Kinetic Control of Regioselectivity in Glycosidase-catalysed Disaccharide Synthesis: Preparation of 2-Acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-D-glucopyranose (*N,N'*-diacetylchitobiose) and 2-Acetamido-6-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-D-glucopyranose

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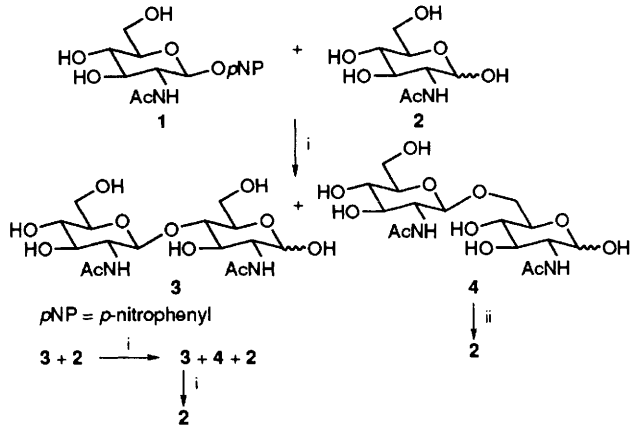
During transfer of the *N*-acetyl-β-D-glucosaminyl (2-acetamido-2-deoxy-β-D-glucopyranosyl) residue from *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide (*p*-nitrophenyl *N*-2-acetamido-2-deoxy-β-D-glucopyranoside) on to *N*-acetyl-D-glucosamine (2-acetamido-2-deoxy-D-glucopyranose) catalysed by the *N*-acetylhexosaminidase from *Aspergillus oryzae*, the major isomer formed was found to depend on the time course of the reaction, 1 → 4 transfer predominating while the *p*-nitrophenyl glycoside was available as donor, but 1 → 6 transfer when the initially-formed 1 → 4 product took over as donor, results that could be interpreted in terms of a constant regioselectivity modulated by selective hydrolysis of the products.

The need to develop increasingly efficient methods of oligosaccharide synthesis, stimulated by recent important developments in glycobiology, has aroused considerable interest in enzymatic methods. Of the two possible basic approaches, using either the biosynthetic oligosaccharide synthetases or the 'reverse hydrolytic' activity of glycosidases,[1–3] the latter has the merit of simplicity and the attraction of using enzymes that are usually stable and often commercially available. Glycosidase-catalysed glycosyl transfer has several advantages over conventional procedures: full control over the configuration at the anomeric centre and the lack of any requirement for protection–deprotection sequences. The most significant problem encountered is lack of regioselectivity. However, the method described below provides an efficient procedure for the synthesis of *N*-acetyl-β-D-glucosamine disaccharides, in which minor products formed in the glycosidase-catalysed reaction are removed by selective enzymatic hydrolysis. In this way, the problem of separating a mixture of regioisomeric disaccharides is converted into the much easier task of isolating a single disaccharide from a mixture with the corresponding monosaccharide.

The *N*-acetylhexosaminidase of *Aspergillus oryzae* has been shown to be an efficient catalyst of *N*-acetyl-β-D-hexosaminyl transfer on to glycosides of glucose and *N*-acetyl-D-glucosamine.[4,5] In extending these studies to *N*-acetyl-β-D-glucosaminyl transfer on to *N*-acetyl-D-glucosamine, a regioselective initial transfer was observed from *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide **1** to the C-4 hydroxy group of *N*-acetyl-D-glucosamine **2** to give the disaccharide **3** (*N,N'*-diacetylchitobiose) the core disaccharide of *N*-linked glycoproteins.[6] The only other transfer product observed was the corresponding (1 → 6) disaccharide **4** formed at 10% of the total transfer rate. (This disaccharide represents the core disaccharide of lipid A[7]). After the donor **1** had been consumed, the disaccharide profile continued to evolve with a relative increase in the concentration of the initial minor product **4**. At the point of disappearance of the glycosyl donor **1**, the ratio of (1 → 4) to (1 → 6) transfer products was 9 : 1. At the extremum during continued evolution of the disaccharide mixture, this ratio was reversed to 8 : 92.

These results are interpretable as shown in Scheme 1.[3] It is assumed only that regioselectivity of glycosyl transfer favours formation of the (1 → 4) product **3** over the (1 → 6) product **4** throughout, by an overall rate factor of approximately 9 : 1. Thus in the first stage, defined as the time interval in which the donor **1** was still present, the (1 → 4) transfer product **3** accumulated together with the (1 → 6) disaccharide **4**. The latter constituted 10% of the disaccharide product at the end of this stage. To obtain the (1 → 4) product **3**, the nitrophenol was removed and the remaining mixture was separated into mono- and di-saccharide fractions on a charcoal–celite column. The disaccharide mixture was incubated with the *N*-acetylhexosaminidase from *Canavalia ensiformis* (Jack bean) which selectively hydrolysed the (1 → 6) isomer **4**. When all of the (1 → 6) product **4** had been hydrolysed, the remaining (1 → 4) isomer **3** was isolated using a charcoal–celite column as before, to give the pure *N,N'*-diacetylchitobiose **3** in 55% yield (0.68 g from 1.00 g of the glycosyl donor **1**), (Scheme 1).

If the reaction was allowed to evolve past the end of the first stage, the concentration of the (1 → 6) product **4** was found to increase, both in absolute terms and relative to the concentration of the (1 → 4) product **3**. This ratio reached a maximum value of 92 : 8. This result can be explained if it assumed that during this second stage, the accumulated (1 → 4) product **3** acted as glycosyl donor, transferring an *N*-acetyl-β-D-glucosaminyl residue to the C-4 hydroxy group of *N*-acetyl-D-glucosamine in a degenerate reaction, and to the C-6 hydroxy group to increase the overall relative and absolute concentration of the (1 → 6) disaccharide **4** (Scheme 1). At the same time, both disaccharides **3** and **4** were undergoing hydrolysis to the monosaccharide **2**, the (1 → 4) product **3** at a faster rate than the (1 → 6) disaccharide **4**. The net effect was the observed increase in concentration, both relative and absolute, of the (1 → 6) disaccharide **4**. When the reaction was terminated at the point of the maximum ratio (**4** : **3**) of the disaccharides, the disaccharide fraction could be isolated by charcoal–celite chromatography as before. The disaccharide fraction was then subjected to hydrolysis catalysed by the *N*-acetylhexosaminidase from *A. oryzae*, which selectively

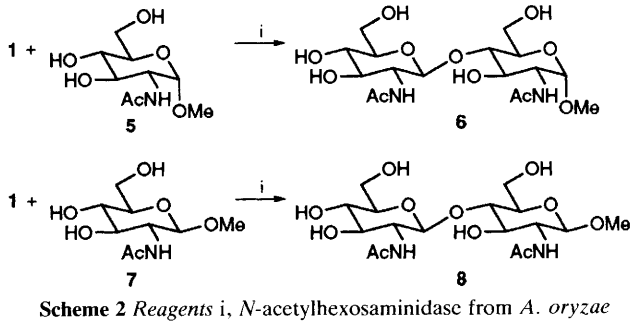


Scheme 1 *Reagents and conditions*: *N*-acetylhexosaminidases from: i, *A. oryzae*; ii, Jack bean. The first enzyme was an ammonium sulfate fraction (85%) of the β-galactosidase from *Aspergillus oryzae* (Sigma). The *N*-acetylhexosaminidase activity of the crude enzyme was 0.011 U mg^{-1} (0.116 U mg^{-1} protein). After ammonium sulfate precipitation and dialysis, the activity was 0.199 U mg^{-1}. A typical reaction was carried out using *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide (7.6×10^{-2} mmol dm^{-3}) and *N*-acetyl-D-glucosamine ($7.72.10^{-1}$ mmol dm^{-3}) in phosphate buffer (0.04 mol dm^{-3}, pH 6.5) at 30 °C.

hydrolysed the (1 → 4) product **3**. The reaction was monitored by HPLC and was terminated at the point of disappearance of the (1 → 4) disaccharide **3**. The sole remaining disaccharide **4** was isolated by charcoal–celite chromatography as before, in 22% yield (136 mg from 0.5 g of the donor **1**). This synthesis was more efficient than direct synthesis from *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide and *N*-acetyl-D-glucosamine using the Jack bean enzyme. Although in this procedure the (1 → 6) product **4** was the sole disaccharide formed, the yield (5%) was much lower than that obtained using the enzyme from *A. oryzae*.

In these reactions, the acceptor, *N*-acetyl-D-glucosamine, was initially present in ten-fold excess. However, this excess was quantitatively recovered from the charcoal–celite column.

The previously observed influence of the nature and stereochemistry of the substituent at the anomeric centre[3–5] was clearly illustrated by parallel experiments in which methyl *N*-acetyl-α- and β-D-glucosaminides were used as acceptors. Methyl *N*-acetyl-α-D-glucosaminide **5** gave the corresponding disaccharide **6** as the only detectable disaccharide product, isolated in 55% yield (Scheme 2). The β-anomer **7** gave a mixture of the corresponding (1 → 4) product **8** (Scheme 2) and an unidentified product in a ratio of 2:1. The (1 → 4) disaccharide **8** was isolated in 24% yield by HPLC. Thus, as previously observed, the α-configuration at the anomeric centre favoured (1 → 4) transfer.



Scheme 2 *Reagents* i, *N*-acetylhexosaminidase from *A. oryzae*

The observation that the overall regioselectivity of glycosidase-catalysed glycosyl transfer is kinetically controlled[3] adds a new dimension to disaccharide synthesis by this procedure. Provided that straightforward methods are available for isolating and purifying the major product, as in the present examples, the glycosidase-catalysed method acquires extra flexibility. The use of the readily available and inexpensive *N*-acetylhexosaminidase from *A. oryzae* thus makes possible the preparation of a variety of disaccharides[4,5] from the easily prepared glycosyl donor **1** in a one-step process that compares favourably in efficiency with multistep non-enzymatic procedures. The availability of the disaccharides **3** and **4** has made it possible, in turn, to study their activities as substrates for *N*-acetyl-β-D-glucosaminyl transfer. Both disaccharides appear to be excellent acceptors.

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