Chemical synthesis of fragments of galactosaminogalactan and pel polysaccharides

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Chapter 6

Summary and Future Prospects

The work described in this Thesis is focused on the assembly of oligosaccharide fragments derived from a fungal polysaccharide, galactosaminogalactan (GAG) and fragments of the exopolysaccharide Pel, generated by *Pseudomonas aeruginosa*. Both polysaccharides are characterized by the presence of α-galactosamine linkages and the occurrence of both *N*-acetyl galactosamine (GalNAc) and galactosamine (GalN) residues makes these complex linear polysaccharides polycationic. In addition, GAG-polysaccharides can contain α-galactose (Gal) residues, while the Pel polysaccharide can contain α-glucosamine (GlcN) and α-*N*-acetyl glucosamine (GlcNAc) monosaccharides. To assemble the corresponding oligosaccharides as effectively as possible, synthetic methodologies, enabling the stereoselective construction of the required *cis*-glycosidic linkages has to be developed. These synthetic fragments will be valuable tools to elucidate the biosynthesis of GAG and

Pel, and characterize the enzymes involved therein. These fragments may also enable avenues to generate potential vaccines.

Aspergillus fumigatus and *Pseudomonas aeruginosa* are biofilm-forming microorganisms, which complicates the treatment of their infections. The polysaccharides GAG and Pel both play important roles in biofilm formation and thus are potential targets in the development of anti-inflammatory therapies. In **Chapter 1** recent knowledge on the plausible biosynthetic pathways and the chemical syntheses of fragments of both polysaccharides are described. Key to the assembly of oligosaccharide fragments is the stereoselective introduction of α-GalN and α-GlcN linkages. An overview of the developed methods for stereoselective synthesis of α-galactosamine and α-glucosamine is described.

Synthesis of GAG oligosaccharides

Chapter 2, **3** and **4** describe the synthesis of GAG oligosaccharides, including homo- and hetero-oligosaccharides as well as an azido-GAG fragment. **Chapter 2** deals with the synthesis of GAG homo-oligomers up to 9- or 12-mers and composed of either Gal, GalN or GalNAc, moieties using effective synthetic methodology. The key feature of the strategy is a three-step chain-elongation cycle: 1) di-*tert*-butylsilylidene (DTBS)-directed α-galactosylation; 2) DTBS-removal with HF/pyridine; and 3) regioselective benzoylation of the primary alcohol group, using benzoyl-hydroxybenzotriazole (BzOBt) as a mild acylating agent. In the deprotection process, the homo-oligomers of Gal (8- and 9-mer) were unexpectedly found to have poor solubility in water, while the homo-oligomers of GalN and GalNAc both proved to be well soluble in water. To investigate the conformation and spatial presentation of the synthetic GAGs, their structural properties were studied by a combination of NMR and computational methods. The oligomers were shown to adopt an elongated, almost straight, structure, stabilized by inter-residue H-bonds, one of which is a non-conventional C-H⋯O hydrogen bond between H5 of the residue (i+1) and O3 of the residue (i). This is the first time that this type of non-conventional C-H⋯O HB is reported for linear oligosaccharide structures, which was revealed by a significant downfield chemical shift for the non-reducing-end H5 protons in the NMR spectra. The structures place the groups at C-2 to the outside of the structure and can readily interact with binding partners, such as biosynthesis enzymes and antibodies.

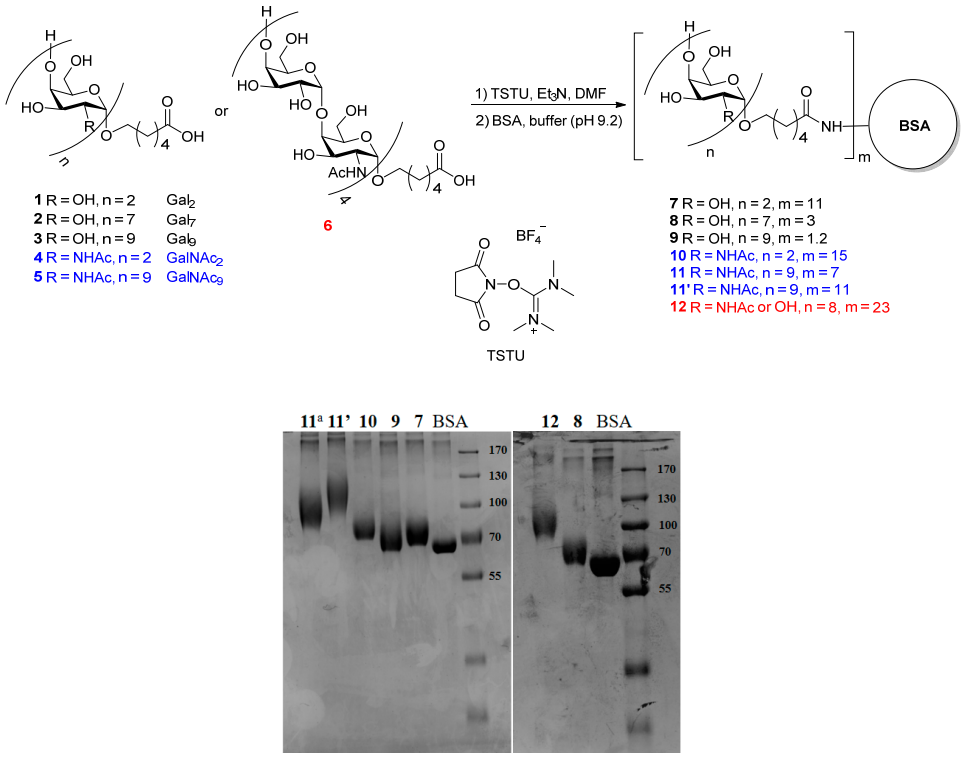
Chapter 3 reports the assembly of four sets of GAG hetero-oligomers, including the hetero-oligomers of α-GalN and α-GalNAc; hetero-oligomers of α-Gal, α-GalN and α-GalNAc; hetero-oligomers of α-Gal and α-GalN; hetero-oligomers of α-Gal and α-GalNAc. To enable the assembly of these hetero-oligosaccharides, the same methodology as described

in Chapter 2 was used. The Gal donor and $GalN_3$ donor served as precursors for Gal and GalN, respectively and a GalNTCA donor served as precursor for the GalNAc moieties. Even though the GalNTCA donor is equipped with a C-2-trichloroacetamide group, intrinsically capable of neighboring group participation, the α-selectivity of the glycosylations of this donor was excellent. Even with a reactive linker alcohol the selectivity was good (α:β=8:1) when the glycosylation was performed at 0 ºC, and lowering the temperature to -20 ºC further increased the selectivity to 14:1 (α:β ratio). The mixed sequence structures were produced uneventfully, showing the developed chemistry to be applicable to any GAG-target. Some of the synthetic fragments were applied for the investigations of the glycosidases Sph3, Ega3 and the *N*-acetyl hydrolase Agd3 involved in GAG biosynthesis. Treatment of GalNAc heptasaccharides with the hydrolase Sph3 resulted in the rapid hydrolysis and accumulation of pentasaccharides, while the hexamer could not be hydrolyzed, indicating that the minimum substrate size of Sph3 is seven and that the enzyme functions as an endo-acting glycoside hydrolase. The hydrolase Ega3 was found to be an endoglycosidase, degrading the non-acetylated α-1,4-$(GalN)_9$ into trisaccharide products. Furthermore, comparative deacetylation experiments with the deacetylase Agd3 suggested that Agd3 is specific for regions of the GAG polymer that are GalNAc/GalN rich and has higher affinity to partially deacetylated polymers.

In recent years, carbohydrate-based vaccines have been widely explored and identified as one of the most effective ways of preventing bacterial and fungal infections.[1-11] Conjugation of a saccharide antigen to a carrier protein converts the saccharide to a T-dependent antigen, increasing immunogenicity from infancy and enabling the development of immunological memory. Although most of the carbohydrate-based vaccines are produced from isolated polysaccharides, the use of synthetic oligosaccharides presents a promising alternative approach. Well-defined oligosaccharides allow more controlled conjugation chemistry compared to native polysaccharides and can be used to study detailed structure-activity relationships.

As depicted in Scheme 1, the synthetic GAG oligomers **1-6** have been successfully conjugated to the carrier protein bovine serum albumin (BSA). The hexanoic acid spacer was first converted to its *N*-hydroxysuccinimide (OSu) ester by using *N*,*N*,*N*',*N*'-tetramethyl-O-(*N*-succinimidyl)-uronium tetrafluoroborate (TSTU).[2] After removal of the solvent, the sugar-OSu esters were directly used without purification to react with the amino groups of BSA in a buffer solution The obtained BSA glycoconjugates were purified by filtration against sodium phosphate buffer. The conjugates were analyzed by SDS-PAGE and mass

spectrometry analysis to estimate the oligomer/BSA molar ratio. Immunization studies of these conjugates are currently ongoing.



Scheme 1: SDS-PAGE of GAG oligomers conjugated to BSA. a) 40 equivents of $(GalNAc)_9$ was used in conjugation reaction. b) 70 Equivents of oligomers were used for the other conjugation reactions.

To explore more details and the dynamics of GAG biosynthesis *in vivo*, the synthesis of an azido-GAG heptamer with a C-2-N_3 group at the non-reducing end is discussed in **Chapter 4**. The DTBS-directed α-galactosaminylation methodology was used to construct α-$GalN_3$ and α-GalNTFA linkages, again with excellent α-stereoselectivity. The reactivity of the used benzoylated GalNTFA donors and acceptors proved to be relatively low, giving moderate or low glycosylation yields. Increasing the concontration of the reaction from 0.05 M to 0.2 M greatly improved the yields of the coupling reactions. The assembled N_3-GAG heptamer is currently being evaluated for cell surface labeling of *A. fumigatus*. Sph3 is expected to have trans-glycosylase activity and may transfer the N_3-GAG to cell surface bound GAG polymers. The azide groups will then be used to visualize the labeled GAG polymers on the cell surface.

Synthesis of Pel heptasaccharides

Chapter 5 covers the synthesis of a library of Pel fragments, containing six α-GalN and α-GalNAc residues and one α-GlcN/GlcNAc moiety at different positions in the saccharide chain. First, a glycosylation study was conducted, using different $GlcN_3$ donors and $GalN_3$ acceptors for the formation of α-$GlcN_3$-(1→4)-$GalN_3$ linkages. Both the MPF-modulated glycosylation method, previously specifically developed to introduce α-glucosamine linkages, and a pre-activation strategy failed to effectively construct the desired 1,2-*cis* linkages. Also, a benzoyl group at the C6-OH of the acceptor did not prove to be beneficial for the wanted stereoselectivity. Thus, a DTBS protected $GlcN_3$ donor and a benzyl group for the protection of C-6-OH in GalN acceptors were chosen for the construction of α-$GlcN_3$-(1→4)-$GalN_3$ linkages. The Bn group was regioselectively introduced under the aegis of Taylor's borinic acid catalyst $Ph_2BO(CH_2)_2NH_2$. With the DTBS-protected $GlcN_3$, $GalN_3$ and GalNHTFA imidate donors and TfOH as promotor, the required 1,2-*cis* GalN and GlcN linkages were stereoselectively formed. Nevertheless, with the elongation of the chains, coupling reaction yields decreased significantly, owing to the low nucleophilicity of the acceptors. Fortunately, the yields of the glycosylation reactions towards the longer chains were optimized using a reverse-addition-sequence strategy, in which the acceptor was pre-mixed with the activator, prior to the addition of the donor glycoside. To generate the final compounds, six synthetic heptasaccharides were deprotected with four different strategies, giving three sets of heptamers: α-GlcN-α-GalN; α-GlcNAc-α-GalNAc and α-GlcN-α-GalNAc-α-GalN. The Bn groups were removed using a Birch reduction to avoid the reduction of C-C double bond in linker part. Unexpectedly, it was found difficult to cleave the TFA groups of fully-protected heptamers in the deprotection protocol. Starting the deprotection procedure with removal of the Bn groups by hydrogenolysis and the intermediate use of a Boc group, to mask the concommitantly released amino groups, the TFA groups could be effectively removed using ammonia hydroxide solution at 60 °C.

The synthetic Pel structures will allow for detailed structural studies by a combination of NMR and computational methods. Comparison of their structural properties with GAG oligomers can be done at the same time, as they only differ in a GlcN/GlcNAc moiety. What's more, these heptasaccharides will be valuable tools for the study of enzymes involved in Pel biosynthesis. Conjugation of heptamers with carrier proteins may produce carbohydrate-based vaccines, which will benefit the development of vaccines against *P. aeruginosa*.

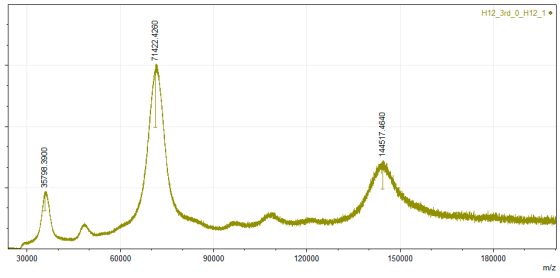
Experimental section

General procedure for conjugation reactions with BSA

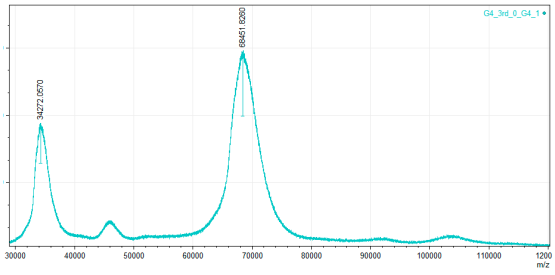
To the solution of carboxylic acid (1.0 eq) in dry DMF (0.01 M), TSTU (1.1 eq) and Et_3N (1.0 eq) were added, which was allowed to stir at rt for 2h. The reaction mixture was concentrated in vacuo and dissolved in butter ($Na_2B_4O_7$ and $NaHCO_3$, pH 9.2). BSA solution (10 mg/ml) was added to the NHS ester solution, which was allowed to stir for 2h at rt. The mixture was diluted to 4 mL with the buffer in a centrifugal filter (5 mL, 10K). After five minutes of centrifugation, the residue was diluted with 4 mL of the buffer. Repeating centrifugation and dilution for another 19 times. Then the BSA-conjugation solution was diluted with sodium phosphate buffer (NaH_2PO_4-Na_2HPO_4, 0.01M, pH 7.4) to 4 mL. After five minutes of centrifugation, the residue was diluted and centrifuged for another 9 times. The BSA-conjugation was finally diluted to 0.5 mg/mL with sodium phosphate buffer and kept at 4 °C.

MALDI-TOF data of BSA-conjugations 7-12:

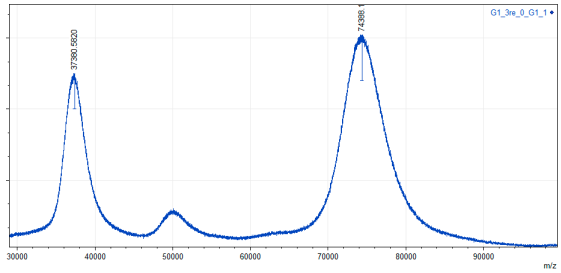
$(Gal_2)_{11}$-BSA **7**



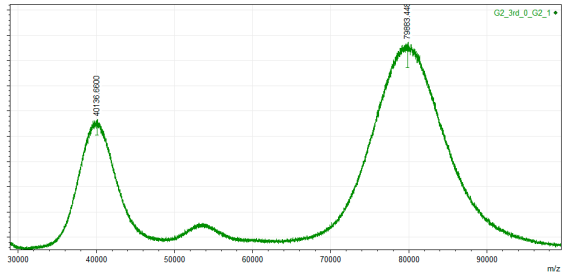
$(Gal_9)_{1.2}$-BSA **9**



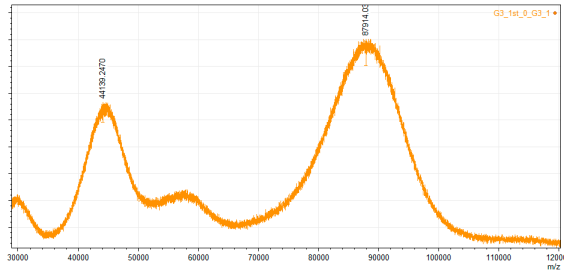
$(GalNAc_2)_{15}$-BSA **10**



$(GalNAc_9)_7$-BSA **11**



$(GalNAc_9)_{11}$-BSA **11'**



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