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Unexpected regioselectivity of *Humicola insolens* Cel7B glycosynthase mutants

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Abstract—Four *Humicola insolens* Cel7B glycoside hydrolase mutants have been evaluated for the coupling of lactosyl fluoride on *O*-allyl N^{I}-acetyl-2^{II}-azido-β-chitobioside. Double mutants Cel7B E197A H209A and Cel7B E197A H209G preferentially catalyze the formation of a β-(1→4) linkage between the two disaccharides, while single mutant Cel7B E197A and triple mutant Cel7B E197A H209A A211T produce predominantly the β-(1→3)-linked tetrasaccharide. This result constitutes the first report of the modulation of the regioselectivity through site-directed mutagenesis for an endoglycosynthase.

Keywords: Glycosynthase; Cellulase; Regioselectivity; Enzymatic synthesis

1. Introduction

Humicola insolens mutant Cel7B E197A is a powerful endoglycosynthase for the synthesis of artificial cellulose and its derivatives.[1–3] In a recent report we have shown by docking simulation that H209 together with A211 are the main residues located in the environment around the position 2 of a β-D-glucosaminyl unit positioned in +1 subsite.[4] Encouraged by this result we have prepared and characterized three mutants of *H. insolens* Cel7B E197A glycosynthase: Cel7B E197A H209A, Cel7B E197A H209G, and Cel7B E197A H209A A211T. These second generation glycosynthase mutants rationally redesigned in +1 subsite were produced in the hope of widening the substrate specificity of this biocatalyst to *N*-acetyl-glucosaminyl acceptors. This strategy has successfully been applied to improve the *N*-acetylgalactosaminyltransferase activity of bovine β-(1→4)-galactosyltransferase T1 by mutation of the Y289 residue,[5] and of human β-(1→3)-galactosyltransferase by mutation of the P234 residue,[6] these two amino acids being involved in the binding of the *N*-acetyl group of the donor substrate UDP-*N*-acetylgalactosamine. However the double and triple mutants of Cel7B did not prove to catalyse the transfer of α-lactosyl fluoride onto N^{I},-N^{II}-diacetylchitobiose, in contrast to cellobiose and N^{I}-acetylchitobiose which remained good acceptors.

The purpose of the present study was to assay the catalytic activity of these cellulase mutants toward N^{I}-acetyl-2^{II}-azido-chitobiose derivative **1**, a precursor for the synthesis of potentially bioactive oligosaccharides such as nodulation factor analogues.

2. Results and discussion

N^{I}-Acetyl-2^{II}-azido-chitobiose derivative **1** was prepared in one step by diazotransfer from N^{I}-acetylchitobiose,[7] a disaccharide easily produced using an engineered *Escherichia coli* strain.[8]

In a preliminary experiment, Cel7B E197A glycosynthase was incubated with an equimolar amount of α-lactosyl fluoride **2** and azido-chitobiose **1**.

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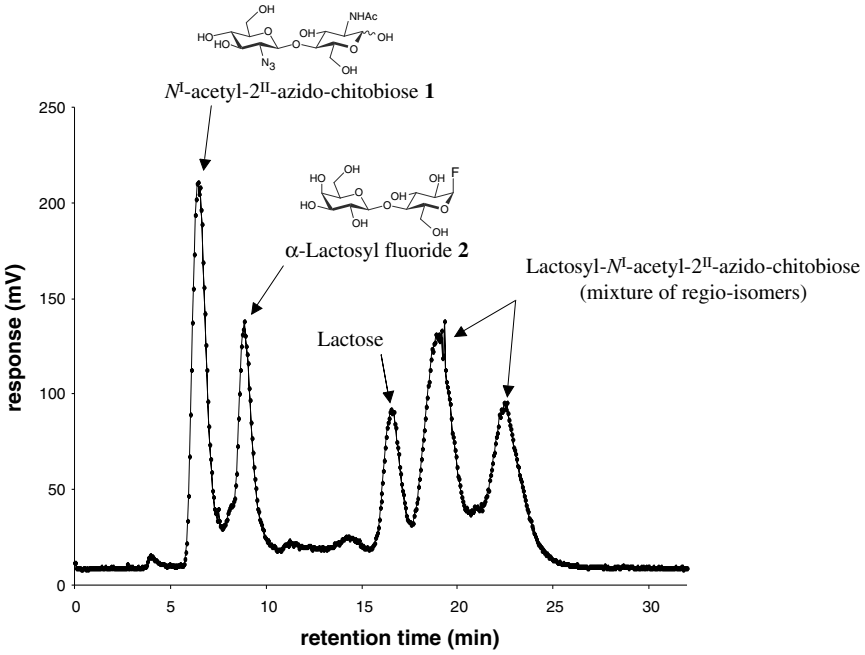

Figure 1. HPLC analysis of the condensation reaction between α-lactosyl fluoride **2** and N^{I}-acetyl-2^{II}-azido-β-chitobiose **1** catalysed by Cel7B E197A glycosynthase.

HPLC-ESIMS analysis of the reaction mixture indicated the formation of two tetrasaccharides instead of a single lactosyl-β-(1→4)-N^{I}-acetyl-2^{II}-azido-chitobiose as expected (Fig. 1). Since Cel7B E197A glycosynthase is able to condense α-lactosyl fluoride onto lactose to give the β-lactosyl-(1↔1)-β-lactoside (unpublished observation), we suspected that the undesired β-lactosyl-(1↔1)-N^{I}-acetyl-2^{II}-azido-β-chitobioside was formed during the reaction. To avoid this postulated glycosylation at C-I of the chitobiosyl derivative, we prepared the *O*-allyl N^{I}-acetyl-2^{II}-azido-β-chitobioside **3** directly from **1** according to the procedure described by Vauzeilles et al.[9] After incubation of an equimolar amount of lactosyl donor **2** and O-1 protected acceptor **3** with Cel7B E197A glycosynthase, TLC analysis of the reaction mixture again indicated the formation of two tetrasaccharides **4** and **5** (Fig. 2, lane C). This result invalidated the hypothesis that Cel7B E197A glycosynthase could catalyze the formation of a (1→1) linkage between a lactosyl unit and the N^{I}-acetyl-2^{II}-azido-chitobiose. The same reaction was run on a preparative scale (0.1 mmol of donor and acceptor substrates), and the two tetrasaccharides **4** and **5** were isolated by silica gel chromatography and fully characterized by ^{1}H and ^{13}C NMR analysis under their acetylated forms **4a** and **5a** (Figs. 3 and 4). Starting from the characteristic H-2 signal of the two glucosaminyl units, and from the two H-1 signals of the glucosyl and galactosyl units, the full assignment of the ^{1}H and ^{13}C NMR spectra could be obtained through ^{1}H–^{1}H COSY and ^{1}H–^{13}C HMQC experiments. For tetrasaccharide **4a**, amongst other signals, H-4^{II} at 3.61 ppm, H-1^{III} at 4.41 ppm with

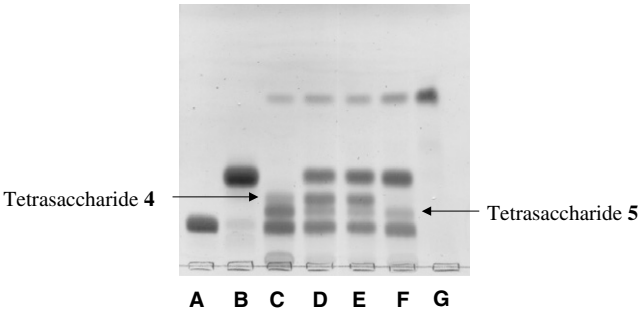

Figure 2. Lactose (A); α-lactosyl fluoride **2** (B); condensation of donor lactosyl fluoride **2** and acceptor **3** catalysed by Cel7B E197A (C), Cel7B E197A H209A (D), Cel7B E197A H209G (E), and Cel7B E197A H209A A211T (F); *O*-allyl N^{I}-acetyl-2^{II}-azido-β-chitobioside **3** (G).

$J_{1,2} = 8.0$ Hz, and C-4^{II} at 76 ppm indicated unambiguously a β-(1→4) linkage between the lactosyl and the N^{I}-acetyl-2^{II}-azido-β-chitobioside units. For tetrasaccharide **5a**, H-4^{II} at 4.83 ppm, H-3^{II} at 3.45 ppm, H-1^{III} at 4.72 ppm with $J_{1,2} = 7.9$ Hz, C-4^{II} at 67.5 ppm, and C-3^{II} at 78.6 ppm indicated unambiguously a β-(1→3) linkage between the lactosyl and the N^{I}-acetyl-2^{II}-azido-β-chitobioside units. TLC analysis showed that tetrasaccharide **5** was predominantly produced (Fig. 2, lane C).[†] After deacetylation, tetrasaccharide

[†] Because of overlapping peaks of starting and reaction products on HPLC chromatograms, analysis of the glycosylation experiments with *O*-allyl chitobioside derivatives was done by TLC analysis. Semi-quantitation of the resulting products was evaluated by comparing the intensity of the spots to a sample reference and validated by the yield of isolated products.

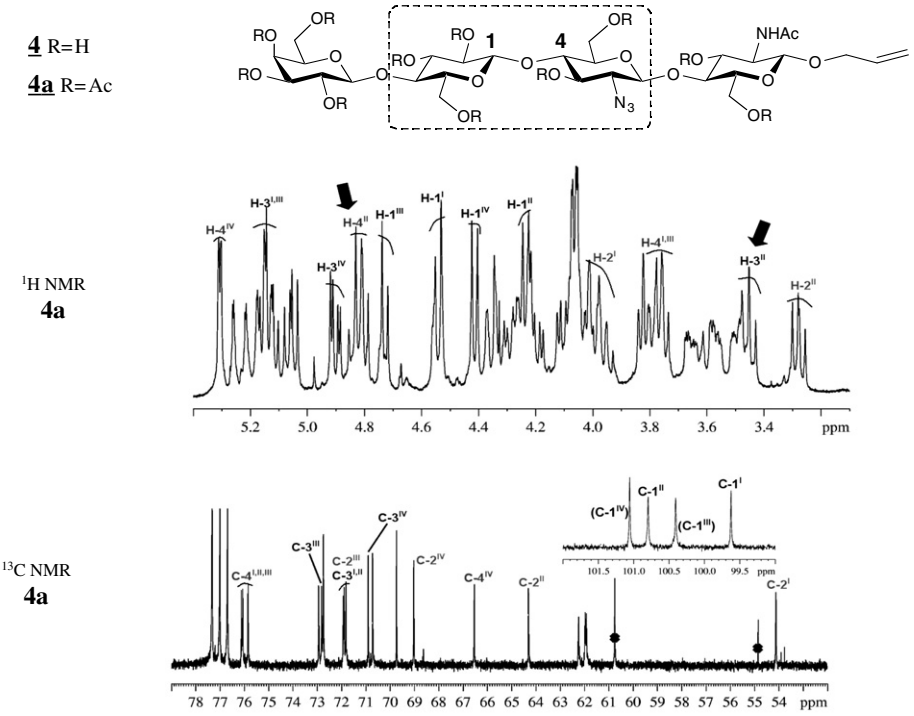

Figure 3. ^{1}H and ^{13}C NMR spectra of tetrasaccharide **4a**.

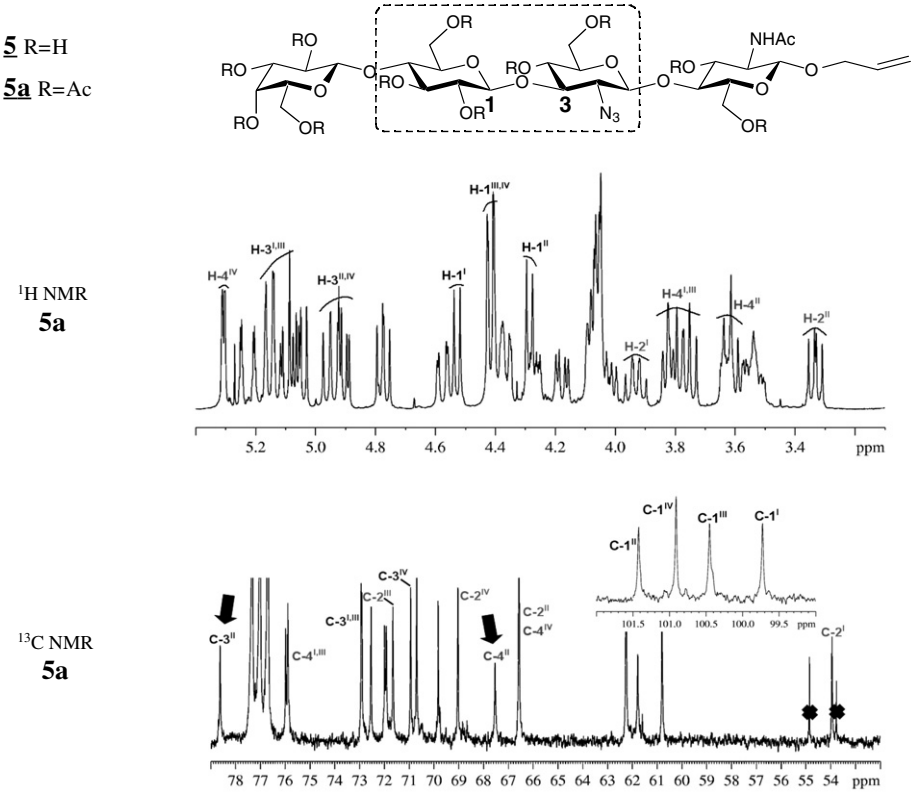

Figure 4. ^{1}H and ^{13}C NMR spectra of tetrasaccharide **5a**.

4 was isolated in 9% yield and tetrasaccharide **5** in 22% yield, indicating that the two tetrasaccharides were produced in an almost 1:3 ratio. From these observations it could be concluded that Cel7B E197A glyco-

synthase was not 100% regioselective in catalyzing the lactosylation of N^{I}-acetyl-2^{II}-azido-chitobiose or the corresponding *O*-allyl glycoside. Although exoglycosynthase are well known to display a wide regioselectivity in glycosylation reactions,[10–15] such a behaviour was only reported for the endoglycosynthase derived from *Pyrococcus furiosus* laminarinase LamA.[16] Cel7B E197A glycosynthase has been previously shown to catalyze exclusively the formation of β-(1→4) bonds from lactosyl or cellobiosyl donors and *gluco*-configured acceptors.[1] Moreover, it was even more unexpected that the predominant product was the β-(1→3)-linked tetrasaccharide, but it should be noted that in a previous observation of the crystalline Cel7B E197S glycosynthase cellobiose complex, the acceptor O-3 is closer than the acceptor O-4 to the acid/base catalytic Glu202 and to the anomeric C-1 of the donor.[17] It is possible that the azido group of the N^{I}-acetyl-2^{II}-azido-chitobiose slightly modified the positioning of the glucosaminyl unit in the +1 subsite, and promoted the formation of the β-(1→3) bond (in Ref. 1 it was shown that N^{I}-acetylchitobiose acts as an acceptor to give a lactosyl-β-(1→4)-N^{I}-acetylchitobiose tetrasaccharide).

In a second set of experiments, the double mutants Cel7B E197A H209A and Cel7B E197A H209G together with the triple mutant Cel7B E197A H209A A211T were assayed in the presence of an equimolar amount of α-lactosyl fluoride **2** and *O*-allyl N^{I}-acetyl-2^{II}-azido-β-chitobioside **3** (Fig. 2, lanes D–F). The double and triple mutants were less active than the Cel7B E197A single mutant (lane C) as estimated by the amounts of unreacted α-lactosyl fluoride **2**. However the regioselectivity of the reaction was dramatically affected by the H209 mutation. Tetrasaccharides **4** and **5** synthesized by Cel7B E197A H209A glycosynthase were isolated in 30% and 9% yield respectively, indicating that the ratio between the two tetrasaccharides (3:1) was completely inverted compared to the single mutant glycosynthase, which implied that the predominant product was the all β-(1→4)-linked tetrasaccharide. Comparison of TLC analysis of the condensation reaction catalyzed by both Cel7B E197A H209 mutants (Fig. 2, lanes D and E) suggested that the ratio between the two tetrasaccharides is similar in both cases. The double and triple mutants were also assayed in the presence of an equimolar amount of α-lactosyl fluoride **2** and cellobiose, the 'natural' acceptor of the glycosynthase. In each case, HPLC analysis (not shown) revealed that a single tetrasaccharide was synthesized, by comparison with a sample of β-lactosyl-(1→4)-cellobiose. When treated with the *H. insolens* wild type Cel7B, analysis of the reaction mixtures indicated the disappearance of the tetrasaccharide and the formation of only lactose and cellobiose, demonstrating that an exclusive β-(1→4) linkage was formed during the condensation reaction.

From these results, we can assume that H209A or H209G mutation probably created a pocket in the +1 subsite such that the 2-azido-glucosyl residue can be appropriately positioned to form a β-(1→4) bond with the lactosyl donor. In contrast, in the triple mutant Cel7B E197A H209A A211T, the +1 subsite has been remodelled in such a manner that the *O*-allyl N^{I}-acetyl-2^{II}-azido-β-chitobioside **3** acceptor can bind in a single mode to catalyse exclusively (as judged by TLC) the formation of the β-(1→3) tetrasaccharide (Fig. 2, lane F).

This work demonstrates that appropriate active site mutations can modulate the regioselectivity of the glycosylation reaction catalyzed by *H. insolens* Cel7B E197A glycosynthase, contributing to the expansion of the constantly growing glycosynthase repertoire.[18]

3. Experimental

3.1. General methods

NMR spectra were recorded on a Bruker AC 300 or a Bruker Avance 400. Carbon and proton chemical shifts (δ) are reported in ppm downfield from TMS. Coupling constants (J) are in Hertz (Hz) and peaks are described as singlet (s), doublet (d), doublet of doublet (dd), triplet (t), multiplet (m), broad (b). Carbon chemical shifts (δ) are reported in ppm using solvent as the internal reference. Complete assignment of the tetrasaccharides **4a** and **5a** was performed using a combination of COSY and HMQC experiments. High-resolution mass spectra (HRESIMS) were recorded on a Micromass ZABSpec-TOF and low-resolution (ESIMS) on a Waters Micromass ZQ spectrometer. HPLC-ESIMS experiments were carried out with a system consisting of a pump system gold LC-125S solvent module Beckmann, a μBondapak 10 μm NH_2 (300 × 3.9 mm) Waters column, and a rheodyne model 7725 injector, with a 20 μL sample loop. The analytes were eluted with a mixture of 80% MeCN in water at a flow rate of 0.5 mL/min. A one-third splitting ratio was delivered at 200 μL/min into the electrospray ionization source. Positive ion ESI mass spectra were collected on a Waters Micromass ZQ spectrometer. The capillary was set to 3.5 kV and the cone voltage was 80 °C, while the ion source temperature was set to 90 °C. Analytical thin-layer chromatography was performed using precoated Silica Gel 60 F254 plates (E. Merck, Darmstadt). The analytes were eluted with 80% MeCN in water, and spots were detected by charring with diluted sulfuric acid containing 0.1% Orcinol. Purification by flash chromatography was performed on silica gel (Geduran SI 60, 40–60 μm, E. Merck). α-Lactosyl fluoride **2** was prepared from β-lactose octaacetate according to the description in Ref. 19, followed by deprotection with catalytic sodium methoxide in MeOH.

N^{I}-Acetylchitobiose was prepared according to the description in Ref. 8. The construction and purification of *H. insolens* Cel7B E197A mutant and Cel7B E197A H209A, Cel7B E197A H209G, Cel7B E197A H209A A211T has been described, respectively, in Refs. 1 and 4.

3.2. N^{I}-Acetyl-2^{II}-azido-chitobiose (1)

A soln of triflic azide (approximately 0.6 M, 6.1 mL) in dichloromethane was prepared according to the description in Ref. 7 starting from 950 mg (14.7 mmol) of sodium azide and 1.2 mL (7.3 mmol) of triflic anhydride. N^{I}-acetylchitobiose (940 mg, 2.45 mmol) and zinc chloride (3.3 mg, 24 μmol) were dissolved in 6.1 mL of deionized water. Triethylamine (1.03 mL, 7.35 mmol), then MeOH (20.4 mL) were added dropwise to the N^{I}-acetylchitobiose soln, to reach a final ratio of 3:10:3 water–MeOH–CH_2Cl_2. The triflic azide soln was added, and the mixture was shaken for 3.5 h at 20 °C, and was then neutralized with sodium hydrogencarbonate (617 mg, 7.35 mmol), and concentrated under diminished pressure in the presence of silica gel (10 g). The resulting solid was applied on top of a silica gel column, and a gradient of MeCN to 90% MeCN in water was used to give the azido derivative **1** (711 mg, 71%); ESIMS: *m*/*z* 431 $[M+Na]^+$; HRESIMS: calcd for $[C_{14}H_{24}N_4O_{10}+Na]^+$: 431.13901, found *m*/*z* 431.1387; ^1H NMR (D_2O): δ 5.15 (s, 1H, H-1^{I}α), 4.66 (d, 1H, $J_{1,2}$ 7.3 Hz, H-1^{I}β), 4.53 (d, 1H, $J_{1,2}$ 7.9 Hz, H-1^{II}); 3.97–3.30 (m, 12 H, H-2^{I,II}, H-3^{I,II}, H-4^{I,II}, H-5^{I,II}, H-6a^{I,II}, H-6b^{I,II}); 1.99 (s, 3H, $COCH_3$); ^{13}C (D_2O): δ 176.0–175.7 (*C*OCH_3); 102.3 (C-1^{II}); 96.1 (C-1^{I}β); 91.8 (C-1^{I}α); 79.9 (C-4^{I}α); 79.4 (C-4^{I}β); 77.3 (C-5^{II}); 76.1 (C-5^{I}β); 75.5 (C-3^{II}); 73.5 (C-3^{I}β); 71.6 (C-5^{I}α); 70.6–70.3 (C-3^{I}α; C-4^{II}); 67.3 (C-2^{II}); 61.7–61.2 (C-6^{I}α/β, C-6^{II}); 57.7 (C-2^{I}β); 55.1 (C-2^{I}α); 23.4–23.1 (CO*C*H_3).

3.3. *O*-Allyl N^{I}-acetyl-2^{II}-azido-β-chitobioside (3)

To a mixture of a N^{I}-acetyl-2^{II}-azido-chitobiose **1** (250 mg, 0.61 mmol), and lithium chloride (77.8 mg, 1.83 mmol) in anhyd *N*,*N*′-dimethylformamide (DMF) (1.25 mL) was added sodium hydride (19 mg, 0.79 mmol). The reaction mixture was stirred under argon at 20 °C for 15 min, then cooled in an ice bath, and allyl bromide (312 μL, 3.7 mmol) was added and the resulting mixture was stirred at 20 °C for 24 h. After cooling in an ice bath, MeOH (500 μL) was added to destroy excess of reagents, and the soln was stirred at 20 °C for 15 min and concentrated under diminished pressure in the presence of silica gel (5 g). The resulting solid was applied on top of a silica gel column, and a gradient of MeCN to 95% MeCN in water was used to give the allyl-chitobioside derivative **3** (137 mg, 50%); ESIMS: *m*/*z* 471 $[M+Na]^+$; HRESIMS: calcd for $[C_{17}H_{28}N_4O_{10}+Na]^+$: 471.17031, found *m*/*z* 471.1703; ^1H NMR (D_2O): δ 5.86 (m, 1H, C*H*=CH_2); 5.23 (m, 1H, CH=CH_2); 4.54–4.51 (2d, $J_{1,2}$ 8.1 Hz, $J_{1,2}$ 8.0 Hz, 2H, H-1^{I}, H-1^{II}); 4.28 (dd, *J* 5.2 Hz, *J* 13.2 Hz, 1H, OCH_2); 4.10 (dd, *J* 6.3 Hz, *J* 13.2 Hz, 1H, OCH_2); 3.97–3.30 (m, 12H, H-2^{I,II}, H-3^{I,II}, H-4^{I,II}, H-5^{I,II}, H-6a^{I,II}, H-6b^{I,II}); 1.98 (s, 3H, $COCH_3$); ^{13}C (D_2O): δ 175.9 (*C*OCH_3); 134.6 (*C*H=CH_2); 119.5 (CH=*C*H_2); 102.3 (C-1^{II}); 101.2 (C-1^{I}); 79.5 (C-4^{I}); 77.3 (C-5^{II}); 76.1 (C-5^{I}); 75.5 (C-3^{II}); 73.5 (C-3^{I}); 71.8 (OCH_2); 70.6 (C-4^{II}); 67.3 (C-2^{II}); 61.7–61.3 (C-6^{I}, C-6^{II}); 56.5 (C-2^{I}); 23.4 (CO*C*H_3).

3.4. Allyl *O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-2-azido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-β-D-glucopyranoside (4) and allyl *O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)-2-azido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-β-D-glucopyranoside (5)

3.4.1. Procedure A using Cel7B E197A glycosynthase. To a soln of lactosyl fluoride **2** (46 mg, 0.134 mmol) and *O*-allyl N^{I}-acetyl-2^{II}-azido-chitobioside **3** (50 mg, 0.111 mmol) in sodium phosphate buffer (9.55 mL, 50 mM, pH 7) was added a soln of *H. insolens* Cel7B E197A glycosynthase (5 mg) in Tris–phosphate buffer (0.45 mL, 200 mM, pH 7.5). The mixture was gently shaken at 40 °C for 22 h, then concentrated under diminished pressure in the presence of silica gel (5 g). The resulting solid was applied on top of a silica gel column, and a gradient of MeCN to 80% MeCN in water was used to give a mixture of tetrasaccharides **4** and **5**, contaminated with lactose. The mixture was dissolved in pyridine (5 mL), and acetic anhydride (2.5 mL) was added together with a catalytic amount of 4-dimethylaminopyridine (1 mg). The soln was stirred for 48 h at 20 °C, protected from light and cooled in an ice bath. MeOH (5 mL) was added to destroy excess of acetic anhydride, and the soln was concentrated under diminished pressure. Flash-column chromatography (CH_2Cl_2 to 2:1 CH_2Cl_2–acetone) of the residue gave a mixture of acetylated tetrasaccharides **4a** and **5a**, separated from peracetylated lactose. This mixture was dissolved in MeOH (3 mL), then treated with sodium methoxide (30 μL, 1 M in MeOH). After 2 h at 20 °C, Amberlite IRN 77 (H^+ form) was added to neutralize the soln, the resin was filtered off, and the filtrate concentrated under diminished pressure in the presence of silica gel. The resulting solid was applied on top of a silica gel column, and a gradient of MeCN to 87.5% MeCN in water was used to give tetrasaccharide **4** (6.9 mg, 9%) and tetrasaccharide **5** (17 mg, 22%).

3.4.2. Procedure B using Cel7B E197A H209A glycosynthase. To a soln of lactosyl fluoride **2** (46 mg, 0.134 mmol) and *O*-allyl N^{I}-acetyl-2^{II}-azido-chitobioside

3 (50 mg, 0.111 mmol) in sodium phosphate buffer (3 mL, 50 mM, pH 7) was added a soln of *H. insolens* Cel7B E197A H209A glycosynthase (5 mg) in Tris–acetate buffer (7 mL, 50 mM, pH 8). The mixture was gently shaken at 40 °C for 13 h, then the same protocol as in procedure A was followed, to give tetrasaccharide **4** (23.1 mg, 30%) and tetrasaccharide **5** (6.7 mg, 9%).

Tetrasaccharide **4**: ESIMS: *m/z* 795 $[M+Na]^+$; ^1H NMR (D_2O): δ 5.81 (m, 1H, C*H*=CH_2); 5.18 (m, 2H, CH=CH_2); 4.51, 4.48 (2d, $J_{1,2}$ 8.2 Hz, $J_{1,2}$ 7.9 Hz, 2H, H-$1^{I,II}$); 4.43 (d, $J_{1,2}$ 7.8 Hz, 1H, H-1^{III}); 4.34 (d, $J_{1,2}$ 7.9 Hz, 1H, H-1^{IV}); 4.24 (m, 1H, OCH_2); 3.99 (m, 1H, OCH_2); 3.92–3.46 (m, 22H, H-$2^{I,II}$, H-$3^{I,II,III,IV}$, H-$4^{I,II,III,IV}$, H-$5^{I,II,III,IV}$, H-$6a^{I,II,III,IV}$, H-$6b^{I,II,III,IV}$); 3.28 (m, 2H, H-$2^{III,IV}$); 1.93 (s, 3H, $COCH_3$); ^{13}C (D_2O): δ 175.5 (*C*OCH_3); 134.5 (*C*H=CH_2); 119.5 (CH=*C*H_2); 104.2, 103.6, 102.1, 101.2 (C-$1^{I,II,III,IV}$); 79.4, 79.3, 79.2 (C-$4^{I,II,III}$); 76.6, 76.2, 76.1, 76.0, 75.4, 74.1, 74.0, 73.8, 73.5, 72.2, 71.8 (C-$2^{III,IV}$, C-$3^{I,II,III,IV}$, C-$5^{I,II,III,IV}$, OCH_2); 69.8 (C-4^{IV}); 66.9 (C-2^{II}); 62.3–61.0 (C-$6^{I,II,III,IV}$); 56.5 (C-2^{I}); 23.4 (CO*C*H_3).

Tetrasaccharide **5**: ESIMS: *m/z* 795 $[M+Na]^+$; ^1H NMR (D_2O): δ 5.79 (m, 1H, C*H*=CH_2); 5.17 (m, 2H, CH=CH_2); 4.64 (d, $J_{1,2}$ 7.9 Hz, 1H, H-1^{III}); 4.50 (2d, $J_{1,2}$ 8.1 Hz, $J_{1,2}$ 8.0 Hz, 2H, H-$1^{I,II}$); 4.35 (d, $J_{1,2}$ 7.8 Hz, 1H, H-1^{IV}); 4.24 (m, 1H, OCH_2); 3.99 (m, 1H, OCH_2); 3.92–3.28 (m, 24H, H-$2^{I,II,III,IV}$, H-$3^{I,II,III,IV}$, H-$4^{I,II,III,IV}$, H-$5^{I,II,III,IV}$, H-$6a^{I,II,III,IV}$, H-$6b^{I,II,III,IV}$); 1.93 (s, 3H, $COCH_3$); ^{13}C (D_2O): δ 175.9 (*C*OCH_3); 134.5 (*C*H=CH_2); 119.5 (CH=*C*H_2); 104.2, 103.7, 102.5, 101.2 (C-$1^{I,II,III,IV}$); 83.1 (C-3^{II}); 79.5 (C-$4^{I,III}$); 77.0, 76.6, 76.1, 76.0, 75.4, 74.1, 74.0, 73.5, 72.2, 71.7 (C-$2^{III,IV}$, C-$3^{I,III,IV}$, C-$5^{I,II,III,IV}$, OCH_2); 69.8, 69.0 (C-$4^{II,IV}$); 67.1 (C-2^{II}); 62.3–61.2 (C-$6^{I,II,III,IV}$); 56.5 (C-2^{I}); 23.4 (CO*C*H_3).

3.5. Allyl *O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-(2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl)-(1→4)-(3,6-di-*O*-acetyl-2-azido-2-deoxy-β-D-glucopyranosyl)-(1→4)-(3,6-di-*O*-acetyl-2-acetamido-β-D-glucopyranoside) (4a)

Tetrasaccharide **4** (19.9 mg, 25.7 μmol) was dissolved in pyridine (2 mL), and acetic anhydride (2 mL) was added together with a catalytic amount of 4-dimethylaminopyridine (1 mg). The soln was stirred for 24 h at 20 °C, protected from light and cooled in an ice bath. MeOH (5 mL) was added to destroy excess of acetic anhydride, and the soln was concentrated under diminished pressure. Flash-column chromatography (toluene–acetone 4:1 to 1:1) of the residue gave the acetylated tetrasaccharide **4a** (24 mg, 75%); ESIMS: *m/z* 1257 $[M+Na]^+$; HRESIMS: calcd for $[C_{51}H_{70}N_4O_{31}+Na]^+$: 1257.39217, found *m/z* 1257.3914; ^1H NMR ($CDCl_3$): 5.81 (m, 1H, C*H*=CH_2); 5.57 (d, *J* 9.2 Hz, 1H, NH); 5.22 (dq, *J* 17.2 Hz, *J* 1.6 Hz, 1H, CH=CH_2); 5.15 (m, 1H, CH=CH_2); 4.26 (m, 1H, OCH_2); 4.01 (m, 1H, OCH_2); 2.32–1.91 (m, 36H, $COCH_3$); ^{13}C NMR ($CDCl_3$): δ 170.9–169.1 (*C*OCH_3); 133.5 (*C*H=CH_2); 117.7 (CH=*C*H_2); 101.0 (C-1^{IV}); 100.8 (C-1^{II}); 100.4 (C-1^{III}); 99.6 (C-1^{I}); 76.1–75.8 (C-$4^{I,II,III}$); 72.9–72.7 (C-3^{III} and C-$5^{I,II,III}$); 71.9–71.8 (C-2^{III} and C-$3^{I,II}$); 70.9 (C-3^{IV}); 70.7 (C-5^{IV}); 69.7 (OCH_2); 69.0 (C-2^{IV}); 66.5 (C-4^{IV}); 64.3 (C-2^{II}); 62.2–61.9 (C-$6^{I,II,III,IV}$); 54.1 (C-2^{I}); 23.2 (NHCO*C*H_3); 20.8–20.4 (CO*C*H_3).

	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
Unit I	4.52 d (8.1)	3.93 m	5.14 t (9.1)	3.79 t (9.5)	3.62 m	4.57 dd (2.0; 12.1)	4.18 dd (4.4; 12.2)
Unit II	4.28 d (8.0)	3.33 dd (8.0; 10.2)	4.95 t (9.9)	3.61 t (9.6)	3.54 m	4.36 m	4.08 m
Unit III	4.41 d (8.0)	4.77 dd (7.8; 9.0)	5.09 t (9.0)	3.75 t (9.1)	3.55 m	4.36 m	4.08 m
Unit IV	4.41 d (8.0)	5.05 dd (7.8; 10.4)	4.90 dd (3.4; 10.4)	5.30 d (2.7)	3.82 t (6.5)	4.05 m	4.05 m

3.6. Allyl *O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-(2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl)-(1→3)-(3,6-di-*O*-acetyl-2-azido-2-deoxy-β-D-glucopyranosyl)-(1→4)-(3,6-di-*O*-acetyl-2-acetamido-β-D-glucopyranoside) (5a)

The same procedure was applied as for **4a**, starting from **5** (17.1 mg, 22.1 mmol), to give **5a** (16.9 mg, 50%); ESIMS: *m/z* 1257 $[M+Na]^+$; HRESIMS: calcd for $[C_{51}H_{70}N_4O_{31}+Na]^+$: 1257.39217, found *m/z* 1257.3918; ^1H NMR ($CDCl_3$): 5.81 (m, 1H, C*H*=CH_2); 5.57 (d, *J* 9.1 Hz, 1H, NH); 5.24 (dq, *J* 17.3 Hz, *J* 1.4 Hz, 1H, CH=CH_2); 5.15 (m, 1H, CH=CH_2); 4.28 (m, 1H, OCH_2); 4.02 (m, 1H, OCH_2); 2.30–1.92 (m, 36H, $COCH_3$). ^{13}C NMR ($CDCl_3$): δ (ppm) 171.0–169.0 (*C*OCH_3); 133.5 (*C*H=CH_2); 117.7 (CH=*C*H_2); 101.4 (C-1^{II}); 100.9 (C-1^{IV}); 100.4 (C-1^{III}); 99.7 (C-1^{I}); 78.6 (C-3^{II}); 76.0–75.9 (C-$4^{I,III}$); 72.9 (C-$3^{I,III}$); 72.5 (C-5^{I}); 72.0–71.9 (C-$5^{II,III}$); 71.6 (C-2^{III}); 70.9 (C-3^{IV}); 70.7 (C-5^{IV}); 69.8 (OCH_2); 69.0 (C-2^{IV}); 67.5 (C-4^{II}); 66.6 (C-4^{IV}); 66.5 (C-2^{II} and C-4^{IV}); 62.2–60.8 (C-$6^{I,II,III,IV}$); 53.9 (C-2^{I}); 23.2 (NHCO*C*H_3); 20.8–20.4 (CO*C*H_3).

	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
Unit I	4.54 d (8.1)	3.96 m	5.12 t (9.0)	3.76 m	3.57 m	4.36 m	4.10 m
Unit II	4.23 d (8.1)	3.27 m	3.45 t (9.3)	4.83 t (9.2)	3.49 m	4.24 m	3.98 m
Unit III	4.72 d (7.9)	4.81 t (8.1)	5.14 t (9.2)	3.77 m	3.66 m	4.54 m	4.19 m
Unit IV	4.41 d (7.9)	5.05 dd (7.8; 10.4)	4.90 dd (3.4; 10.4)	5.30 d (5.3)	3.82 m	4.06 m	4.06 m

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