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Glycosidase-catalysed oligosaccharide synthesis: preparation of *N*-acetylchitooligosaccharides using the β-*N*-acetylhexosaminidase of *Aspergillus oryzae*

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Abstract

The β-*N*-acetylhexosaminidase of *Aspergillus oryzae* catalyses the formation of 2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-D-glucopyranose (di-*N*-acetylchitobiose) and 2-acetamido-6-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-D-glucopyranose from *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside and 2-acetamido-2-deoxy-D-glucopyranose. The ratio of the two disaccharides is time-dependent. The ratio of (1 → 4)- to (1 → 6)-isomers is a maximum (~ 9:1) at the point of disappearance of the glycosyl donor. If left to evolve, the ratio changes to 92:8 in favour of the (1 → 6)-isomer. Either the (1 → 4)- or the (1 → 6)-isomer can be isolated by treating the appropriately enriched disaccharide mixture with the β-*N*-acetylhexosaminidase of Jack bean (*Canavalia ensiformis*) or the β-*N*-acetylhexosaminidase of *A. oryzae*, respectively. Di-*N*-acetylchitobiose [GlcNAc(β1-4)GlcNAc] is an efficient donor of 2-acetamido-2-deoxy-D-glucopyranosyl units in reactions catalysed by the *N*-acetylhexosaminidase of *A. oryzae*. Di-*N*-acetylchitobiose itself acts as acceptor to give tri-*N*-acetylchitotriose [GlcNAc(β1-4)GlcNAc(β1-4)GlcNAc]. As the trisaccharide accumulates it, in turn, acts as acceptor giving tetra-*N*-acetylchitotetraose [GlcNAc(β1-4)GlcNAc(β1-4)GlcNAc(β1-4)GlcNAc]. The product mixture consisting of mono-, di-, tri-, and tetrasaccharides is conveniently separated by charcoal–Celite chromatography.

Keywords: Glycosidase; *N*-Acetylhexosaminidase; Chitooligosaccharides; Enzymatic synthesis

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1. Introduction

The complexities of oligosaccharide synthesis have stimulated much interest in enzymatic methods [1–3]. There are two basic approaches: it is possible to use either the biosynthetic enzymes (glycosyl transferases) or glycosidases. Glycosidase-catalysed reactions have the merit of simplicity and a requirement for substrates less complex than those required for syntheses catalysed by glycosyl transferases. We report here the use of the β-*N*-acetylhexosaminidase of *A. oryzae* to catalyse the formation of the chitooligosaccharides di-*N*-acetylchitobiose, tri-*N*-acetylchitotriose, and tetra-*N*-acetylchitotetraose from *N*-acetyl-D-glucosamine and *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside. Di-*N*-acetylchitobiose is the core disaccharide of N-linked glycoproteins [4]. The (1 → 6)-isomer is the core disaccharide of lipid A [5,6]. Oligosaccharides consisting of β-(1 → 4)-linked *N*-acetyl-D-glucosamine residues (chitooligosaccharides) are of considerable interest as lysozyme substrates [7–9]. They have also been used in biological studies of lectins [10–13], as antitumour agents [14], and for their immunopotentiating effect [15,16].

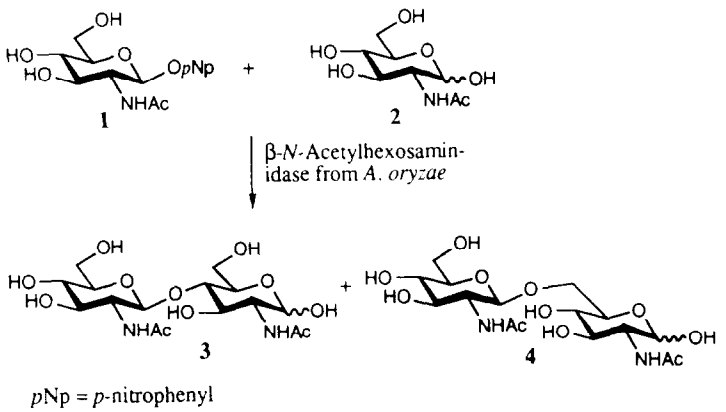
The tetrasaccharide is the core oligosaccharide of Nod factors that are involved in the symbiotic association between nitrogen-fixing bacteria and leguminous plants [17]. The free tetrasaccharide has not been synthesised by conventional chemical procedures. Chitooligosaccharides traditionally have been obtained from the hydrolysis of chitin or chitosan, by either chemical [18–20] or enzymatic [21] methods.

Enzymatic methods of synthesis have also been effective. Thus a chitinase from *Trichoderma reesei* catalysed glycosyl transfer from one molecule of tetra-*N*-acetylchitotetraose to another molecule to give the corresponding hexa-*N*-acetylchitohexaose and di-*N*-acetylchitobiose. Chain elongation from di-*N*-acetylchitobiose was catalysed by lysozyme in a medium of high ionic strength to give an oligomer mixture containing significant amounts of hexamer and heptamer [22].

Recently, in a preliminary communication [23], we showed that di-*N*-acetylchitobiose (**3**) can be synthesised efficiently from *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**1**) and *N*-acetyl-D-glucosamine (**2**) under catalysis by the β-*N*-acetylhexosaminidase from *Aspergillus oryzae*, which was used as an ammonium sulfate fraction from the commercially available β-galactosidase from this microorganism (Scheme 1). Transfer to the 4-hydroxyl group of the acceptor predominated during the early part of the reaction with a small amount (10%) of transfer to the 6-hydroxyl group to give the (1 → 6)-disaccharide **4**. However, at later stages, after all of the donor *p*-nitrophenyl glycoside **1** had disappeared, the mixture continued to evolve and **4** became the major product. The (1 → 4)- and (1 → 6)-disaccharides were isolated by selective hydrolysis of the minor disaccharide component using the *N*-acetylhexosaminidase from Jack bean [(1 → 6)-selective] and *Aspergillus oryzae* [(1 → 4)-selective], respectively. Full details of these preparations are given in the Experimental section.

2. Results and discussion

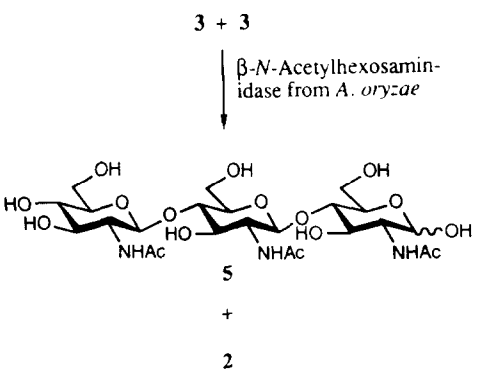
The disaccharide di-*N*-acetylchitobiose [GlcNAc(β1-4)GlcNAc, **3**] (Scheme 2) proved to be an excellent substrate for the *N*-acetylhexosaminidase from *Aspergillus*



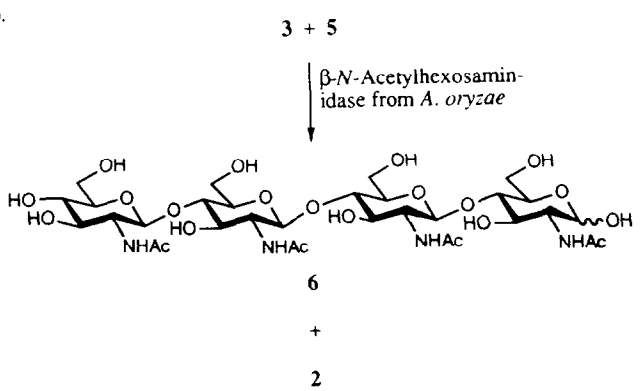
*p*Np = *p*-nitrophenyl

Scheme 1.

a.



b.



Scheme 2.

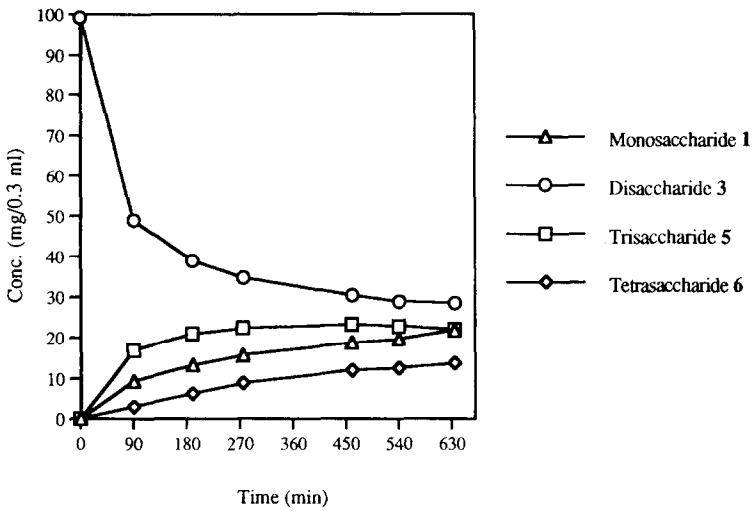


Fig. 1. Evolution of the product mixture generated on incubation of di-*N*-acetylchitobiose (**3**) with the β-*N*-acetylhexosaminidase from *Aspergillus oryzae*.

oryzae, which catalysed *N*-acetyl-β-D-glucosaminyl transfer from this donor to suitable acceptors more efficiently than from *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside. Accordingly, incubation of di-*N*-acetylchitobiose (acting both as glycosyl donor and glycosyl acceptor) with the enzyme gave rise to tri-*N*-acetylchitotriose (**5**) (Scheme 2a). This, in turn, proved to be an excellent substrate and accepted another *N*-acetylglucosaminyl residue to give the corresponding tetrasaccharide, tetra-*N*-acetylchitotetraose (**6**) (Scheme 2b). Evolution of the product mixture was followed by HPLC (Fig. 1). The impression given by these plots is that the disaccharide is readily cleaved, whereas the tri- and tetra-saccharides are resistant to cleavage. However, the movement towards an apparent equilibrium mixture is governed by a set of at least 12 individual reversible reactions (1–12, below). In these equations, the first reactant is the glycosyl donor and the second, the glycosyl acceptor [monomer = *N*-acetyl-D-glucosamine, dimer = di-*N*-acetylchitobiose (**3**), etc.]. On the product side, the first product is the residue from the glycosyl donor and the second is the transfer product.

$$\text{dimer} + \text{dimer} = \text{monomer} + \text{trimer} \quad (1)$$

$$\text{dimer} + \text{monomer} = \text{monomer} + \text{dimer} \quad (2)$$

$$\text{dimer} + \text{trimer} = \text{monomer} + \text{tetramer} \quad (3)$$

$$\text{dimer} + H_2O = 2 \times \text{monomer} \quad (4)$$

$$\text{trimer} + \text{monomer} = 2 \times \text{dimer} \quad (5)$$

$$\text{trimer} + \text{dimer} = \text{dimer} + \text{trimer} \quad (6)$$

$$\text{trimer} + \text{trimer} = \text{dimer} + \text{tetramer} \quad (7)$$

$$\text{trimer} + H_2O = \text{monomer} + \text{dimer} \quad (8)$$

$$\text{tetramer} + \text{monomer} = \text{trimer} + \text{dimer} \quad (9)$$

$$\text{tetramer} + \text{dimer} = 2 \times \text{trimer} \quad (10)$$

$$\text{tetramer} + \text{trimer} = \text{trimer} + \text{tetramer} \quad (11)$$

$$\text{tetramer} + H_2O = \text{trimer} + \text{monomer} \quad (12)$$

In each set of four reactions, in which the donor is dimer, trimer, or tetramer, respectively, there is one degenerate reaction (products = reactants). There is also one hydrolysis reaction. Transfer reactions leading to changes in concentrations of dimer, trimer, and tetramer are reactions (1), (3), (5), (7), (9), and (10). The hydrolysis reactions also lead to changes in the concentrations of these species. The reactions involving the tri- and tetra-saccharides obviously assume more importance as the concentrations of these substances increase. The product mixture evolves in a general manner that has long been familiar in the enzymatic degradation of carbohydrate polymers. Initially, these lead to an accumulation of monomer. However, as the concentration of monomer increases, oligomer formation begins with the monomer and subsequently dimers, trimers, etc., acting as acceptors of glycosyl units instead of water. The final product consists of an equilibrium, or near equilibrium mixture of these oligomeric species. This is the phenomenon classically described as 'reverse hydrolysis'.

Elucidation of the kinetic parameters of the individual reactions (1) to (12) would be a complex task. Accordingly, the evolution of the mixture is best determined by HPLC so that it can be halted when the concentration of the desired product has reached a maximum. This is a simple matter since the concentrations of trimer and tetramer change only slowly as they approach their maximum values (Fig. 1).

During formation of the (1 → 4)- and (1 → 6)-disaccharides from **1** and **2** (Scheme 1), the reaction mixture developed to contain a ratio of **3**:**4** of 9:1 at the point of disappearance of the initial donor. On continued evolution, this ratio changed to 8:92 in favour of the (1 → 6)-disaccharide, as noted above [23].

The reaction thus showed three phases: first, decay of the *p*-nitrophenyl glycoside leading to a predominance of the (1 → 4)-linked disaccharide; second, net conversion of the (1 → 4)- into the (1 → 6)-linked disaccharide; and third, slow conversion of both disaccharides into the monomer. At first sight, this was surprising, so we attempted to simulate the behaviour by numerical integration of simplified rate equations. It was assumed that the *p*-nitrophenyl glycoside **1** and both disaccharides **3** and **4** act as donor substrates to give a common glycosyl enzyme intermediate (EI) and that *N*-acetyl-D-glucosamine (**2**) and water act as acceptor substrates, with the former partitioning between reaction at the 4-and 6-positions, leading to the following scheme.

$$\mathbf{1} + E \rightarrow EI + p\text{NpOH} \quad (13)$$

$$\mathbf{3} + E \rightarrow EI + \mathbf{2} \quad (14)$$

$$\mathbf{4} + E \rightarrow EI + \mathbf{2} \quad (15)$$

$$EI + \mathbf{2} \rightarrow E + \mathbf{3} \quad (16)$$

$$EI + \mathbf{2} \rightarrow E + \mathbf{4} \quad (17)$$

$$EI + H_2O \rightarrow E + \mathbf{2} \quad (18)$$

For simplification, the binding equilibrium and the catalytic steps, for each donor, were combined to give equations 13 to 15. Three assumptions were made. The first was that the three donors compete for binding to the enzyme, but are always at equilibrium, so that the rate of processing of each substrate is determined by its success in the competition and its k_{cat}. The second was that the concentrations of donors are high enough to ensure saturation kinetics throughout, so that the concentration of free enzyme can be ignored. A third assumption was that reaction of the glycosyl enzyme intermediate is faster than its formation and thus its concentration is low. This leads to the following equations,

$$\text{rate of reaction 13} = \frac{k_{1cat} \times E_0}{K_{1m} \times \left(\frac{[1]}{K_{1m}} + \frac{[3]}{K_{3m}} + \frac{[4]}{K_{4m}} \right)} [1]$$

$$\text{rate of reaction 14} = \frac{k_{3cat} \times E_0}{K_{3m} \times \left(\frac{[1]}{K_{1m}} + \frac{[3]}{K_{3m}} + \frac{[4]}{K_{4m}} \right)} [3]$$

$$\text{rate of reaction 15} = \frac{k_{4cat} \times E_0}{K_{4m} \times \left(\frac{[1]}{K_{1m}} + \frac{[3]}{K_{3m}} + \frac{[4]}{K_{4m}} \right)} [4]$$

where k_{ncat} and K_{nm} are the catalytic rate and Michaelis–Menten constant, respectively, for substrate n; and [1], [3], and [4] are the concentrations respectively of compounds **1**, **3**, and **4**.

With these assumptions and reasonable estimates of the various equilibrium and rate constants, the numerical integration led to the three-phase pattern of behaviour observed experimentally, as shown in Fig. 2. Although this simplified scheme contained too many unknown parameters for meaningful attempts to fit the data, it is nevertheless shown to be able to account for the data.

Procedures for the isolation and purification of the disaccharides **3** and **4** have already been described [23]. For the present study, the product mixture was readily separated into its individual components by charcoal–Celite chromatography [24], which gives excellent separation of mono-, di-, tri- and tetra-saccharides. Since tri-*N*-acetylchitotriose (**5**) and tetra-*N*-acetylchitotetraose (**6**) were respectively the only oligosaccharides of their class in the product mixture from incubation of the disaccharide **3** with the enzyme, their isolation and purification were readily achieved.

The molecular recognition characteristics of the enzyme are of interest. The *p*-nitrophenyl glycoside **1** binds at the active site in such a manner that *N*-acetylglucosaminyl transfer either to the C-6 or C-4 hydroxyl group is possible. However, it is noteworthy [23] that on replacing **2** by the corresponding methyl α-glycoside **7** (Scheme 3a), transfer is directed exclusively to the 4-position to give the corresponding disaccharide **8** in 51% yield. If the acceptor is the corresponding methyl β-glycoside **9**, the major product is again the (1 → 4)-transfer product **10** which is produced together with an as yet unidentified product in a ratio of 2:1, but with lower efficiency (Scheme 3b). When

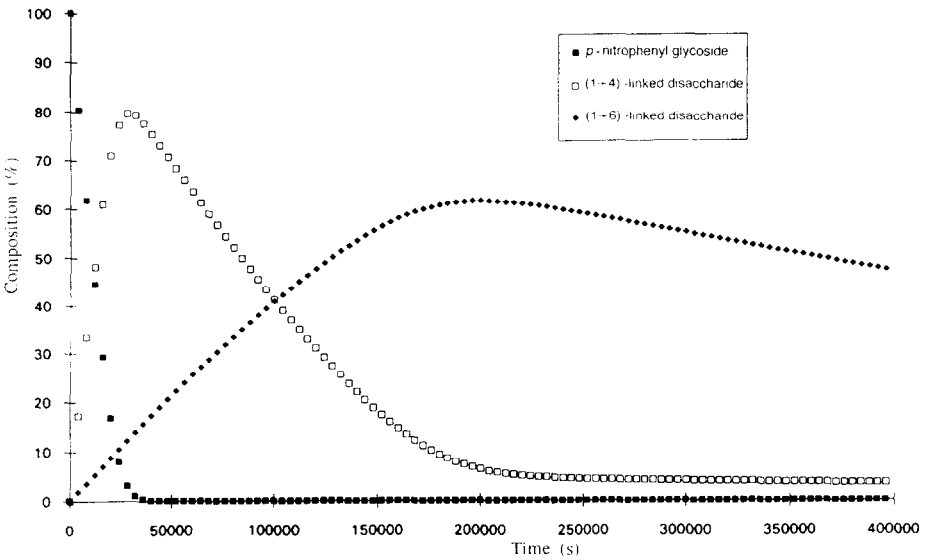
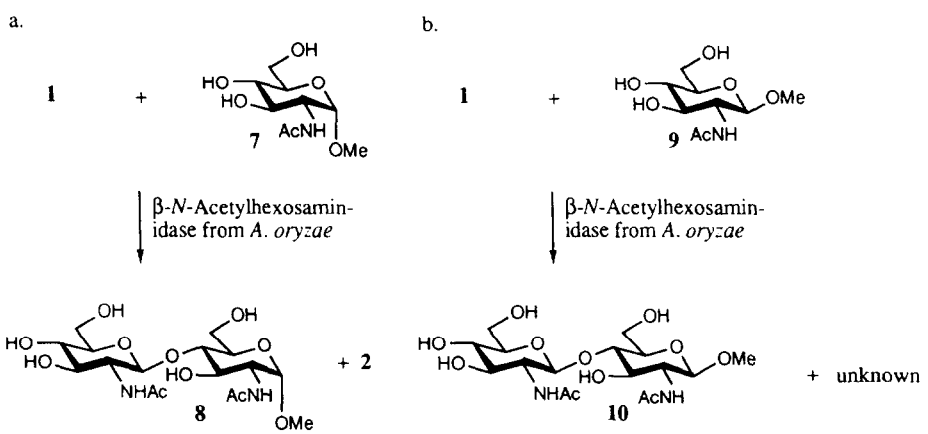


Fig. 2. Simulation of the kinetics of formation of GlcNAc(β1-4)GlcNAc (**3**) and GlcNAc(β1-6)GlcNAc (**4**) from *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**1**) and 2-acetamido-2-deoxy-D-glucopyranose (**2**) on incubation with the β-*N*-acetylhexosaminidase from *Aspergillus oryzae*.

di-*N*-acetylchitobiose (**3**) or tri-*N*-acetylchitotriose (**5**) is bound at the acceptor site, exclusive transfer to the 4-hydroxyl group of the non-reducing terminal unit is again observed. Clearly, the α-OMe group in the glycoside **7** and the reducing *N*-acetylglucosamine unit in di-*N*-acetylchitobiose and the corresponding monosaccharide unit in the trisaccharide **5** restrict the conformation of the bound acceptor so that transfer only to the 4-hydroxyl group is possible.



Scheme 3.

Formation of the tetramer would clearly be more efficient if the trimer were used as acceptor. Experiments to develop this approach to the tetramer and higher oligomers are in hand.

3. Experimental

General.—^{1}H NMR spectra were determined at 250 or 400 MHz using a Bruker AC 250 or WH 400 spectrometer, respectively. ^{13}C NMR spectra were determined at 62.89 or 100.62 MHz using the same instruments. Mass spectra were determined with a Kratos MS 80 or VG Analytical Quattro 2 mass spectrometer. Optical rotations were determined using an AA-1000 polarimeter (Optical Activity Ltd). with a 2-dm cell. β-Galactosidase from *Aspergillus oryzae*, β-*N*-acetylhexosaminidase (Jack bean), and authentic samples of tri-*N*-acetylchitotriose and tetra-*N*-acetylchitotetraose were obtained from the Sigma Chemical Company. Celite 535 was obtained from Fluka and activated charcoal (Darco G-60, 100 mesh) was obtained from the Aldrich Chemical Company. TLC was carried out using Silica Gel 60 GF_{254} (Merck) with the solvent system propan-1-ol–nitromethane–water (10:5:4). Oligosaccharides were visualised by spraying with 10% H_2SO_4 and charring. HPLC analyses were carried out using a Gilson HPLC instrument with a Hypersil 5 APS (aminopropyl silica) column (20 × 4.6 mm) with UV detection at 210 nm and 4:1 MeCN–H_2O as eluent at a flow rate of 1 mL min^{-1}. Kinetic simulations were carried out using the programme Facsimile (AEA Technology, Harwell, U.K.).

Enzyme preparation.—Crude β-galactosidase from *Aspergillus oryzae* (Sigma grade IX, 50 g) was suspended in 800 mL of sodium phosphate buffer (50 mM, pH 6.5) and 1 mM EDTA. The suspension was brought to 85% saturation with 447 g of $(NH_4)_2SO_4$ (Fluka) and stirred overnight at 4°C. The precipitate was removed by centrifugation at 4000 *g* for 30 min. The supernatant solution was brought to 100% saturation and stirred overnight at 4°C. The precipitate, recovered by centrifugation at 4000 *g* for 30 min, was redissolved in buffer and dialysed overnight against the same buffer at 4°C. The sample volume was reduced after dialysis using a Centriprep (Amicon) ultrafiltration device. The total protein was assayed by a commercial implementation (Bio-Rad) of the Bradford method [25] using bovine serum albumin (BSA) as a standard. The increase in activity following this procedure was as previously described [23].

*2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-*D*-glucopyranosyl chloride.*—A solution of 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-α-D-glucopyranose (10 g, 25.7 mmol) in dry Ac_2O (30 mL) was saturated with dry HCl gas for 8 h at 0°C. The mixture was left at room temperature for 16 h. The solution was further saturated with HCl gas for 7 h at 0°C and left in a stoppered vessel at room temperature for 5 days. The mixture was diluted with $CHCl_3$ (125 mL) and cooled. The organic solution was washed with cold water (2 × 100 mL), saturated aq sodium hydrogen carbonate (2 × 100 mL), and water (2 × 100 mL). The organic phase was separated, dried ($MgSO_4$), filtered, and concentrated to a syrup. The residue was triturated with dry diethyl ether to give 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride (8.29 g, 88%); mp 118–119°C (lit.[26] mp 124–126°C, lit. [27] mp 126–127°C); $[\alpha]_D^{26}$ +120.6° (*c* 1.03, $CHCl_3$) {lit. [26] $[\alpha]_D^{20}$ + 113° ($CHCl_3$), lit. [27] $[\alpha]_D^{18}$ +118° ($CHCl_3$)}.

p-*Nitrophenyl 2-acetamido-3,4,6-tri*-O-*acetyl-2-deoxy-β*-D-*glucopyranoside*.—A solution of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride (3.51 g, 9.6 mmol), tetrabutylammonium hydrogen sulfate (3.27 g, 9.6 mmol), and *p*-nitrophenol (2.67 g, 19.2 mmol) in a mixture of CH_2Cl_2 (35 mL) and 1 M NaOH (35 mL) was stirred vigorously for 1.4 h. The mixture was extracted with $CHCl_3$ (4 × 75 mL), and washed with 1 M NaOH (4 × 200 mL), water (until the yellow colour was removed), and saturated aq NaCl (300 mL). The organic phase was dried (Na_2SO_4) and evaporated under reduced pressure to give *p*-nitrophenyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (3.35 g, 74%), which was crystallised from MeOH–EtOAc; mp 234–235°C (dec.) (lit. [26] mp 236–239°C, lit. [28] mp 237–238°C); $[\alpha]_D^{27}$ −45° (*c* 0.8, pyridine) {lit. [26] $[\alpha]_D^{20}$ −47° (pyridine)}.

p-*Nitrophenyl 2-acetamido-2-deoxy-β*-D-*glucopyranoside* (**1**).—The above acetate (2.45 g, 5.2 mmol) was suspended in dry MeOH (70 mL). The solution was warmed to 37°C. Methanolic NaOMe (1 M, 2.5 mL) was added and the mixture was stirred until dissolution was complete (~ 5 min). The mixture was allowed to stand at 5°C overnight. The precipitated solid was filtered off and the filtrate was evaporated under reduced pressure to small volume. The solid that precipitated was filtered off and combined with the first batch of product. The combined material was recrystallised from water and suspended in acetone (50 mL). The mixture was stirred for 30 min, and the product was filtered off and dried to give the glycoside **1** (1.68 g, 94%); mp 205°C (lit. [28] mp 206°C, lit. [26] mp 210–212°C); $[\alpha]_D^{27}$ −14.1° (*c* 1, Me_2SO) {lit. [28] $[\alpha]_D$ −13.2° (Me_2SO)}.

2-Acetamido-4-O-*(2-acetamido-2-deoxy-β*-D-*glucopyranosyl)-2-deoxy*-D-*glucopyranose (di*-N-*acetylchitobiose*, **3**).—Glycoside **1** (1.0 g, 2.92 mmol) and *N*-acetyl-D-glucosamine (**2**) (6.4 g, 29 mmol) were suspended in phosphate buffer (0.04 M, pH 6.5, 25 mL). The mixture was heated at 45–50°C for 2–3 min (water bath) and at 30°C for 5 min. The enzyme solution (1.0 mL, 76.3 mg protein/mL, 18.2×10^{-3} U/mg protein, prepared as described above) was added and the mixture was incubated for 40 h at 30°C. The reaction was stopped by heating in a boiling water bath for 5 min. By HPLC it was determined that the ratio of the (1 → 4)- to the (1 → 6)-isomers of the disaccharide product was 9:1. The mixture was applied to a column (45 cm × 3 cm) made up from activated carbon (Darco G-60, 100 mesh) and Celite 535 (1:1, 70 g). The column was eluted with 5:95 EtOH–water to remove monosaccharide and then with 10:90 EtOH–water to recover the disaccharide mixture. The eluate was evaporated to dryness under reduced pressure. The residue was dissolved in phosphate buffer (0.04 M, pH 6.5, 6 mL) and the mixture was incubated at 30°C with the β-*N*-acetylhexosaminidase from Jack bean (0.35 mL, 1 mg protein/mL, 56 U/mg protein) for 45 h. At this point no remaining (1 → 6)-disaccharide was detectable by HPLC The reaction was stopped by heating the mixture in a boiling water bath for 5 min. The disaccharide fraction was isolated by charcoal–Celite chromatography as above, to give **3** (0.68 g, 55%) as a white solid, crystallised from aqueous MeOH; mp 260–263°C (lit. [19] mp 260–262°C); $[\alpha]_D^{21}$ + 16.3° (*c* 0.4, H_2O) {lit. [19] $[\alpha]_D$ + 17.2° (H_2O), lit. [29] $[\alpha]_D^{22}$ + 16.3° (H_2O)}; ^{1}H-NMR (D_2O): δ 1.96 (s, 3 H, Me), 1.99 (s, 3 H, Me), 3.39–3.87 (m, 12 H), 4.50 (d, 0.39 H, $J_{1',2'}$ 8.43 Hz, H-1′ β-anomer), 4.51 (d, 0.61 H, $J_{1',2'}$ 8.43 Hz, H-1′ α-anomer), 4.62 (d, 0.39 H, $J_{1,2}$ 8.15 Hz, H-1 β-anomer), 5.11 (d, 0.61 H, $J_{1,2}$ 2.60 Hz, H-1

α-anomer); ^{13}C-NMR (D_2O): δ 22.68 (Me, reducing end α-anomer), 22.91 (Me, non-reducing end), 22.98 (Me, reducing end β-anomer), 54.49 (C-2 α-anomer), 56.39 (C-2′), 56.86 (C-2 β-anomer), 60.84 (C-6 α- and β-anomer), 61.32 (C-6′), 70.10 (C-3, α-anomer), 70.49 (C-4′), 70.76 (C-5, α-anomer), 73.34 (C-3, β-anomer), 74.26 (C-3′), 75.37 (C-5, β-anomer), 76.69 (C-5′), 80.16 (C-4, β-anomer), 80.63 (C-4, α-anomer), 91.23 (C-1, α-anomer), 95.63 (C-1, β-anomer), 102.29 (C-1′), 175.24 (C=O, reducing end α-anomer), 175.40 (C=O, non-reducing end), 175.51 (C=O, reducing end β-anomer); *m/z* (FAB) 447 $(M + Na)^+$, 425 $(M + H)^+$.

*2-Acetamido-6-O-(2-acetamido-2-deoxy-β-*D*-glucopyranosyl)-2-deoxy-*D*-glucopyranose* (**4**).—Glycoside **1** (0.5 g, 1.46 mmol) and **2** (3.2 g, 14.5 mmol) were suspended in phosphate buffer (0.04 M, pH 6.5, 13 mL). The mixture was heated at 45–50°C for 2–3 min (water bath) and at 30°C for 5 min. The enzyme solution (0.5 mL; 76.3 mg protein/mL, 18.2×10^{-3} U/mg protein) was added and the mixture was incubated for 215 h at 30°C. By HPLC it was determined that the ratio of the (1 → 6)- to the (1 → 4)-isomers of the disaccharide product was 92:8. The reaction was stopped by heating the mixture in a boiling water bath for 5 min. The disaccharide fraction was isolated by charcoal–Celite chromatography, as above. It was dissolved in phosphate buffer (0.04 M, pH 6.5, 6 mL) and the mixture was incubated at 30°C with the β-*N*-acetylhexosaminidase of *A. oryzae* (0.1 mL) for 2 h. At this point no remaining (1 → 4)-disaccharide was detectable by HPLC. The reaction was stopped by heating the mixture to 85–90°C for 5 min. The disaccharide fraction was isolated by charcoal–Celite chromatography as above, to give **4** (0.136 g, 22%) as a white solid, crystallised from EtOH; mp 198–200°C (lit. [30] mp 200°C, lit. [31] mp 199–201°C); $[\alpha]_D^{21}$ +8.2° (*c* 0.3, H_2O) {lit. [30] $[\alpha]_D^{25}$ +8° (H_2O), lit. [31] $[\alpha]_D$ +10.1° (H_2O)}; ^{1}H-NMR (D_2O): δ 1.96 (s, 3 H, Me), 1.98 (s, 3 H, Me), 3.22–3.90 (m, 11 H), 4.00–4.11 (m, 1 H), 4.46 (d, 0.4 H, $J_{1',2'}$ 8.13 Hz, H-1′, β-anomer), 4.47 (d, 0.6 H, $J_{1',2'}$ 8.40 Hz, H-1′, α-anomer), 4.60 (d, 0.4 H, $J_{1,2}$ 8.43 Hz, H-1, β-anomer), 5.09 (d, 0.6 H, $J_{1,2}$ 3.20 Hz, H-1, α-anomer); ^{13}C-NMR (D_2O): δ 22.60 (Me, reducing end α-anomer), 22.86 (Me, reducing end β-anomer), 22.92 (Me, non-reducing end), 54.71 (C-2, α-anomer), 56.17 (C-2′), 57.38 (C-2, β-anomer), 61.39 (C-6′), 69.22 (C-6, α-anomer), 69.47 (C-6, β-anomer), 70.57 (C-4′ and C-4 β-anomer), 70.74 (C-4, α-anomer), 71.15 (C-3, α-anomer), 71.37 (C-5, α-anomer), 74.39 (C-3′), 74.56 (C-3, β-anomer), 75.52 (C-5, β-anomer), 76.52 (C-5′), 91.54 (C-1, α-anomer), 95.66 (C-1, β-anomer), 102.34 (C-1′), 102.45 (C-1′), 175.16 (C=O), 175.33 (C=O), 175.39 (C=O); *m/z* (FAB) 447 $(M + Na)^+$, 425 $(M + H)^+$.

*Preparation of tri-*N*-acetylchitotriose* (**5**) *and tetra-*N*-acetylchitotetraose* (**6**).—To a solution of **3** (550 mg) in phosphate buffer (pH 6.5, 0.04 M, 1.5 mL) was added β-*N*-acetylhexosaminidase (80 μL, 17.07×10^{-3} U/mg protein, 242.5 mg protein/mL). The mixture was incubated for 25 h, heated in a boiling water bath for 5 min, and applied to a column (45 cm × 3 cm) made up from activated carbon (Darco G-60, 100 mesh) and Celite 535 (1:1, 70 g). The column was eluted with 5:95 EtOH–water (600 mL) to remove *N*-acetyl-D-glucosamine, 10:90 EtOH–water (1000 mL) to give **3** (220 mg), 15:85 EtOH–water (700 mL) to give **5** (128 mg), and 20:80 EtOH–water (400 mL) to give **6** (41 mg).

Tri-*N*-Acetylchitotriose (**5**); $[\alpha]_D^{28}$ +2.90° (*c* 0.69, H_2O) {lit. [19] $[\alpha]_D^{17}$ +2.5°

(H_2O)}; ^{1}H-NMR (D_2O): δ 1.96, 1.98, and 1.99 (3 s, each 3 H, 3 × Me), 3.34–3.87 (m, 18 H), 4.50 (d, 2 H, J 8.43 Hz, H-1′ and H-1″), 4.61 (d, 0.43 H, $J_{1,2}$ 7.85 Hz, H-1β), 5.11 (d, 0.57 H, $J_{1,2}$ 2.33 Hz, H-1α); ^{13}C-NMR (D_2O): δ 22.55 (Me, reducing end α-anomer), 22.78 (2 × Me, non-reducing end), 22.84 (Me, reducing end β-anomer), 54.30 (C-2, α-anomer), 55.70 (C-2′), 56.25 (C-2″), 56.78 (C-2, β-anomer), 60.65 (C-6), 60.79 (C-6′), 61.20 (C-6″), 69.91 (C-3, α-anomer), 70.36 (C-4″), 70.68 (C-5, α-anomer), 72.82 (C-3′), 73.14 (C-3, β-anomer), 74.10 (C-3″), 75.16 (C-5′), 75.27 (C-5, β-anomer), 76.57 (C-5″), 79.78 (C-4, β-anomer), 79.81 (C-4′), 80.28 (C-4, α-anomer), 91.11 (C-1, α-anomer), 95.49 (C-1, β-anomer), 101.94 (C-1′), 102.13 (C-1″), 175.13 (reducing end CO, α-anomer), 175.26 (CO), 175.29 (CO), 175.41 (reducing end CO, β-anomer); the ^{1}H and ^{13}C NMR spectra were identical with those of an authentic sample; m/z (FAB) 628 $(M + H)^+$.

Tetra-*N*-acetylchitotetraose (**6**); $[\alpha]_D^{22}$ −4.2° (*c* 0.64, H_2O) {lit.[19] $[\alpha]_D$ −4.1° (H_2O)}; ^{1}H-NMR (D_2O): δ 1.96, 1.98, 1.99, 1.99 (4 s, 12 H, 4 × Me), 3.35–3.87 (m, 24 H), 4.51 (d, 3 H, J 8.1 Hz, H-1′, H-1″, H-1‴), 4.62 (d, 0.29 H, $J_{1,2}$ 7.6 Hz, H-1β), 5.11 (d, 0.71 H, $J_{1,2}$ 1.5 Hz, H-1α); ^{13}C-NMR (D_2O): δ 22.54 (Me, α-anomer), 22.78 (3 × Me), 22.84 (Me, β-anomer), 54.31 (C-2, α-anomer), 55.68 (C-2′), 55.73 (C-2″), 56.25 (C-2‴), 56.78 (C-2, β-anomer), 60.64 (C-6), 60.70 (C-6″), 60.80 (C-6′), 61.20 (C-6‴), 69.90 (C-3, α-anomer), 70.36 (C-4‴), 70.67 (C-5, α-anomer), 72.75 (C-3′), 72.79 (C-3″), 73.14 (C-3, β-anomer), 74.10 (C-3‴), 75.18 (C-5′ and C-5″), 75.26 (C-5, β-anomer), 76.57 (C-5‴), 79.57 (C-4″), 79.80 (C-4′ and C-4, β-anomer), 80.29 (C-4, α-anomer), 91.11 (C-1, α-anomer), 95.49 (C-1, β-anomer), 101.91 (C-1″, C-1′), 102.14 (C-1‴), 175.14 (reducing end, C=O, α-anomer), 175.29 (3 × C=O, non-reducing ends), 175.41 (reducing end C=O, β-anomer); the ^{1}H and ^{13}C NMR spectra were identical with those of an authentic sample; m/z (FAB) 853 $(M + Na)^+$, 831 $(M + H)^+$.

Methyl 2-acetamido-4-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-α-D-glucopyranoside (**8**).—Glycoside **1** (0.1 g, 0.29 mmol) and methyl 2-acetamido-2-deoxy-α-D-glucopyranoside (**7**) (0.35 g, 1.49 mmol) were suspended in phosphate buffer (0.04 M, pH 6.5, 2.8 mL). The mixture was heated at 45–50°C for 2–3 min (water bath) and at 30°C for 5 min. The enzyme solution (purified) (0.9 mL, 435 × 10^{-3} U/mg protein) was added and the mixture was incubated for 120 h at 30°C after which time no glycosyl donor remained as determined by HPLC The reaction was stopped by heating the mixture in a boiling water bath for 5 min. By HPLC it was determined that only a single disaccharide product was present. The mixture was applied to a charcoal–Celite column. The column was eluted with 5:95 EtOH–water to remove monosaccharide and then with 10:90 EtOH–water to recover **8** (0.065 g, 51%); $[\alpha]_D^{22}$ +56° (*c* 0.4, H_2O); ^{1}H-NMR (D_2O): δ 1.95 (s, 3 H, Me), 1.99 (s, 3 H, Me), 3.29 (s, 3 H, OMe). 3.38–3.88 (m, 12 H), 4.50 (d, 1 H, $J_{1',2'}$ 8.43 Hz, H-1′), 4.67 (d, 1 H, $J_{1,2}$ 3.18 Hz, H-1); ^{13}C-NMR (D_2O): δ 22.54 (Me), 22.79 (Me), 53.80 (C-2), 55.83 (OMe), 56.27 (C-2′), 60.71 (C-6), 61.21 (C-6′), 70.39 (C-3 and C-4′), 70.75 (C-5), 74.14 (C-3′), 76.57 (C-5′), 80.44 (C-4), 98.32 (C-1), 102.16 (C-1′), 175.10 (C=O), 175.26 (C=O); m/z (FAB) 461 $(M + Na)^+$.

Methyl 2-acetamido-4-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside (**10**).—Glycoside **1** (25 mg, 0.073 mmol) and methyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**9**) (100 mg, 0.43 mmol) were suspended in phosphate

buffer (0.04 M, pH 6.5, 0.75 mL). The mixture was heated at 45–50°C for 2–3 min (water bath) and at 30°C for 5 min. The enzyme solution (0.2 mL, 52 mg protein/mL, 5.7×10^{-3} U/mg protein) was added and the mixture was incubated for 48 h at 30°C after which time no glycosyl donor remained as determined by HPLC The reaction was stopped by heating the mixture in a boiling water bath for 5 min. By HPLC it was determined that two products were formed in a ratio of 66:34. The released *p*-nitrophenol was extracted with diethyl ether (4 × 5 mL) and the remaining solution was evaporated to dryness under reduced pressure The product was purified by HPLC [Hypersil 5 APS column (25 × 20 mm), UV detection at 210 nm, with 80:20 MeCN–H_2O as eluent at a flow rate of 5 mL min^{-1}] to give **10** (7.7 mg); $[\alpha]_D^{24}$ −23.16° (*c* 0.23, H_2O) {lit. [32] $[\alpha]_D^{25}$ −27° (*c* 0.5, H_2O)}; ^{1}H-NMR (D_2O); δ 1.97 (s, 3 H, Me), 2.01 (s, 3 H, Me), 3.44 (s, 3 H, OMe), 3.40–3.84 (m, 12 H), 4.37 (d, 1 H, $J_{1,2}$ 8.13 Hz, H-1), 4.52 (d, 1 H, $J_{1',2'}$ 8.43 Hz, H-1'); ^{13}C-NMR (D_2O): δ 22.79 (Me), 22.82 (Me), 55.52 (C-2), 56.25 (C-2'), 57.79 (OCH_3), 60.80 (C-6), 61.19 (C-6'), 70.35 (C-4'), 73.27 (C-3), 74.11 (C-3'), 75.17 (C-5), 76.57 (C-5'), 80.14 (C-4), 102.15 (C-1'), 102.52 (C-1), 175.29 (C=O), 175.37 (C=O); *m/z* (FAB) 461 $(M + Na)^+$.

Kinetic simulations.—In the kinetic simulation using the programme Facsimile [33] the concentrations of **1** and **2** were taken at their experimental values. The values taken for the Michaelis and rate constants were as follows. $K_{1m} = 0.001$ mol L^{-1}, $K_{3m} = 0.005$ mol L^{-1}, and $K_{4m} = 0.05$ mol L^{-1}; $k_{1cat} = 0.5$ s^{-1}, $k_{3cat} = 0.5$ s^{-1}, and $k_{4cat} = 0.05$ s^{-1}; $k_{16} = 100.0$ dm^3 mol^{-1} s^{-1}, $k_{17} = 10.0$ L mol^{-1} s^{-1}, and $k_{18} = 4.0$ s^{-1}. These values ensure that the concentrations of both free enzyme and glycosyl enzyme intermediate are low.

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