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From β-glucanase to β-glucansynthase: glycosyl transfer to α-glycosyl fluorides catalyzed by a mutant endoglucanase lacking its catalytic nucleophile

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Abstract Removal of the catalytic nucleophile Glu134 of the retaining 1,3-1,4-β-glucanase from *Bacillus licheniformis* by mutation to alanine yields an enzyme with no glycosidase activity. The mutant is able to catalyze the regio- and stereospecific glycosylation of α-laminaribiosyl fluoride with different glucoside acceptors through a single-step inverting mechanism. The main advantage of the mutant as glycosylation catalyst with respect to the kinetically controlled transglycosylation using the wild-type enzyme is that the reaction products cannot be hydrolyzed by the mutant enzyme, and glycosylation yields rise to 90%.

Key words: Glycosynthase; Enzymatic glycosylation; β-Glucanase; Glycosyl fluoride; Nucleophile residue

1. Introduction

The potential of retaining glycosidases as tools for the regio- and stereospecific synthesis of glycosides has been realized by many research groups since the early part of this century [1]. The two main approaches to glycosidase-catalyzed synthesis of glycosidic linkages involve direct reversal of hydrolysis (equilibrium-controlled synthesis) and trapping of a glycosyl-enzyme intermediate (kinetically controlled process) [2]. The equilibrium approach struggles with an unfavorable thermodynamic balance of about 4 kcal/mol for the synthesis-hydrolysis process. High concentration of sugars, addition of organic cosolvents and elevated reaction temperatures are a requirement to achieve significant transformation, yields generally not exceeding 15%. On the other hand, oligosaccharides, aryl glycosides and glycosyl fluorides have been used as donors for the kinetically controlled synthesis. The approach depends on the more rapid trapping of an activated glycosyl-enzyme intermediate by the glycosyl acceptor than by water (Scheme 1a). Although under the right conditions glycoside formation is favored kinetically, hydrolysis either of the intermediate or of the resulting product is favored thermodynamically, and practical yields generally range from 20 to 40%.

A novel approach based on the rational design of the enzyme's catalytic machinery is currently emerging (Scheme 1b). Withers and coworkers [3] have reported that a specifically mutated β-glucosidase lacking its catalytic nucleophile, in conjunction with activated glycosyl donors of the opposite anomeric configuration of that of the normal substrate, can efficiently synthesize oligosaccharides, but does not hydrolyze them. While this first report uses an exo-glycosidase, thus being limited to monosaccharide donors, here we communicate that the methodology also applies to endo-glycosidases where oligosaccharides of different degree of polymerization can act as glycosyl donors.

As part of an ongoing program on structure/function studies of bacterial glucanases, a *Bacillus* 1,3-1,4-β-glucanase (EC 3.2.1.73) [4–7] was chosen as a model endo-glycosidase. The enzyme hydrolyzes β-glucans containing mixed β-1,3 and β-1,4 glycosidic linkages, such as lichenin and cereal β-glucans. It is a retaining glycosidase acting by general acid/base catalysis in a double displacement mechanism [5]. Two glutamic acid residues, Glu138 and Glu134, have been identified in the *B. licheniformis* isozyme as the catalytic acid/base and the nucleophile respectively [6], the role of each residue being assessed by chemical rescue of inactive mutants with exogenous nucleophiles [7]. We have recently reported that the wild-type enzyme shows significant transglycosylation under kinetically controlled conditions with β-glycosyl fluorides [8,9]. The process is regio- and stereospecific, yielding a new β-1,4 linkage. Practical yields are up to 40%.

2. Materials and methods

2.1. General

MALDI-TOF mass spectra were acquired on a Bruker (Bremen, Germany) BIFLEX spectrometer equipped with a pulsed nitrogen laser (337 nm), in the reflectron, positive ion mode, using a 19 kV acceleration voltage and a 20 kV reflector voltage. 2,5-Didroxybenzoic acid (Aldrich Chemicals Co.) was used as ionization matrix. NMR spectra were recorded on a Varian Gemini-300 spectrometer. Proton chemical shifts (δ in ppm) were referenced to internal Me_4Si for solutions in $CDCl_3$ and to an external reference for solutions in D_2O or d_6-Me_2SO.

2.2. Enzymes

Recombinant wild-type and E134A mutant 1,3-1,4-β-glucanases from *B. licheniformis* expressed in *Escherichia coli* were prepared as previously reported [7]. Purity was higher than 95% as judged by SDS-polyacrylamide gel electrophoresis according to Laemmli [10]. Enzyme concentrations were determined by UV spectrophotometry using $\varepsilon_{280} = 14.5$ mg^{-1} ml cm^{-1} (3.55×10^5 M^{-1} cm^{-1}) [11].

2.3. Glycosyl donor

α-Laminaribiose octaacetate [8] (500 mg, 0.737 mmol) was stirred in a sealed plastic tube with 70% HF-py (1 ml) under Ar for 8 h at room temperature. After diluting with CH_2Cl_2 (5 ml), usual work-up and column chromatography (Merck Silica Gel 60 (0.040–0.063 mm), eluted with cyclohexane-EtOAc 1:1) yielded 2,4,6-tri-*O*-acetyl-3-*O*-

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Abbreviations: 1,3-1,4-β-glucanase, 1,3-1,4-β-D-glucan 4-glucanohydrolase; MU, 4-methylumbelliferyl; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; MES, 2-[*N*-morpholino]ethanesulfonic acid; TES, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid

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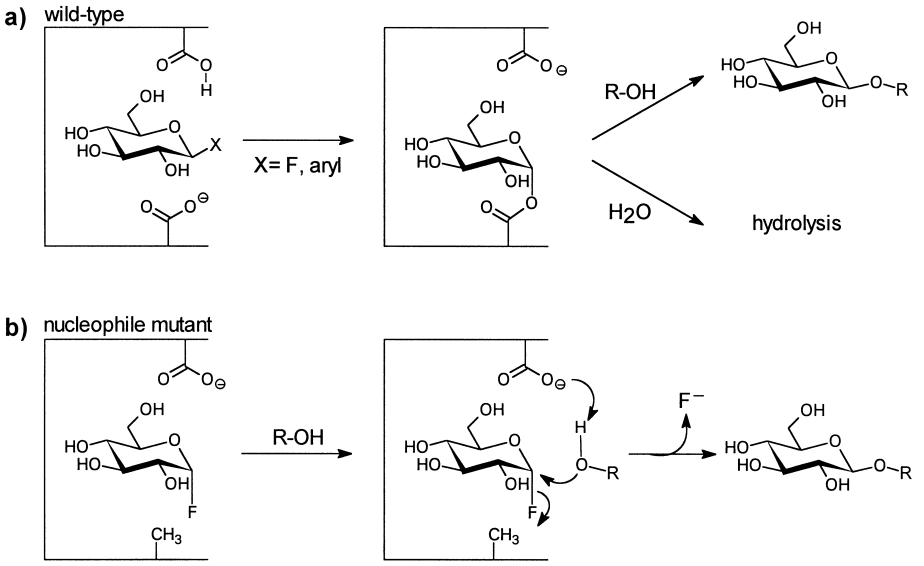


Scheme 1.

(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-α-D-glucopyranosyl fluoride (156 mg, 33%). ^{1}H NMR ($CDCl_3$) δ 5.66 (dd, 1H, *J* 53.1, 2.7 Hz), 4.94 (ddd, 1H, *J* 24.9, 9.9, 2.7 Hz), 4.66 (d, 1H, *J* 8.1 Hz). The compound was stirred with freshly prepared 30 mM NaOMe in MeOH (50 ml) for 3 h at 0°C. The crude was neutralized with excess Amberlite IR-120 (H^+), and the resin was removed by filtration. Evaporation of the solvent gave amorphous α-laminaribiosyl fluoride (**1**). The product was homogeneous on TLC (R_f 0.83, 4:3:10 MeOH-H_2O-EtOAc). ^{1}H NMR (D_2O) δ 5.70 (dd, 1H, *J* 53.4, 3.0 Hz), 4.74 (d, 1H, *J* 7.8 Hz). Compound **1** was dissolved in deionized H_2O (8 ml), filtered (0.22 μm) and stored at −20°C. Concentration of the stock solution was determined by measuring the total amount of fluoride ion by capillary electrophoresis [12] after hydrolysis with 20 mM H_2SO_4 at 100°C for 10–20 min. The average fluoride concentration after three determinations was 26.2 ± 0.9 mM. Free fluoride concentration in the stock solution was below the limit of detection by capillary electrophoresis (< 1 ppm). No decomposition of the aqueous solution of **1** was observed after thawing (0°C) and refreezing several times over a 3-month period.

2.4. Glycosyl acceptors

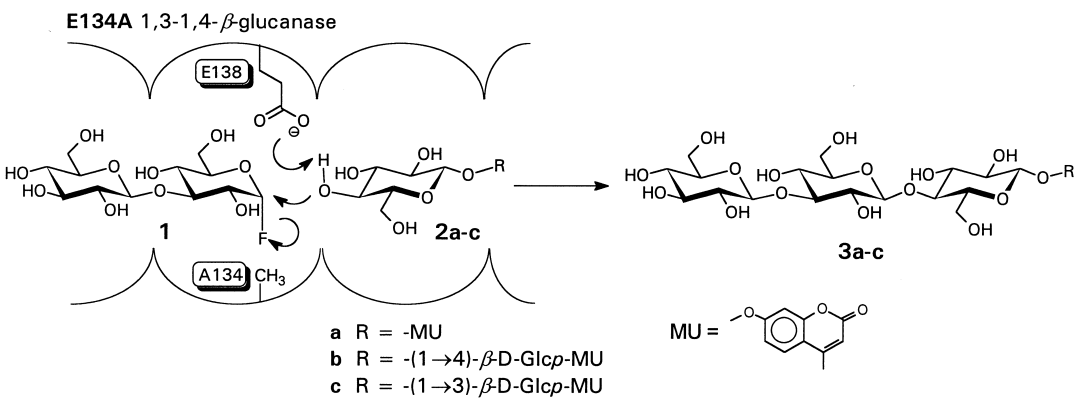
4-Methylumbelliferyl β-D-glucopyranoside (**2a**), 4-methylumbelliferyl β-cellobioside (**2b**) and 4-methylumbelliferyl β-laminaribioside (**2c**) were prepared as previously described [13,14]. 4-Nitrophenyl β-D-galactopyranoside (**2d**) and 4-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**2e**) were from Fluka.

2.5. Enzymatic glycosylations

Analytical runs consisted of fluoride **1** (2 mM), glycosyl acceptor (10 mM), E134A β-glucanase (1–4 μM) and $CaCl_2$ (0.5–1 mM) in citrate (30 mM)-phosphate (30 mM) or TES (80 mM) buffer, pH 7.5 ± 0.5. Samples were withdrawn at regular intervals, diluted with H_2O (1:10) and analyzed by HPLC. Chromatographic conditions: 3.9×150 mm (4 μm) Nova-Pak C18 (Waters) column, 18:82 MeOH/H_2O 1 ml/min, 37°C, λ 316 nm. Retention times (min): G-MU (5.4), G4G-MU (7.1), G3G-MU (6.1), G3G4G-MU (7.0), G3G4G4G-MU (7.3), G3G4G3G-MU (8.6). For pH studies, 80 mM TES (pH 5.5–6.6) and 80 mM MES (pH 7–8) buffers, 0.4 mM $CaCl_2$, 2 mM **1**, 9 mM **2a**, 4.6 μM enzyme were used.

2.6. Preparative synthesis of 4-methylumbelliferyl β-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranoside (3a)

A solution of 4-methylumbelliferyl β-D-glucopyranoside (**2a**, 50 mg, 0.148 mmol), α-laminaribiosyl fluoride (**1**, 10 mg, 0.029 mmol) and E134A β-glucanase (0.5 mg, 20 pmol) in 6.1 mM citrate/80 mM phosphate buffer (6.1 ml, pH 7.3) containing $CaCl_2$ (0.5 mM) was incubated at 35°C for 2 days. The reaction mixture was centrifuged and the crude extract was directly loaded onto a Lichroprep RP-18 (Lobar-A, Merck) column. Elution was performed with water (250 ml) followed by a gradient H_2O → 20% MeOH/H_2O. Unreacted acceptor (41 mg, 82%) was collected first, followed by the title trisaccharide. Concentration under reduced pressure and freeze-drying yielded **3a** as a white amorphous solid (17 mg, 88%). ^{1}H NMR (D_2O) δ 7.70 (d, 1H, *J* 8.8 Hz), 7.11 (dd, 1H, *J* 8.8, 2.1 Hz), 7.05 (d, 1H, *J* 2.1 Hz), 6.24 (q, 1H, *J* 0.9 Hz), 5.24 (d, 1H, *J* 7.5 Hz), 4.77 (d, 1H, *J* 7.8 Hz), 4.60 (d, 1H, *J* 7.8 Hz), 2.43 (d, 3H, *J* 0.9 Hz). MALDI-TOF (*m/z*) 685 $[M+Na]^+$.



Scheme 2.

3. Results and discussion

Replacement of the essential nucleophile (Glu134) in the *B. licheniformis* 1,3-1,4-β-glucanase by an alanine residue yields an enzyme with no glycosidase activity [7]. The mutant is, however, able to catalyze the regio- and stereospecific glycosylation of α-laminaribiosyl fluoride (**1**) with different glucoside acceptors. Condensation between fluoride **1** and the chromophoric acceptor 4-methylumbelliferyl β-D-glucopyranoside (**2a**) catalyzed by E134A 1,3-1,4-β-glucanase was chosen as the model reaction (Scheme 2). HPLC analysis of the reaction mixture revealed formation of essentially only one new chromogenic compound with a retention time compatible with a 4-methylumbelliferyl trisaccharide. MALDI-TOF mass spectrometry confirmed the expected molecular weight of the new compound (*m/z* 685 $[M+Na]^+$). Redigestion of a transglycosylation mixture with wild-type *B. licheniformis* 1,3-1,4-β-glucanase produced quantitative recovery of the initial 4-methylumbelliferyl monosaccharide acceptor and laminaribiose. It indicates that the product contains a new β-1,4-glucosidic bond, since the wild-type enzyme is highly specific for cleav-

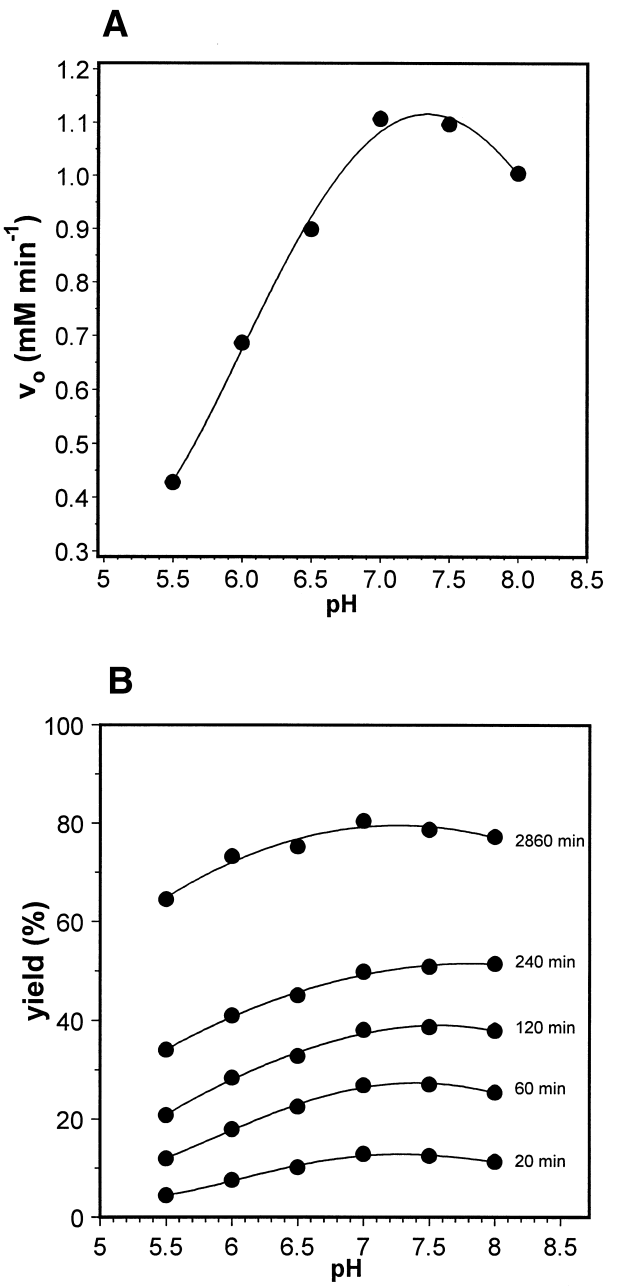


Fig. 1. pH dependence of the enzymatic condensation **1**+**2a** catalyzed by E134A mutant 1,3-1,4-β-glucanase. A: Initial glycosylation rates. B: Yields in glycosylation product after different reaction times as indicated. Conditions: 80 mM TES (pH 5.5–6.5) or MES (pH 7–8), 0.4 mM $CaCl_2$, 2 mM **1**, 9 mM **2a**, 4.6 μM enzyme.

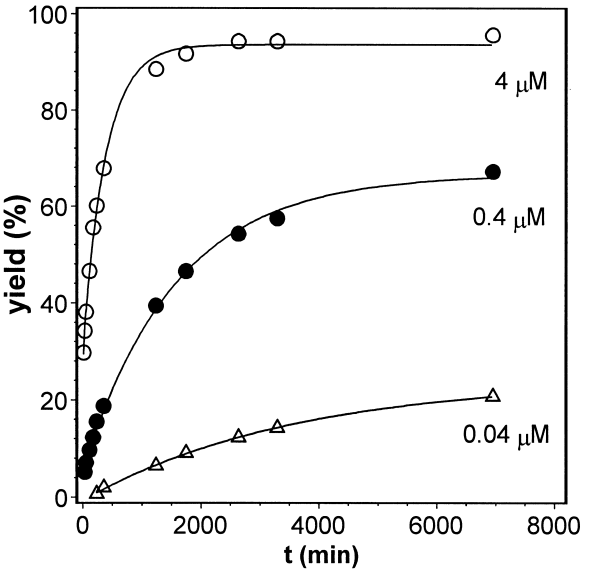
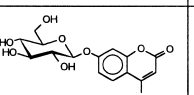
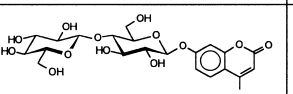
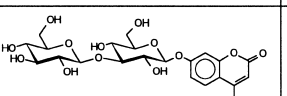
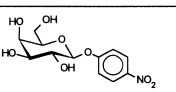
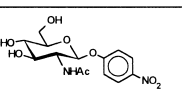


Fig. 2. Reaction course of the enzymatic condensation **1**+**2a** catalyzed by E134A mutant 1,3-1,4-β-glucanase at different enzyme concentrations. Conditions: 30 mM phosphate-30 mM citrate buffer pH 7.0, 0.75 mM **1**, 11.3 mM **2a**, 0.04–4 μM enzyme.

Table 1
Initial enzyme-catalyzed glycosylation rates of different glycosyl acceptors with fluoride **1**

Acceptor		Relative initial rates [a]
2a		1.00
2b		1.20
2c		0.43
2d		0.00
2e		0.00

[a]Rates were determined by HPLC. Reaction products were characterized by MALDI-TOF and by redigestion with wild-type 1,3-1,4-β-glucanase to the original chromophoric acceptor and laminaribiose.

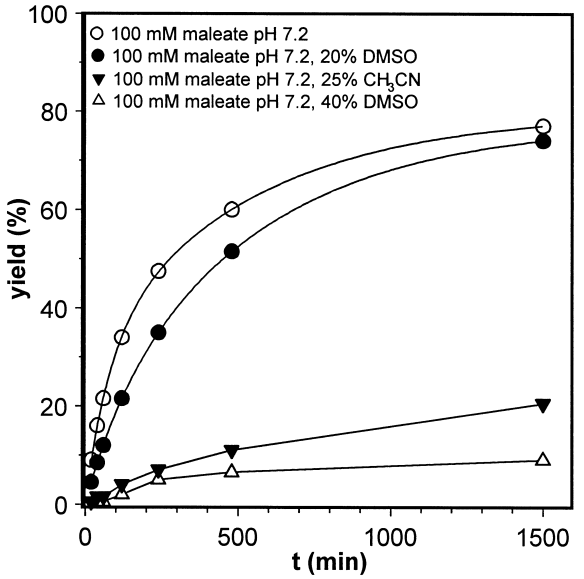


Fig. 3. Effect of organic cosolvents in the enzymatic condensation **1**+**2a** catalyzed by E134A mutant 1,3-1,4-β-glucanase. Conditions: 2.2 mM **1**, 10 mM **2a**, 3.3 μM enzyme, 100 mM maleate buffer pH 7.2, 1 mM $CaCl_2$, organic cosolvent as shown.

age of β-1,4 linkages on 3-*O*-substituted glucopyranose units [15,16,5]. Final evidence for a β-Glc*p*-(1→3)-β-Glc*p*-(1→4)-β-Glc*p*-MU structure (**3a**) was provided by ^{1}H NMR after isolation of the product from a preparative run (see Section 2).

The optimal temperature for transglycosylation was around 35 ± 5°C. Although the wild-type *B. licheniformis* enzyme shows maximum glycosidase activity at 55°C, the E134A mutant is less thermostable and precipitates after a few hours at this temperature. Chemical hydrolysis of the fluoride substrate was also reduced by working only slightly above room temperature. Maximum yields were obtained at pH 7.5 ± 0.5, where the initial transglycosylation rate was maximal (Fig. 1). A molar ratio of ca. 1:5:0.002 fluoride/acceptor/enzyme gave a 90% yield (measured by HPLC, Fig. 2) of trisaccharide after 2–3 days of incubation under optimal conditions (88% isolated from a preparative run).

Organic cosolvents (DMSO, CH_3CN), which are commonly used in the kinetically controlled oligosaccharide synthesis using wild-type enzymes to reduce the hydrolytic reaction [2], did not improve glycosylation yields, rather they were detrimental as shown in Fig. 3.

Table 1 shows the relative reactivities of several glycosyl acceptors. The disaccharide 4-methylumbelliferyl β-cellobioside (**2b**) was transferred to fluoride **1** slightly more efficiently than the monosaccharide **2a**, as expected for an endoglucanase. However, a significant decrease in reactivity was found for the β-laminaribioside **2c**, which is probably competing with α-laminaribiosyl fluoride for the same binding sites in the enzyme's active cleft [14]. This differential reactivity may also reflect the substrate specificity of the binding subsites on the non-reducing end of the enzyme. Subsites +1/+2 may prefer a cellobiosyl rather than a laminaribiosyl unit as far as barley β-glucan locates the former into these subsites in the productive enzyme-substrate complex [4,16]. As expected, *p*-nitrophenyl-β-D-galactopyranoside (**2d**), a 4-epimer of **2a**, was not glycosylated enzymatically, and neither was the 2-acetamido-2-deoxy derivative **2e**.

The obvious advantage of E134A β-glucanase as glycosylation catalyst with respect to the previously reported kinetic approach using the wild-type enzyme [8,9] is that reaction products cannot be hydrolyzed by the mutant enzyme, and glycosylation yields rise to 90%. However, long reaction times or high concentrations of enzyme are still necessary to achieve high glycosylation yields. As compared with the wild-type two-step hydrolysis mechanism, condensation is postulated to occur by a single SN_2-like displacement of the fluoride leaving group. For the 1,3-1,4-β-glucanase enzyme, the residue Glu138 may play the role of general base, selectively enhancing the nucleophilicity of a 4-OH group in the Glc*p* acceptor. A downward shift in the pK_a of this residue with respect to the wild-type enzyme (pK_a 7.0 for the free enzyme, pK_a 7.3 for an enzyme-trisaccharide substrate, and pK_a 8.5 for the β-glucan-substrate complex [4]) seems a prerequisite to justify transglycosylation activity even at pH values under 5.5. Evidence has been recently provided that the pK_a of the general acid in retaining glycosidases is tuned by the charge on the nucleophile residue, enabling the former one to act as a general acid or base at different instants along the reaction coordinate [17]. In this sense, substitution of the catalytic nucleophile E134 (kinetic pK_a 5.5 [4]) by a non-charged residue (Ala) may produced the desired shift in the pK_a of E138. Finally, although direct substitution of fluoride ion by an activated glucopyranose OH is chemically plausible, fluoride activation by another acidic residue on the active site cannot be totally ruled out at this point. Additional studies to unambiguously characterize the transglycosylation mechanism are currently in progress.

In conclusion, the ability of the E134A mutant of 1,3-1,4-β-glucanase to act as a β-glucansynthase extends the recently reported 'glycosynthase' approach for exo-enzymes (β-glucosidase from *Agrobacterium* [3]) to endoglycosidases, thus broadening the application of the method as a convenient strategy for oligosaccharide synthesis.

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