Thermus thermophilus Glycosynthases for the Efficient Synthesis of Galactosyl and Glucosyl β-(1→3)-Glycosides

Jullien Drone,[a] Hui-yong Feng,[b] Charles Tellier,[a] Lionel Hoffmann,[a] Vinh Tran,[a] Claude Rabiller,[a] and Michel Dion*[a]

Keywords: Glycosynthase / Transglycosidation / Thermophilic β-glycosidase / *Thermus thermophilus* / β-(1→3)-Disaccharides / Molecular modeling

Inverting mutant glycosynthases were designed according to the Withers strategy, starting from wild-type *Thermus thermophilus* retaining Tt-β-Gly glycosidase. Directed mutagenesis of catalytic nucleophile glutamate 338 by alanine, serine, and glycine afforded the E338A, E338S, and E338G mutant enzymes, respectively. As was to be expected, the mutants were unable to catalyze the hydrolysis of the transglycosidation products. In agreement with previous results, the E338S and E338G catalysts were much more efficient than E338A. Moreover, our results showed that these enzymes were inactive in the hydrolysis of the α-D-glycopyranosyl fluorides used as donors, and so suitable experimental conditions, under which the rate of spontaneous hydrolysis of the donor was considerably lower than that of enzymatic transglycosidation, provided galactosyl and glucosyl β-(1→3)-glycosides in yields of up to 90%. The structure of native Tt-β-Gly available in the Protein Data Bank offers a good basis for interpretation of our results by means of molecular modeling. Thus, in the case of the E338S mutant, a lower energy of the system was obtained when the donor and the acceptor were in the right position to form the β-(1→3)-glycosidic bond.

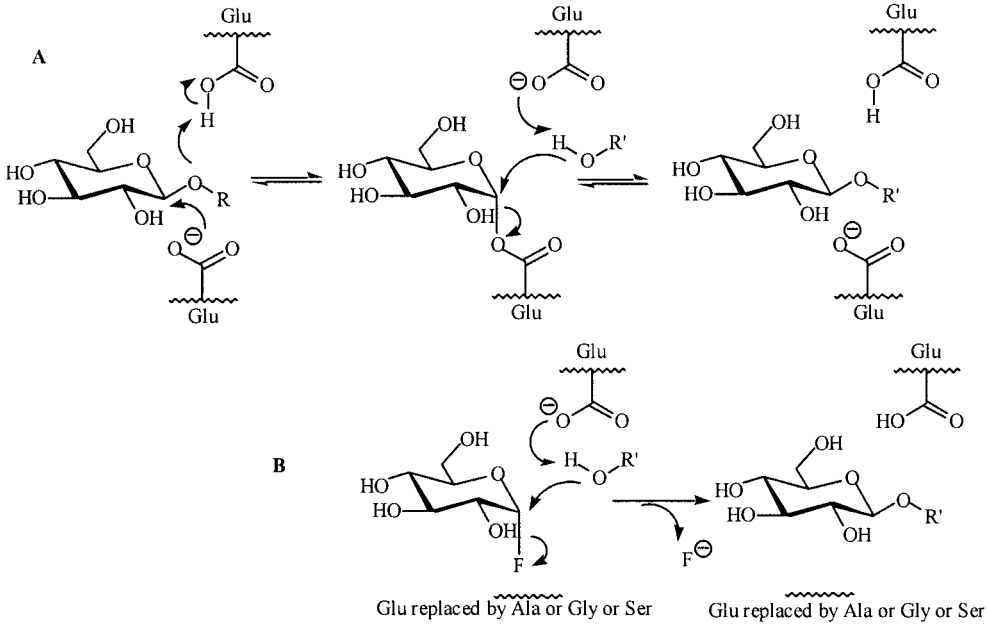
Introduction

A number of carbohydrate ligands play important roles in many biological mechanisms. Their use as drugs and, to a degree, understanding of their action depend on the availability of the oligosaccharides involved in the processes, and so the transfer activity exerted by inexpensive retaining glycosidases has provided an efficient approach for the synthesis of saccharides. One of the drawbacks of this method was the incomplete regioselectivity, which resulted in mixtures of regioisomers not easy to separate. Another obstacle involved the hydrolysis of the substrate and of the products, which remained in competition with transglycosidation. Moreover, when working with an *exo*-glycosidase, two types of glycosides were usually synthesized: those resulting from the reaction between donor and acceptor and those produced from the self-condensation of the donor.[1–3] An impressive advancement of the method was achieved after the elucidation of the mechanism of retaining β-glucosidases by Withers et al.[4–6] A pair of carboxylic acids acting as catalytic residues were shown to be involved in the process. One functioned as an acid/base and the other as a nucleophile to create the ester glycosyl enzyme intermediate (glycosylation step). As shown in Scheme 1, this activated complex was then able to react with the nucleophiles present in the reaction medium/water (hydrolysis) and the hydroxy groups of carbohydrates (transglycosylation). As pointed out by Withers, the similar transition states (oxocarbenium ions) involved either in inversion or in retention of glycosidases offered the potential to convert the latter into the former by appropriate mutations. Thus, a mutant of a retaining *exo* β-glucosidase from *Agrobacterium*, devoid of any action of E358 as a nucleophile (E358A mutant), showed a high transglycosidation activity with use of α-glucosyl fluoride as a donor in the presence of various acceptors. Good transglycosidation yields were obtained, since this "nucleophile-free" enzyme was unable to hydrolyze the reaction product.[7] A similar approach developed at the same time by Planas et al. on an *endo* β-glucanase also came to the same conclusions.[8–12] The "glycosynthase" concept was born (see Scheme 1), and the development of the idea was to result in the design of efficient mutants for the synthesis of oligosaccharides.[13–22] Improvements in the catalytic properties of glycosynthases from *Agrobacterium sp.* were also recently obtained by directed evolution.[23]

In our continuing efforts to provide new glycosidases for the selective synthesis of oligosaccharides, we have cloned and overexpressed (in *E. coli*) a β-glycosidase (Tt-β-Gly) from the thermophilic species *Thermus thermophilus*.[24,25]

[a] Biotechnologie, Biocatalyse, Biorégulation (UMR CNRS 6204), Faculté des Sciences et des Techniques,
2, rue de la Houssinière, BP 92208, 44322 Nantes, cedex 3, France,
Fax: +33-51-12-56-37
E-mail: michel.dion@univ-nantes.fr
[b] Institute of Biosciences and Biotechnology, University of Science and Technology,
Shijiazhuang, Hebei, China

 DOI: 10.1002/ejoc.200500014



Scheme 1. Transglycosidation mechanism: A) with retaining glycosidases, and B) with glycosynthases.

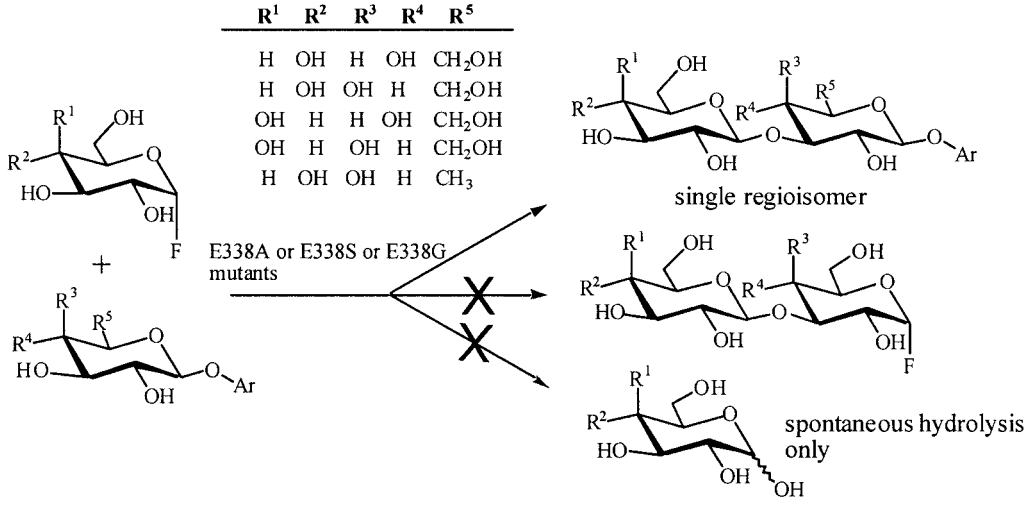
This enzyme catalyzed the synthesis of β-(1→3)-disaccharides by transglycosidation in reasonable yields (≈ 40–50%) with use of *o*-nitrophenyl β-D-Gal*p* (or Glc*p*) as a glycosyl donor.[1] However, the enzyme mainly catalyzed the self-condensation reaction and consequently the yield of the condensation disaccharide remained relatively moderate. The aim of this paper is to evaluate mutants, according to the "glycosynthase " concept, capable of synthesizing oligosaccharides in higher yields. Since only a few glycosidases exhibit such regioselectivity, an enzyme capable of efficiently catalyzing the synthesis of the β-(1→3)-glycosidic bonds would be of great interest in view of the biological importance of the saccharides incorporating such structures. Such a glycosidic bond is present in the Thomsen–Friedenreich antigen determinant β-Gal*p*-(1→3)-α-Gal*p*-NAc-O–Ser and in the antigen H type 1 (L-Fuc*p*-α-(1→2)-D-Gal*p* β-(1→3)-D-Glc*p*-NAc.[26] In addition, the D-Glc*p*-β-(1→3)-D-Glc*p* disaccharide is also known to act as an immunosuppressant.[27] The Glc-β-(1→3)-D-Glc linkage is involved in the structure of hexasaccharides acting as elicitors of the defense response in plants,[28] particularly in tobacco.[29]

Results and Discussion

The gene *tt*-β-*gly*, encoding a β-glycosidase, has been cloned from *Thermus thermophilus* and overexpressed into *E. coli*.[25] The amount of this enzyme can reach up to 50% of the total protein fraction in the cells. After lysis of the cell membranes, simple heating at 70 °C destroys all the glycosidasic activities normally present in the mesophilic species, apart from that of Tt-β-Gly. In this work, the soluble fraction from the heated cells was used as a biocatalyst without any further purification. Kinetic studies[24] have shown that the hydrolytic activity with *p*-nitrophenyl glycosides as substrates is higher for β-D-fucoside than for β-D-glucoside and β-D-galactoside (the respective values for k_{cat}/K_m are 247, 227, and 14.8 $mM^{-1} s^{-1}$).

Although the native enzyme showed good transglycosidation activity, the possibility of improving this property by site-directed mutagenesis still remained open. More particularly, according to the findings of Withers and Planas,[7,12] replacement of catalytic nucleophile glutamate 338 by alanine, serine, or glycine should provide glycosidases devoid of their hydrolytic activity towards the products of the reactions. Furthermore, the inverting mutants thus obtained are able to accept α-D-activated donors, such as the readily available α-D-glycopyranosyl fluorides (e.g., α-GlcF and α-GalF). Moreover, the use of such substrates generally avoids the concurrent self-condensation of the donor. Mutants E338A, E338G, and E338S were therefore produced, and their transglycosidation potentials were tested with α-GlcF or α-GalF as donors and different acceptors. Preliminary experiments in the presence of *p*-nitrophenyl α-D-glycopyranosides indicated that these donors were not recognized as substrates for the mutant enzymes, which in addition did not catalyze the self-condensation of α-glycosyl fluorides. This property is probably due to a steric clash of the axial fluorine at site +2. Furthermore, water was not accepted as a nucleophile by the three mutants (see Scheme 2). This rather surprising, but very interesting, property is highlighted in Figure 1 for the case of the E338G mutant.

The great similarity between the kinetic hydrolysis curves obtained in the presence or in the absence of the enzyme implied that this mutant had lost its ability to accept water



Scheme 2. Reactions catalyzed by Tt-β-Gly glycosynthase mutants.

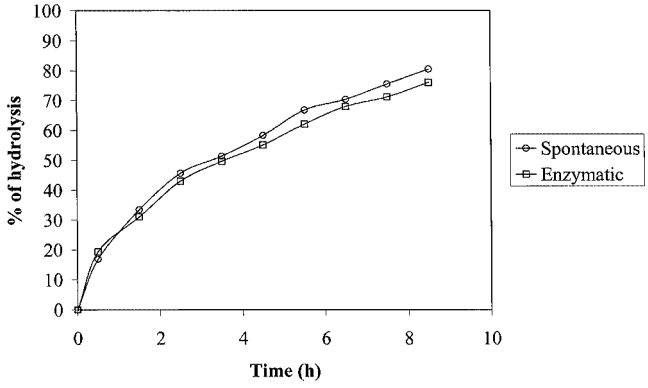


Figure 1. Comparison between spontaneous and enzymatically catalyzed hydrolysis of α-glucosyl fluoride {experimental conditions: E338G glycosynthase, T = 55 °C, pH = 7.8, [α-GlcF] = 50 mmol L^{-1}}.

as a nucleophile. The same was true for the two other mutants (data not shown).

This property should be usable to improve the transglycosidation yields if the rate of the transglycosidation reaction could be enhanced relative to that of spontaneous hydrolysis of the fluoride. Thus, in a first set of experiments, the potentials of the three mutants were tested under conditions allowing quantitative comparisons (pH = 7.8 buffer solutions containing 50 mmol L^{-1} of glycosyl fluoride as a donor, 50 mmol L^{-1} of an acceptor, and 4.1 mg of enzyme per mmol of donor, see Table 1).

The yields were determined by proton NMR spectra integration measurements on the crude reaction mixtures obtained at the time at which the donor was almost consumed. The separation of the components was achieved by silica gel liquid chromatography and the structures of the products were determined by standard one- and two-dimensional NMR proton and carbon spectra. Whatever the donor and the mutant used, the only disaccharides produced were of the β-(1→3) type. Attempts to obtain trisaccharides by use of double amounts of donor were unsuccessful, disaccharides always being synthesized as single products. Thus, on comparison with native Tt-β-Gly, which also gave the β-(1→6)-regioisomer, the three mutants looked completely regioselective. Meanwhile, trisaccharide synthesis

Table 1. Transglycosidation yields (%) obtained with *Thermus thermophilus* mutant glycosynthases for a donor/acceptor ratio = 1.

			E338A		E338S		E338G	
Entry	Donor	Acceptor	β-1,3	β-1,*x*	β-1,3	β-1,*x*	β-1,3	β-1,*x*
1	α-GlcF	β-D-GlcPh	46	0	86	0	89	0
2		β-D-GlcPh[c]	77	0			96	0
3		β-D-GalPh	10	0	77	0		
4		β-D-Fuc*p*NP	0	0	39	0		
5		β-Cell*p*NP	0	0	31	37[a]	30	35[a]
6		β-Cell*p*NP[c]					31	52[a]
7		Cell	0	0		50[b]		
8		Lac	0	0		37[b]		
9		β-D-GlcMe			10	0		
10	α-GalF	β-D-GlcPh	13	0	86	0		
11		β-D-GalPh	0	0	18	0		
12		β-Cell*p*NP				20		
13		Mal	0	0		0	0	0

[a] Mixture of β-(1→6)-regioisomer as a major compound (>90%) and another unknown compound. [b] Complex mixture of α,β-anomeric regioisomers. [c] A three times higher enzyme concentration (relative to the other experiments) was used.

was achieved by use of β-(1→4)-disaccharides as acceptors, but the regioselectivity was not so good and several regioisomers were obtained. This was the case, for instance, with *p*-nitrophenyl β-D-cellobioside (β-Cell*p*NP), from which equivalent amounts of *p*-nitrophenyl β-D-glucopyranosyl-(1→3)-cellobioside and its (1→6)-regioisomer were present, in addition to a small amount of a third unknown species. The experimental results collected in Table 1 also indicate that the E338A mutant, although providing single β-(1→3)-disaccharides (in moderate yields) displays a rather narrow tolerance for acceptors. This result, in agreement with previous experiments,[13] can be explained by hydrophobicity induced by the methyl of alanine 338. Conversely, mutation by serine is known to afford much more efficient mutants, although the free space between the anomeric carbon of a substrate and the hydroxymethyl group of serine is reduced. The best results with such mutants have been explained in terms of a favorable interaction between the CH_2OH group and the fluorine of the glycosyl fluoride, which could occur in the glycosylation transition state, resulting in an enhanced transglycosylation reaction rate. This trend was also observed for the Tt-β-Gly E338S mutant, for which yields of up to 80% were observed with β-D-GlcPh or β-D-GalPh as acceptors (Table 1, Entries 1 and 3). Moreover, this enzyme catalyzed the transglycosidation reaction with a broad spectrum of acceptors, such as fucosides or β-(1→4)-disaccharides (Table 1). It was noteworthy that Tt-β-Gly mutants behaved similarly to Withers Abg glycosynthases. The transglycosidation reaction rates induced by the alanine mutant were always lower than those of the serine and glycine counterparts. Our preliminary observations thus suggested the conclusion that Tt-β-Gly glycosynthases did not catalyze the hydrolysis of α-GlcF or α-GalF. As a result, we carried out a second series of experiments with three times the amount of enzyme (12.3 mg of enzyme per mmol of donor). The results shown in Table 1 clearly indicate an enhancement of the transglycosidation yields. This was particularly the case with the E338A mutant, for which a yield of 77% (instead of 46%, Table 1, Entry 2) was obtained with β-D-GlcPh as an acceptor. Similar results were also observed for the transglycosidation yields in the presence of β-Cell*p*NP with E338G glycosynthase as a catalyst. This mutant also produced a nearly quantitative yield (96% of phenyl β-D-glucopyranosyl-(1→3)-β-D-glucopyranoside) with the most favorable β-D-GlcPh acceptor (Table 1, Entry 1). It should be highlighted that these mutants also need acceptors bearing an aromatic aglycon group (see Table 1, Entries 1 and 9). Thus, in the presence of the E338S mutant, the replacement of β-D-GlcPh by β-D-GlcMe resulted in a dramatic drop in the transglycosylation yield from 77% to 10%. Similarly, free disaccharides such as lactose were not good acceptors for the mutants. Maltose, an α-(1→4)-disaccharide, was the worst acceptor, with no transglycosylation product present in the reaction medium. Moreover, *p*NP–GlcNAc, although possessing an aromatic aglycon group, was not recognized by the E338S mutant. This behavior also showed a significant difference from that of Abg glycosynthase E358S, which produced D-glucopyranosyl-(1→4)-β-D-*N*-acetylglucopyranoside in good yields from α-GlcF and *p*NP–GlcNAc.[7]

In order to provide an explanation of this behavior, we used molecular modeling techniques. Since the X-ray structure of the native Tt-β-Gly was available (PDB code: lug6), we performed in silico mutation of glutamate 338 with a serine residue to obtain a potentially valid model for the E338S mutant enzyme, since only one residue had been replaced, on the assumption that the local environment had been preserved. All possible side chain orientations for the serine residue were tested and that of lowest energy was kept for further calculations. We then docked the ligands into the active site. For this process, we used a three-dimensional structure that is a covalent intermediate between the β-glycosidase of *Bacillus polymyxa* (PDB code: le4i) and 2-deoxy-2-fluoroglucose. Because of the close similarity between these two enzymes (both belonging to Glycoside Hydrolase Family 1), superimposition of the complex with the E338S mutant model, followed by superimposition of α-GlcF with its 2-deoxy-2-fluoroglucose analogue in the complex, provided a good starting localization for the α-GlcF. This position was then optimized by a molecular mechanics scheme. In a second step, we placed the second ligand, β-D-GlcPh, roughly inside the E338S mutant model active site according to the supposed transglycosidation reaction mechanism. We then performed systematic manual translations and rotations according to the interaction energy between β-D-GlcPh and the E338S mutant model. The best position found for β-D-GlcPh was that shown in Figure 2. According to this result, the need for a phenyl group could

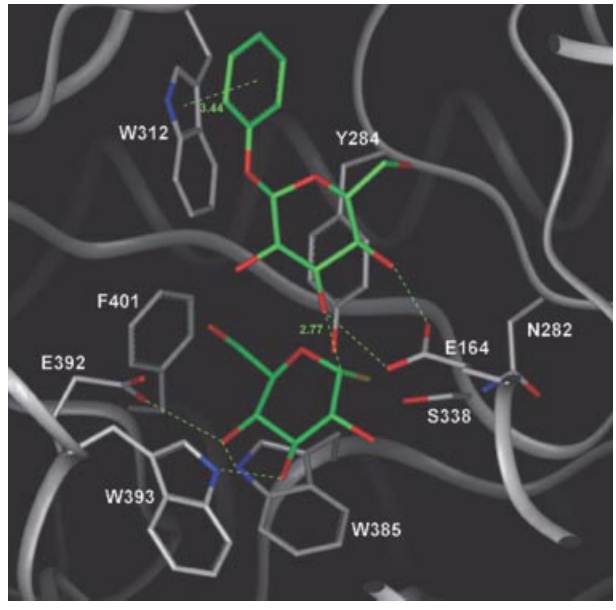


Figure 2. Molecular modeling, in the active site, of the disposition of reactants before the transglycosidation reaction catalyzed by E338S glycosynthase, with α-GlcF as donor and β-GlcPh as acceptor. An energy minimum was obtained when stacking occurred between the phenyl group of β-GlcPh and tryptophan 312. Moreover, the relative dispositions of the reactants, stabilized by several hydrogen bonds, were in agreement with the formation of a β-(1→3) glycosidic bond.

be explained by the important stacking between this phenyl group and the tryptophan 312 residue, which was mainly responsible for the energy stabilization. Moreover, the relative positions of the two monosaccharides stabilized by several hydrogen bonds are consistent with the complete β-(1→3)-regioselectivity observed for this reaction. In contrast, when the acceptors are cellobiose or lactose the site +2 is occupied by a glucosyl unit. As a result, a lower conformational constraint is probably imposed on the orientation of the disaccharide within the active site by Trp 312. This may explain the relatively low regioselectivity observed with these acceptors.

Conclusion

In summary, we have shown that three Tt-β-Gly glycosynthases, designed according to the strategy developed by Withers, circumvented the drawbacks of wild-type retaining glycosidases in terms of their transglycosidation ability, since:

a) no hydrolytic activity towards the product and the substrate was detectable,
b) the regioselectivity was almost total, at least for the synthesis of β-(1→3)-disaccharides, and
c) no donor self-condensation products were synthesized.

Optimization of the reaction to produce yields of up to 90% was also possible through the use of appropriate amounts of enzymes to enhance transglycosidation rates relative to those of spontaneous hydrolysis of the glycosyl fluorides. An interpretation of the properties of the mutant glycosynthases based on molecular modeling studies has also been proposed. Moreover, directed evolution of *Thermus thermophilus* glycosidase is currently under investigation in order to increase its catalytic activities and expand its substrate repertoire.

Experimental Section

General: Directed mutagenesis of gene *tt*β*Gly* was carried out by PCR with overlapping extension, and cloning was done into the pBtac2 vector at sites *Eco*RI-*Pst*I. The primers Nphi1/Nphi2 (5′-gtcacggcgaacggggccgcctac; 5′-gtaggcggccccgttcgccgtgac), Nphi3/Nphi4 (5′-gtcacgtctaacggggccgcctac, 5′-gtaggcggccccgttagacgtgac), and Nphi5/Nphi6 (5′-gtcacgggtaacggggccgcctac, 5′gtaggcggccccgttacccgtgac) were used to generate the mutant enzymes E338A, E338S, and E338G, respectively. Overexpression of the different β-glycosidases was performed in *E. coli* strain XL1 Blue MRF′. After overnight cultivation in LB medium, cells were harvested, lysed, and heated for 1 h at 70 °C to remove *E. coli* thermolabile proteins. Supernatants of centrifuged extracts were used as biocatalysts in transglycosylation reactions.

Chemicals supplied by Aldrich were used without further purification. The courses of the reactions were followed by TLC (Merck F254 precoated silica gel 60 sheets) and proton NMR spectroscopy. The components of the reaction mixtures were separated by silica gel chromatography. Complete analysis of the NMR ^{1}H and ^{13}C resonances and subsequent structure assignments were carried out by use of standard 2D sequences (COSY HH and HC correlations). The spectra were recorded with a Bruker AX 500 spectrometer operating at 500 MHz for ^{1}H and 126 MHz for ^{13}C or on a Bruker AX 400 spectrometer operating at 400 MHz for ^{1}H and 100.6 MHz for ^{13}C. In all cases, chemical shifts in ppm are quoted from the resonance of methyl protons of sodium 3-(trimethylsilyl)propanesulfonate (DSS) used as an internal reference.

Determination of Yields by Proton NMR Spectroscopy

Use of E338S and E338G Glycosynthases: The donor (30 μmol) and the acceptor (30 μmol) were dissolved in ammonium/hydrogen carbonate buffer in D_2O (pH = 7.8, 510 μL, 150 mmol L^{-1}) and the mutant solution, prepared as described above (90 μL, 125 μg of enzyme, 100 mmol L^{-1} phosphate buffer in H_2O, pH = 7.0), was added. The reactions were allowed to proceed at 55 °C until the donor had completely disappeared. The reaction mixture was then subjected to proton NMR spectroscopy. Experiments with threefold amounts of enzymatic solutions were carried out similarly, but the reaction mixture was evaporated under reduced pressure and redissolved in D_2O before NMR analysis.

Use of E338A Glycosynthase: The donor (30 μmol) and the acceptor (30 μmol) were dissolved in ammonium hydrogen carbonate buffer in D_2O (pH = 7.8, 550 μL of 150 mmol L^{-1}), and the mutant solution, prepared as described above (50 μL, 125 μg of enzyme), was added. The reactions were allowed to proceed at 55 °C until the donor had completely disappeared. The reaction mixture was then subjected to ^{1}H NMR spectroscopy. Experiments with threefold amounts of enzymatic solutions were carried out similarly but the reaction mixture was evaporated under reduced pressure and redissolved in D_2O before NMR analysis.

Kinetics of Hydrolysis of α-Glycosyl Fluorides: Experiments were performed directly in the NMR tube at 55 °C. ^{1}H NMR spectra were recorded each hour for 9 h.

Enzymatic Hydrolysis: α-GlcF or -GalF (30 μmol) was dissolved in ammonium hydrogen carbonate buffer in D_2O (pH = 7.8, 510 μL of 150 mmol L^{-1}). The glycosynthase E338G solution, prepared as described above (90 μL, 125 μg of enzyme), was then added.

Spontaneous Hydrolysis: α-GlcF or -GalF (30 μmol) was dissolved in ammonium hydrogen carbonate buffer in D_2O (pH = 7.8, 600 μL, 150 mmol L^{-1}).

General Optimized Procedure for the Synthesis of β-(1→3) Disaccharides with Use of the Tt-β-Gly E338S Mutant: α-D-Glucopyranosyl (or -galactopyranosyl) fluoride (18.2 mg, 0.1 mmol) and an appropriate acceptor (0.1 mmol) were dissolved in ammonium carbonate buffer (pH = 7.8, 2 mL, 150 mmol L^{-1}). The Tt-β-Gly E338S mutant (0.126 mg, 0.9 mL), prepared as described above, was then added and the mixture was stirred overnight at 55 °C. After solvent removal under vacuum, the residue was purified by flash chromatography to afford the corresponding disaccharides.

Phenyl β-D-Glucopyranosyl-(1→3)-β-D-glucopyranoside: Flash chromatography (AcOEt/MeOH/water, 17:2:1) gave the disaccharide (33 mg, 75%) as a white solid. M.p. 77 °C. $[\alpha]_D^{20}$ = –39.1 (c = 1.067, H_2O). R_f 0.62 (AcOEt/MeOH/water, 7:2:1). HRMS (CI/glycerol) calcd. for $C_{18}H_{27}O_{11}$ [M + H]$^+$: 419.1475, found 419.1578. NMR: see Table 2, Table 3, and Table 4.

Phenyl β-D-Glucopyranosyl-(1→3)-β-D-galactopyranoside: Flash chromatography (AcOEt/MeOH/water, 15:2:1) gave the disaccharide (31 mg, 70%) as a white solid. M.p. 142 °C. $[\alpha]_D^{20}$ = +6.5 (c = 0.467, H_2O). R_f = 0.51 (AcOEt/MeOH/water, 7:2:1). HRMS (CI/glycerol) calcd. for $C_{18}H_{27}O_{11}$ [M + H]$^+$: 419.1475; found 419.1604. NMR: see Table 5, Table 6, and Table 7.

Table 2. Phenyl β-D-glucopyranosyl-(1→3)-β-D-glucopyranoside: ^{1}H NMR (500 MHz, D_2O), chemical shifts (δ in ppm).

	H-1	H-2	H-3	H-4	H-5	H-6A	H-6B
I	5.16	3.78	3.86	3.62	3.65	3.77	3.93
II	4.78	3.39	3.54	3.43	3.50	3.73	3.93
δ(aromatic) = 7.15 and 7.41 ppm							

Table 3. Phenyl β-D-glucopyranosyl-(1→3)-β-D-glucopyranoside: ^{1}H-^{1}H coupling constants (Hz, solvent D_2O).

	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6A}$	$J_{5,6B}$	$J_{6A,6B}$
I	7.9	9.2	8.4	9.9	5.1	2.0	–12.4
II	8.0	9.4	9.1	9.8	6.1	2.2	–12.3

Table 4. Phenyl β-D-glucopyranosyl-(1→3)-β-D-glucopyranoside: ^{13}C NMR (126 MHz, D_2O), chemical shifts (δ in ppm).

	C-1	C-2	C-3	C-4	C-5	C-6
I	102.7	75.4	86.9	70.7	78.4	63.2
II	105.5	76.2	78.3	72.3	78.7	63.4
δ(aromatic) = 119.3, 126.1, 132.7 and 159.2 ppm						

Table 5. Phenyl β-D-glucopyranosyl-(1→3)-β-D-galactopyranoside: ^{1}H NMR (400 MHz, D_2O), chemical shifts (δ in ppm).

	H-1	H-2	H-3	H-4	H-5	H-6A	H-6B
I	5.11	3.98	3.92	4.25	3.87	3.71–3.82	3.71–3.82
II	4.71	3.40	3.52	3.43	3.47	3.71–3.82	3.90
δ(aromatic) = 7.15 and 7.41 ppm							

Table 6. Phenyl β-D-glucopyranosyl-(1→3)-β-D-galactopyranoside: ^{1}H-^{1}H coupling constants (Hz, solvent D_2O)

	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6A}$	$J_{5,6B}$	$J_{6A,6B}$
I	7.6	9.8	3.2	0.9	5.5	6.4	n.d.
II	7.9	9.4	9.7	9.7	2.3	5.0	n.d.

Table 7. Phenyl β-D-glucopyranosyl-(1→3)-β-D-galactopyranoside: ^{13}C NMR (100.8 MHz, D_2O), chemical shifts (δ in ppm).

	C-1	C-2	C-3	C-4	C-5	C-6
I	103.2	72.4	84.8	70.9	77.7	63.4
II	106.4	76.0	78.3	78.5	72.2	63.3
δ(aromatic) = 119.3, 126.1, 132.7 and 159.2 ppm						

Phenyl β-D-Galactopyranosyl-(1→3)-β-D-glucopyranoside: Flash chromatography (AcOEt/MeOH/water, 17:2:1) gave the disaccharide (30 mg, 68%) as white needles. M.p. 134 °C. $[a]_D^{20}$ = –30.5 (c = 0.967, H_2O). R_f = 0.49 (AcOEt/MeOH/water, 7:2:1). HRMS (CI/glycerol) calcd. for $C_{18}H_{27}O_{11}$ $[M]^+$: 419.1475; found 419,1557. NMR: see Table 8, Table 9, and Table 10.

Table 8. Phenyl β-D-galactopyranosyl-(1→3)-β-D-glucopyranoside: ^{1}H NMR (500 MHz, D_2O), chemical shifts (δ in ppm).

	H-1	H-2	H-3	H-4	H-5	H-6A	H-6B
I	5.16	3.77	3.87	3.62	365	3.77	3.93
II	4.70	3.63	3.69	3.93	3.73	nd	3.80
δ(aromatic) = 7.15 and 7.41 ppm							

Table 9. Phenyl β-D-galactopyranosyl-(1→3)-β-D-glucopyranoside: ^{1}H-^{1}H coupling constants (Hz, solvent D_2O).

	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6A}$	$J_{5,6B}$	$J_{6A,6B}$
I	7.9	8.4	9.0	9.9	5.3	2.2	–12.4
II	7.7	9.9	3.4	0.8	3.8	8.1	–11.7

Table 10. Phenyl β-D-galactopyranosyl-(1→3)-β-D-glucopyranoside: ^{13}C NMR (100.8 MHz, D_2O), chemical shifts (δ in ppm).

	C-1	C-2	C-3	C-4	C-5	C-6
I	102.7	75.3	87.0	70.8	78.4	63.3
II	106.0	74.0	75.3	71.3	78.0	63.8
δ(aromatic) = 119.3, 126.1, 132.7 and 159.2 ppm						

4-Nitrophenyl β-D-Glucopyranosyl-(1→3)-β-D-fucopyranoside: Flash chromatography (AcOEt/MeOH/water, 15:2:1) gave the disaccharide (13 mg, 31%) as a white solid. M.p. 80 °C. $[a]_D^{20}$ = –32.9 (c = 0.480, H_2O). R_f = 0.61 (AcOEt/MeOH/water, 7:2:1). NMR: see Table 11, Table 12, and Table 13.

Table 11. 4-Nitrophenyl β-D-glucopyranosyl-(1→3)-β-D-fucopyranoside: ^{1}H NMR (400 MHz, [D_5]pyridine), chemical shifts (δ in ppm).

	H-1	H-2	H-3	H-4	H-5	H-6A	H-6B
I	5.61	4.89	4.43	4.27	4.10	1.58 (CH_3)	–
II	5.52	4.13	4.30	4.41	4.08	4.44	4.61
δ(aromatic) = 7.33 and 8.25 ppm							

Table 12. 4-Nitrophenyl β-D-glucopyranosyl-(1→3)-β-D-fucopyranoside: ^{1}H-^{1}H coupling constants (Hz, solvent [D_5]pyridine).

	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6A}$	$J_{5,6B}$	$J_{6A,6B}$
I	7.7	8.2	n.d.	n.d.	6.4 (CH_3)	–	–
II	7.8	8.0	8.7	n.d.	n.d.	2.5	–11.6

Table 13. 4-Nitrophenyl β-D-glucopyranosyl-(1→3)-β-D-fucopyranoside: ^{13}C NMR (100.8 MHz, [D_5]pyridine), chemical shifts (δ in ppm).

	C-1	C-2	C-3	C-4	C-5	C-6
I	103.4	72.2	85.9	73.5	73.7	18.6
II	108.1	77.5	80.1	80.1	80.3	64.5
δ(aromatic) = 118.6, 127.7, 144.4 and 164.9 ppm						

Acknowledgments

Thanks are due to the French Ministry of Education and Research for a grant to one of us (J. D.) and to the CNRS for financially supporting this work. D. Maume (Ecole Vétérinaire de Nantes) is gratefully acknowledged for mass spectra experiments.

[1] V. Chiffoleau-Giraud, P. Spangenberg, M. Dion, C. Rabiller, *Eur. J. Org. Chem.* **1999**, 757–763.
[2] P. Spangenberg, V. Chiffoleau-Giraud, C. Andre, M. Dion, C. Rabiller, *Tetrahedron: Asymmetry* **1999**, *10*, 2905–2912.
[3] P. Spangenberg, C. Andre, V. Langlois, M. Dion, C. Rabiller, *Carbohydr. Res.* **2002**, *337*, 221–228.
[4] S. G. Withers, K. Rupitz, D. Trimbur, R. A. J. Warren, *Biochemistry* **1992**, *31*, 9979–9985.
[5] J. C. Gebler, D. E. Trimbur, R. A. J. Warren, R. Aebersold, M. Namchuk, S. G. Withers, *Biochemistry* **1995**, *34*, 14547–14553.
[6] Q. Wang, R. W. Graham, D. Trimbur, R. A. J. Warren, S. G. Withers, *J. Am. Chem. Soc.* **1994**, *116*, 11594–5.
[7] L. F. Mackenzie, Q. Wang, R. A. J. Warren, S. G. Withers, *J. Am. Chem. Soc.* **1998**, *120*, 5583–5584.
[8] M. Faijes, J. K. Fairweather, H. Driguez, A. Planas, *Chem. Eur. J.* **2001**, *7*, 4651–4655.
[9] M. Faijes, X. Perez, O. Perez, A. Planas, *Biochemistry* **2003**, *42*, 13304–18.
[10] M. Faijes, T. Imai, V. Bulone, A. Planas, *Biochem. J.* **2004**, *380*, 635–641.
[11] J. K. Fairweather, M. Faijes, H. Driguez, A. Planas, *ChemBioChem* **2002**, *3*, 866–873.
[12] C. Malet, A. Planas, *FEBS Lett.* **1998**, *440*, 208–212.
[13] C. Mayer, D. L. Zechel, S. P. Reid, R. A. Warren, S. G. Withers, *FEBS Lett.* **2000**, *466*, 40–44.
[14] J. F. Tolborg, L. Petersen, K. J. Jensen, C. Mayer, D. L. Jakeman, R. A. J. Warren, S. G. Withers, *J. Org. Chem.* **2002**, *67*, 4143–4149.
[15] G. Perugino, A. Trincone, M. Rossi, M. Moracci, *Trends Biotechnol.* **2004**, *22*, 31–37.
[16] M. Jahn, J. Marles, R. A. J. Warren, S. G. Withers, *Angew. Chem. Int. Ed.* **2003**, *42*, 352–354.
[17] M. Jahn, H. Chen, J. Muellegger, J. Marles, R. A. J. Warren, S. G. Withers, *Chem. Commun.* **2004**, 274–275.
[18] M. Moracci, A. Trincone, G. Perugino, M. Ciaramella, M. Rossi, *Biochemistry* **1998**, *37*, 17262–70.
[19] J.-L. Viladot, F. Canals, X. Batllori, A. Planas, *Biochem. J.* **2001**, *355*, 79–86.
[20] H. Lin, H. Tao, V. W. Cornish, *J. Am. Chem. Soc.* **2004**, *126*, 15051–15059.
[21] O. Nashiru, D. L. Zechel, D. Stoll, T. Mohammadzadeh, R. A. J. Warren, S. J. Withers, *Angew. Chem. Int. Ed.* **2001**, *40*, 417–420.
[22] M. Jahn, D. Stoll, R. A. J. Warren, L. Szabo, P. Singh, H. J. Gilbert, V. M.-A. Ducros, G. J. Davies, S. J. Withers, *Chem. Commun.* **2003**, 1327–1329.
[23] S. G. Withers, M. Jahn, in *PCT Int. Appl.*, Wo, **2004**, p. 36.
[24] L. Fourage, M. Dion, B. Colas, *Glycoconjugate J.* **2000**, *17*, 377–383.
[25] M. Dion, L. Fourage, J.-N. Hallet, B. Colas, *Glycoconjugate J.* **1999**, *16*, 27–37.
[26] K. Fukase, T. Yasukochi, Y. Suda, M. Yoshida, S. Kusumoto, *Tetrahedron Lett.* **1996**, *37*, 6763–6766.
[27] Y. Yamamoto, S. Murosaki, H. Kusaka, in: *Jpn. Kokai Tokkyo Koho* (Takeda Shokuhin Kogyo K. K., Japan), **1997**, p. 13.
[28] P. Albersheim, A. G. Darvill, M. McNeil, B. S. Valent, J. K. Sharp, E. A. Nothnagel, K. R. Davis, N. Yamazaki, D. J. Gollin, W. S. York, W. F. Dudman, J. E. Darvill, A. Dell, *Oligosaccharins: naturally occurring carbohydrates with biological regulatory functions,* in Structure and Function of Plant Genomes (Eds.: O. Ciferri, L. Dure, III), Plenum, New York, **1983**, pp. 293–312.
[29] O. Klarzynski, B. Plesse, J.-M. Joubert, J.-C. Yvin, M. Kopp, B. Kloareg, B. Fritig, *Plant Physiol.* **2000**, *124*, 1027–1037.

Received: January 10, 2005

