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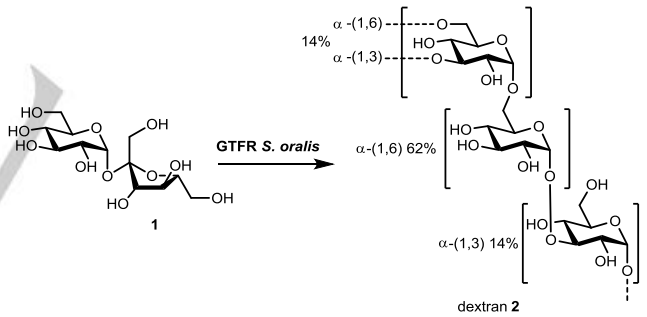
Extending the scope of GTFR Glucosylation reactions with tosylated substrates for rare sugars synthesis

Julian Görl[a], Christian Possiel[a], Christoph Sotriffer[b], Jürgen Seibel*[a]

Functionalized rare sugars were synthesized with 2-, 3- and 6-tosylated glucose derivatives as acceptor substrates by transglucosylation with sucrose and the glucansucrase GTFR from *Streptococcus oralis*. The 2- and 3-tosylated glucose derivatives yielded the corresponding 1,6-linked disaccharides (isomaltose analogues) whereas the 6-tosylated glucose derivatives resulted in 1,3-linked disaccharides (nigerose analogue) with high regioselectivity in up to 95% yield. Docking studies provide insights into the binding mode of the acceptors suggesting two different orientations which are responsible for the change in regioselectivity.

Glycoconjugates play a crucial role in biological recognition processes in particular viral and bacterial infection.[2] Since the synthesis of oligosaccharides is still a challenge for modern science, researchers try to allocate modular assembly systems to obtain tailor-made oligosaccharides.[3] The presence of several chemically similar hydroxyl-groups results in many reaction steps involving different strategies of orthogonal protection group chemistry. Thus, the control of regio- and stereochemistry is the key challenge in chemical synthesis of oligosaccharides.[4] Glucansucrases have been shown to be promising tools for the synthesis of glycoconjugates.[5] They belong to the glucosyl hydrolase family GH70 (CAZy database)[6] and are expressed extracellularly by bacteria such as *Streptococcus mutans*, *S. oralis* or *Lactobacillus reuteri*.[7] Glucansucrases take part in biofilm formation by synthesizing exopolysaccharides (EPS) from sucrose **1** as donor (Scheme 1).[7] In contrast to glycosyl transferases, most GH70 enzymes are readily available and their substrate (sucrose **1**) is cheap. They produce a variety of α-linked glucosyl-polymers with different lengths and linkages among which are dextran (**2**, mainly α-1,6), reuteran (α-1,4), alternan (alternating α-1,3 and α-1,6) and mutan (α-1,3).[8] Beside polymer formation and hydrolysis glucansucrases are able to glucosylate numerous acceptors such as alditols, aliphatic alcohols and natural aromatic compounds.[9] This reaction type makes them a valuable tool in the chemo-enzymatic synthesis of glycoconjugates since highly selective transfer reactions can be accomplished without the need of protection groups. However, their product promiscuity can be contra productive for the synthesis of tailor-made carbohydrates. To overcome this limitation protein- and substrate engineering is frequently used to fine-tune enzymatic conversion.[3c, 10] In earlier works our research group succeeded in altering the product diversification by mutagenesis of the glucansucrase from *Streptococcus oralis* ATCC10557[11] (GTFR, EC 2.4.1.5) shifting the product to more α-(1,3) linked, insoluble polymer or enhancing isomaltose production.[12] The transfer reaction of an enzyme can be strongly influenced by substituents blocking certain hydroxyl groups of the acceptor (substrate engineering). Hellmuth *et al.* used lactose as an acceptor for the glucansucrase GTFR which mainly produces a trisaccharide presenting an α-(1,2) linkage due to the steric blockage of the 3- and 6-positions by the galactose-moiety.[1, 12] The use of 6-tosylated glucose derivatives (e.g. **12**, Scheme 2c) as acceptors lead to the formation of α-(1,3)-linked disaccharides in 62-95% yield. The tosyl group can be readily substituted by various thio-sugars to form trisaccharides.[1] In this work we extended this concept by the usage of 2- and 3-tosylated glucose derivatives as acceptors yielding the corresponding α-(1,6)-glucosylated products. Performed docking studies provide insights into the basic principles of the glucansucrase's acceptor reactions suggesting that two different orientations are responsible for its regioselectivity.



Scheme 1 GTFR from *S. oralis* produces a water soluble dextran with α-(1,6) and α-(1,3) linkages (about 62 and 14%, respectively) with minor branching (14%).[12]

For the synthesis of the 2-tosylated-glucoside 1-*O*-allyl-2-*O*-*p*-toluenesulfonyl-α-D-glucopyranoside (allyl 2-Ts-α-D-Glc*p*, **7**), *O*-allyl-D-glucose was synthesized from glucose **3** according to literature[13] (Scheme 2a). The crude mixture of α- and β-allyl D-glucose was subsequently converted into 1-*O*-allyl-4,6-*O*-benzylidene-D-glucopyranoside.[14] At this stage α- and β-anomer were separated by column chromatography to yield **4** in 44% overall yield (starting from glucose **3**). The tosylation of **4** affords a mixture of 2- and 3-tosylated products (**5** and **6**) which can be isolated by column chromatography in yields of 50% and 12% respectively. Subsequent deprotection of **5** with 70% AcOH yielded **7** in quantitative yields.

Due to the low yields of the 3-tosylated derivate an alternative way starting from di-*O*-isopropyliden α-D-glucofuranose **8** was

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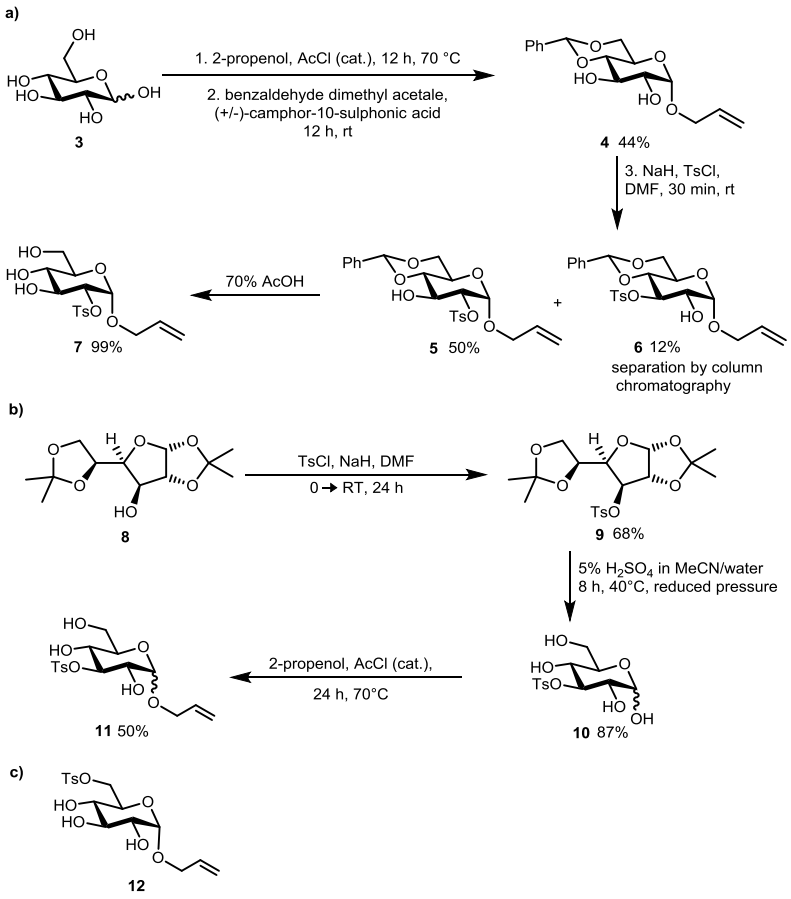
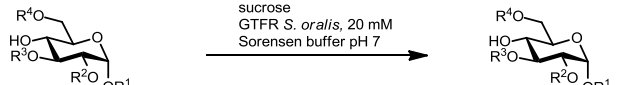


Scheme 2 a) Synthesis of the acceptor 1-*O*-allyl-2-*O*-*para*-toloenesulphonyl-α-D-glucopyranoside **7**, b) Synthesis of 3-tosyl-D-glucopyranose **10** and subsequent allylation to 1-*O*-allyl-3-tosyl-D-glucopyranoside **11**, c) structure of the acceptor 1-*O*-allyl-6-*O*-*para*-toloenesulphonyl-α-D-glucopyranoside **12** (previous work)[1].

developed (Scheme 2b). The 3-position was tosylated in 68% yields. The subsequent acidic deprotection of **9** was performed under reduced pressure to shift the equilibrium to the product side by removing acetone from the reaction mixture while maintaining the reaction temperature at 40 °C (the product quickly decomposes at high temperatures or basic conditions). 3-*O*-*p*-toluenesulphonyl glucopyranoside **10** (3-Ts-D-Glc*p*) was obtained in 87% yield. An optional allylation gave 1-*O*-allyl-3-*O*-*p*-toluenesulphonyl glucopyranoside **11** (allyl 3-Ts-D-Glc*p*) as a mixture of α- and β-anomers in 50% yields. 3-Ts-D-Glc*p* is readily soluble in water whereas for the allylated acceptors, enzymatic reactions were performed in 10% DMSO.

The glucansucrase GTFR was incubated with allyl 2-Ts-α-D-Glc*p* **7** and sucrose **1** (10 eq.) as a donor substrate. The enzyme glucosylated allyl 2-Ts-α-D-Glc*p* **7** to give the isomaltose derivate allyl α-D-Glc*p*-(1,6) allyl 2-Ts-α-D-Glc*p* **13** (Scheme 3) in 91% yield (determined via HPLC, 62% isolated yield). The GTFR prefers the transfer of glucose to the tosylated acceptors to the production of the dextran polymer. After the consumption of the acceptor the enzyme continues with the dextran formation while **13** is not degraded in the process. In contrast to the previous reaction, using allyl 3-Ts-D-Glc*p* **11** as an acceptor gave only very poor yields of glucosylated product which could not be isolated in sufficient yields (Scheme 3). However, the acceptor without the allyl-group (3-Ts-D-Glc*p,* **10**) yielded the corresponding isomaltose-derivate α-D-glucopyranosyl-(1,6)-3-*O*-*p*-toluoenesulfonyl-D-glucopyranose (α-D-Glc*p*-(1,6) 3-Ts-D-Glc*p* **14**) in 88% yield (determined via HPLC, 40% isolated yield, Scheme 3). Purification of **14** was not trivial due to the easy loss of the tosyl group. The obtained decomposition products could not be fully characterized but ESI mass spectrometry (m/z = 347.095 $[M+Na]^+$, calc. 347.0949) suggest an intramolecular substitution reaction forming an epoxide.

The blockage of the favoured hydroxyl-group of the acceptor with an orthogonal group (such as other sugar moieties, azides, tosylates) probably forces the acceptor to occupy different orientations towards the donor causing a change in the regioselectivity. For further investigation, docking studies were performed to gain insights into potential binding modes of the acceptors. Since no crystal structure of GTFR is known to date an *N*-truncated and mutated (D1025N) variant of GTF-180a from *Lactobacillus reuteri* 180[15] was used as template (PDB identifier: 3HZ3, numbering in brackets) in the docking process. GTF-180a has 47% sequence identity (61% positives, 5% gaps, protein-BLAST tool) and forms similar α-1,6 and 1,3-linked glucan with branches[16]. In addition, Leemhuis *et al.* recently compared the active site clefts of several glucansucrases suggesting that α-(1,6)-linking with minor branching GTFs exhibit similarly formed clefts.[7] As a member of the α-amylase superfamily[17], the GTFR enzyme converts sucrose via a double-displacement mechanism[18] Glu554 (Glu1063) serves as proton source and facilitates the attack of Asp516 (D1025N) as nucleophile on the anomeric centre of the glc*p*-moiety of sucrose. A covalent enzyme-substrate complex (ES-complex) is formed and the product is obtained by nucleophilic attack of an acceptor on C1 releasing the free enzyme.[18] In order to imitate the attack of the acceptor on the bound glucose, an ES-complex was simulated from the crystal structure 3HZ3 which contains sucrose



Acceptor	R^1	R^2	R^3	R^4	Solvent	Yield [%] (isolated)	Product	R^1	R^2	R^3	R^4
7	allyl	Ts	H	H	buffer/dmso (9:1)	91 (62)	**13**	allyl	Ts	H	αGlc
10	H	H	Ts	H	buffer	88 (40)	**14**	H	H	Ts	αGlc
11	allyl	H	Ts	H	buffer/dmso (9:1)	-	-	-	-	-	-
12	allyl	H	H	Ts	buffer/dmso (9:1)	95 (95)[1]	**15**	allyl	H	αGlc	Ts

Scheme 3 Overview of the performed reactions with the GTFR enzyme and the tosylated acceptor substrates. Reaction and isolated (in brackets) yields are given.

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bound in the active site due to a single mutation of Asp1025 to asparagine. The fru*f*-moiety was deleted (PyMOL) and the resulting structure was subsequently used as a template in AutoDock 3.0. The dockings were then compared with the

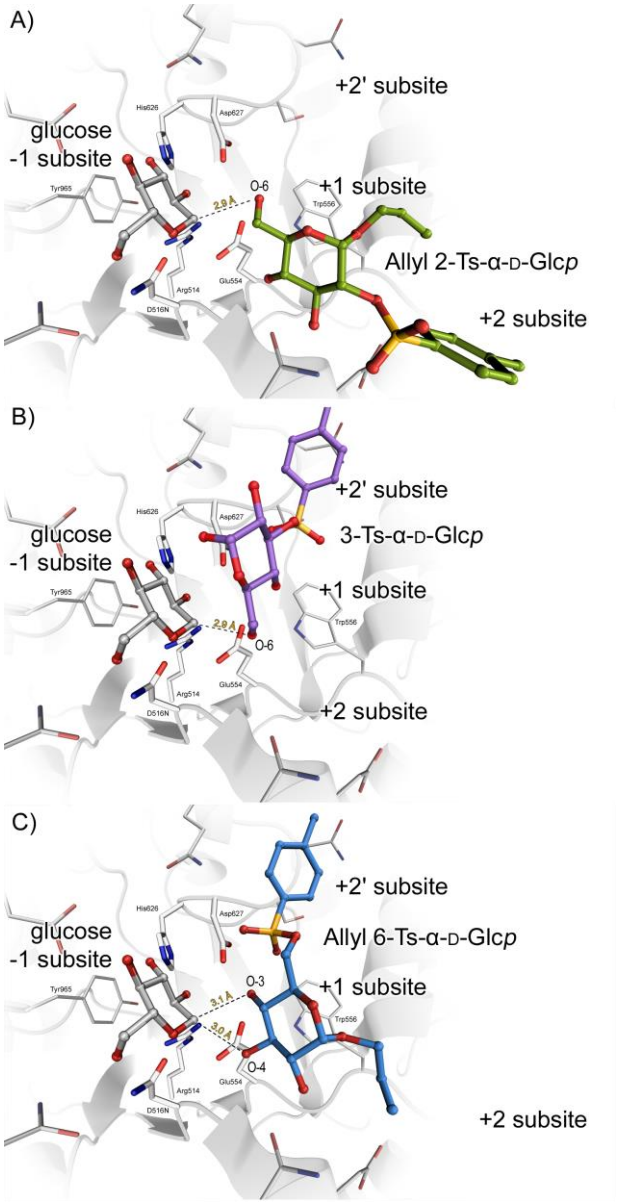

Figure 1 Docking studies of tosylated acceptor substrates with the GTFR enzyme. A) The best docking result of allyl 2-Ts-α-D-Glc*p* (-6.11 kcal·mol^{-1}) occupies the +2 subsite, with a distance of 2.9 Å between C1 of the simulated ES complex and O6; B) the best docking mode of 3-Ts-α-D-Glc*p* (-5.68 kcal·mol^{-1}) occupies the +2' subsite instead; C) the best docking mode of ally 6-Ts-α-D-Glc*p* (-6.61 kcal·mol^{-1}) occupies the same subsite as the 3-tosylated sugar; figure generated with PyMOL (PyMOL Molecular Graphics System, Version 1.8; Schrödinger, LLC; 2015)

subsites identified through analysis of the GTF-180a crystal structure in complex with maltose by Vujivic-Zahar *et al*.[15a] (PDB identifier: 3KLL, Figure S1A).

In a first docking study the goal was to confirm the results achieved experimentally by Hellmuth *et al.*[1] using 6-tosylated glucose derivatives as substrates yielding α-1,3 linked disaccharides. Docking of allyl 6-Ts-α-D-Glc*p* **12** provided a variety of different overlapping orientations among the top ranks in which the glucosyl-scaffold occupies the +1 subsite (Figure 1A, S1B). The best result in terms of the estimated free energy of binding (-6.61 kcal·mol^{-1}) corresponds to a position in which O3 is pointing to C1 of the protein-bound glucose, at a distance of 3.0 Å. The next-closest group of the tosylated ligand is 4-OH, with a 3.1 Å distance to C1. Both OH-groups of the ligand appear to be interacting favorably with Glu554 (Glu1063, general acid/base). The 2-OH is forming a hydrogen bond with Asn520 (Asn1029) and the tosyl group is placed in a shallow pocket (Figure 1A, S1B) formed by the residues Aps627 (Asp1136) to Val633 (Gln1142) which was identified as the +2 subsite before (Figure S1A).[15a]

Docking of allyl 2-Ts-α-D-Glc*p* **7** provided essentially three different orientations of the ligand among the top-ranked results: two in which the tosyl-group is placed in the +1 subsite and a third in which the 6-OH of the allyl 2-Ts-α-D-Glc*p* is pointing towards the C1 of the protein-bound glucose (Figure 1B, S1C). In terms of the docked-energy score, the latter is only marginally (0.1 to 0.5 kcal·mol^{-1}) less favorable than the former two and may thus be considered as an energetically degenerate top-ranked result. The orientation suggests that productive binding for reaction is readily possible. In this binding mode (Figure 1B), the distance between the O6 of the incoming ligand and the C1 of the bound glucose is 2.9 Å, which is close to the lower limit that can be expected in non-covalent docking. In comparison to the 6-tosylated sugar, the tosyl-group of allyl 2-Ts-Glc*p* occupies a different pocket (+2' subsite, Figure 1B, S1C). The ligand appears well accommodated in a cleft between Asn520 (Asn1029) and Trp556 (Trp1065), with the sulfone group forming potential hydrogen bonds with the Tyr469 (Ala978) backbone and the Asn520 (Asn1029) side chain which was suggested by Vujicic-Zagar *et al.* to be the +2' subsite.[15a]

Docking of allyl 3-Ts-Glc*p* **11** (α- and ß-anomer) with the same settings as the other ligands did not provide any binding orientation with a free OH-group in proximity to C1 of the protein-bound glucose. The closest distance of a hydroxyl-group was 3.3-3.5 Å and the calculated binding energies are considerably less favorable (about 2 kcal·mol^{-1}). The observations are in agreement with the experiment yielding only low amounts of glucosylated product. In contrast to the docking with the corresponding allylated derivate, docking of 3-Ts-Glc*p* **10** (α-anomer) provided two different docking modes: one in which the tosyl group is pointing towards the anomeric carbon of bound glucose (not shown) and one with the 6-OH pointing towards C1 at a distance of 2.8-2.9 Å (-5.68 kcal·mol^{-1}). The latter docking mode occupies the same cleft as the best docking modes for allyl 6-Ts-Glc*p* (+2 subsite, Figure 1C, S1D). Thus it is reasonable to assume that the allyl-group causes an additional steric hindrance that disables effective glucosylation of the allylated derivate.

Our goal was to utilize the glucansucrase GTFR as a tool in chemo-enzymatic synthesis for rare sugars and sugar building blocks. The decoration of the acceptor glucose with *p*-toluenesulfonyl groups in 2-, 3- and 6-positions allows the control of the regioselectivity of the transglucosylation reaction. The synthesized nigerose and isomaltose derivatives equipped with *p*-toluenesulfonyl groups are ideal functional building blocks for follow-up reactions via S_N2 substitutions with different nucleophiles i.e. thioacetate[15b], azides, hydroxylates and for synthesis of bacterial rare sugar building blocks.[19] In addition the docking studies provided a plausible model of putative binding modes of the acceptors that may be responsible for the strict regioselectivity of the enzymatic reactions.

Experimental Section

Experimental details can be found in the Supporting Information

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Keywords: biocatalysis • substrate engineering • glucansucrase • substrate promiscuity • rare sugar

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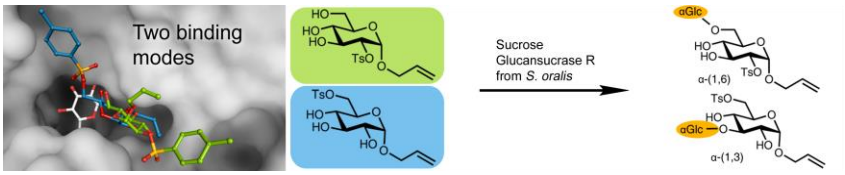

Substrate directed synthesis of rare sugars. Tosylated glucose derivates were used as acceptors of the glucansucrase R from *S. oralis*. The tosyl groups allow only distinct orientations of the acceptor in the active cleft (docking experiments) resulting in highly regioselective glucosylated products.

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