Specificity Studies of *Bacillus* 1,3-1,4-β-Glucanases and Application to Glycosynthase-Catalyzed Transglycosylation

Jon K. Fairweather,[b] Magda Faijes,[a] Hugues Driguez,[b] and Antoni Planas*[a]

Bacillus *1,3-1,4-β-glucanases hydrolyze 1,3-1,4-β-gluco-oligosaccharides with a retaining mechanism. The binding-site cleft of these endoglycosidases is composed of six subsites (−4 to +2) of which subsite −3 makes the largest contribution to transition state stabilization. The specificity of this subsite is here analyzed for both glycosidase and glycosynthase activities in the wild-type and the nucleophile-less E134A mutant* Bacillus licheniformis *enzymes. A D-galactosyl residue on the nonreducing end of a trisaccharide substrate is accepted by the enzyme and binds at subsite −3 in the productive enzyme–substrate complex. The wild-type enzyme catalyzes the hydrolysis of the substrate Glcβ4Glcβ3GlcβMU (Glc = glucosyl, MU = 4-methylumbelliferyl) with a k_{cat}/K_M value only 1.3-fold higher than for the Galβ4Glcβ3GlcβMU (Gal = galactosyl) substrate. The corresponding α-fluorides act as good donors for the glycosynthase condensation reaction with mono- and disaccharide acceptors catalyzed by the E134A mutant. Whereas self-condensation and elongation products are also obtained as minor compounds with the Glcβ4Glcβ3GlcαF donor, nearly quantitative yields of single condensation products are obtained with the Galβ4Glcβ3GlcαF donor, in which the axial configuration of the 4-OH group on the nonreducing end prevents self-condensation and elongation reactions.*

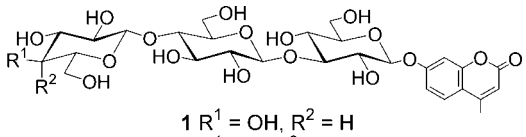
KEYWORDS:

carbohydrates · enzymes · glycosidase · glycosynthase · hydrolases

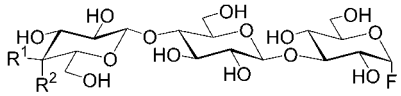
Introduction

The bacterial 1,3-1,4-β-glucanases (EC 3.2.1.73), or lichenases, are highly specific to β-1,4 glycosidic bond cleavage at 3-*O*-substituted glucopyranosyl residues in mixed linked β-1,3-1,4-gluco-oligosaccharides and polysaccharides (barley β-glucan and lichenan).[1–3] *Bacillus* 1,3-1,4-β-glucanases have been studied in detail in one of our laboratories through mechanistic, specificity, and structure–function analyses (for a recent review, see Ref.[4]). These enzymes are family-16 glycosyl hydrolases (according to the current classification based on sequence similarities[5]) of 25–30 kDa molecular mass with a monomeric jelly-roll β-sandwich structure[6] and an open binding site cleft typical of endodepolymerases.

All known substrates of 1,3-1,4-β-glucanases (polysaccharides and purposely synthesized oligosaccharides for kinetic studies[7–9]) are gluco-oligomers. Subsite mapping studies have shown that the binding-site cleft contains four subsites on the nonreducing side of the scissile glycosidic bond of the substrate. Subsite −3 makes the largest contribution to binding and transition state stabilization.[10] We here report the synthesis of a substrate analogue used to evaluate the specificity of this subsite. The analogue has a galactosyl unit in the position that will occupy subsite −3 in the enzyme–substrate complex (compound **2**, Scheme 1) and the kinetic parameters of the enzyme-catalyzed hydrolysis of this analogue are compared to those of the normal substrate of the homologous series (compound **1**), which places a glucosyl unit in that subsite. If a galactosyl residue on the nonreducing end of the substrate is tolerated by the enzyme, this trisaccharide structure may be used as a glycosyl donor in glycosynthase reactions catalyzed by the E134A mutant of 1,3-1,4-β-glucanase.



1 R^1 = OH, R^2 = H
2 R^1 = H, R^2 = OH



3 R^1 = OH, R^2 = H
4 R^1 = H, R^2 = OH

Scheme 1. *Substrates for 1,3-1,4-β-glucanase used in this study.*

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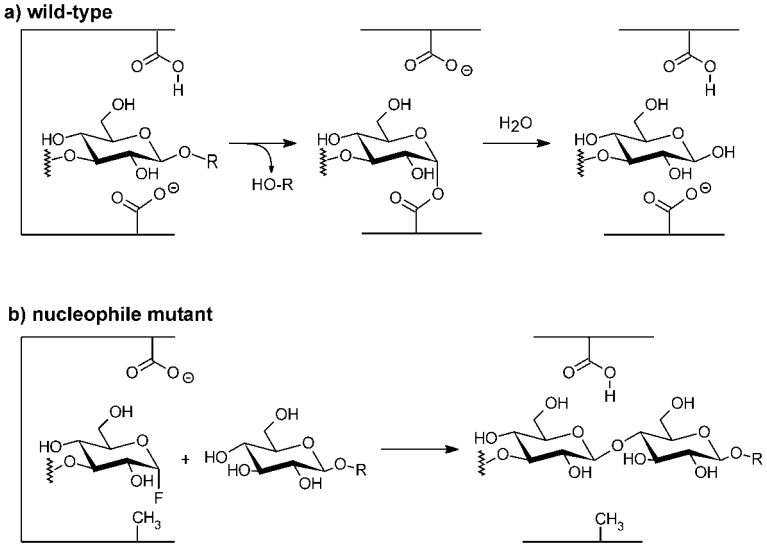
Glycosynthases are engineered retaining glycosidases in which mutation of the catalytic nucleophilic residue renders the enzymes hydrolytically inactive but able to catalyze transglycosylation reactions of glycosyl fluoride donors that have the opposite anomeric configuration to that of the normal substrate of the parental wild-type enzymes (Scheme 2). The glycosynthases methodology was introduced for the exo-acting enzyme β-glucosidase from *Agrobacterium faecalis*[11] and was also developed on the endo-acting 1,3-1,4-β-glucanase from *Bacillus licheniformis*[12] in 1998, and since then it has been successfully applied to few other enzymes (cellulase from *Humicola insolens*,[13] β-mannosidase from *Cellumonas fimi*,[14] β-glycosidase from *Sulfolobus solfataricus*,[15] and 1,3-β-glucanase from *Hordeum vulgare*[16]). High transglycosylation yields (typically 60–90% in isolated products) are obtained in contrast to the conventional kinetically controlled transglycosylation by wild-type enzymes. In some cases, the glycosyl fluoride donor and the transglycosylation product may also act as acceptors, provided that the configuration of the condensable hydroxy group on the nonreducing end of the donor has the same stereochemistry as the normal acceptor. This situation leads to self-condensation of the donor or elongation of the transglycosylation product, respectively. Different strategies to control these undesired reactions have been proposed: 1) selection of a donor with a different configuration of the hydroxy group that normally acts as acceptor,[11, 13] 2) use of a temporary protecting group on the polymerizable hydroxy group of the donor,[17, 18] or 3) addition of an excess of acceptor to decrease the probability that the donor acts as an acceptor.[18]



Scheme 2. *Hydrolase and glycosynthase activities of retaining glycosidases. a) Glycosidic bond hydrolysis catalyzed by a wild-type glycosidase. b) Glycosynthase reaction catalyzed by a non-nucleophilic mutant glycosidase.*

We analyze here the behavior of a "blocked" donor with respect to the glycosynthase activity of the E134A mutant 1,3-1,4-β-glucanase from *B. licheniformis*. If compound **2**, which has a galactosyl unit on the nonreducing end, is a good substrate for the wild-type enzyme, the corresponding α-glycosyl fluoride **4** may be used as a glycosyl donor in glycosynthase reactions catalyzed by the E134A 1,3-1,4-β-glucanase. Compound **4** could condense with an acceptor but self-condensation and elongation reactions would be prevented by the 4^{III}OH axial configuration of the donor, whereas these reactions occur to some extent with the homologous Glcβ4Gluβ3GluαF (Glc = glucosyl, Glu = glutamate) donor (**3**).

Results and Discussion

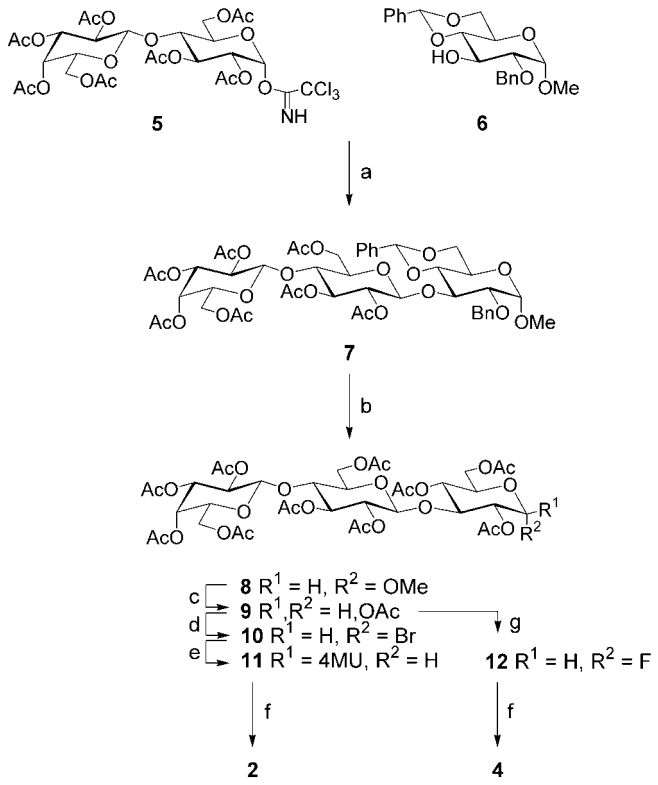
Chemical synthesis of substrates 2 and 4

We adopted a straightforward approach to the synthesis of the trisaccharide motif Galβ4Glcβ3Glc common to the substrates of interest (**2** and **4**, Scheme 3, Gal = galactosyl). Condensation of lactosyl trichloroacetimidate **5**[19] with the alcohol **6**[20] gave the trisaccharide **7** in good yield (81%). Hydrogenolysis followed by acetylation yielded **8**, which was further subjected to acetolysis to provide a trisaccharide template **9** from which derivatives could be produced. In one case, the bromide **10**, conveniently generated by using HBr/AcOH, was glycosylated with 4-methylumbelliferone under phase transfer catalytic conditions[7] to give the glycoside **11** in moderate yield (47%) with some elimination product also obtained (22%). Transesterification under Zemplen conditions gave Galβ4Glcβ3GlcβMU (**2**). Alternatively, treatment of trisaccharide **9** with HF/pyridine afforded exclusively the α-fluoride **12** in good yield (78%). Conventional *O*-deacetylation with sodium methoxide in methanol then afforded Galβ4Glcβ3GlcαF (**4**).

Hydrolase specificity of the wild-type enzyme

The second-order rate constants k_{cat}/K_M (k_{cat} = the catalytic constant, K_M = the Michaelis constant) for the hydrolysis of the 4-methylumbelliferyl glycosides **1** and **2** catalyzed by the wild-type 1,3-1,4-β-glucanase from *B. licheniformis* were determined at pH 7.02 and 35 °C. Kinetic results are shown in Figure 1. No saturation was reached since substrate concentrations ([S]) were below K_M values. k_{cat}/K_M values were calculated as the slope of a plot of reaction velocity v versus [S] at low substrate concentration. The enzyme-catalyzed hydrolysis of both substrates gives a ratio k_{cat}/K_M (**1**):k_{cat}/K_M (**2**) of 1.3, which means that an equatorial or axial 4-OH group in subsite -3 has almost no effect on substrate binding. This small difference corresponds to a $\delta\Delta G$ value, given by $-RT\ln(\{k_{cat}/K_M(\mathbf{1})\}/\{k_{cat}/K_M(\mathbf{2})\})$, of only 0.16 kcal·mol^{-1} for transition state destabilization of the Gal substrate over the normal Glu substrate. This result is in agreement with current structural information.

Subsite mapping studies on the 1,3-1,4-β-glucanase of *B. licheniformis*[10] and *B. macerans*[21] concluded that subsite -3 has the largest contribution to binding and transition state stabilization. The modeled structure of an enzyme–substrate



Scheme 3. *Synthesis of the Galβ4Gluβ3Glu* substrates **2** and **4**. a) Trimethylsilyl trifluoromethanesulfonate (*TMSOTf) in CH_2Cl_2; b) i) H_2, Pd/C (MeOH), ii) Ac_2O/pyridine/dimethylaminopyridine; c) H_2SO_4, Ac_2O (AcOH); d) HBr (AcOH); e) 4-methylumbelliferone, Bu_4NI, NaOH (1 M in $CHCl_3$); f) NaOMe (MeOH); g) HF (pyridine). MU = 4-methylumbelliferyl.*

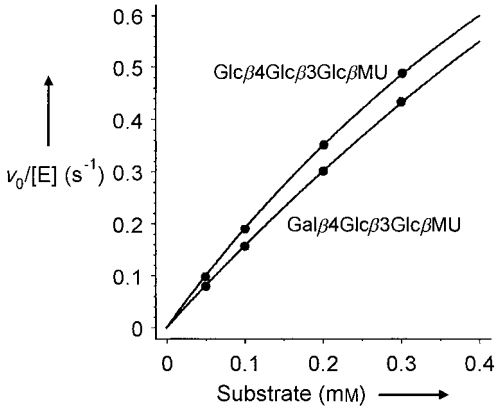


Figure 1. *Kinetics of the enzyme-catalyzed hydrolysis of substrates **1** and **2**. v_0 = initial rate of reaction, [E] = enzyme concentration.*

complex[22] showed more protein–carbohydrate contacts in subsite −3 than in any other subsite. Moreover, mutational analysis of enzyme–substrate interactions provided experimental evidence in agreement with the model and quantified the energetic contribution of hydrogen-bonding interactions.[21] The model proposes that Glu61 forms a bidentate H bond with the 6-OH (Glc[−2]) and 2-OH (Glc[−3]) groups, Asn24 hydrogen bonds with the 6-OH (Glc[−3]) group, and the 3-OH (Glc[−3]) group is within H-bonding distance of the Tyr22 and Arg63 side chains (Figure 2). The 4-OH of the Glc residue in subsite −3 does

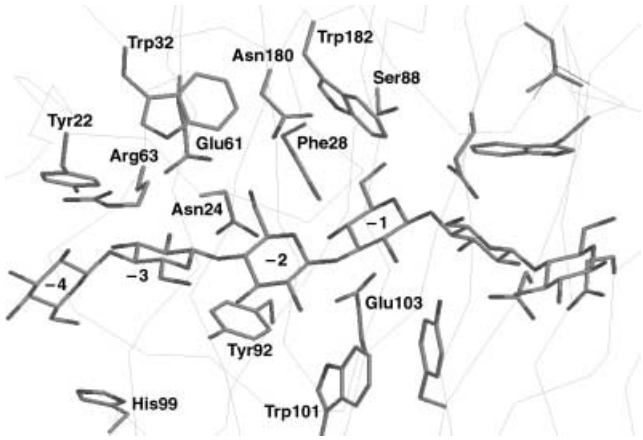


Figure 2. *Modeled enzyme–substrate complex. The protein–carbohydrate interactions in subsite −3 are shown. Amino acid numbering corresponds to the* B. macerans *1,3-1,4-β-glucanase.*[4]

not participate in any hydrogen bond with any residue of the protein in the *gluco* (equatorial) configuration and there is room in the pocket to accommodate a *galacto* (axial) configuration.

Glycosynthase reactions catalyzed by E134A 1,3-1,4-β-glucanase

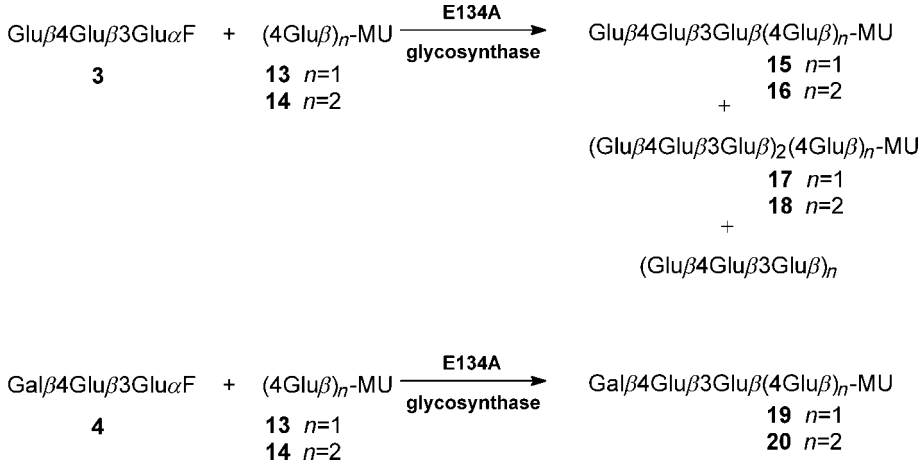
The fact that the trisaccharide unit Galβ4Gluβ3Glu binds productively in subsites −3 to −1 validates its use in a glycosynthase reaction. To this end, the reactions of the two trisaccharide donors Gluβ4Gluβ3GluαF (**3**) and Galβ4Gluβ3GluαF (**4**) with two acceptors (**13** and **14**, Scheme 4) were compared and both initial rates of transglycosylation and yields in condensation products were evaluated. Reactions were performed in maleate buffer (pH 7.0) with $CaCl_2$ (0.1 mM) at 35 °C, conditions known to be optimal for the E134A glycosynthase.[12] Kinetic results are summarized in Table 1. Initial rates of condensation and elongation were determined at both 1:1 and 1:5 donor/acceptor molar ratios. Yields at the indicated times are also given. These values are rather low because the reactions were performed at low enzyme concentrations in order to monitor initial formation of products. Reactions were monitored by HPLC with a UV detector and so only products that contained the MU chromophore (condensation and elongation products) were detected, not the products of the eventual self-condensation of the donor (in the case of **3**).

Reaction of donor **3** with the cellobioside acceptor **14** in a 1:1 molar ratio with 0.25 μM enzyme (entry 1, Table 1) gave the condensation product **16** at an initial rate of 0.21 s^{-1}. The octasaccharide **18** (the elongation product that arises from a second condensation of the donor with the initial condensation product **16**) was also formed at a lower rate (0.04 s^{-1}) and accumulated to give 5% yield after 24 h. When the donor/acceptor ratio was increased to 1:5 (entry 3), the condensation rate was faster (0.9 s^{-1}; 66% yield after 24 h) but elongation still occured and compound **18** accumulated to give a 9% yield after 24 h.

Table 1. *Initial rates and product yields of the glycosynthase reactions of donors **3** and **4** catalyzed by E134A 1,3-1,4-β-glucanase.*[a]

Entry	Donor	Acceptor	D:A[b]	[E] [μM]	Condensation			Elongation		
					Product	v_0/[E] [s^{-1}]	Yield [%] (time)	Product	v_0/[E] [s^{-1}]	Yield [%] (time)
1	Gluβ4Gluβ3GluαF (**3**)	Gluβ4Glu-MU (**14**)	1:1	0.25	**16**	0.21	20 (24 h)	**18**	0.04	5 (24 h)
2			1:1	12.3	**16**		25 (20 min)	**18**		15 (20 min)
3			1:5	0.25	**16**	0.90	66 (24 h)	**18**	0.12	9 (24 h)
4			1:5	12.9	**16**		55 (20 min)	**18**		8 (20 min)
5		Glu-MU (**13**)	1:1*	2.5	**15**	0.12	20 (4 h)	**17**	0.08	10 (4 h)
6			1:5	10.1	**15**		51 (3 h)	**17**		5 (3 h)
7	Galβ4Gluβ3GluαF (**4**)	Gluβ4Glu-MU (**14**)	1:1	0.25	**20**	0.23	45 (24 h)	–	–	—
8			1:1	12.3	**20**		46 (20 min)	–		—
9			1:5	0.25	**20**	0.89	71 (24 h)	–	–	—
10			1:5	12.3	**20**		76 (20 min)	–		—
11		Glu-MU (**13**)	1:1*	2.5	**19**	0.26	40 (1.5 h)	–	–	—

[a] Conditions: maleate buffer (50 mM, pH 7.0), $CaCl_2$ (0.1 mM), 35 °C. [b] Donor to acceptor molar ratio. [Donor] = 2 mM, except for entries marked *, where [Donor] = 3 mM.



Scheme 4. *Glycosynthase reactions catalyzed by E134A 1,3-1,4-β-glucanase from* B. licheniformis.

The equivalent reaction with the Gal donor **4** and acceptor **14** showed initial rates of condensation that were essentially the same as with the Glu donor **3** (entry 7, Table 1), but no elongation product was formed at all. The reactions at 1:5 donor/acceptor ratio and 12.3 μM enzyme (entries 4 and 10) show a condensation yield with the Gal donor **4** of 76% after 20 min reaction, whereas the yield is 55% with the Glu donor **3**, for which 8% forms the elongation product. The remaining 13% yield difference between the donors may correspond to self-condensation of **3** but this was not determined.

The glucoside **13** is a poorer acceptor (slower rate) in the reaction with the Glu donor **3** than with the Gal donor **4** (entries 5 and 11, Table 1). Whereas the condensation yield with **4** was 40%, the overall yield of condensation plus elongation with the Glu donor **3** was only 30%. In this case, self-condensation of **3** may be more important than in the previous series of results because the monosaccharide acceptor **13** has weaker binding to the enzyme than the disaccharide **14** and the donor itself is able to compete more effectively for the acceptor subsites of the enzyme, which leads to self-condensation. No competing self-condensation is obtained with the Gal donor since self-condensation is blocked by the 4^{III}OH axial configuration but also because binding of the donor to the acceptor subsites would be weaker than with the Glu donor, which results in a faster rate of condensation.

Preparative glycosynthase reactions were performed with the cellobioside acceptor **14** and both α-glycosyl fluoride donors **3** and **4** by using a larger amount of enzyme, as detailed in the Experimental Section. Reactions were completed after 20–30 min. The yield of isolated condensation product **20** was nearly quantitative for the Gal donor **4**. The structures of the condensation products **16** and **20** were established by MS and NMR spectroscopy as detailed in the Experimental Section. The newly formed glycosidic bond is a β1,4 bond for both the Glu and Gal donors; in the ^{1}H NMR spectra, a new signal in the anomeric region assignable to an H-1 proton in a β1,4-linkage (δ4.6–4.5 ppm, J = 7.8 Hz) is observed and the signal corresponding to C-4^{II} appears in the δ = 79.2–78.7 ppm region of the ^{13}C NMR spectra, which is characteristic of a β1,4 linkage. Moreover, the condensation products **16** and **20**, as well as **15** and **19**, were substrates of the wild-type 1,3-1,4-β-glucanase. This result provides an additional proof of the regiochemistry of the glycosidic bond produced by the glycosynthase since the *B. licheniformis* enzyme is strictly cleavage specific and hydrolyzes β1,4 linkages on 3-*O*-substituted glucopyranosyl units.[3]

Conclusions

1,3-1,4-β-Glucanase from *B. licheniformis* accepts both D-galactosyl and D-glucosyl residues in subsite −3 of the binding-site cleft and catalyzes the hydrolysis of the substrate Glcβ4Glcβ3GlcβMU with a k_{cat}/K_M value only 1.3-fold higher than that for the Galβ4Glcβ3GlcβMU substrate.

The glycosynthase reaction catalyzed by the E134A 1,3-1,4-β-glucanase mutant is regio- and stereospecific for formation of β-glycosidic linkages with the 4-OH group of the acceptor in a

gluco (equatorial) configuration. Reaction of the glycosyl donor Glcβ4Glcβ3GlcαF with an acceptor gives self-condensation and elongation products in addition to the donor–acceptor condensation product (major product). The equatorial 4-OH group on the nonreducing end of either the donor or the condensation product can act as an acceptor for another molecule of the fluoride donor. The 4-OH group of the Galβ4Glcβ3GlcαF donor is in the axial configuration, which prevents self-condensation of the donor as well as elongation of the condensation product and preparative yields are nearly quantitative.

Experimental Section

General methods: Optical rotations were performed with a Perkin-Elmer 341 Polarimeter in a microcell (1 mL, 10-cm path length) in the specified solvents at ambient temperature. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC-300 or a Varian Gemini-300 (300 MHz for ^{1}H and 75.5 MHz for ^{13}C) spectrometer, either in deuteriochloroform ($CDCl_3$) with residual $CHCl_3$ ($\delta_H = 7.24$) and $CDCl_3$ ($\delta_C = 77.0$) as internal standards or in d_6-dimethylsulfoxide ([D$_6$]DMSO) or deuteriumoxide (D_2O) with residual DMSO ($\delta_H = 2.49$) and [D$_6$]DMSO ($\delta_C = 39.7$) as internal standards, at ambient temperature (298 K) unless specified otherwise. Where necessary, assignment of signals in NMR spectra was aided by COSY experiments. Mass spectra were recorded either at high resolution (HRMS) on a VG-ZAB or low resolution (MS) on a Nermag R-1010C spectrometer by using the FAB technique with NaCl as the matrix, or on a VG Platform for ESI. Flash chromatography (FC) was performed on Merck silica gel (40–63 μm) under positive pressure with the specified eluents. Reversed-phase chromatography was performed on Sep-Pak (C18) cartridges (300 mg) with the eluents specified. All solvents used were of analytical grade. Evaporation of the solvents was effected under reduced pressure on a rotary evaporator. The progress of the reactions was monitored by analytical TLC on commercially available silica gel 60 F_{254} precoated aluminium plates (E. Merck, Darmstadt). Compounds were visualized (TLC) by charring with 5% sulfuric acid in MeOH/water and/or by visualization under UV light. All reactions that involved organic solvents were performed under argon. The term workup refers here to dilution with water, extraction into organic solvent (specified), sequential washing of the organic extract with aq HCl (1 M, where appropriate), saturated aq $NaHCO_3$ and NaCl solutions, followed by drying over anhydrous $MgSO_4$, filtration, and evaporation of the solvent.

Enzyme and substrates: Wild-type and E134A mutant 1,3-1,4-β-glucanases from *Bacillus licheniformis* were expressed and purified as reported elsewhere.[12, 23] Both enzymes were lyophilized for storage (at $-20\,^\circ$C) and dissolved in the reaction buffer prior to use. In addition to the compounds **2** and **4** here described (see below), other substrates were prepared as previously reported: Gluβ4Gluβ3GluMU (**1**),[7] Gluβ4Gluβ3GluαF (**3**),[12] and Gluβ4GluMU (**14**).[24] 4-Methylumbelliferyl-β-D-glucoside (**13**) was from Fluka.

Syntheses of substrates 2 and 4:

Methyl (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-(2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl)-(1→3)-2-*O*-benzyl-4,6-*O*-benzylidene-α-D-glucopyranoside (**7**): A mixture of the lactosyl trichloroacetimidate **5**[19] (934 mg, 1.20 mmol), the alcohol **6**[20] (335 mg, 0.90 mmol), and molecular sieves (powdered 4 Å; 200 mg) in CH_2Cl_2 (20 mL), was stirred at room temperature for 30 min. TMSOTf (0.040 mL, 0.22 mmol) was then introduced with continued stirring (5 min), followed by addition of Et_3N (0.10 mL). The mixture was filtered, the solvent evaporated, and the residual oil subjected to flash chromatography (30–50% EtOAc/petrol ether) to yield the trisaccharide **7** (22 mg, 81%) as a colorless oil; $[\alpha]_D$: -11.6° ($c = 0.7$, $CHCl_3$); ^{1}H NMR ($CDCl_3$): $\delta = 1.91$–2.06 (10 s, 30 H; $COCH_3$), 3.22–3.28 (m, 1 H; H-5^{III}), 3.32 (s, 3 H; OCH_3), 3.47 (dd, $^3J(1,2) = 3.8$ Hz, $^3J(2,3) = 9.3$ Hz, 1 H; H-2^I), 3.54 (dd, $^3J(3,4) = {}^3J(4,5) = 9.1$ Hz, 1 H; H-4^{II}), [3.63–3.82 (m), 3.99–4.04 (m) and 4.12–4.20 (m), 10 H; H-1^{II}, H-3^I, H-5^{I,II}, H-6^{I-III}], 4.31 (d, $^3J(1,2) = 7.7$ Hz, 1 H; H-1^{III}), 4.45 (d, $^3J(1,2) = 3.8$ Hz, 1 H; H-1^I), [4.46 (d, $^2J = 12.0$ Hz, 1 H,) and 4.72 (d, $^2J = 12.0$ Hz, 1 H,); CH_2Ph], 4.83 (dd, $^3J(1,2) = {}^3J(2,3) = 7.7$ Hz, 1 H; H-2^{II}), 4.86 (dd, $^3J(2,3) = 10.4$ Hz, $^3J(3,4) = 3.5$ Hz, 1 H; H-3^{III}), 4.97 (dd, $^3J(1,2) = 7.8$ Hz, $^3J(2,3) = 9.1$ Hz, 1 H; H-2^{III}), 5.01 (dd, $^3J(2,3) = 10.4$ Hz, $^3J(3,4) = 8.0$ Hz, 1 H; H-3^{II}), 5.08 (dd, $^3J(3,4) = {}^3J(4,5) = 9.1$ Hz, 1 H; H-4^I), 5.28 (dd, $^3J(3,4) = 3.4$ Hz, $^3J(4,5) = 0.8$ Hz, 1 H; H-4^{III}), [7.22–7.28 (m, 6 H) and 7.38–7.42 (m, 4 H); Ph] ppm; ^{13}C NMR ($CDCl_3$): $\delta = 20.43$–20.78 (7 C; $COCH_3$), 55.32 (OCH_3), 60.75, 61.78, 61.93 (C-6^{I-III}), 66.58 (C-4^{III}), 68.99, 70.58, 70.92, 72.68, 72.42, 73.22, 73.86, 75.95, 77.88, 78.82, 80.63 (12 C; C-2^{I-III}, C-3^{I-III}, C-4^{I,II}, C-5^{I-III}, CH_2Ph), 98.72, 100.40, 101.03, 101.55 (C-1^{I-III}, CHPh), 126.17–129.16, 137.37, 137.89 (12 C; Ph), 168.83–170.27 (7 C; CO) ppm; HR MS (FAB): *m/z*: calcd for $C_{47}H_{58}O_{23}$: 1013.3267 $[M+Na]^+$; found: 1013.3258.

Methyl (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-(2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl)-(1→3)-2,4,6-tri-*O*-acetyl-α-D-glucopyranoside (**8**): Palladium on charcoal (30 mg; 10% palladium by mass) was added to a solution of the trisaccharide **7** (318 mg, 0.32 mmol) and glacial acetic acid (50 μL) in MeOH (15 mL) and the mixture was vigorously stirred under an atmosphere of hydrogen for 12 h). The mixture was then filtered, evaporated, and co-evaporated (toluene) and the residue was treated with pyridine (5 mL), Ac_2O (4 mL), and *N*,*N*-dimethylaminopyridine (30 mg) and the mixture stirred for 2 h. MeOH (2 mL) was added and the solvent coevaporated (toluene). The residual oil was subjected to flash chromatography (40–80% EtOAc/petrol ether) to yield the decaacetate **8** (271 mg, 90%) as a colorless foam; $[\alpha]_D = +23.3^\circ$ ($c = 1.0$, $CHCl_3$); ^{1}H NMR ($CDCl_3$) $\delta =$ 1.93–2.41 (10 s, 30 H, COMe), 3.37 (s, 3 H, OMe), 3.54 (ddd, $^3J(4,5) = 9.2$ Hz, $^3J(5,6) = 1.8$, 4.7 Hz, 1 H; H-5^I), 3.75 (dd, $^3J(3,4) = 9.5$, $^3J(4,5) = 9.1$ Hz, 1 H; H-4^{II}), 3.79–3.84 (m, 1 H; H-5^{III}), 3.87 (ddd, $^3J(4,5) = 10.2$ Hz, $^3J(5,6) = 2.4$ and 4.6 Hz, 1 H; H-5^{II}), [4.01–4.12 (m, 5 H), 4.16 (dd, $^3J(5,6) = 4.6$ Hz, $^2J(6,6) = 12.4$ Hz, 1 H) and 4.37 (dd, $^3J(5,6) = 1.9$ Hz, $^2J(6,6) = 12.0$ Hz, 1 H); H-3^I, H-6^{I-III})], 4.43 (d, $^3J(1,2) = 7.9$ Hz, 1 H; H-1^{II}), 4.61 (d, $^3J(1,2) = 8.0$ Hz, 1 H; H-1^{III}), 4.77 (dd, $^3J(1,2) = 8.2$ Hz, $^3J(2,3) = 9.5$ Hz, 1 H; H-2^{III}), [4.81–4.86 (m, 2 H) and 4.89–4.96 (m, 2 H); H-1^I, H-2^I, H-3^{II,III}), 5.06 (dd, $^3J(1,2) = 7.8$ Hz, $^3J(2,3) = 10.4$ Hz, 1 H; H-2^I), 5.09 (dd, $^3J(3,4) = 8.9$ Hz, $^3J(4,5) = 9.2$ Hz, 1 H; H-4^I), 5.31 (dd, $^3J(3,4) = 3.2$ Hz, $^3J(4,5) = 0.8$ Hz, 1 H; H-4^{III}) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 20.31$–20.93 (10 C; COMe), 55.38 (OMe), 60.72, 62.09, 62.21 (C-6^{I-III}), 66.57 (C-4^{III}), 67.28, 68.03, 69.10, 70.73, 70.90, 71.53, 72.50, 72.83, 73.21, 76.09, 76.19 (C-2^{I-III}, C-3^{I-III}, C-4^{I,II}, C-5^{I-III}), 96.73 (C-1^I), 100.54, 101.05 (C-1^{II,III}), 169.04–170.64 (10 C; CO) ppm; MS (FAB, positive mode): *m/z*: 961 $[M+Na]^+$.

(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-(2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl)-(1→3)-1,2,4,6-tetra-*O*-acetyl-D-glucopyranose (**9**): H_2SO_4 (0.14 mL) was added to a solution of the methyl glycoside **8** (251 mg, 0.27 mmol) in Ac_2O (7.0 mL) and AcOH (3.0 mL) and the mixture was stirred at room temperature (16 h). NaOAc (1 g) was added with continued stirring then the mixture was filtered through Celite and the solvent evaporated. The residue was dissolved in CH_2Cl_2 (10 mL) and subjected to workup and flash chromatography (40–70% EtOAc/petrol) to yield an anomeric mixture of acetates **9** (184 mg, 71%) as a colorless oil (α:β, 9:1); partial ^{1}H NMR ($CDCl_3$): $\delta = 5.54$ (d, $^3J(1,2) = 8.2$ Hz, 1 H; H-1^I–β), 5.36 (d, $^3J(1,2) = 3.6$ Hz, 1 H; H-1^I-α) ppm; ^{13}C NMR ($CDCl_3$): δ (α-anomer) = 20.22–20.76 (11 C; CH_3), 60.68, 61.58, 61.99 (C-6^{I-III}), 66.49, (C-4^{III}), 67.31, 69.07, 69.82, 70.63, 70.79, 71.20, 71.38, 72.49, 73.00, 75.83, 76.12

(C-2^{I-III}, C-3^{I-III}, C-4^{I,II}, C-5^{I-III}), 89.06 (C-1^{I}), 100.61, 100.96 (C-1^{II,III}), 168.55 – 170.56 (10 C; CO); HR MS (FAB): *m/z*: calcd for $C_{40}H_{50}O_{27}Na$: 1005.2490 $[M+Na]^+$; found: 1005.2505.

(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1 →4)-(2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl)-(1 →3)-2,4,6-tri-*O*-acetyl-D-glucopyranosyl bromide (**10**): HBr (1.0 mL, 2.7 mmol; 30% in AcOH) was added to a cooled (0 °C) solution of the acetate **9** (55 mg, 56 μmol) in CH_2Cl_2 (5.0 mL) and the mixture was allowed to warm to room temperature (6 h). The solution was further diluted (CH_2Cl_2), poured onto an ice – water suspension and subjected to workup and flash chromatography (50% EtOAc/petrol ether) to yield the bromide **10** (48 mg, 86%) as a colorless foam; $[\alpha]_D = +44.4°$ ($c = 1.0$, $CHCl_3$); ^{1}H NMR ($CDCl_3$): $\delta = 1.92 - 2.15$ (10 s, 30 H, CH_3), 3.59 – 3.65 (m, 1 H, H-5^{I}), 3.72 – 3.85 (m, 2 H, H-3^{I}, H-4^{I}), [4.00 – 4.24 (m, 7 H) and 4.41 (dd, $^3J(5,6) = 1.8$ Hz, $^2J(6,6) = 11.2$ Hz, 1 H); H-5^{II,III}, H-6^{I-III})], 4.42 (d, $^3J(1,2) = 7.8$ Hz, 1 H; H-1^{II}), 4.63 (d, $^3J(1,2) = 8.0$ Hz, 1 H; H-1^{III}), 4.75 (dd, $^3J(1,2) = 4.0$ Hz, $^3J(2,3) = 9.7$ Hz, 1 H; H-2^{I}), 4.77 (dd, $^3J(1,2) = 8.2$ Hz, $^3J(2,3) = 9.3$ Hz, 1 H; H-2^{III}), 4.01 (dd, $^3J(2,3) = 8.9$ Hz, $^3J(3,4) = 3.3$ Hz, 1 H; H-3^{III}), 5.05 (dd, $^3J(2,3) = 8.9$ Hz, $^3J(3,4) = 10.0$ Hz, 1 H; H-3^{II}), 5.06 (dd, $^3J(1,2) = 7.7$ Hz, $^3J(2,3) = 10.4$ Hz, 1 H; H-2^{II}), 5.10 (dd, $^3J(3,4) = {}^3J(4,5) = 9.2$ Hz, 1 H; H-4^{I}), 5.31 (dd, $^3J(3,4) = 3.8$ Hz, $^3J(4,5) = 0.8$ Hz, 1 H; H-4^{III}), 6.47 (d, $^3J(1,2) = 4.0$ Hz, 1 H; H-1^{I}) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 20.31 - 20.77$ (10 C; CH_3), 60.76, 61.07, 62.07 (C-6^{I-III}), 66.57, 66.62, 69.07, 70.72, 70.86, 71.56, 72.39, 72.65, 73.02, 75.92 (12 C; C-2^{I-III}, C-3^{I-III}, C-4^{I-III}, C-5^{I-III}), 87.27 (C-1^{I}), 100.61, 100.99 (C-1^{II,III}), 168.93 – 170.48 (10 C; CO) ppm.

4-Methylumbelliferyl (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1 →4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl)-(1 →4)-2,4,6-tri-*O*-acetyl-β-D-glucopyranoside (**11**): A vigorously stirred mixture of the bromide **10** (48 mg, 48 μmol), 4-methylumbelliferone (35 mg, 200 μmol), tetra-*n*butylammonium iodide (20 mg, 48 μmol) in $CHCl_3$ (4.0 mL), and NaOH (1.5 mL, 1.5 mmol; 1 M) was heated under reflux for 6 h. The mixture was diluted with $CHCl_3$ (5 mL) and the phases separated. The organic extract was washed with NaOH (1 M) and saturated NaCl solution, dried ($MgSO_4$), filtered, and evaporated. Flash chromatography (50 – 80% EtOAc/petroleum ether) of the residue yielded two products:

3-*O*-[(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)-(1 →4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl)]-2,4,6-tri-*O*-acetyl-1,5-anhydro-D-arabino-hex-1-enitol (10 mg, 22%) was isolated as a colorless oil; ^{1}H NMR ($CDCl_3$): $\delta = 1.94 - 2.12$ (10 s, 30 H, CH_3), 3.59 – 3.88 (m, 3 H, H-4^{II}, H-5^{II,III}), [4.02 – 4.17 (m, 5 H), 4.29 – 4.37 (m, 2 H) and 4.53 (dd, $^3J(5,6) = 2.0$ Hz, $^3J(6,6) = 11.9$ Hz, 1 H); H-3^{I}, H-5^{II}, H-6^{I-III}], 4.47 (d, $^3J(1,2) = 7.7$ Hz, 1 H; H-1^{II}), 4.62 (d, $^3J(1,2) = 7.7$ Hz, 1 H; H-1^{III}), 4.82 (dd, $^3J(1,2) = 8.0$ Hz, $^3J(2,3) = 9.3$ Hz, 1 H; H-2^{II}), 4.94 (dd, $^3J(2,3) = 10.4$ Hz, $^3J(3,4) = 3.5$ Hz, 1 H; H-3^{III}), 5.08 (dd, $^3J(1,2) = 7.9$ Hz, $^3J(2,3) = 10.4$ Hz, 1 H; H-2^{III}), 5.15 (dd, $^3J(1,2) = {}^3J(2,3) = 9.1$ Hz, 1 H; H-4^{I}), 5.31 – 5.35 (m, 2 H; H-3^{II}, H-4^{III}), 6.55 (s, 1 H; H-1^{I}) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 20.47 - 20.76$ (10 C; CH_3), 60.84, 61.44, 67.73 (C-6^{I-III}), 66.67 (C-4^{III}), 68.29, 69.13, 70.77, 70.98, 71.95, 72.15, 72.77, 72.94, 74.19, 76.09 (11 C; C-2^{I-III}, C-3^{I-III}, C-4^{I,II},C-5^{I-III}), 100.84, 101.13 (C-1^{II,III}), 126.10, 137.95 (C-1^{I}, C-2^{I}), 169.107 – 170.42 (10 C; CO) ppm; HR MS (FAB): *m/z*: calcd for $C_{38}H_{50}O_{25}Na$: 929.2539 $[M+Na]^+$; found 929.2538.

Glycoside **11** (25 mg, 47%) was obtained as a colorless oil; ^{1}H NMR ($CDCl_3$): $\delta = 1.92 - 2.12$ (30 H, $COCH_3$), 2.37 (d, $^4J(3',CH_3) = 1.1$ Hz, 3 H; CH_3), [3.57 – 3.62 (m, 1 H), 3.74 – 3.86 (m, 3 H), 3.95 (dd, $^3J(2,3) = {}^3J(3,4) = 9.1$ Hz, 1 H), 4.01 – 4.23 (m, 5 H), and 4.38 (dd, $^3J(5,6) = 2.0$ Hz, $^3J(6,6) = 11.9$ Hz, 1 H); H-3^{I}, H-4^{II}, H-5^{I-III}, H-6^{I-III}], 4.42 (d, $^3J(1,2) = 7.9$ Hz, 1 H; H-1^{III}), 4.58 (d, $^3J(1,2) = 7.0$ Hz, 1 H; H-1^{II}), 4.80 (dd, $^3J(1,2) = 8.2$ Hz, $^3J(2,3) = 9.5$ Hz, 1 H; H-2^{II}), 4.91 (dd, $^3J(2,3) = 10.4$ Hz, $^3J(3,4) = 3.5$ Hz, 1 H; H-3^{III}), 4.98 (dd, $^3J(1,2) = {}^3J(2,3) = 9.5$ Hz, 1 H; H-2^{I}), 4.99 (d, $^3J(1,2) = 7.9$ Hz, 1 H; H-1^{I}), 5.06 (dd, $^3J(1,2) = 7.9$ Hz, $^3J(2,3) = 10.4$ Hz, 1 H; H-2^{III}), 5.11 (dd, $^3J(3,4) = {}^3J(4,5) = 9.1$ Hz, 1 H; H-4^{I}), 5.25 (dd, $^3J(2,3) = 9.3$ Hz, $^3J(3,4) = 8.0$ Hz, 1 H; H-3^{II}), 5.31 (dd, $^3J(3,4) = 3.2$ Hz, $^3J(4,5) = 0.8$ Hz, 1 H; H-4^{III}), 6.15 (d, $^4J(3',CH_3) = 1.1$ Hz, 1 H; H-3′), 6.84 – 6.88 (m, 2 H; H-8′, H-6′), 7.45 – 7.49 (m, 1 H; H-5′) ppm; ^{13}C NMR ($CDCl_3$) $\delta = 20.33 - 20.81$ (10 C; $COCH_3$), 60.71, 62.02, 62.26 (C-6^{I-III}), 66.56 (C-4^{III}), 68.00, 69.11, 70.71, 70.86, 71.26, 72.26, 72.49, 72.69, 73.07, 76.02, 78.78 (C-2^{I-III}, H-3^{I-III}, H-4^{I,II}, H-5^{I-III}), 98.36, 100.86, 101.07 (C-1^{I-III}), 103.65 (C-8′), 113.10 (C-3′), 114.09 (C-6′), 115.40 (C-4a′), 125.65 (C-5′), 152.12 (C-4′), 154.81 (C-1a′), 159.25 (C-7′), 160.66 (C-2′), 168.77 – 169.94 (10 C; $COCH_3$) ppm; HR MS (FAB): *m/z*: calcd for $C_{48}H_{58}O_{28}Na$: 1105.3012 $[M+Na]^+$; found: 1105.3028.

4-Methylumbelliferyl β-D-galactopyranosyl-(1 →4)-β-D-glucopyranosyl)-(1 →3)-β-D-glucopyranoside (**2**): NaOMe (50 μL; 0.10 M in MeOH) was added to a cold (0 °C) solution of the decaacetate **11** (21 mg, 19 μmol) in MeOH (5.0 mL) and the solution was slowly warmed to room temperature over 2 h, during which time a precipitate was formed. Water was added and the solution was treated with the Amberlite IR-120 resin (H^+ form) until it reached neutral pH value. Filtration, evaporation of the solvent, and lyophilization of the residue yielded an off-white colored powder that was further purified by reversed-phase chromatography to afford the title glycoside **2** (9 mg, 70%); ^{1}H NMR ([D$_6$]DMSO): $\delta = 2.33$, (br s, 3 H; CH_3), 3.15 – 3.80 (m, 18 H; H-2^{I-III}, H-3^{I-III}, H-4^{I-III}, H-5^{I-III}, H-6^{I-III}), 4.19 (d, $^3J(1,2) = 7.1$ Hz, 1 H; H-1^{III}), 4.55 (d, $^3J(1,2) = 8.0$ Hz, 1 H; H-1^{II}), 5.05 (d, $^3J(1,2) = 7.5$ Hz, 1 H; H-1^{I}), 6.37 (q, $^3J = 1.3$ Hz, 1 H; H-3′), 6.97 – 7.04 (m, 2 H; H-8′, H-6′), 7.63 – 7.68 (m, 1 H; H-5′) ppm; ^{13}C NMR ([D$_6$]DMSO): $\delta = 19.26$ (CH_3), 61.13, 61.52, 61.85 (C-6^{I-III}), 68.98, 69.42, 71.76, 73.35, 73.76, 74.31, 75.33, 75.93, 76.43, 77.12, 80.05 (C-2^{I-III}, C-3^{II,III}, C-4^{I-III}, C-5^{I-III}), 86.05 (C-3^{I}), 100.39, 103.95, 104.26 (C-1^{I-III}), 104.58 (C-8′), 112.64 (C-3′), 115.06 (C-6′), 115.91 (C-4a′), 127.93 (C-5′), 155.20 (C-4′), 156.19 (C-1a′), 160.86 (C-7′), 163.37 (C-2′); HR MS (FAB): *m/z*: calcd for $C_{28}H_{38}O_{18}Na$: 685.1956 $[M+Na]^+$; found: 685.1956.

(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)-(1 →4)-(2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl)-(1 →3)-2,4,6-tri-*O*-acetyl-β-D-glucopyranosyl fluoride (**12**): HF (5.0 mL; 70% in pyridine) was added to a cooled (0 °C) polyethylene vessel that contained the heptaacetate **9** (87 mg, 90 μmol) and the mixture was kept at 4 °C for 12 h. CH_2Cl_2 (6.0 mL) was added and the combined solution poured onto a stirred suspension of ice in aq NH_3 (60 mL; 3 M). The solution was subjected to workup (CH_2Cl_2) and flash chromatography (40 – 60% EtOAc/petrol ether) to afford the fluoride **12** (65 mg, 78%) as a colorless oil; ^{1}H NMR ($CDCl_3$): $\delta = 1.94 - 2.18$ (10 s, 30 H, CH_3), [3.56 – 3.63 (m, 1 H), 3.73 – 3.86 (m, 2 H), 4.00 – 4.24 (m, 7 H), and 4.40 (dd, $^3J(5,6) = 1.9$ Hz, $^3J(6,6)$ 11.2 Hz, 1 H); H-3^{I}, H-4^{II}, H-5^{I-III}, H-6^{I-III}], 4.44 (d, $^3J(1,2) = 7.9$ Hz, 1 H; H-1^{II}), 4.62 (d, $^3J(1,2) = 8.2$ Hz, 1 H; H-1^{III}), 4.79 (dd, $^3J(1,2) = 8.1$ Hz, $^3J(2,3) = 9.6$ Hz, 1 H; H-2^{III}), 4.88 (ddd, $^3J(1,2) = 2.9$ Hz, $^3J(2,3) = 11.1$ Hz, $^3J(2,F) = 24.8$ Hz, 1 H; H-2^{I}), 5.04 (dd, $^3J(2,3) = 11.3$ Hz, $^3J(3,4) = 8.0$ Hz, 1 H; H-3^{II}), 5.08 (dd, $^3J(1,2) = 7.9$ Hz, $^3J(2,3) = 10.2$ Hz, 1 H; H-2^{II}), 5.12 (dd, $^3J(3,4) = 9.5$ Hz, $^3J(4,5) = 8.9$ Hz, 1 H; H-4^{I}), 5.32 (dd, $^3J(3,4) = 3.2$ Hz, $^3J(4,5) = 0.8$ Hz, 1 H; H-4^{III}), 5.64 (dd, $^3J(1,2) = 2.7$ Hz, $^3J(1,F) = 53.2$ Hz, 1 H; H-1^{I}) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 20.37 - 20.71$ (10 C,CH_3), 60.76, 61.41, 62.17 (C-6^{I-III}), 66.59, 66.88, 69.13, 70.76, 70.90, 71.52, 72.69, 73.14, 75.72, 76.03 (C-2^{II,III}, C-3^{I-III}, C-4^{I-III}, C-5^{II,III}), 70.09 (d, $^4J(5,F) = 3.8$ Hz, C-5^{I}), 72.21 (d, $^3J(2,F) = 24.0$ Hz, C-2^{I}), 100.74, 101.09 (C-1^{II,III}), 104.01 (d, $^2J(1,F) = 210$ Hz, C-1^{I}), 169.06 – 170.58 (10 C; CO); HR MS (FAB): *m/z*: calcd for $C_{38}H_{51}FO_{25}$: 949.2601 $[M+Na]^+$; found: 949.2608.

β-D-Galactopyranosyl-(1 →4)-β-D-glucopyranosyl-(1 →3)-α-D-glucopyranosyl fluoride (**4**): NaOMe (0.10 mL; 1.0 M in MeOH) was added to a solution of the hexaacetate **12** (54 mg, 58 μmol) in MeOH (10 mL) and the mixture stirred and allowed to warm from 0 °C to RT over 2 h. The solution was treated with Amberlite IR-120 resin (H^+ form) until it reached neutral pH, filtered, the solvent evaporated, and the residual

oil lyophilized (H_2O) to yield the fluoride **4** as a colorless foam (31 mg, 99%). MS (FAB, positive mode): *m/z*: 529 $[M+Na]^+$.

Kinetics of hydrolysis by the wild-type 1,3-1,4-β-glucanase: Kinetic measurements were performed by following the changes in UV absorbance caused by the release of 4-methylumbelliferone by using matched 1-cm path length cells in a Varian Cary 4 spectrophotometer with a Peltier temperature control system that maintained the cells at 35 °C. The rates of the enzyme-catalyzed hydrolyses were determined by incubation of the substrate at the appropriate concentration in citrate–phosphate buffer (citric acid (6.5 mM), Na_2HPO_4 (87 mM), pH 7.2, $CaCl_2$ (0.1 mM)) for 5 min in the thermostated cell holder. Reactions were initiated by the addition of the enzyme and the absorbance change at $\lambda = 365$ nm ($\Delta\varepsilon = 5440\ M^{-1}cm^{-1}$[10]) was monitored. The concentration of stock solutions of substrates Gluβ4Gluβ3GluMU (**1**) and Galβ1Gluβ1GluMU (**2**) were determined by UV spectrophotometry by using the molar extinction coefficient reported for 4-methylumbelliferyl glycosides ($\varepsilon_{316nm} = 13\,600\ M^{-}cm^{-1}$[24]).

Kinetics of glycosynthase reactions by E134A 1,3-1,4-β-glucanase: Glycosyl donor and acceptor were dissolved in maleate buffer (50 mM; pH 7.0) that contained $CaCl_2$ (0.1 mM), and preincubated at 35 °C for 5 min. Reactions were initiated by addition of E134A 1,3-1,4-β-glucanase (final volume 0.3 mL). Concentrations for the different experiments are indicated in Table 1. The reaction mixtures were incubated at 35 °C, and aliquots were taken at different time intervals and diluted 1:10 in deionized H_2O for HPLC analysis (NovaPak C18 column, 1 mL min^{-1}, 16% MeOH in H_2O, UV detector at $\lambda = 316$ nm). Initial rates v_0 were calculated from the linear portion of the HPLC progress curve (area versus time) and expressed as v_0/[E] in s^{-1} (Table 1). Reaction yields were calculated from the peak areas in the chromatogram at the indicated times (Table 1).

Preparative glycosynthase-catalyzed reactions:

a) donor **3** + acceptor **14**: The enzyme E134A 1,3-1,4-β-glucanase (1 mg) was added to a solution of **3** (11.2 mg, 0.022 mmol, 1 equiv) and **14** (50 mg, 0.099 mmol, 4.5 equiv) in phosphate buffer (3.7 mL; 100 mM, pH 7.0) that contained $CaCl_2$ (0.1 mM) and the solution was incubated at 35 °C for 30 min. The reaction mixture was loaded directly onto a reversed-phase chromatography column (Lichroprep RP-18, Lobar-A, Merck) and eluted with H_2O (150 mL) followed by a 0–25% gradient of MeOH in H_2O. The excess acceptor **14** (41 mg) was eluted first.

The pentasaccharide 4-methylumbelliferyl β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranoside (**16**) was isolated as a white powder after lyophilization (11 mg, 50%); ^{1}H NMR (D_2O, 30 °C): $\delta = 2.41$ (s, 3H; CH_3), 3.30–4.10 (m, 30H; H-2^{I-V}, H-3^{I-V},H-4^{I-V}, H-5^{I-V}, H-6^{I-V}), [4.52 (d, $^3J(1,2) = 7.8$ Hz, 1H), 4.56 (d, $^3J(1,2) = 8.1$ Hz, 1H), and 4.60 (d, $^3J(1,2) = 8.1$ Hz, 1H); H-1^{II,III,V}], 4.80 (d, $^3J(1,2) = 8.1$ Hz, 1H; H-1^{IV}), 5.20 (d, $^3J(1,2) = 7.8$ Hz, 1H; H-1^{I}), 6.21 (s, 1H; H-3′), 7.02 (d, $^3J(8',6') = 2.1$ Hz, 1H; H-8′), 7.10 (dd, $^3J(5',6') = 9.0$ Hz, $^3J(6',8') = 2.4$ Hz, 1H; H-6′), 7.67 (d, $^3J(5',6') = 9.0$ Hz, 1H; H-5′) ppm; ^{13}C NMR (D_2O, 30 °C): $\delta = 18.6$ (CH_3), 60.4–61.2 (C-6^{I-V}), 68.6, 70.1 (C-4^{III,V}), 73.2–76.6 (C-2^{I-V}, C-3^{I,II,IV,V}, C-5^{I-V}), 79.2, 79.1, 78.7 (C-4^{I,II,IV}), 84.5 (C-3^{III}), 100.2–104.2 (C-1^{I-V}, C-8′), 112.0 (C-3′), 114.6 (C-6′), 115.9 (C-4a′), 127.3 (C-5′), 154.5 (C-4′), 156.8 (C-1a′), 160.1 (C-7′), 165.3 (C-2′) ppm; MS (ES): *m/z*: calcd for $C_{40}H_{58}O_{28}Na$: 1009.31 $[M+Na]^+$; found: 1009.30.

The octasaccharide **18** (elongation product) was obtained in 9% yield (3 mg); ^{1}H NMR (D_2O, 30 °C) $\delta = 2.50$ (s, 3H; CH_3), 3.15–4.10 (m, 48H; H-2^{I-VIII}, H-3^{I-VIII},H-4^{I-VIII}, H-5^{I-VIII}, H-6^{I-VIII}), 4.46–5.00 (H-1^{II,VIII}), 5.30 (d, $^3J(1,2) = 7.8$ Hz, 1H; H-1^{I}), 6.34 (s, 1H; H-3′), 7.18 (d, $^3J(5',6') = 6.3$ Hz, 1H; H-6′), 7.81 (d, $^3J(6',5') = 9.3$ Hz, 1H; H-5′); MS (ES): *m/z*: calcd for $C_{58}H_{88}O_{43}Na$: 1495.46 $[M+Na]^+$; found: 1495.48.

b) donor **4** + acceptor **14**: E134A 1,3-1,4-β-Glucanase (0.5 mg) was added to a solution of **4** (4.62 mg, 0.0091 mmol, 1 equiv) and **14** (21.2 mg, 0.04 mmol, 4.6 equiv) in phosphate buffer (1.5 mL, 100 mM, pH 7.0) that contained $CaCl_2$ (0.1 mM). The solution was incubated at 35 °C for 20 min. Reversed-phase chromatography of the reaction mixture (Lichroprep RP-18, Lobar-A, Merck, eluted with H_2O (150 mL) followed by a 0–25% gradient of MeOH in H_2O give first the excess acceptor **14** (17 mg), followed by the condensation product 4-methylumbelliferyl β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranoside (**20**; 9 mg, 98%); ^{1}H NMR (D_2O, 30 °C) $\delta = 2.45$ (s, 3H; CH_3), 3.25–4.10 (m, 30H; H-2^{I-V}, H-3^{I-V},H-4^{I-V}, H-5^{I-V}, H-6^{I-V}), 4,46 (d, $^3J(1,2) = 7.5$ Hz, 1H; H-1^{V}), [4.56 (d, $^3J(1,2) = 8.1$ Hz, 1H) and 4.59 (d, $^3J(1,2) = 8.1$ Hz ,1H); H-1^{II,III}], 4.79 (1H; H-1^{IV}), 5.26 (d, $^3J(1,2) = 7.8$ Hz, 1H; H-1^{I}), 6.27 (d, $^4J(3',\ CH_3) = 1.2$ Hz, 1H; H-3′), 7.09 (d, $^3J(8',6') = 2.4$ Hz, 1H; H-8′), 7.14 (dd, $^3J(5',6') = 8.7$ Hz, $^3J(6',8') = 2.4$ Hz, 1H; H-6′), 7.74 (d, $^3J(5',6') = 9.0$ Hz, 1H; H-5′) ppm; ^{13}C NMR (D_2O, 30 °C): $\delta = 18.7$ (CH_3), 60.5–61.6 (C-6^{I-V}), 68.6(C-4^{III}), 69.2 (C-4^{V}), 71.6–76.0 (C-2^{I-V}, C-3^{I,II,IV,V}, C-5^{I-V}), 79.0 (C-4^{I,II,IV}), 84.6 (C-3^{III}), 100.1–104.3 (C-1^{I-V}, C-8′), 112.1 (C-3′), 114.7 (C-6′), 116.0 (C-4a′), 127.5 (C-5′), 154.7 (C-4′), 156.8 (C-1a′), 160.2 (C-7′), 164.9 (C-2′) ppm; MS (ES): *m/z*: calcd for $C_{40}H_{58}O_{28}Na$: 1009.31 $[M+Na]^+$; found: 1009.30.

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