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Published on Web 05/11/2009

Design of α-Transglucosidases of Controlled Specificity for Programmed Chemoenzymatic Synthesis of Antigenic Oligosaccharides

Elise Champion,[†,‡,¶] Isabelle André,[†,‡,¶] Claire Moulis,[†,‡,¶] Julien Boutet,[⊥,#] Karine Descroix,[⊥] Sandrine Morel,[†,‡,¶] Pierre Monsan,[†,‡,¶,||] Laurence A. Mulard,[⊥] and Magali Remaud-Siméon*[,†,‡,¶]

Université de Toulouse; INSA, UPS, INP, LISBP, 135 avenue de Rangueil, F-31077 Toulouse, France, CNRS UMR 5504, F-31400 Toulouse, France, INRA UMR 792, Ingénierie des Systèmes Biologiques et des Procédés, F-31400 Toulouse, France, Institut Universitaire de France, 103 boulevard Saint-Michel, F-75005 Paris, France, Institut Pasteur, Unité de Chimie des Biomolécules, CNRS URA 2128, 28 rue du Dr. Roux, F-75015 Paris, France, and Université Paris Descartes, 4 avenue de l'Observatoire, F-75006 Paris, France

Received January 13, 2009; E-mail: remaud@insa-toulouse.fr

Abstract: Combined with chemical synthesis, the use of biocatalysts holds great potential to open the way to novel molecular diversity. We report *in vitro* chemoenzymatic pathways that, for the first time, take advantage of enzyme engineering to produce complex microbial cell-surface oligosaccharides and circumvent the chemical boundaries of glycochemistry. Glycoenzymes were designed to act on nonnatural conveniently protected substrates to produce intermediates compatible with a programmed chemical elongation. The study was focused on the synthesis of oligosaccharides mimicking the O-antigen motif of *Shigella flexneri* serotypes 1b and 3a, which could be used for the development of multivalent carbohydrate-based vaccines. A semirational engineering approach was successfully applied to amylosucrase, a transglucosidase that uses a low cost sucrose substrate as a glucosyl donor. The main difficulty was to retain the enzyme specificity toward sucrose, while creating a new catalytic function to render the enzyme able to regiospecifically glucosylate protected nonnatural acceptors. A structurally guided library of 133 mutants was generated from which several mutants with either completely new specificity toward methyl α-L-rhamnopyranoside or a tremendously enhanced one toward allyl 2-acetamido-2-deoxy-α-D-glucopyranoside acceptors were isolated. The best variants were used to synthesize glucosylated building blocks. They were then converted into acceptors and potential donors compatible with chemical elongation toward oligosaccharide fragments of the O-antigens of the two targeted serotypes. This is the first report of a successful engineering of an α-transglycosidase acceptor binding site that led to new specificities. It demonstrates the potential of appropriate combinations of a planned chemoenzymatic pathway and enzyme engineering in glycochemistry.

1. Introduction

Recent developments in glycochemistry, providing more straightforward access to well-defined carbohydrates, have opened the way to significant progress in the fields of glycobiology and glycotherapeutics.[1,2] Nevertheless, despite these accomplished advancements,[3−5] chemical approaches toward specific effective microbial oligosaccharides still need considerable effort.[1,3−5] Yet limited to the synthesis of short oligosaccharides, metabolically engineered cell factories are seen as an attractive methodology.[6] Enzymes have also emerged as practical alternatives to chemical synthesis,[7] including in glycochemistry,[8,9] but examples of *in vitro* enzymatic synthesis of microbial carbohydrates remain scarce. Despite the increasing number of available carbohydrate-active enzymes—glycosyltransferases and transglycosidases—the lack of appropriate enzymatic tools with requisite substrate specificity has prevented extensive exploration of chemoenzymatic routes to complex oligosaccha-

[†] Université de Toulouse; INSA.
[‡] CNRS UMR 5504.
[¶] INRA UMR 792.
[⊥] Institut Pasteur.
[#] Université Paris Descartes.
[||] Institut Universitaire de France.

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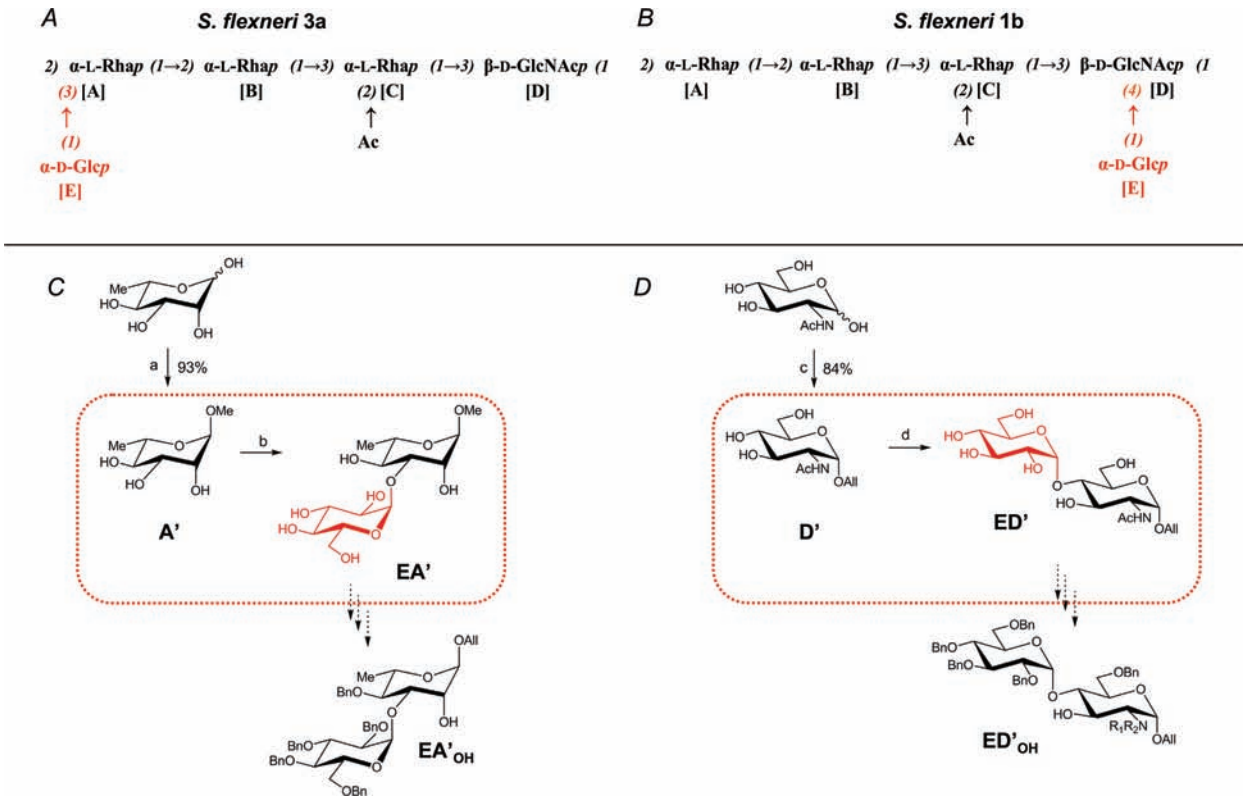

Figure 1. Chemoenzymatic routes toward **EA**- and **ED**-containing oligosaccharides: the example of *S. flexneri* (A) Repeating unit of *S. flexneri* 3a O-antigen. (B) Repeating unit of *S. flexneri* 1b O-antigen. (C) Programmed chemoenzymatic synthesis for *S. flexneri* 3a oligosaccharides: **A′** as the selected acceptor to be enzymatically glucosylated (encircled in red) following enzyme engineering, and proposed building block $\mathbf{EA'_{OH}}$ for the synthesis of *S. flexneri* oligosaccharides bearing the **EA** substitution pattern. Acceptor **A′** was obtained by Fisher glycosylation of L-rhamnose in methanol (81% from crystallization, 93% following column chromatography). (D) Programmed chemoenzymatic synthesis for *S. flexneri* 1b oligosaccharides: **D′** as the selected acceptor to be enzymatically glucosylated (encircled in red) following enzyme engineering, and proposed building block $\mathbf{ED'_{OH}}$ for the synthesis of *S. flexneri* oligosaccharides bearing the **ED** substitution pattern. Acceptor **D′** was obtained by Fisher glycosylation of *N*-acetyl-D-glucosamine (84% from crystallization). (a) MeOH, Dowex X8-200 ion-exchange resin (H^+), reflux; (b) I228Y mutant, sucrose; (c) AllOH, $BF_3{\cdot}OEt_2$, 90 °C; (d) F290K mutant, sucrose. Rha*p* = L-Rhamnopyranosyl, Glc*p*NAc = 2-Acetamido-2-deoxy-D-glucopyranosyl, Glc*p* = D-Glucopyranosyl, R_1,R_2 = protecting groups.

rides. Membrane Leloir-type glycosyltransferases and their nucleotide activated donors are not all easily available.[8] Alternatively, transglycosidases proceed from diverse non-nucleotide sugar donors and transfer to a broad variety of acceptors.[10−12] Nonetheless, enzyme availability is often critical, especially when considering the regio- and stereospecificity required for the synthesis of a given target molecule.[10,13] Moreover, *in vitro* enzymatic glycosylation mostly involves fully deprotected acceptors. Thus, it often comes at the latest stages in the synthesis,[8,14] although exceptions exist.[15,16]

Protein engineering has proven to be very efficient to generate biocatalysts, including glycoenzymes with novel substrate specificities.[17,18] Along this line, we investigate herein the applicability of glycoenzyme engineering to obtain complex bacterial carbohydrates by *in vitro* chemoenzymatic synthesis involving an enzymatic glycosylation step at an early stage of the pathway. The approach is exemplified for the synthesis of fragments of *Shigella flexneri* 3a and 1b O-antigens. *S. flexneri* are enteroinvasive Gram negative bacteria responsible for endemic shigellosis,[19] and vaccines ensuring multiserotype coverage are in demand.[19,20] New glycovaccines based on synthetic oligosaccharides mimicking the bacterial lipopolysaccharides, the major targets for antibody-mediated protection, are proposed as promising alternatives to live-attenuated candidate vaccines.[21,22] The most known *S. flexneri* O-antigen repeats share a linear trirhamnose-*N*-acetyl-glucosamine tetrasaccharide backbone (designated **ABCD**, Figure 1).[23] The serotype is defined by *O*-acetyl and glucosyl branching onto this backbone. In particular, the α-D-glucosylation pattern

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usually involves either L-rhamnopyranose or *N*-acetyl-D-glucosamine residues[23,24] as in *Shigella flexneri* 3a and 1b serotypes, which are among the most prevalent ones. Here, we focus on the enzymatic synthesis of both the α-D-glucopyranosyl-(1→4)-*N*-acetyl-β-D-glucosaminyl (**ED**) branching pattern shared by serotypes 1a and 1b[23,24] and the α-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl (**EA**) side chain, common to serotypes 2b, 3a, 5a, 5b, and X[23,24] (Figure 1A and 1B). Protein engineering technologies and organic chemistry were used to tailor biocatalysts with the requisite functions and evaluate the efficiency of such chemoenzymatic routes, respectively.

2. Results

2.1. Programmed Chemoenzymatic Pathway. For the synthesis of the pentasaccharides mimicking the O-antigen fragments of *S. flexneri* 3a and 1b, we envisioned a route starting from the enzymatic glucosylation of conveniently protected L-rhamnose (**A**) and *N*-acetyl-D-glucosamine (**D**) respectively. This paved the way for the subsequent chemical elongation (Figure 1A and 1B). The choice of the protecting groups obeyed two criteria: easy synthetic access and possible site-selective chemical elongation of the glucosylation product at either the reducing or nonreducing end. On this basis, methyl α-L-rhamnopyranoside (**A′**), a crystalline material easily obtained[25] from L-rhamnose (**A**), was selected (Figure 1C). Indeed, conversion of the glucosylation product α-D-Glc*p*-(1→3)-α-L-Rha*p*-OMe[26] (**EA′**) into allyl (2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl)-(1→3)-4-*O*-benzyl-α-L-rhamnopyranoside (**EA′$_{OH}$**)[27] was thought feasible. Acting as an acceptor and as a precursor to a trichloroacetimidate donor, disaccharide **EA′$_{OH}$** was used as an exquisite intermediate in the synthesis of several *S. flexneri* 5a,[28] 3a, and X O-antigen fragments.[29,30] Similarly, allyl 2-*N*-acetyl-2-deoxy-α-D-glucopyranoside (**D′**), a crystalline material prepared in one step from *N*-acetyl-D-glucosamine (**D**) (Figure 1D), was selected based on the assumption that the 3$_{D'}$-OH group would be easily differentiated at the disaccharide level, by introduction of a 2,3-oxazolidinone moiety. Regioselective differentiation of the 3$_{D'}$-OH of α-D-Glc*p*-(1→4)-α-D-Glc*p*NAc-OAll (**ED′**) is indeed a prerequisite to any specific chain elongation at this position as present in *S. flexneri* 1a and 1b O-antigens.

For the enzymatic glucosylation step, we favored the use of transglucosidases, named glucansucrases. They belong to GH families 13 and 70[37] and catalyze the synthesis of α-glucan chains by successive transfers of α-D-glucopyranosyl units from sucrose without any mediation of sugar nucleotides (Figure 2). Using the high energy of the sucrose glycosidic bond to catalyze the condensation reaction, they stand among the most efficient transglucosidases in the glycoside-hydrolase family. Depending on their regiospecificity, different types of glucosidic linkage

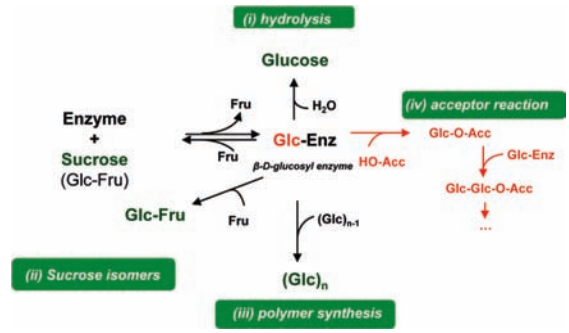

Figure 2. Reactions catalyzed by glucansucrases. Enz: enzyme; Acc: exogenous acceptor; Fru: fructose; Glc: glucose.

are found in the resultant polymer. Notably, the polymerization reaction can be redirected toward the glucosylation of exogenous acceptors, when the latter are well recognized.[9,12,31,32] A preliminary study carried out with four recombinant glucansucrases, selected for their very distinct specificities (namely their ability to form α-1,2, α-1,3, α-1,4, and α-1,6 glucosidic bonds, respectively) revealed that none could readily catalyze the glucosylation of methyl α-L-rhamnopyranoside (**A′**) and *N*-acetyl-D-glucosamine (**D**) with the required regiospecificity and within satisfactory yields.[33] Note that screening was run with commercially available *N*-acetyl-D-glucosamine (**D**) rather than allyl 2-acetamido-2-deoxy-α-D-glucopyranoside (**D′**), as we assumed that the allyl aglycone would not significantly affect acceptor recognition. We later showed that this was indeed the case and, therefore, that the hypothesis was valid. Engineering novel transglucosidases with improved acceptor substrate specificity was thus necessary. Recombinant *Neisseria polysaccharea* amylosucrase (wtAS) is the only glucansucrase whose three-dimensional structure is available as a complex with either sucrose or the natural reaction product.[34,35] To allow structurally guided engineering, it was thus chosen as a scaffold to tailor appropriate enzymatic glucosylation tools.

2.2. Engineering of Amylosucrase. As shown in Figures 3A and 3B, the wtAS active site has a 15 Å deep and narrow pocket like architecture with seven well-defined subsites spanning from −1 to +6 according to the glycoside-hydrolase nomenclature.[36] The subsite −1 is responsible for the enzyme specificity toward sucrose and is occupied by the glucosyl unit which will be transferred during the reaction. During the transglucosylation, the subsite +1 plays a dual role: it is involved in the recognition of the departing fructosyl ring from sucrose donor as well as in the recognition and correct positioning of the acceptor molecule to ensure a proper and regioselective glucosylation. Those are the main concerns that we addressed through a semirational engineering approach, which consisted of (i) mapping the binding site residues important for functional plasticity and identifying the most promising positions to be modified to favor acceptor recognition without

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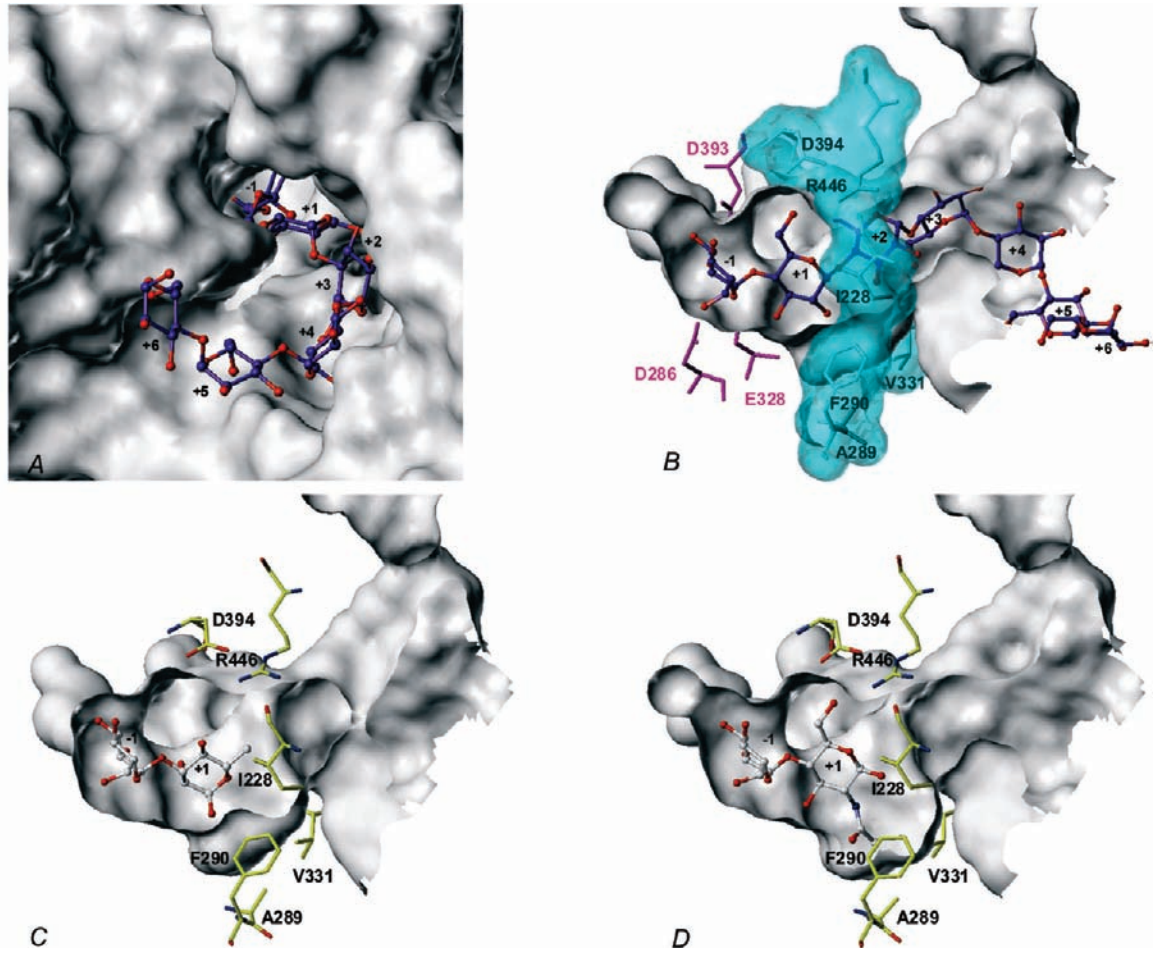

Figure 3. View of amylosucrase active site in complex with maltoheptaose (PDB: 1MW0). (A) Amylosucrase is viewed as a Connoly surface, as calculated by the MOLCAD module implemented in Sybyl7.3. (B) Cross view of the active site. Residues forming the catalytic triad (D286, E328, and D393), located at the bottom of the pocket, are colored in magenta. The seven positions identified for site-directed mutagenesis (I228, A289, F290, I330, V331, D394, and R446) are shown in cyan color. I330 is not shown in the figure, as it is located on the opposite side. (C) Docking mode of **EA′**. (D) Docking mode of **ED**.

interfering with sucrose binding and (ii) proceeding to systematic site-directed mutagenesis on the amino acid targets. The identification of these mutation positions relied on docking studies of disaccharides α-D-Glc*p*-(1→3)-α-L-Rha*p*-OMe (**EA′**) and α-D-Glc*p*-(1→4)-D-Glc*p*NAc (**ED**) in the active site of wtAS.

2.2.1. Structural Analysis of wtAS:EA′ Complex. The most energetically favored conformation of α-D-Glc*p*-(1→3)-α-L-Rha*p*-OMe (**EA′**) in the enzyme pocket was considered for detailed structural analysis (Figure 3C). The α-D-glucopyranosyl moiety (**E**) of **EA′** is more tightly bound to the enzyme at subsite −1 than methyl α-L-rhamnopyranoside (**A′**) at subsite +1. Comparison of the docking modes adopted by **EA′** with crystallographic maltose (α-D-Glc*p*-(1→4)-D-Glc*p*) at subsite +1 revealed a "flip" of the +1 pyranose ring, due to the inversion in the ring chair conformation adopted by **A′** ($^1C_4$ vs $^4C_1$, respectively) and the altered glycosidic linkage (α-1→3 vs α-1→4, respectively). As a result of these main structural differences, the network of interactions with the wtAS was drastically impaired, particularly at subsite +1. Four amino acids (Ile228, Ile330, Asp394, and Arg446), found in close contact with **EA′**, were chosen as targets for site-directed mutagenesis. In the search for a catalytically productive binding mode of methyl α-L-rhamnopyranoside (**A′**), we reasoned that introduction of less hindered hydrophobic residues at positions 228 and 446 could bring beneficial van der Waals interactions with the 6-methyl group of methyl α-L-rhamnopyranoside (**A′**) and assist a better positioning of the acceptor in the catalytic pocket. In addition, introducing longer polar side chains at positions 394 and 330 should help recovering some stabilizing hydrogen bonds with the acceptor.

2.2.2. Structural Analysis of wtAS:ED Complex. The docking mode adopted by α-D-Glc*p*-(1→4)-D-Glc*p*NAc (**ED**) in the wtAS binding site resembled that observed by crystallography for maltose at subsites −1 and +1 (Figure 3D). The unique difference between maltose and α-D-Glc*p*-(1→4)-D-Glc*p*NAc (**ED**) is the *N*-acetyl group at C-2_D. Docking results indicated that to accommodate *N*-acetyl-D-glucosamine (**D**) at subsite +1, the hydrophobic residues (Ala289, Phe290, Ile330, Val331) which form a pocket surrounding the *N*-acetyl group had to move away to provide sufficient space for the ligand. Replacing these residues by smaller polar amino acids might also provide additional beneficial hydrogen bond interactions to reshape the pocket for *N*-acetyl-D-glucosamine (**D**) specific recognition.

In summary, 7 positions out of the 18 residues that constitute the first shell of subsite +1 were judged to be not critical for sucrose binding but beneficial for target acceptor glucosylation. They were selected for mutagenesis: I228, A289, F290, I330, V331, D394, and R446 (Figure 3B) and systematically mutated

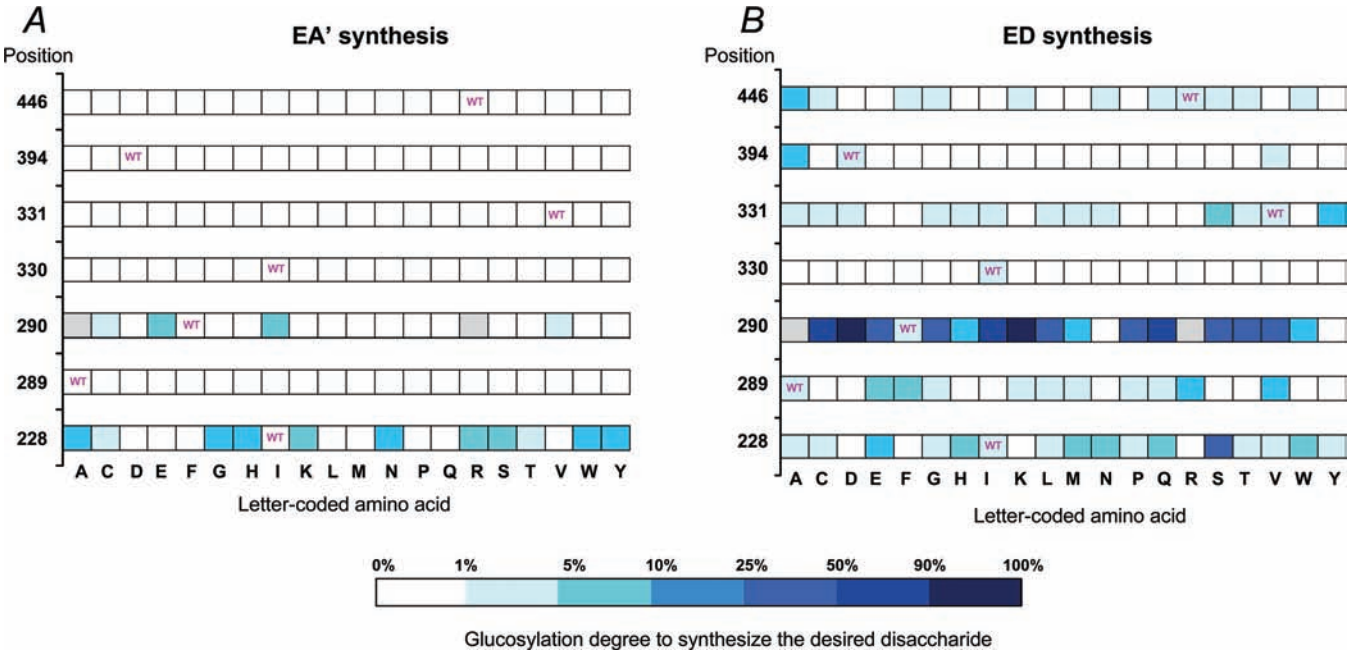

Figure 4. Screening of the library of AS monomutants for their ability to synthesize the desired disaccharide: (A) **EA′**. (B) **ED**. Mutants were tested on both acceptors (Acc) **A′** and **D**. Rows indicate the seven mutated positions, and columns represent the 20 possible amino acid mutations including the corresponding wt amino acid (colored in magenta). Glucosylation degree = $[\text{Monoglucosylated Acc}]_{t_f}/([\text{Suc}]_{t_0} - [\text{Suc}]_{t_f}) \times 100$. t_0 and t_f are the initial and final reaction times, respectively. Suc = Sucrose.

by the 19 other possible residues to create a small size library focused on subsite +1 of AS variants.

2.3. Screening of Mutants Adapted to Target Acceptor Glucosylation. A library of 133 monomutants (7 × 19) was thus obtained. 64% were active on sucrose (data not shown). Mutants were tested against the acceptors methyl α-L-rhamnopyranoside (**A′**) and *N*-acetyl-D-glucosamine (**D**) in microtiter format experiments combined with HPLC screening (Figure 4).

In contrast to wtAS that was unable to catalyze the glucosylation of methyl α-L-rhamnopyranoside (**A′**), 15 mutants displayed a totally novel specificity toward this acceptor (Figure 4A). Furthermore, these mutants showed the correct regiospecificity allowing the synthesis of α-D-Glc*p*-(1→3)-α-L-Rha*p*-OMe (**EA′**). In particular, position 228 was critical for **EA′** enzymatic synthesis as 11 of the 19 corresponding mutants formed the desired disaccharide. Mutations at position 290 were also beneficial since 4 mutants could form **EA′**, albeit in lower yield (≤5%). Mutations at positions 289, 330, 331, 394, and 446 failed to promote **A′** glucosylation.

Remodelling of subsite +1 led to marked improvements for the glucosylation of *N*-acetyl-D-glucosamine (**D**): 30 mutants catalyzed α-D-Glc*p*-(1→4)-D-Glc*p*NAc (**ED**) formation with a glucosylation degree over 5% (5−10% for 8 mutants, 10−50% for 17 mutants, over 50% for 5 mutants), while wtAS only reached a glucosylation degree of 2% under the same reaction conditions (Figure 4B). Position 290 was clearly strategic to improve recognition of *N*-acetyl-D-glucosamine (**D**). Notably, F290D and F290K yielded **ED** with glucosylation degrees over 90%, a 45-fold increase compared to that of wtAS. Mutations at positions 228, 289, 331, 394, and 446 also improved **ED** synthesis.

2.4. Characterization of I228Y and F290K Mutants. Mutants I228Y, yielding α-D-Glc*p*-(1→3)-α-L-Rha*p*-OMe (**EA′**), and F290K, providing α-D-Glc*p*-(1→4)-D-Glc*p*NAc (**ED**), were produced and purified to homogeneity to determine their product reaction profile and their kinetic parameters. They were both compared to that obtained with wtAS. With sucrose alone, I228Y transglucosylation activity (maltooligosaccharide and amylose synthesis) was abolished, sucrose hydrolysis being the main reaction (Supplementary Figure S1). In the presence of α-L-Rha*p*-OMe (**A′**) acceptor, 47% of the sucrose glucosyl residues were transferred onto **A′** to form α-D-Glc*p*-(1→3)-α-L-Rha*p*-OMe (**EA′**) and traces of diglucosylated α-L-rhamnopyranoside (Table 1A). The other part of the glucosyl moieties was transferred onto water via sucrose hydrolysis.

In contrast with I228Y, F290K retained the ability to synthesize maltooligosaccharides (up to DP 20) in the presence of sucrose alone, like the parent enzyme. When α-D-Glc*p*NAc-OAll (**D′**) was added, maltooligosaccharide production was fully suppressed in favor of **D′** glucosylation (Supplementary Figure S2). Indeed, the totality of the sucrose glucosyl residues was transferred onto **D′,** providing in majority α-D-Glc*p*-(1→4)-α-D-Glc*p*NAc-OAll (**ED′**) (56%) and a small amount of the diglucosylated form (α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*NAc-OAll, $\mathbf{E_1ED'}$). These values, close to those obtained with *N*-acetyl-D-glucosamine (**D**) acceptor, confirm the high specificity of the mutant for glucosaminyl acceptors and the little influence of the allyl aglycone on the glucosylation outcome (Table 1B).

The mutants were further compared to wtAS by determining steady-state kinetic parameters of transglucosylation reactions of both wtAS and mutants. Kinetic measurements indicated a poor affinity of both I228Y and F290K for sucrose (data not shown). k_{cat}/K_m values were thus determined by linear regression of the initial rates of transglucosylation measured as variable substrates (sucrose donor or acceptor) (Table 1). When acceptors (α-L-Rha*p*-OMe (**A′**) for I228Y, α-D-Glc*p*NAc-OAll (**D′**) for F290K) were present, the catalytic efficiency toward the acceptors tremendously increased compared to that observed for wtAS.

α-D-Glc*p*-(1→3)-α-L-Rha*p*-OMe (EA′) as Starting Material for the Synthesis of *S. flexneri*3a O-antigen Building Blocks. Disaccharide **EA′** was purified from the crude methyl α-L-rhamnopyranoside (**A′**) glucosylation mixture as its peracetate

Table 1. Reaction Characterization and Kinetic Parameters Determination (Values are the Mean of Three Independent Measurements; Error Range Is within 5 to 10%)

A: Transglucosylation of A'			
		wtAS	I228Y
Characterization of acceptor transglucosylation[a]	A' conversion degree (%)	0	44
	% Monoglucosylated –A'	0	41
	% Diglucosylated –A'	0	3
	% Glc units transferred onto A'	0	47
Kinetic Parameters of acceptor transglucosylation	Initial rate[b] of Glc units transferred onto A' (μmoles of product formed/min/g_{enzyme})	<5[c]	210
	Initial rate[b] of Glc units transferred onto natural acceptors[d] (μmoles of product formed/min/g_{enzyme})	610	65
	$k_{cat}/K_{m,Suc}$[e] ($mM^{-1}min^{-1}$)	$<4.10^{-4}$ [c]	0.080
	$k_{cat}/K_{m,A'}$[f] ($mM^{-1}min^{-1}$)	$<4.10^{-4}$ [c]	0.085

B: Transglucosylation of D'			
		wtAS	F290K
Characterization of acceptor transglucosylation[a]	D'conversion degree (%)	6	78
	% Monoglucosylated D'	3	56
	% Diglucosylated D'	3	22
	% Glc units transferred onto D'	9	100
Kinetic Parameters of acceptor transglucosylation	Initial rate[b] of Glc units transferred onto D' (μmoles of product formed/min/g_{enzyme})	290	6690
	Initial rate[b] of Glc units transferred onto natural acceptors[d] (μmoles of product formed/min/g_{enzyme})	630	<5[c]
	$k_{cat}/K_{m,Suc}$[e] ($mM^{-1}min^{-1}$)	0.074	3.630
	$k_{cat}/K_{m,D'}$[f] ($mM^{-1}min^{-1}$)	0.122	15.880

[a] At initial time (t_0): Sucrose (Suc) = Acceptor (Acc) = 146 mM. At final time (t_f = 24 h), sucrose was fully consumed. Glc = Glucosyl. Acceptor conversion degree = $([Acc]t_0 - [Acc]_f)/[Acc]_{t_0}$ where [Acc] = Molar acceptor concentration. % Monoglucosylated Acceptor = [Monoglucosylated Acc]t_f /$[Acc]_{t_0}$. % Diglucosylated acceptor = [Diglucosylated Acc]t_f/$[Acc]_{t_0}$. % Glc units transferred onto acceptor derivatives = [Glucosyl units transferred onto acceptor derivatives]/[Glucosyl units transferrable from initial sucrose]. [b] Initial rate was determined at a concentration of 250 mM for both donor and acceptor. [c] The HPLC limit of detection is in the nmole range. [d] Natural acceptors = H_2O, sucrose isomers, $(Glc)_n$. See Figure 2. [e] Catalytic efficiency $k_{cat}/K_{m,Suc}$ was determined by measuring the initial rate of monoglucosylated acceptor formation at a constant acceptor concentration (250 mM). [f] Catalytic efficiency $k_{cat}/K_{m,Acc}$ was determined by measuring the initial rate of monoglucosylated acceptor formation at a constant sucrose concentration (250 mM).

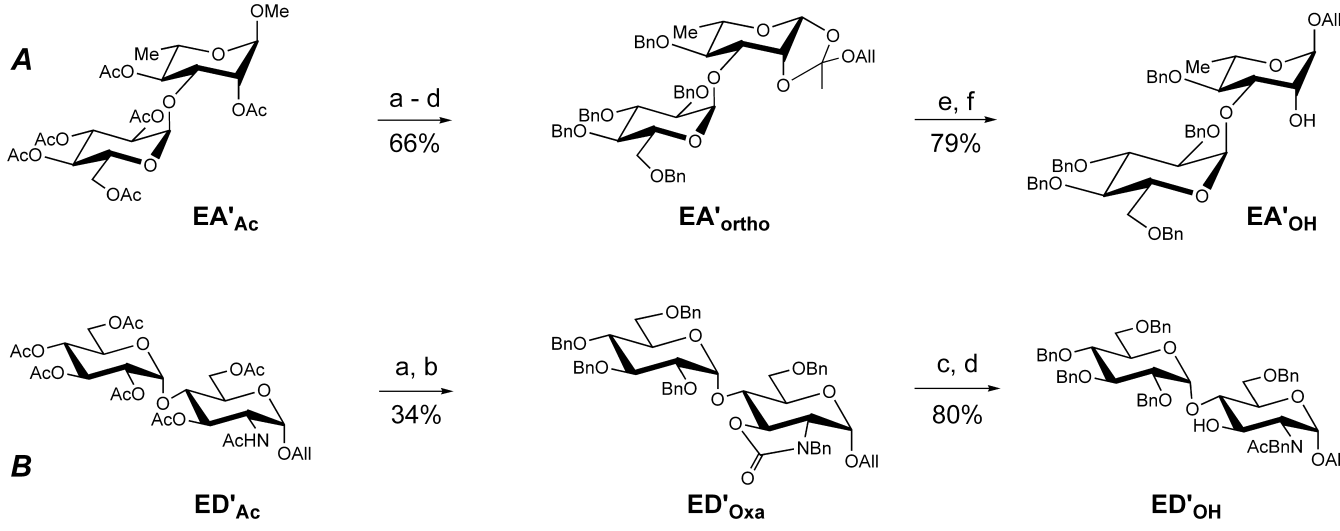

Figure 5. (A) Chemoenzymatic synthesis of key building block $\mathbf{EA'_{OH}}$: highlights. (a) HBr/AcOH; (b) AllOH, lutidine, CH_2Cl_2; (c) K_2CO_3, MeOH; (d) NaH, BnBr, DMF; (e) TMSOTf, CH_2Cl_2; (f) MeONa, MeOH. (B) Chemoenzymatic synthesis of building block $\mathbf{ED'_{OH}}$: highlights. (a) $Ba(OH)_2$, H_2O; (b) (i) CCl_3CH_2OCOCl, NaOMe, MeOH, (ii) BnBr; NaH; DMF; (c) 1 M aq NaOH, 1,4-dioxane; (d) Ac_2O, pyridine.

derivative $\mathbf{EA'_{Ac}}$, a necessary intermediate in the chemical conversion of **EA′** into $\mathbf{EA'_{OH}}$. The peracetylated derivative $\mathbf{EA'_{Ac}}$ was then converted into orthoester $\mathbf{EA'_{ortho}}$ in four steps (one purification from $\mathbf{EA'_{Ac}}$, in 66% yield). Orthoester $\mathbf{EA'_{ortho}}$ allowed the selective masking of positions $1_{A'}$ and $2_{A'}$ (Figure 5A). It served as a key intermediate for the hydroxyl groups, except those at positions $1_{A'}$ and $2_{A'}$, which had permanent benzyl protection. The absence of interference during the elongation process was therefore warranted. Indeed, as anticipated, careful monitoring of orthoester opening into the fully protected allyl glycoside followed by transesterification provided the known acceptor $\mathbf{EA'_{OH}}$ in 79% yield.

2.6. α-D-Glc*p*-(1→4)-α-D-Glc*p*NAc-OAll (ED′) as Starting Material for the Synthesis of *S. flexneri* 1b O-antigen Building Blocks. Regarding the use of α-D-Glc*p*-(1→4)-α-D-Glc*p*NAc-OAll (**ED′**) as a starting building block, a first purification step, consisting of peracetylation of the acceptor reaction mixture enabled the removal of the fructose and

diglucosylated derivatives (α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*NAc-OAll, $\mathbf{E_1ED'}$) and led to intermediate $\mathbf{ED'_{Ac}}$. The latter was saponified to give allyl glycoside $\mathbf{ED'_{amino}}$ (84%) bearing a free amino group at position $2_{D'}$. Accessing the $\mathbf{ED'_{amino}}$ intermediate was the key point in the whole strategy. Indeed, having a 2_D-NH_2 allowed the chemoselective differentiation of the vicinal hydroxyl group at position $3_{D'}$ (Figure 5B). The potency of such a strategy was demonstrated previously in an elegant chemoenzymatic synthesis of sialyl Lewis X derivatives.[15] Herein, allyl glycoside $\mathbf{ED'_{amino}}$ was turned into the per-*O*,*N*-benzylated $2_{D'}$,$3_{D'}$-oxazolidinone $\mathbf{ED'_{oxa}}$ (34%). Oxazolidinone cleavage and subsequent *N*-acetylation gave acceptor $\mathbf{ED'_{OH}}$ (80%) bearing a free hydroxyl group at position $3_{D'}$ and a participating group at position $2_{D'}$, as originally planned. In support of the overall strategy, the chemical transformation of **ED′** into an acceptor and potential donor $\mathbf{ED'_{OH}}$ (5 steps) was thus shown feasible.

3. Discussion

Our results show that enzyme engineering technologies can be efficiently exploited to circumvent sugar organic synthesis boundaries and create newly designed glycoenzymes entering into a programmed chemoenzymatic pathway. This strategy has never been proposed before although it would be of major interest to develop new synthetic pathways. The semirational enzyme engineering proposed here allowed for the first time the design of α-transglucosidases with new and tremendously enhanced specificities toward non-natural protected acceptors, compatible with subsequent chemical elongation, thus validating the concept.

The first step of the envisioned synthetic pathway relied on the production of two α-D-glucosyl-based disaccharide motifs, α-D-Glc*p*-(1→3)-α-L-Rha*p*-OMe (**EA′**) and α-D-Glc*p*-(1→4)-α-D-Glc*p*NAc-OAll (**ED′**), compatible with chemical chain elongation at both ends. Enzymatic glucosylation was found attractive to overcome the poor α/β-stereoselectivity of the chemical glucosylation process.[26,27] The disaccharide synthesis required the specific glucosylation of two conveniently protected monosaccharides, i.e., α-L-Rha*p*-OMe (**A′**) and α-D-Glc*p*NAc-OAll (**D′**). This issue was an *a priori* critical limitation. In the absence of any known *in vitro* system mimicking the serotype-specific S*higella* regioselective glucosylation system, we turned to glucansucrases. Given that no native glucansucrase was able to synthesize the desired disaccharides in significant yields,[33] enzyme engineering had to be considered. A number of original glycosyltransferases[18,63] and promiscuous glycosidases[38−41] are issued from rational and combinatorial engineering. Engineering glycosynthases from β-glycosidases also emerged as a powerful way to generate modified transferases which use fluoride donors.[42−44] However, the engineering of the nucleophile in α-retaining enzymes seems to be more tricky as only one α-glycosynthase has been disclosed.[45] In a similar way, there is also no example of acceptor specificity engineering of α-transglycosidases described to date. Although novel regiospecificities of enzymes of the GH 70 family have been successfully engineered toward natural acceptors,[46] their adaptation to a non-natural acceptor substrate has not yet been reported. Their natural promiscuity was a good indicator of the acceptor binding site plasticity. In the absence of any applicable high throughput screening methods to isolate the desired variants,[47,48] a structure-based engineering strategy was thus adopted to redesign amylosucrase acceptor substrate specificity. The construction and screening of a small size library of variants focused on 7 positions of the subsite +1 first shell (Figure 3B) led to a high frequency of positive hits (45 mutants of interest out of 133 screened), outlining the efficiency of the semirational approach used to broaden acceptor specificity for this class of enzymes. These results emphasize the merits of targeted randomization leading to small size and smart libraries when changes of specificity are searched.[49] A totally new specificity for methyl α-L-rhamnopyranoside (**A′**) glucosylation and a remarkable improvement (×130) for allyl 2-*N*-acetyl-2-deoxy-α-D-glucopyranoside (**D′**) glucosylation were obtained with mutants I228Y and F290K, respectively. Both mutants displayed a very high catalytic efficiency of acceptor glucosylation compared to that of wtAS. During the acceptor reaction, there is competition between the natural acceptors of wtAS (water, glucose, fructose, maltooligosaccharides, Figure 2) and the exogenous acceptor molecule. Reshaping the acceptor binding site has directed transglucosylation toward exogenous acceptor glucosylation at the detriment of the natural reactions and enhanced the rate of the enzyme deglucosylation, which follows the β-D-glucosyl-enzyme formation.

All 7 targeted positions are located on flexible loops that connect β-strands with α-helices of the wtAS $(\beta/\alpha)_8$ catalytic barrel and encircle subsite +1. Interestingly, key positions 290 and 228 which are in close vicinity are also conserved among known amylosucrases. Both of them tolerate a wide range of amino acid substitutions that result in increased non-natural acceptor glucosylation degrees (Figure 4). Docking of α-D-Glc*p*-(1→4)-α-D-Glc*p*NAc-OAll (**ED′**) in the active site of F290K indicated a favorable binding compared to wtAS, with the predicted free energy of binding stabilized by up to 3 kcal/mol. Docking of α-D-Glc*p*-(1→3)-α-L-Rha*p*-OMe (**EA′**) in the I228Y active site necessitated the rearrangement of several residues in the vicinity of I228, including F290, thus suggesting close residue interdependence at these positions.

The mutants were used to synthesize gram amounts of target disaccharides, **EA′** and **ED′**, next purified as their peracetylated analogues. Interestingly, peracetylated $\mathbf{EA'_{Ac}}$ and $\mathbf{ED'_{Ac}}$ served as useful intermediates in the conversion of **EA′** and **ED′** into

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EA′$_{OH}$ and **ED′$_{OH}$**, respectively. The efficient transformation (7 steps, 3 purifications) of intermediate **EA′$_{Ac}$** into the known building block **EA′$_{OH}$**, used in the synthesis of *S. flexneri* oligosaccharides on several occasions,[27,29] validates the proof of concept. Indeed, as a whole, this newly disclosed chemoenzymatic protocol to **EA′$_{OH}$** comprises one enzymatic step involving a low cost donor (sucrose), an easily available acceptor (**A′**), and eight additional chemical steps. It compares favorably with the full chemical synthesis of **EA′$_{OH}$** (11 steps) since the number of chemical steps is lowered. Furthermore, the proposed chemoenzymatic route substitutes the use of chemically obtained 2,3,4,6-tetra-*O*-benzyl-D-glucopyranose by sucrose as a glucosyl unit donor. The enzymatic glucosylation yield of **A′** (44%) is still low compared to that of **D′** (78%). However, having demonstrated the feasibility of the whole strategy, we are now considering additional engineering and recombinations of monomutants to further improve the enzymatic conversion of **A′**. This chemoenzymatic process may also be seen as a new alternative to the chemical synthesis of *S. flexneri* 1a O-antigen fragments[29] which involved a sophisticated prearranged-glycoside mediated intramolecular glucosylation strategy developed to ensure the correct stereochemical outcome.[50] Needless to say that the chemoenzymatic strategy can be extended to other applications since building block **EA′$_{OH}$** could serve as an intermediate in the synthesis of any oligosaccharide having the →3)-[α-D-Glc*p*-(1→3)]-α-L-Rha*p*-(1→ branching pattern.

Well-illustrating the potential of enzyme semirational engineering, this is to our knowledge the first report on α-transglycosidase engineering, purposely tailored toward non-natural acceptors. Additionally, mutants selected in this study were shown to glucosylate other protected acceptors (allyl α-L-rhamnopyranoside, 2-*N*-trichloroacetyl-D-glucosamine, methyl 2-acetamido-2-deoxy-α-D-glucopyranoside, methyl 2-acetamido-2-deoxy-β-D-glucopyranoside, and allyl 2-acetamido-2-deoxy-β-D-glucopyranoside) with highly increased degrees of conversion compared to the wild-type enzyme (Supplementary Table S3), thus offering additional synthetic route alternatives. In addition, enzymes disclosed herein might serve in the synthesis of selected fragments of several microbial polysaccharides comprising the α-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl and/or α-D-glucopyranosyl-(1→4)-*N*-acetyl-β-D-glucosaminyl moieties in their repeating unit.[51−54] Overall, the concept validation demonstrated herein offers new opportunities in the synthesis of complex oligosaccharides providing that appropriate combinations of donors, acceptors, and transglucosidases are defined based on compatibility with chemical chain elongation.

4. Experimental Section

4.1. Bacterial Strains, Plasmids, and Chemicals. Plasmid pGST-AS, derived from the pGEX-6P-3 (GE Healthcare Biosciences) and containing the *Neisseria polysaccharea* amylosucrase (AS) encoding gene,[55] was used for the construction of the AS single mutant library. *Escherichia coli* JM109 was used as the host for the plasmid library transformation, gene expression, and large-scale production of the selected mutants. Sucrose, *N*-acetyl-D-glucosamine (**D**), and glycogen were purchased from Sigma-Aldrich (Saint-Louis, MO, USA). L-Rhamnose (**A**) was purchased from Carbosynth (Newbury, Berkshire, UK). Methyl α-L-rhamnopyranoside (**A′**)[25] and allyl 2-acetamido-2-deoxy-α-D-glucopyranoside (**D′**)[56] were prepared according to known protocols. Reference disaccharides α-D-Glc*p*-(1→3)-α-L-Rha*p*-OMe (**EA′**),[26] β-D-Glc*p*-(1→3)-α-L-Rha*p*-OMe,[57] and α-D-Glc*p*-(1→4)-α-L-Rha*p*-OMe[58] were synthesized at the Unité de Chimie des Biomolécules, Institut Pasteur, France. Disaccharides α-D-Glc*p*-(1→4)-D-Glc*p*NAc (**ED**) and α-D-Glc*p*-(1→6)-D-Glc*p*NAc were enzymatically synthesized and characterized at LISBP, Université de Toulouse, France.[33] Ampicillin, lysozyme, and isopropyl β-D-thiogalactopyranoside (IPTG) were purchased from Euromedex (Souffelweyersheim, France), and *Dpn*I restriction enzyme was purchased from New England Biolabs (Beverly, MA, USA). Oligonucleotides were synthetized by Eurogenetec (Liege, Belgium). DNA extraction (QIASpin) and purification (QIAQuick) columns were purchased from Qiagen (Chatsworth, CA).

4.2. Construction, Expression, and Screening of Mutant Library. Single mutagenesis, focused on subsite +1 amino acids selected from ligand docking, was carried out with the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer instructions and using pGST-AS G537D as a vector template. It was checked that this mutation had no impact on the native enzyme catalytic properties. The bacterial strains, plasmids, chemicals, and complementary primers used are listed in Supplementary Tables S1 and S2.

PCR amplification was carried out with Pfu DNA polymerase (2.5 U) for 16 cycles (95 °C, 30 s; 55 °C, 30 s; 72 °C, 12 min). DNA was digested with Dpn*I* to eliminate the methylated parental template and purified using a Qiaquick spin column, following the manufacturer recommendations. *E. coli* JM109 was transformed with the plasmid and plated on LB agar supplemented with 100 μg/mL ampicillin. For each construction, two clones were isolated and their corresponding plasmids stored at −20 °C. Seventeen mutants (I228A, I228V, I228Y, A289D, F290D, F290K, F290Q, I330A, I330D, I330E, I330F, I330T, I330W, V331A, V331S, D394V and R446K) were sequenced on the entire gene by Millegen (Labège, France) or Cogenics (Meylan, France) and showed no other mutations.

The protocol was established to enable the rapid identification of (i) clones able to glucosylate **A′** and (ii) clones for which **D** glucosylation was improved. To obtain larger amounts of enzymes and facilitate detection of glucosylated compounds by HPLC, the mutants were produced in 96-Deep Well Format plates. Storage microplates containing monomutants were thawed and replicated to inoculate a starter culture in 96-well microplates containing, in each well, 150 μL of LB medium supplemented with ampicillin (100 μg/mL). After growth for 24 h at 30 °C under agitation (200 rpm), plates were duplicated into 96-Deep Well plates containing, in each well, 1.1 mL of LB medium supplemented with ampicillin (100 μg/mL) and IPTG (1 mM) to induce Glutathion S-transferase-Amylosucrase fusion protein (GST-AS) expression. Cultures were then grown for 24 h at 30 °C under agitation (200 rpm). Plates were centrifuged (20 min, 3000 *g*, 4 °C), and the supernatant was removed. The cell pellet was resuspended in 200 μL of lysozyme (0.5 mg/mL), followed by freezing at −80 °C for 8 to 12 h. After thawing at room temperature, 100 μL of sucrose and 100 μL of acceptor (each at a final concentration of 73 mM) were added to each well. The enzymatic reaction was incubated at 30 °C for 24 h under agitation. The Deep Well plates were then centrifuged (20 min, 3000 *g*, 4 °C), and 300 μL of the supernatant were transferred to a filter microplate (Glass fiber membrane, PS, 0.25 mm pore) to be clarified. Supernatant filtration was carried out by centrifugation

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of the filter microplate (5 min, 2000 *g*, 4 °C) into a novel microplate for screening.

Efficiency of the glucosylation reaction was evaluated by HPLC analysis of the acceptor reaction product synthesized when using either **A′** or **D** as acceptors using a Dionex P 680 series pump, a Shodex RI 101 series refractometer, a Dionex UVD 340 UV/vis detector, and autosampler HTC PAL. HPLC analyses were performed using two columns: (1) a Biorad HPLC Carbohydrate Analysis column (HPX-87K column (300 mm × 7.8 mm)) maintained at 65 °C, using ultrapure water as eluent with a flow rate of 0.6 mL/min; (2) a reversed phase analytical column (Synergi C18RPFusion, 4 μm, 30 mm × 4.6 mm) kept at room temperature, with 1 mL/min of ultrapure water as eluent. The HPX-87K column was used to determine sucrose consumption by RI detection and concomitant **ED** formation by UV_{220nm} detection. The C18RP fusion column served to detect **EA′** formation. The acceptor conversion degree was calculated as follows: Conversion degree = ([Acceptor]t_0 − [Acceptor]t_f)/[Acceptor]t_0 × 100, where [Acceptor] represents the molar acceptor concentration, and t_0 and t_f are the initial and final reaction times, respectively.

4.3. Production, Purification and Characterization of the Selected Variants: I228Y and F290K. Production and purification of AS variants were performed as previously described.[55,59] Since pure GST-AS possesses the same function and the same efficiency as pure AS, enzymes were purified to the GST-AS fusion protein stage (96 kDa). All assays were performed at 30 °C in 50 mM Tris buffer, pH = 7.0. I228Y was tested on **A′**, F290K on **D′**, and wtAS on both **A′** and **D′** acceptors. The specific activity of the purified enzymes was determined by measuring the initial rate of released fructose under standard conditions (146 mM sucrose). Fructose concentration was determined using the dinitrosalycilic acid (DNS) method.[60] One unit of AS variant corresponds to the amount of enzyme that catalyzes the production of 1 μmol of fructose per min under the assay conditions.

4.4. Comparison of Products Synthesized by Wild-Type (wtAS) and AS Variants. Reactions were performed in the presence of 146 mM sucrose alone or supplemented with 146 mM acceptor. The purified wild-type or mutated GST-AS was employed at 0.5 U/mL. The reactions were stopped after 24 h 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. In addition to HPLC analyses (see above), different analyses were also performed to compare the product profiles synthesized by wtAS and AS variants (I228Y and F290K): soluble and insoluble oligosaccharides produced during the reaction were identified by HPAEC using a Dionex Carbo-Pack PA100 column at 30 °C. Before analysis, the insoluble fraction was solubilized in 1 M aq KOH at a final total sugar concentration of 10 g/L. Mobile phase (150 mM aq NaOH) was set at a 1 mL/min flow rate with a sodium acetate gradient (6 to 500 mM over 120 min). Detection was performed using a Dionex ED40 module with a gold working electrode and a Ag/AgCl reference electrode. Note that acceptors **A′**, **D′**, and their derivatives are not oxidable products and thus are not detectable by HPAEC. Therefore, sucrose, glucose, fructose, **A′**, **D′**, and their glucosylation products were quantified by HPLC (see above).

4.5. Determination of Kinetic Parameters. Enzyme assays were carried out in a total volume of 2 mL containing pure enzyme (115 μg, 2.6 mg and 106 μg when using wtAS, I228Y, and F290K, respectively). Catalytic efficiency (k_{cat}/K_m) of wtAS and AS variants was determined with (i) sucrose alone and with (ii) both sucrose and acceptor **A′** (or **D′**) as variable substrates. In the presence of sucrose, as a sole substrate whose concentration varied between 0 and 600 mM, $k_{cat}/K_{m,Suc}$ values were determined by measuring the released fructose. Fructose formation reflects the consumption of sucrose. In the presence of sucrose and acceptor, both catalytic efficiencies, $k_{cat}/K_{m,Suc}$ and $k_{cat}/K_{m,A'}$ (or $k_{cat}/K_{m,D'}$) values were determined by the rate of formation of the desired disaccharide. For the determination of $k_{cat}/K_{m,Suc}$, the **A′** (or **D′**) concentration was held constant at 250 mM and the sucrose concentration was varied between 0 and 600 mM. For the determination of $k_{cat}/K_{m,A'}$ (or $k_{cat}/K_{m,D'}$), sucrose concentration was held constant at 250 mM and **A′** (or **D′**) concentration was varied between 0 and 250 mM. Experiments were performed until reaching sucrose and **A′** (or **D′**) solubility limits.

Initial velocities were fitted to the Michaelis−Menten equation using Sigma-Plot. When saturation was not achieved with the mutant, efficiency was calculated by linear regression analysis of a velocity versus substrate concentration plot. Aliquots (200 μL) were removed between 0 and 60 min (at which time product formation was still linear with respect to time), heated at 95 °C for 5 min, and centrifuged at 18 000 *g* for 5 min. The final mixtures were filtered on a 0.22 μm membrane and analyzed using HPLC material previously described.

4.6. Preparative Synthesis of the Acceptor Reaction Product (see also below for the general purification and analysis protocols). To characterize the products of **D** glucosylation, the synthesis was carried out on a preparative scale, using a 10 mL mixture containing sucrose (146 mM), **D** (146 mM), and 1.5 U/mL of purified F290K mutant. The mixture was incubated at 30 °C for 24 h. Then, the reaction mixture was centrifuged (4800 rpm, 20 min, 4 °C) to remove proteins and filtered on a 0.22 μm membrane. Mono- and diglucosylated acceptor reaction products were separated by reversed phase preparative chromatography using C18 columns (Bischoff Chromatography). Ultrapure water was used as eluent at a constant flow rate of 50 mL/min. Glucose detection was carried out with a refractometer (Bischoff). Each peak was collected separately, concentrated, and reinjected into the analytical HPLC system described above to ascertain purity. Mass spectrometry, HPLC, and NMR analysis of analytical samples of the isolated glucosylation products showed that the first eluting product corresponds to the α-D-glucopyranosyl-(1→4)-*N*-acetyl-D-glucosamine, a disaccharide previously obtained using wtAS and characterized elsewhere.[33] The second eluting product corresponds to a trisaccharide tentatively identified as a diglucosylated **D**.

Oligosaccharides issued from **D′** glucosylation were produced in a 100 mL batch containing sucrose (146 mM), and **D′** (146 mM), using 4 U/mL of nonpurified F290K extract (sonication supernatant) at 30 °C for 24 h. The products of glucosylation were identified following silica gel column chromatography (CH_3CN-H_2O, 9:1) which gave first a mixture of compounds containing **ED′**, and then as the second eluting product, the pure trisaccharide **E_1ED′**. The mixture of compounds eluting first was peracetylated in pyridine containing a large excess of acetic anhydride. The residue was purified by column chromatography (cyclohexane−EtOAc, 6:4) to give a major compound **ED′$_{Ac}$** (see below) which was identified following transesterification into **ED′** using methanolic sodium methoxide and subsequent silica gel chromatography, eluting with 10% H_2O in CH_3CN.

4.6.1. Allyl α-D-Glucopyranosyl-(1→4)-2-acetamido-2-deoxy-α-D-glucopyranoside (ED′). R_f = 0.3 (CH_3CN-H_2O, 9:1), $[\alpha]_D$ = +16.4 (c = 1.1, water); ^{1}H NMR (D_2O) δ 5.95 (m, 1H, CH=), 5.38 (d, 1H, $J_{1,2}$ = 3.9 Hz, H-1_E), 5.34 (m, 1H, =CH_2), 5.25 (m, 1H, =CH_2), 4.91 (d, 1H, $J_{1,2}$ = 3.5 Hz, H-1_D), 4.20 (m, 1H, OCH_2), 4.02 ($m_{overlapped}$, 1H, OCH_2), 4.01 ($dd_{overlapped}$, 1H, $J_{3,4}$ = 8.4 Hz, H-3_D), 3.94 (dd, 1H, $J_{2,3}$ = 10.8 Hz, H-2_D), 3.89−3.67 (m, 7H, H-4_D, H-5_D, H-$6a_D$, H-$6b_D$, H-5_E, H-$6a_E$, H-$6b_E$), 3.66 (pt, 1H, H-3_E), 3.56 (dd, 1H, $J_{2,3}$ = 9.9 Hz, H-2_E), 3.40 (pt, 1H, $J_{3,4}$ = $J_{4,5}$ = 9.5 Hz, H-4_E), 2.02 (s, 3H, Ac); ^{13}C NMR (D_2O) δ 174.5 (C=O), 133.7 (CH=), 118.1 (=CH_2), 99.9 (C-1_E), 96.0 (C-1_D), 77.6 (C4_D), 72.9 (C-3_E), 72.8 (C-5_E), 71.8 (C-2_E), 71.5 (C-3_D), 70.6 (C-5_D), 69.4 (C-4_E), 68.6 (OCH_2), 60.6 (2C, C-6_D, C-6_E), 53.5 (C-2_D), 21.9 (Ac). HRMS (ESI^+) for $C_{17}H_{30}NO_{11}$ ($[M + H]^+$, 424.1819) found m/z 424.1821.

4.6.2. Allyl α-D-Glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-α-D-glucopyranoside (E_1ED′). R_f = 0.3 (CH_3CN-H_2O, 9:1). $[\alpha]_D$ = +15.0 (c = 1.0, water); ^{1}H NMR

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(D_2O) δ 5.91 (m, 1H, CH═), 5.35−5.32 (m, 2H, H-1_E, H-1_{E1}), 5.29 (m, 1H, ═CH_2), 5.20 (m, 1H, ═CH_2), 4.86 (d, 1H, $J_{1,2}$ = 3.5 Hz, H-1_D), 4.15 (m, 1H, OCH_2), 4.01−3.93 (m, 2H, H-3_D, OCH_2), 3.92−3.85 (m, 2H, H-2_D, H-3_E), 3.83−3.58 (m, 12H, H-4_D, H-5_D, H-6a_D, H-6b_D, H-4_E, H-5_E, H-6a_E, H-6b_E, H-3_{E1}, H-5_{E1}, H-6a_{E1}, H-6b_{E1}), 3.56 ($dd_{overlapped}$, 1H, $J_{1,2}$ = 4.0 Hz, $J_{2,3}$ = 10.0 Hz, H-2_E), 3.52 ($dd_{overlapped}$, 1H, $J_{1,2}$ = 3.8 Hz, $J_{2,3}$ = 9.8 Hz, H-2_{E1}), 3.35 (pt, 1H, $J_{3,4}$ = $J_{4,5}$ = 9.3 Hz, H-4_{E1}), 1.97 (s, 3H, Ac); ^{13}C NMR (D_2O) δ 174.5 (C═O), 133.7 (CH═), 118.1 (═CH_2), 99.7 (C-1_E, $J_{C,H}$ = 171.6 Hz), 99.9 (C-1_{E1}, $J_{C,H}$ = 172.2 Hz), 96.0 (C-1_D), 77.7 (C-4_D), 76.9 (C-4_E), 73.4 (C-3_E), 72.9 (C-3_{E1}), 72.8 (C-5_{E1}), 71.8, 71.6 (2C, C-2_E, C-2_{E1}), 71.5 (C-3_D), 71.3 (C-5_E), 70.5 (C-5_D), 69.4 (C-4_{E1}), 68.6 (OCH_2), 60.6 (3C, C-6_D, C-6_E, C-6_{E1}), 53.5 ($C2_D$), 21.9 (Ac). HRMS (ESI^+) for $C_{23}H_{39}NO_{16}$ ([M + Na]$^+$, 608.2167) found *m/z* 608.2172.

Along the same line, oligosaccharides issued from **A′** glucosylation were produced in a 500 mL batch containing sucrose (146 mM) and **A′** (100 mM), using 0.25 U/mL of nonpurified I228Y extract at 30 °C for 24 h. Column chromatography of the crude material provided three fractions. The intermediate fraction composed of a mixture of compounds which included **EA′** was peracetylated in pyridine containing a large excess of acetic anhydride. The residue was purified by column chromatography (toluene−EtOAc, 7:3) to give a major compound **EA′$_{Ac}$** (see below) which was identified following transesterification into **EA′** using methanolic potassium carbonate and subsequent reversed phase chromatography, eluting with H_2O. The structure of **EA′** was confirmed by mass spectrometry and NMR analysis.

4.6.3. Methyl α-D-Glucopyranosyl-(1→3)-α-L-rhamnopyranoside (EA′). NMR data were identical to those of **EA′** issued from chemical synthesis.[26,61] HRMS (ESI^+) for $C_{13}H_{24}O_{10}$ ([M + Na]$^+$, 363.1267) found *m/z* 363.1304.

4.7. Molecular Modeling. All molecular modeling calculations were performed using the Sybyl 7.3 software (Tripos Inc., Saint Louis, MO, USA). The coordinates of amylosucrase were taken from the 2.0 Å resolution crystal structures of amylosucrase from *N. polysaccharea* in complex with sucrose (PDB: 1JGI) and maltoheptaose, a reaction product (PDB: 1MW0). All hydrogen atoms were added to the enzyme, and their positions optimized with the Tripos force field. **EA′** and both anomers of **ED** disaccharides were subjected to a systematic grid search study of the glycosidic linkage conformation. Starting from energy-minimized disaccharide conformations, a two-dimensional systematic conformational search was performed by rotating the two torsion angles defining the glycosidic linkages, Φ and Ψ, by 20° steps: Φ = O5′−C1′−O3−C3′ and Ψ = C1′−O3−C3′−C2′ for **EA′** and Φ = O5′−C1′−O4−C4 and Ψ = C1′−O4−C4−C3 for **ED**. The MM3 force field implemented in Sybyl 7.3 software was used for this purpose together with the energy parameters appropriate for carbohydrates. The geometries were optimized at each point of the grid with the driver option that keeps fixed the atoms defining the torsion angles. The relaxed conformational maps (data not shown) obtained for all disaccharides were then used to locate the different energy minima that were subsequently fully relaxed. The lowest energy conformations identified on the disaccharides potential energy maps were used as starting structures to be docked in the binding site of AS. This was performed by superimposing the glucosyl unit of the disaccharides at subsite −1 onto the glucosyl unit of the crystallographic maltoheptaose. Each of these AS/disaccharide complexes was optimized by means of the appropriate energy parameters. The annealing method implemented in Sybyl 7.3 software was used to optimize the complexes. Two shells of amino acids were considered: a 12 Å shell centered on the binding site was taken into account for the energy calculations. A 6 Å shell region closest to the carbohydrate was defined as the hot region to be optimized. The positions of all atoms included in this region were optimized using Powell's method.

Flexible docking of product disaccharides (**EA′** and **ED**) in AS mutants of interest was performed using the latest version of the automated Autodock 4.0 software (Scripps Institute, La Jolla, CA, USA) and the specific empirical free energy model developed for carbohydrate−protein docking.[62] All torsion angles of the disaccharides were considered flexible during the search as well as the side chains of eight amino acid residues of the enzyme: I228, F229, F290, I330, V331, D394, R446 and A289. A Lamarckian genetic algorithm (LGA) combining a modified pseudo-Solis and Wets local search routine was used to determine the docked conformations. For each ligand, the population was set to 100 with 2 500 000 energy evaluations. A 60 × 60 × 60-point grid map was used, centered on the ligand midpoint.

4.8. Chemical Conversion of A′ I228Y Glucosylation Product and D′ F290K Glucosylation Product into Building Blocks Acting as Potential Donors and/or Acceptors. TLC was performed on precoated slides of Silica Gel 60 F_{254} (Merck). Detection was effected with UV light and/or by charring in 5% sulfuric acid in ethanol. Preparative chromatography was performed by elution from columns of Silica Gel 60 (particle size 0.040−0.063 mm). NMR spectra were recorded at 30 °C (400 MHz for ^{1}H, 100 MHz for ^{13}C). Proton-signal assignments were made by first-order analysis of the spectra, as well as analysis of 2D ^{1}H−^{1}H correlation maps (COSY). The ^{13}C NMR assignments were supported by 2D ^{13}C−^{1}H correlations maps (HSQC and HMBC). Optical rotations were measured for $CHCl_3$ and water solutions at 25 °C, as indicated, with a Bellingham (BS+) automatic polarimeter, Model ADP 220. Electrospray ionization/time-of-flight (ESI-TOF) mass spectra were recorded in the positive-ion mode with 1:1 acetonitrile/water containing 0.1% formic acid as the ESI-TOF spectrometer solution.

4.8.1. Methyl (2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl)-(1→3)-2,4-di-*O*-acetyl-α-L-rhamnopyranoside (EA′$_{Ac}$). The crude mixture issued from the enzymatic glucosylation of **A′** (9.1 g, 50 mmol) by use of nonpurified I228Y extract was purified by column chromatography (CH_3CN−H_2O, 9:1) to give contaminated unreacted acceptor (7.7 g) as the first eluting product, followed by the target disaccharide **EA′** contaminated by the more polar fructose (17.5 g) as the second eluting product. Acetic anhydride (100 mL) was added dropwise to a solution of contaminated **EA′** (17.5 g) in anhydrous pyridine (100 mL) stirred at 0 °C. The solution was stirred overnight at room temperature. TLC (toluene−EtOAc, 6:4) showed the complete disappearance of the starting materials and the presence of three less polar products. The mixture was concentrated under reduced pressure, and volatiles were eliminated by repeated coevaporation with toluene. The residue was purified by column chromatography (toluene−EtOAc, 7:3) to give **EA′$_{Ac}$** (4.9 g) as a colorless amorphous solid. R_f = 0.45 (toluene−EtOAc, 6:4); $[\alpha]_D$ = +5.6 (c = 1.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 5.30 (dd, 1H, $J_{3,4}$ = 9.4 Hz, H-3_E), 5.18 (dd, 1H, $J_{2,3}$ = 3.2 Hz, H-2_A), 5.11 (d, 1H, H-1_E), 5.08 (dd, 1H, $J_{3,4}$ = 9.8 Hz, H-4_A), 5.05 (dd, 1H, $J_{4,5}$ = 9.4 Hz, H-4_E), 4.91 (dd, 1H, $J_{1,2}$ = 3.4 Hz, $J_{2,3}$ = 10.3 Hz, H-2_E), 4.61 (d, 1H, $J_{1,2}$ = 1.7 Hz, H-1_A), 4.15 ($dd_{overlapped}$, 1H, $J_{5,6a}$ = 4.3 Hz, $J_{6a,6b}$ = 12.4 Hz, H-6a_E), 4.13 ($dd_{overlapped}$, 1H, $J_{5,6bE}$ = 2.5 Hz, H-6b_E), 4.07−4.02 (m, 2H, H-5_E, H-3_A), 3.72 (dq, 1H, $J_{4,5}$ = 9.8 Hz, H-5_A), 3.33 (s, 1H, OCH_3), 2.13, 2.12, 2.06, 2.00, 1.98, 1.94 (6s, 18H, 6Ac), 1.21 (d, 3H, $J_{5,6}$ = 6.3 Hz, H-6_A); ^{13}C NMR ($CDCl_3$) δ 170.5, 170.0, 169.9, 169.8, 169.6, 169.3 (6C, 6C═O), 98.5 (C-1_A, $^1J_{CH}$ = 166.5 Hz), 93.6 (C-1_E, $^1J_{CH}$ = 174.6 Hz), 72.1 (C-3_A), 71.8 (C-4_A), 69.7 (2C, C-2_E, C-3_E), 68.5 (C-4_E), 67.9 (C-2_A), 67.7 (C-5_E), 66.6 (C-5_A), 61.6 (C-6_E), 55.0 (OCH_3), 20.9, 20.8, 20.7, 20.6, 20.5, 20.4 (6C, 6Ac), 17.5 (C-6_A). HRMS (ESI^+) for $C_{25}H_{36}O_{16}$ ([M + Na]$^+$, 615.1901) found *m/z* 615.1861, ([M + NH_4]$^+$, 610.2347) found *m/z* 610.2309.

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4.8.2. (2,3,4,6-Tetra-*O*-benzyl-α-D-glucopyranosyl)-(1→3)-1,2-*O*-allyloxyethylidene-4-*O*-benzyl-β-L-rhamnopyranose (EA′$_{ortho}$). R_f = 0.5 (cyclohexane−EtOAc, 7:3); $[\alpha]_D$ = +4.5 (c = 1.2, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 7.49−7.09 (m, 25H, CH_{Ph}), 5.96 (m, 1H, CH═), 5.38 (d, 1H, $J_{1,2}$ = 2.3 Hz, H-1$_A$), 5.37 (d, 1H, $J_{1,2}$ = 3.5 Hz, H-1$_E$), 5.30 (m, 1H, J_{trans} = 17.2 Hz, ═CH_2), 5.18 (m, 1H, J_{cis} = 10.4 Hz, ═CH_2), 5.06 (d, 1H, J = 11.0 Hz, H_{Bn}), 4.98 (d, 1H, J = 10.1 Hz, H_{Bn}), 4.93−4.87 (m, 3H, H_{Bn}), 4.77 (d, 1H, J = 11.8 Hz, H_{Bn}), 4.72 (dd, 1H, H-2$_A$), 4.66 (d, 1H, J = 10.1 Hz, H_{Bn}), 4.58 (d, 1H, J = 12.1 Hz, H_{Bn}), 4.51 (d, 1H, J = 11.0 Hz, H_{Bn}), 4.38 (d, 1H, J = 12.1 Hz, H_{Bn}), 4.17 (pt$_{overlapped}$, 1H, $J_{3,4}$ = 9.3 Hz, H-3$_E$), 4.12 (m$_{overlapped}$, 2H, H_{All}), 4.06 (m$_{overlapped}$, 1H, H-5$_E$), 4.02 (dd$_{overlapped}$, 1H, $J_{2,3}$ = 4.0 Hz, $J_{3,4}$ = 9.2 Hz, H-3$_A$), 3.76−3.72 (m, 2H, H-2$_E$, H-4$_E$), 3.67 (dd$_{overlapped}$, 1H, $J_{5,6aE}$ = 4.0 Hz, H-6a$_E$), 3.65 (dd, 1H, H-4$_A$), 3.58 (dd, 1H, $J_{5,6bE}$ = 1.8 Hz, $J_{6aE,6bE}$ = 10.8 Hz, H-6b$_E$), 3.36 (dq, 1H, $J_{4,5}$ = 9.3 Hz, H-5$_A$), 1.83 (s, 3H, CH_{3ortho}), 1.42 (d, 3H, $J_{5,6}$ = 6.3 Hz, H-6$_A$); ^{13}C NMR ($CDCl_3$) δ 138.7−137.6 (C_{Ph}), 134.2 (CH═), 128.8−127.5 (CH_{Ph}), 124.0 (C_{ortho}), 116.5 (═CH_2), 97.6 (C-1$_A$, $^1J_{CH}$ = 173.6 Hz), 92.1 (C-1$_E$, $^1J_{CH}$ = 168.2 Hz), 82.2 (C-3$_E$), 78.9 (C-2$_E$), 78.6 (C-4$_A$), 77.5 (C-4$_E$), 76.2, 75.7, 75.0 (3C, C_{Bn}), 74.9 (C-2$_A$), 73.9 (C-3$_A$), 73.3, 72.1 (2C, C_{Bn}), 70.3 (2C, C-5$_E$, C-5$_A$), 68.3 (C-6$_E$), 63.6 (C_{All}), 25.1 (CH_{3ortho}), 17.9 (C-6$_A$); HRMS (ESI$^+$) for $C_{52}H_{58}O_{11}$ ([M + Na]$^+$, 881.3877) found *m*/*z* 881.3655.

4.8.3. Allyl 2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-α-D-glucopyranoside (ED′$_{Ac}$). The crude mixture issued from the enzymatic glucosylation of **D′** (3.80 g, 14.54 mmol) was purified by silica gel column chromatography (CH_3CN-H_2O, 9:1) to give a mixture of **ED′** and fructose (4.16 g) and the pure trisaccharide **E$_I$ED′** (2.53 g, 4.32 mmol). Acetic anhydride (80 mL, 725 mmol) was added dropwise to a solution of the above-mentioned disaccharide **ED′** mixed to fructose in anhydrous pyridine (80 mL). The resulting mixture was stirred at room temperature under argon. After 2 days, TLC showed the complete disappearance of the starting materials. The mixture was concentrated under reduced pressure, and volatiles were eliminated by repeated coevaporation with toluene. The residue was purified by column chromatography (cyclohexane−EtOAc, 6:4) to give **ED′**$_{AC}$ (2.96 g,) as a colorless amorphous solid. Compound **ED′**$_{AC}$ had R_f = 0.6 (CH_2Cl_2−MeOH, 95:5); $[\alpha]_D$ = +13.0 (c = 1.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 5.90 (m, 1H, CH═), 5.71 (d, 1H, J = 9.7 Hz, NH), 5.45 (d, 1H, J_{1-2} = 4.0 Hz, H-1$_E$), 5.35 (dd, 1H, $J_{3,4}$ = 9.6 Hz, H-3$_E$), 5.34−5.23 (m, 3H, ═CH_2, H-3$_D$), 5.04 (pt, 1H, $J_{4,5}$ = 9.8 Hz, H-4$_E$), 4.85 (dd, 1H, $J_{2,3}$ = 10.5 Hz, H-2$_E$), 4.79 (d, 1H, $J_{1,2}$ = 3.6 Hz, H-1$_D$), 4.42 (dd, 1H, $J_{5,6a}$ = 2.0 Hz, $J_{6a,6b}$ = 12.0 Hz, H-6a$_D$), 4.23−4.15 (m, 4H, H_{All}, H-2$_D$, H-6b$_D$, H-6a$_E$), 4.04−3.91 (m, 5H, H_{All}, H-4$_D$, H-5$_D$, H-5$_E$, H-6b$_E$), 2.13, 2.08, 2.01, 2.00, 1.98, 1.91 (7s, 21H, 7Ac); ^{13}C NMR ($CDCl_3$) δ 171.6, 170.6, 170.5, 170.2, 170.0, 169.4 (7C, 7C═O), 133.0 (CH═), 118.7 (═CH_2), 96.1 (C-1$_D$), 95.5 (C-1$_E$), 74.1 (C-3$_D$), 72.4 (C-4$_D$), 70.1 (C-2$_E$), 69.4 (C-3$_E$), 68.8 (C_{All}), 68.4 (C-5$_E$), 68.0 (C-5$_D$), 67.9 (C-4$_E$), 62.8 (C-6$_D$), 61.4 (C-6$_E$), 52.2 (C-2$_D$), 23.1, 21.0, 20.8, 20.7, 20.6, 20.5 (7C, 7Ac). HRMS (ESI$^+$) for $C_{29}H_{41}NO_{17}$ ([M + Na]$^+$, 698.2272) found *m*/*z* 698.2277.

4.8.4. Allyl 2,3,4,6-Tetra-*O*-benzyl-α-D-glucopyranosyl-(1→4)-6-*O*-benzyl-2-benzylamino-2-*N*,3-*O*-carbonyl-2-deoxy-α-D-glucopyranoside (ED′$_{oxa}$). R_f = 0.3 (cyclohexane−EtOAc, 4:1). $[\alpha]_D$ = +6.3 (c = 2.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 7.46−7.16 (m, 30H, CH_{Ph}), 5.79 (m, 1H, CH═), 5.56 (d, 1H, $J_{1,2}$ = 3.5 Hz, H-1$_E$), 5.26 (m, 2H, ═CH_2), 5.02 (d$_{overlapped}$, 1H, H_{Bn}), 4.87−4.80 (m, 3H, H_{Bn}), 4.79−4.72 (m$_{overlapped}$, 3H, H-1$_D$, H-3$_D$, H_{Bn}), 4.56−4.49 (m, 4H, H_{Bn}), 4.47 (m, 2H, H_{Bn}), 4.35 (pt$_{overlapped}$, 1H, H-4$_D$), 4.34 (d$_{overlapped}$, 1H, H_{Bn}), 4.05 (m, 1H, H_{All}), 3.92−3.88 (m, 2H, H-5$_D$, H-3$_E$), 3.81 (dd, 1H, $J_{5,6a}$ = 3.5 Hz, $J_{6a,6b}$ = 11.0 Hz, H-6a$_D$), 3.73−3.66 (m, 4H, H_{All}, H-6b$_D$, H-4$_E$, H-5$_E$), 3.65 (dd$_{overlapped}$, 1H, H-2$_E$), 3.57 (dd, 1H, $J_{5,6a}$ = 2.7 Hz, $J_{6a,6b}$ = 10.8 Hz, H-6a$_E$), 3.43 (dd, 1H, $J_{5,6b}$ = 1.4 Hz, H-6b$_E$), 3.37 (dd, 1H, $J_{1,2}$ = 2.7 Hz, $J_{2,3}$ = 11.8 Hz, H-2$_D$); ^{13}C NMR ($CDCl_3$) δ 158.7 (C═O), 138.9, 138.5, 138.1, 137.9, 137.7, 135.3 (6C, C_{Ph}), 133.1 (CH═), 128.8, 128.7, 128.5, 128.4, 128.3, 128.2, 127.9, 127.8, 127.6, 127.5, 127.3 (30C, C_{Ph}), 118.3 (═CH_2), 95.4 (C-1$_E$), 94.4 (C-1$_D$), 81.8 (C-3$_E$), 79.5 (C-2$_E$), 77.4 (C-4$_E$), 76.9 (C-3$_D$), 75.6, 75.1, 73.5, 73.1 (5C, C_{Bn}), 72.1 (C-5$_D$), 71.3 (C-5$_E$), 70.9 (C-4$_D$), 69.1 (C_{All}), 68.4 (C-6$_D$), 68.2 (C-6$_E$), 61.2 (C-2$_D$), 48.7 (NCH_2Ph). HRMS (ESI$^+$) for $C_{58}H_{61}NO_{11}$ ([M + Na]$^+$, 970.4142) found *m*/*z* 970.4107.

4.8.5. Allyl 2,3,4,6-Tetra-*O*-benzyl-α-D-glucopyranosyl-(1→4)-6-*O*-benzyl-2-benzylacetamido-2-deoxy-α-D-glucopyranoside (ED′$_{OH}$). R_f = 0.2 (2:1 cyclohexane−EtOAc). $[\alpha]_D$ = +7.9 (c = 1.2, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.37−7.15 (m, 42H, CH_{Ph}), 5.50−5.40 (m, 1.4H, CH═), 5.10−4.76 (m, 13H, H-1$_D$, H-1$_E$, H-2$_D$, ═CH_2, H_{Bn}), 4.75−4.65 (m, 3.4H, H_{Bn}, NCH_2Ph), 4.60−4.38 (m, 9.3H, H_{Bn}), 4.27−4.12 (m, 2H, H-3$_D$, H_{All}), 4.04−3.91 (m, 3H, H-3$_D$, H_{All}), 3.91−3.81 (m, 1.4H, H-5$_E$), 3.80−3.50 (m, 12.2H, H-2$_E$, H-4$_D$, H-4$_E$, H-6a$_D$, H-6b$_D$, H-6a$_E$), 3.50−3.38 (m, 1.4H, H-6b$_E$), 2.31 (s, 1.2H, Ac), 1.99 (s, 3H, Ac); ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 173.9, 172.5 (C═O), 139.8, 139.4, 138.5, 138.2, 137.8, 136.9 (C quat. arom.), 133.5 (CH═), 128.8, 128.7, 128.6, 128.4, 128.3, 128.0, 127.9, 127.7, 127.6, 127.5, 127.4, 126.6, 126.4, 125.9 (C_{Ph}), 117.8, 117.3 (═CH_2), 100.8 (C-1$_E$), 98.0, 97.7 (C-1$_D$), 83.8 (C-4$_D$), 82.2 (C-3$_E$), 79.4 (C-2$_E$), 77.7 (C-4$_E$), 75.7, 75.6, 75.0, 74.2, 73.5, 73.3, 73.1 (C_{Bn}), 71.3 (C-5$_E$), 70.0 (C-5$_D$), 69.1 (C-6$_D$), 69.0 (C-3$_D$), 68.9 (C_{All}), 68.3 (C-6$_E$), 56.4 (C-2$_D$), 48.5 (NCH_2Ph), 22.6 (Ac). HRMS (ESI$^+$) of $C_{59}H_{66}NO_{11}$ ([M + H]$^+$, 964.4636) *m*/*z* 964.4813.

Acknowledgment. Authors thank Hugues Driguez, Sébastien Fort, and Antoni Planas for insightful discussions and Laurent Bornaghi for proofreading the manuscript. This work was supported by the French National Research Agency (ANR Project OPTIGLUC 2005−2008). E.C. and J.B. thank the French Ministry of Research for financial support.

Supporting Information Available: Primers used to generate the 19 monomutants for the positions 228, 289, 290, 330, 331, 394, and 446 in Table S1; Sequence of XXX codon used to replace each selected amino acids by the 19 other ones in Table S2; Glucosylation of other protected acceptors in Table S3; Comparison of Dionex HPAEC product profiles obtained for wild-type AS and variant I228Y for glucosylation of α-L-Rha*p*-OMe (**A′**) in Figure S1; Comparison of Dionex HPAEC product profiles obtained for wtAS and variant F290K for glucosylation of α-D-Glc*p*NAc-OAll (**D′**) in Figure S2. This material is available free of charge via the Internet at http://pubs.acs.org.

JA900183H