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Acceptor-dependent regioselectivity of glycosynthase reactions by *Streptomyces* E383A β-glucosidase

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Abstract—The nonnucleophilic mutant E383A β-glucosidase from *Streptomyces* sp. has proven to be an efficient glycosynthase enzyme, catalyzing the condensation of α-glucosyl and α-galactosyl fluoride donors to a variety of acceptors. The enzyme has maximal activity at 45 °C, and a pH-dependence reflecting general base catalysis with an apparent kinetic pK_a of 7.2. The regioselectivity of the new glycosidic linkage depends unexpectedly on the acceptor substrate. With aryl monosaccharide acceptors, β-(1→3) disaccharides are obtained in good to excellent yields, thus expanding the synthetic products available with current *exo*-glycosynthases. With xylopyranosyl acceptor, regioselectivity is poorer and results in the formation of a mixture of β-(1→3) and β-(1→4) linkages. In contrast, disaccharide acceptors produce exclusively β-(1→4) linkages. Therefore, the presence of a glycosyl unit in subsite +II redirects regioselectivity from β-(1→3) to β-(1→4). To improve operational performance, the E383A mutant was immobilized on a Ni^{2+}-chelating Sepharose resin. Immobilization did not increase stability to pH and organic solvents, but the operational stability and storage stability were clearly enhanced for recycling and scaling-up.

Keywords: Glycosynthase; Oligosaccharide synthesis, enzymatic; Glycosylation; Immobilization; β-Glucosidase; *Streptomyces* sp; Enzyme specificity

1. Introduction

Engineering a retaining glycosidase into a glycosynthase involves mutation of the catalytic nucleophile to a smaller nonnucleophilic amino acid residue and the use of activated glycosyl donors (i.e., glycosyl fluorides) with the opposite anomeric configuration than that of the normal substrate of the parental wild type enzyme (Scheme 1).[1,2] Because the mutant is hydrolytically inactive, the condensation product is not hydrolyzed and accumulates to achieve high yields. Therefore, glycosynthases share the advantages of glycosidases in transglycosylation reactions (availability, stability, and use of simple glycoside donors), but overcome their major drawback (low synthetic yields due to product hydrolysis).[3–5]

The glycosynthase methodology has been explored for several (but yet limited number) retaining glycosidases belonging to different families (as classified on the bases of sequence similarities in the CAZY database (http://afmb.cnrs-mrs.fr/CAZY/)).[6] For *endo*-glycosidases, their derived glycosynthases are highly regiospecific, catalyzing the formation of a unique glycosidic bond matching the specificity shown by the parental wt-hydrolase enzyme.[2,7–11] *exo*-Glycosidases have a more relaxed specificity. Several Family 1 glycosidases have shown to function as glycosynthases upon mutation of the catalytic nucleophile, but their regioselectivity is substantially different.[1,12–14] This approach will be considered as a general synthetic methodology when an extensive repertoire of glycosynthases covering different regio and stereoselectivities will become available.

β-Glucosidase from *Streptomyces* sp. (a Family 1 glycosyl hydrolase) has a broad substrate specificity,[15–17] and it is a new candidate to explore its abilities as glycosynthase. The wt enzyme is highly active on glucosyl and

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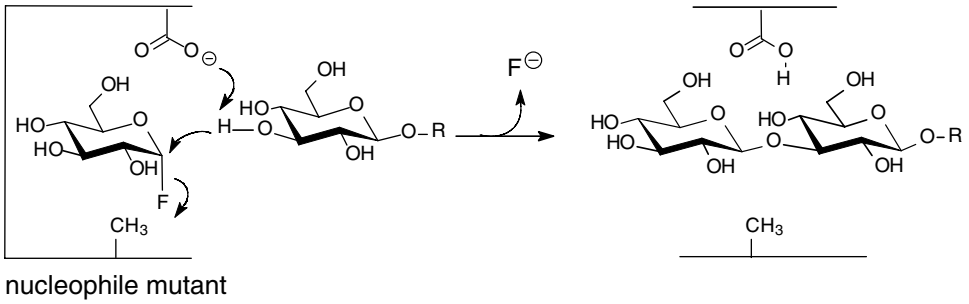


Scheme 1. Glycosynthase reaction catalyzed by a nucleophile mutant of retaining glycosidases.

fucosyl glycosides and, to a lesser extent, on galactosyl and xylosyl derivatives. Besides, it hydrolyzes either β-(1→2) (sophorose), β-(1→4) (cellobiose) or β-(1→3) (laminaribiose) linked disaccharides with similar activity. Under kinetically controlled transglycosylation conditions, these three glycosidic linkages are also formed.[18] Since −I and +I subsites accept a variety of glycosidic units and linkages, the glycosynthase mutants could in principle couple a wide combination of donors and acceptors and broaden the spectrum of di- and trisaccharides already obtained with currently existing glycosynthases.

On the basis of sequence alignment, site-directed mutagenesis, and chemical rescue experiments, Glu383 was identified as the catalytic nucleophile of the β-glucosidase from *Streptomyces* sp.[16] In this paper, we explore the properties of the E383A mutant as a glycosynthase to define its activity and specificity for a wide combination of donors and acceptors. Additionally, the enzyme was immobilized to increase its stability and improve operational conditions for recycling and scaling-up.

2. Results and discussion

Glycosynthase activity of the E383A mutant β-glucosidase from *Streptomyces* sp. was first assayed by reaction between α-glucosyl fluoride (**1**) and *p*-nitrophenyl glucoside (**4**), which produced a disaccharide (*m*/*z* 486.1 $[M+Na]^+$). It was chosen as the model reaction to set optimal glycosynthase conditions and further explore the specificity with different donor and acceptor saccharides.

2.1. Enzyme kinetics: pH and temperature dependence of the glycosynthase activity

Initial rates of product formation (v_o) between donor **1** and acceptor **4** were determined by HPLC analysis. The reactions were done at constant acceptor (5 mM) and donor (0.9 mM) concentrations in phosphate buffer either at pH 7 over the temperature range from 25 to 55 °C, or at 35 °C over the pH range from 5.5 to 10. The results are presented in Figure 1.

The temperature profile shows a maximal activity at 45 °C. But at this temperature, spontaneous hydrolysis of the fluoride donor measured by capillary electrophoresis (data not shown) is significant. The ratio between glycosynthase activity and spontaneous hydrolysis was maximal at 35 °C, so this temperature was chosen for all coming experiments.

The plot v_o versus pH (Fig. 1B) corresponds to general base catalysis with residual glycosynthase activity (A_{res}) at pH 5.5, and maximum (B_{max}) activity above

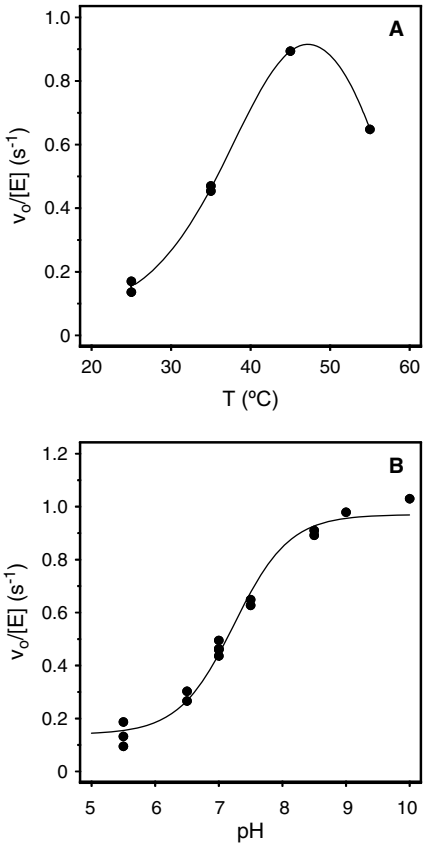


Figure 1. Temperature and pH dependencies of glycosynthase-catalyzed condensation of donor **1** and acceptor **4** by E383A β-glucosidase in 50 mM phosphate buffer and constant ionic strength (0.15 M): (A) temperature profile, [enzyme] = 8.5 nM, [donor (**1**)] = 0.8 mM, [acceptor (**4**)] = 5.7 mM, pH 7.0; (B) pH profile, [enzyme] = 8.1–14.8 nM, [donor (**1**)] = 0.9 mM, [acceptor (**4**)] = 5.0 mM, 35 °C.

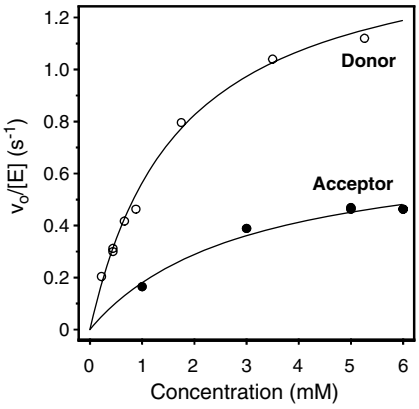


Figure 2. Glycosynthase kinetics of E383A β-glucosidase with donor **1** and acceptor **4** in 50 mM phosphate buffer, pH 7 and 35 °C: (●) kinetics for the acceptor at fixed 0.9 mM donor concentration, (○) kinetics for the donor at fixed 4.9 mM acceptor concentration. [Enzyme] = 4.8–7.6 nM.

pH > 8. Data were fitted to Eq. 1 to give an apparent kinetic pK_a value of 7.24.

$$\frac{v}{[E]} = A_{res} + \frac{B_{max}}{1 + 10^{(pK_a - pH)}} \quad (1)$$

This pK_a reflects ionization of Glu178 as the general base in the glycosynthase mechanism (Scheme 1). It has been proved for another glycosynthase, the E134A mutant (1→3)-(1→4)-β-glucanase from *Bacillus licheniformis*, that the same residue acting as the general acid/base catalyst in the wild type hydrolase activity is indeed the general base in the glycosynthase reaction.[7]

p-Nitrophenyl glucoside (**4**) showed significant substrate inhibition of the wt hydrolase activity (K_I 1.4 mM, K_M 0.15 mM).[16] To evaluate the potential inhibitory effect on the glycosynthase reaction, kinetics with varying acceptor concentration and fixed α-glucosyl fluoride (**1**) donor (0.9 mM) at pH 7.0 and 35 °C were analyzed. The Michaelis–Menten curve (Fig. 2) shows no inhibition up to 6 mM, with apparent kinetic parameters $k_{cat}^{app} = 0.72 \pm 0.08\ s^{-1}$ and $K_M^{app} = 3.0 \pm 0.8$ mM. Kinetic parameters for the donor were also determined at constant (4.9 mM) acceptor concentration (Fig. 2): $k_{cat}^{app} = 1.53 \pm 0.06\ s^{-1}$ and $K_M^{app} = 1.7 \pm 0.2$ mM.

2.2. Donor and acceptor specificity

The wt enzyme has a broad substrate specificity, being able to hydrolyze aryl β-glucosides, fucosides, galactosides and xylosides, as well as β-(1→4), β-(1→3) and β-(1→2) glycosidic linkages.[15,16] The transglycosylation activity of the wild type enzyme with *p*-nitrophenyl β-D-galactoside as donor and D-xylose as acceptor yielded β-(1→3) (40%), β-(1→2) (26%) and β-(1→4) (4%) disaccharides.[18]

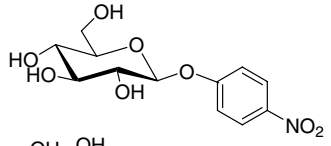
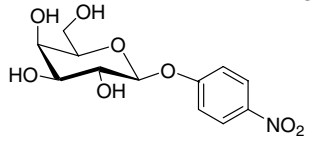
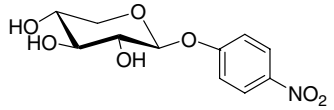
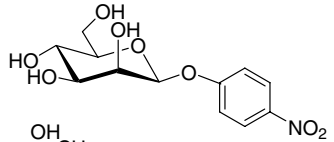
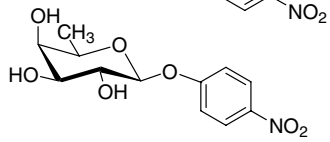
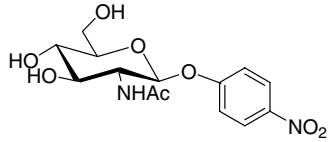
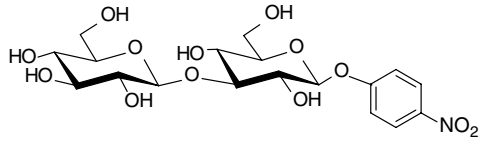
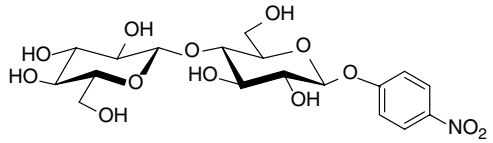
To define donor and acceptor glycosynthase specificity of the E383A β-glucosidase, three donors, α-glucosyl, α-galactosyl and α-xylosyl fluorides (**1**–**3**), were assayed, and a set of different monosaccharides (**4**–**9**) and disaccharides (**10**–**11**) were evaluated as acceptors (Tables 1 and 2). Donor and acceptor at 1–5 M ratio were incubated with the enzyme (0.01–35 μM, depending on substrates reactivity) at pH 7.0 and 35 °C. The excess of acceptor minimizes elongation reactions where the glycosyl donor may react with the condensation product leading to oligomers. Two sets of reactions were carried out: analytical scale reactions at low enzyme concentration to determine initial rates of product formation by HPLC, and preparative reactions at higher enzyme concentration to evaluate product yields after longer reaction times and to determine the regioselectivity of the newly formed glycosidic bond by NMR spectroscopy.

As shown in Tables 1 and 2, α-glucosyl and galactosyl fluorides are good donors, but not α-xylosyl fluoride, where the absence of the 5-hydroxymethyl group has a dramatic effect and no glycosynthase activity with any acceptor was detected. The importance of this substituent in subsite −I of the enzyme binding site has been observed in the wt hydrolase activity of this and other Family 1 β-glycosidases, with 15–19 kJ mol^{-1} transition state destabilization on C-6-deoxygenated substrates as compared to their oxygenated homologues.[16,19–21] Likewise, α-xylosyl fluoride was not a donor for the glycosynthase derived from *Agrobacterium* β-glucosidase,[1] but remarkably, directed evolution has recently overcome this limitation to afford an efficient catalyst for xylooligosaccharides synthesis.[22]

Relative reactivities were evaluated from initial rates of condensation (Tables 1 and 2). The glucosyl donor **1** reacts five-times faster than the galactosyl donor **2** with the *p*-nitrophenyl glucoside acceptor, in agreement with kinetics of the wild type hydrolase activity.[15] For the acceptors, the sequence of reactivities is Glu > Gal ≈ Xyl ≈ Man ≫ Fuc. The fucoside acceptor reacts slowly with α-glucosyl fluoride (**1**), and no reaction was detected with the galactosyl donor **2** under the same conditions. *p*-Nitrophenyl cellobioside (**10**) is a better acceptor than the laminaribioside **11**, but both gave lower transglycosylation initial rates than the glucoside acceptor **4**. This is probably the consequence of competition of the disaccharide acceptors for the donor subsite. However, direct comparison to monosaccharides cannot be done because of the different regioselectivity of the reactions, as discussed below.

Preparative reactions were finalized when no increase of product concentration was detected by HPLC. Reaction mixtures were directly purified by reverse-phase chromatography to isolate the condensation products. Good yields were obtained with some donor–acceptor combinations. With α-glucosyl fluoride donor yields varied from 30% to 100% depending on the acceptor, and with α-galactosyl fluoride they were slightly lower (14–77%). All compounds were characterized by MS

Table 1. Glycosynthase reactions of E383A β-glucosidase using α-glucosyl fluoride (**1**) donor and different acceptors

	Acceptor	Disaccharide product (% reaction yield)[a]	$v/[E] \times 10^2$ (s^{-1})[b]	Reactivity[c] (%)
4		β-(1→3) (96)	46.14	100
5		β-(1→3) (100)	1.80	3.9
6		β-(1→3) (54) β-(1→4) (28)	0.28	0.6
7		β-(1→3) (53)	0.45	1
8		β-(1→3) (30)	0.02	0.04
9		Nr	—	—
10		β-(1→4) (80)	9.72	21
11		β-(1→4)	0.70	1.5

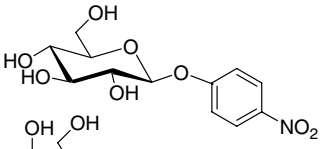
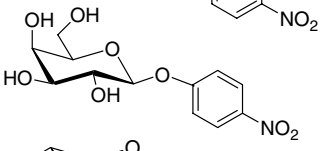
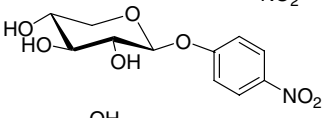
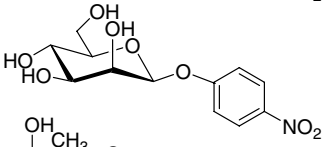
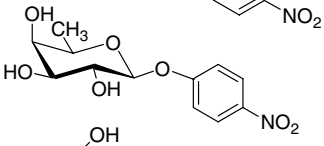
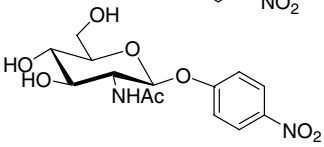
[a] Preparative reactions in 50 mM phosphate buffer, pH 7.0, 35 °C, at 1 to 5 donor:acceptor molar ratio, and 2–28 μM enzyme concentration. Yields in isolated product after purification.
[b] Initial rates of product formation (by HPLC). Conditions: 50 mM phosphate buffer, pH 7.0, 35 °C. [donor] = 0.9 mM, [acceptor] = 5 mM.
[c] Reactivity expressed as percentage of initial rates relative to acceptor **4**. Nr: no reaction.

and NMR spectroscopy. Connectivities of the new glycosidic linkages were assigned by comparison to published ^{13}C NMR spectra,[23,24] by calculating the spectra taking into account the effect of O-glycosidation on ^{13}C chemical shifts,[25,26] or by two-dimensional ^{1}H and ^{13}C NMR experiments (see Section 3).

With monosaccharide acceptors, the E383A glycosynthase gives specifically β-(1→3) disaccharides (Tables 1 and 2). *p*-Nitrophenyl β-laminaribioside, glucosyl-β-(1→3)-galactoside and galactosyl-β-(1→3)-glucoside were obtained with nearly quantitative yields. Condensation between glucosyl and galactosyl donors and *p*-nitrophenyl mannoside acceptor gave the corresponding β-(1→3) disaccharides in 50% and 60%, respectively. Lower yields were obtained for *p*-nitrophenyl galactosyl-β-(1→3)-galactoside and glucosyl-β-(1→3)-fucoside products (<30%).

The β-(1→3) regioselectivity of the E383A glucosynthase was partially lost with the xyloside acceptor **6**. Condensation of glucosyl and galactosyl donors with *p*-nitrophenyl xyloside yielded β-(1→3) and β-(1→4) regioisomers. It suggests that the 5-hydroxymethyl group of the saccharide acceptor in subsite +I directs regioselectivity to β-(1→3) linkages, whereas its absence results in a relaxed selectivity. A similar but inverse behaviour was observed with the glycosynthase mutants

Table 2. Glycosynthase reactions of E383A β-glucosidase using α-galactosyl fluoride (**2**) donor and different acceptors

	Acceptor	Disaccharide product (% reaction yield)[a]	$v/[E]\times 10^2$ (s^{-1})[b]	Reactivity[c] (%)
4		β-(1→3) (77)	9.44	100
5		β-(1→3) (14)	0.017	0.2
6		β-(1→3) (25) β-(1→4) (12)	0.034	0.3
7		β-(1→3) (47)	0.38	4.0
8		Nr	—	—
9		Nr	—	—

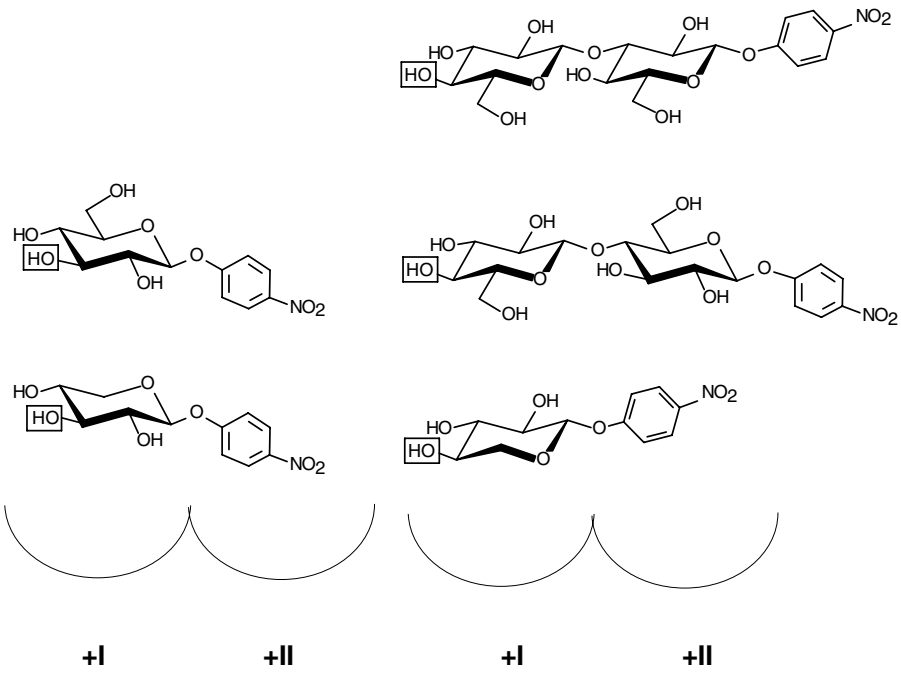
[a] Preparative reactions in 50 mM phosphate buffer, pH 7.0, 35 °C, at 1 to 5 donor:acceptor molar ratio, and 2–28 μM enzyme concentration. Yields in isolated product after purification.
[b] Initial rates of product formation (by HPLC). Conditions: 50 mM phosphate buffer, pH 7.0, 35 °C. [donor] = 0.9 mM, [acceptor] = 5 mM.
[c] Reactivity expressed as percentage of initial rates relative to acceptor **4**. Nr: no reaction.

of β-glucosidase from *Agrobacterium*.[1,22] Aryl glycoside acceptors were condensed via β-(1→4) linkages in all cases, except for the β-xyloside acceptor, which gave β-(1→3) products.

In contrast to monosaccharide, condensation with disaccharide acceptors resulted in the formation of β-(1→4) glycosidic linkage. *p*-Nitrophenyl cellotrioside was obtained in 80% yield by reaction between α-glucosyl fluoride (**1**) and the cellobioside acceptor **10** as confirmed by NMR spectroscopy. In the same way, transfer to the laminaribioside acceptor **11** yielded the trisaccharide *p*-nitrophenyl glucosyl-β-(1→4)-laminaribioside, which was identified by coinjection with a synthetic sample[27] in HPLC. In both reactions, a second minor compound appeared after six hours in 3% yield. For the cellobioside acceptor **10**, it was corroborated not to be the regioisomer of the cellotrioside Glcβ3Glcβ4Glc-pNP, by coinjection with an independent standard.[7] And for the laminaribioside acceptor **11**, this second product was identified as Glcβ4Glcβ4Glcβ3Glc-pNP. These minor compounds are tetrasaccharides proposed to be elongation products coming from a second coupling of the α-glucosyl fluoride donor with the first trisaccharide product via β-(1→4) linkage.

Therefore, the regioselectivity of glycosidic bond formation is switched from β-(1→3) with monosaccharide acceptors to β-(1→4) with disaccharides. It suggests that enzyme–ligand interactions in subsite +II have a decisive effect on the orientation of the acceptor. For 4-nitrophenyl β-D-glucopyranoside, the aryl group occupies subsite +II and the glycosyl unit in subsite +I presents the 3-OH group to be glycosylated (Scheme 2). In contrast, when a glucosyl unit occupies subsite +II (cellobiose and laminaribiose), the glucosyl residue in subsite +I presumably binds in an alternative mode, presenting the 4-OH group for glycosylation. Although the orientation of the pyranose ring in subsite +II for the cellobioside and laminaribioside acceptors differs, the presence of this glucose residue instead of the aryl group favours formation of a β-(1→4) linkage in both cases.

Compared to the other *exo*-glycosynthases, the E383A glycosynthase has a different specificity than other Family 1 glycosynthases. β-Glucosidase from *Agrobacterium*[1] produces exclusively β-(1→4) linkages with a variety of mono- and disaccharide acceptors, with the exception of a xylosyl acceptor that gives a β-(1→3) bond. Recently, it has been shown that the regioselectivity of this glycosynthase can be modified by changes in



Scheme 2. Binding modes of monosaccharide and disaccharide acceptors according to the regioselectivity observed for the E383A β-glucosidase. The hydroxyl group to be glycosylated is indicated.

the acceptor; that is, 6-benzyl or 6-benzoyl glucopyranose as acceptor yielded β-(1→2) disaccharides as a consequence of rotation of the glycosyl moiety into subsite +1 because subsite +II accommodates the aromatic substituent in O-6.[28] On the other hand, β-(1→3) glycosylation is mainly obtained with glycosynthases from *Sulfolobus solfataricus*,[12] *Pyrococcus furiosus*, *Thermosphaera aggregans*[13] and *Thermus thermophilus*.[14]

2.3. Immobilized glycosynthase enzyme

Since yields obtained in the preparative glycosynthase reactions looked promising (Table 1), the E383A β-glucosidase was immobilized to improve its operational performance for large-scale oligosaccharide synthesis. Taking advantage of the N-terminal His-tag fused to the enzyme to simplify the protein purification protocol,[16] immobilization was performed with Chelating Sepharose resin previously charged with Ni^{2+}. Quantitative immobilization was assured using a four-fold excess of resin relative to its nominal loading capacity.

Reaction between α-glucosyl fluoride (**1**) and *p*-nitrophenyl glucoside (**4**) using the immobilized enzyme was completed after 5 h. The corresponding β-(1→3) disaccharide was obtained in 95% yield.

The soluble enzyme is rather unstable at 4 °C and should be stored at −20 °C in 45% glycerol for long periods.[15] The glycosynthase activity of the immobilized enzyme was checked after long-term storage (storage stability) and after multiple cycles of conversion (operational stability). Several immobilized enzyme samples were stored at 4 °C for different time periods, before being incubated with donor **1** and acceptor **4** at 35 °C. The reaction was stopped after 5 h by centrifugation and the condensation product was quantified by HPLC. Table 3 presents the % of activity relative to the initial activity. The yield in product formation is the same as that obtained with the soluble enzyme but the immobilized form retains at least 80% activity after 55 days of storage. Moreover, when recycled and reused, complete operational stability was found after nine cycles (Table 3).

Glycosynthase activities of soluble and immobilized enzymes were comparable in the presence of organic co-solvents. Both maintained 100% activity up to 10%

Table 3. Storage and operational stabilities of the immobilized E383A β-glucosidase[a]

Operational stability				Storage stability	
Cycle	Activity	Cycle	Activity	Day	Activity
1	100	5	104	0	100
2	96	6	92	1	93
3	103	7	102	12	94
4	95	8	100	54	83
		9	93		

[a] Transglycosylation activities of the immobilized glycosynthase (10 μM) with donor **1** and acceptor **4** during nine cycles of reuse (operational stability) and after 54 days of storage (storage stability). Activities are expressed as percentage of the initial rate.

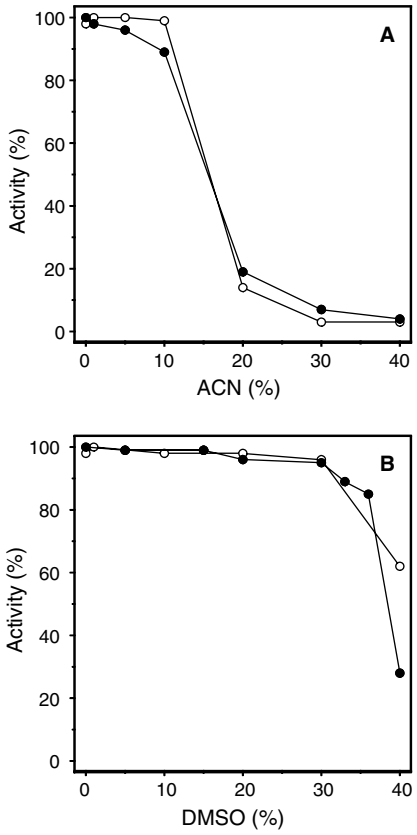


Figure 3. Glycosynthase yields of immobilized E383A β-glucosidase (●) and soluble E383A β-glucosidase (○) with donor **1** (1 mM) and acceptor **4** (5 mM) in phosphate buffer (50 mM, pH 7) and MeCN (A) or Me_2SO (B) after 20 h of reaction.

and 30% of MeCN and Me_2SO, respectively (Fig. 3). Likewise, the pH profiles were comparable (data not shown).

2.4. Conclusions

The E383A glycosynthase from *Streptomyces* sp. offers an unexpected regioselectivity compared to the other glycosynthases so far reported. For monosaccharide acceptors, the enzyme produces β-(1→3) disaccharides in excellent yields. Particularly, the disaccharides Glcβ3Man and Galβ3Man here obtained have not been reported for any other glycosynthase. In contrast, β-(1→4) linkages are obtained with disaccharide acceptors. Therefore, interactions in subsite +II of the enzyme seem to play a major role in directing regioselectivity.

Although immobilization does not increase stability in organic solvents, the enzyme is remarkably more stable than the soluble form when stored at 4 °C in phosphate buffer at pH 7.0, and can be reused for several cycles. Therefore, the immobilized form guarantees the availability of this enzyme for the scale-up synthesis of oligosaccharides and glycoconjugates.

3. Experimental

3.1. General

Unidimensional ^1H and ^{13}C NMR spectra were recorded on a Varian Gemini-300 spectrometer, and two-dimensional spectra (COSY, TOCSY, NOESY, HSQC and HMBC) were recorded on a Inova-500 spectrometer. Proton chemical shifts (δ in ppm) were referenced to internal Me_4Si for solns in $CDCl_3$ and to an external reference for solns in D_2O or Me_2SO-d_6. Mass spectra were determined on a VG Platform spectrometer in the FAB ionization mode: m/z for the peaks (100%) corresponding to $[M+Na]^+$ are given.

3.2. Enzyme

E383A mutant β-glucosidase from *Streptomyces* was expressed in *Escherichia coli* and purified as published.[16] Enzyme concentrations were determined by UV spectrophotometry using $\varepsilon_{280} = 107{,}000\ M^{-1}\ cm^{-1}$.

3.3. Glycosyl fluoride donors 1–3

α-Glycosyl fluorides were prepared according to standard procedures[29,30] by treatment of the corresponding peracetylated monosaccharide with hydrogen fluoride in pyridine, purification of the peracetylated α-glycosyl fluorides by flash chromatography and O-deacetylation with NaOMe in MeOH to afford compounds **1**–**3** in 70–80% yield.

The donors were dissolved in deionized D_2O, filtered and stored at −20 °C. Concentrations of the stock solns were determined by measuring the total amount of fluoride ion after hydrolysis with 20 mM H_2SO_4 at 100 °C for 10 min and neutralization with NaOH using a selective fluoride electrode (Sentek, UK interfaced with a CiberScan Bench pH/Ion meter from Eutech Instruments).

3.4. Glycosyl acceptors

4-Nitrophenyl β-D-glucopyranoside (**4**), 4-nitrophenyl β-D-galactopyranoside (**5**), 4-nitrophenyl β-D-xylopyranoside (**6**), 4-nitrophenyl β-D-mannopyranoside (**7**), 4-nitrophenyl β-D-fucopyranoside (**8**) and 4-nitrophenyl 2-acetamido-2-deoxy-glucose (**9**) were from Fluka. 4-Nitrophenyl β-cellobioside (**10**) was prepared as described,[31] and 4-nitrophenyl β-laminaribioside (**11**) was obtained by glycosynthase reaction of E383A β-glucosidase as described below.

3.5. Glycosynthase kinetics

Glycosyl donor and acceptor at appropriate concentrations in phosphate buffer (50 mM, ionic strength

0.15 M with added KCl, pH 7.0) were pre-incubated at 35 °C for 5 min. After addition of E383A β-glucosidase (0.01–35 μM, final vol of 0.3 mL), the reactions were kept at 35 °C. Aliquots were withdrawn at regular time intervals, diluted with deionized water (1:10), and analyzed by HPLC (NovaPak C18 column, 4 μm, 3.9 × 150 mm from Waters, 1 mL min^{-1} or Zorbax Eclypse XDB C-18 column, 1.8 μm, 4.6 × 50 mm, from Agilent, 1.2 mL min^{-1}, 5–16% MeOH in water, UV detector at 300 nm). Chromatographic peaks were identified by co-injection with independent standards or by spectroscopic analysis after preparative synthesis. Initial rates (v_o) were calculated from the linear progress curve of product formation (normalized area vs time) and expressed as v_o/[E] in s^{-1}.

3.6. Preparative glycosynthase-catalyzed syntheses

A soln of donor (5–20 mg, 1 equiv), acceptor (5 equiv) and E383A β-glucosidase (2–28 μM) was incubated at 35 °C in phosphate buffer (50 mM, pH 7.0, 1–6 mL). The reactions were monitored by HPLC as indicated above. After 24–48 h, the reaction mixture was filtered and directly loaded onto a Lichroprep RP-18 Lobar-A column (E. Merck), which was eluted with a gradient MeOH–water from 0% to 3–20% (v/v). Unreacted acceptor eluted first, followed by the condensation product. It was freeze dried and lyophilized for storage. The results are summarized in Tables 1 and 2. Yields and spectral data of the condensation products are:

3.6.1. 4-Nitrophenyl β-D-glucopyranosyl-(1→3)-β-D-glucopyranoside (11). From donor **1** (5.1 mg) and acceptor **4** (43 mg); 12.4 mg (96%); FABMS: *m/z* 486.12 $[M+Na]^+$; ^1H NMR (D_2O, 25 °C): δ 8.29 (d, $J_{3',2'} = J_{5',6'}$ 9.5 Hz, 2H, H-3′, H-5′), 7.27 (d, 2H, H-2′, H-6′), 5.31 (d, 1H, $J_{1,2}$ 7.5 Hz, H-1^I), 4.80 (d, 1H, $J_{1,2}$ 8.0 Hz, H-1^{II}), 3.95–3.35 (m, 12H, H-$2^{I,II}$, H-$3^{I,II}$, H-$4^{I,II}$, H-$5^{I,II}$, H-$6a^{I,II}$, H-$6b^{I,II}$); ^{13}C NMR (D_2O, 25 °C): δ 162.4 (C-1′), 143.3 (C-4′), 126.8 (C-3′, C-5′), 117.2 (C-2′, C-6′), 103.5 (C-1^{II}), 99.9 (C-1^I), 84.6 (C-3^I), 76.7–76.2 (C-3^{II}, C-$5^{I,II}$), 74.1, 73.2 (C-$2^{I,II}$), 70.3 (C-4^{II}), 68.5 (C-4^I), 61.4, 61.1 (C-$6^{I,II}$). HPLC (16% MeOH): t_R 5.14 min.

β-(1→3) Connectivity: δ_H at 4.80 ppm characteristic of H-1 in a β-(1→3)-linked glucose, and δ_C at 84.6 ppm corresponding to O-glycosylated C-3 and at 68.5 ppm characteristic of unsubstituted C-4 next to O-glycosylated C-3.[23,25]

3.6.2. 4-Nitrophenyl β-D-glucopyranosyl-(1→3)-β-D-galactopyranoside. From donor **1** (5.1 mg) and acceptor **5** (40 mg); 13.1 mg (100%); FABMS: *m/z* 486.12 $[M+Na]^+$. ^1H NMR (D_2O, 25 °C): δ 8.28 (d, $J_{3',2'} = J_{5',6'}$ 9.5 Hz, 2H, H-3′, H-5′), 7.27 (d, $J_{2',3'} = J_{6',5'}$ 9.5 Hz, 2H, H-2′, H-6′), 5.28 (d, 1H, $J_{1,2}$ 7.5 Hz, H-1^I), 4.74 (d, 1H, $J_{1,2}$ 7.5 Hz, H-1^{II}), 4.31 (d, 1H, $J_{4,3}$ 2.7 Hz, H-4^I), 4.08–3.40 (m, 11H, H-$2^{I,II}$, H-$3^{I,II}$, H-4^{II}, H-$5^{I,II}$, H-$6a^{I,II}$, H-$6b^{I,II}$). ^{13}C NMR (D_2O, 25 °C): δ 162.4 (C-1′), 143.2 (C-4′), 126.7 (C-3′, C-5′), 117.1 (C-2′, C-6′), 104.4 (C-1^{II}), 100.3 (C-1^I), 82.6 (C-3^I), 76.5–76.0 (C-3^{II}, C-$5^{I,II}$), 74.0 (C-2^{II}), 70.1, 70.1 (C-2^I, C-4^{II}), 68.7 (C-4^I), 61.3, 61.2 (C-$6^{I,II}$). COSY (D_2O, 25 °C) selected data: 4.15, 3.82 (H-4^I, H-3^I); NOESY (D_2O, 25 °C) selected data: 4.59, 3.82 (H-1^{II}, H-3^I); TOCSY (D_2O, 25 °C) selected data: 5.13, 3.82 (H-1^I, H-3^I); HSQC (D_2O, 25 °C) selected data: 3.82, 82.6 (H-3^I, C-3^I); HMBC (D_2O, 25 °C) selected data: 4.59, 82.6 (H-1^{II}, C-3^I). HPLC (10% MeOH): t_R = 10.85 min.

β-(1→3) Connectivity: confirmed by the presence of a correlation of H-1^{II} and C-3^I (4.59, 82.6) in the HMBC spectrum.

3.6.3. 4-Nitrophenyl β-D-glucopyranosyl-(1→4)-β-D-xylopyranoside. From donor **1** (5.2 mg) and acceptor **6** (37.6 mg); 3.1 mg (28%); FABMS: *m/z* 456.15 $[M+Na]^+$. ^1H NMR (D_2O, 25 °C): δ 8.29 (d, $J_{3',2'} = J_{5',6'}$ 9.5 Hz, 2H, H-3′, H-5′), 7.27 (d, $J_{2',3'} = J_{6',5'}$ 9.5 Hz, 2H, H-2′, H-6′), 5.27 (d, 1H, $J_{1,2}$ 7.5 Hz, H-1^I), 4.57 (d, 1H, $J_{1,2}$ 8.0 Hz, H-1^{II}), 4.23–3.27 (11H, H-$2^{I,II}$, H-$3^{I,II}$, H-$4^{I,II}$, H-$5a^I$, H-$5b^I$, H-5^{II}, H-$6a^{II}$, H-$6b^{II}$). ^{13}C NMR (D_2O, 25 °C) δ: 162.2 (C-1′), 143.3 (C-4′), 126.8 (C-3′, C-5′), 117.1 (C-2′, C-6′), 101.9 (C-1^{II}), 100.5 (C-1^I), 76.8–76.2 (C-3^{II}, C-4^I, C-5^{II}), 74.2–73.2 (C-$2^{I,II}$, C-3^I), 70.3 (C-4^{II}), 63.8 (C-5^I), 61.4 (C-6^{II}). HPLC (16% MeOH): t_R = 7.77 min.

β-(1→4) Connectivity: δ_H at 4.57 ppm characteristic of H-1 in a β-(1→4)-linked glucose, and δ_C at 76 ppm corresponding to O-glycosylated C-4.[23,24,18]

3.6.4. 4-Nitrophenyl β-D-glucopyranosyl-(1→3)-β-D-xylopyranoside. From donor **1** (5.2 mg) and acceptor **6** (37.6 mg); 6.7 mg (54%); FABMS: *m/z* 456.11 $[M+Na]^+$. ^1H NMR (D_2O, 25 °C): δ 8.28 (d, $J_{3',2'} = J_{5',6'}$ 9.5 Hz, 2H, H-3′, H-5′), 7.26 (d, $J_{2',3'} = J_{6',5'}$ 9.5 Hz, 2H, H-2′, H-6′), 5.26 (d, 1H, $J_{1,2}$ 7.0 Hz, H-1^I), 4.8 (d, 1H, $J_{1,2}$ 8.0 Hz, H-1^{II}), 4.13–3.4 (m, 11H, H-$2^{I,II}$, H-$3^{I,II}$, H-$4^{I,II}$, H-$5a^I$, H-$5b^I$, H-5^{II}, H-$6a^{II}$, H-$6b^{II}$). ^{13}C NMR (D_2O, 25 °C): δ 162.2 (C-1′), 143.3 (C-4′), 126.8 (C-3′, C-5′), 117.1 (C-2′, C-6′), 103.4 (C-1^{II}), 100.4 (C-1^I), 84.1 (C-3^I), 76.7, 76.2 (C-3^{II}, C-5^{II}), 74.1, 72.9 (C-$2^{I,II}$), 70.3 (C-4^{II}), 68.3 (C-4^I), 65.6 (C-5^I), 61.4 (C-6^{II}). HPLC (16% MeOH): t_R = 14.37 min.

β-(1→3) Connectivity: δ_H at 4.8 ppm characteristic of H-1 in a β-(1→3)-linked glucose, and δ_C at 84.1 ppm corresponding to O-glycosylated C-3 and at 68.3 ppm assigned to unsubstituted C-4 next to O-glycosylated C-3.[23,24,18]

3.6.5. 4-Nitrophenyl β-D-glucopyranosyl-(1→3)-β-D-mannopyranoside. From donor **1** (5.2 mg) and acceptor **7** (43.2 mg); 7.1 mg (53%); FABMS: *m/z* 486.14 $[M+Na]^+$. ^1H NMR (D_2O, 25 °C): δ 8.28 (d, $J_{3',2'} = J_{5',6'}$ 9.3 Hz, 2H, H-3′, H-5′), 7.24 (d, $J_{2',3'} = J_{6',5'}$ 9.3 Hz, 2H, H-2′, H-6′), 5.53 (s, 1H, H-1^I), 4.65 (d, 1H, $J_{1,2}$ 7.8 Hz, H-1^{II}), 4.47 (d, 1H, $J_{2,3}$ 2.5 Hz, H-2^I), 4.10–3.40 (11H, H-2^{II}, H-$3^{I,II}$, H-$4^{I,II}$, H-$5^{I,II}$, H-$6a^{I,II}$, H-$6b^{I,II}$). ^{13}C NMR (D_2O, 25 °C): δ 162.0 (C-1′), 143.1 (C-4′), 126.7 (C-3′, C-5′), 116.9 (C-2′, C-6′), 100.9 (C-1^{II}), 97.6 (C-1^I), 80.2 (C-3^I), 77.0–76.2 (C-3^{II}, C-$5^{I,II}$), 73.6 (C-2^{II}), 70.2 (C-4^{II}), 68.4 (C-2^I), 65.5 (C-4^I), 61.5, 61.4 (C-$6^{I,II}$). COSY (D_2O, 25 °C) selected data: 4.31, 3.83 (H-2^I, H-3^I); NOESY (D_2O, 25 °C) selected data: 4.50, 3.83 (H-1^{II}, H-3^I); HSQC (D_2O, 25 °C) selected data: 3.83, 80.2 (H-3^I, C-3^I); HMBC (D_2O, 25 °C) selected data: 4.50, 80.2 (H-1^{II}, C-3^I). HPLC (5% MeOH): $t_R = 12.97$ min.

β-(1→3) Connectivity: confirmed by the presence of a correlation of H-1^{II} and C-3^I (4.50, 80.2) in the HMBC spectrum.

3.6.6. 4-Nitrophenyl β-D-glucopyranosyl-(1→3)-β-D-fucopyranoside. From donor **1** (5.1 mg) and acceptor **8** (41 mg); 3.8 mg (30%); FABMS: *m/z* 470.13 $[M+Na]^+$. ^1H NMR (D_2O, 25 °C): δ 8.13 (d, $J_{3',2'} = J_{5',6'}$ 9.0 Hz, 2H, H-3′, H-5′), 7.12 (d, $J_{2',3'} = J_{6',5'}$ 9.0 Hz, 2H, H-2′, H-6′), 5.12 (d, 1H, $J_{1,2}$ 7.0 Hz, H-1^I), 4.58 (d, 1H, $J_{1,2}$ 7.5 Hz, H-1^{II}), 3.97–3.26 (m, 10H, H-$2^{I,II}$, H-$3^{I,II}$, H-$4^{I,II}$, H-$5^{I,II}$, H-$6a^{II}$, H-$6b^{II}$), 1.32 (d, 3H, $J_{6,5}$ 6.5 Hz, H-6^I). ^{13}C NMR (D_2O, 25 °C): δ 162.4 (C-1′), 143.2 (C-4′), 126.7 (C-3′, C-5′), 117.0 (C-2′, C-6′), 104.5 (C-1^{II}), 100.0 (C-1^I), 82.7 (C-3^I), 76.5, 76.2 (C-3^{II}, C-5^{II}), 74.0–69.8 (C-$2^{I,II}$, C-$4^{I,II}$, C-5^I), 61.2 (C-6^{II}), 16.0 (C-6^I). COSY (D_2O, 25 °C) selected data: 3.97, 3.83 (H-4^I, H-3^I). HSQC (D_2O, 25 °C) selected data: 3.83, 82.7 (H-3^I, C-3^I). HMBC (D_2O, 25 °C) selected data: 4.58, 82.7 (H-1^{II}, C-3^I). HPLC (16% MeOH): $t_R = 11.37$ min.

β-(1→3) Connectivity: confirmed by the presence of a correlation of H-1^{II} and C-3^I (4.58, 82.7) in the HMBC spectrum.

3.6.7. 4-Nitrophenyl β-D-galactopyranosyl-(1→3)-β-D-glucopyranoside. From donor **2** (10.2 mg) and acceptor **4** (85.7 mg); 19.9 mg (77%); FABMS: *m/z* 486.123 $[M+Na]^+$. ^1H NMR (D_2O, 25 °C): δ 8.27 (d, $J_{3',2'} = J_{5',6'}$ 9.5 Hz, 2H, H-3′, H-5′), 7.25 (d, $J_{2',3'} = J_{6',5'}$ 9.5 Hz, 2H, H-2′, H-6′), 5.31 (d, 1H, $J_{1,2}$ 6.0 Hz, H-1^I), 4.73 (d, 1H, $J_{1,2}$ 7.0 Hz, H-1^{II}), 4.00–3.60 (m, 12H, H-$2^{I,II}$, H-$3^{I,II}$, H-$4^{I,II}$, H-$5^{I,II}$, H-$6a^{I,II}$, H-$6b^{I,II}$). ^{13}C NMR (D_2O, 25 °C): $\delta = 162.3$ (C-1′), 143.3 (C-4′), 126.7 (C-3′, C-5′), 117.1 (C-2′, C-6′), 104.0 (C-1^{II}), 99.9 (C-1^I), 84.6 (C-3^I), 76.5–71.9 (C-$2^{I,II}$, C-3^{II}, C-$5^{I,II}$), 69.3 (C-4^{II}), 68.6 (C-4^I), 61.8, 61.1 (C-$6^{I,II}$). HPLC (10% MeOH): $t_R = 8.13$ min.

β-(1→3) Connectivity: δ_H at 4.73 ppm characteristic of H-1 in a β-(1→3) linkage, and δ_C at 84.6 ppm corresponding to O-glycosylated C-3 and at 68.6 ppm characteristic of unsubstituted C-4 next to O-glycosylated C-3.[23–25]

3.6.8. 4-Nitrophenyl β-D-galactopyranosyl-(1→3)-β-D-galactopyranoside. From donor **2** (10.8 mg) and acceptor **5** (89 mg); 3.6 mg (14%); FABMS: *m/z* 486.123 $[M+Na]^+$. ^1H NMR (D_2O, 25 °C): δ 8.13 (d, $J_{3',2'} = J_{5',6'}$ 9.5 Hz, 2H, H-3′, H-5′), 7.13 (d, $J_{2',3'} = J_{6',5'}$ 9.5 Hz, 2H, H-2′, H-6′), 5.14 (d, 1H, $J_{1,2}$ 8.0 Hz, H-1^I), 4.53 (d, 1H, $J_{1,2}$ 7.5 Hz, H-1^{II}), 4.17 (dd, 1H, $J_{4,3} = J_{4,5}$ 3.5 Hz, H-4^I), 3.92–3.39 (11H, H-$2^{I,II}$ H-$3^{I,II}$, H-$4^{I,II}$, H-$5^{I,II}$, H-$6a^{I,II}$, H-$6b^{I,II}$). ^{13}C NMR (D_2O, 25 °C): δ 162.5 (C-1′), 143.2 (C-4′), 126.8 (C-3′, C-5′), 117.2 (C-2′, C-6′), 105.0 (C-1^{II}), 100.3 (C-1^I), 82.5 (C-3^I), 75.9–70.1 (C-$2^{I,II}$, C-3^{II}, C-$5^{I,II}$), 69.2 (C-4^{II}), 68.8 (C-4^I), 61.6, 61.3 (C-$6^{I,II}$). NOESY (D_2O, 25 °C) selected data: 5.14, 3.89 (H-1^I, H-3^I), 4.53, 3.89 (H-1^{II}, H-3^I). HMBC (D_2O, 25 °C) selected data: 4.53, 82.5 (H-1^{II}, C-3^I). HPLC (5% MeOH): $t_R =$ 11.12 min.

β-(1→3) Connectivity: confirmed by the presence of a correlation of H-1^{II} and C-3^I (4.53, 82.5) in the HMBC spectrum.

3.6.9. 4-Nitrophenyl β-D-galactopyranosyl-(1→4)-β-D-xylopyranoside. From donor **2** (12.6 mg) and acceptor **6** (79.4 mg); 3.6 mg (12%); FABMS: *m/z*: 456.112 $[M+Na]^+$. ^1H NMR (D_2O, 25 °C): δ 8.29 (d, $J_{3',2'} = J_{5',6'}$ 9.5 Hz, 2H, H-3′, H-5′), 7.27 (d, $J_{2',3'} = J_{6',5'}$ 9.5 Hz, 2H, H-2′, H-6′), 5.26 (d, 1H, $J_{1,2}$ 7.5 Hz, H-1^I), 4.49 (d, 1H, $J_{1,2}$ 7.5 Hz, H-1^{II}), 4.22–3.5 (m, 11H, H-$2^{I,II}$, H-$3^{I,II}$, H-$4^{I,II}$, H-$5a^I$, H-$5b^I$, H-5^{II}, H-$6a^{II}$, H-$6b^{II}$). ^{13}C NMR (D_2O, 25 °C): δ 162.2 (C-1′), 143.3 (C-4′), 126.8 (C-3′, C-5′), 117.1 (C-2′, C-6′), 102.5 (C-1^{II}), 100.5 (C-1^I), 76.8–74.3 (C-3^{II}, C-4^I, C-5^{II}), 73.3–71.3 (C-$2^{I,II}$, C-3^I), 69.3 (C-4^{II}), 63.8 (C-5^I), 61.8 (C-6^{II}). HPLC (16% MeOH): $t_R = 6.60$ min.

β-(1→4) Connectivity: δ_H at 4.49 ppm characteristic of H-1 in a β-(1→4) glycosidic linkage, and δ_C at 76 ppm corresponding to O-glycosylated C-4.[23,24,18]

3.6.10. 4-Nitrophenyl β-D-galactopyranosyl-(1→3)-β-D-xylopyranoside. From donor **2** (12.6 mg) and acceptor **6** (79.4 mg); 7.5 mg (25%); FABMS: *m/z* 456.112 $[M+Na]^+$. ^1H NMR (D_2O, 25 °C): δ 8.29 (d, $J_{3',2'} = J_{5',6'}$ 9.5 Hz, 2H, H-3′, H-5′), 7.27 (d, $J_{2',3'} = J_{6',5'}$ 9.5 Hz, 2H, H-2′, H-6′), 5.28 (d, 1H, $J_{1,2}$ 5.5 Hz, H-1^I), 4.71 (d, 1H, $J_{1,2}$ 6.5 Hz, H-1^{II}), 4.12–3.55 (m, 11H, H-$2^{I,\,II}$, H-$3^{I,II}$, H-$4^{I,II}$, H-$5a^I$, H-$5b^I$, H-5^{II}, H-$6a^{II}$, H-$6b^{II}$). ^{13}C NMR (D_2O, 25 °C): δ 162.2 (C-1′), 143.3 (C-4′), 126.8 (C-3′, C-5′), 117.2 (C-2′, C-6′), 103.9 (C-1^{II}), 100.4 (C-1^I), 84.3 (C-3^I), 76.0–71.9

(C-$2^{I,II}$, C-3^{II}, C-5^{II}), 69.2 (C-4^{II}), 68.4 (C-4^{I}), 65.5 (C-5^{I}), 61.7 (C-6^{II}). HPLC (16% MeOH): $t_R = 11.06$ min.

β-(1→3) Connectivity: δ_H at 4.71 ppm characteristic of H-1 in a β-(1→3)-linked glucose, and δ_C at 84.3 ppm corresponding to O-glycosylated C-3 and at 68.4 ppm assigned to unsubstituted C-4 next to O-glycosylated C-3.[23,24,18]

3.6.11. 4-Nitrophenyl β-D-galactopyranosyl-(1→3)-β-D-mannopyranoside. From donor **2** (10.3 mg) and acceptor **7** (89 mg); 12.3 mg (47%); FABMS: *m/z* 486.123 $[M+Na]^+$. ^1H NMR (D_2O, 25 °C): δ 8.28 (d, $J_{3',2'} = J_{5',6'}$ 9.5 Hz, 2H, H-3′, H-5′), 7.24 (d, $J_{2',3'} = J_{6',5'}$ 9.5 Hz, 2H, H-2′, H-6′), 5.53 (d, 1H, H-1^{I}), 4.60 (d, 1H, $J_{1,2}$ 7.0 Hz, H-1^{II}), 4.45 (d 1H, $J_{2,3}$ 2.5 Hz, H-2^{I}), 4.08 (dd, 1H, $J_{3,2}$ 2.5 Hz, $J_{3,4}$ 9.5 Hz, H-3^{I}), 4.02–3.6 (m, 10H, H-2^{II}, H-3^{II}, H-$4^{I,II}$, H-$5^{I,II}$, H-$6a^{I,II}$, H-$6b^{I,II}$). ^{13}C NMR (D_2O, 25 °C): δ 162.1 (C-1′), 143.1 (C-4′), 126.7 (C-3′, C-5′), 116.9 (C-2′, C-6′), 101.5 (C-1^{II}), 97.6 (C-1^{I}), 80.2 (C-3^{I}) 77.0–73.3 (C-3^{II}, C-$5^{I,II}$), 71.4 (C-2^{II}), 69.3 (C-4^{II}), 68.6 (C-2^{I}), 65.6 (C-4^{I}), 61.8, 61.5 (C-$6^{I,II}$). COSY (D_2O, 25 °C) selected data: 4.31, 3.83 (H-2^{I}, H-3^{I}); NOESY (D_2O, 25 °C) selected data: 4.45, 3.83 (H-1^{II}, H-3^{I}); HMBC (D_2O, 25 °C) selected data: 4.45, 80.2 (H-1^{II}, C-3^{I}). HPLC (16% MeOH): $t_R = 4.12$ min.

β-(1→3) Connectivity: confirmed by the presence of a correlation of H-1^{II} and C-3^{I} (4.45, 80.2) in the HMBC spectrum.

3.6.12. 4-Nitrophenyl β-cellotrioside. From donor **1** (45 mg) and acceptor **10** (263 mg); 125 mg (80%); FABMS: *m/z* 648.18 $[M+Na]^+$. ^1H NMR (D_2O, 25 °C): δ 8.28 (d, $J_{3',2'} = J_{5',6'}$ 9.3 Hz, 2H, H-3′, H-5′), 7.26 (d, $J_{2',3'} = J_{6',5'}$ 9.3 Hz, 2H, H-2′, H-6′), 5.31 (d, 1H, $J_{1,2}$ 7.5 Hz, H-1^{I}), [4.58 (d, 1H, $J_{1,2}$ 7.8 Hz), 4.53 (d, 1H, $J_{1,2}$ 7.8 Hz) H-$1^{II,III}$], 4.00–3.20 (m, 18H, H-$2^{I–III}$, H-$3^{I–III}$, H-$4^{I–III}$, H-$5^{I–III}$, H-$6a^{I–III}$, H-$6b^{I–III}$). ^{13}C NMR (DMSO, 25 °C): 162.3 (C-1′), 143.3 (C-4′), 126.8 (C-3′, C-5′), 117.1 (C-2′, C-6′), 103.2–99.9 (C-$1^{I–III}$), 79.0, 78.7 (C-$4^{I,II}$), 76.6–73.2 (C-$2^{I–III}$, C-$3^{I–III}$, C-$5^{I–III}$), 70.1 (C-4^{III}), 61.2–60.3 (C-$6^{I–III}$). HPLC (14% MeOH): $t_R = 6.44$ min.

β-(1→4) Connectivity: δ_H at 4.53 ppm characteristic of H-1 in β-(1→4) glycosidic linkages, and δ_C at 76 ppm corresponding to O-glycosylated C-4.[23,24,31]

3.7. Enzyme immobilization

Chelating Sepharose (500 μL, Amersham Biosciences 17-0575-01) was washed with 2 vol of deionized water (2 × 5 mL) and then saturated with 0.8 M $NiSO_4$ soln. The resin was washed with deionized water (5 × 5 mL) to remove unbounded Ni^{2+} and dried under diminished pressure for 5 min. Dried resin (20 mg) was resuspended in phosphate buffer (50 mM, pH 7) and E383A mutant soln (40–170 μg) was added. After 15 min, the suspension was centrifuged. Immobilization yields were quantified determining unbound enzyme by spectrophotometry.

3.8. Immobilized glycosynthase reactions

α-Glucosyl fluoride (**1**) as donor and *p*-nitrophenyl glucoside (**2**) as acceptor at 1–5 M ratio were added to a suspension containing immobilized E383A (40–200 μg) in phosphate buffer (50 mM, pH 7, final vol 300 μL). The reactions were kept at 35 °C, terminated by filtration or centrifugation, diluted with deionized H_2O (1:10) and analyzed by HPLC as before.

In order to determine the storage stability, several samples of immobilized E383A (162 μg enzyme/20 mg resin) were stored at 4 °C. After different times of storage, the remaining glycosynthase activity was determined.

With respect to the operational stability, three samples of immobilized E383A (108 μg enzyme/20 mg resin) were prepared and the glycosynthase activity was measured after nine cycles of substrates conversion. Between reactions, the immobilized enzyme was kept at 4 °C suspended in phosphate buffer for 19 h.

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