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Ruijgrok, G.; Wu, D.; Overkleeft, H.S.; Codee, J.D.C.

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Synthesis and application of bacterial exopolysaccharides

Gijs Ruijgrok, Dung-Yeh Wu, Herman S. Overkleeft and Jeroen D. C. Codée

Abstract

Exopolysaccharides are produced and excreted by bacteria in the generation of biofilms to provide a protective environment. These polysaccharides are generally generated as heterogeneous polymers of varying length, featuring diverse substitution patterns. To obtain well-defined fragments of these polysaccharides, organic synthesis often is the method of choice, as it allows for full control over chain length and the installation of a pre-determined substitution pattern. This review presents several recent syntheses of exopolysaccharide fragments of *Pseudomonas aeruginosa* and *Staphylococcus aureus* and illustrates how these have been used to study biosynthesis enzymes and generate synthetic glycoconjugate vaccines.

Addresses

Leiden Institute of Chemistry, Leiden University, Einsteinweg 55, 2333, CC Leiden, the Netherlands

Corresponding author: Codée, Jeroen D.C. (jcodee@chem.leidenuniv.nl)

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Keywords

Glycosylation, Biofilm, Stereoselectivity, Polysaccharides.

Introduction

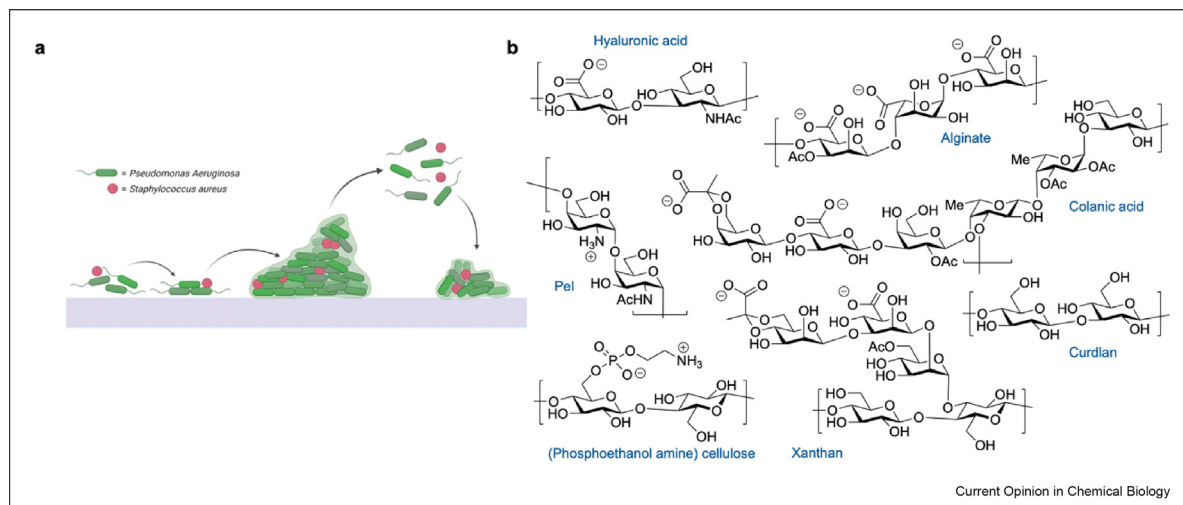
Using one of the first-ever microscopes, Antonie van Leeuwenhoek discovered bacterial biofilms in dental plaque, reporting on these in a 1684 communication to the Royal Society of London: “The number of these animalcules in the scurf of a man’s teeth are so many that I believe they exceed the number of men in a kingdom.” By the attachment to surfaces, bacteria, or animalcules as Van Leeuwenhoek termed them, survive through the

generation of a protective environment, the biofilm, in which bacterial colonies are encased by an extracellular polymeric matrix ([Figure 1](#)) [1]. Here, bacteria reside in a protective living milieu, protecting them from dehydration and adversaries such as the immune system of their host and providing resistance against mechanical stress [2,3]. Biofilms play a profound role in human health as they sustain bacterial infections. They protect bacteria from antibiotics [4,5] and enable bacteria to survive on medical devices such as catheters, ventilators and prosthetic joints. The extracellular biofilm matrix contains proteins, extracellular DNA (eDNA) and exopolysaccharides (EPS) [6,7]. The exopolysaccharides play important roles in the attachment to surfaces, scaffolding the biofilm matrix, and the retention of water and ions. Depending on growth conditions and needs, different exopolysaccharides may be expressed and polysaccharides may be chemically modified [8,9].

The vast majority of bacteria produce biofilms, and the structures of the exopolysaccharides in these biofilms are incredibly diverse [10]. This review describes some recent chemical syntheses of well-defined fragments of exopolysaccharides that have been employed in biochemical and immunological studies to showcase the applicability of these fragments. Key challenges in the assembly of bacterial EPS fragments are the rare monosaccharide constituents, the *cis*-glycosidic linkages and the presence of (labile) non-stoichiometric functional groups, such as acetyl esters (See [Figure 1a](#) for examples) [11,12]. The generation of long fragments is confronted by the lower reactivity of large oligosaccharide building blocks, challenges associated with the structural characterization of large complex molecules and difficulties with the final deprotection steps, as a large amount of protecting groups have to be removed simultaneously.

The ESKAPE pathogens, *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter*, are highly infectious antibiotic-resistant pathogens, that pose a great threat to human health. Here we primarily focus on *P. aeruginosa* and *S. aureus*, which are responsible for most health care-associated infections. Both pathogens have been shown to form polymicrobial biofilms. The studies

Figure 1



a) Biofilm formation. Planktonic bacteria adhere to surfaces and establish cell-cell contacts. Production of exopolysaccharides is a key step in the generation of a mature biofilm, from which bacteria can be liberated in the dispersion stage to start another colony. **b)** Selected examples of exopolysaccharides are shown, highlighting the structural variation present in these polysaccharides.

illustrate well how well-defined synthetic polysaccharide fragments can be used in epitope mapping studies and semi-synthetic vaccines and to probe the biosynthetic enzymes [13–15]. Understanding enzymatic biomachinery at the molecular level opens up avenues to interfere with their assembly and thus offers new avenues for therapeutic intervention. For example, different approaches have been reported to use the bacterial hydrolases, involved in the biosynthesis of the exopolysaccharides, to degrade the biofilms and sensitize the bacterial colonies for antibiotic treatment [16*,17].

Synthetic biofilm polysaccharide fragments

Pseudomonas aeruginosa

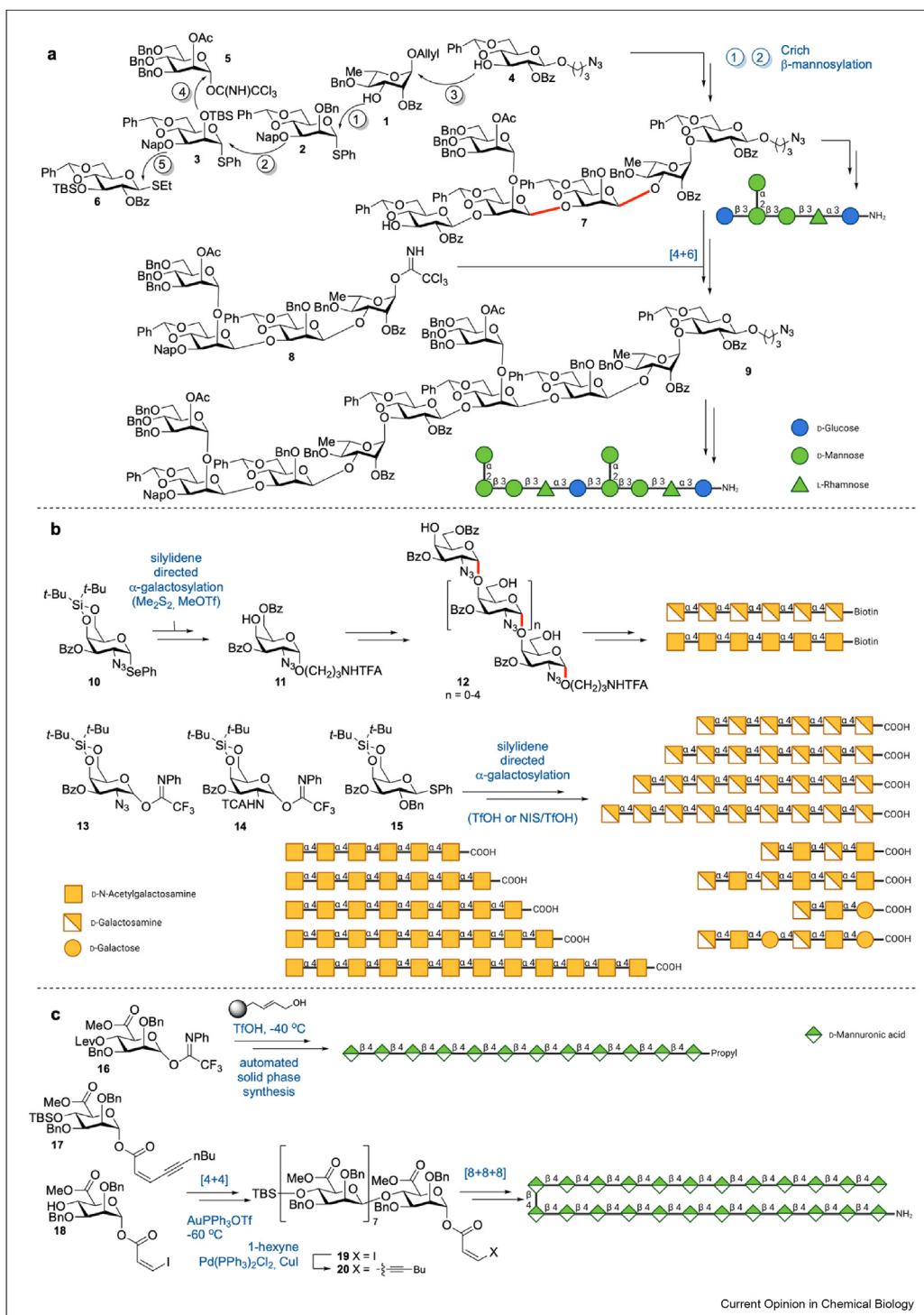
Pseudomonas aeruginosa is a life-threatening bacterium that mainly infects patients with an impaired immune system, patients suffering from burn wounds and patients with cystic fibrosis [18]. It is a Gram-negative bacterium that produces three distinct exopolysaccharides: alginate, Psl and Pel. Each of these exopolysaccharides has a different role and are found in different stages during infection and biofilm formation [19–22]. Psl is a neutral oligosaccharide which consists of a repeating pentameric saccharide, which is built up from D-mannose, L-rhamnose and D-glucose [21]. Besides its function as a biofilm backbone, it acts as a ‘molecular glue’, promoting attachment to cells [23,24], and it can function as a trail for bacterial exploration and microcolony formation [25]. Psl also influences the expression of cyclic-di-GMP, which serves as an activator for exopolysaccharide biosynthesis [26].

Pel is a positively charged polymer built up from dimeric repeats of α -1,4-linked galactosamine and *N*-acetylgalactosamine [27*]. The positive charge enables cell-cell contacts, the adherence to surfaces, crosslinking of negatively charged polymers such as alginate and extracellular DNA (eDNA) through ionic interactions [28] and helps in protecting the bacteria from cationic antimicrobials, such as aminoglycoside antibiotics [29].

Alginate is an anionic polymer, composed of β -1,4-linked D-mannuronic and α -L-guluronic acids [30]. The C-2 and C-3 hydroxy groups of the mannuronic acid residues can be acetylated to a varying degree, and these esters have been shown to play a role in initial surface colonization [31]. Alginate is a major constituent of mature *P. aeruginosa* biofilms and transitions to a mucoid phenotype in the lungs of cystic fibrosis (CF) patients, playing a major role in chronic infections [32].

Well-defined synthetic fragments of exopolysaccharides have been used as synthetic antigens to probe the interaction with antibodies and to interrogate biosynthesis enzymes. Figure 2a shows the building blocks used by Boons and co-workers to assemble a Psl deca-saccharide [33]. Building on the Crich β -mannosylation chemistry [34*], employing benzylidene-protected mannosyl building blocks, the critical *cis*-mannosidic linkages were constructed. Of note, the presence of the bulky C-2-*O*-TBS ether in **3** — required as an orthogonal protecting group to enable regioselective unmasking and attachment of the α -mannose appendages — posed no significant problem in forging the sterically demanding β -mannosidic linkage. The deca-saccharide was obtained in

Figure 2



Recent syntheses of *P. aeruginosa* exopolysaccharides. **a)** Assembly of a Psl-decasaccharide by Boons and co-workers, used for antigen mapping studies. The challenging β -mannosyl linkages were constructed using Crich β -mannosylation chemistry. **b)** Assembly of Pel and GAG oligosaccharides hinges on the use of di-*tert*-butyl silyl-protected galactosamine building blocks to ensure the stereoselective construction of the α -galactosyl linkages. **c)** Mannuronic acid donors have allowed for the synthesis of large alginate structures. The excellent stereoselectivity of the mannuronic acid donors has allowed for the reliable installation of the β -mannuronic acid bonds both in an automated solid phase approach and using large oligosaccharide building blocks.

a [4 + 6] coupling strategy. The deca-saccharide and constituting tetramer and hexamer fragments were used to probe the binding of three different monoclonal antibodies, identified using single-chain variable fragment phage libraries, derived from antibodies originating from healthy donors and patients recovering from a *Pseudomonas* infection: WapR-001, WapR-016 and Cam-003, the latter of which has shown highest opsonophagocytic activity [35**]. WapR-001 recognized all sequences, while WapR-016 only bound the hexasaccharide, indicating that the natural polysaccharide could terminate in this sequence. The Cam-003 mAb did not show binding to any of the synthetic fragments, which may be explained by the fact that it targets either a conformational epitope, not present in the synthetic fragments, or that other structural elements are required. Indeed, when the natural polysaccharide is treated with mild alkali binding with Cam-003 is abolished, pointing to the presence of esters in (parts of) the natural Psl.

Figure 2b shows the synthetic endeavors undertaken to assemble Pel structures [36*,37*,38,39]. The key challenge in the synthesis of Pel fragments is the stereoselective installation of the α -1,4-linkages. Around the same time, Kazakova et al. [36] and Zhang et al. [37] reported a similar strategy to generate libraries of Pel structures. Both groups employed Kiso's di-*tert*-butylsilylidene (DTBS) galactosylation methodology [40**], to achieve the stereoselective construction of the glycosidic linkages. This was combined with a regioselective protection step of the 4,6-diol, liberated by the removal of the DTBS group after the glycosylation reactions. Kazakova et al. [36] achieved the synthesis of a GalNAc and GalN hexasaccharide, whereas Zhang et al. [37] generated structures up to the dodecasaccharide level as well as structures featuring alternating GalN and GalNAc monosaccharides, that were introduced by the combination of azide and trichloroacetyl-protected GalN building blocks. In addition, structures were generated featuring α -1,4-galactose residues, as these constitute the galactosaminogalactan (GAG) polysaccharide of the invasive pathogenic fungus *Aspergillus fumigatus* [41]. The hexasaccharides of Kazakova et al. were used to determine the GAG epitopes recognized by anti-GAG antibodies in sera of patients with allergic bronchopulmonary (ABPA) or chronic pulmonary aspergillosis (CPA), showing that the level of antibodies that recognized the GalNAc and GalN hexamers was significantly higher in the blood of aspergillosis patients, with the acetylated fragments binding most antibodies. The larger and alternating Pel structures were used to interrogate different biosynthesis enzymes. It was found that the *A. fumigatus* glycosyl hydrolases Sph3 and Ega3 are both endoglycosidases and that Sph3 requires longer (>7 monosaccharides in length) substrates and cleaves GalNAc linkages while Ega3 degrades GalN bonds [42,43]. Using the synthetic fragments, the activity of

Agd3, the *A. fumigatus* esterase, was characterized revealing that this metal-dependent hydrolase also requires large (>6 residues) substrates [44].

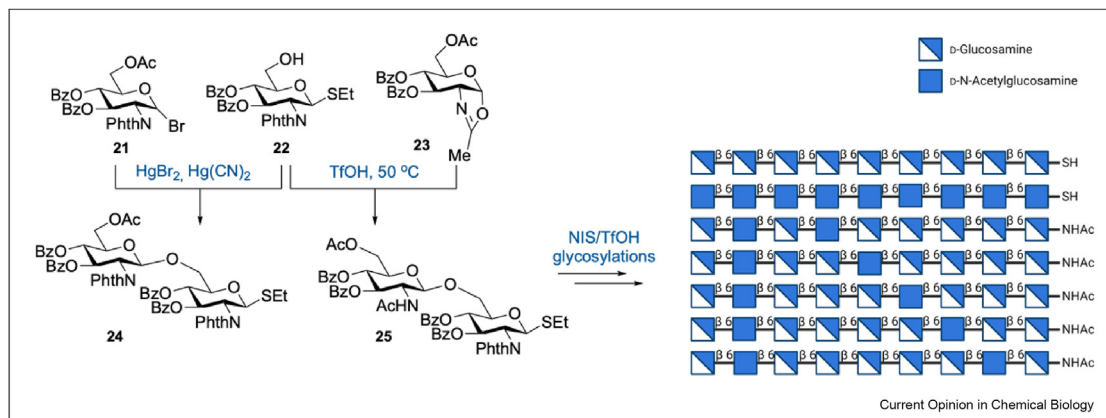
Figure 2c summarizes synthetic efforts undertaken to generate well-defined alginate fragments. Building on the β -selectivity of mannuronic acid (ManA) donors, several approaches have been reported to generate mannuronic acid alginates [45**,46,47]. Using an automated solid phase synthesis approach alginates up to the dodecamer length have been assembled using *N*-phenyltrifluoroacetamide donor **16**, showcasing the high reliability in constructing the β -mannuronic acid linkages [48*]. More recently block couplings have been performed to generate a 24-mer through a [8 + 8+8] approach. For this latter approach, an active-latent strategy was devised in which latent *Z*-(3)-iodoacrylate synthons could be transformed to a *Z*-ynenoate donor, through a Sonogashira coupling reaction [49*]. The donors could effectively be activated using an excess of a gold(I) activator. The latter synthetic alginates have been used for epitope mapping studies and as probes for biosynthesis enzymes. Quite strikingly, in a preliminary study, in which mice were immunized with inactivated *P. aeruginosa* and Freund's adjuvant, ELISA analysis of the pooled sera showed that a small ManA-tetrasaccharide was recognized best by the raised antibodies. These results will have to be corroborated in follow-up studies.

The assembly of alginate by *P. aeruginosa* is achieved using an ensemble of 10 enzymes, that build and export the polysaccharides [21]. A poly-ManA is assembled by Alg8 using GDP-ManA donors, and while the polymer traverses through the periplasmic space, the polymers can be epimerized (by AlgG) and acetylated (by the concerted action of AlgI, J, F and X). A lyase (AlgL) can cleave the polymeric fragments. Using the synthetic fragments, AlgX has been identified as the enzyme that transfers the acetyl ester to the growing polymers [50,51]. Through a combination of X-ray studies, activity assays with active site mutants, docking efforts and mass spectrometry-based affinity assays the mode of action of the epimerase AlgG has been elucidated at the atomic level [52]. An electropositive groove in the AlgG enzyme can accommodate a ManA 9-mer to properly align the ManA residue destined for epimerization, which happens in a processive fashion to epimerize every other ManA residue to generate repetitive [ManA-GulA] dimer blocks.

Poly N-acetylglucosamine (PNAG)

Poly- β -1,6-N-acetylglucosamine (PNAG) is produced by various bacteria and fungi [53]. Partially deacetylated (dPNAG) is the major exopolysaccharide present in biofilms of *S. aureus*, *Bacillus subtilis*, *E. coli* and *A. baumannii* [54,55]. The positively charged amine groups are essential for cell-surface attachment, bacterial virulence

Figure 3



Various PNAG oligosaccharides have been assembled to unravel binding interactions with (monoclonal) antibodies.

and protection against positively charged antimicrobial peptides and proteins [56]. Inhibitors of the polysaccharide deacetylase have been forwarded as potential therapeutic agents, but potent inhibitors have not been disclosed yet [57]. Anti-PNAG antibodies are part of the natural antibody repertoire of humans and animals, but these generally offer little immune protection, while it has been shown that antibodies capable of recognizing dPNAG are capable of complement activation and can be opsonic [58**]. A human monoclonal antibody F598, isolated from an individual recovered from an invasive *S. aureus* infection, is in development for passive immune protection [59]. Using synthetic PNAG fragments, the binding mode of the antibody and PNAG was studied, revealing the binding cleft formed by the heavy and light chains to accommodate a (d)PNAG stretch of at least five monosaccharides in length.[60*] Molecular modeling showed that a PNAG polysaccharide spanning 40 monosaccharides can bind both fab-fragments of the antibody.

Various syntheses of short (d)PNAG have been reported, and Figure 2 presents the assembly of a set of dPNAG nonsaccharides, as developed by Nifantiev and co-workers. They have used building blocks having either an *N*-acetyl group or an *N*-phthaloyl group to discriminate between the acetamides and free amino groups in the end products. Thioglycoside building blocks were combined in chemoselective glycosylation reactions with either oxazolidinone or bromide donors to generate larger building blocks, that were then used to assemble the set of PNAG fragments shown in Figure 3 [60,61]. These nonsaccharides have, amongst others, been used to generate a glycoconjugate vaccine modality using tetanus toxoid (TT) as a carrier protein [62,63*]. This model vaccine was used to elicit mouse and rabbit

antibodies that were shown to be capable of inducing killing of several *S. aureus* and *E. coli* strains.

Conclusion

Most bacteria survive in a biofilm habitat, in which polysaccharides play essential structural and functional roles. These polysaccharides have been an inspiration for the development of novel synthetic methodologies to access well-defined fragments of these polysaccharides. Innovative glycosylation methodology has been used to construct the challenging glycosidic linkages encountered in these glycans and effective strategies have been devised to generate relatively long fragments, including convergent active-latent block couplings and automated solid phase approaches. The synthetic glycans have been key to unravel the mode of action of biofilm polysaccharide biosynthesis enzymes as well as the generation of vaccine modalities. With more effective chemistry continuously being developed to assemble rare monosaccharide building blocks and our increasing knowledge of glycosylation chemistry the future will see the generation of longer and more complex structures. Libraries of oligosaccharides with varying substitution patterns will be generated for epitope mapping and receptor and enzyme interaction studies. Well-defined, large oligomers may be used in the generation of structures comprising several biosynthesis enzymes, where the oligomers can function as a scaffold for the different proteins. The development of biosynthesis inhibitors, inspired by the polysaccharide structure will open up new avenues for therapeutic development. With numerous biofilm polysaccharides not being targeted yet, there seem to be many opportunities for synthetic chemistry to deliver powerful molecular tools and make a significant contribution to

the exploration and exploitation of bacterial biofilm biosynthesis machinery.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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- ** of outstanding interest

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