Highly Efficient Synthesis of $\beta(1 \rightarrow 4)$-Oligo- and -Polysaccharides Using a Mutant Cellulase

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Abstract: This report describes an efficient chemoenzymatic synthesis of a variety of regioselectively modified $\beta(1\rightarrow4)$-oligo- and -polysaccharides. This successful approach was based on: (i) the use of a "glycosynthase" which is a Glu-197-Ala nucleophile mutant of the retaining cellulase endoglucanase I (Cel7B) from *Humicola insolens* and (ii) the rational design of modified acceptor and donor molecules through a careful examination of information given by the X-ray structures of wild type and mutated enzymes. The mutant was able to catalyze, in high yield, the regio- and stereoselective glycosylation of α-glycobiosyl fluorides both unsubstituted and modified with various mono- and disaccharide acceptors, as well as the polymerization of these donors through a single-step inverting mechanism.

Introduction

The use of enzymes is a theoretically straightforward approach for the formation of glycosidic bonds in oligo- and polysaccharides, and the potential of glycoside hydrolases as tools for the synthesis of glycosides has been realized in many laboratories.[1] These enzymes as well as the requisite donor substrates (aryl glycosides, glycosyl fluorides, or disaccharides) are readily available and inexpensive. Retaining glycosidases, which hydrolyze glycosides through a glycosyl-enzyme intermediate which is formed by nucleophilic attack of one catalytic amino acid (the nucleophile) onto the anomeric carbon of the donors, are known to be effective for oligosaccharide synthesis. New oligosaccharides can be formed when a sugar competes with water as acceptor during the deglycosylation step.[2] The transglycosylation catalyzed by *endo*-enzymes is always highly regioselective, and under appropriate conditions (organic cosolvent and pH) oligosaccharides are isolated in good yields (up to 60%).[3]

Very recently, a new and highly efficient approach based on protein engineering of retaining β-glucosidase has been developed by Withers and co-workers.[4] As expected, a glycoside hydrolase in which the carboxylate nucleophile was replaced by alanine was unable to form the glycosyl enzyme intermediate and had no hydrolytic activity. However, a mimic of this intermediate, an α-glycosyl fluoride, was recognized and used for transglycosylation reactions, with the advantage that the reaction products cannot be hydrolyzed and were accumulated almost quantitatively. This original concept was also applied successfully by Planas and Malet to an *endo*-glycosidase, the 1,3-1,4-β-glucanase from *Bacillus licheniformis*, which gave tri- and tetrasaccharides in very high yield.[5]

To overcome the problems related to chemical syntheses of cellooligosaccharides and cellulose having $\beta(1\rightarrow4)$-glycosidic linkages, Kobayashi and co-workers developed a transglycosylation approach using β-cellobiosyl or β-lactosyl fluorides, catalyzed by partially purified cellulases in hydroorganic medium.[1a−c,g] We also applied the same methodology for the preparation of hemithiocellodextrins[6] and for the synthesis of a bifunctionalized fluorogenic tetrasaccharide.[3] In the most recent example, the enzymatic condensation was achieved by a recombinant endocellulase Cel7B (formerly called endoglucanase I) from the fungus *Humicola insolens*, an enzyme which was known to have high transglycosylation activity.[7] In a mixture of acetonitrile and maleate buffer, condensation of β-lactosyl fluoride onto a cellobiosyl acceptor gave the expected tetrasaccharide in 60% yield. The same strategy was, however, unsuccessful for the synthesis of higher oligomers, when

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(1) (a) Kobayashi, S.; Kashiwa, K.; Kawasaki, T.; Shoda, S−i. *J. Am. Chem. Soc*. **1991**, *113*, 3079−3084. (b) Shoda, S−i.; Kawasaki, T.; Obata, K.; Kobayashi, S. *Carbohydr. Res*. **1993**, *249*, 127−137. (c) Karthaus, O.; Shoda, S−i.; Takano, H.; Obata, K.; Kobayashi, S. *J. Chem. Soc., Perkin Trans 1* **1994**, 1851−1857. (d) Singh, S.; Scigelova, M.; Crout, D. H. G. *Chem. Commun*. **1996**, 993−994. (e) Kobayashi, S.; Kiyosada, T.; Shoda, S−i. *J. Am. Chem. Soc*. **1996**, *118*, 13113−13114. (f) Murata, T.; Tashiro, A.; Itoh, T.; Usui, T. *Biochim. Biophys. Acta* **1997**, 1335, 326−334. (g) Okamoto, E.; Kiyosada, T.; Shoda, S−i.; Kobayashi, S. *Cellulose* **1997**, *4*, 161−172. (h) Vic, G.; Tran, C. H.; Schigelova, M.; Crout, D. H. G. *Chem. Commun*. **1997**, 169−170. (i) Viladot, J.-L.; Moreau, V.; Planas, A.; Driguez, H. *J. Chem. Soc., Perkin Trans 1* **1997**, 2383−2387. (j) Hrmova, M.; Fincher, G. B.; Viladot, J. L.; Planas, A. Driguez, H. *ibid*. **1998**, 3571−3576. (k) Fujita, M.; Shoda, S−i; Kobayashi, S. *J. Am. Chem. Soc*. **1998**, *120*, 6411−6412. (l) Li, J.; Robertson, D. E.; Short, J. M., Wang, P. B. *Biorg. Med. Chem*. **1999**, *9*, 35−38. (m) Macmanus, D. A.; Grabowska, U.; Biggadike, K.; Bird, M. I.; Davies, S.; Vulfson, E. N.; Gallagher, T. *J. Chem. Soc., Perkin Trans 1* **1999**, 295−305.
(2) Sinnott, M. L. *Chem. Rev*. **1990**, *90*, 1171−1202.
(3) Armand, S.; Drouillard, S.; Schülein, M.; Henrissat, B.; Driguez, H. *J. Biol. Chem*. **1997**, *272*, 2709−2713.
(4) Mackenzie, L. F.; Wang, Q.; Warren, R. A. J.; Withers, S. G. *J. Am. Chem. Soc*. **1998**, *120*, 5583−5584.
(5) Malet, C.; Planas, A. *FEBS Lett.* **1998**, *440*, 208−212.
(6) Moreau, V.; Driguez, H. *J. Chem. Soc., Perkin Trans 1* **1996**, 525−527.
(7) Schou, C.; Ramussen, G.; Kaltoft, M.-B.; Henrissat, B.; Schülein, M. *Eur. J. Biochem*. **1993**, *217*, 947−953.

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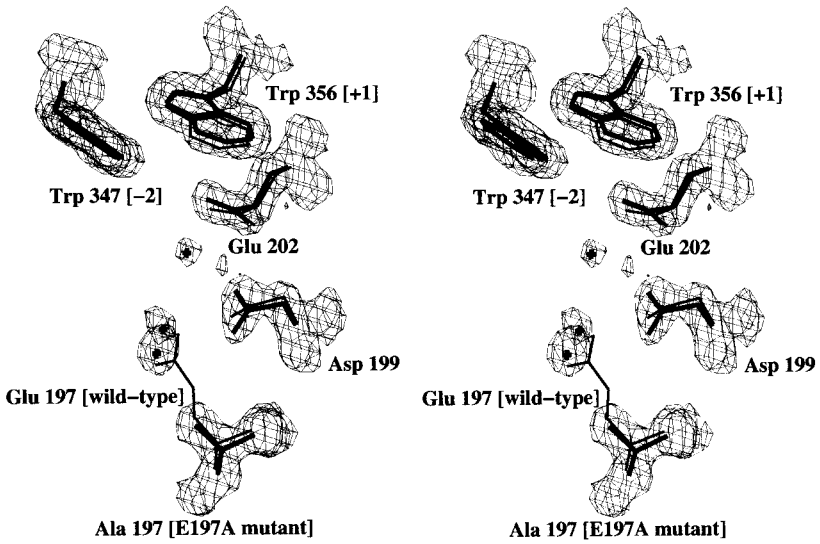


Figure 1. Stereo representation of the observed electron density around the Glu197Ala mutation. The refined coordinates of the mutant structure are shown in bold, and the overlapped wild-type coordinates, in faint lines. The two structures are almost identical except for the inclusion of two solvent molecules in the space provided by the Glu197Ala mutation. The map shown is a maximum likelihood and $\sigma_A$weighted $2F_{obs} - F_{calc}$ synthesis contoured at 0.44 e/Å^3.

cellotrioside was used as acceptor. This molecule competes with lactosyl fluoride as a donor and a cellobiosyl-enzyme intermediate was formed which led to an untractable mixture of products with both nonreducing end galactosyl or glucosyl units (unpublished results).

To overcome these problems we have developed a "glycosynthase" mutant of Cel7B. The Glu-197-Ala mutant has been expressed and purified and its X-ray structure determined at 1.75 Å resolution. The Cel7B glycosynthase is a highly efficient catalyst for the synthesis of novel $\beta(1 \rightarrow 4)$-oligo- and -polysaccharides.

Results and Discussion

Specific Mutagenesis of Cel7B from *Humicola insolens.* Glu 197 has been identified in Cel7B from *Humicola insolens* as the catalytic nucleophile.[8] The Glu-197-Ala variant was made by site directed mutagenesis (Chameleon, Stratagene), directly in the expression plasmid pHW704, an *Escherichia coli* plasmid in which the transcription of Cel7B is governed by an *Aspergillus* α-amylase promoter and glucoamylase terminator. The Glu-197-Ala modification was verified by complete sequencing of the gene. The plasmid harboring pCE10 was transformed into *Aspergillus oryzae* JaL228, together pToC104, encoding the selectable marker amdS, encoding an acetamidase, and plated with acetamide as the sole nitrogen source.[9]

The amdS transformants were screened for expression of the inactive Cel7B variant by Ouchterlony method on fermentation samples, applying a polyclonal Ab raised against wild-type Cel7B. The highest expressing transformant was reisolated three times, and stored as LaC2922. The transformed *A. oryzae* was grown in a fermentor, and the extracellular expressed protein was purified to homogeneity using cationic (S-Sepharose) chromatography. The purified protein gave a single band in SDS-PAGE of 50 kDa and had no activity on CMC.

Crystal Structure of the Ala Mutant of Cel7B from *H. insolens.* The Cel7B Glu-197-Ala mutant crystallizes in space group $P2_1$ with cell-dimensions $a = 49.53$ Å, $b = 73.98$ Å, $c = 59.87$ Å, and $\beta = 103.81°$. Synchrotron data collected from a single crystal at 100 K have an overall R_{merge} ($R_{merge} = \Sigma_{hkl} \Sigma_i |I_{hkli} - \langle I_{hkl} \rangle| / \Sigma_{hkl} \Sigma_i \langle I_{hkl} \rangle$) of 0.075, a mean $I/\sigma I$ of 19.7 and a mean multiplicity of 4.9 observations/reflection. The data are complete. In the outer resolution shell (1.81−1.75 Å) the corresponding values are R_{merge} 0.45, $I/\sigma I$ 3.3, multiplicity 4.8, and are again 100% complete. Structure solution by molecular replacement, using the native *H. insolens* Cel7B structure, was trivial and maximum likelihood refinement proceeded smoothly. The final model coordinates have a crystallographic *R*-factor of 0.16, with an R_{free} of 0.20. They consist of 3104 protein atoms (residues 1 to 398), 595 water molecules (with temperature factors ≤60 Å^2) and 28 atoms resulting from single *N*-acetylglucosamine residues *N*-linked to Asn 89 and Asn 247.

Crystal structures of Cel7B enzymes from *H. insolens*,[8] *Fusarium oxysporum*,[10] and *Trichoderma reesei*[11] reveal their close architectural similarity with an active site located in a long (50 Å) and deep (20 Å) open groove with the active site nucleophile located about two-thirds of the way along the cleft. The structure of the Glu-197-Ala mutant is virtually identical to the native *H. insolens* Cel7B structure; the only changes are in the vicinity of the mutation where the solvent structure is slightly altered (Figure 1). The mutation of Glu-197-Ala liberates a volume of approximately 120 Å^3 which is more than sufficient to accommodate the axial fluoride of the $\beta(1\rightarrow4)$-disaccharide donor. Two solvent water molecules occupy this newly formed cavity in the uncomplexed Cel7B glycosynthase structure. One can also imagine that other "short" nucleophile mutations such as glutamate to serine or cysteine would also generate enough space to accommodate an axial fluoride.

The high degree of sequence similarity (57%) between the *H. insolens* and *F. oxysporum* enzymes suggest that the same interactions may occur in the active site of both enzymes. The structure of Cel7B from *F. oxysporum* complexed to both a non-hydrolyzable thiooligosaccharide substrate analogue and cello-

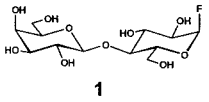
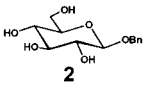
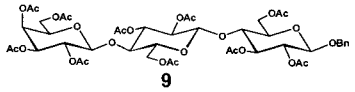
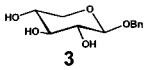
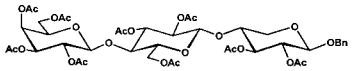
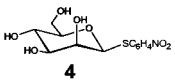
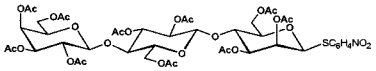
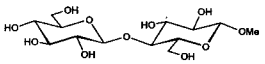
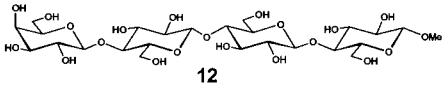
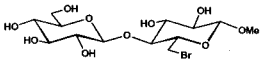
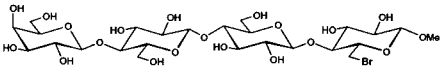
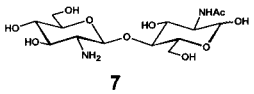
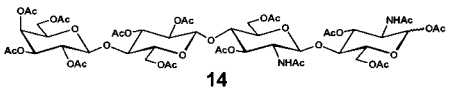
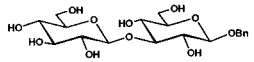
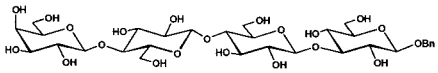
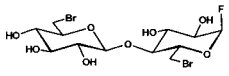
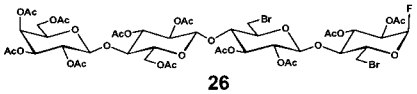
(8) Mackenzie, L. F.; Sulzenbacher, G.; Divne, C.; Jones, T. A.; Wöldike, H.; Schülein, M.; Withers, S. G.; Davies, G. J. *Biochem J.* **1998**, *335*, 409−416.

(9) Kelly, J. M.; Hynes, M. *EMBO J.* **1985**, *4*, 475−479.

(10) (a) Sulzenbacher, G.; Driguez, H.; Henrissat, B.; Schülein, M.; Davies, G. J. *Biochemistry* **1996**, *35*, 15280−15287. (b) Sulzenbacher, G.; Schülein, M.; Davies, G. J. *ibid.* **1997**, *36*, 5902−5911

(11) Kleywegt, G. J.; Zou, J.-Y., Divine, C.; Davies, G. J.; Sinning, J.; Stählberg, J.; Srisoduk, M., Teeri, T. T.; Jones, T. A. *J. Mol. Biol.* **1997**, *272*, 383−397.

Table 1. Obtention of β-(1→4)-Oligosaccharides by Enzymatic Condensation of Lactosyl Fluoride **1** on Mono- and Disaccharide Acceptors **2**−**8**, **16e** Using Cel7B Glu197Ala

Donor	Acceptors	Isolated product	Yield
 **1**	 **2**	 **9**	83%
	 **3**	 **10**	51%
	 **4**	 **11**	61%
	 **5**	 **12**	100%
	 **6**	 **13**	100%
	 **7**	 **14**	65%
	 **8**	 **15**	100%
	 **16e**	 **26**	80%

biose has shown that the three hydroxyl groups of −1 subsite sugar strongly interact with Asp 173, Gln 175, and Trp 347 (*F. oxysporum* numbering). There are no interactions with the protein involving the 6-OH in both the −2 and +1 glucosyl residues. Indeed, the −2 subsite 6-OH is extremely solvent exposed and frequently disordered.[10] It was thus anticipated that the mutated enzyme could not only be used as a "glycosynthase" for the synthesis of natural oligo- and polysaccharides, but that the lack of steric restriction on the C6−OH interactions could be exploited for the synthesis of substituted β(1→4)-glucans.

Enzymatic Reaction Catalyzed by Ala Mutant of Cel7B from *H. insolens*. The scope of the enzyme condensation catalyzed by the Ala mutant of Cel7B from *H. insolens* was first explored using α-lactosyl fluoride **1**[12] as a donor in the presence of a range of mono- and disaccharide acceptors **2**−**8**, **16e** (Table 1) in ammonium carbonate solution at pH 8.0 or in phosphate buffer at pH 7.0. The transfer of the fluoride **1** on β-D-mannopyranoside **4**[13] and β-D-xylopyranoside **3**[14] was slightly less efficient than on the β-D-glucoside **2**[15] but resulted in higher yields than reported using a commercially available enzyme in hydro-organic solvents.[1c] The present yields of acetylated purified products **9**−**11** were respectively 83, 51, and 61%. These results confirm those of the X-ray structure of the complexed wild type enzyme in which the 6-OH and 2-OH of the glucosyl unit in subsite +1 were not involved in key polar interactions. It must be emphasized that only β(1→4) linkages were formed whatever monosaccharide acceptors were involved. This conclusion arises from the lack of the characteristic signals at C-2 or C-3 of 2- or 3-*O*-substituted glycosyl units in ^{13}C NMR of unprotected compounds obtained by de-*O*-acetylation of **9** and **10**, at 79−85 ppm.[16] In the ^{1}H NMR spectrum of the tetraacetylated *p*-nitrophenyl 1-thio-β-D-mannoside, the triplet signal at δ 5.26 was assigned to H-4.[13] The complete interpretation of the ^{1}H NMR spectrum of the trisaccharide **11** shows that H-4 was shifted upfield to δ 3.86. On the other hand, the position of C-4 at δ 65.46 for the acetylated mannoside was missing in the ^{13}C NMR spectrum of **11** and as expected for a

(12) Jünnemann, J.; Thiem, J.; Pedersen, C. *Carbohydr. Res*. **1993**, *249*, 91−94.

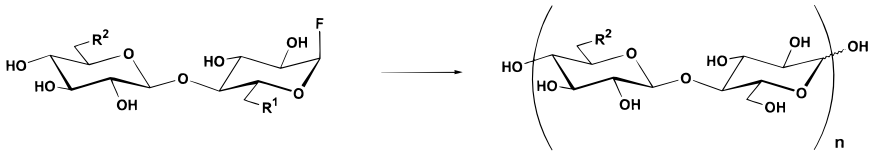
(13) Blanc-Muesser, M.; Defaye, J.; Driguez, H. *ibid*. **1978**, *67*, 305−328.

(14) Ballou, C. E.; Roseman, S.; Link, K. P. *J. Am. Chem. Soc*. **1951**, *73*, 1140−1144.

(15) Fischer, E.; Helferich, B. *Justus Liebigs Ann. Chem.* **1911**, *71*, 68−71.

(16) Bock, K.; Pedersen, C.; Pedersen, H. *Adv. Carbohydr. Chem. Biochem.* **1984**, *42*, 193−224.

Scheme 1. Enzymatic Polymerization of Cellobiosyl Fluorides **16a**−**16d** Using Cel7B Glu197Ala



16a-e **17a-d**

	R^1	R^2
16a,17a	OH	OH
16b,17b	OH	Br
16c,17c	OH	NH_2
16d,17d	OH	α-*S*-xylopyranosyl
16e	Br	Br

4-*O*-substituted D-mannosyl residue was found at δ 73.60. This is in contrast with the $\beta(1\rightarrow3)$ reaction product obtained when xyloside was used with the Ala mutant of β-glucosidase from *Agrobacterium* sp. [4]

Several $\beta(1\rightarrow4)$- and $\beta(1\rightarrow3)$-disaccharides were also used as acceptors. Methyl β-cellobioside **5**,[17] methyl 6-bromo-6-deoxy-β-cellobioside **6**,[18] *N*-acetyl-chitobiose **7**,[19] and benzyl β-laminaribioside **8**[20] gave the expected tetrasaccharides **12**−**15** in excellent yields (Table 1).[21] Only *N*,*N*^{II}-diacetyl-chitobiose, a close analogue of **7** with an additional acetyl group which should fit in +1 subsite, was found to be unable to act as an acceptor molecule.

These results demonstrate that the acceptor subsites of Glu-197-Ala mutant of Cel7B from *H. insolens* may accommodate various mono- and disaccharides structurally different from the natural cellobiosyl unit. As already observed and expected for an endoglucanase, disaccharides are better acceptors than monosaccharides for transglycosylation reaction.[5]

In a second set of experiments, the polymerization reaction of cellobiosyl fluoride **16a** and various derivatives **16b**−**16e** catalyzed by the Glu-197-Ala mutant of Cel7B was investigated. (Scheme 1). Mild de-*O*-acetylation of the known acetylated α-cellobiosyl fluoride[12] gave the corresponding compound **16a** which was incubated with enzyme, in phosphate buffer (0.1M, pH 7.0) at 40 °C for 24 h. As the reaction proceeded a white crystalline precipitate was formed.The structure of the insoluble material **17a** (Scheme 1) was investigated by electron and X-ray diffraction analysis, together with ^{1}H- and ^{13}C NMR in 0.2 M sodium hydroxide (NaOD) at room temperature and in dimethyl sulfoxide $(CD_3)_2SO$ at 353 K, respectively. The spectra are identical to those of low molecular weight cellulose II obtained by us using phosphorolytic synthesis.[22]

Polymerization reactions were then attempted using α-cellobiosyl fluorides modified at C-6^{II} as suggested from the X-ray data of the wild-type enzyme. The 6^{II}-bromo-6^{II}-deoxy-α-cellobiosyl fluoride **16b**, 6^{II}-amino-6^{II}-deoxy-α-cellobiosyl fluoride **16c**, and 6^{II}-*S*-(α-D-xylopyranosyl)-6^{II}-thio-α-cellobiosyl fluoride **16d** were selected.

Scheme 2 outlines the synthesis of these compounds. The synthesis began with the mild and selective bromination of the free primary hydroxyl group of 1,6-anhydro-β-cellobiose (**18a**)[23] by a method described for the bromination of methyl α-D-glucoside.[24] Thus **18a** reacted with carbon tetrabromide and triphenylphosphine in pyridine for 2 h at 50 °C. After addition of acetic anhydride, the expected bromide **18b** was isolated in 54% yield. Acetolysis of the 1,6-anhydro ring was performed by treatment of **18b** with acetic anhydride in the presence of triethylsilyl triflate. The anomeric mixture of acetates was converted via the corresponding α-bromide, followed by the action of silver acetate in acetic acid−acetic anhydride mixture into the hepta-*O*-acetyl-6^{II}-bromo-6^{II}-deoxy-β-cellobiose **19**, the precursor for the synthesis of the corresponding fluoride **20**. The fluorination was achieved in 90% yield using commercially available pyridine-hydrogen fluoride reagent, as already described in the maltose and cellobiose series.[12] The substituted α-cellobiosyl fluoride **20** was de-*O*-acetylated with sodium methoxide in methanol at 0 °C to give the corresponding unprotected pure compound **16b** in quantitative yield. This fluoride **20** was the key compound for other modified α-cellobiosyl fluorides. Treatment with sodium azide in *N*,*N*-dimethylformamide generated the 6^{II}-azido-6^{II}-deoxy derivative **21** in 88% yield.[25] After de-*O*-acetylation with sodium methoxide in methanol at 0 °C and hydrogenation in ethanol using Pd/C as catalyst, the fluoride **16c** was obtained (90% yield over the two steps). The fluoride derivative **16d** was also prepared from **20**, since the synthesis of 1,6-thiooligosaccharides is easy and effective by displacement of a 6-halide derivative with a 1-thiolate glycose as a donor.[26] The β-D-xylosyl chloride **22**[27] was treated with the tetrabutylammonium salt of triphenylmethanethiol in toluene[28] to produce stereoselectively the α-thio-

(17) Newth, F. H.; Nicholas, S. D.; Smith, F.; Wiggins, L. F. *J. Chem. Soc* **1949**, 2550−2553.

(18) Takeo, K.; Fukatsu, T.; Yasato, T. *Carbohydr. Res.* **1982**, *107*, 71−90.

(19) Drouillard, S.; Armand, S.; Davies, G. J.; Vorgias, C. E.; Henrissat, B. *Biochem. J.* **1997**, *328*, 945−949.

(20) Obtained by deacetylation of acetylated benzyl β-laminaribioside prepared as described in Thiem J.; Horst, K. *Chem. Ber.* **1979**, *112*, 1046−1056.

(21) To ensure that only $\beta(1\rightarrow4)$ linkages were formed during the reaction catalyzed by the "glycosynthase", all the new isolated oligo- and polysaccharides were subjected to enzymatic hydrolysis by the wild-type enzyme Cel7B from *H. insolens* and their hydrolysis products were characterized by TLC using autenthic samples.

(22) Samain, E.; Lancelon-Pin, C.; Ferigo, F.; Moreau, V.; Chanzy, H.; Heyraud, A.; Driguez, H. *Carbohydr. Res.* **1995**, *271*, 217−226.

(23) Fujimaki, I.; Ichikawa, Y.; Kuzuhara, H. *ibid.* **1982**, *101*, 148−151.

(24) Anisuzzaman, A. K. M.; Whistler, R. L. *ibid.* **1978**, *61*, 511−518.

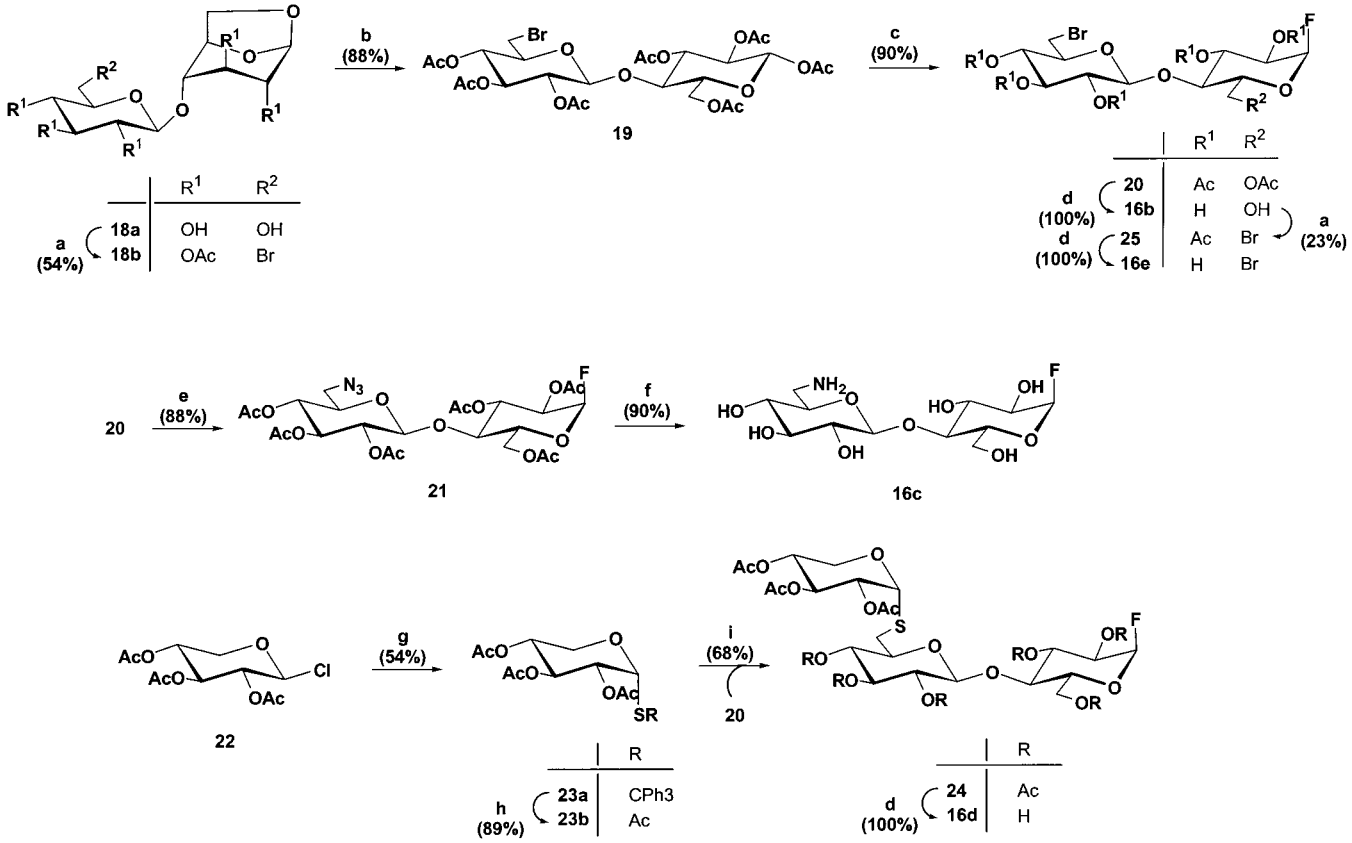
(25) As already reported in the glucose series, under these conditions, no β-D-glycosyl azide was detected (Horneman A. M.; Lundt, I. *J. Carbohydr. Chem.* **1995**, *14*, 1−8).

(26) (a) Hasegawa, A.; Terada, T.; Ogawa, H.; Kiso, M. *J. Carbohydr. Chem.* **1992**, *11*, 319−331. (b) Bennet, S.; von Itzstein, M.; Kiefel, M. *J. Carbohydr. Res.* **1994**, 259, 293−299.

(27) Holland, C. V.; Horton, D.; Jewell, J. S. *J. Org. Chem.* **1967**, *32*, 1818−1821.

(28) Blanc-Muesser, M.; Vigne, L.; Driguez, H. *Tetrahedron Lett.* **1990**, 3869−3870.

Scheme 2. Syntheses of Cellobiosyl Fluorides **16b**−**16e**[a]



[a] (a) PPh_3, CBr_4, pyridine, 0 to 50 °C, 2 h, then Ac_2O, 3 h. (b) (1) TESTf, AC_2O, 0 °C, 30 min, aq. $NaHCO_3$, 30 min; (2) HBr−AcOH, CH_2Cl_2, 0 °C, 30 min then rt, 1 h; (3) AgOAc, Ac_2O, AcOH, rt, 12 h; (c) HF−pyridine, 0 °C, 1.5 h; (d) (1) NaOMe, MeOH, 0 °C, 4 h; (2) H^+ resin; (e) NaN_3, DMF, 80 °C, 12 h; (f) (1) d; (2) 10% Pd/C, H_2, EtOH, 2 h; (g) Bu_4NSTr, toluene, rt, 24h; (h) (1) PhHgOAc, MeOH−CH_2Cl_2, rt, 1 h; (2) H_2S, Ac_2O, pyridine, CH_2Cl_2, rt, 12h; (i) DTE, cysteamine, HMPA, rt, 7h.

glycoside **23a** (54% yield). Well-known two-step procedures[28] led to **23b** from **23a** in 89% yield. Selective de-*S*-acetylation and activation, using cysteamine in HMPA in the presence of DTE,[29] and reaction with glycosyl acceptor **20** produced the desired trisaccharide fluoride **24** in 68% yield, but **20** was recovered in 30% yield. De-*O*-acetylation as already described, afforded **16d** in quantitative yield. This compound is the thio-analogue fluoride of the trisaccharide α-D-Xyl*p*-(1→6)-*β*-D-Glc*p*-(1→4)-D-Glc, one of the building blocks of xyloglucan, a widely distributed polymer which interacts intimately with cellulose in primary cell walls of higher plants,[30] and is a substrate for wild-type Cel7B.[31]

In the presence of a catalytic amount (10 μM) of the Glu-197-Ala mutant of Cel7B, the fluorides **16b**−**16d** were incubated in buffers at 40 °C for 24 h. Water insoluble polymers **17b** and **17d** and the soluble one **17c** (at pH 7.0) were characterized by NMR, MS, or GPC and enzymatic hydrolysis.[21]

An attempt at polymerization of **16e**, readily obtained from **16b** via the acetylated dibromo compound **25** (Scheme 2), failed. However, this molecule was an acceptor when incubated in the presence of lactosyl fluoride **1**, and the expected tetrasaccharide **26** (Table 1) was isolated in 80% yield.

Conclusions

This work clearly shows the benefit of a multi-disciplinary approach for the rational design of new tools in organic synthesis. The 1.75 Å X-ray structure of the Glu-197-Ala mutant reveals its structural integrity with no change in the position of critical substrate-binding residues such as Trp 347 and Trp 356. The catalytic acid/base, Glu 202, (functioning as a Brønsted base for the enzymatic condensation) is found in an unchanged, and hence catalytically viable, position. Initial modeling, using the wild-type *H. insolens* Cel7B structure and the thio-oligosaccharide and cellobiose complexes of the closely related Cel7B structures from *F. oxysporum* correctly indicated that the −2 subsite would tolerate substantial substitutions, particularly at the C6 position, which has been successfully exploited for the synthesis of new glucans.

Experimental Section

General. NMR spectra were recorded on a Bruker AC 300, Bruker Avance 400 or Varian Unity 500. Proton chemical shifts (δ) are reported in ppm downfield from TMS. Coupling constants (J) are in hertz (Hz) with singlet (s), doublet (d), doublet of doublet (dd), triplet (t), multiplet (m), broad (b). Carbon chemical shifts (δ) are reported in ppm with internal reference of solvent. Complete assignment of the oligomers **11** and **15** was performed using a combination of COSY or TOCSY 1D, HMQC, and HMBC experiments. One bond $^{13}C-^{1}H$ correlations were obtained from HMQC data, and the position of the glycosidic linkages was determined using HMBC data. High-resolution mass spectra (HRMS) were recorded on VG ZAB and low-resolution (MS) on a Nermag R-1010C spectrometers. Multidetection steric exclusion

(29) Blanc-Muesser, M.; Driguez, H. *J. Chem. Soc., Perkin Trans 1* **1988**, 3345−3351.

(30) Perrin, R. M.; De Rocher, A. E.; Bar-Peled, M.; Zeng, W.; Norambuena, L.; Orellana, A.; Raikhel, N. V.; Keegstra, K. *Science* **1999**, *284*, 1976−1978.

(31) Vargas-Rechia, C.; Reicher, F.; Sierakowski, M. R.; Heyraud, A.; Driguez, H.; Lienart, Y. *Plant Physiol.* **1998**, *116*, 1013−1021.

chromatography was performed on 150 C Waters chromatograph equipped with a multiangle DAWN DSP-F light scattering detector (laser wavelength 632.8 nm), an ambient flow cell for aqueous solution K5, and the ASTRA 4 software (Wyatt technology Corporation, Santa Barbara, CA). Optical rotations were measured with a Perkin-Elmer 341 polarimeter. Melting points were measured on a Büchi 535 apparatus. Microanalyses were performed by the "Laboratoire Central d'analyses du CNRS" (Vernaison).

Evolution of reactions was monitored by analytical thin-layer chromatography using silica gel 60 F254 precoated plates (E. Merck, Darmstadt).

Di-*N*,*N*^{II}-acetyl chitobiose was commercially available. All reactions in organic medium were carried out under argon using freshly distilled solvents. After workup, organic phases were dried over anhydrous Na_2SO_4.

X-ray Structure Determination. The Glu-197-Ala mutant of Cel7B was purified as described for the CE8 double mutant.[32] Crystals were grown by the hanging-drop vapor-phase diffusion technique in 4 μL hanging-drops. The protein concentration was 20 mg·mL^{-1} in 20 mM Tris-HCl buffer with 20−30% (w/v) poly(ethylene glycol) 4000 as precipitant. 5% (v/v) DMSO was included to help prevent the formation of disordered arrays of plates. X-ray data to 1.75 Å resolution were collected on the EMBL BW7A beam line ($\lambda = 0.9998$ Å) at the DORIS storage ring in Hamburg from a single crystal in a stream of N_2 gas at 100 K. A 300 mm Mar-Research Imaging Plate Scanner was used as detector. Data processing and reduction were carried out with the HKL program suite,[33] and all further computing used the CCP4 suite of programs[34] unless otherwise stated.

The structure was solved by molecular replacement using the native *H. insolens* Cel7B structure as the search model (PDB code 2A39). The program AMORE[35] was used in conjunction with data in the resolution range 20−4 Å and an outer radius of Patterson integration of 30 Å. Structure solution revealed just one significant solution. For refinement, 5% of the observations were immediately set aside for cross validation analysis[36] and were used to monitor various refinement strategies such as geometric and temperature-factor restraint values, the insertion of solvent water and as the basis for the maximum likelihood refinement using REFMAC.[37] Manual corrections of the model using the X-FIT routines of the program QUANTA (Molecular Simulations Inc., San Diego, CA) were interspersed with cycles of maximum-likelihood based refinement. Water molecules were added in an automated manner using ARP[38] and verified manually prior to coordinate deposition. Hydrogen scattering from "riding" hydrogen atoms was included and justified by a decrease in the R_{cryst} and R_{free} of 1.2%/1.2%, respectively. Coordinates have been deposited with the Protein Databank.[39]

Benzyl (2,3,4,6-Tetra-*O*-acetyl-*β*-D-galactopyranosyl)-(1→4)-(2,3,6-tri-*O*-acetyl-*β*-D-glucopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-*β*-D-glucopyranoside (9). Benzyl glucoside **2** (34 mg, 0.125 mmol) and then Cel7B Glu197Ala (0.45 mg) were added to a solution of lactosyl fluoride **1** (43 mg, 1 equiv) in phosphate buffer (0.1M, pH 7.0, 1 mL). The reaction mixture was gently shaken in an oven at 40 °C. After 48 h, the solution was filtered on C18 Cartridge Sep-Pak Plus (Waters) and then concentrated to dryness and acetylated (acetic anhydride-pyridine 1:1 v/v, 10 mL). After 12 h of stirring at 25 °C, the reaction was quenched by adding MeOH at 0 °C and concentrated in vacuo. The residue was dissolved in CH_2Cl_2 and washed with 20% aqueous $KHSO_4$ and saturated aqueous $NaHCO_3$. The organic layers were concentrated and purified by flash chromatography (EtOAc−petroleum ether 6:4 v/v) to generate the title trisaccharide **9** (105 mg, 83%): $[\alpha]^{25}_D$ −31.5 (*c* 1.33, $CHCl_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 169.6−169.2 (CO), 136.6 (Cq arom), 128.3−127.6 (C arom), 101.0 (C-1^{I}), 100.3 (C-1^{II}), 99.0 (C-1^{III}), 76.3, 75.9 (C-4$^{I,\ II}$), 72.9, 72.6, 72.4, 71.8, 71.6, 70.8, 70.65, 70.6 (C-2$^{I,\ II}$, C-3$^{I,\ II,\ III}$, C-5$^{I,\ II,\ III}$, CH_2Ph), 69.0 (C-2^{III}), 66.5 (C-4^{III}), 62.2, 61.7, 60.7 (C-6$^{I,\ II,\ III}$), 20.7−20.5 (CH_3); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.28−7.22 (m, 5H, H arom), 5.30 (d, 1H, $J_{3,\ 4}$ = 3.3 Hz, H-4^{III}), 5.13−4.76 (m, 7H, H-2$^{I,\ II,\ III}$, H-3$^{I,\ II,\ III}$, CH_2Ph), 4.58−4.31 (m, 6H, H-1$^{I,\ II,\ III}$, CH_2Ph, H-6$^{I,\ II}$a), 4.05 (m, 4H, H-6$^{I,\ II}$b, H-6^{III}a, H-6^{III}b), 3.84−3.71 (m, 3H, H-4$^{I,\ II}$, H-5^{III}), 3.57−3.50 (m, 2H, H-5$^{I,\ II}$), 2.11−1.93 (m, 30H, CH_3); FABMS: *m*/*z* 1038 [M + Na]$^+$; Anal. Calcd for $C_{45}H_{58}O_{26}$: C,53.25; H,5.76. Found: C, 52.48; H, 5.89.

Benzyl (2,3,4,6-Tetra-*O*-acetyl-*β*-D-galactopyranosyl)-(1→4)-(2,3,6-tri-*O*-acetyl-*β*-D-glucopyranosyl)-(1→4)-2,3-di-*O*-acetyl-*β*-D-xylopyranoside (10). Benzyl xyloside **3** (22 mg,0.092 mmol) and then Cel7B Glu197Ala (0.5 mg) were added to a solution of lactosyl fluoride **1** (31.5 mg, 1 equiv) in phosphate buffer (0.1M, pH 7.0, 1 mL). The reaction mixture was gently shaken in an oven at 40 °C. After 48 h, the solution was filtered on C18 Cartridge Sep-Pak Plus (Waters), and then concentrated to dryness and acetylated (acetic anhydride−pyridine 1:1 v/v, 10 mL). After 12 h of stirring at 25 °C, the reaction mixture was quenched by adding MeOH at 0 °C and concentrated in vacuo. The residue was dissolved in CH_2Cl_2 and washed with 20% aqueous $KHSO_4$ and saturated aqueous $NaHCO_3$. The organic layers were concentrated and purified by flash chromatography (EtOAc−petroleum ether 6:4 v/v) to generate the title trisaccharide **10** (26.5 mg, 51%): $[\alpha]^{25}_D$ −49.0 (*c* 1.0, $CHCl_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 169.9−169.0 (CO), 136.9 (Cq arom), 128.4−127.5 (C arom), 101.0, 100.0, 99.5 (C-1$^{I,\ II,\ III}$), 76.0, 75.4 (C-4$^{I,\ II}$), 72.8, 72.7, 71.73, 71.68, 70.9, 70.8, 70.7, 70.4 (C-2$^{I,\ II}$, C-3$^{I,\ II,\ III}$, C-5$^{II,\ III}$, CH_2Ph), 69.0 (C-2^{III}), 66.6 (C-4^{III}), 62.6, 62.0, 60.8 (C-5^{I}, C-6$^{II,\ III}$), 20.7−20.4(CH_3), Anal. Calcd for $C_{42}H_{54}O_{24}$: C, 53.50; H, 5.77. Found: C, 53.68; H, 5.88.

***p*-Nitrophenyl (2,3,4,6-Tetra-*O*-acetyl-*β*-D-galactopyranosyl)-(1→4)−(2,3,6-tri-*O*-acetyl-*β*-D-glucopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-1-thio-*β*-D-mannopyranoside (11).** Lactosyl fluoride **1** (21.7 mg, 0.063 mmol) and *p*-nitrophenyl 1-thio-*β*-D-mannopyranoside **4** (22.6 mg, 1 equiv) in ammonium carbonate solution (0.1M, pH 8.0, 1 mL) were incubated with Cel7B Glu197Ala (0.6 mg) at 40 °C for 48 h. The condensation product which precipitated in the reaction medium was isolated by centrifugation and removal of the supernatant, washed with H_2O, dried in vacuo over sodium pentoxide at 40 °C for 24 h, and then acetylated and purified as described for compound **9**, to give **11** (41.0 mg, 61%): $[\alpha]^{25}_D$ −53 (*c* 0.58, $CHCl_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 169.6−168.9 (CO), 146.6, 143.1 (Cq arom), 129.3, 123.8 (C arom), 101.0 (C-1^{III}), 100.0 (C-1^{II}), 83.4 (C-1^{I}), 77.1 (C-5^{I}), 75.8 (C-4^{II}), 73.6 (C-4^{I}), 72.74 (C-3^{II}), 72.69 (C-5^{II}), 71.9 (C-2^{II}), 71.5 (C-3^{I}), 70.8 (C-3^{III}), 70.6 (C-5^{III}), 70.4 (C-2^{I}), 69.0 (C-2^{III}), 66.5 (C-4^{III}), 62.7 (C-6^{I}), 62.1 (C-6^{II}), 60.6 (C-6^{III}), 20.7−20.3 (CH_3); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.09 et 7.52 (2d, 4H, J = 9.1 Hz, H arom), 5.60 (d, 1H, $J_{2,\ 3}$ = 2.9 Hz, H-2^{I}), 5.30 (d, 1H, $J_{3,\ 4}$ = 2.9 Hz, H-4^{III}), 5.13 (t, 1H, $J_{2,\ 3}$ = 9.1 Hz, H-3^{II}), 5.13−5.03 (m, 3H, H-1^{I}, H-2^{III}, H-3^{I}), 4.91 (dd, 1H, $J_{2,\ 3}$ = 10.6 Hz, H-3^{III}), 4.78 (dd, 1H, $J_{1,\ 2}$ = 7.6 Hz, H-2^{II}), 4.54 (d, 1H, $J_{1,\ 2}$ = 8 Hz, H-1^{II}), 4.46−4.43 (m and d, 2 H, $J_{1,\ 2}$ = 7.6 Hz, H-1^{III}, H-6^{I}a), 4.35 (dd, 1H, $J_{5,\ 6}$ = 1.8 Hz, $J_{a,\ b}$ = 12 Hz, H-6^{II}a), 4.18−4.04 (m, 4H, H-6^{I}b, H-6^{II}b, H-6^{III}a, H-6^{III}b), 3.87−3.73 (m, 4H, H-4$^{I,\ II}$, H-5$^{I,\ III}$), 3.56 (m, 1H, H-5^{II}), 2.16−1.93 (m, 30H, CH_3); HRFABMS calcd for $C_{44}H_{55}NO_{27}S$ (M + Na)$^+$ 889.2590, found 889.2595.

Methyl *β*-D-Galactopyranosyl-(1→4)-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyl -(1→4)-*β*-D-glucopyranoside (12). Lactosyl fluoride **1** (30 mg, 0.087 mmol) and methyl *β*-cellobioside **5** (31.0 mg, 1 equiv) in phosphate buffer (0.1M, pH 7.0, 1 mL) were incubated with Cel7B Glu197Ala (0.5 mg) at 40 °C. After 24 h, the solution was

(32) Davies, G. J.; Lewis, R. J.; Ducros, V.; Borchert, T.; Schülein, M. *J. Biotechnol.* **1997**, *57*, 91−100.

(33) (a) Otwinowski, Z. Oscillation data reduction program. In *Data Collection and Processing: proceedings of the CCP4 study weekend*; Sawyer, L., Isaacs, N., Bailey, S., Eds.; Science and Engineering Research Council: Daresbury, UK, 1993. (b) Otwinowski, Z.; Minor, W. Processing of X-ray diffraction data collected in oscillation mode. In *Methods in Enzymology: Macromolecular Crystallography, part A*; Carter, C. W. J., Sweet, R. M., Eds.; Academic Press: London & New York, 1997; Vol. 276, pp 307−326.

(34) Collaborative Computational Project Number 4, 1994. The CCP4 suite: programs for protein crystallography. *Acta Crystallogr.* **1994,** *D50*, 760−763.

(35) Navaza, J. *Acta Crystallogr.* **1994**, *A50*, 157−163.

(36) Brünger, A. T. *Nature* **1992**, *355*, 472−475.

(37) Murshudov, G. N.; Vagin, A. A.; Dodson, E. J. *Acta Crystallogr.* **1997**, *D 53*, 240−255.

(38) Lamzin, V. S.; Wilson, K. S. *Acta Crystallogr.* **1993**, *D49*, 129−147.

(39) Bernstein, F. C.; Koetzle, T. F.; Williams, G. J. B.; Meyer, E. T., Jr.; Brice, M. D.; Rodgers, J. R.; Kennard, O.; Shimanouchi, T.; Tasumi, M. *J. Mol. Biol.* **1977**, *112*, 535−542

filtered on C18 Cartridge Sep-Pak Plus (Waters) and then freeze-dried. The expected tetrasaccharide **12**[1b] was obtained in quantitative yield (59 mg): $[\alpha]^{25}_D$ −1 (*c* 0.32, H_2O); ^{13}C NMR (D_2O, 75 MHz) δ 103.9, 103.7, 103.1 (2C) (C-$1^{I, II, III, IV}$), 79.4, 79.1, 79.0 (C-$4^{I, II, III}$), 76.2, 75.6, 75.1, 74.9, 74.8, 73.7, 73.3, 71.7 (C-$2^{I, II, III, IV}$, C-$3^{I, II, III, IV}$, C-$5^{I, II, III, IV}$), 69.3 (C-4^{IV}), 61.8−60.8 (C-$6^{I, II, III, IV}$), 58.0 (OCH_3); FABMS: *m*/*z* 703 $[M + Na]^+$.

Methyl β-D-Galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl -(1→4)-6-bromo-6-deoxy-β-D-glucopyranoside (13). Lactosyl fluoride **1** (9.0 mg, 0.026 mmol) and methyl 6-bromo-6-deoxy-β-cellobioside **6** (11.0 mg, 1 equiv) in phosphate buffer (0.1M, pH 7.0, 1 mL) were incubated with Cel7B Glu197Ala (0.6 mg) at 40 °C. After 7 h, the solution was filtered on C18 Cartridge Sep-Pak Plus (Waters) and then freeze-dried. The expected tetrasaccharide **13** was obtained in quantitative yield (19.3 mg): $[\alpha]^{25}_D$ +3 (*c* 0.93, H_2O); ^{13}C NMR (D_2O, 75 MHz) δ 103.9, 103.7, 103.2, 103.1 (C-$1^{I, II, III, IV}$), 80.9 (C-4^{I}), 79.0, 78.97 (C-$4^{II, III}$), 76.2, 75.6, 74.9, 74.8, 74.7, 73.76, 73.66, 73.6, 73.3, 71.8 (C-$2^{I, II, III, IV}$, C-$3^{I, II, III, IV}$, C-$5^{I, II, III, IV}$), 69.4 (C-4^{IV}), 61.8, 60.8, 60.7 (C-$6^{II, III, IV}$), 58.2 (OCH_3), 33.2 (C-6^{I}); HRFABMS calcd for $C_{25}H_{43}BrO_{20}$ $(M + Na)^+$ 765.1429, found 765.1435.

2-Acetamido-1,3,6-tri-*O*-acetyl-2-deoxy-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)−(2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl)-(1→4)-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-α,β-D-glucopyranose (14). Lactosyl fluoride **1** (10.0 mg, 0.029 mmol) and *N*-acetyl-chitobiose **7** (11.1 mg, 1 equiv) in ammonium carbonate solution (0.1M, pH 8.0, 1 mL) were incubated with Cel7B Glu197Ala (0.6 mg) at 40 °C for 24 h. Then the solution was evaporated, dried in vacuo over sodium pentoxide at 40 °C for 24 h, acetylated as described for compound **9** and purified by flash column chromatography using a mixture CH_2Cl_2-MeOH (95:5 v/v) as eluent. The tetrasaccharide **14** was obtained in 65% yield (23.6 mg): ^{13}C NMR ($CDCl_3$, 75 MHz) δ 170.0−169.0 (CO), 101.8, 101.0, 101.3 (C-$1^{II, III, IV}$), 92.3, 90.3 (C-$1^{I\ \alpha,\beta}$), 77.1, 75.9, 75.7, 72.7, 71.8, 70.8, 70.6, 69.0, 66.5, 62.2, 62.0, 61.4, 60.6, 53.7, 51.5, 51.0 (C-$2^{I, II, III, IV}$ to C-$5^{I, II, III, IV}$), 20.8−20.5 (CH_3); HRFABMS calcd for $C_{52}H_{72}O_{33}N_2$ $(M + Na)^+$ 1275.3915, found 1275.3914.

Benzyl β-D-Galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)-β-D-glucopyranoside (15). Lactosyl fluoride **1** (12.0 mg, 0.035 mmol) and benzyl laminaribioside **8** (15.0 mg, 1 equiv) in phosphate buffer (0.1M, pH 7.0, 1 mL) were incubated with Cel7B Glu197Ala (0.6 mg) at 40 °C. After 6 h, the solution was filtered on C18 Cartridge Sep-Pak Plus (Waters) and then freeze-dried. The expected tetrasaccharide **15** was obtained in quantitative yield (26.0 mg): $[\alpha]^{25}_D$ −16 (*c* 0.5, H_2O); ^{13}C NMR (D_2O, 125 MHz) δ 137.4 (Cq arom), 129.5−129.3 (C arom), 103.82 (C-1^{IV}), 103.47 (C-1^{II}), 103.26 (C-1^{III}), 101.86 (C-1^{I}), 85.25 (C-3^{I}), 78.98 (C-4^{III}), 78.85 (C-4^{II}), 76.50 (C-5^{I}), 75.76 (C-5^{III}), 75.70 (C-5^{II}), 75.12 (C-3^{III}), 74.98 (C-3^{II}), 74.12 (C-2^{II}), 73.80 (C-2^{I}), 73.78 (C-2^{III}), 72.3 (CH_2Ph), 71.80 (C-2^{IV}), 69.45 (C-4^{IV}), 69.10 (C-4^{I}), 62.82 (C-6^{IV}), 61.59 (C-6^{I}), 60.89 (C-6^{III}), 60.74 (C-6^{II}); HRFABMS calcd for $C_{31}H_{48}O_{21}$ $(M + Na)^+$ 779.2586, found 779.2589.

2,3,4-Tri-*O*-acetyl-6-bromo-6-deoxy-β-D-glucopyranosyl-(1→4)-2,3-di-*O*-acetyl-1,6-anhydro-β-D-glucopyranose (18b). To a solution of 1,6-anhydro-β-cellobiose **18a** (281 mg, 0.87 mmol) in pyridine (20 mL) at 0 °C was added triphenylphosphine (911 mg, 4 equiv) and tetrabromomethane (578 mg, 2 equiv). After stirring the solution for 30 min at 0 °C and 2 h at 50 °C, acetic anhydride (10 mL) was added. After an additional 3 h, the reaction mixture was worked up as described for compound **9** and purified by flash chromatography (EtOAc−petroleum ether 8:2 v/v) to give **18b** (280 mg, 54%) which was crystallized in MeOH, mp 140−141 °C, $[\alpha]^{25}_D$ −34.5 (*c* 0.73, $CHCl_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) 100δ 169.4−168.9 (CO), 0.3, 98.9 (C-$1^{I, II}$), 76.9, 73.7, 73.2, 72.7, 71.3, 70.6, 69.6, 68.5 (C-$2^{I, II}$, C-$3^{I, II}$, C-$4^{I, II}$, C-$5^{I, II}$), 64.9 (C-6^{I}), 30.6 (C-6^{II}), 20.9−20.5 (CH3); ^1H NMR ($CDCl_3$, 300 MHz) δ 5.41 (s, 1H, H-1^{I}), 5.22−5.03 (m, 2H, H-3^{I}, H-3^{II}), 5.03−4.96 (m, 2H, H-2^{II}, H4^{II}), 4.90 (d, 1H, $J_{1,2}$ = 8.2 Hz, H-1^{II}), 4.56 (m, 2H, H-2^{I}, H-5^{I}), 3.93 (d, 1H, $J_{a,b}$ = 7.7 Hz, H-6^{I}a), 3.77 (m, 2H, H-5^{II}, H-6^{I}b), 3.53 (s, 1H, H-4^{I}), 3.44 (dd, 1H, $J_{5,6}$ = 2.7 Hz, $J_{a,b}$ = 11.5 Hz, H-6^{II}a), 3.33 (dd, 1H, $J_{5,6}$ = 6.6 Hz, H-6^{II}b), 2.12−1.96 (m, 15H, CH_3); DCIMS: *m*/*z* 616 $[M + H + NH_3]^+$; Anal. Calcd for $C_{22}H_{29}BrO_{14}$: C,44.23; H,4.89; Br, 13.38. Found: C, 43.95; H, 5.10; Br,. 13.26.

2,3,4-Tri-*O*-acetyl-6-bromo-6-deoxy-β-D-glucopyranosyl-(1→4)-1,2,3,6-tetra-*O*-acetyl-β-D-glucopyranose (19). A catalytic amount of triethylsilyl triflate (20 μL) was added, dropwise via a syringe, to a solution of compound **18b** (1.5 g, 2.5 mmol) in acetic anhydride (20 mL) cooled at 0 °C. The mixture was stirred at 0 °C for 30 min, and then saturated aqueous $NaHCO_3$ was added to quench the reaction, and the products were extracted with CH_2Cl_2. The organic layers were dried, concentrated and coevaporated with toluene. HBr (30% w/v in AcOH, 5 mL) was added to the crude anomeric mixture in CH_2Cl_2 (10 mL) at 0 °C. After stirring at 0 °C for 30 min and then at room temperature for an additional 1 h, the reaction mixture was diluted with CH_2Cl_2, washed with ice-cold H_2O and ice-cold saturated aqueous $NaHCO_3$ (3×), dried, and concentrated. The resulting bromide and AgOAc (840 mg, 2 equiv) in a mixture of Ac_2O−AcOH (20 mL, 1:1 v/v) were stirred in the dark overnight at room temperature. The mixture was diluted with CH_2Cl_2 and filtered through a Celite bed, and the filtrate was washed with saturated aqueous $NaHCO_3$ (3×), dried, and concentrated. Filtration through silica gel (EtOAc−petroleum ether 7:3 v/v) gave compound **19** (1.53 g, 88% three steps) which was crystallized in EtOH, mp 156 °C, $[\alpha]^{25}_D$ −2.5 (*c* 0.81, $CHCl_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 169.3−166.2 (CO), 99.8 (C-1^{II}), 91.5 (C-1^{I}), 75.2 (C-4^{I}), 73.4, 73.3, 72.5, 71.9, 71.4, 70.5, 70.4 (C-$2^{I, II}$, C-$3^{I, II}$, C-$5^{I, II}$, C-4^{II}), 61.5 (C-6^{I}), 30.2 (C-6^{II}), 20.9−20.3 (CH_3); ^1H NMR ($CDCl_3$, 300 MHz) δ 5.57 (d, 1H, $J_{1,2}$ = 8.2 Hz, H-1^{I}), 5.20−4.78 (m, 5H, H-2^{I}, H-2^{II}, H-3^{I}, H-3^{II}, H-4^{II}), 4.47 (d, 1H, $J_{1,2}$ = 8 Hz, H-1^{II}), 4.39 (dd, 1H, $J_{5,6}$ = 1.6 Hz, $J_{a,b}$ = 12.2 Hz, H-6^{I}a), 4.02 (dd, 1H, $J_{5,6}$ = 4.6 Hz, H-6^{I}b), 3.80 (t, 1H, $J_{2,3} = J_{3,4}$ = 9 Hz, H-4^{I}), 3.62−3.56 (m, 2H, H-5^{I}, H-5^{II}), 3.40 (dd, 1H, $J_{5,6}$ = 2.6 Hz, $J_{a,b}$ = 11.5 Hz, H-6^{II}a), 3.25 (dd, 1H, $J_{5,6}$ = 7 Hz, H-6^{II}_b), 2.10−1.95 (m, 21H, CH_3); DCIMS: *m*/*z* 718 $[M + H + NH_3]^+$, 641 [M − OAc]; Anal. Calcd for $C_{26}H_{35}BrO_{17}$: C,44.65; H,5.04; Br, 11.42. Found: C, 44.64; H, 5.13; Br,. 11.07.

2,3,4-Tri-*O*-acetyl-6-bromo-6-deoxy-β-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl fluoride (20). A solution of compound **19** (1.4 g, 2.0 mmol) in HF-pyridine (10 mL, 7:3 v/v) was stirred at 0 °C for 1.5 h in a plastic vial. The mixture was diluted with CH_2Cl_2 and poured into ice-cold aqueous NH_3 (3 M); the organic layer was washed with saturated aqueous $NaHCO_3$ (3×), dried, and concentrated. Crystallization from EtOH− Et_2O gave pure **20** (1.2 g, 90%): mp 177−178 °C; $[\alpha]^{25}_D$ +40 (*c* 1.1, $CHCl_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 170.0−168.7 (CO), 103.6 (d, $J_{C,F}$ = 230 Hz; C-1^{I}), 99.8 (C-1^{II}), 77.1, 74.7, 73.5, 72.6, 71.5, 70.6, 70.2, 68.3 (C-$2^{I, II}$, C-$3^{I, II}$, C-$4^{I, II}$, C-$5^{I, II}$), 60.9 (C-6^{I}), 30.2 (C-6^{II}), 20.8−20.4 (CH_3); ^1H NMR ($CDCl_3$, 300 MHz) δ 5.62 (dd, 1H, $J_{1,2}$ = 2.7 Hz, $J_{1,F}$ = 53 Hz, H-1^{I}), 5.42 (t, 1H, $J_{2,3} = J_{3,4}$ = 9.8 Hz, H-3^{I}), 5.10 (t, 1H, $J_{2,3} = J_{3,4}$ = 9.8 Hz, H-3^{II}), 4.89 (m, 3H, H-2^{I}, H-2^{II}, H-4^{II}), 4.50 (m, 2H, H-1^{II}, H-6^{I}a), 4.10 (m, 2H, H-5^{I}, H-6^{I}b), 3.83 (t, 1H, $J_{4,5}$ = 9.8 Hz, H-4^{I}), 3.63 (m, 1H, H-5^{II}), 3.42 (dd, 1H, $J_{5,6}$ = 2.4 Hz, $J_{a,b}$= 11.5 Hz, H-6^{II}a), 3.26 (dd, 1H, $J_{5,6}$ = 7.3 Hz, H-6^{II}b), 2.10−1.93 (m,18H, CH_3); FABMS: *m*/*z* 683 $[M + Na]^+$; Anal. Calcd for $C_{24}H_{32}BrFO_{15}$: C,43.71; H,4.89; Br, 12.12. Found: C, 43.87; H, 5.02; Br,. 12.13.

2,3,4-Tri-*O*-acetyl-6-azido-6-deoxy-β-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl fluoride (21). Compound **20** (282 mg, 0.43 mmol) was dissolved in *N*,*N*-dimethylformamide (20 mL) which contained sodium azide (298 mg, 11 equiv).The mixture was heated at 80 °C for 12 h and then concentrated in vacuo. The residue was dissolved in CH_2Cl_2, and the organic layer was washed with H_2O and dried and concentrated. Filtration through silica gel (EtOAc−petroleum ether 1:1 v/v) gave compound **21** (235 mg, 88%) which was crystallized in EtOH, mp 155−156 °C, $[\alpha]^{25}_D$ +28 (*c* 0.51, $CHCl_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 169.9−168.7 (CO), 103.5 (d, $J_{C,F}$ = 230 Hz; C-1^{I}), 99.8 (C-1^{II}), 74.3, 72.8, 72.5, 71.5, 70.63, 70.58, 70.4, 70.1, 69.2, 68.6 (C-$2^{I, II}$, C-$3^{I, II}$, C-$4^{I, II}$, C-$5^{I, II}$), 61.0 (C-6^{I}), 50.8 (C-6^{II}), 20.6−20.3 (CH3); ^1H NMR ($CDCl_3$, 300 MHz) δ 5.62 (dd, 1H, $J_{1,2}$ = 2.4 Hz, $J_{1,F}$ = 53 Hz, H-1^{I}), 5.44 (t, 1H, $J_{2,3} = J_{3,4}$ = 9.8 Hz, H-3^{I}), 5.13 (t, 1H, $J_{2,3} = J_{3,4}$ = 9.3 Hz, H-3^{II}), 4.95−4.80 (m, 3H, H-2^{I}, H-2^{II}, H-4^{II}), 4.57−4.50 (m, 2H, H-1^{II}, H-6^{I}a), 4.10 (m, 2H, H-5^{I}, H-6^{I}b), 3.87 (t, 1H, $J_{4,5}$ = 9.3 Hz, H-4^{I}), 3.60 (m, 1H, H-5^{II}), 3.33 (m, 2H, H6^{II}a, H-6^{II}b), 2.10−1.95 (m,18H, CH_3); DCIMS: *m*/*z* 639 $[M + H + NH_3]^+$; Anal. Calcd for $C_{24}H_{32}FN_3O_{15}$: C,46.38; H,5.19; F, 3.06, N, 6.76. Found: C, 46.67; H, 5.37; F, 2.87; N, 6.59.

Triphenylmethyl 2,3,4-Tri-*O*-acetyl-1-*S*-α-D-xylopyranoside (23a). A solution of triphenylmethanethiol (8 g, 29 mmol) and *N*-tetrabutylammonium hydroxide (37 mL, 25% in MeOH, 29 mmol) in a mixture MeOH-toluene (60 mL, 1:1 v/v) was evaporated to dryness then coevaporated with toluene (30 mL, 3×). The residual thiolate was dissolved in toluene (75 mL), and then tri-*O*-acetyl-β-D-xylopyranosyl chloride **22** (7.94 g, 27 mmol) was added. The solution was stirred under argon for 24 h and then concentrated. The residue was purified by flash-chromatography (EtOAc−petroleum ether 1:3 v/v), to give after recrystallization in Et_2O−petroleum ether **23a** (8.44 g, 54%): mp 93−95 °C; $[\alpha]^{25}_D$ +117 (*c* 1, $CHCl_3$); ^{13}C NMR (75 MHz) δ 169.4, 169.2 and 169.0 (CO), 144.2, 129.6, 127.6 and 126.8 (C arom), 81.8 (C-1), 69.8, 68.5 and 67.8 (C-2, C-3, C-4), 62.5 (C-5), 20.4 and 20.5 (CH_3); ^1H NMR (300 MHz, $CDCl_3$) δ 7.20−7.38 (m, 15 H, 3 H arom), 5.11 (t, 1 H, $J_{2,3} = J_{3,4} = 7$ Hz, H-3), 4.74 (dd, 1 H, $J_{1,2}$ 4.1 Hz, H-2), 4.69 (m, 1 H, H-4), 4.60 (d, 1 H, H-1), 3.99 (dd, 1 H, $J_{4,5ax}$ 7.15 Hz, $J_{5ax,5eq}$ 12.4 Hz H-5ax), 3.57 (dd, 1 H, $J_{4,5eq}$ 4.5 Hz, H-5eq), 2.09, 2.01 and 1.92 (3 s, 9 H, 3 CH_3); Anal. Calcd for $C_{30}H_{30}O_7S$: C, 67.39; H, 5.65; S, 5.99. Found: C, 67.60; H, 5.47; S, 5.80.

***S*-Acetyl-2,3,4-tri-*O*-acetyl-α-D-xylopyranose (23b).** Phenylmercury(II) acetate (6.57 g, 19.49 mmol) was added to a solution of compound **23a** (9 g, 16.86 mmol) in a mixture of MeOH−CH_2Cl_2 (300 mL, 2:1 v/v). The reaction mixture was stirred for 1 h at room temperature and then concentrated in vacuo. The residue, coevaporated with toluene (50 mL), was dissolved in CH_2Cl_2 (270 mL), and then pyridine (36 mL) and Ac_2O (18 mL) were added. Hydrogen sulfide was bubbled into the solution for 15 min, and the resulting cloudy mixture was stirred overnight at room temperature. The precipitated mercuric salts were removed by filtration through a bed of Celite, and the resulting clear filtrate was concentrated. Flash-column chromatography (EtOAc−petroleum ether 1:3 v/v) of the residue gave compound **23b** which crystallized in Et_2O−petroleum ether (5 g, 89%): mp 70 °C; $[\alpha]^{25}_D$ +63 (*c* 1, $CHCl_3$); ^{13}C NMR (75 MHz) δ 191.5 (S*C*OCH$_3$), 169.0, 169.2 and 169.3 (3 O*C*OCH$_3$), 78.0 (C-1), 69.4, 68.8, 67.7 (C-2, C-3, C-4), 62.9 (C-5), 30.9 (SCO*C*H3), 20.1 and 20.3 (3 OCO*C*H$_3$); ^1H NMR (300 MHz, $CDCl_3$) δ 5.85 (d, 1 H, $J_{1,2}$ 4.1 Hz, H-1), 4.95 (m, 2 H, H-2, H-3), 4.75 (m, 1 H, H-4), 3.80 (dd, 1 H, $J_{4,5eq}$ 5.1 Hz, $J_{5ax,5eq}$ 12 Hz, H-5eq), 3.50 (dd, 1 H, $J_{4,5ax}$ 8.9 Hz, H-5ax), 2.25 (s, 3 H, SAc), 1.85, 1.87 and 1.88 (3 s, 9 H, CH_3); Anal. Calcd for $C_{13}H_{18}O_8S$: C, 46.69; H, 5.42; S, 9.59. Found: C, 46.72; H, 5.51; S, 9.34.

(2,3,4,-Tri-*O*-acetyl-α-D-xylopyranosyl)-(1→6)-(2,3,4-tri-*O*-acetyl-β-D-glucopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl fluoride (24). Compound **23b** (153 mg,0.45 mmol) was added to a solution of bromide **20** (100 mg,0.3 equiv) in HMPA (2.5 mL) with dithioerythritol (23 mg, 1 equiv) and cysteamine (20 mg, 1.7 equiv). The reaction mixture was stirred for 7 h at room temperature and then precipitated in ice-cold water (10 mL). After filtration through a bed of Celite, the precipitate was washed with H_2O and then dissolved in CH_2Cl_2. The organic phase was washed with H_2O, dried, concentrated, and purified by flash chromatography (EtOAc−petroleum ether 1:1 v/v) to give **24** (86 mg, 68%): $[\alpha]^{25}_D$ +80 (*c* 1.73, $CHCl_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 169.7−169.3 (CO), 103.5 (d, $J_{C,F} = 230$ Hz, C-1), 99.8 (C-1^{II}), 82.4 (C-1^{III}), 74.5, 74.4, 72.6, 71.6, 70.8, 70.6, 70.5, 70.2, 69.3, 68.8, 68.7 (C-2$^{I,\ II,\ III}$, C-3$^{I,\ II,\ III}$, C-4$^{I,\ II,\ III}$, C-5$^{I,\ II}$), 61.1, 59.4 (C-6^{I}, C-5^{III}), 31.0 (C-6^{II}), 20.7−20.4 (CH_3); ^1H NMR ($CDCl_3$, 300 MHz) δ 5.64 (dd, 1H, $J_{1,2} = 2.4$ Hz, $J_{1,F} = 53$ Hz, H-1^{I}), 5.58 (d, 1H, $J_{1,2} = 5.5$ Hz, H-1^{III}), 5.45 (t, 1H, $J_{2,3} = J_{3,4} = 9.5$ Hz, H-3^{I}), 5.26 (t, 1H, $J_{2,3} = J_{3,4} = 9.2$ Hz, H-3^{III}), 5.10 (t, 1H, $J_{2,3} = J_{3,4} = 9.3$ Hz, H-3^{II}), 4.92−4.78 (m, 5H, H-2$^{I,\ II,\ III}$, H-4$^{II,\ III}$), 4.48 (m, 2H, $J_{1,2} = 8$ Hz, H-1^{I}, H-6^{I}a), 4.07 (m, 2H, H-5^{I}, H-6^{I}b), 3.88 (m, 2H, H-4^{I}, H-5^{III}a), 3.76 (dd, 1H, $J_{4,5} = 5.6$ Hz, $J_{a,b} = 11.3$ Hz, H-5^{III}b), 3.53 (m, 1H, H-5^{II}), 2.72 (dd, 1H, $J_{5,6} = 2.9$ Hz, $J_{a,b} = 14$ Hz, H-6^{II}a), 2.54 (dd, 1H, $J_{5,6} = 7.3$ Hz, H-6^{II}b), 2.1−1.93 (m, 27H, CH_3); DCIMS: *m*/*z* 888 $[M + H + NH_3]^+$; HRFABMS calcd for $C_{35}H_{47}FO_{22}S$ $(M + Na)^+$ 893.2161, found 893.2156.

Unreacted fluoride **20** was also recovered (30 mg, 30%).

Preparation of the potential donors 16a − 16e. β-D-Glucopyranosyl-(1→4)-α-D-glucopyranosyl fluoride (16a) and 6-Bromo-6-deoxy-β-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl fluoride (16b): Hepta-*O*-acetyl-α-cellobiosyl fluoride or 2,3,6,2^{II},3^{II},4^{II}-hexa-*O*-acetyl-6^{II}-bromo-6^{II}-deoxy-α-cellobiosyl fluoride (**20**), 100 mg each were de-*O*-acetylated by treatment with sodium methoxide (1 M, 50 μL) in methanol (10 mL) for 4 h at 0 °C. The mixture was neutralized with Amberlite IR 120 (H^+) resin, the resin was removed by filtration, and the filtrate concentrated. Freeze-dried compounds **16a** or **16b** were obtained in quantitative yield.

Compound **(16a):** ^{13}C NMR (D_2O, 75 MHz) δ 107.8 (d, $J_{C,F} = 223$ Hz; C-1^{I}), 103.3 (C-1^{II}), 78.1, 76.8, 76.3, 74.0, 73.7, 71.8, 71.5, 70.3 (C-2$^{I,\ II}$, C-3$^{I,\ II}$, C-4$^{I,\ II}$, C-5$^{I,\ II}$), 61.4, 60.2 (C-6$^{I,\ II}$); ^1H NMR (D_2O, 300 MHz) δ 5.58 (dd, 1H, $J_{1,2} = 2.7$ Hz, $J_{1,F} = 53.3$ Hz, H-1^{I}), 4.40 (d, 1H, $J_{1,2} = 7.9$ Hz, H-1^{II}), 3.8−3.2 (m, 12H)

Compound **(16b):** FABMS: *m*/*z* 429 $[M + Na]^+$.

6-Amino-6-deoxy-β-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl fluoride (16c). Acetylated 6^{II}-azido-6^{II}-deoxy-α-cellobiosyl fluoride **21** (100 mg, 0.16 mmol) was de-*O*-acetylated as described above, giving the free fluoride which was used in the next step without further characterization. This compound (20 mg, 0.054 mmol) in EtOH (5 mL) was hydrogenolyzed on 10% Pd/C (20 mg) for 12 h under atmospheric pressure. The catalyst was filtered off, the filtrate was concentrated, and the residue was freeze-dried to give **16c** (16.8 mg, 90%) which was characterized by its DCIMS spectrum *m*/*z* 344 $[M + H + NH_3]^+$.

α-D-Xylopyranosyl-(1→6)-β-D-glucopyranosyl)-(1→4)-α-D-glucopyranosyl fluoride (16d). Compound **24** was de-*O*-acetylated as already described to give **16d** in quantitative yield: FABMS: *m*/*z* 513 $[M + Na]^+$.

6-Bromo-6-deoxy-β-D-glucopyranosyl-(1→4)-6-bromo-6-deoxy-β-α-D-glucopyranosyl fluoride (16e). Compound **16b** (58.5 mg, 0.144 mmol) was dissolved in pyridine (5 mL) and treated as described for the synthesis of **18b**, and then the reaction mixture was acetylated under standard conditions. Workup and flash chromatography (EtOAc−petroleum ether 4:6 v/v) provided pure **25** in 23% yield: $[\alpha]^{25}_D$ +40 (*c* 0.79, $CHCl_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 169.2−168.6 (CO), 103.6 (d, $J_{C,F} = 229$ Hz, C-1^{I}), 99.7 (C-1^{II}), 76.0 (C-4^{I}), 73.3, 72.7, 71.6, 70.7, 70.6, 70.5, 68.2 (C-2$^{I,\ II}$, C-3$^{I,\ II}$, C-5$^{I,\ II}$, C-4^{II}), 31.5, 30.2 (C-6$^{I,\ II}$), 21.3−20.4 (CH3); ^1H NMR ($CDCl_3$, 300 MHz) δ 5.69 (dd, 1H, $J_{1,2} = 2.7$ Hz, $J_{1,F} = 53$ Hz, H-1^{I}), 5.47 (t, 1H, $J_{2,} = J_{3,4} = 10$ Hz, H-3^{I}), 5.16 (t, 1H, $J_{2,} = J_{3,} = 9.3$ Hz, H-3^{II}), 5.01−4.88 (m, 3H, H-2, H-2^{II}, H-4^{II}), 4.74 (d, 1H, $J_{1,2} = 8$ Hz, H-1^{II}), 4.05 (m, 1H, H-5^{I}), 3.95 (t, 1H, $J_{4,5} = 9.3$ Hz, H-4^{I}), 3.67 (m, 3H, H-5^{II}, H-6^{I}a, H-6^{I}b), 3.46 (dd, 1H, $J_{5,6} = 2.5$ Hz, $J_{a,b} = 11.3$ Hz, H-6^{II}a), 3.31 (dd, 1H, $J_{5,\ 6} = 6.7$ Hz, H-6^{II}b), 2.10−1.97 (m, 15H, CH_3); FABMS: *m*/*z* 703 $[M + Na]^+$.

Unreacted compound **20** was recovered in 68% yield.

Standard de-*O*-acetylation of compound **25** gave the corresponding derivative **16e.**

Enzymatic synthesis of modified celluloses. Cellobiosyl fluoride **16a** (100 mg) in phosphate buffer (0.1M, pH 7.0, 6 mL) was incubated with Cel7B Glu197Ala (2.5 mg) at 40 °C. After 24 h, the resulting suspension was recovered by centrifugation and the solid was washed with H_2O (3×). The wet precipitate was used for electron microscopy or X-ray analyses as already described.[22] When dried under reduced pressure at 40 °C in the presence of phosporous pentoxide, a white powder of **17a** was obtained (81 mg) with ^{13}C and ^1H NMR spectra identical to those already reported. [22]

6^{II}-Bromo-6^{II}-deoxy-α-cellobiosyl fluoride **16b** (34 mg) in phosphate buffer (0.1M, pH 7.0, 1.5 mL) was incubated with Cel7B Glu197Ala (1.5 mg) at 40 °C for 24 h. A precipitate appeared (23 mg) and the supernatant was freeze-dried and the residue was purified using steric exclusion chromatography on a Biogel P2 column with water as eluent.

FABMS spectra with *m*/*z* 815 $[M + Na]^+$and *m*/*z* 1203 $[M + Na]^+$ allowed the characterization of corresponding tetra- and hexasaccharides **17b** respectively. The precipitate was assumed to consist of higher oligomers of **17b**.

6^{II}-Amino-6^{II}-deoxy-α-cellobiosyl fluoride **16c** (60 mg) in carbonate solution (0.1M, pH 8.0, 2 mL) was incubated with Cel7B Glu197Ala (1.2 mg) at 40 °C for 36 h. The solution was freeze-dried, a sample (2 mg·mL^{-1}) was filtered through Satorius membranes (0.45 and 0.2 μm) and analyzed at 30 °C by steric exclusion chromatography on Synchropak CATSEC 100 and 1000 columns using solution of AcOH (0.3M) containing AcONa (0.2 M)) with a flow rate of 0.2 mL·min^{-1}. The d*n*/d*c* value of 0.185 mL·g^{-1} found for chitosan under the same conditions was applied.[40] The weight average molecular weight of **17c** is 5.000 ± 1.000.

6^{II}-Thioxylosyl -α-cellobiosyl fluoride **16d** (25.5 mg) in phosphate buffer (0.1 M, pH 7.0, 1 mL) was incubated with Cel7B Glu197Ala (0.5 mg) at 40 °C for 24 h. Insoluble **17d** was obtained (18.4 mg) after centrifugation of the incubation mixture and H_2O washings as already described. ^{1}H NMR (NaOD, 300 MHz) δ 5.13 (d, 1H, $J_{1,2} = 4.4$ Hz, H-1^{III}), 4.3 (m, 2H, H-$1^{I,\ II}$), 3.74−2.6 (m, 17H).

$6,6^{II}$-Dibromo-α-cellobiosyl fluoride **16e** (7.0 mg, 0.0015 mmol) was found unchanged under the above conditions. In the presence of lactosyl fluoride **1** (1 eq), incubation, acetylation, workup and purification as described for the synthesis of **9** gave the expected tetrasaccharide **26** (15 mg, 80%): $[\alpha]^{25}_D$ +1.5 (*c* 0.45, $CHCl_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 170.0−169.5 (CO), 103.7 (d, $J_{C,F} = 222$ Hz, C-1^I), 100.9, 100.0, 99.7 (C-$1^{II,\ III,\ IV}$), 77.1, 76.1, 75.7, 73.6, 72.9, 72.8, 72.4, 71.8, 71.7, 70.8, 70.6, 69.0, 68.3, 66.5 (C-$2^{I,\ II,\ III,\ IV}$ to C-$5^{\ I,\ II,\ III,\ IV}$), 62.1, 60.7 (C-$6^{III,\ IV}$), 31.5, 31.0 (C-$6^{I,\ II}$), 21.0−20.5 (CH_3); HRFABMS calcd. for $C_{46}H_{61}Br_2FO_{29}$ $[M + Na]^+$: 1279.1547; found: 1279.1544.

(40) Brugnerotto, J.; Rinaudo, M., personal communication.

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Supporting Information Available: Coordinates for the structures described in this paper have been deposited with the Protein Data Bank with accession code 1DYM. Data for determination of weight average molecular weight for **17c** (PDF). An X-ray crystallographic file in CIF format. This material is available free of charge via the Internet at http://pubs.acs.org.

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