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CARBOHYDRATE RESEARCH

Note

Enzymatic synthesis of 2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-2-deoxy-D-glucopyranose and 2-acetamido-6-*O*-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-2-deoxy-D-glucopyranose catalysed by the β-*N*-acetylhexosaminidase from *Aspergillus oryzae*

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We recently described the enzymatic synthesis of the *N*-acetyl-D-glucosamine disaccharides GlcNAc(β1-4)GlcNAc (di-*N*-acetylchitobiose, **3**) and GlcNAc(β1-6)GlcNAc (**4**) [1,2]. The synthesis was based on *N*-acetyl-D-glucosaminyl transfer from *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**1**) to *N*-acetyl-D-glucosamine (**2**) catalysed by the *N*-acetylhexosaminidase from *Aspergillus oryzae*, a cheap enzyme readily available as a minor activity in the commercially available β-galactosidase from *A. oryzae* (Scheme 1). Formation of the two disaccharides was kinetically controlled. By monitoring the evolution of the disaccharide mixture, products could be isolated at times when either the (1 → 4)- or the (1 → 6)-disaccharide predominated.

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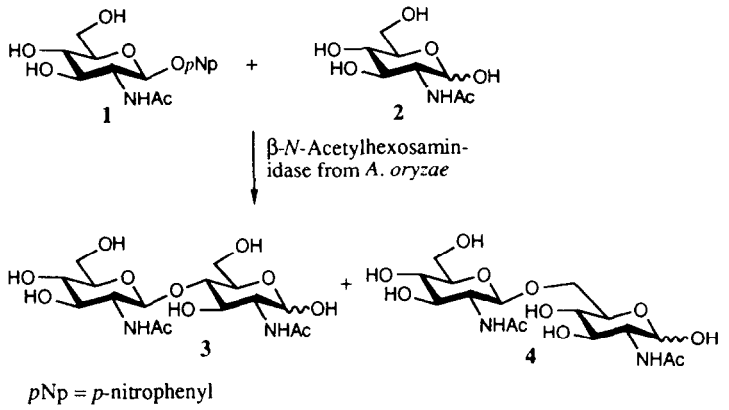
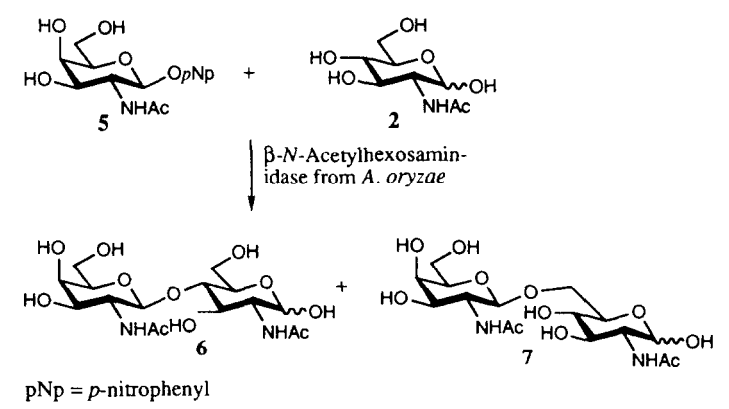


Scheme 1.

It is now found that, following the same procedure, the corresponding GalNAcGlcNAc disaccharides can be prepared in even higher yields than the GlcNAcGlcNAc disaccharides. Thus *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside (**5**) was incubated with **2** in the presence of the β-*N*-acetylhexosaminidase from *Aspergillus oryzae* (Scheme 2). After 56 h, it was found by HPLC that the ratio of the (1 → 4)- and (1 → 6)-disaccharides, **6** and **7**, respectively, was 92:8. Traces (< 2%) of the corresponding GlcNAcGlcNAc dimers were also formed. The disaccharide fraction was separated from other products by charcoal–Celite chromatography. It was then incubated with the β-*N*-acetylhexosaminidase from Jack bean (*Canavalia ensiformis*) which selectively hydrolysed the minor (1 → 6)-component GalNAc(1-6)GlcNAc (**7**), and the GlcNAc(β1-4)GlcNAc (**3**) and GlcNAc(β1-6)GlcNAc (**4**) by-products. The only remaining disaccharide in the mixture, GalNAc(β1-4)GlcNAc (**6**), was isolated by charcoal–Celite chromatography as before in 72% yield. This yield is based on the glycosyl donor **5**. When the reaction was allowed to continue for 20 days, it was found



Scheme 2.

by HPLC that the product mixture had evolved to contain a mixture of **6** and **7** in a ratio of 14:86. However, the mixture also contained higher levels (18% of the disaccharide fraction) of the GlcNAcGlcNAc disaccharides **3** and **4** in a ratio of 24:76. The disaccharide mixture was isolated as before and incubated again with the β-*N*-acetylhexosaminidase from *A. oryzae* for 40 h. This brought about selective hydrolysis of the minor components **6** and **3**. The reaction was stopped by boiling the incubation mixture briefly. Hydrolysis was then continued using the β-*N*-acetylhexosaminidase from Jack bean for 24 h. This enzyme is principally a β-*N*-acetylglucosaminidase and catalysed the selective hydrolysis of the remaining GlcNAc(β1-6)GlcNAc by-product. The remaining disaccharide, GalNAc(β1-6)GlcNAc (**7**), which is only slowly hydrolysed by the Jack bean enzyme, was isolated by charcoal–Celite chromatography in 33% yield.

By these simple procedures, the disaccharides **6** and **7** are readily prepared. The method is readily adapted to scale-up to multigramme levels, as we have demonstrated [2] for the GlcNAcGlcNAc disaccharides **3** and **4**.

The formation of **3** and **4** can be attributed to 'reverse hydrolysis', in which GlcNAc (**2**) itself acts as glycosyl donor. This reaction occurs at a relatively low rate but attains significance during production of GalNAc(β1-6)GlcNAc (**7**) over the long (20 days) incubation times involved.

1. 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. High-resolution mass spectra were determined on a VG Analytical ZAB-E mass spectrometer. Optical rotations were determined using an AA-1000 polarimeter (Optical Activity Ltd). with a 2-dm cell. β-Galactosidase from *Aspergillus oryzae* and β-*N*-acetylhexosaminidase (Jack bean) were obtained from the Sigma Chemical Company. For the preparative experiments described below, an ammonium sulfate fraction of the β-galactosidase was prepared as previously described [2]. Celite 535 was obtained from Fluka, and activated charcoal (Darco G-60, 100 mesh) and *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside were 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 (10:9:2). 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 (aminopropylsilica) column (20 × 4.6 mm) with UV detection at 210 nm and 4:1 MeCN–H_2O as eluant at a flow rate of 1 mL min^{-1}.

2-Acetamido-4-O-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-2-deoxy-D-glucopyranose (**6**).—*p*-Nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside (**5**) (0.4 g, 1.17 mmol) and *N*-acetyl-D-glucosamine (**2**) (2.56 g, 11.57 mmol) were suspended in citrate–phosphate buffer (0.05 M, pH 4.5, 10 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 mL, 242.5 mg protein/mL, 5.84×10^{-3} U/mg protein) was added to the reaction mixture which was incubated at 30 °C for 56 h. By HPLC it was determined that the ratio of **6** to

GalNAc(β1-6)GlcNAc (**7**) was 92:8 (more than 98%) and that GlcNAc(β1-4)GlcNAc (**3**) and GlcNAc(β1-6)GlcNAc (**4**) together represented less than 2% of the product mixture. The reaction was stopped by heating the mixture in a boiling water bath for 5 min. It was then applied to a charcoal–Celite column as previously described [2]. The column was eluted first with 5:95 EtOH–water to remove the monosaccharides and then with 10:90 EtOH–water to recover the disaccharide mixture. The fraction containing the disaccharides was evaporated to dryness under reduced pressure. The residue was redissolved in phosphate buffer (0.04 M, pH 6.5, 6 mL) and incubated with the β-*N*-acetylhexosaminidase from Jack bean (0.3 mL, 1 mg protein/mL, 56 U/mg protein) at 30 °C for 60 h to hydrolyse **7** and traces of **3** and **4**. The reaction was stopped by heating in the boiling water bath for 5 min. The disaccharide fraction was isolated by charcoal–Celite chromatography as above to give **6** (0.352 g, 72%); $[\alpha]_D^{25}$ +42.3° (*c* 0.52, H_2O); ^1H NMR (D_2O): δ 1.97 (s, 3 H, Me), 2.00 (s, 3 H, Me), 3.44–3.90 (m, 12 H), 4.46 (d, $J_{1',2'}$ 8.40 Hz, H-1′, β-anomer), 4.47 (d, $J_{1',2'}$ 8.44 Hz, H-1′, α-anomer), 4.64 (d, $J_{1,2}$ 8.28 Hz, H-1, β-anomer), 5.13 (d, $J_{1,2}$ 2.92 Hz, H-1, α-anomer); ^{13}C NMR (D_2O): δ 22.55 (Me, reducing end α-anomer), 22.85 (Me, non-reducing end β-anomer), 53.24 (C-2′), 54.22 (C-2, α-anomer), 56.64 (C-2, β-anomer), 60.71 (C-6, α-anomer), 60.84 (C-6, β-anomer), 61.62 (C-6′), 68.28 (C-4′), 70.00 (C-3, α-anomer), 70.64 (C-5, α-anomer), 71.36 (C-3′), 73.24 (C-3, β-anomer), 75.25 (C-5, β-anomer), 76.01 (C-5′), 79.72 (C-4, β-anomer), 80.20 (C-4, α-anomer), 91.09 (C-1, α-anomer), 95.51 (C-1, β-anomer), 102.40 (C-1′), 175.11 (C=O, reducing end, α-anomer), 175.38 (C=O, reducing end, β-anomer), 175.43 (C=O, non-reducing end). FABMS: Found *m/z* 447.1601 $(M + Na)^+$; $C_{16}H_{28}N_2O_{11}$ requires 447.1591.

*2-Acetamido-6-*O*-(2-acetamido-2-deoxy-*β*-*D*-galactopyranosyl)-2-deoxy-*D*-glucopyranose* (**7**).—Glycoside **5** (0.4 g, 1.17 mmol) and **2** (2.56 g, 11.57 mmol) were suspended in citrate–phosphate buffer (0.05 M, pH 4.5, 10 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 mL, 242.5 mg protein/mL, 5.84×10^{-3} U/mg protein) was added to the mixture which was incubated at 30 °C for 20 days. By HPLC it was determined that the ratio of **6** to **7** was 14:86 (82% of product mixture). GlcNAc(β1-4)GlcNAc (**3**) and GlcNAc(β1-6)GlcNAc (**4**) (24:76) were also present, representing 18% of the product mixture. The reaction was stopped by heating the mixture in a boiling water bath for 5 min. It was then applied to a charcoal–Celite column as before. The column was eluted first with 5:95 EtOH–water to remove the monosaccharides and then with 10:90 EtOH–water to recover the disaccharide mixture. The disaccharide fraction was evaporated to dryness under reduced pressure. The residue was redissolved in phosphate buffer (0.04 M, pH 6.5, 6 mL) and incubated with the β-*N*-acetyhexosaminidase from *A. oryzae* (0.2 mL, as above) at 30 °C for 40 h to hydrolyse **6** and **3**. The reaction was stopped by heating the mixture in a boiling water bath for 5 min. The solution was then incubated with the β-*N*-acetylhexosaminidase from Jack bean (0.2 mL, as above) at 30 °C for 24 h to hydrolyse **4**. The disaccharide fraction was purified by charcoal–Celite chromatography as above to give **7** (0.166 g, 33%); $[\alpha]_D^{25}$ +30.36° (*c* 0.49, H_2O); ^1H NMR (D_2O): δ 1.97 (s, 3 H, Me), 1.99 (s, 3 H, Me), 3.32–3.90 (m, 11 H), 4.04–4.14 (m, 1 H), 4.42 (d, $J_{1',2'}$ 8.48 Hz, H-1′, α-anomer), 4.43 (d, $J_{1',2'}$ 8.48 Hz, H-1′, β-anomer), 4.62 (d, $J_{1,2}$ 8.40 Hz, H-1, β-anomer), 5.11 (d, $J_{1,2}$ 3.48 Hz, H-1,

α-anomer); ^{13}C NMR (D_2O): δ 22.53 (Me), 22.81 (Me), 22.93 (Me), 53.02 (C-2′), 54.68 (C-2, α-anomer), 57.34 (C-2, β-anomer), 61.66 (C-6′), 68.49 (C-4′), 69.12 (C-6, β-anomer), 69.36 (C-6, α-anomer), 70.54 (C-4′, β-anomer), 70.71 (C-4, α-anomer), 71.13 (C-3, α-anomer), 71.35 (C-5, α-anomer), 71.57 (C-3′), 74.55 (C-3, β-anomer), 75.52 (C-5, β-anomer), 75.79 (C-5′), 91.51 (C-1, α-anomer), 95.63 (C-1, β-anomer), 102.85 (C-1′), 102.93 (C-1′), 175.14 (C=O), 175.39 (C=O), 175.50 (C=O), 175.56 (C=O). FABMS: Found m/z 447.1593 $(M + Na)^+$; $C_{16}H_{28}N_2O_{11}$ requires 447.1591.

Acknowledgements

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