbohydrates of pathogens have been synthesized, coupled to carrier proteins, and proved to elicit protective antibodies in animal models.[16] Synthetic oligosaccharides, besides being possible vaccine candidates, can help to elucidate the structural moieties of the biological polysaccharide that are essential to induce antibodies.[17] This step is crucial for the design of a new generation of improved and safer vaccines obtained either from chemical synthesis or bacterial source.

Accordingly we decided to investigate synthetic oligosaccharides as candidates for a *C. difficile* conjugate vaccine. PSII is a polyanionic polysaccharide composed of a hexaglycosyl phosphate repeating unit: [-6)-*β*-D-Glc*p*-(1−3)-*β*-D-Gal*p*NAc-(1−4)-*α*-D-Glc*p*-(1−4)-[*β*-D-Glcp-(1-]-*β*-D-Gal*p*NAc-(1−3)-*α*-D-Man*p*-(1-P].[12] Besides *C. difficile*, other pathogenic bacteria display negatively charged polysaccharides (carboxylated or phosphorylated) on their surface. The presence of phosphodiester-linked repeating units is a structural motif widely occurring in many pathogens, such as *Haemophilus influenzae* type b, serogroup A meningococcus, and serotypes 6 (A, B and C) and 19 (A and F) of *Streptococcus pneumonia*.[18]

Although a variety of studies have been conducted to identify the critical structural parameters that are important for polysaccharide and conjugate vaccines antigenicity and immunogenicity, to our knowledge little is known about the immunodominance of the negatively charged groups in polyanionic polysaccharide antigens.[19] Therefore, in conjunction with the question whether the single repeating unit, conjugated to a carrier protein, is sufficient to elicit anti PSII antibodies in mice, we thought it important to also address the question whther phosphorylation of the synthetic oligosaccharide is necessary.

We previously disclosed the first synthesis of the phosphorylated hexasaccharide PSII repeating unit **1** bearing a linker for conjugation to the carrier protein, and its nonphosphorylated counterpart **2** (Figure 1).[20] Synthetic glycans **1** and **2** are suitable candidates to determine the contribution of phosphorylation at the nonreducing end terminal residue of PSII in the polysaccharide immunogenicity. According to our retrosynthetic scheme (Figure 1), the two structures **1** and **2** can be prepared from a common tetrasaccharide AB(D)C intermediate **4**, which we considered *per se* an interesting fragment to be investigated. Since the *β*-D-Glc*p*-(1−3)-*β*-D-Gal*p*NAc-(1−4)- (CB) arm is repeated twice in the hexasaccharide repeating unit, we were interested in understanding whether this polysaccharide sub unit could still be immunogenic.

The tetrasaccharide AB(D)C **3** would also make it possible to examine more thoroughly the branching point of the hexaglycosyl unit, as it has been reported that branches can play a crucial role determining or deleting the immunogenicity of short oligosaccharides.[17,21] Accordingly, we have developed the synthesis of the tetrasaccharide subunit **3**, *via* the AB(D)C intermediate **5**.

The preparation of a nonphosporylated hexasaccharide and its conjugation to the nontoxic mutant of diphteria toxin CRM_{197},[22] a carrier protein widely used in commercial vaccines, was recently reported by Oberli *et al.*,[23] who demonstrated the immunogenicity of the glycan by the presence of its own specific IgG antibodies in the serum of immunized mice. Moreover, specific IgA antibodies in the stool of hospital patients infected with *C. difficile* recognized the synthetic PSII hexasaccharide hapten. However, no proof about the

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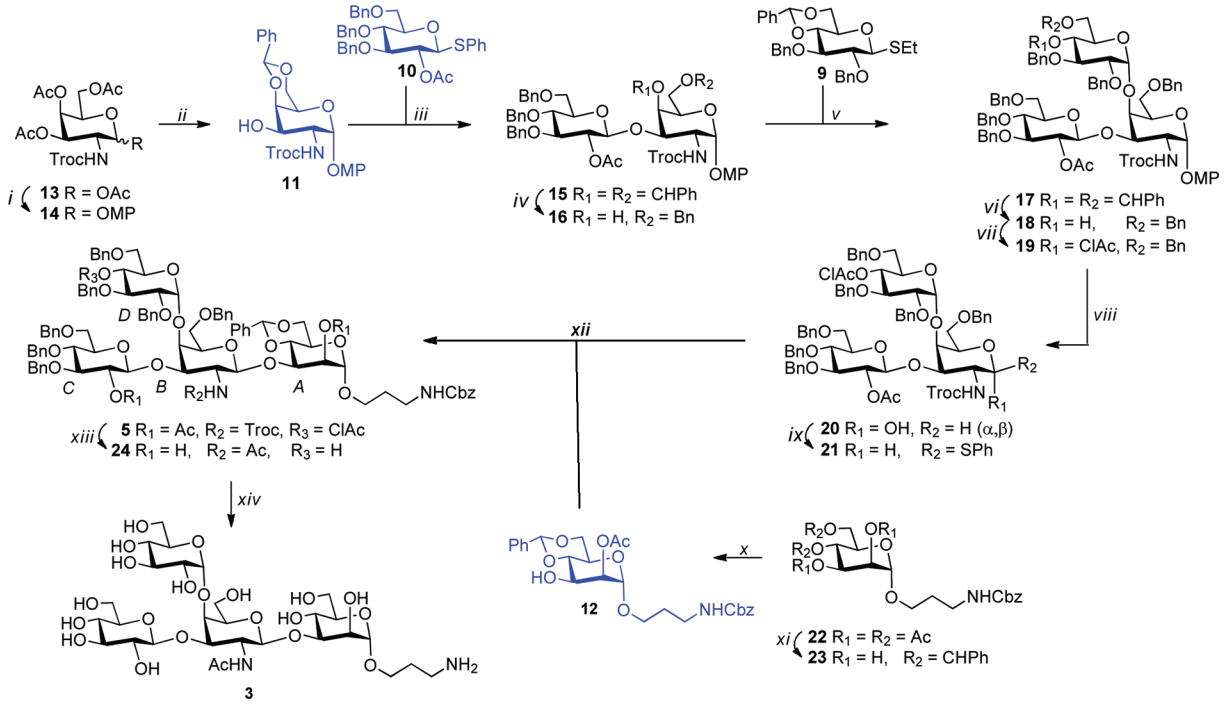
Scheme 1. Synthesis of PSII Tetrasaccharide Fragment[a]



[a]Reagents and conditions: (i) *p*-methoxyphenol, $BF_3{\cdot}Et_2O$, CH_2Cl_2, 93%; (ii) NaOMe, MeOH; $PhCH(OMe)_2$, *p*-TsOH, CH_3CN, 83% (over 2 steps); (iii) NIS, TfOH, CH_2Cl_2, 98%; (iv) $Me_3N{\cdot}BH_3$, $BF_3{\cdot}Et_2O$, CH_3CN, 87%; (v) NIS, TfOH, toluene−dioxane, 82%; (vi) $Me_3N{\cdot}BH_3$, $BF_3{\cdot}Et_2O$, CH_3CN, 99%; (vii) $ClAc_2O$, pyridine−CH_2Cl_2, 91%; (viii) CAN, CH_3CN-H_2O, 62%; (ix) Ac_2O, pyridine; PhSH, $BF_3{\cdot}Et_2O$, CH_2Cl_2, 77%; (x) NaOMe, MeOH; $PhCH(OMe)_2$, *p*-TsOH, CH_3CN, 50% (over 2 steps); (xi) $CH_3C(OCH_2CH_3)_3$, *p*-TsOH, CH_3CN; $AcOH-H_2O$, 67% (over 2 steps); (xii) **21**, NIS, TfOH, CH_2Cl_2, 65%; (xiii) 3 M NaOH, THF; Ac_2O, MeOH, 81% (over 2 steps); (xiv) H_2, 10% Pd−C, AcOH, EtOH, 74%.

immunogenicity of the native PSII polysaccharide and whether synthetic fragments can be used in place of it has been hitherto reported.[24]

In the present paper we investigate the role of the phosphate group in the hexaglycosyl repeating unit **1** in order to antigenically represent the PSII polysaccharide, exploiting a combination of chemical synthesis with physicochemical and immunological techniques that were applied to compare native PSII and related chemically synthesized structures **1**, **2**, and **3**. The PSII polysaccharide was isolated and purified from bacterial growth in view of the coupling to the carrier protein. The native polymer and the three synthetic glycans **1**, **2** and **3** were conjugated to CRM_{197}, and the immunogenicity of the resulting glycoconjugates was tested in mice. Polyclonal antibodies raised from immunization with the CRM_{197} conjugates of the synthetic oligosaccharides **1**, **2**, **3** and native PSII were then utilized to verify by confocal microscopy imaging the accessibility of surface epitopes in *C. difficile* cell samples. Analyzed strains were selected on the basis of a fast screening through solid state NMR analysis of a wide range of *C. difficile* bacterial samples collected from clinical settings.

RESULTS AND DISCUSSION

PSII Production, Purification, and Characterization. Five strains (M68, M120, R20291, 630, Nt2023) were tested to check PSII polysaccharide expression. The strains were grown, and after inactivation and extraction, supernatants were analyzed by ^1H NMR. The spectra obtained were consistent with the published structure,[12] and anomeric protons of unit A at 5.44 ppm, D at 4.95 ppm, B at 4.76 ppm, and B′ at 4.60 ppm (Man, α-Glc and GalNAc, respectively) were clearly detectable, confirming the PSII expression in all the analyzed strains, with a minor extent in 630 strain.

R20291 strain, also known as Stoke Mandeville (ribotype 027) was selected as the best producer and used for subsequent fermentation development. The polysaccharide was released by acidolysis, followed by filtration for inactivation of any spores (see Supporting Information, Paragraph 1.2). The purification process was mainly based on fractionation by precipitation with $CaCl_2$/EtOH and enzymatic treatments to remove protein, nucleic acid, and peptidoglycan contaminants, followed by different chromatographic steps to remove low molecular weight oligosaccharides and contaminants (see Supporting Information, Figure S1). As a first step 1% $CaCl_2$ and 20% EtOH were added to precipitate the protein contaminants that were removed by centrifugation; subsequent increase of the EtOH concentration to 80% led to PSII precipitation. PSII was then treated with DNase and RNase for nucleic acid removal, and mutanolysin, a muralytic enzyme that cleaves β-*N*-acetylmuramyl-(1,4)-*N*-acetylglucosamine linkages, for peptidoglycan removal (>1%, see Supporting Information, Paragraph 1.2).

At this stage, after fractionation by precipitation with $CaCl_2$/EtOH to remove the enzymes, an anionic exchange chromatography was carried out to separate the low molecular weight (avDP 2−6) from the higher molecular weight species (DP> 7). The material, analyzed by the phenol−sulphuric acid test for saccharide content and by NMR to determine the averaged degree of polymerization (avDP), was concentrated by tangential flow filtration (TFF) through a 5 kDa cutoff membrane and diafiltered in order to reach the appropriate volume and buffer composition for the next step. The

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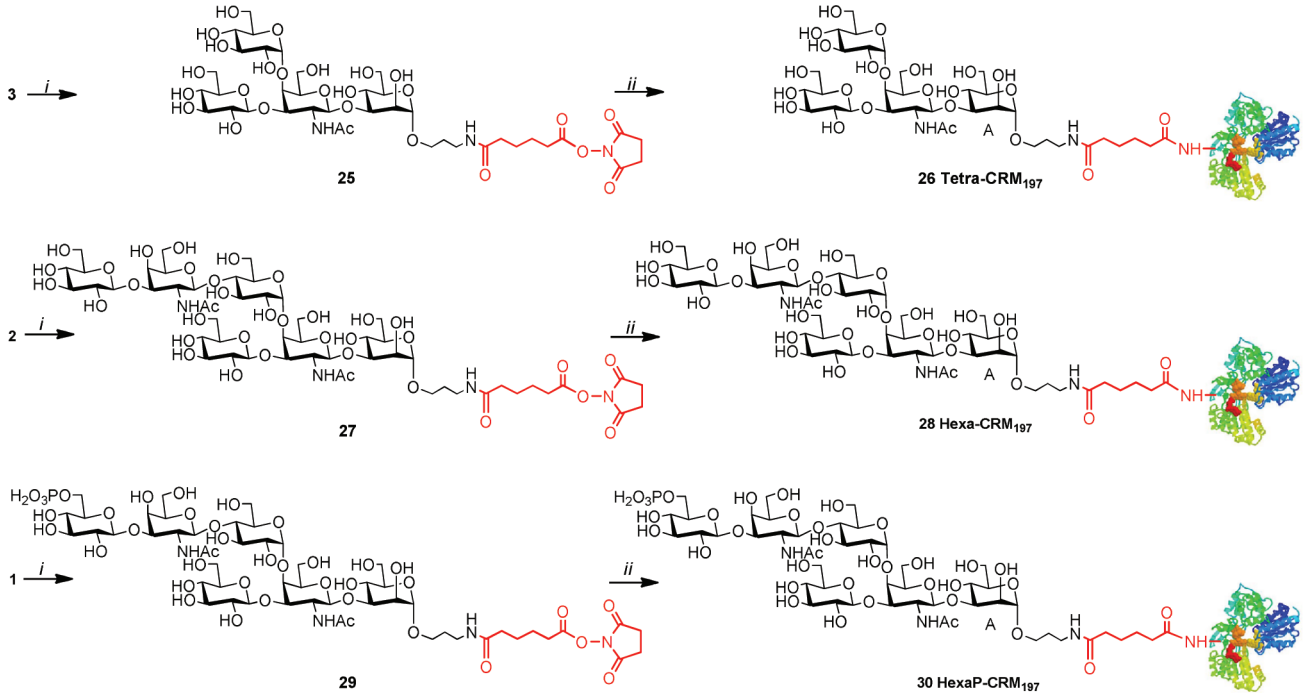
Scheme 2. Conjugation of Synthetic Glycans[a]



[a]Reagents and conditions: (i) di-*N*-hydroxy-succinimidyl adipate ester, Et_3N DMSO; (ii) CRM_{197}, 100 mM NaPi, pH 7.

polyanionic PSII was then loaded onto a cationic exchange chromatography to remove positively charged contaminants. Finally, size exclusion chromatography was performed to remove the impurities of low molecular weight.

Protein contamination was determined by MicroBCA assay, Bradford assay, and aminoacid (AA) analysis; comparing the results obtained by the different assays, we found that AA analysis and the Bradford assay are more suitable to assess the purity of this polysaccharide (see Supporting Information, Table S1 and Figure S2).

The structure of PSII released by acidolysis and purified was verified by ^1H and ^{31}P NMR analysis. $^1H-^{31}P$ HMBC NMR indicated that the phosphate-C-1-α-mannose linkage was less stable in comparison with the C-6-β-glucose-phosphate, and thus the phosphate group remained linked to the C-6 position of Glc C′ unit (see Supporting Information, Paragraph 1.3 and Figure S3).

The ^{31}P NMR profile of the polysaccharide showed two spin systems, which were assigned to the phosphodiester (P_{de}) and phosphomonoester (P_{me}) signals at 0.5 and −1.5 ppm, respectively. avDP was calculated by the integral ratios of selected proton signals (avDP = $[H_{1A}/(H_{1A}\text{-}\alpha + H_{1A}\text{-}\beta) + 1]$) and phosphorus signals (avDP = $[(P_{de}/P_{me}) + 1]$).

Design and Synthesis of PSII Related Oligosaccharides. The observation that the phosphate group was bound to the C-6 of Glc C′ unit suggested the design of the synthesis of the phosphorylated hexasaccharide repeating unit **1** in order to mimic the nonreducing end terminal repeating unit of native PSII and its nonphosphorylated counterpart **2** to determine the effect of the charged group. As shown in Figure 1 (strategy A), the two hexasaccharides **1** and **2** can be assembled by a (4 + 2) convergent approach from a common tetrasaccharide intermediate AB(D)C **4**. By this strategy the challenging insertion of the 1,2-cis glycosidic linkage between residues D and B is accomplished in an early stage of our multistep synthesis. Tetrasaccharide **4** is prepared in turn from the four monosaccharide building blocks **6**, **7**, **8**, and **9** by first assembling the linear trisaccharide ABC and then inserting the α-Glc D unit. Synthon **6** can present a benzyl group at C-6 position for the preparation of compound **2**, whereas this position can be differentiated with an acetate ester for the selective introduction of the phosphate group in hexasaccharide **1**.

Besides the development of strategy A, which has been extensively discussed in our previous report,[20] we explored an alternative approach leading to the tetrasaccharide AB(D)C **5** starting from building blocks **9**, **10**, **11**, and **12** (Figure 1, strategy B). In this case the branched trisaccharide B(D)C **17** is initially assembled to be later coupled to the monosaccharide acceptor **12** (Scheme 1). For the synthesis of the disaccharide unit CB we envisioned the use of a *p*-methoxyphenyl (MP) moiety for temporary protection of the anomeric position of **14** to be later activated as trichloroacetimidate donor.[25]

For this purpose, glycosylation with **13**[26] of *p*-methoxyphenol in the presence of $BF_3{\cdot}Et_2O$ afforded exclusively **14**, with unexpected α configuration. The subsequent deacetylation under Zemplen conditions, followed by 4,6-*O*-benzylidene formation, provided the galactosamine acceptor **11**. Glycosylation of **11** with donor **10**,[27] promoted by NIS-TfOH, afforded disaccharide **15** in 98% yield, which was subjected to regioselective opening of the benzylidene acetal to furnish acceptor **16** (87% yield). The glycosylation of **16** with ethylthioglycoside **9**[28] permitted the stereoselective introduction of the α-linkage and provided trisaccharide **17** in high yield (82%).[20] After regioselective acetal ring opening of compound **17** and chloroacetylation of the resulting alcohol **18**, the *p*-methoxyphenyl group was removed with ammonium cerium(IV) nitrate (CAN) to give **20**.[25] Acetylation of the anomeric position and conversion to phenyl thioglycoside gave the trisaccharide donor **21**.

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The mannosyl acceptor **12**, bearing the tether for conjugation, was prepared by methanolysis of **22**[20] followed by 4,6-*O*-benzylidene protection to give diol **23**. Regioselective acetylation of the axial 2-hydroxy group of **23** gave the mannosyl acceptor **12**, which was glycosylated with the trisaccharide donor **21** to afford the target tetrasaccharide **5** in 65% yield. Eventually, tetrasaccharide **5** was fully deprotected in three steps, including Troc cleavage with simultaneous esters saponification, N-acetylation (81% yield over two steps), and hydrogenolysis (74% yield), providing target **3**, which was used to explore its immunogenicity in comparison with the other PSII fragments.

Conjugation to CRM_{197}. The conjugation of the synthesized PSII fragments **1**, **2**, and **3** was achieved by reaction of the linker-equipped oligosaccharide with an excess of di-*N*-hydroxysuccinimidyl adipate in DMSO (Scheme 2), in order to obtain the formation of the half ester exclusively, which was condensed with the amino groups of the protein in phosphate buffer (pH 7).[21] Glycoconjugates using different molar carbohydrate/protein ratios were prepared, and the corresponding saccharide loadings determined by MALDI-TOF mass spectrometry analysis are reported in Table 1.

Table 1. Characteristics of the Synthetic Glycoconjugates

compd	stoichiometry[a]	saccharide loading (mol/mol)	conjugation efficiency (%)
26a	40	15.5	39
26b	30	9.8	33
28a	40	13.6	34
28b	30	9.8	33
30	40	10.4	26

[a]Moles saccharide/moles protein used for conjugation, on the basis of spectrophotometric determination of active ester in the oligosaccharide sample.

The number of sugar chains linked to the carrier proteins were in agreement with SDS-PAGE profiles (see Supporting Information, Paragraph 3.1 and Figure S4) and showed that the shorter and uncharged glycan **3** could be covalently attached more efficiently to the protein than the longer and charged hexasaccharide **1**, according to a trend that has been previously observed for similar synthetic oligosaccharides.[29]

Considering that the reducing Man end was not phosphorylated, PSII was coupled to CRM_{197} by a selective modification of this sugar to generate aldehyde groups. Reduction of PSII with $NaBH_4$, followed by a controlled periodate oxidation, gave the aldehyde groups for subsequent coupling to the lysine residues of CRM197 *via* reductive amination (Scheme 3).

PSII oligomers with different avDPs (8, 11, and 21) were used for glycoconjugates preparation (see Supporting Information, Paragraph 3.2). The complete disappearance of unglycosylated CRM_{197} (see Supporting Information, paragraph 3.3 and Figure S4) confirmed that the reaction conditions enable the efficient conjugation of the protein. The purification of the conjugates was performed by size exclusion chromatography that permitted efficient separation from the unbound saccharide. Total and free saccharide and protein contents of glycoconjugates are shown in Table 2. After purification, no signals of either residual aldehyde or hydrated aldehyde were detected in the ^1H NMR spectrum of the glycoconjugates, indicating that no further quenching of these highly reactive groups was needed. The degrees of glycosylation ranged from 0.2 to 0.3 (w/w), which correspond to 1−2 mols of PSII (considering a molecular weight of 9−24 kDa) coupled to the protein.

Immunological Evaluation of Glycoconjugates. PSII-CRM_{197} conjugate and neoglycoconjugates from synthetic glycans were evaluated for their ability to elicit anti PSII antibodies. With this aim, groups of 8 Balb/C mice were immunized with 2.5 μg carbohydrate based doses of Tetra- (**26a**), Hexa- (**28b**), HexaP- (**30**), and PSII-CRM_{197} (**33b**) conjugates, respectively. The biomolecules were formulated with the adjuvant MF59, an oil in water emulsion frequently used for seasonal flu vaccination.[30] MF59 has been proven to induce effective long-term protection in the elderly, which is the same target population estimated for a *C. difficile* vaccine.[31,32] Adjuvant alone in phosphate buffer (PBS) was used as a negative control.

Sera were analyzed by ELISA for their content of anti PSII IgG. PSII-HSA, prepared with the same method described for the CRM_{197} conjugate, was used for the coating of the plates. Only CRM_{197} conjugates obtained from the native polysaccharide and the phosphorylated hexasaccharide **1** were able to induce IgG antibodies which bind to PSII (Figure 2).

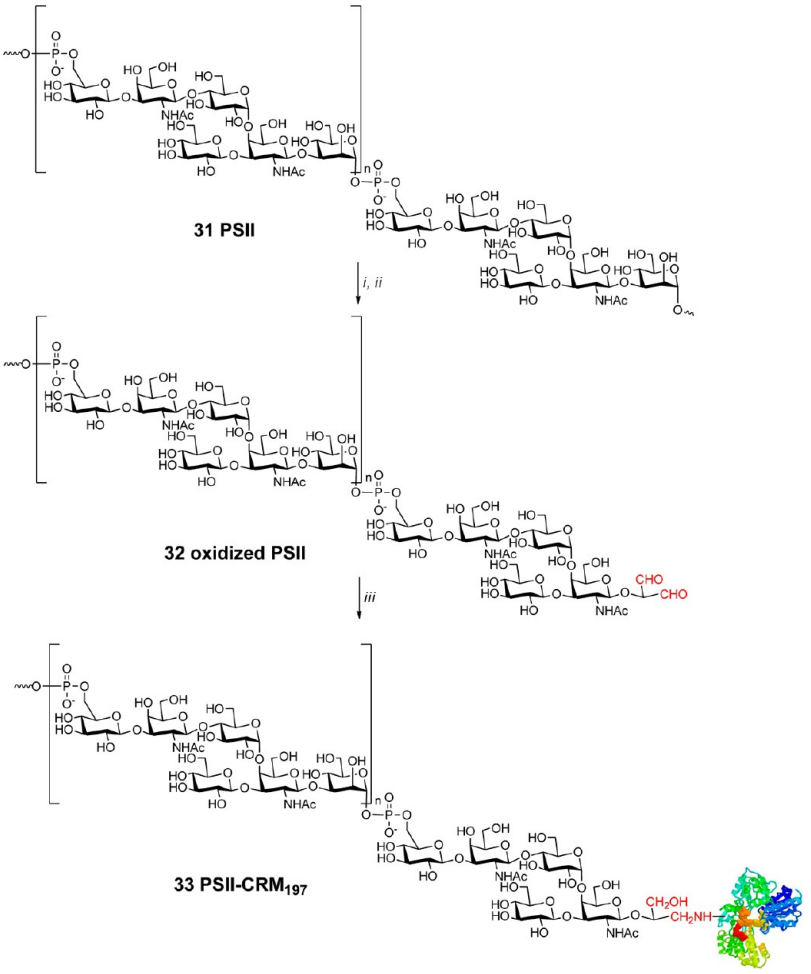
In addition, PSII and phosphorylated glycan **1** conjugates were also the only compounds in the set of analyzed glycoconjugates that elicited low levels of anti-PSII IgM antibodies (see Supporting Information, Paragraph 4 and Figure S6). The tetrasaccharide **3** and the nonphosphorylated hexasaccharide **2**, albeit eliciting self-specific antibodies, showed neither IgG nor IgM anti-PSII titers, demonstrating that the presence of the charged phosphate is crucial to induce antibodies recognizing the polysaccharide.

Specificity of the raised IgG toward PSII polysaccharide was assessed by competitive ELISA using PSII as inhibitor (see Supporting Information, Figure S7). As it has been reported that the nonphosphorylated hapten **2** was recognized by IgA antibodies isolated from patients infected with *C. difficile*,[23] sera from mice immunized with the PSII-CRM_{197} conjugate were used to check their capability to bind the synthetic PSII fragments conjugated to a non CRM_{197} protein (for dot-blot analysis, see Supporting Information Figure S8).

Unlike tetrasaccharide **3**, which showed no binding, sera containing anti-PSII antibodies were found to bind to both **1** and its nonphosphorylated counterpart **2**. Antibodies directed against the PSII polysaccharide can thereby detect the presence of both the phosphorylated and the nonphosporylated hapten, but only the synthetic hexasaccharide **1** induces antibodies that recognize the native PSII. The truncation of the C′B′ arm from the repeating unit results in loss of immunogenicity, and sera of mice immunized with PSII conjugates do not contain anti-tetrasaccharide antibodies. Therefore, the phosphorylated hexasaccharide **1** represents the minimal structure able to mimic the native PSII, and the phosphate group (in the form of either mono or diester) is likely part of the minimal conformational epitope.

Confocal Immunofluorescence Microscopy and Solid State NMR on Clinical Isolates. In order to investigate whether antibodies induced by PSII and the synthetic glycans **1**, **2**, and **3** conjugated to CRM_{197} could recognize the native PSII directly on the *C. difficile* surface, fixed bacteria were analyzed by immunofluorescence. The R20291 strain was chosen for this analysis, as it is the source of PSII purification and the main polysaccharide producer among the strains analyzed. We found that bacteria were labeled with antibodies against the purified

Scheme 3. Conjugation of PSII[a]



[a]Reagents and conditions. *i.* $NaBH_4$, H_2O; *ii.* $NaIO_4$, H_2O; *iii.* $NaCNBH_3$, pH 6.5.

Table 2. Characteristics of PSII-CRM_{197} Conjugates

compd	DP	saccharide/protein (w/w)	saccharide loading (mol/mol)
33a	8	0.31	2.07
33b	21	0.24	0.61
33c	11	0.26	1.31

PSII and the phosphorylated hexasaccharide **1** CRM_{197} conjugates (Figure 3a,b); on the contrary, an extremely weak labeling was observed with antibodies against the tetrasaccharide **3** and the nonphosphorylated hexasaccharide **2** CRM_{197} conjugates (Figure 3c,d).

Since the bacteria were not uniformly labeled, it can be hypothesized that the heterogeneity of the surface structures, containing different carbohydrate antigens in addition to PSII[18] could result in bacterial subpopulations with variable staining. However, in agreement with the immunogenicity data, this evidence strongly suggests that the purified PSII and the phosphorylated hexasaccharide **1** CRM_{197} conjugates induce antibodies recognizing accessible epitopes on the bacterial polysaccharide surface.

As the presence of PSII polysaccharide has been demonstrated in only three strains,[12] we considered its expression within a larger collection of strains an important point to be evaluated in view of a possible vaccine application. In this regard we analyzed by ^{1}H HR-MAS NMR a panel of 40 clinical isolates belonging to 6 ribotypes (see Supporting Information), in addition to R20291 and 630 strains, using the purified PSII polysaccharide NMR solution spectrum collected as a positive control (Figure 4). The anomeric region of the ^{1}H HR-MAS

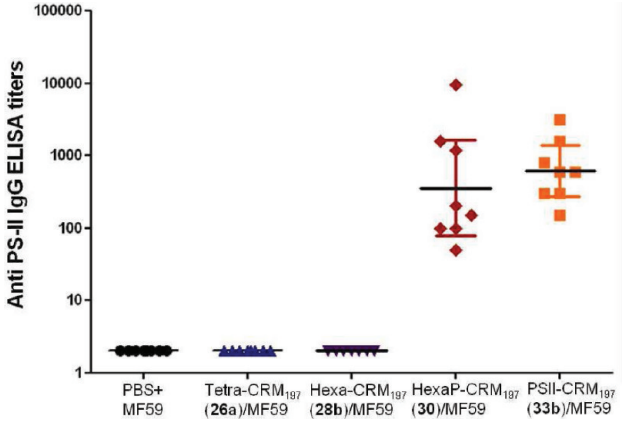


Figure 2. IgG levels detected in individual post 3 sera (sera collected two weeks after the third immunization) of Balc/C mice against PSII-HSA conjugate coating; each dot represents single mouse sera; horizontal dark bars indicate geometric mean titers of each group with 95% statistical confidence intervals as colored bars.

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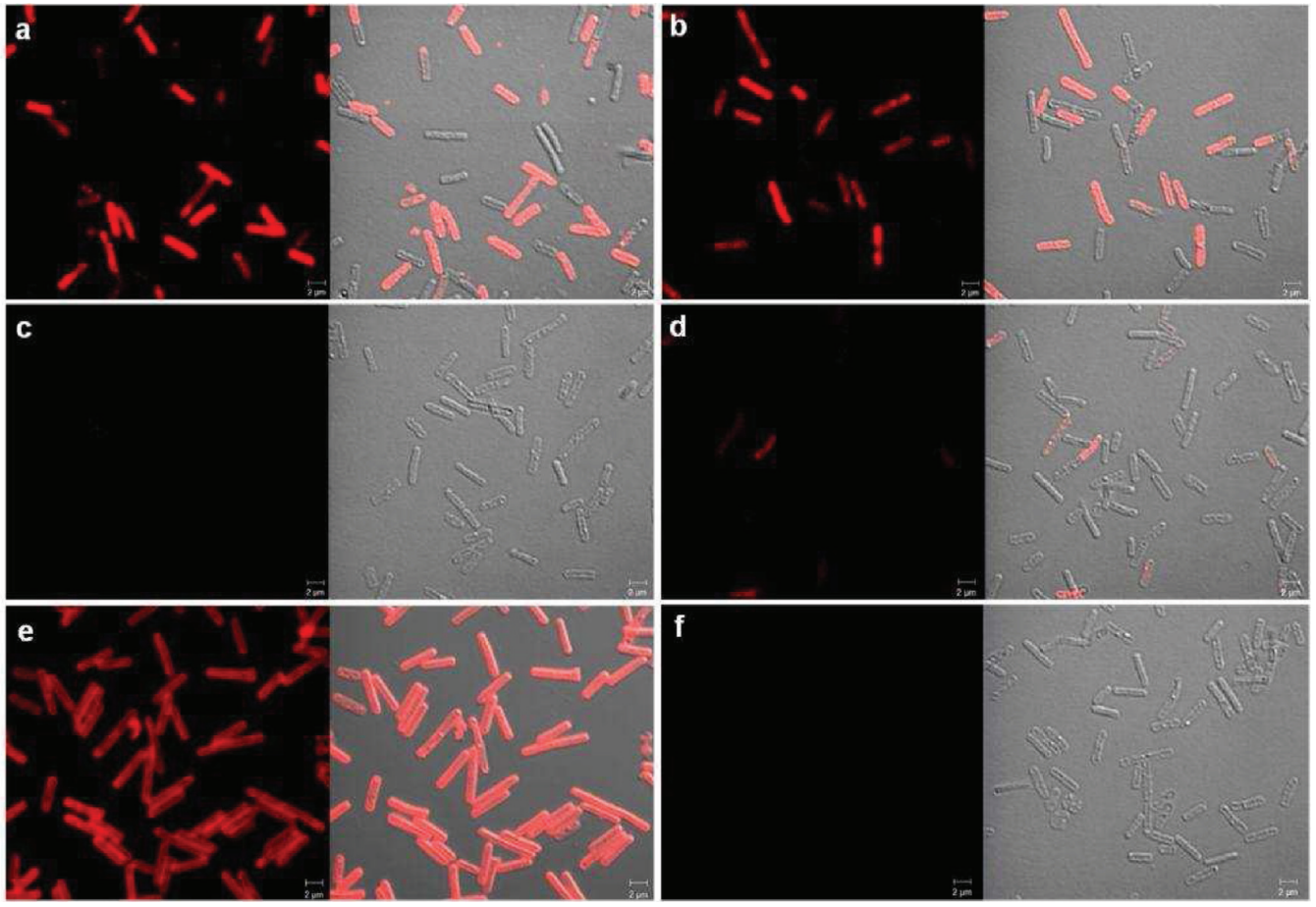

Figure 3. Confocal immunofluorescence microscopy of the R20291 strain using the following sera: α-PSII-CRM$_{197}$ (a), α-Hexa-P-CRM$_{197}$ (b), α-Tetra-CRM$_{197}$ (c), and α-Hexa-CRM$_{197}$ (d). Labeling was visualized using secondary anti-IgG antibodies conjugated to red-fluorescent Alexa-Fluor568. Positive and negative controls were sera against whole fixed bacteria (e) and an unrelated CRM$_{197}$ conjugate (f), respectively. The fluorescence images (left panels) were merged to light microscopy images to show overlaps (right panels).

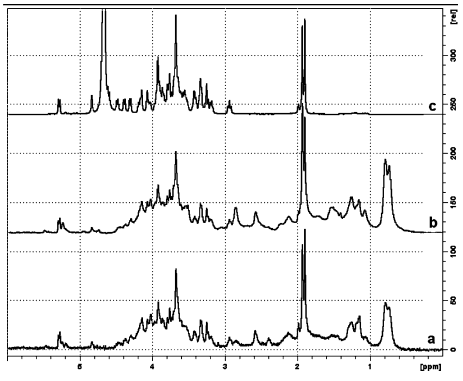

Figure 4. Comparison of the ^{1}H HR-MAS NMR (400 MHz, 90% H_2O + 10% D_2O, 298 K) spectra collected from the two different strains R20291 (a) and B1 (b) and the PSII ^{1}H NMR (400 MHz, D_2O, 298 K) spectrum of R20291 measured in solution (c).

NMR spectra (signals at 5.40, 4.95, 4.70, 4.60 ppm) was used as fingerprint to identify PSII in the heterogeneous phase of the analyzed samples from inactivated cells, except for strains 630 (012 ribotype) and 014 where anomeric signals were not clearly detectable.

To support the NMR data and evaluate the presence of the surface polysaccharide in the context of intact bacteria, a representative strain for each ribotype (001, 012, 014, 018, 078 and 126) was selected for confocal immunofluorescence studies. Sera against PSII-CRM$_{197}$ and HexaP-CRM$_{197}$ conjugates were able to stain polysaccharide structures on the surface of strains belonging to ribotypes 001, 018, 078 and 126, whereas no staining was detected on strains belonging to 012 (630 strain and an additional clinical isolate) and 014 ribotypes. However we could confirm the presence of PSII in the 630 strain by GC−MS analysis.

The reasons for this different behavior revealed by confocal microscopy remain to be clarified. Inspection of publicly available genome sequences shows that the putative capsule biosynthesis locus (CD2768−CD2780) is well conserved across strains belonging to ribotypes 012, 027, 001, and 078 (strains 630, R202291, ATCC43255, and QCD-23m63 respectively),[33−35] leading to excluding that such differences can be ascribed to the absence of specific genes.

We could hypothesize that PSII in the 012 and 014 ribotypes was not efficiently accessible to antibodies, due to the presence of other surface structures. Alternatively, the inability to detect PSII could be exclusively due to the specific experimental conditions, which do not reflect the *in vivo* regulation mechanisms in these strains.

Conclusion. It is becoming more and more evident that *C. difficile* is the cause for many nosocomial infections, especially in the U.S. and Canada. In the past decade, the organism has evolved to become more virulent and resistant to antibiotics.

We investigated the possibility of using the PSII polysaccharide, isolated from the bacterial surface of the hypervirulent epidemic strain BI/NAP1/027, as a potential antigen for a glycoconjugate vaccine. To achieve a better understanding of the relevant epitopes in the polysaccharide and highlight possible synthetic candidates, we applied a combination of chemical synthesis with physicochemical and immunochemical techniques. In particular we focused on elucidating the role of the phosphate group in PSII repeating unit.

A procedure for PSII purification was established, and native polysaccharide and synthetic glycans **1**, **2** and **3** were conjugated to CRM_{197}. Immunological evaluation of the glycoconjugates in mice highlighted the *on/off* role of the phosphate group to mimic PSII, as only the conjugated hexasaccharide **1** and the native polysaccharide were able to elicit comparable IgG titers against PSII.

By immunofluorescence microscopy we assessed that surface PSII expressed by R20291 strain is accessible *in vitro* by sera against PSII-CRM_{197} and HexaP-CRM_{197} conjugates, thus confirming the ELISA data. Concurrent utilization of sera from mice immunized with conjugated native polysaccharide and synthetic glycans proved that the staining in confocal microscopy can be correlated only to the presence of PSII on the bacterial cell wall, thereby excluding any possible surface contaminant.

PSII expression was finally analyzed on a panel of clinical isolates by ^{1}H HR-MAS NMR and confocal immunofluorescence microscopy. The presence of the surface PSII polysaccharide was revealed for ribotypes 027, which is endemic in North America,[4] and in strains belonging to ribotypes 001, 078 and 126, known to have significant incidence in causing severe nosocomial disease in Europe.[6]

Therefore, by a combination of chemical synthesis, immuchemistry, solid state NMR, and confocal microscopy we can identify the native PSII and the phosphorylated synthetic hexasaccharide repeating unit **1** as two possible candidates for either a glycoconjugate or a multiantigen vaccine against *C. difficile*. Our finding highlights for the first time, by well-defined synthetic structures, that phosphorylation of the terminal residue of short length fragments of phosphodiester-linked glycopolymers is an essential determinant for their immunogenicity. Noteworthy, a single synthetic repeating unit phosphorylated at the terminal residue was in our case sufficient to mimic the bacterial native phosphopolymer. Since phosphodiester linkages occur in the surface-exposed polysaccharides of many pathogenic bacteria, we expect that these results will open new perspectives in the design and selection of carbohydrate antigens to be explored as vaccine candidates.

■ ASSOCIATED CONTENT

Supporting Information

Materials and methods for PSII isolation, purification, and characterization; experimental procedures and characterization of new compounds, NMR spectra of compounds **14**, **11**, **15**, **17**, **19**, **23**, **12**, and **3**; procedures for preparation of glycoconjugates and their characterization; immunochemical analysis; methods for confocal immunofluorescence microscopy and HR-MAS NMR. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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