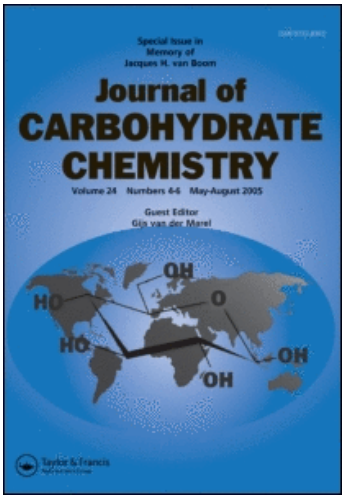

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Synthesis of L-Rhamnose and *N*-Acetyl-D-Glucosamine Derivatives Entering in the Composition of Bacterial Polysaccharides by Use of Glucansucrases

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Transglucosylation reactions using sucrose as glucosyl donor and either *N*-acetyl-D-glucosamine, L-rhamnose, or methyl α-L-rhamnopyranoside as acceptors were carried out with recombinant glucansucrases from families 70 and 13 of glycoside-hydrolases. Depending on the enzyme specificity, various carbohydrate structures were synthesized and characterized including α-D-glucopyranosyl-(1→6)-*N*-acetyl-D-glucosamine, α-D-glucopyranosyl-(1→4)-*N*-acetyl-D-glucosamine, α-D-glucopyranosyl-(1→1)-β-L-rhamnopyranoside, α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→1)-β-L-rhamnopyranoside, methyl α-D-glucopyranosyl-(1→4)-α-L-rhamnopyranoside, and methyl α-D-glucopyranosyl-(1→3)-α-L-rhamnopyranoside. Disaccharides were obtained with yields going up to 64%. The structural diversity generated as well as the obtained yields appear to be related to enzyme active site architecture, which can be modulated and improved by enzyme engineering. Several of the obtained disaccharides

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enter in the composition of surface polysaccharides of pathogenic bacteria, among which is *Shigella flexneri.* Our results outline the potential of glucansucrases in the chemo-enzymatic synthesis of complex carbohydrates of biological interest whose chemical synthesis may be seen as a limitation.

Keywords Glucansucrase, Enzymatic glucosylation, L-Rhamnose, *N*-Acetyl-D-glucosamine, Bacterial polysaccharide

INTRODUCTION

Two categories of natural enzymes catalyze glycosyl transfer onto sugar acceptors: glycosyltransferases of the Leloir pathway, which use expensive nucleotide-activated sugars as glycosyl donor, and transglycosidases, often associated with low yields of the glycosylated targets. For both types, reactivity toward acceptors is strictly controlled by the enzyme selectivity and specificity. In this regard, glucansucrases (GS, EC 2.4.1), which are transglycosidases found in glycoside hydrolases (GH) families 13 and 70 according to the CAZY classification,[1] are very attractive synthetic tools. They naturally catalyze glucan synthesis and the concomitant release of fructose from sucrose donor, a largely available and low-cost substrate. The type, number, and organization of the glucosidic linkages displayed in the glucan polymer is strongly dependant on glucansucrase specificities.[2–10] An interesting particularity of glucansucrases is their ability to catalyze the glucosylation of nonnatural acceptors (Fig. 1). Glucansucrases are indeed able to transfer glucosyl residues onto

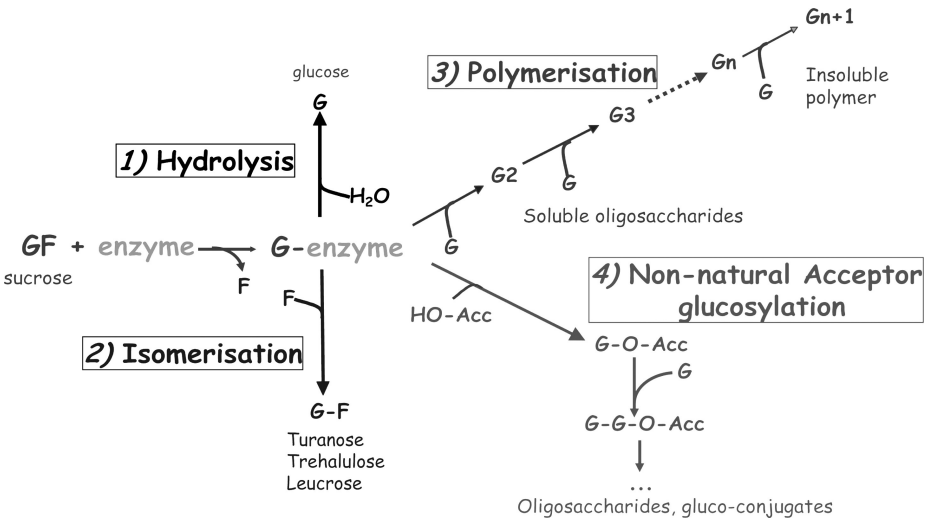

Figure 1: Reactions catalyzed by glucansucrases. G: glucosyl; F: fructosyl or fructose; AccOH: nonnatural acceptor.

a large variety of acceptors, including sugar[11–18] and nonsugar[11,16,19–24] molecules. In this process, acceptor recognition, glucosylation yields, and resulting products are strongly dependent on glucansucrase stereo- and regiospecificities.

In the present study, we have investigated the glucosylation of commercially available *N*-acetyl-D-glucosamine and L-rhamnose, as well as of methyl α-L rhamnopyranoside,[25,26] prepared in one step from the latter, by use of several recombinant or native glucansucrases, displaying distinct specificities (Table 1). The interest of using enzymatically produced disaccharides as starting building blocks for the synthesis of fragments of the O-antigen part of various *Shigella flexneri* lipopolysaccharides is highlighted.

RESULTS AND DISCUSSION

Glucansucrases are α-retaining transglucosidases that follow a double displacement mechanism, in which a β-D-glucosyl enzyme covalent intermediate is first formed from sucrose substrate.[27–29] In a second step, the glucosyl moiety is transferred to an acceptor, which can be either (1) water leading to the production of glucose, (2) fructose to form sucrose isomers, or (3) the glucose moiety released from sucrose hydrolysis to form soluble oligosaccharides and α-glucans. The major reaction of these enzymes is the synthesis of glucan polymer. However, an exogenous hydroxylated acceptor well recognized by the enzyme may be glucosylated at the detriment of polymer synthesis (reaction 4 in Fig. 1). Depending on the enzyme regiospecificity, distinct types of glucosidic linkage are found in the formed polymer or in the nonnatural glucosyl derivative (Fig. 1).

In order to take advantage of the ability of glucansucrases to glucosylate nonnatural acceptors in a regio- and stereospecific manner, five recombinant glucansucrases were tested for the glucosylation of *N*-acetyl-D-glucosamine (D-Glc*p*NAc), L-rhamnose (L-Rha*p*), and methyl α-L-rhamnopyranoside (α-L-Rha*p*OMe). As shown in Table 1, these enzymes catalyze the synthesis of diverse types of glucosidic linkage and were chosen on purpose to attempt the synthesis of structurally distinct disaccharides. They correspond to engineered forms of the dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F (DSR-S_1, DSR-S_2, and DSR-S_3),[30,31] the truncated alternansucrase from *L. mesenteroides* NRRL B-1355 (ASR),[32] and the amylosucrase from *Neisseria polysaccharea* (AS).[7] Notably, mutant enzymes DSR-S_2 and DSR-S_3 carry mutations in the region downstream the second aspartic acid of the catalytic triad, known to participate in the distortion of the glucosyl enzyme and in the positioning of the acceptor, which could potentially alter the enzyme stereo- and regiospecificity.[31]

Table 1: Origin and specificity of the recombinant glucansucrases selected for the glucosylation of the target acceptors: D-Glc*p*NAc, L-Rha*p*, and α-L-Rha*p*OMe.

Enzyme name abbreviation	Recombinant Enzyme	Origin	EC	GH Family	Type of glucosidic linkages	Ref
DSR-S_1	DSR-S vardel Δ4N	Truncated form of DSR-S dextransucrase from *L. mesenteroides* NRRL B-512F	2.4.1.5	GH70	$\alpha - 1,6$	(30)
DSR-S_2	DSR-S vardel Δ4N SEV663YDA	Mutant of DSR-S vardel Δ4N	2.4.1.5	GH70	α - 1,6	(31)
DSR-S_3	DSR-S vardel Δ4N SEV663NNS	Mutant of DSR-S vardel Δ4N	2.4.1.5	GH70	α - 1,6	(31)
ASR	ASR-C-APY del	Truncated form of ASR alternansucrase from *L. mesenteroides* NRRL B-1355	2.4.1.140	GH70	α - 1,6 and α-1,3 (mainly alternating)	(32)
AS	AS	*N. polysaccharea* amylosucrase	2.4.1.4	GH13	α - 1,4	(7)

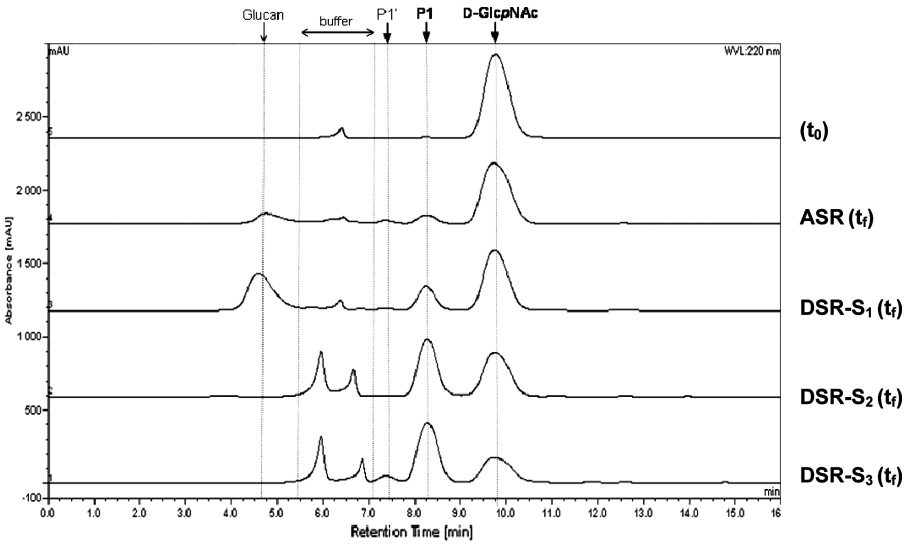

Figure 2: Analysis of D-Glc*p*NAc glucosylation by four different GH70 glucansucrases. Comparison of HPLC chromatograms (with $UV_{\lambda=220nm}$ detection) at the starting time of the reaction (t_0) and after total sucrose consumption (t_f =24h); **P1** and **P1**′: acceptor reaction products.

Glucosylation of *N*-acetyl-D-glucosamine

Enzyme Screening

Acceptor reactions were first carried out using sucrose as donor and D-Glc*p*NAc as acceptor in equimolar ratio. The HPLC profiles of the acceptor reaction products synthesized by DSR-S_1, its two variants (DSR-$S_2$and DSR-S_3),and ASR were all highly similar (Fig. 2). It can thus be assumed that all tested GH70 glucansucrases produced the same compound, herein called **P1** (t_R = 8.2 min). Interestingly, the main acceptor reaction product obtained by action of AS, product **P2** (t_R = 8 min), displayed a retention time slightly differing from that of **P1,** indicating that a structurally distinct product was synthesized by transglucosylation (Fig. 3). Other glucosylation products were detected (**P1**′ and **P2**′ in Fig. 2 and 3, respectively), although in lower amounts that did not allow further characterization.

Structural Characterization

The structure of **P1**, which was synthesized using DSR-S_2mutant, was analyzed by HRMS and NMR (supporting materials 1 and 5; details given in Experimental section).

HRMS analysis of **P1** indicated a molecular weight of 383 Da, corresponding to a monoglucosylated form of D-Glc*p*NAc.The ^{1}H NMR spectrum of **P1** shows two doublets at 4.87 ppm and 4.88 ppm, assigned to the anomeric proton

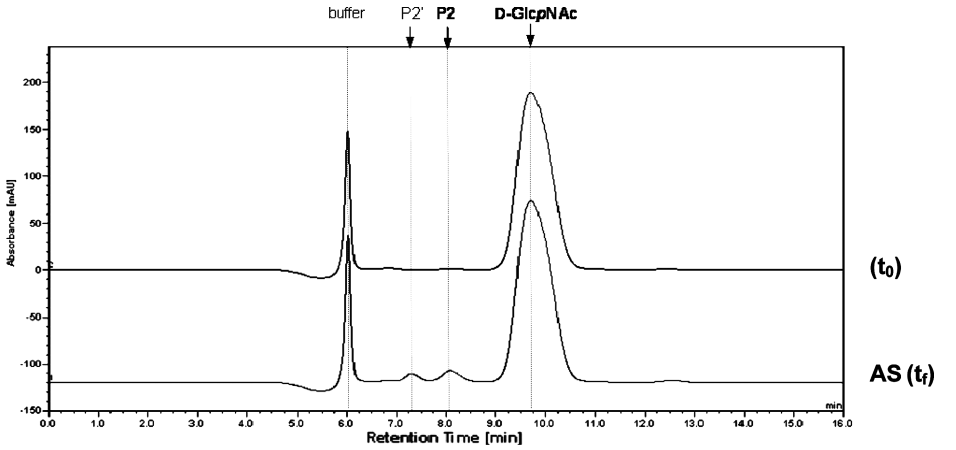

Figure 3: Analysis of D-Glc*p*NAc glucosylation by GH13 glucansucrase, amylosucrase from *N. polysaccharea*. Comparison of HPLC chromatograms (with $UV_{\lambda=220nm}$ detection) at the starting time of the reaction (t_0) and after total sucrose consumption ($t_f = 24$ h); **P2** and **P2′**: acceptor reaction products.

of the D-glucosyl residue, and two doublets at 4.66 ppm and 5.13 ppm, which by comparison with the D-Glc*p*NAc spectrum were assigned to the anomeric proton of β- and α-D-Glc*p*NAc, respectively. Signal integration indicates a disaccharide structure in good agreement with MS data. Moreover, both $H1_{Glc}$ doublets exhibit weak J_{1-2}coupling constants (3.4 and 3.5 Hz), which are characteristic of an α-D-glucosidic linkage, in agreement with the known stereospecificity of the DSR-S_2mutant. Besides, H-1_{Glc}:C-$6_{GlcNAc}$and H-6_{GlcNAc}:C-1_{Glc} cross-peaks in the HMBC spectrum ascertain the presence of an α-(1→6) linkage. All ^{1}H and ^{13}C signal assignments are listed in the Experimental section. From these assignments, it can be concluded that **P1** is a new disaccharide corresponding to α-D-glucopyranosyl-(1→6)-*N*-acetyl-D-glucosamine (Fig. 4).

Analogously, with a molecular weight of 383 Da determined by HRMS analysis, **P2** is also a disaccharide (supporting materials 2 and 6; details given in Experimental section). Moreover, NMR analysis indicates two major differences in the ^{13}C spectra of **P2** in comparison to that of **P1**. Differences involve δ_{C6}, lowered by 4.5 ppm, and δ_{C4}, which is increased by at least 7.2 ppm. Taking into account the H-1_{Glc}:C-$4_{GlcpNAc}$ and H-$4_{GlcpNAc}$:C-1_{Glc} cross-peaks in the HMBC spectrum, all data converge to suggest that **P2** corresponds to the yet unknown regioisomer, namely α-D-glucopyranosyl-(1→4)-*N*-acetyl-D-glucosamine (Fig. 4).

Glucosylation Reaction

As shown in Table 2, for all tested enzymes, D-Glc*p*NAc conversion degrees were composed between 6% and 64% with yields of monoglucosylated D-Glc*p*NAc products ranging from 4% to 61% based on quantitative HPLC

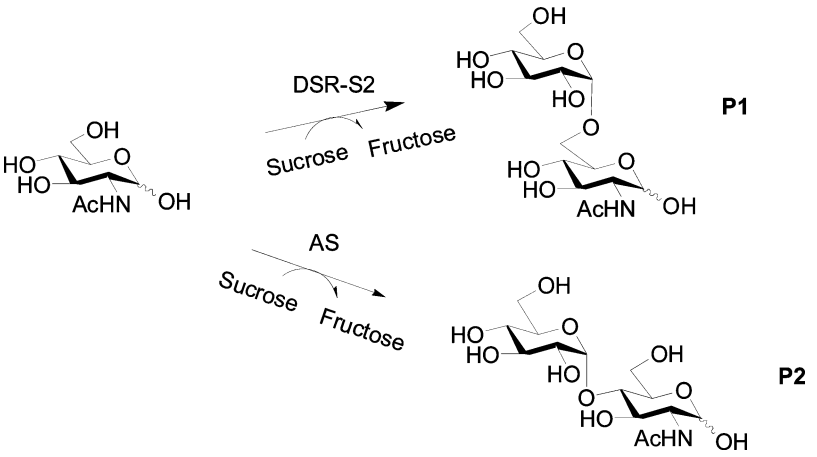

Figure 4: Structure of disaccharides **P1** and **P2** obtained by glucansucrase-mediated glucosylation of *N*-acetyl-D-glucosamine.

analysis. Acceptor glucosylation, which is in competition with polymer synthesis, is thus highly dependent on acceptor recognition by the enzyme. Notably, conversion degrees and monoglucosylated acceptor yields are almost equal, indicating that **P1** and **P2** are the two major products formed, respectively.

D-Glc*p*NAc glucosylation by the native alternansucrase from *L. mesenteroides* NRRL B-1355 was previously reported.[11] The acceptor conversion degree was very low and the glucosylation products were not characterized. We showed here that all tested GH family 70 enzymes produced the same α-(1→6)-linked disaccharide **P1** upon D-Glc*p*NAc glucosylation. These enzymes thus conserve their same natural regiospecificity toward D-Glc*p*NAc. DSR-S_1 and its two variants were the most efficient enzymes for the synthesis of **P1**. Interestingly, mutations introduced downstream the second aspartic acids of the catalytic triad of the enzymes did not alter the enzyme regiospecificity, but had a beneficial effect on the conversion degree, which increased by more

Table 2: Transglucosylation of D-Glc*p*NAc using different recombinant glucansucrases at acceptor:sucrose molar ratio of 1:1 (146 mM).

	Recombinant enzyme				
	DSR-S_1	**DSR-S_2**	**DSR-S_3**	**ASR**	**AS**
D-Glc*p*NAc conversion degree[a] (%)	26	54	64	14	6
Monoglucosylated D-Glc*p*NAc yield[b] (%)	24 (**P1**)	54 (**P1**)	61 (**P1**)	13 (**P1**)	4 (**P2**)

At final time (24 h), sucrose is fully consumed by all variants.
[a]The conversion degree of the acceptor was calculated using the formula: $((\text{D-Glc}pNAc)_{initial} - (\text{D-Glc}pNAc)_{t24h}) / (\text{D-Glc}pNAc)_{initial} \times 100$.
[b]% Monoglucosylated D-Glc*p*NAc = (Monoglucosylated D-Glc*p*NAc)t_f/(consumed sucrose) $t_f \times 100$.

than twofold for both DSR-S_2 and DSR-S_3mutants compared to DSR-S_1. Located close to the catalytic residues, mutations introduced in DSR-S_2 and DSR-S_3may favor binding of the nonnatural acceptor, suggesting that engineering of glucansucrases can greatly enhance the ability of these enzymes for the synthesis of novel oligosaccharides.

Glucosylation of L-rhamnose

Enzyme Screening

Acceptor reactions were carried using an equimolar L-rhamnose:sucrose ratio (Table 3). Among family 70 glucansucrases, only DSR-S_2 and ASR were able to glucosylate L-rhamnose. Interestingly, they synthesized several coproducts, notably leucrose and isomaltose,[31–33] but the competition with polymer synthesis was not in favor of the disaccharide formation (supporting material 10). Consequently, the conversion degree and the yield of each independent product were very low, thus preventing further characterization. Regarding glucosylation reactions carried out with AS, two major products were obtained (**P3** and **P4**) concomitantly with turanose and trehalulose products[34] (supporting material 9). **P3** and **P4** were produced in larger amounts and purified for structural characterization.

Optimization of P3 and P4 synthesis by AS

To improve **P3** and **P4** synthesis, the influence of acceptor:sucrose ratio on the conversion degree was first studied at two sucrose concentrations (146 mM and 292 mM) (Table 4). In both cases, increasing the acceptor concentration favors acceptor glucosylation at the cost of polymer formation, and yields higher amounts of products. However, L-rhamnose conversion degree decreases concomitantly. Maintaining similar acceptor:sucrose ratio but varying the initial sucrose concentration did not significantly affect the glucosylation yields. Therefore, in search for the best compromise between glucosylation yield and L-rhamnose conversion degree, we retained a sucrose concentration of 292 mM and an acceptor:sucrose molar ratio of 2.5 for preparative scale-up.

Table 3: Transglucosylation of L-rhamnose using different recombinant glucansucrases at acceptor:sucrose ratio of 1:1 (146 mM).

	Recombinant enzyme				
	DSR-S_1	**DSR-S_2**	**DSR-S_3**	**ASR**	**AS**
L-rhamnose conversion degree[a] (%)	<1	10	<1	6	6.5
Number of new products	0	5	0	4	2 (**P3** + **P4**)

[a]The conversion degree of the acceptor was calculated using the formula ((Acceptor)$_{initial}$ − (Acceptor)$_{t24h}$) / (Acceptor)$_{initial}$ × 100. At final time (24 h), sucrose was fully consumed.

Table 4: Effect of the acceptor:sucrose molar ratio (A:S) on L-rhamnose glucosylation catalyzed by amylosucrase.

	A:S Ratio ([S]) = 146 mM)				A:S Ratio ([S] = 292 mM)			
	1	**2.5**	**5**	**10**	**0.5**	**1**	**2.5**	**5**
L-Rhamnose (mM)	146	365	730	1460	146	292	730	1460
Conversion degree[a] (%)	6.5	4.4	3.1	2.1	8.0	6.6	4.8	3.1
Monoglucosylated L-rhamnose (**P3**) yield[b] (%)	4.4	8.4	12.6	17.5	2.7	4.5	8.9	12.3
Diglucosylated L-rhamnose (**P4**) yield[c](%)	2.1	2.6	3.1	4.1	1.4	2.1	3.0	2.9
% L-Rha*p* converted into **P3**[d]	4.4	3.4	2.5	1.7	5.3	4.5	3.6	2.5
% L-Rha*p* converted into **P4**[e]	2.1	1.0	0.6	0.4	2.7	2.1	1.2	0.6

[a]The conversion degree of the acceptor was calculated using the formula: $((\text{Acceptor})_{\text{initial}} - (\text{Acceptor})_{t24h})/(\text{Acceptor})_{\text{initial}} \times 100$.
[b]Monoglucosylated rhamnose yield is the molar ratio: $(\text{Glc-Acceptor})_{t24h}/(\text{consumed sucrose})_{t24h} \times 100$. At final time, sucrose is fully consumed.
[c]Di-glucosylated rhamnose yield is the molar ratio: $(\text{Glc}_2\text{-Acceptor})_{t24h}/(\text{consumed sucrose})_{t24h} \times 100$. At final time, sucrose is fully consumed.
[d]% L-Rha*p* converted into P3 is the molar ratio: $(\text{Glc-Acceptor})_{t24h}/(\text{Acceptor})_{t0} \times 100$.
[e]% L-Rha*p* converted into P4 is the molar ratio: $(\text{Glc}_2\text{-Acceptor})_{t24h}/(\text{Acceptor})_{t0} \times 100$.

Structural Characterization of P3 and P4

P3 and **P4** were separated by preparative HPLC. HRMS data of **P3** indicates a molecular weight of 326 Da corresponding to the mass of a monoglucosylated L-rhamnose (supporting materials 3 and 7; details given in Experimental section). The 1D and 2D L-rhamnose NMR spectra were used as reference (data not shown). L-Rhamnose is found in a $^1C_4$ pyranose ring conformation, as revealed by a weak $J_{1,2}$ coupling constant. Signals at 4.76 ppm and 5.00 ppm were assigned to the anomeric proton in β and α form, respectively.

Two anomeric signals of equal intensity, only, are observed in the ^{1}H NMR spectrum of **P3**. Aided by the chemical shifts (see Experimental section) and $^1J_{C,H}$ values of the anomeric carbons, signals at 5.19 ppm ($^1J_{C1,H1} = 173.9$ Hz) and 4.78 ppm ($^1J_{C1,H1} = 161.3$ Hz) were assigned to the anomeric protons of D-glucosyl and L-rhamnosyl residues in α and β configuration, respectively. No signal corresponding to the α/β-anomerization of a reducing disaccharide could be found. In contrast, the chemical shift attributed to C-1_{Rha} in the ^{13}C NMR spectrum suggested that the two sugar moieties were linked by their anomeric carbons through an ($\alpha 1 \rightarrow \beta 1$) linkage, as confirmed by HMBC analysis showing two inter-residual scalar couplings, C-1_{Rha}:H-1_{Glc} and C-1_{Glc}:H-1_{Rha}, respectively.

In agreement with MS data, which strongly suggest that **P4** is a trisaccharide of 488 Da made of two hexoses and a deoxyhexose, the ^{1}H NMR

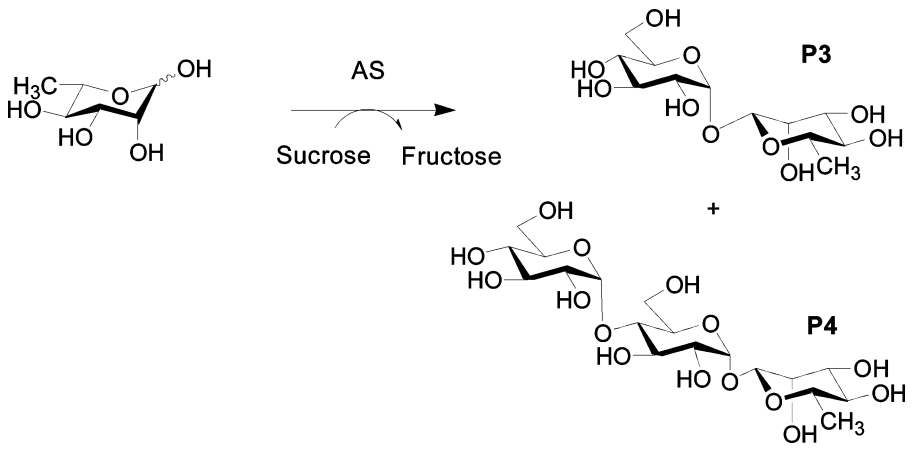

Figure 5: Structures of disaccharide **P3** and trisaccharide **P4** obtained by AS-mediated glucosylation of L-rhamnose.

spectrum of **P4** indicates three doublets of equal intensity in the anomeric region (supporting materials 4 and 8; details given in Experimental section). In support of this assumption, the ^{13}C NMR spectrum showed a single signal in the C-6_{Rha} region (16–17 ppm), and two signals in the C-6_{Glc} region (60–61 ppm), indicating the presence of one L-rhamnosyl ring and two D-glucosyl rings. The HMBC spectrum showed strong cross-peaks between an H-1_{Glc} and a C-4_{Glc}, and between an H4_{Glc} and a C1_{Glc}, respectively, indicating the presence of an α-(1→4)-linkage between the two glucosyl residues. Besides, as for **P3**, NMR analysis revealed that the glucosyl moiety incorporated first was linked to the L-rhamnosyl residue through an (α1→ β1) linkage. The whole sets of **P3** and **P4** ^{1}H and ^{13}C chemical shifts are given in the experimental section and in supporting materials 3–4 and 7–8. Finally, **P3** and **P4** are new oligosaccharides identified as the α-D-glucopyranosyl-(1→1)-β-L-rhamnopyranoside and α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→1)-β-L-rhamnopyranoside, respectively (Fig. 5).

Glucosylation of methyl α-L-Rhamnopyranoside

The acceptor reaction was carried out using an equimolar acceptor:sucrose ratio and a sucrose concentration of 146 mM. HPLC analysis of the reaction mixtures revealed the presence of two main products (**P5** and **P6**) (supporting materials 11 and 12). NMR data of these compounds were in agreement with those of chemically synthesized methyl α-D-glucopyranosyl-(1→4)-α-L-rhamnopyranoside (**P5**)[35,36] and methyl α-D-glucopyranosyl-(1→3)-α-L-rhamnopyranoside (**P6**)[37] used as reference compounds. Using the family 70 glucansucrases, methyl α-L-rhamnopyranoside conversion degrees ranging from 3% to 17% were obtained, indicating a poor recognition of this

Table 5: Transglucosylation of α-L-Rha*p*OMe using different recombinant glucansucrases at acceptor:sucrose ratio of 1 (146 mM).

	Recombinant enzyme				
	DSR-S_1	**DSR-S_2**	**DSR-S_3**	**ASR**	**AS**
Conversion degree[a]	3	5	6	17	<1
α−D-Glcp-(1→3)-α-L-RhapOMe (P6) yield[b] (%)	0	4	3	3	0
α−D-Glcp-(1→4)-α-L-RhapOMe (P5) yield[b] (%)	<1	<1	3	10	0

[a]The conversion degree of the acceptor was calculated according to the formula $((Acceptor)_{initial} - (Acceptor)_{t24h}) / (Acceptor)_{initial} \times 100$.
[b]The percentage of monoglucosylated acceptor was determined by the ratio $(Glc\text{-}Acceptor)_{t24h} / (consumed\ sucrose)_{t24h} \times 100$. At final time, sucrose was totally consumed.

acceptor. The best conversion was obtained for ASR, forming preferentially methyl α-D-glucopyranosyl-(1→4)-α-L-rhamnopyranoside and, to a lesser extent, methyl α-D-glucopyranosyl-(1→3)-α-L-rhamnopyranoside. Two additional peaks present on the chromatogram were putatively assigned to the homologous trisaccharides but were not further characterized. Noteworthy, AS was unable to use methyl α-L-rhamnopyranoside as an acceptor. Indeed, the anomeric hydroxyl group, which was the preferred AS-mediated glucosylation site of L-rhamnose, is blocked in methyl α-L-rhamnopyranoside, thus preventing the enzyme from transferring the glucosyl residue onto it, or onto any other hydroxyl groups of α-L-Rha*p*OMe (Table 5).

We have shown in this paper that glucosylation of *N*-acetyl-D-glucosamine and L-rhamnose catalyzed by glucansucrases leads to the synthesis of various disaccharides, and even trisaccharides. To our knowledge, these oligosaccharides were never reported before. All involve the formation of 1,2-*cis* glucosides, which are difficult to obtain by chemical synthesis due to lack of efficient control of the stereochemical outcome of the glucosylation step. Providing an enzymatic access to such compounds is thus of interest.

A number of studies have shown that introducing an enzymatic step in the synthesis of complex oligosaccharides could be of great advantage. The potency of such alternatives has been demonstrated in bacterial oligosaccharide synthesis.[38–40] Occasionally, the strategy was used in the field of glycovaccines.[41–44] The disaccharides that were more specifically targeted in this work were α-D-glucopyranosyl-(1→6)-*N*-acetyl-D-glucosamine, α-D-glucopyranosyl-(1→4)-*N*-acetyl-D-glucosamine, and a set of α-D-glucosyl-α-L-rhamnopyranose. Noteworthy, such disaccharides are components of various *S. flexneri* O-antigen repeating units.[45–47]

Oligosaccharide fragments of the O-antigens of *S. flexneri* serotypes 5a, 2a, and 3a, among others, have been chemically synthesized,[36,37,48,49] a number as their methyl glycosides using an appropriately protected [α-D-Glcp-(1→3)]-α-L-Rha*p*-OMe acceptor.[50,51]

In the case of *S. flexneri* 2a, several of those synthetic haptens were shown to be immunogenic in an animal model when administered as oligosaccharide-protein conjugates.[52] With the view of providing alternative synthetic pathways to complex oligosaccharides, the enzymatically produced disaccharides could serve as building blocks involved at an early stage of a chemo-enzymatic synthesis of selected *S. flexneri* oligosaccharides. Demonstrating the feasibility of such strategy may open the way to a new diversity of potent chemo-enzymatic routes integrating an enzymatic step at the early stage of the synthesis of glycosylated targets, as nicely illustrated for the synthesis of sialylated oligosaccharides and conjugates.[53–55] However, to be of interest in a chemo-enzymatic pathway, enzymatic transglucosylation must be efficient and high yielding. With DSR-S_1, ASR, and AS recombinant glucansucrases tested in our work, we have obtained low yields of transglucosylation. Considering the case of *N*-acetyl-D-glucosamine glucosylation, the use of DSR-S_1 led to the synthesis of α-D-glucopyranosyl-(1→6)-*N*-acetyl-D-glucosamine with a conversion degree of 24% yield. Noteworthy, the use of the triple mutant DSR-S_3, mutated in the region close to the catalytic residues, allowed increasing the conversion degree up to 64%, demonstrating that enzyme engineering significantly improved the transglucosylation activity toward the poorly recognized nonnatural acceptor. In particular, amino acids in close vicinity with catalytic residues appear to be key mutagenesis targets. Overall, data presented here serve as a basis to apply semi-rational engineering to selected glucansucrases in order to modulate their selectivity and/or regioselectivity and identify new variants able to produce the appropriate glucosylated building blocks in amounts compatible with their use in multistep synthesis.

EXPERIMENTAL

Materials

Choice of Recombinant Glucansucrases

For DSR-S and ASR glucansucrases, the enzyme constructs chosen for this study are, respectively, DSR-S vardel Δ4N (DSR-S_1)and ASR C-del APY (ASR). Both enzymes are truncated variants from *L. mesenteroides* NRRL B-512F dextransucrase and from *L. mesenteroides* NRRL B-1355 alternansucrase. They were constructed to reduce the glucansucrase degradation occurring during heterologous enzyme expression by *Escherichia coli* and have previously been shown to display the same behavior than the wild-type enzyme in terms of specificity and synthesized products.[30,32]

DSR-S vardel Δ4N variants (DSR-S_2:Δ4N SEV663YDA, DSR-S_3:DSR-S SEV663NNS[31]) are mutated in the active site downstream the catalytic aspartic acid D662. These mutants were designed by sequence alignment

analyses with glucansucrases of various linkage specificities, and described to have a particular influence on acceptor binding, as they preferentially catalyze the formation of disaccharides to the detriment of dextran formation.

All recombinant enzymes were produced in *E. coli* as reported elsewhere.[7,30,32] Nonpurified DSR-S and ASR (sonication supernatants) stored at –20°C were used for enzymatic assays. Purified AS,[7,30,32] conserved at –80°C, was used for enzymatic reactions.

Chemical Material

Sucrose, L-rhamnose, and *N*-acetyl-D-glucosamine were purchased from Sigma-Aldrich. Methyl α-L-rhamnopyranoside[25,26] and reference compounds methyl α-D-glucopyranosyl-(1→3)-α-L-rhamnopyranoside[37]and methyl α-D-glucopyranosyl-(1→4)-α-L-rhamnopyranoside[35] were synthesized chemically at the Unité de Chimie des Biomolécules - Institut Pasteur.

Acceptor Reaction Assay

The glucosylation reaction was performed in the enzyme optimal buffer: in Tris-HCl (50 mM, pH = 7.5) for AS assays, in sodium acetate buffer (AcONa) (20 mM, pH = 5.4) for ASR assays, and in AcONa (50 mM, [$CaCl_2$] = 0.05 g/L, pH = 5.2) for DSR-S assays. The reaction mixture (1 mL) was carried out at 30°C with sucrose and acceptor in equimolar ratio (146 mM). Enzymes were used at 1 U/mL. For DSR-S and ASR, one unit is defined as the amount of enzyme that catalyzes the formation of 1 μmol of fructose/min at 30°C, in enzyme buffer and 292 mM sucrose. For AS, activity was determined under the same conditions, except that sucrose was used at a concentration of 146 mM. All the acceptor reactions were stopped after total sucrose consumption by heating at 95°C for 5 min. The final mixture was centrifuged at 18,000 g for 10 min and filtered on a 0.22-μm membrane before HPLC analysis.

Glucosyl Acceptor Production

In order to characterize glucosylated products of L-rhamnose and *N*-acetyl-D-glucosamine, acceptor reactions were conducted at preparative scale.

P1 from *N*-acetyl-D-glucosamine glucosylation by DSR-S_2 (0.8 U/mL) was synthesized in 50 mL mixture reaction (500 mM in sucrose, 100 mM in acceptor); **P2** from *N*-acetyl-D-glucosamine glucosylation by purified AS (1 U/mL) was produced in 100 mL mixture reaction (292 mM in sucrose, 730 mM in acceptor). **P3** and **P4** from L-rhamnose glucosylation by purified AS (1 U/mL) were produced in 60 mL mixture reaction (292 mM in sucrose, 730 mM in acceptor). After a 24 h reaction time at 30°C, the media were centrifugated at 4800 rpm for 20 min at 4°C to remove proteins and filtered for a better clarification. The purification of the glucosylated products was performed on a preparative octadecyl reverse-phase chromatography column (C18 column)

(Bischoff Chromatography). Ultra-pure water was used as eluent at a constant flow rate of 50 mL/min. Glucosyl detection was carried out with a refractometer, and each peak was collected separately, concentrated, and reinjected into an analytical HPLC system to check the purity of the compounds. Purified **P1**, **P2**, **P3**, and **P4** were used as reference to determine yield of products obtained from glucansucrases (Tables 2 and 4).

Analytical Methods

High-Performance Liquid Chromatography (HPLC)

The HPLC analysis device consisted of a Dionex P 680 series pump, a Shodex RI 101 series refractometer, a Dionex UVD 340 UV/Vis detector, and an autosampler HTC PAL. Five columns were employed to separate the acceptor reaction products and to determine the acceptor conversion degree and product yields: (i) Biorad HPLC Carbohydrate Analysis columns: AMINEX HPX-87C at 80°C (elution with ultra-pure water at 0.6 mL/min); (ii) HPX-87K columns (300 × 7.8 mm) at 65°C (elution with ultra-pure water at 0.6 mL/min); (iii) C18 column Bischoff Prontosil Eurobond, 5 μm (elution with ultra-pure water at rt and 1 mL/min); (iv) C30: Bischoff Prontosil Eurobond, 5 μm, 250 × 4.0 mm (elution with ultra-pure water at rt and 1 mL/min); and (v) C18RP: Sinergi Fusion RP Phenomenex, 4 μm, 250 × 4.6 mm (elution with ultra-pure water at rt and 1 mL/min).

High-Resolution Mass Spectrometry (HRMS) and Nuclear Magnetic Resonance (NMR)

Accurate mass determination was carried out using an Autospec mass spectrometer arranged in an EBE geometry (Micromass, Manchester, UK). The instrument was operated at 8 kV accelerating voltage in positive mode. The cesium gun was set to 35 keV energy and 1 μL of sample was mixed in the tip of the probe with a glycerol or dithiothreitol/dithioerythritol matrix. NMR analyses: ^{1}H (400.130 MHz), ^{13}C (100.612 MHz); HSQC and HMBC were registered on a Bruker-ARX 400 spectrometer equipped with an ultra-shim system. Samples were dissolved in deuterium oxide at c.a. 80 g/L and experiments were performed at 300K.

α-D-glucopyranosyl-(1→6)-N-acetyl-D-glucosamine (P1)

HRMS (FAB): Calcd for $C_{14}H_{26}NO_{11}$: 384.1506 [MH^+], Found: 384.1518
β-anomer: ^{1}H NMR (400.130 MHz, D_2O) δ: 1.96 (s, 3H, COCH3), 3.32 (t, 1H, H-4′), 3.46 (m, 1H, H-2′), 3.49 (m, 1H, H-3), 3.58–3.92 (m, 8H, H-2, H-4, H-5, H-6, H-3′, H-5′, H-6′), 4.66 (d, 1H, $J_{1,2} = 11.2$ Hz, H-1), 4.87 (d, 1H, H-1′)

^{13}C NMR (100.612 MHz) δ: 22.16 (CH_3), 56.58 (C2), 60.43 (C6′), 65.65 (C6), 69.47 (C4′), 69.97 (C4), 71.45 (C2′), 71.78 (C5′), 73.03 (C3′), 74.08 (C3), 74.30 (C5), 95.05 (C1), 97.97 (C1′), 174.47 (CO)

$\alpha-$anomer: ^{1}H NMR (400.130 MHz, D_2O) δ: 1.96 (s, 3H, COCH3), 3.32 (t, 1H, H-4′), 3.46 (m, 1H, H-2′), 3.48 (m, 1H, H-3), 3.65–3.92 (m, 9H, H-5′, H-3′, H-6′, H-5, H-2, H-4, H-6), 4.88 (d, 1H, H-1′), 5.13 (d, 1H, $J_{1,2} = 4.8$ Hz, H-1)

^{13}C NMR (100.612 MHz) δ: 22.62 (CH_3), 57.09 (C2), 60.97 (C6′), 61.00 (C6), 69.78 (C4′), 72.06 (C2′), 73.14 (C5′), 73.27 (C3′), 74.79 (C3), 75.06 (C5), 77.22 (C4), 90.91 (C1), 97.91 (C1′), 175.22 (CO)

$\alpha-$D-glucopyranosyl-(1→4)-N-acetyl-D-glucosamine (P2)

HRMS (FAB): Calcd for $C_{14}H_{25}NO_{11}Na$: 406.1325 $[M+Na]^+$, Found: 406.1356

β-anomer: ^{1}H NMR (400.130 MHz, D_2O) δ: 2.02 (s, 3H, COCH3), 3.40 (t, 1H, H-4′), 3.56 (m, 1H, H-2′, H-5), 3.66–3.79 (m, 5H, H-3′, H-2, H-4, H-5′, H-3), 3.82 (m, 4H, H-6, H-6′), 4.70 (d, 1H, $J_{1,2} = 8$ Hz, H-1β), 5.39 (d, 1H, H-1′)

^{13}C NMR (100.612 MHz) δ: 22.62 (CH_3), 57.09 (C2), 60.97 (C6′), 61.00 (C6), 69.78 (C4′), 72.06 (C2′), 73.14 (C5′), 73.27 (C3′), 74.79 (C3), 75.06 (C5), 77.22 (C4), 95.25 (C1), 99.87 (C1′), 175.22 (CO)

α-anomer: ^{1}H NMR (400.130 MHz, D_2O) δ: 2.02 (s, 3H, COCH3), 3.40 (t, 1H, H-4′), 3.56 (m, 1H, H-2′), 3.66–3.82 (m, 7H, H-4, H-3′, H-5′, H-6, H-6′), 3.89 (dd, 1H, H-2), 3.94 (m, 1H, H-5), 4.00 (dd, 1H, H-3), 5.18 (d, 1H, $J_{1,2} = 3.6$ Hz, H-1α), 5.40 (d, 1H, H-1′)

^{13}C NMR (100.612 MHz) δ: 22.34 (CH_3), 54.36 (C2), 60.97 (C6′), 61.00 (C6), 69.78 (C4′), 70.57 (C5), 71.62 (C3), 72.17 (C2′), 73.14 (C5′), 73.30 (C3′), 77.94 (C4), 91.13 (C1), 100.15 (C1′), 174.98 (CO)

$\alpha-$D-glucopyranosyl-(1→1)-β-L-rhamnopyranoside (P3)

HRMS (FAB): Calcd for $C_{12}H_{22}O_{10}K$: 365.0850 $[M+K]^+$, Found: 365.0861

^{1}H NMR (400.130 MHz, D_2O) δ: 1.23 (d, 3H, $J_{5,6} = 6.7$ Hz, H-6), 3.32–3.33 (m, 2H, H-4, H-5), 3.35 (d, 1H, $J_{3,4} = 10$ Hz, H-4′), 3.49 (ddd, 1H, $J_{2,3} = 10$ Hz, H-2′), 3.55 (m, 1H, H-5′), 3.58 (m, 1H, H-3), 3.64 (dd, 1H, H-3′), 3.65 (dd, 1H, H-6′), 3.76 (dd, 1H, H-6′), 3.94 (d, 1H, $J_{2,3} = 3,2$ Hz, H-2), 4.78 (d, 1H , $J_{1,2} = 1.2$ Hz, H-1), 5.19 (d, 1H, $J_{1,2} = 3.7$ Hz, H-1′)

^{13}C NMR (100.612 MHz) δ: 16.82 (C6), 60.63 (C6′), 69.55 (C4′), 71.00 (C2), 71.13 (C2′), 72.16 (C4), 72.62 (C5), 72.67 (C3), 72.73 (C5′), 72.93 (C3′), 94.91 (C1), 95,23 (C1′)

α−D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→1)-β-L-rhamnopyranoside (P4)

HRMS (FAB): Calcd for $C_{18}H_{32}O_{15}K$: 527.1378 $[M+K]^+$, Found: 527.1366

^{1}H NMR (400.130 MHz, D_2O) δ: 1.22 (d, 3H, $J_{5,6} = 6.7$ Hz, H-6), 3.31–3.32 (m, 3H, H-4, H-5, H-4′), 3.47–3.61 (m, 6H, H-2′H-2″H-3, H-3′ H-4″,H-5″), 3.70–3.74 (m, 5H, H-5′, H-6′, H-6″), 3.93 (m, 2H, H-2, H-3″), 4.79 (d, 1H, $J_{1,2} = 1.2$ Hz, H-1), 5.20 (d, 1H, $J_{1,2} = 3.7$ Hz, H-1′), 5.31 (d, 1H, $J_{1,2} = 3.6$ Hz, H-1″)

^{13}C NMR (100.612 MHz) δ: 16.77 (C6), 60.64 (C6, C6′), 69.47 (C4?), 70.97 (C2), 71.00 (C2′), 71.12 (C5′), 71.86 (C2″), 72.15 (C4), 72.69 (C5), 72.72 (C3), 72.87 (C3′), 73.02 (C5″), 73.42 (C3?), 76.78 (C4′), 94.96 (C1), 95.05 (C1′), 99.83 (C1″) (C4), 91.13 (C1), 100.15 (C1′), 174.98 (CO)

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