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# Progress toward developing a carbohydrate-conjugate vaccine against *Clostridium difficile* ribotype 027: synthesis of the cell-surface polysaccharide PS-I repeating unit†‡

**Christopher E. Martin,[ab] Markus W. Weishaupt[ab] and Peter H. Seeberger*[ab]**

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***Clostridium difficile* strain ribotype 027 is a hypervirulent pathogen that is responsible for recent, severe outbreaks of serious nosocomial infections. As a foundation for the development of a preventative carbohydrate-based vaccine, we have synthesized a pentasaccharide cell wall repeating unit from PS-I unique to this strain, by the linear assembly of four monosaccharide building blocks.**

*Clostridium difficile* is a Gram-positive, spore-forming anaerobic bacterium that colonizes the intestinal tract of humans thus leading to *C. difficile* infections (CDI).[1] CDI have become the most commonly diagnosed cause of hospital-acquired diarrhea, particularly in risk groups including the elderly and immunodeficient patients, as well as those receiving antibiotic treatment. A steep rise in the incidence of CDI over the past decade is attributed to the emergence of the hypervirulent, and now predominant strain ribotype 027 that causes epidemic outbreaks with increased morbidity, mortality and high rates of relapse.[2] These infections significantly increase the treatment costs of patients, particularly in the case of recurring CDI.[3] Prevention of CDI is therefore desirable, and vaccination of risk groups may be a useful and cost-efficient means to avoid future infections. Although models have shown that vaccination against *C. difficile* should be economically feasible,[4] a vaccine has not yet been developed.

Carbohydrates exposed on the cell-surface of pathogens are often immunogenic and constitute potential candidates for vaccine development. When covalently connected to a carrier protein, a carbohydrate antigen can elicit long lasting, T-cell-dependent protection.[5] The chemical structures of two *C. difficile* cell-surface polysaccharides, PS-I and PS-II were recently elucidated[6] Initially, the focus was directed towards the PS-II hexasaccharide repeating unit antigen[7] because it is common to several *C. difficile* strains. PS-I consists of a pentasaccharide phosphate repeating unit with the structure [→ 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] and, so far, has been found to be expressed by *C. difficile* ribotype 027 only.[6] The branched pentasaccharide repeating unit **1** of PS-I is shown in Fig. 1. Units A and B are 1 → 2 linked α-Glc residues, wherein A is equipped with the aminopentyl linker at the reducing terminus, and B constitutes the branching point. B connects to α-Rha residue D at *C*-3 and β-Glc residue C at *C*-4. Finally, residue D′ is the terminal α-Rha, linked to *C*-3 of C.

Here we report the first total synthesis of pentasaccharide **1**, the synthetic challenge was met by devising a linear strategy using a set of building blocks **2–7** (Fig. 1). This linear strategy allowed for the installation of the challenging 1,2-*cis* glycosidic linkages of both residues A and B early in the synthetic pathway by using building blocks **2** and **3**. Formation of the linkage between C and the AB fragment was investigated using three different glycosylating agents of C, namely thioglycoside

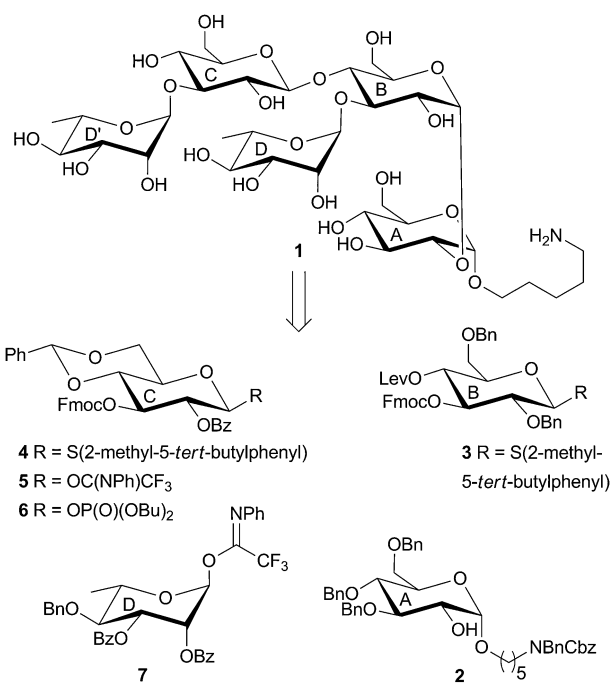


**Fig. 1** Retrosynthesis of the PS-I pentasaccharide repeating unit **1**.

[a] *Max Planck Institute of Colloids and Interfaces, Department of Biomolecular Systems, Am Mühlenberg 1, 14476 Potsdam, Germany. E-mail: peter.seeberger@mpikg.mpg.de*
[b] *Freie Universität Berlin, Institute for Chemistry and Biochemistry, Arnimallee 22, 14195 Berlin, Germany*

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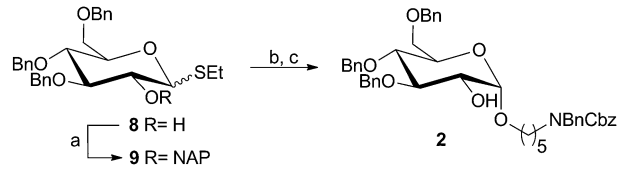
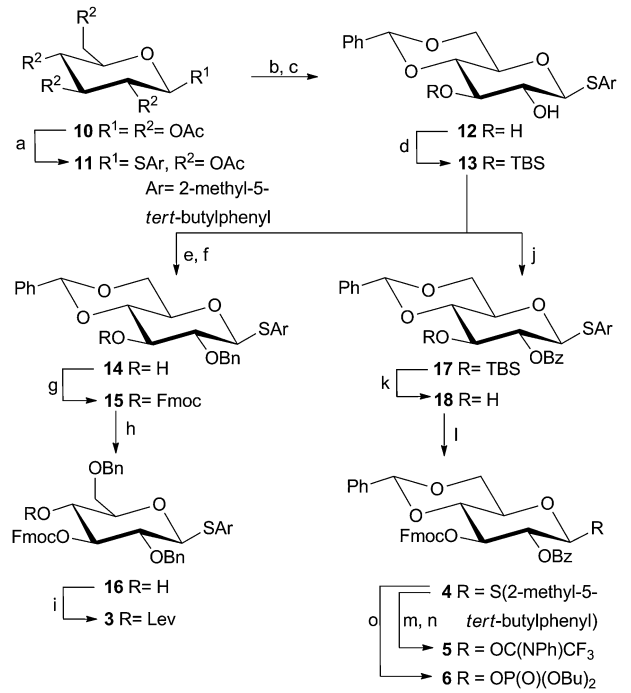



**Scheme 1** Synthesis of building block **2**. Reagents and conditions: (a) NaH, NAPBr, DMF, 0 °C to rt, 92%; (b) $HO(CH_2)_5NBnCbz$, NIS, TfOH, toluene/dioxane, −40 °C to −20 °C; (c) DDQ, DCM, $H_2O$, 35% over 2 steps.

**4**, *N*-phenyl trifluoroacetimidate **5** and glycosyl phosphate **6**. In order to minimize the number of deprotection and glycosylation steps, both Rha residues D and D′ were added to the intermediate trisaccharide ABC in a single bis-glycosylation reaction with building block **7**.

The synthesis of building block **2** is depicted in Scheme 1. The latent amine at the reducing end was introduced by union of thioglucoside **9** and the linker.[8] Thereby, 2-naphthylmethyl (NAP) served as a non-participating temporary protecting group at *C*-2 in order to achieve formation of the α-glycosidic linkage. Subsequent 3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) mediated cleavage of the NAP ether resulted in building block **2**.[9]

All four building blocks **3–6** originate from the key intermediate **13** (Scheme 2). This common precursor is synthesized in four steps from glucose pentaacetate **10**.[10] We found that use of the nontoxic and odorless 2-methyl-5-*tert*-butylthiophenol group[11] ensures exclusive β-anomer formation of thioglucosides and results in storage-stable monomer units. The adaptable thiol group is readily converted to phosphates or imidates, thus allowing creation of the series of glycosylating agents **3–6**. The protecting group pattern of **13** enables the orthogonal protection of all hydroxyl groups of the hexose ring. This feature was exploited in the synthesis of building block **3**, where a non-participating benzyl group was installed at *C*-2 of intermediate **14** to favor the formation of the α-glycosidic linkage between the A and B fragments. Subsequent placement of the 3-*O*-Fmoc-protection furnished compound **15**. Finally, regioselective opening of the 4,6-*O*-benzylidene acetal with TES–TfOH and protection of the free 4-hydroxyl gave orthogonally-protected building block **3**.

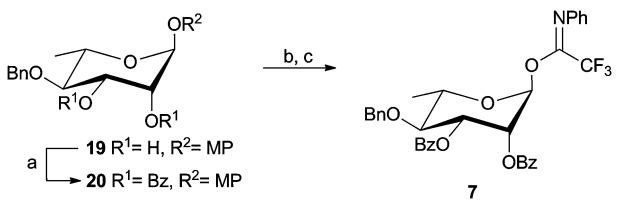



**Scheme 2** Synthesis of monosaccharide building blocks **3–6**. Reagents and conditions: (a) 2-methyl-5-*tert*-butylthiophenol, $BF_3{\cdot}OEt_2$, DCM, 85%; (b) NaOMe, MeOH, rt; (c) benzaldehyde dimethyl acetal, CSA, MeCN, 87% over 2 steps; (d) TBS-Cl, imidazole, DMF, 0 °C, 69%; (e) NaH, BnBr, DMF, 0 °C to rt; (f) 1 M TBAF in THF, 0 °C to rt, 93% over 2 steps; (g) Fmoc-Cl, pyridine, DCM, 95%; (h) TES, TfOH, DCM, 4 Å MS, −78 °C, 73%; (i) $Lev_2O$, pyridine, DCM, 3 days, 79%; (j) BzCl, DMAP, pyridine, 70 °C, 88%; (k) TBAF·$3H_2O$, AcOH, DMF, 35 °C, 91%; (l) Fmoc-Cl, pyridine, DCM, 96%; (m) NIS, AgOTf, TTBP, MeCN, $H_2O$; (n) $CF_3C(NPh)Cl$, $Cs_2CO_3$, DCM, 67% over 2 steps; (o) $HOPO(OBu)_2$, NIS/TfOH, DCM, 4 Å MS, 0 °C, 81%.

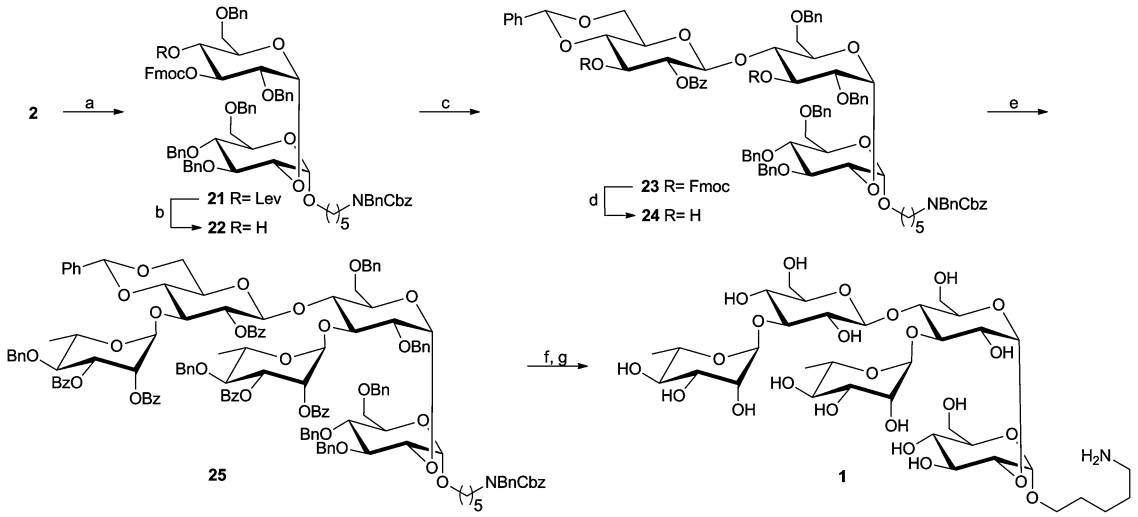
Preparation of differentially-protected thioglycoside **4** from **13** followed a similar route. In anticipation of the formation of a 1,2-*trans* linkage between the B and C saccharide fragments, a participating benzoyl group was installed at the *C*-2 hydroxyl of intermediate **17**. During TBAF-mediated desilylation of **17**, careful control of the TBAF:AcOH ratio was essential to prevent benzoyl-migration from *C*-2 to *C*-3. Fmoc-protected thioglycoside **4** was further diversified to glycosyl imidate **5** and glycosyl phosphate **6**. Building blocks **4–6** were individually evaluated for their reactivity towards the AB disaccharide fragment in assembly of the ABC trisaccharide. The terminal D and D′ fragments of the pentasaccharide were provided by building block **7**. Synthesis of this rhamnosyl unit commenced with the bis-benzoylation of *p*-methoxyphenyl glycoside **19** (Scheme 3).[12]

CAN-mediated removal of the anomeric *p*-methoxyphenyl group yielded the free lactol that was immediately converted into rhamnosyl *N*-phenyl trifluoroacetimidate **7**.[13]

Assembly of the pentasaccharide target was achieved in seven linear steps by combining the monosaccharide building blocks in sequence (Scheme 4). The 1,2-*cis* glycosidic linkage between residues A and B was formed using nucleophile **2** and glycosylating agent **3**. Disaccharide **21** was obtained in good yield and stereoselectivity when NIS and TfOH in $Et_2O$ was employed as promoter system. Selective cleavage of the levulinic ester with hydrazine hydrate in pyridine/AcOH, did not compromise the integrity of the Fmoc-group but cleanly produced disaccharide acceptor **22**. Installation of the next glycosidic linkage between C and the AB fragment to form trisaccharide **23** was attempted using the different building blocks **4–6** and the three reactions were screened for their




**Scheme 3** Synthesis of rhamnosyl building block **7**. Reagents and conditions: (a) BzCl, DMAP, pyridine, DCM, 0 °C to rt, 97%; (b) CAN, MeCN, $H_2O$; (c) $CF_3C(NPh)Cl$, $Cs_2CO_3$, DCM, 74% over 2 steps.




**Scheme 4** Synthesis of **1**. Reagents and conditions: (a) **3**, NIS/TfOH, $Et_2O$, −35 °C to −10 °C, 70%; (b) $N_2H_4{\cdot}H_2O$, AcOH/pyridine, DCM, 94%; (c) **6**, TMSOTf, DCM, 4 Å MS, −35 °C to −7 °C; (d) $NEt_3$, DCM, rt, 38% over 2 steps; (e) **7**, TMSOTf, DCM, 4 Å MS, −30 °C to −15 °C, 81%; (f) NaOMe, THF/MeOH, 50 °C; (g) $H_2$, 10% Pd/C, MeOH, $H_2O$, AcOH, 61% over 2 steps.

efficiency. Use of thioglycoside **4** and *N*-phenyl trifluoroacetimidate **5** resulted in the formation of only traces of the desired product. However, glycosyl phosphate **6** proved to be a superior glycosylating agent for the synthesis of **23**, although purification was achieved only following Fmoc cleavage to yield **24**. Finally, conversion of diol **24** to fully protected pentasaccharide **25** was achieved by a single bis-glycosylation using rhamnosyl-imidate **7** in the presence of TMSOTf to add both rhamnose residues. Final deprotection of **25** required two transformations: saponification of the benzoate esters and catalytic hydrogenation of the aromatic groups gave pentasaccharide **1**.

Comparison of NMR data of synthetic pentasaccharide **1** and native PS-I[6] showed overall good agreement, chemical shifts of signals corresponding to residues B, C and D were nearly identical with those reported. Deviations were observed for terminal sugars A and D′ due to the phosphate linkages at *C*-1 of A and *C*-4 of D′ in the native repeating units that were not present in the synthetic structure (see ESI for more detail†).

In summary, the first synthesis of the *C. difficile* cell-surface PS-I pentasaccharide repeating unit **1** was achieved employing a linear strategy based on six building blocks **2–7**. Glycosyl phosphate **6** proved to be a significantly better glycosylating agent than identically protected thioglycoside **4** or glycosyl imidate **5**. The terminal amine linker serves as an attachment point for immobilization to microarray surfaces, or for conjugation to carrier proteins. Immunization analysis of synthetic oligosaccharide glycoconjugates is currently underway.

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