ORGANIC LETTERS

2011
Vol. 13, No. 3
378−381

# First Synthesis of *C. difficile* PS-II Cell Wall Polysaccharide Repeating Unit

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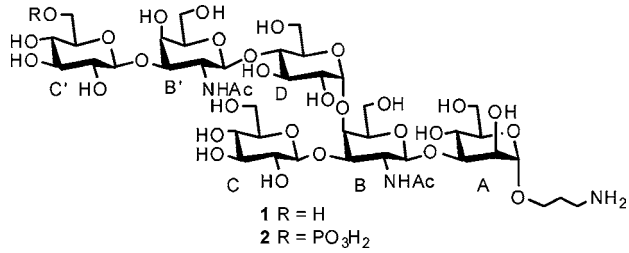
**Received October 28, 2010**

## ABSTRACT




***Clostridium difficile*** **is the most commonly diagnosed cause of nosocomial diarrhea with increasing incidence and mortality among elderly and hospitalized patients. We report the first synthesis of the surface polysaccharide PS-II repeating unit and its nonphosphorylated analogue, with a linker for conjugation, via a (4 + 2) convergent approach from a common AB(D)C tetrasaccharide intermediate.**

*Clostridium difficile* is a Gram-positive spore-forming anaerobic bacterium, which is considered the most important definable cause of nosocomial diarrhea.[1,2] Since its description in 1978 as a cause of antimicrobial-associated diarrhea, colitis, and pseudomembranous colitis (PMC), the interest in this pathogen is growing due to its impact on morbidity and mortality in the elderly and among hospitalized patients.[3]

The incidence of *C. difficile* infection (CDI) is rapidly increasing in the US and Canada,[4] where a recent study reported an attack rate of 22.5 cases per 1000 hospital admissions, which was associated with a significantly high mortality rate of 6.9%.[3,5] The most virulent strain is considered the ribotype 027 or North American pulsotype 1 (NAP1), which caused outbreaks in 16 European countries in 2008.[2] Current treatments for CDI are suboptimal, with up to 20% of treated patients failing to respond to antibiotics and relapses occurring in up to 25% of cases after initial clinical resolution.[6] Health-related costs for afflicted people are significant and estimated as $4000/case in the US.[7] Treatment failures and recurrences with antibiotics are emphasizing the need for the discovery of new preventative agents using vaccination based on either protein or carbohydrate antigens.[8]

Monteiro's group recently analyzed the cell wall polysaccharide of *C. difficile* rybotype 027 and two additional strains, MOH900 (classified as NAP2) and MOH718.[9] Two different structures were identified, named PS-I and PS-II. However, PS-II is the only structure occurring in all strains. This finding strongly suggests that PS-II is a conserved surface antigen,

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10.1021/ol1026188 
**Published on Web 12/29/2010**

which may be advantageous in the development of a carbohydrate-based anti *C. difficile* vaccine. PS-II is composed of a hexasaccharide 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-Glc*p*-(1→3]-*β*-D-Gal*p*NAc-(1→3)-α-D-Man*p*-(1→P].

To the best of our knowledge, the immunogenicity of such polysaccharide and consequently the possibility of preparing a glycoconjugate vaccine have not hitherto been investigated. The synthetic approach would be considerably appropriate to obtain well-defined structures to be tested as anti *C. difficile* vaccines and to circumvent fermentation and isolation of the polysaccharide from spore-forming bacteria.[10]

Here we report the first synthesis of the hexasaccharide PS-II repeating unit **2** and its nonphosphorylated analogue **1** (Figure 1). Both oligosaccharides were synthesized with

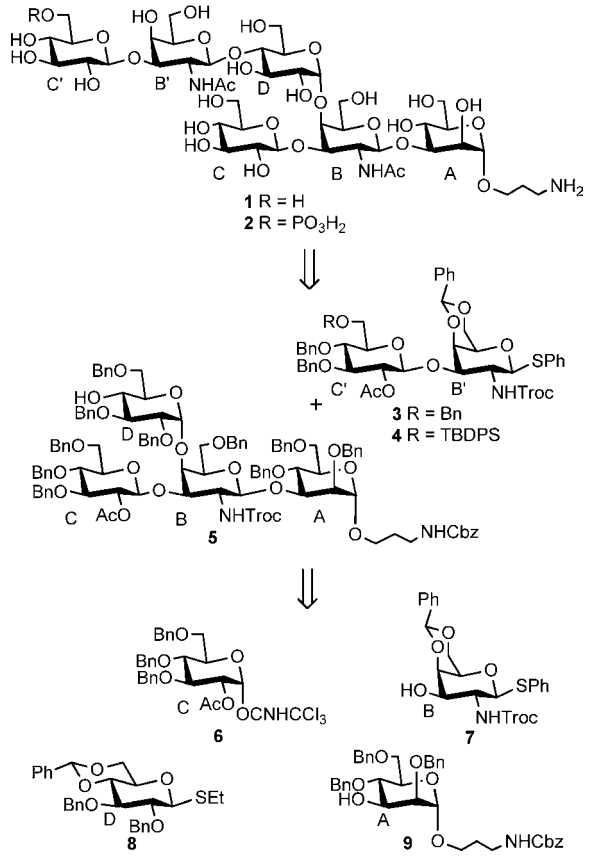



**Figure 1.** Retrosynthetic analysis of PS-II hexasaccharide.

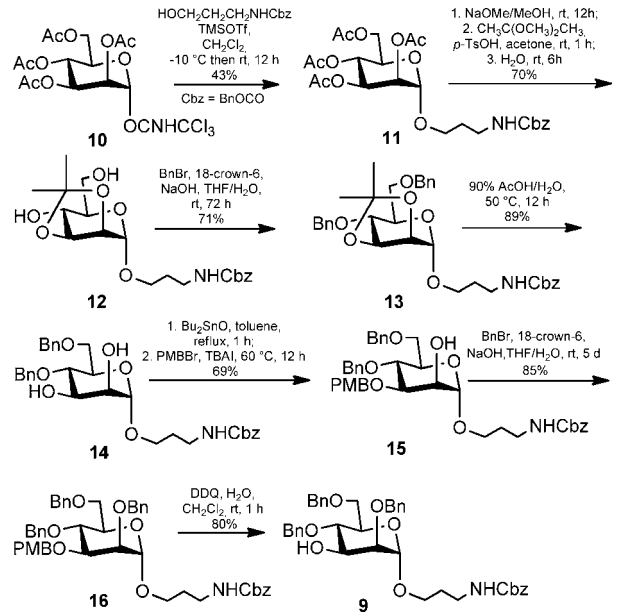
an *O*-linked aminopropyl spacer at the reducing end suitable for conjugation to a carrier protein, which is a fundamental step to make poorly immunogenic carbohydrates able to induce a T cell dependent response.[11] According to our retrosynthetic analysis, target hexasaccharides **1** and **2** can be assembled by a (4 + 2) convergent approach from a common tetrasaccharide intermediate AB(D)C **5** (Figure 1). This strategy features disaccharide **3** as a key intermediate both for the synthesis of tetrasaccharide **5** and the construction of hexasaccharide **1**. In addition, the challenging insertion of the 1,2-*cis* glycosidic linkage between residues D and B could be carried out in an early stage of our work.

On the other hand, the preparation of the phosphorylated hexasaccharide **2** required disaccharide donor **4**, which differs from **3** by a further selectively removable group at the primary hydroxyl of the C′ unit.

We employed the *N*-trichloroethoxycarbonyl (Troc) participating group for the amino group protection in the galactosamine units of **3** and **4**[12] to ensure the formation of 1,2-*trans* glycosidic linkages.

The synthesis of 2,4,6-tri-*O*-benzylated mannoside **9**, bearing the α-oriented anomeric linker, was carried out as illustrated in Scheme 1.

**Scheme 1.** Synthesis of the Mannoside Building Block **9**




After glycosylation of commercially available benzyl *N*-(3-hydroxypropyl)carbamate with donor **10**,[13] the resulting mannoside **11**[14] was deacetylated and converted into the 2,3-*O*-isopropylidene derivative **12** by 2,3:4,6-bis-isopropylidenation followed by selective monohydrolysis.[15] Benzylation of diol **12**, using NaOH as a base under phase-transfer conditions in order to prevent concomitant *N*-benzylation of the linker,[13] and subsequent isopropylidene acetal hydrolysis

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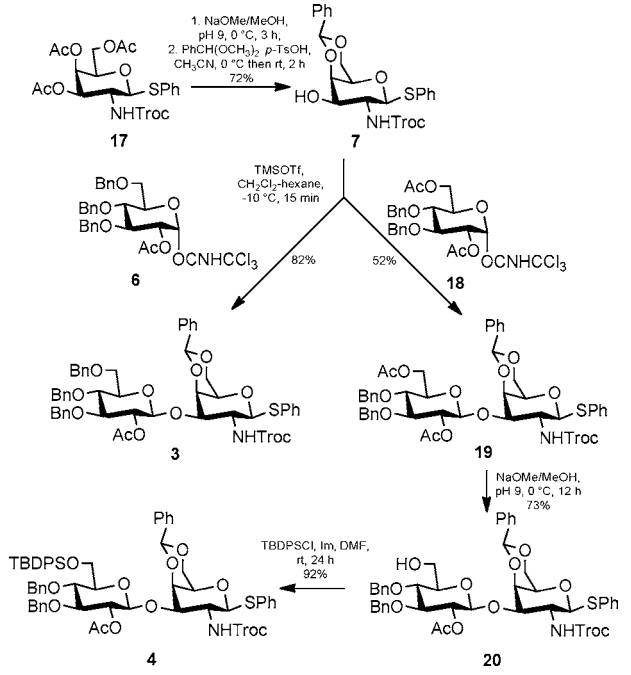
afforded compound **14**. Regioselective *p*-methoxybenzylation at 3-OH of **14** using the stannylene acetal protocol allowed the benzylation of the axial 2-hydroxyl.[16] Finally, standard oxidation of **16** with 3-dichloro-5,6-dicyano-1,4-benzoquinone provided the 3-OH mannosyl acceptor **9**.

The syntheses of disaccharide donors **3** and **4** were achieved starting from the *N*-Troc galactosamine acceptor **7** (Scheme 2),

**Scheme 2.** Syntheses of the Disaccharide Donors **3** and **4**



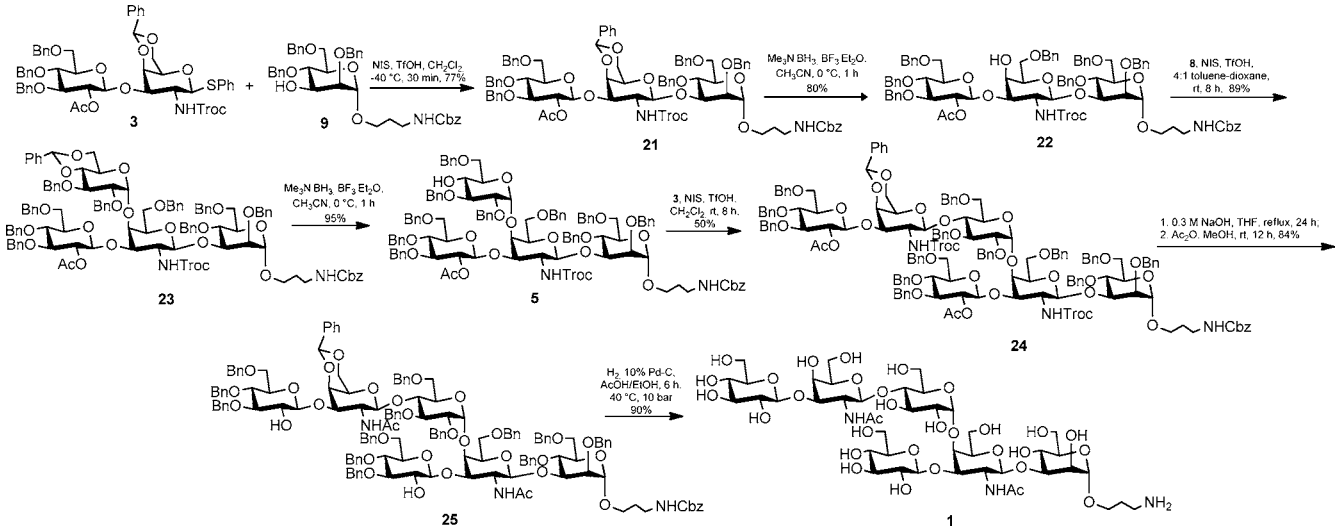

obtained from the known compound **17**[17] by deacetylation and introduction of the 4,6-*O*-benzylidene acetal.

Glycosylation of monosaccharide acceptor **7** with trichloroacedimidate donor **6**[18] in the presence of TMSOTf as a Lewis acid required dichloromethane−hexane as a solvent mixture to circumvent formation of the 1-*N*-trichloroacetamide,[19] which was the predominant byproduct when the reaction was performed in only dichloromethane. Disaccharide **3** could be obtained in a satisfactory 82% yield, whereas the preparation of the closely related compound **19** from donor **18**[20] proceeded in lower yield (52%) since the formation of substantial amount of the 1-*N*-trichloroacetamide byproduct could not be avoided. Disaccharide **19** was then regioselectively 6-*O*-deacetylated by mild transesterification with NaOMe at pH 9 and 0 °C, allowing the straightforward introduction of the *tert*-butyldiphenylsilyl protecting group to afford compound **4**.

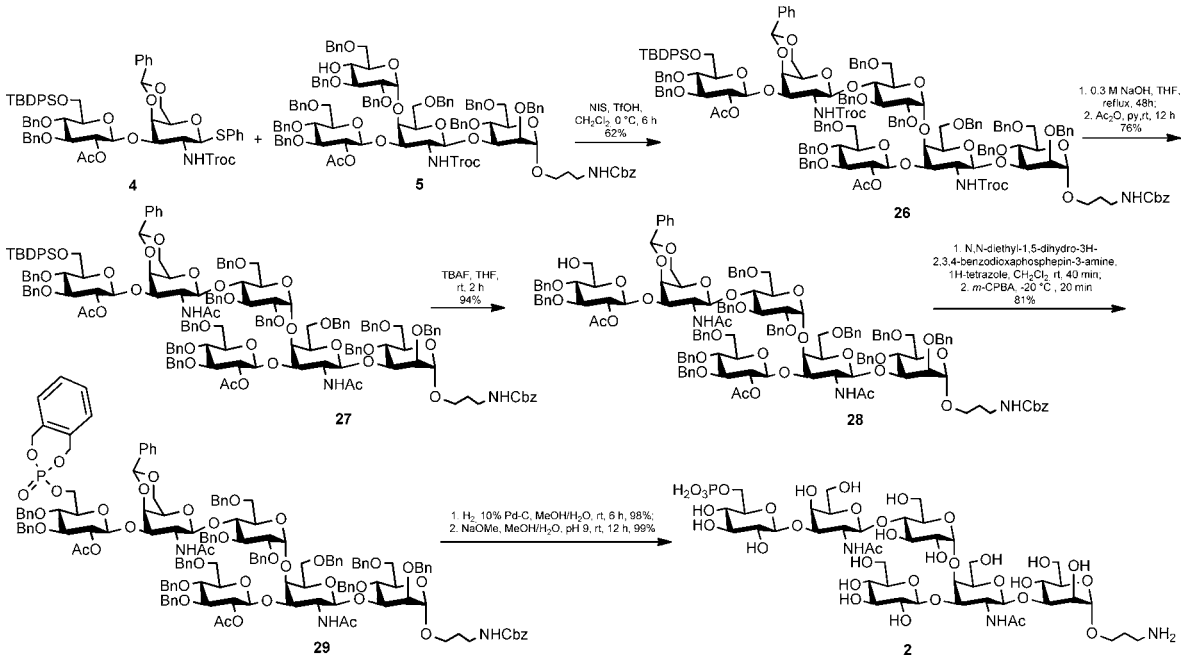
Thioglycoside **3** was used as a donor for glycosylation of the acceptor **9** promoted by NIS-TfOH, giving trisaccharide **21** in 77% yield (Scheme 3).

Compound **21** was subjected to regioselective opening of the benzylidene acetal by borane−trimethylamine complex and $BF_3{\cdot}Et_2O$[21] to directly furnish the trisaccharide acceptor **22** (80% yield). Gratifyingly, the glycosylation of **22** with ethylthioglycoside **8**[22] in toluene−dioxane using NIS−TfOH as promoters permitted the stereoselective introduction of the α-linkage and provided tetrasaccharide **23** in 89% yield. The α configuration of the newly formed glycosidic bond was confirmed in $^1$H NMR spectrum ($CDCl_3$, 400 MHz) by a doublet appearing at 5.11 ppm corresponding to H-1$^D$ with $J_{1,2}$ = 2.3 Hz (see the Supporting Information). A second regioselective ring-opening step provided efficiently tetrasaccharide acceptor **5** in 95% yield. Hexasaccharide **24**, leading to the nonphosphorylated analogue of PS-II repeating unit, was then completed by glycosylation of the tetrasaccharide **5** with the disaccharide donor **3** in moderate 50% yield (Scheme 3). Conversion of the Troc group into acetamide was carried out by basic hydrolysis, which led to concomitant removal of the *O*-acetyl esters, followed by *N*-acetylation.

**Scheme 3.** Assembling of the Hexasaccharide **1**



**Scheme 4.** Assembly of the Hexasaccharide **2**




Hydrogenation of hexasaccharide intermediate **25** by flow chemistry, utilizing a 10% Pd−C cartridge, gave the first target molecule **1**.

Since charged groups are often important epitopes for bacterial polysaccharides, the role of the phosphate group occurring in the PS-II repeating unit is an important issue to be addressed.

Accordingly, the synthesis of phosphorylated hexasaccharide **2** was approached by glycosylation of tetrasaccharide acceptor **5** with disaccharide donor **4** in 62% yield (Scheme 4).

After Troc group removal with 0.3 M NaOH from hexasaccharide **26**, the foregoing oligosaccharide was acetylated with acetic anhydride−pyridine to give **27**.

Removal of the silyl protection by means of tetrabutylammonium fluoride (TBAF) afforded hexasaccharide **28**, suitable for the phosphate group introduction with *N*,*N*-diethyl-1,5-dihydro-3*H*-2,3,4-benzodioxaphosphepin-3-amine and 1*H*-tetrazole, followed by oxidation with *m*-chloroperbenzoic acid (*m*-CPBA),[23] furnishing hexasaccharide **29** in 81% yield. A sharp peak in the $^{31}$P NMR spectrum ($CDCl_3$, 162 MHz) at −0.36 ppm showed the introduction of the protected phosphate, which was confirmed by ESI MS (see the Supporting Information). Final deprotection was performed in nearly quantitative yield by hydrogenation using flow chemistry followed by mild Zemplen transesterification of the acetyl esters. The structures of the purified hexasaccharides **1** and **2** were consistent with the native PS-II repeating unit,[12] the main difference being the presence of the linker connected to the anomeric position of the mannosyl residue in the synthetic molecules (see the Supporting Information).

In conclusion, the first synthesis of the *C. difficile* PS-II hexasaccharide repeating unit has been successfully achieved. In order to study the role of the phosphate group, both the natural phosphate-containing hexasaccharide repeating unit and its nonphosphorylated counterpart have been synthesized. These compounds bear an α-*O*-linked aminopropyl spacer at the anomeric position of the reducing end to allow their conjugation to a carrier protein. Ongoing immunological studies on the glycoconjugates prepared from the synthesized molecules and the native polysaccharide will provide information about the feasibility of a carbohydrate-based vaccine against *C. difficile*.

**Acknowledgment.** We gratefully acknowledge Dr. A. Vivi from NMR Center, University of Siena, for recording the 600 MHz NMR spectra.

**Supporting Information Available:** Experimental procedures and spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

OL1026188

(14) The moderate yield of **11** is due to the formation of variable amounts of the orthoester which, besides rearranging to the desired product **11**, was partially hydrolyzed during the reaction course.

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