Inhibitors and probes targeting PslG

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CHAPTER 1

Introduction

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterium that mainly infects immunocompromised individuals, in particular patients suffering from cystic fibrosis, cancer, urinary tract infections, burn wounds and COPD.[1,2] *P. aeruginosa* is a major cause of nosocomial infections, causing both acute, life-threatening infections and chronic infections. *P. aeruginosa* is capable of forming protective biofilms further complicating the problem of infection.[3] *P. aeruginosa* is one of the high priority multi-drug resistant ESKAPE pathogens, *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter*. Treatment of *P. aeruginosa* is difficult due to its environmental adaptability, owing to its metabolic versatility, its capacity to form biofilms, the different mechanisms of antibiotic resistance it employs and the variation of virulence factors it produces.[1,2,4] *P. aeruginosa* biofilms consist of proteins, extracellular DNA (eDNA), lipids and exo-polysaccharides (Figure 1) including three distinct exo-polysaccharides: Pel, alginate, and Psl. The expression of the type(s) of exo-polysaccharide is subject to the stage of infection and biofilm formation.[5]

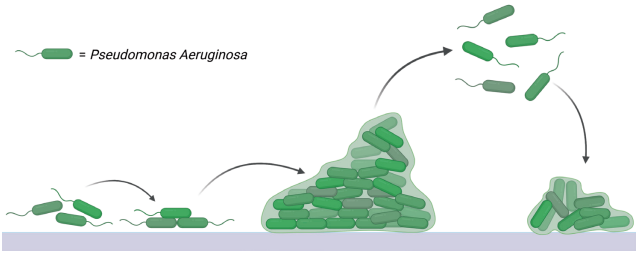

Figure 1. Biofilm formation. Initially bacteria adhere to a surface. Subsequently, a biofilm is formed which protects the bacteria from the environment.

Alginate is an anionic polymer composed of β-D-mannuronic acid and α-L-guluronic acid residues linked in a 1,2-cis fashion (Figure 2). The D-mannuronic acid residues are acetylated at either the C-2 or C-3 position to a varying degree. Alginate is mainly found in patients with cystic fibrosis. Early evidence suggests *P. aeruginosa* produces little alginate in these patients, while larger amounts are produced in later stages of the infection.[6] In this way the bacteria transition to a mucoid phenotype, from a non-mucoid phenotype, which leads to chronic infections.[5,7] Having alginate in their biofilm protects the bacteria from dehydration, antibiotics, disinfectants and the host immune system.

The biosynthetic machinery to produce alginate is the best studied among the three exopolysaccharides formed by *P. aeruginosa*, and is composed of 13 gene products, *algD844KEGXLIJFA* and *algC*.[8,9] Based on experimental results and gene sequence analysis most enzymatic functions have been determined or postulated.[8,10] AlgA, AlgC and AlgD are involved in the synthesis of the monosaccharide building block, GDP-D-mannuronate from fructose-6-phosphate. This precursor can then be polymerized by mannuronosyl transferase, Alg8. AlgI, AlgJ, AlgF and AlgX acetylate the formed polymannuronic acid polymers, while AlgG epimerizes some non-acetylated D-mannuronic acid residues to L-guluronic acid residues. AlgL is a polysaccharide lyase located in the periplasm that is able to cleave the alginate polymers through a β-elimination reaction of the non-acetylated mannuronic acid residues. AlgL is responsible for clearing accumulated alginate to maintain homeostasis in the bacterial cell periplasm.[11] AlgK and AlgE are involved in the secretion of the produced polymer, with AlgK thought to play a role in the localization of AlgE, which then transports the alginate polymer through the outer membrane. Lastly, Alg44 initiates Alg8-mediated polymannuronic acid biosynthesis in a c-di-GMP-dependent manner.[8,10]

To date, several groups have developed synthetic methodologies to produce pure, well-defined alginate fragments.[12–15] These fragments can be used to study interactions with the bio-machinery enzymes to unravel their mode of action at the molecular level, which may pave the way to new treatment options by inhibiting the biosynthesis enzymes, and also to generate antibodies or determine the binding epitopes of anti-bodies obtained from recovering patients.

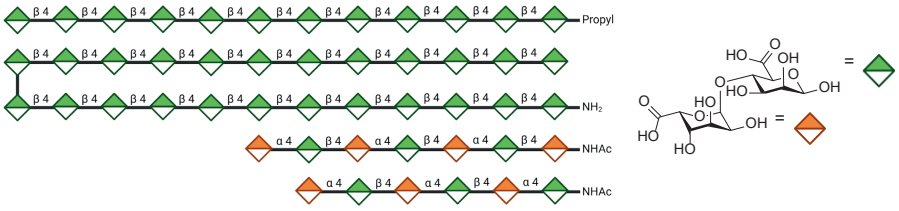

Figure 2. Several synthetic alginate oligosaccharides, which have been used to study biological functions of alginate. Moreover, they can be used to generate antibodies against alginate or to determine existing binding epitopes of anti-bodies. So far, there have been no fragments synthesized with a varying acetylation pattern.[14–17]

The exact structure of Pel has only recently (2022) been fully elucidated. Pel is a polymer containing galactosamine (GalN) and *N*-acetylgalactosamine (GalNAc) residues linked in an α-1,4 fashion.[16] While the Pel structure is predominantly composed of [GalN-GalNAc] dimer repeating units, it contains more galactosamine residues than *N*-acetylgalactosamine residues. The GalN residues provide the Pel polymer with its cationic character.[16] This allows for many interactions, including ones with negatively charged biofilm components such as extracellular DNA (eDNA). Pel is involved in initiation of surface attachment, biofilm integrity and antibiotic resistance, specifically against cationic antibiotics.[5,7,17]

Pel is produced by enzymes encoded from seven genes which are termed *pelA-G*. This operon is conserved in other bacterial species, implying that Pel is present in biofilms produced by other bacterial species as well.[8] Based on experimental analysis and bioinformatics data, a model for Pel biosynthesis has been proposed.[8,18] Among the *pelA-G* genes there is no postulated enzyme that is involved in the production of a Pel precursor suggesting that Pel is synthesized using carbohydrate-nucleotide donors also used in other metabolic processes. PelF is the only predicted glycosyltransferase within the Pel operon and also the only enzyme predicted to be in the periplasm. PelF, PelD, PelE and PelG form a complex which has been termed the Pel polysaccharide synthase complex.[19] In this complex, PelF polymerizes UDP-GalNac, and the resulting polymer is then transported across the inner cell membrane by the combined action of PelD, PelE, and PelG. PelD is able to bind c-di-GMP and, like with Alg44, polymer synthesis is halted when there is no binding with c-di-GMP, suggesting a regulatory role.[19] PelB is partly located in the periplasm and partly in the outer membrane. The section located in the periplasm interacts with PelA which partially de-acetylates the poly-GalNAc polymers.[20] PelA is an enzyme with a dual mode of action: it acts as a deacetylase as well as a glycoside hydrolase, and interactions between PelA and PelB increases the deacetylase activity while decreasing the hydrolysis activity.[20] Excretion of the thus formed Pel polymers then occurs through the combined action of PelC and PelB.[8,21,22]

Despite the fact that the exact structure of Pel has only recently been elucidated, several synthetic oligosaccharides haven been synthesized that resemble Pel (Figure 3).[23–25] It has to be noted, that some of these synthtetic oligosaccharides have been used to investigate galactosaminogalactans (GAGs), an exopolysaccharide produced by the pathogenic fungus *Aspergillus fumigatus*. These synthetic fragments can aid further studies into the role of Pel and the functions of the Pel-biosynthesis enzymes, as well as to determine antibody binding epitopes and to generate antibodies against Pel.[23–25]

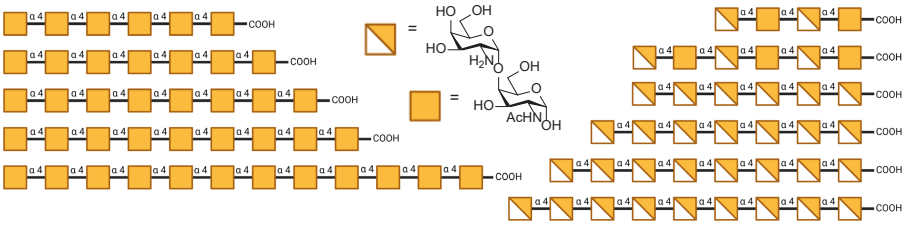

Figure 3. Well-defined synthetic fragments that resemble parts of Pel polysaccharides. The synthetic oligosaccharides are composed of only GalN, only GalNAc, or of an alternating pattern of these two monosaccharides.[21–23] While a dimeric repeat of GalN-GalNAc is considered to be the repeating dimer unit, the natural polysaccharide contains more GalN than GalNAc.

Psl is a neutral oligosaccharide which consists of a repeating pentameric saccharide, which contains D-mannose, L-rhamnose and D-glucose (Figure 4).[26,27] Psl has several biological functions: it acts as a biofilm backbone, as a 'molecular glue' promoting attachment to cells,[28,29] and as a trail for bacterial exploration and microcolony formation.[30] Psl also influences the expression of c-di-GMP, an important messenger molecule that upregulates biofilm production.[5,31] Lastly, it shields the bacteria from the host immune system and antibiotics.[32,33]

The biosynthesis of Psl is effected by proteins which are encoded on the Psl operon, which includes a total of 12 genes, termed *pslA-L*.[34–36] The Psl pathway has not been fully elucidated yet. However a model has been proposed, based on evidence that the Psl biosynthesis machinery resembles a isoprenoid lipid-based biosynthesis mechanism of *E. coli* employed for group 1 capsular and extracellular polysaccharides.[8] PslB is the only encoded enzyme that is involved in carbohydrate nucleotide precursor production. It is responsible for forming GDP-mannose from fructose-6-phosphate (similar to AlgA) and gene deletion studies have found that *pslB* is not required for the biosynthesis of Psl. This indicates that its function can be overtaken by enzymes from central carbohydrate-nucleotide precursor synthesis. PslF, PslH, PslI and PslC are all postulated glycosyl transferases, this also corresponds to the number of different

glycosidic linkages present in Psl (Figure 4). PslA, PslE, PslJ, PslK, and PslL all have inner membrane spanning domains. Based on sequence analysis, PslL is suggested to have an acyl transferase domain, but its role in Psl synthesis remains unknown. PslD is the only enzyme located in the outer membrane, however it needs PslE in order to localize in the outer membrane. Despite the location of PslD in the outer membrane it is still not known how exactly Psl is exported through the outer membrane since PslD appears to lack functional domains to do so. It has been postulated that the Psl export is an isoprenoid lipid carrier dependent mechanism, where PslA is responsible for assembling the Psl repeating unit on an isoprenoid lipid.[8,37,38] Lastly, PslG is a glycoside hydrolase mainly localized in the inner membrane, where it is thought to have a similar function as PelA and AlgL. Later studies have revealed that PslG is not necessary for the production of Psl, although this is still under debate.[27,38–41]

Psl fragments have been chemically synthesized (Figure 4) to investigate binding of monoclonal antibodies, first by Li *et al*. (2013, up to a decamer) and later (2020, up to a pentamer) by Demeter *et al*.[42,43] During epitope mapping in the study from Li *et al*. it was noticed that one anti-body did not bind any synthetic fragment, indicating that there still might be some unelucidated post translational modification on Psl.

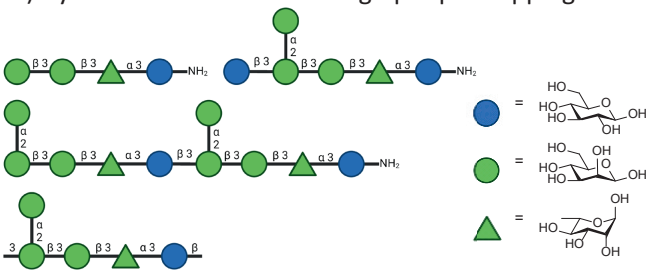

Figure 4. The chemically synthesized Psl fragments. Li *et al*. synthesized up to the decamer and also made smaller fragments, a tetra-, penta-, hexasaccharide, whereas Demeter *et al*. synthesized the pentasaccharide.[32,33]

Numerous studies have been conducted with the aim to unravel the role of PslG, but the function of this enzyme remains enigmatic.[27,38–41] Several studies have shown that exogenous addition of PslG leads to higher antibiotic susceptibility of *P. aeruginosa*[39,40,44] but it has also been shown that genetic deletion of PslG reduces the total amount of produced Psl by 80%.[38,41] PslG is mainly localized in the inner membrane and small amounts of PslG are found in the periplasm where it is thought to degrade periplasmic, and potentially toxic, Psl.[38,40] More recently an in-depth analysis of PslG has led to several new observations. Lack of PslG was shown to reduce initial attachment, increase microcolony formation, generate a higher roughness of the biofilm, less Psl network in the mature biofilms and a higher chance of increased c-di-GMP levels in daughter cells after division.[41] On

the molecular level, the exact mode of action of Psl hydrolysis by PslG also remains unknown. This Thesis were aimed to shed more light on this mechanism, with a particular focus on its substrate specificity. Since PslG is reported to act as a retaining endoglycosidase, activity-based protein profiling (ABPP) was considered a good starting point for this purpose. In the next sections, the general concept of ABPP and how this technique is used to probe retaining glycosidases is discussed, after which the research chapters within this Thesis are outlined.

Activity-based protein profiling

Activity-based protein profiling (ABPP) is a technique that allows the discovery and functional annotation of enzymes in complex biological samples, even in live cells. ABPP uses activity-based probes (ABPs), which consist of a warhead, a linker, and a reporter group (Figure 5). The warhead is generally an electrophile or binding element with a photoreactive group that forms a covalent and irreversible chemical bond with the enzyme (or enzyme family) of interest. After covalent binding, the reporter group is used to investigate the enzyme. The reporter group can be a biotin (for retrieval and proteomics identification of ABP-bound enzymes) or a fluorophore (for fluorescence detection of reacted enzymes). The reporter tag can also be an azide or alkyne, so that after binding of the warhead to the target, a biotin or fluorophore can be added using click chemistry.[45] There are many reported ABPs that are able to label individual enzymes and also entire enzyme families, sometimes even multiple classes of enzymes.[46] These classes of enzymes include serine hydrolases, cysteine proteases, metallohydrolases, kinases, nucleotide binding proteins, glycosidases and phosphatases.[46]

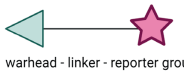

Figure 5. Typical components of an ABP.

ABPP can be performed in several different ways to investigate enzyme activities in complex biological samples (Figure 6).[46] In comparative ABPP methods an ABP is added to a complex biological sample, after which all proteins that have reacted with the ABP can be visualized on SDS PAGE gels or (when using a biotin-ABP) identified by pull-down – proteomics experiments.[46] Using this method active proteins can be identified under varying biological conditions. Usage of fluorescent ABPs allows even the tracing of protein movements inside living cells.[46,47] In competitive ABPP, inhibitors can be discovered and their selectivity determined. In such an experiment, first a biological sample is incubated with a candidate-inhibitor, or a library of such compounds. Subsequently, an ABP is

added after which the enzymes that were inhibited can be identified using in-gel fluorescence analysis or LC-MS/MS.

Glycoside hydrolases

Glycoside hydrolases (GHs) are a group of enzymes that hydrolyse glycosidic linkages. GHs can be divided in several subgroups based on the mechanism, exo- or endo specificity, on the chemical reaction that is catalysed or on their primary sequence. The latter categorization is used by the Cazy database where enzymes are divided in different glycoside hydrolase families based on their sequence similarity.[48]

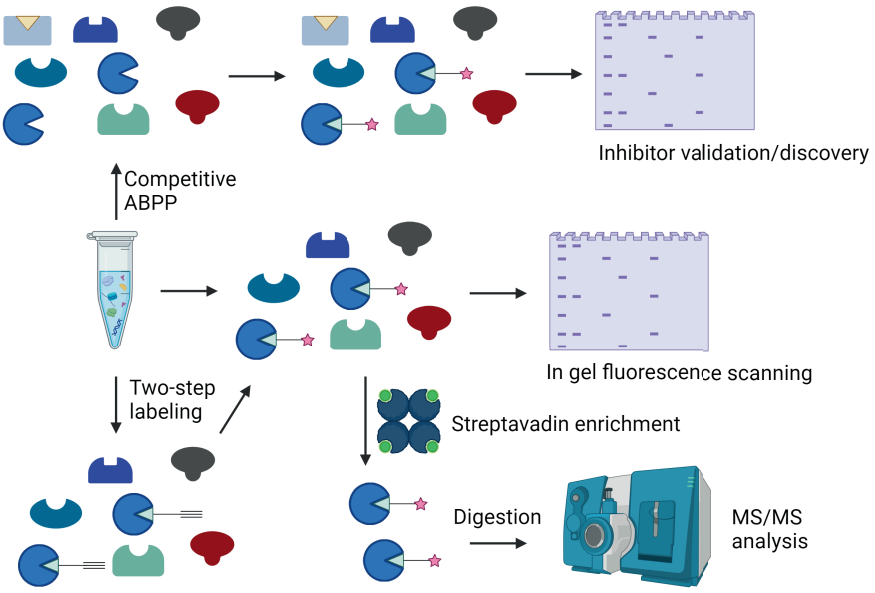

Figure 6. Schematic representation of ABPP experiments. In comparative ABPP, a biological sample is incubated with an ABP, so that proteins can be identified using gel fluorescence scanning or MS/MS analysis.[42] The latter experiments can also be done using a two-step labeling approach, where the functional tag is introduced using click chemistry after binding of the ABP to the target. In competitive ABPP a biological sample is incubated with a (potential) inhibitor (yellow triangle) and with subsequent addition of an ABP, inhibitors can be discovered or validated, and inhibitor specificity can be determined.

The two main types of mechanisms, retaining and inverting, both follow S_N2 mechanisms (Figure 7) as first postulated by Koshland in 1953.[49] Both retaining and inverting enzymes use a general acid/base residue for catalysis, and these residues are commonly a glutamic acid or aspartic acid. The inverting mechanism is initiated when the substrate enters the enzyme and a Michaelis-Menten complex is formed (Figure 7A). Then water enters the active site, and in a concerted manner, the enzyme deprotonates the water molecule while it attacks the anomeric position, with concomitant expulsion of the leaving group, which is protonated by the enzyme. This results in a transition state as shown in Figure 7A, and leads to the product with an inverted configuration of the anomeric centre with respect to the original substrate. The acid/base residue are optimally positioned and are ~9.5 Å apart to accommodate the substrate and the water molecule.[50] The retaining mechanism is different, with the two catalytic residues ~5 Å apart, leaving no room for a water molecule in addition to the substrate.[50] One of the two residues acts as a nucleophile while the other functions as the acid/base (Figure 7B). [50] After the substrate enters the active-site, the nucleophilic residue in the enzyme active site (most commonly an aspartate or glutamate, but others have also been identified)[48] attacks the anomeric centre, while the leaving group is protonated this leads to a covalent enzyme-substrate complex via transition state 1. The complex is then hydrolysed by a water molecule, in a subsequent S_N2-type substitution, resulting in the product with retention of the configuration at the anomeric position.

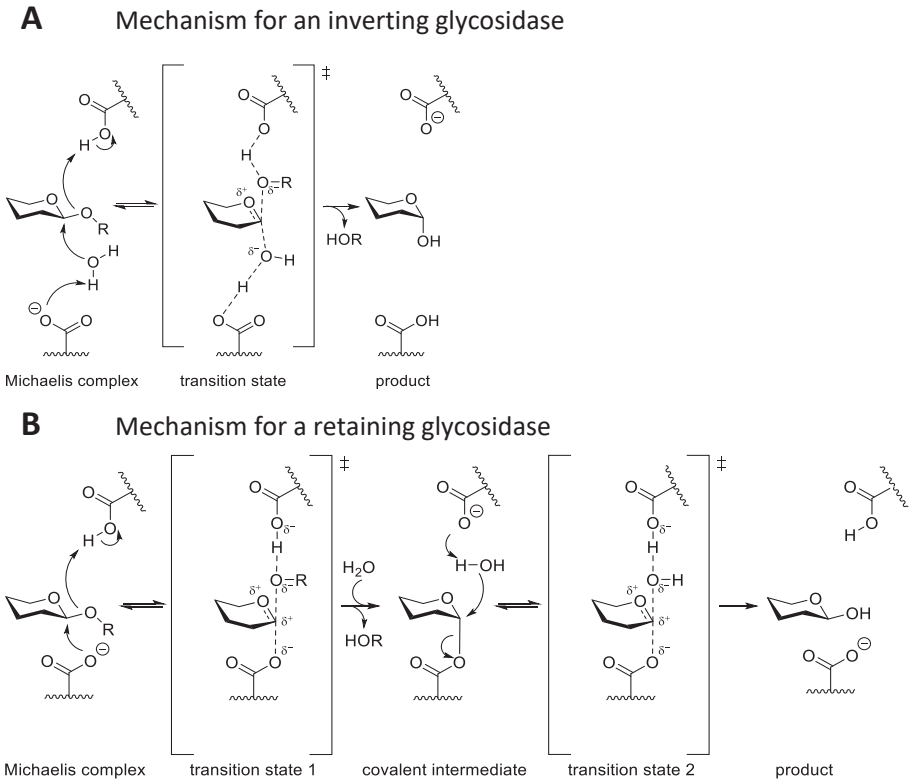

Figure 8. Mechanism of action of inverting (top) and retaining (bottom) glycosidases. Hydroxyls have been omitted for clarity.

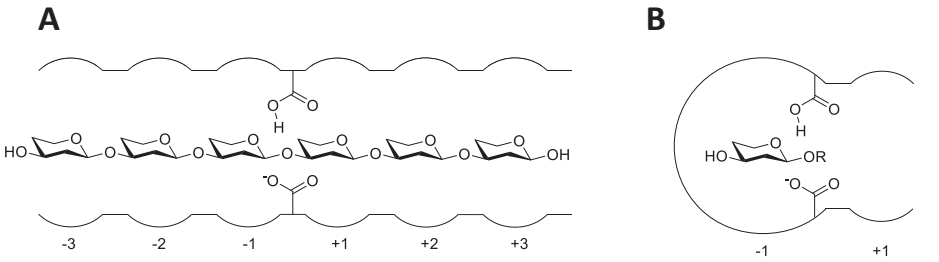

Figure 7. **A**) An endo-acting enzyme with the nomenclature subsite numbering. **B**) An exo-acting enzyme with the nomenclature subsite numbering. The subsite numbering is centered around the cleavage site in the substrate, the '-' subsites are towards the non-reducing end of the scissile bond and the '+' subsites are towards the reducing end of the scissile bond.

The endo/exo classification for glycosidases is based on the position where they cleave their substrate (Figure 8).[51] Endoglycosidases can cleave somewhere within the substrate oligosaccharide chain and usually requires multiple residues in their subsites for recognition (Figure 8A), whereas exoglycosidases cleave off terminal residues from their substrates (Figure 8B). While the active site of exoglycosidases is commonly pocket shaped, the active site of endoglycosidases forms a binding cleft.

Retaining glycosidases are excellent targets for ABPP due to the covalent intermediate they form with their substrate. This intermediate has inspired the design of mechanism-based inhibitors. One of the most used class of mechanism-based, covalent and irreversible retaining glycosidase inhibitors is based on the natural product, cyclophellitol (Figure 9A).[52] Cyclophellitol (**1**) is a β-D-glucopyranose mimic where the endocyclic oxygen is replaced with a carbon atom and an epoxide bridges the 1 and 7 position.[49] Epoxides are good electrophilic traps that react with the retaining glycosidase active site nucleophile to form a covalent and irreversible bond (Figure 9C).[54,55] Once the cyclophellitol-based compound enters the active site, the epoxide is protonated by the general acid-base active site residue and displaced by the active site nucleophile. This forms an enzyme-substrate ester, which compared to the acylal linkage that emerges during substrate processing is much more stable (lack of the endocyclic oxygen, preventing expulsion of the leaving group).[54] This mode of action makes cyclophellitol (**1**) an excellent starting point for the development of retaining glycosidase-targeting ABPs (Figure 9B). In earlier reports and

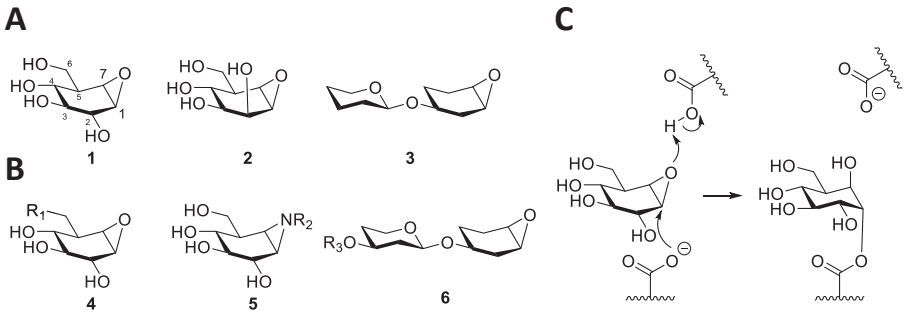

Figure 9. **A**) The structure of cyclophellitol (**1**), mannose-cyclophellitol (**2**) and an example of a disaccharide cyclophellitol (**3**). **B**) Examples of how cyclophellitol is transformed into an ABP. In **4** the primary alcohol is replaced with an azide for click chemistry, in **5** an aziridine allows for installation of a tag at the reducing-end of cyclophellitol, lastly a disaccharide (**6**) is elongated at the natural elongation position with a tag. **C**) the mechanism of action of cyclophellitol.

specifically for exo-acting glycosidases, the alcohol on the 6-position was exchanged with an azide in order to attach a fluorophore or biotin using click chemistry.[56] Later, utilizing an aziridine as warhead instead of an epoxide, the linker was attached on the aziridine, so that interactions with exo-acting enzymes are not disturbed at the non-reducing end.[57] Alternatively, cyclophellitol-like compounds can be derivatized with additional carbohydrates to target endoglycosidases.[58] Usually, the tag is positioned at the site where the saccharide would be elongated with a carbohydrate.

This technology was recently applied to study the mode of action of PslG.[59] PslG was postulated to have endo-mannosidase activity, where Psl was cleaved between the D-mannose and L-rhamnose residues (position 2 in Figure 10).[39] The study by de Boer[54] describes the construction of trisaccharide ABP **7** based on the Psl structure and with a D-mannose configured cyclophellitol warhead. Trisaccharide **7** was tested against purified recombinant PslG using ESI-MS, but no mechanism-based reaction could be observed. PslG was then crystallized and the crystals were soaked with probe **7**. Analysis of the crystal structure showed non-covalent binding of the C8 linker in a hydrophobic pocket at the edge of the enzyme. This result suggested the annotation of the substrate activity of PslG to be wrong, and the work described in this Thesis aims to investigate whether an alternative cleavage site is employed by this enzyme.

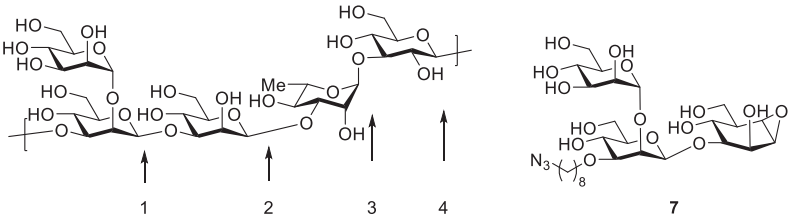

Figure 10. The glycosidic linkages that can possibly be cleaved by PslG (1-4), using probe **7** cleavage site 2 was investigated by de Boer.[54]

Thesis outline

In this Thesis a variety of oligosaccharide structures are synthesized, based on the Psl structure. Part of the oligosaccharides are functionalised with cyclophellitol or a derivative thereof, or with a deoxy-nojirimycin. These synthetic oligosaccharides are used to unambiguously establish the glycosidase specificity of PslG. In **Chapter 2** three ABPs are synthesized. All three ABPs are pseudo-trisaccharides based on the Psl motif and contain a cyclophellitol derivative as warhead. The tag is positioned on the alcohol of the C-3 position at the non-

reducing end. All three probes are tested against PslG in order to determine its preferred cleavage site in Psl. In **Chapter 3** a Psl-pentasaccharide and -decasaccharide are synthesized, to serve as a substrate for PslG to unequivocally determine the position where Psl is cleaved by PslG. By treating the Psl fragments with PslG and subsequent mass analysis of the fragments the cleavage site is determined. In **Chapter 4** three inhibitors are synthesized based on the cleavage site of PslG in Psl, with the size of the inhibitors ranging from a trisaccharide up to a pentasaccharide. The inhibitors are tested against PslG and used for crystallization experiments to further investigate the catalytic site. In **Chapter 5** two putative competitive PslG inhibitors are synthesized, namely a trisaccharidic and pentasaccharidic deoxynojirimycin derivative with the aim to deliver competitive and non-covalent inhibitors for the enzyme. **Chapter 6** summarizes the work performed in Chapters 2-5 and provides an outlook on potential future research directions.

References

1. Botelho, J., Grosso, F. & Peixe, L. Antibiotic resistance in *Pseudomonas aeruginosa* – mechanisms, epidemiology and evolution. *Drug Resist. Updat.* **44**, 100640 (2019).
2. Qin, S. *et al. Pseudomonas aeruginosa*: pathogenesis, virulence factors, antibiotic resistance, interaction with host, technology advances and emerging therapeutics. *Signal Transduct. Target. Ther.* **7**, 1–27 (2022).
3. O'Toole, G., Kaplan, H. B. & Kolter, R. Biofilm formation as microbial development. *Annu. Rev. Microbiol.* **54**, 49–79 (2000).
4. Jurado-Martín, I., Sainz-Mejías, M. & McClean, S. *Pseudomonas aeruginosa*: an audacious pathogen with an adaptable arsenal of virulence factors. *Int. J. Mol. Sci.* **22**, 3128 (2021).
5. Ma, L. Z., Wang, D., Liu, Y., Zhang, Z. & Wozniak, D. J. Regulation of biofilm exopolysaccharide biosynthesis and degradation in *Pseudomonas aeruginosa*. *Annu. Rev. Microbiol.* **76**, 413–433 (2022).
6. Nivens, D. E., Ohman, D. E., Williams, J. & Franklin, M. J. Role of alginate and its o acetylation in formation of *Pseudomonas aeruginosa* microcolonies and biofilms. *J. Bacteriol.* **183**, 1047–1057 (2001).
7. Thi, M. T. T., Wibowo, D. & Rehm, B. H. A. *Pseudomonas aeruginosa* biofilms. *Int. J. Mol. Sci.* **21**, 8671 (2020).
8. Franklin, M., Nivens, D., Weadge, J. & Howell, P. Biosynthesis of the *Pseudomonas aeruginosa* extracellular polysaccharides, alginate, Pel, and Psl. *Front. Microbiol.* **2**, (2011).

9. Ramsey, D. M. & Wozniak, D. J. Understanding the control of *Pseudomonas aeruginosa* alginate synthesis and the prospects for management of chronic infections in cystic fibrosis. *Mol. Microbiol.* **56**, 309–322 (2005).
10. Gheorghita, A. A. *et al.* Structure of the AlgKX modification and secretion complex required for alginate production and biofilm attachment in *Pseudomonas aeruginosa*. *Nat. Commun.* **13**, 7631 (2022).
11. Gheorghita, A. A. *et al.* The *Pseudomonas aeruginosa* homeostasis enzyme AlgL clears the periplasmic space of accumulated alginate during polymer biosynthesis. *J. Biol. Chem.* **298**, (2022).
12. Zhang, Q. *et al.* Acceptor reactivity in the total synthesis of alginate fragments containing α-L-guluronic acid and β-D-mannuronic acid. *Angew. Chem. Int. Ed.* **54**, 7670–7673 (2015).
13. Chi, F.-C., Kulkarni, S. S., Zulueta, M. M. L. & Hung, S.-C. Synthesis of alginate oligosaccharides containing L-guluronic acids. *Chem. – Asian J.* **4**, 386–390 (2009).
14. Walvoort, M. T. C. *et al.* Automated solid-phase synthesis of β-mannuronic acid alginates. *Angew. Chem. Int. Ed.* **51**, 4393–4396 (2012).
15. Zhang, L. *et al.* Promoter-controlled synthesis and antigenic evaluation of mannuronic acid alginate glycans of *Pseudomonas aeruginosa*. *Org. Lett.* **24**, 8381–8386 (2022).
16. Le Mauff, F. *et al.* The Pel polysaccharide is predominantly composed of a dimeric repeat of α-1,4 linked galactosamine and N-acetylgalactosamine. *Commun. Biol.* **5**, 1–7 (2022).
17. Jennings, L. K. *et al.* Pel is a cationic exopolysaccharide that cross-links extracellular DNA in the *Pseudomonas aeruginosa* biofilm matrix. *Proc. Natl. Acad. Sci.* **112**, 11353–11358 (2015).
18. Whitfield, G. B. *et al.* Discovery and characterization of a Gram-positive Pel polysaccharide biosynthetic gene cluster. *PLOS Pathog.* **16**, e1008281 (2020).
19. Whitfield, G. B. *et al.* Pel polysaccharide biosynthesis requires an inner membrane complex comprised of PelD, PelE, PelF, and PelG. *J. Bacteriol.* **202**, e00684-19 (2020).
20. Marmont, L. S. *et al.* PelA and PelB proteins form a modification and secretion complex essential for Pel polysaccharide-dependent biofilm formation in *Pseudomonas aeruginosa*. *J. Biol. Chem.* **292**, 19411–19422 (2017).
21. Marmont, L. S. *et al.* Oligomeric lipoprotein PelC guides Pel polysaccharide export across the outer membrane of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci.* **114**, 2892–2897 (2017).
22. Colvin, K. M. *et al.* PelA deacetylase activity is required for Pel polysaccharide synthesis in *Pseudomonas aeruginosa*. *J. Bacteriol.* **195**, 2329–2339 (2013).
23. Kazakova, E. D. *et al.* Biotinylated oligo-α-(1 → 4)-D-galactosamines and their N-acetylated derivatives: α-stereoselective synthesis and immunology application. *J. Am. Chem. Soc.* **142**, 1175–1179 (2020).

24. Zhang, Y. *et al.* Synthesis and structural analysis of *Aspergillus fumigatus* galactosaminogalactans featuring α-galactose, α-galactosamine and α-N-acetyl galactosamine linkages. *Angew. Chem. Int. Ed.* **59**, 12746–12750 (2020).
25. Wang, L., Zhang, Y., Overkleeft, H. S., van der Marel, G. A. & Codée, J. D. C. Reagent controlled glycosylations for the assembly of well-defined Pel oligosaccharides. *J. Org. Chem.* **85**, 15872–15884 (2020).
26. Kocharova, N. A., Knirel, Y. A., Shashkov, A. S., Kochetkov, N. K. & Pier, G. B. Structure of an extracellular cross-reactive polysaccharide from *Pseudomonas aeruginosa* immunotype 4. *J. Biol. Chem.* **263**, 11291–11295 (1988).
27. Byrd, M. S. *et al.* Genetic and biochemical analyses of the *Pseudomonas aeruginosa* Psl exopolysaccharide reveal overlapping roles for polysaccharide synthesis enzymes in Psl and LPS production. *Mol. Microbiol.* **73**, 622–638 (2009).
28. Byrd, M. S., Pang, B., Mishra, M., Swords, W. E. & Wozniak, D. J. The *Pseudomonas aeruginosa* exopolysaccharide Psl facilitates surface adherence and NF-κB activation in A549 cells. *mBio* **1**, 10.1128/mbio.00140-10 (2010).
29. Ma, L., Jackson, K. D., Landry, R. M., Parsek, M. R. & Wozniak, D. J. Analysis of *Pseudomonas aeruginosa* conditional Psl variants reveals roles for the Psl polysaccharide in adhesion and maintaining biofilm structure postattachment. *J. Bacteriol.* **188**, 8213–8221 (2006).
30. Zhao, K. *et al.* Psl trails guide exploration and microcolony formation in *Pseudomonas aeruginosa* biofilms. *Nature* **497**, 388–391 (2013).
31. Irie, Y. *et al.* Self-produced exopolysaccharide is a signal that stimulates biofilm formation in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci.* **109**, 20632–20636 (2012).
32. Mishra, M. *et al. Pseudomonas aeruginosa* Psl polysaccharide reduces neutrophil phagocytosis and the oxidative response by limiting complement-mediated opsonization. *Cell. Microbiol.* **14**, 95–106 (2012).
33. Tseng, B. S. *et al.* The extracellular matrix protects *Pseudomonas aeruginosa* biofilms by limiting the penetration of tobramycin. *Environ. Microbiol.* **15**, 2865–2878 (2013).
34. Matsukawa, M. & Greenberg, E. P. Putative exopolysaccharide synthesis genes influence *Pseudomonas aeruginosa* biofilm development. *J. Bacteriol.* **186**, 4449–4456 (2004).
35. Jackson, K. D., Starkey, M., Kremer, S., Parsek, M. R. & Wozniak, D. J. Identification of psl, a locus encoding a potential exopolysaccharide that is essential for *Pseudomonas aeruginosa* PAO1 biofilm formation. *J. Bacteriol.* **186**, 4466–4475 (2004).
36. Friedman, L. & Kolter, R. Two genetic loci produce distinct carbohydrate-rich structural components of the *Pseudomonas aeruginosa* biofilm matrix. *J. Bacteriol.* **186**, 4457–4465 (2004).
37. Vandana & Das, S. Genetic regulation, biosynthesis and applications of extracellular polysaccharides of the biofilm matrix of bacteria. *Carbohydr. Polym.* **291**, 119536 (2022).

38. Wu, H., Wang, D., Tang, M. & Ma, L. Z. The advance of assembly of exopolysaccharide Psl biosynthesis machinery in *Pseudomonas aeruginosa*. *MicrobiologyOpen* **8**, e857 (2019).
39. Yu, S. *et al.* PslG, a self-produced glycosyl hydrolase, triggers biofilm disassembly by disrupting exopolysaccharide matrix. *Cell Res.* **25**, 1352–1367 (2015).
40. Baker, P. *et al.* Characterization of the *Pseudomonas aeruginosa* glycoside hydrolase PslG reveals that its levels are critical for Psl polysaccharide biosynthesis and biofilm formation. *J. Biol. Chem.* **290**, 28374–28387 (2015).
41. Zhang, J. *et al.* Intracellular glycosyl hydrolase PslG shapes bacterial cell fate, signalling, and the biofilm development of *Pseudomonas aeruginosa*. *eLife* **11**, e72778 (2022).
42. Li, H. *et al.* Epitope mapping of monoclonal antibodies using synthetic oligosaccharides uncovers novel aspects of immune recognition of the Psl exopolysaccharide of *Pseudomonas aeruginosa*. *Chem. – Eur. J.* **19**, 17425–17431 (2013).
43. Demeter, F., Chang, M. D.-T., Lee, Y.-C., Borbás, A. & Herczeg, M. An efficient synthesis of the pentasaccharide repeating unit of *Pseudomonas aeruginosa* Psl exopolysaccharide. *Synlett* **31**, 469–474 (2020).
44. Ostapska, H. *et al.* Preclinical evaluation of recombinant microbial glycoside hydrolases as antibiofilm agents in acute pulmonary *Pseudomonas aeruginosa* Infection. *Antimicrob. Agents Chemother.* **66**, e00052-22 (2022).
45. Kolb, H. C. & Sharpless, K. B. The growing impact of click chemistry on drug discovery. *Drug Discov. Today* **8**, 1128–1137 (2003).
46. Cravatt, B. F., Wright, A. T. & Kozarich, J. W. Activity-based protein profiling: from enzyme chemistry to proteomic chemistry. *Annu. Rev. Biochem.* **77**, 383–414 (2008).
47. Riera, R. *et al.* Single-molecule imaging of glycan–lectin interactions on cells with Glyco-PAINT. *Nat. Chem. Biol.* **17**, 1281–1288 (2021).
48. Drula, E. *et al.* The carbohydrate-active enzyme database: functions and literature. *Nucleic Acids Res.* **50**, D571–D577 (2022).
49. Koshland Jr., D. E. Stereochemistry and the mechanism of enzymatic reactions. *Biol. Rev.* **28**, 416–436 (1953).
50. McCarter, J. D. & Stephen Withers, G. Mechanisms of enzymatic glycoside hydrolysis. *Curr. Opin. Struct. Biol.* **4**, 885–892 (1994).
51. Davies, G. & Henrissat, B. Structures and mechanisms of glycosyl hydrolases. *Structure* **3**, 853–859 (1995).
52. Atsumi, S. *et al.* Production, isolation and structure determination of a novel β-glucosidase inhibitor, cyclophellitol, from Phellinus sp. *J. Antibiot. (Tokyo)* **43**, 49–53 (1990).
53. Hansen, F. G., Bundgaard, E. & Madsen, R. A short synthesis of (+)-cyclophellitol. *J. Org. Chem.* **70**, 10139–10142 (2005).
54. Gloster, T. M., Madsen, R. & Davies, G. J. Structural basis for cyclophellitol inhibition of a β-glucosidase. *Org. Biomol. Chem.* **5**, 444–446 (2007).

55. Willems, L. I. *et al.* From covalent glycosidase inhibitors to activity-based glycosidase probes. *Chem. – Eur. J.* **20**, 10864–10872 (2014).
55. Witte, M. D. *et al.* Ultrasensitive in situ visualization of active glucocerebrosidase molecules. *Nat. Chem. Biol.* **6**, 907–913 (2010).
57. Kallemeijn, W. W. *et al.* Novel activity-based probes for broad-spectrum profiling of retaining β-exoglucosidases in situ and in vivo. *Angew. Chem.* **124**, 12697–12701 (2012).
58. Boer, C. de *et al.* Glycosylated cyclophellitol-derived activity-based probes and inhibitors for cellulases. *RSC Chem. Biol.* **1**, 148–155 (2020).
59. de Boer, C. *Inhibitors and probes targeting endo-glycosidases*. (2021).