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# Trisaccharide synthesis by glycosyl transfer from *p*-nitrophenyl β-D-*N*-acetylgalactosaminide on to disaccharide acceptors catalysed by the β-*N*-acetylhexosaminidase from *Aspergillus oryzae*

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**Abstract**

The β-*N*-acetylhexosaminidase from *Aspergillus oryzae* catalysed the transfer of β-D-*N*-acetylgalactosaminyl residues from *p*-nitrophenyl β-D-*N*-acetylglucosaminide on to disaccharide acceptors consisting of thioethyl glycosides of α-D-Glc-(1 → 4)-β-D-Glc, β-D-Glc-(1 → 4)-β-D-Glc and β-D-Glc-(1 → 6)-β-D-Glc. The principle of 'anomeric control' was exemplified by the results which showed that an α-linkage between the units of the acceptor favoured exclusively the formation of a new (1 → 4)-linkage, whereas the β-configuration in the acceptor led to a mixture of (1 → 4)- and (1 → 3)-linked products, as observed for simple glycosides of monosaccharide acceptors. With the thioethyl β-lactoside as acceptor, β-D-Gal-(1 → 6)-β-D-Gal-(1 → 4)-β-D-GlcSEt was formed, owing to the action of residual β-D-galactosidase activity in the *N*-acetylhexosaminidase on the thioethyl β-lactoside acting as both donor and acceptor. © 1998 Elsevier Science Ltd.

*Keywords:* *N*-Acetylhexosaminidase; Trisaccharides; *N*-Acetylgalactosaminide transfer

## 1. Introduction

Glycosidase-catalysed glycosyl transfer has proven to be very effective in oligosaccharide synthesis [1]. The application of this method is limited only by the availability of glycosidases with the appropriate activity (high rate of glycosyl transfer to the carbohydrate acceptor relative to the rate of transfer to water). Of the enzymes described up until the present, the *N*-acetylhexosaminidase from *Aspergillus oryzae* has proven to be particularly effective [2–9]. It is able to catalyse the transfer of both *N*-acetylglucosaminyl and *N*-acetylgalactosaminyl residues to a variety of acceptors giving products exclusively with the β-configuration. In studies of disaccharide synthesis, it was observed that with alkyl glycosides as acceptors, the regiochemistry of glycosyl transfer was dependent on the configuration at the anomeric centre in the acceptor. This 'anomeric control', first

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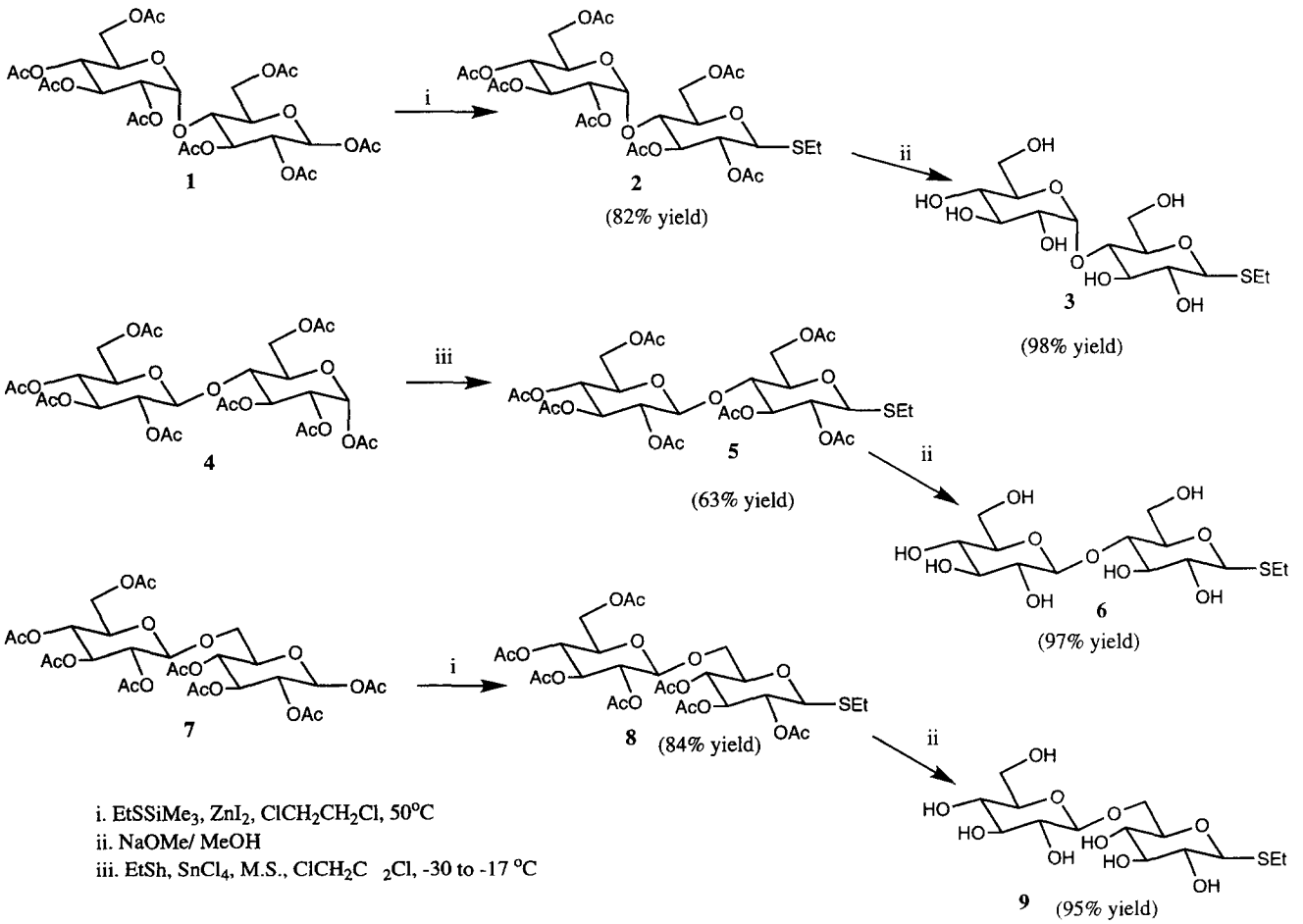
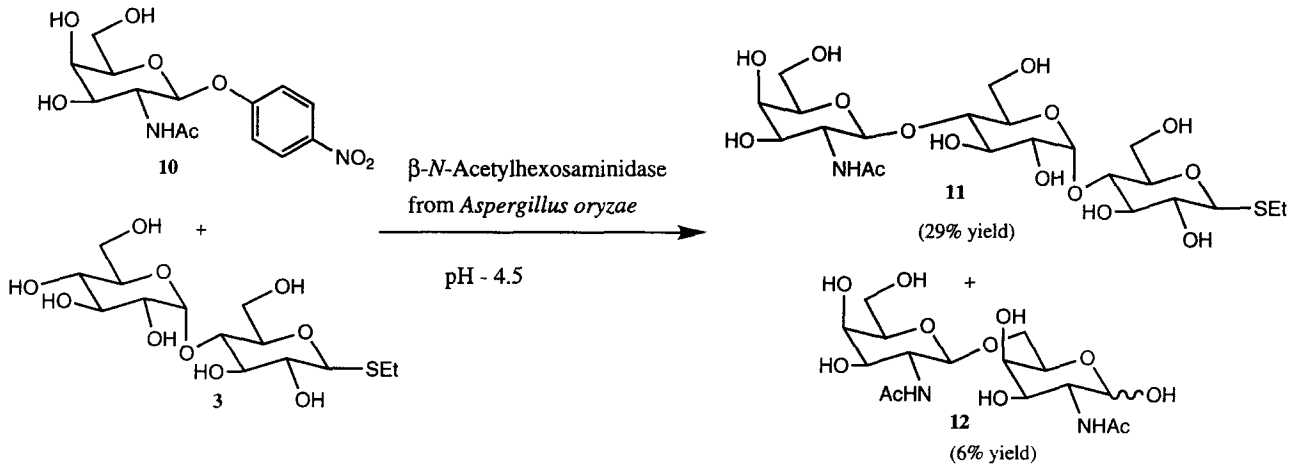
described by Nilsson [10], was very striking [2] making it possible to bias transfer either to C-4 or to C-3 in the acceptor by altering the anomeric configuration of the acceptor from $\beta$ to $\alpha$. (Concomitant transfer to the 6-OH in the acceptor was also observed.) Indications from experiments in which transfer was made to the same monosaccharide residue but in either a mono- or disaccharide acceptor indicated that in general, transfer was more effective when the acceptor was a component of a disaccharide. This was attributed to the better recognition of disaccharide acceptors relative to monosaccharide acceptors and indicated that the acceptor binding site on the enzyme accommodated at least a disaccharide derivative. Against this background we undertook a study of glycosyl transfer onto disaccharide acceptors. It was expected that transfer would be more effective than with monosaccharide acceptors. It was also of interest to determine if the principle of anomeric control extended to disaccharide acceptors in which the alkyl groups of monosaccharide glycosides were replaced by monosaccharide units. As acceptors, the $\beta$-thioethyl glycosides of maltose, cellobiose and gentiobiose (**3**, **6** and **9**, respectively) were prepared. The thioethyl glycosides were used so that products could be used via conventional thioglycoside activation as building blocks for the synthesis of larger oligosaccharides.

## 2. Results and discussion

The $\beta$-thioethyl glycosides were prepared directly from the peracetates of the chosen disaccharides in good to excellent yield (Scheme 1). As a donor, the *p*-nitrophenyl $\beta$-glycoside of GalNAc was used. (Previous experience had shown that the results obtained for GlcNAc and *N*-acetylgalactosaminyl transfers were very similar.) With the maltose thioglycoside as acceptor, the (1 → 4)-linked trisaccharide was produced, together with a small amount of the disaccharide obtained by GalNAc transfer onto hydrolysed




Scheme 1.

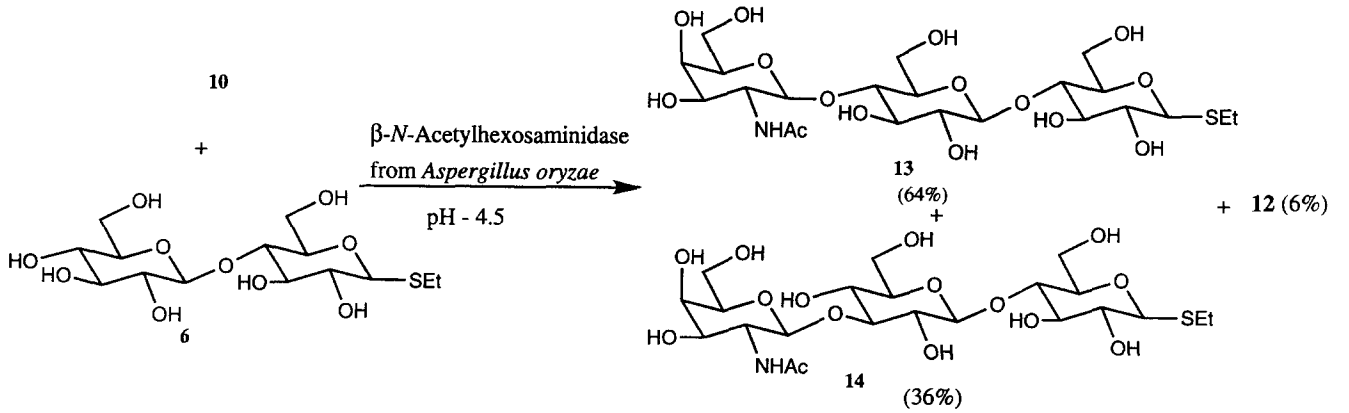



Scheme 2.

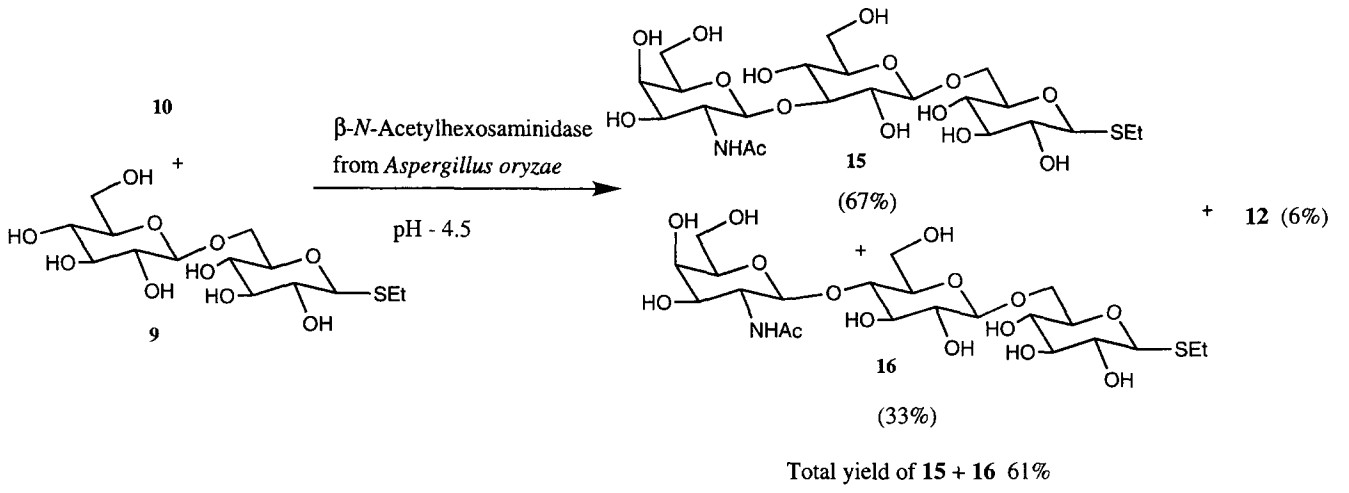
donor (Scheme 2). This was consistent with the results obtained with simple glycosides in which the α-configuration in the acceptor was found to favour transfer to the 4-OH group. None of the (1 → 3)-linked trisaccharides was detected. The major, (1 → 4)-linked, product was obtained in 29% yield and was readily separated from the minor disaccharide product by charcoal–Celite chromatography. With the maltose thioglycoside as acceptor, a mixture of (1 → 4)-linked and (1 → 3)-linked trisaccharides was obtained in 48% overall yield (Scheme 3). Although the (1 → 4)-linked product predominated, the formation of a significant amount of (1 → 3)-linked trisaccharides was consistent with the anomeric control observed with monosaccharide glycosides. The isomeric products were readily separated by charcoal–Celite chromatography.

Transfer to the gentiobiose thioglycoside was similarly expected to give a mixture of (1 → 4)- and (1 → 3)-linked products since the internal glycosidic linkage has the β-configuration. This was borne out by the experiment, in which a mixture of (1 → 3)- and (1 → 4)-linked trisaccharides was formed in 61% overall yield with the (1 → 3)-linked product predominating (Scheme 4). Again, the products were separated by charcoal–Celite chromatography.

A further experiment was attempted in which the β-thioethyl glycoside (**17**) of lactose [11] was used as acceptor. The result of this experiment illustrated an important factor that has to be taken into account when glycosidase-catalysed synthesis is attempted with acceptors larger than monosaccharides or monosaccharide glycosides—namely the possible intervention of competing activities in incompletely purified enzymes. The β-*N*-acetylhexosaminidase used in the present experiments is isolated as a minor component of the commercially available β-galactosidase of *A. oryzae*. Although the enzyme had been extensively purified, it contained residual β-galactosidase activity that accepted the intended acceptor as




Scheme 3.

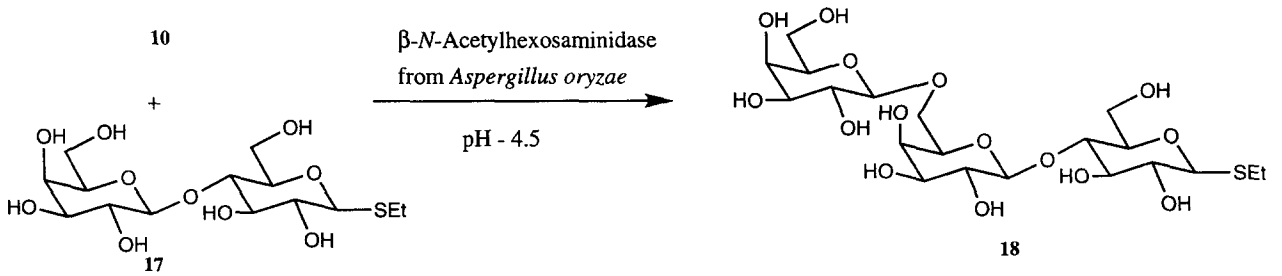



Scheme 4.

both donor and acceptor to give the product of $1 \rightarrow 6$ transfer of a β-galactosyl residue onto the lactose thioglycoside (Scheme 5). This was the sole product apart from a minor amount of an unidentified compound. The principle of anomeric control is clearly valid as a qualitative guide to the regiochemistry of glycosyl transfer catalysed by the *N*-acetylhexosaminidase. However, it does not apply when the acceptor has a GlcNAc residue at the non-reducing terminus since in these cases exclusive transfer to the 4-position is observed in the synthesis of oligomers of *N*-acetylglucosamine up to and including the hexamer. [5,6]

Because structural information is not available for the *N*-acetylhexosaminidase, it is not possible to interpret the regioselectivity of glycosyl transfer by docking studies. However, a qualitative inspection of energy minimised (PCMODEL) structures of α-and β-Glc (taken as models of corresponding glycosides and disaccharides) shows that if the $^4C_1$ conformation of α-D-glucose is compared with the $^3C_O$ conformation of β-D-Glc, the arrangement of hydroxyl oxygen atoms is very similar. Two views (Chem 3D) of these two conformations are shown in Fig. 1. Views (a) and (c) are of the α-anomer and views (b) and (d) are corresponding representations of the β-anomer. The arrangement of *O*-2,3,4,6 in the $^4C_1$ conformation of the α-anomer (Fig. 1a,c) corresponds rather closely to the arrangement of *O*-6,4,3,2, respectively, in the $^3C_O$ conformation of the β-anomer (Fig. 1b,d). The oxygen–oxygen distances are rather similar [O $(2 \rightarrow 3) = 2.89$ Å, O $(3 \rightarrow 4) = 2.88$ Å, O $(4 \rightarrow 6) = 2.68$ Å]. Accordingly, it is possible that with minor adjustments similar binding modes at the active site might be possible for the α-anomer in the $^4C_1$ conformation and the β-anomer in the $^3C_O$ conformation. In these conformations O-4 in the α-anomer and O-3 in the β-anomer occupy similar positions. Given that the position occupied by these atoms places them at the correct point for nucleophilic attack on the glycosyl–enzyme intermediate, the switch in regioselectivity between the α-and β-anomers is explained. More detailed information on the active site architecture will be required before it is possible to say why a switch from the α-to the β-configuration requires the substrate to be bound in these alternative conformations.

The syntheses described above show that the glycosidase-catalysed method is capable of ready extension to the preparation of trisaccharides to give prod-




Scheme 5.

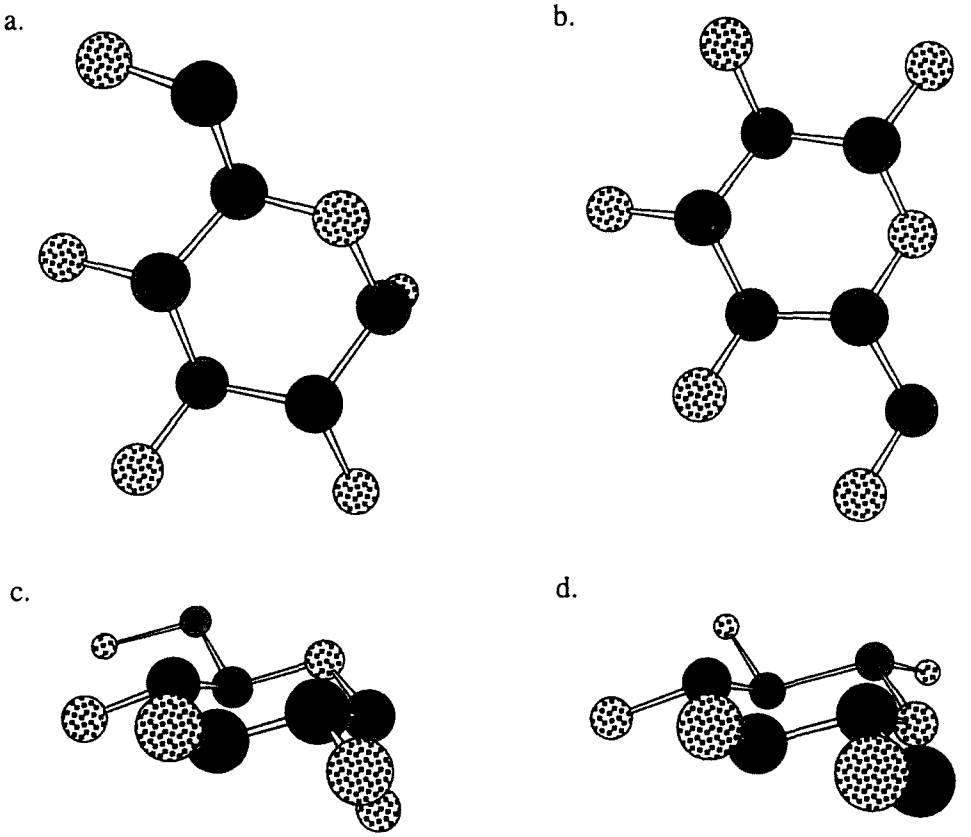



Fig. 1. Views of the $^4C_1$ (a), (c) and $^3C_O$ (b), (d) conformations of α-D-glucopyranose and β-D-glucopyranose respectively.

ucts analogous to those obtained in glycosyl transfers to corresponding monosaccharides. This lends weight to the prediction that glycosidases can be regarded as a toolkit for oligosaccharide synthesis with specificities determined principally by the nature of the terminal disaccharide component in acceptors equal to or larger than disaccharides or disaccharide glycosides.

## 3. Experimental

*General.*—$^1$H NMR spectra were determined at 250 MHz or 400 MHz using either Bruker AC 250 or WH 400 spectrometers respectively. $^{13}$C NMR spectra were determined at either 62.89 or 100.62 MHz using the same instruments. Mass spectra were determined with a Bruker 9.4 T BioApex FTICR mass spectrometer (electrospray source). Optical rotations were determined using an AA-1000 polarimeter (Optical Activity) with a 2-dm cell. Optical rotations are given in units of $10^{-1}$ deg cm$^2$ g$^{-1}$. β-Galactosidase from *A. oryzae* was obtained from Sigma Chemical. Celite 535 was obtained from Fluka and activated charcoal (Darco G-60, 100 mesh) was obtained from Aldrich Chemical. Thin layer chromatography 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. Silica Gel 60 for column chromatography was obtained from Merck.

*Ethyl 4-O-(2, 3, 4, 6-tetra-O-acetyl-α-D-glucopyranosyl)-(1 → 4)-2,3,6-tri-O-acetyl-1-thio-β-D-glucopyranoside* **2**.—β-D-Maltose octaacetate **1** (8 g, 11.8 mmol) was dissolved in 1,2-dichloroethane (150 mL), containing molecular sieves 4 Å (8 g) and the mixture was stirred at −30 °C under nitrogen gas. Ethanethiol (1.44 g, 23.8 mmol) was added and temperature was maintained at −30 °C for 1 h. Tin(IV) chloride (3.55 g, 13.62 mmol) was added and the temperature was slowly brought to −17 °C (during 1.1 h). The reaction was quenched by the addition of saturated $NaHCO_3$ (120 mL). The mixture was stirred for 10 min and the layers were separated. The aqueous layer was extracted with dichloromethane (3 × 80 mL). The organic layer was washed with water (2 × 100 mL), dried ($MgSO_4$) and evaporated under reduced pressure. The residue was purified by chromatography over silicon dioxide eluting with ethyl acetate:toluene (1:3) to give **2** as a colourless solid (6.75 g, 84%); $[\alpha]_D^{24}$ +46.9° (*c* 2.59, $CHCl_3$); $^1$H NMR ($D_2O$): δ 1.22 (t, 3 H, *J* 7.44 Hz,

$SCH_2CH_3$), 1.95, 1.96, 1.97, 1.98, 2.00, 2.06 and 2.08 (each s, 3 H, $COCH_3$), 2.56–2.71 (m, 2 H, $SCH_2CH_3$), 3.62–3.67 (m, 1 H, H-5), 3.88–3.96 (m, 2 H, H-4 and H-5′), 4.00 (dd, 1 H, *J* 12.40 and 2.12 Hz, H-6′a), 4.15–4.21 (m, 2 H, H-6b and H-6′b), 4.14 (dd, 1 H, *J* 12.12 and 2.64 Hz, H-6a), 4.50 (d, 1 H, *J* 10.08 Hz, H-1), 4.79–4.84 (m, 2 H, H-2 and H-2′), 5.00 (dd, 1 H, *J* 10.04 and 9.68 Hz, H-4′), 5.24 (t, 1 H, *J* 9 Hz, H-3), 5.30 (dd, 1 H, *J* 10.32 and 9.72 Hz, H-3′), 5.36 (d, 1 H, *J* 4 Hz, H-1′); $^{13}$C NMR ($D_2O$): δ 14.76, 20.39 (3 × $CH_3$), 20.49 (2 × $CH_3$), 20.63, 20.72, 24.13, 61.32, 62.87, 67.82, 68.34, 69.11, 69.78, 70.60, 72.54, 75.90, 76.22, 82.93, 95.39, 169.22, 169.44, 169.72, 169.94, 170.23, 170.32, 170.36; HRMS: Found *m/z* 703.1881. $C_{28}H_{40}NaO_{17}S$ requires 703.1878.

*Ethyl (α-*D*-glucopyranosyl)-(1 → 4)-1-thio-β-*D*-glucopyranoside* **3**.—To a solution of acetate **2** (5 g, 7.35 mmol) in dry methanol (60 mL) was added NaOMe (1 M, 3mL). The mixture was stirred at room temperature for 4 h, neutralised with Amberlyst 15, filtered and evaporated under reduced pressure. The residue was purified over activated carbon: Celite (1:1) eluting with 20% ethanol to give **2** as a colourless solid (2.75 g, 98%); $[\alpha]_D^{24}$ +60.51° (*c* 1.56, $H_2O$); $^1$H NMR ($D_2O$): δ 1.27 (t, 3 H, *J* 7.25 Hz, $SCH_2CH_3$), 2.64–2.86 (m, 2 H, $SCH_2CH_3$), 3.30–3.43 (m, H), 3.54–3.93 (m, H), 4.54 (d, 1 H, *J* 9.88 Hz, H-1), 5.39 (d, 1 H, *J* 3.80 Hz, H-1′); $^{13}$C NMR ($D_2O$): δ 15.43, 25.03, 61.37, 61.76, 70.21, 72.57, 72.96, 73.58, 73.73, 77.60, 78.54, 79.30, 85.78, 100.49; HRMS: Found *m/z* 409.1145. $C_{14}H_{26}NaO_{10}S$ requires 409.1139.

*Ethyl (2,3,4, 6-tetra-*O*-acetyl-β-*D*-glucopyranosyl)-(1 → 4)-2,3,6-tri-*O*-acetyl-1-thio-β-*D*-glucopyranoside* **5**.—A solution of α-D-cellobiose octaacetate **4** (8 g, 11.8 mmol) in 1,2-dichloroethane (150 mL) was heated at 50 °C. Zinc iodide (26.3 g) and ethylthiotrimethylethylsilane (7.64 mL, 47.2 mmol) were added and the reaction mixture was stirred at 50 °C for 7 h. The reaction mixture was filtered through a pad of Celite and washed with saturated $NaHCO_3$ (2 × 100 mL) and water (2 × 75 mL). The organic layer was dried ($MgSO_4$), filtered and evaporated under reduced pressure. The residue was purified by chromatography over silicon dioxide with ethyl acetate:toluene (3:7) as eluent. Crystallisation from chloroform:light petroleum gave **3** (5.02 g, 63%); $[\alpha]_D^{24}$ −28.74° (*c* 1.26, $CHCl_3$); $^1$H NMR ($D_2O$): δ 1.24 (t, 3 H, *J* 7.44 Hz, $SCH_2CH_3$), 1.96, 1.99, 2.00, 2.01, 2.03, 2.07 and 2.10 (all s, 3 H, $COCH_3$), 2.58–2.73 (m, 2 H, $SCH_2CH_3$), 3.59 (ddd, 1 H, *J* 1.96, 5.32 and 9.94 Hz, H-5), 3.63 (ddd, 1 H, *J* 2.32, 4.30 and 9.80 Hz, H-5′), 3.74 (dd, 1 H, *J* 9.44 and 9.64 Hz, H-4), 4.02 (dd, 1 H, *J* 2.24 and 12.64 Hz, H-6′a), 4.07 (dd, 1 H, *J* 5.36 and 12 Hz, H-6′b), 4.35 (dd, 1 H, *J* 4.36 and 12.64 Hz, H-6′b), 4.45 (d, 1 H, *J* 14.60 Hz, H-1), 4.48 (d, 1 H, *J* 12.36 Hz, H-1′), 4.49 (dd, 1 H, *J* 2.12 and 2.64 Hz, H-6a), 4.88–4.95 (m, 2 H, H-2 and H-2′), 5.04 (dd, 1 H, *J* 9.52 and 9.64 Hz, H-3′), 5.12 (dd, 1 H, *J* 9.24 and 9.36 Hz, H-4′), 5.17 (t, 1 H, *J* 9.24 Hz, H-3); $^{13}$C NMR ($D_2O$): δ 14.78, 20.41 (4 × $COCH_3$), 20.53, 20.61, 20.71, 24.34, 61.40, 67.62, 70.07, 71.46, 71.84, 72.80, 73.41, 76.36, 76.63, 83.36, 100.69, 168.91, 169.17, 169.52, 169.62, 170.08, 170.14, 170.35; Found: *m/z* 703.1883. $C_{28}H_{40}NaO_{17}S$ requires 703.1878.

*Ethyl β-*D*-glucopyranosyl)-(1 → 6)-1-thio-β-*D*-glucopyranoside* **6**.—Acetate **5** (4.4 g, 6.47 mmol) was deacetylated and purified as above to give **6** as a colourless solid (2.42 g, 97%); $[\alpha]_D^{24}$ −36.12° (*c* 1.93, $H_2O$); $^1$H NMR ($D_2O$): δ 1.23 (t, 3 H, *J* 7.55 Hz, $SCH_2CH_3$), 2.60–2.83 (m, 2 H, $SCH_2CH_3$), 3.23–3.94 (m, 12 H), 4.46 (d, 1 H, *J* 7.85 Hz, H-1′), 4.51 (d, 1 H, *J* 10.18 Hz, H-1); $^{13}$C NMR ($D_2O$) δ: 15.40, 25.03, 61.02, 61.42, 70.29, 72.57, 72.86, 74.00, 76.33, 76.55, 79.32, 79.48, 85.77, 103.36; HRMS: Found *m/z* 409.1121. $C_{14}H_{26}NaO_{10}S$ requires 409.1139.

*Ethyl (2, 3, 4, 6-tetra-*O*-acetyl-β-*D*-glucopyranosyl) (1 → 6)-2, 3, 6-tri-*O*-acetyl-1-thio-β-*D*-glucopyranose* **8**.—β-D-Maltose octaacetate **7** (4.5g, 6.64 mmol) was dissolved in 1,2-dichloroethane (100 mL). Molecular sieves (4 Å, 4.5 g) were added and the mixture was stirred at −30 °C under nitrogen gas. Ethanethiol (1.05 mL, 14.2 mmol) was added and the temperature was maintained at −30 °C for 1 h. Tin(IV) chloride (0.95 mL, 8.09 mmol) was added and the temperature was slowly brought to −17 °C during 1.3 h. The reaction was quenched by the addition of saturated $NaHCO_3$ (100 mL). The mixture was stirred for 10 min, the layers were separated and the aqueous layer was extracted with dichloromethane (3 × 80 mL). The organic layer was washed with water (2 × 175 mL), dried ($MgSO_4$) and evaporated under reduced pressure. The residue was purified by chromatography over silicon dioxide with ethyl acetate:toluene (1:3) as eluent to give **8** as a colourless solid (3.8 g, 84%); $[\alpha]_D^{24}$ −20.98° (*c* 1.25, $CHCl_3$); $^1$H NMR ($D_2O$): δ 1.23 (t, 3 H, *J* 7.44 Hz, $SCH_2CH_3$), 1.95, 1.96 1.98, 1.99, 2.01, 2.03 and 2.05 (all s, 3 H, $COCH_3$), 2.59–2.73 (m, 2 H, $SCH_2CH_3$), 3.56 (dd, 1 H, *J* 7.16 and 11 Hz, H-6′), 3.62–3.67 (m, 2 H, H-5 and H-5′), 3.82 (dd, 1 H, *J*

2.04 and 11.02 Hz, H-6′), 4.08 (dd, 1 H, *J* 2.28 and 12.34 Hz, H-6), 4.23 (dd, 1 H, *J* 4.80 and 12.34 Hz, H-6), 4.43 (d, 1 H, *J* 10.08 Hz, H-1), 4.54 (d 1 H, *J* 7.96 Hz, H-1′), 4.85 (t, 1 H, *J* 9.76 Hz, H-4), 5.03 (t, 1 H, *J* 9.56 Hz, H-2′), 5.14 (t, 1 H, *J* 9.32 Hz, H-3′), 5.16 (t, 1 H, *J* 9.24 Hz, H–); $^{13}$C NMR ($D_2O$): δ 14.69, 20.41 (4 × $CH_3$), 20.54 (3 × $CH_3$), 23.90, 61.61, 68.07, 68.30, 68.84, 69.75, 70.88, 71.74, 72.57, 73.61, 77.12, 83.03, 100.55 169.17, 169.23 (2 × CO), 169.94, 170.02, 170.42; HRMS: Found *m/z* 703.1849. $C_{28}H_{40}NaO_{17}S$ requires 703.1878.

*Ethyl (β-D-glucopyranosyl)-(1 → 6)-1-thio-β-D-glucopyranoside* **9**.—Acetate **8** (3.8 g, 5.59 mmol) was deacetylated and purified as above to give **9** as a colourless solid (2.05 g, 95%); $[\alpha]_D^{24}$ −44.65° (*c* 2.59, $H_2O$); $^1$H NMR ($D_2O$): δ 1.24 (t, 3 H, *J* 7.28 Hz, $SCH_2CH_3$), 2.65–2.80 (m, 2 H, $SCH_2CH_3$), 3.25–3.37 (m, H), 3.54–3.93 (m, 3 H), 3.41–3.49 (m, 4 H), 3.57–3.62 (m, H), 3.68 (dd, 1 H, *J* 6 and 12.26 Hz, H), 3.79 (dd, 1 H, *J* 5.64 and 11.80 Hz, H), 3.88 (dd, 1 H, *J* 2 and 12.28 Hz, H), 4.16 (dd, 1 H, *J* 1.64 and 11.60 Hz, H), 4.47 (d, 1 H, *J* 7.96 Hz, H-1′), 4.52 (d, 1 H, *J* 9.92 Hz, H-1); $^{13}$C NMR ($D_2O$): δ 15.26, 25.03, 61.39, 69.39, 70.00, 70.28, 73.75, 76.32, 76.57, 77.77, 79.40, 85.94, 100.33. HRMS: Found *m/z* 409.1141. $C_{14}H_{26}NaO_{10}S$ requires 409.1139.

*Ethyl (2-acetamido-2-deoxy-β-D-galactopyranosyl)-(1 → 4)-(α-D-glucopyranosyl)-(1 → 4)-1-thio-β-D-glucopyranoside* **11**.—*p*-Nitrophenyl *N*-acetyl-β-D-galactopyranoside **10** (0.1 g, 0.29 mmol) and **3** (0.65 g, 1.68 mmol) were suspended in citrate–phosphate buffer (0.05 M, pH 4.5, 2 mL). The mixture was heated at 45–50 °C for 2–3 min (water bath) and 30 °C for 5 min. β-*N*-Acetylhexosaminidase from *A. oryzae* (0.46 mg protein/mL, 68 U/mg protein, 45 μL) was added to the reaction mixture which was incubated at 30 °C for 19 h. The reaction was stopped by heating in a boiling water bath for 5 min. The mixture was purified by carbon–Celite chromatography eluted first with 5%, 10% and 20% ethanol to remove the monosaccharide, disaccharide **12** and disaccharide **9**, followed by 30% ethanol to elute trisaccharide **11** (49 mg, 29%) $[\alpha]_D^{24}$ +58.23° (*c* 1.61, $H_2O$); $^1$H NMR ($D_2O$): δ 1.23 (t, 3 H, *J* 7.44 Hz, $SCH_2CH_3$), 2.02 (s, 3 H, $COCH_3$), 2.64–2.79 (m, 2 H, $SCH_2CH_3$), 3.30 (dd, 1 H, *J* 9.24 and 9.80 Hz, H-2), 3.51–3.62 (m, 5 H), 3.64–3.80 (m, 9 H), 3.84–3.91 (m, 3 H), 4.46 (d, 1 H, *J* 8.40 Hz, H-1″), 4.51 (d, 1 H, *J* 9.88 Hz, H-1), 5.34 (d, 1 H, *J* 3.96 Hz, H-1′); $^{13}$C NMR ($D_2O$): δ 15.93, 22.87, 24.81, 53.25, 60.57, 61.42, 61.64, 68.28, 71.33, 71.72, 71.90, 72.23, 72.75, 76.00, 77.45, 78.28, 79.01 (C-4), 79.48 (C-4″), 85.60, (C-1), 99.94 (C-1′), 102.33 (C-1″), 175.40 (C=O); HRMS: Found *m/z* 612.1935. $C_{22}H_{39}NNaO_{15}S$ requires 612.1932.

*Ethyl (2-acetamido-2-deoxy-β-D-galactopyranosyl)-(1 → 4)-(β-D-glucopyranosyl)-(1 → 4)-β-D-glucopyranoside* **13** and ethyl 1-thio-(2-acetamido-2-deoxy-β-D-*galactopyranosyl)-(1 → 3)-(β-D-glucopyranosyl)-(1 → 4)-1-thio-β-D-glucopyranoside* **14**.—*p*-Nitrophenyl *N*-acetyl-β-D-galactopyranoside **10** (0.1 g, 0.29 mmol) and **9** (0..65 g, 1.68 mmol) were suspended in citrate–phosphate buffer (0.05 M, pH 4.5, 2 mL). The mixture was heated at 45–50 °C for 2–3 min (water bath) and 30 °C for 5 min. β-*N*-Acetylhexosaminidase from *A. oryzae* (0.46 mg protein/mL, 68 U/mg protein, 35 μL) was added to the reaction mixture which was incubated at 30 °C for 21 h. The reaction was stopped by heating in a boiling water bath for 5 min. The mixture was purified by column chromatography on carbon–Celite, with elution as above for trisaccharide **11** with 5%, 10% and 20% ethanol to remove the monosaccharide and disaccharides **12** and **9** followed by 35% ethanol to elute trisaccharide **13** (57 mg) and 40% ethanol to elute the trisaccharide **14** (26 mg) in 48% overall yield. For trisaccharide **13**: $[\alpha]_D^{24}$ +1.92° (*c* 0.94, $H_2O$); $^1$H NMR ($D_2O$): δ 1.19 (t, 3 H, *J* 7.28 Hz, $SCH_2CH_3$), 1.98 (s, 3 H, $COCH_3$), 2.60–2.75 (m, 2 H, $SCH_2CH_3$), 3.20–3.89 (m, H), 4.42 (d, 1 H, *J* 8.60 Hz, H–), 4.44 (d, 1 H, *J* 8.64 Hz, H–), 4.48 (d, 1 H, *J* 9.92 Hz, H–); $^{13}$C NMR ($D_2O$): δ 15.38, 23.04, 24.98, 53.37, 60.86, 60.94, 61.79, 68.43, 71.46, 72.84, 73.53, 75.03, 75.40, 76.15, 76.47, 79.07, 79.47, 79.66, 85.74 (C-1), 102.53 (C–), 103.01 (C–), 175.61 (C=O); HRMS: Found *m/z* 612.1892 $(M + Na)^+$ requires 612.1932. For trisaccharide **14**: $[\alpha]_D^{24}$ +4.02 (*c* 0.73, $H_2O$); $^1$H NMR ($D_2O$): δ 1.17 (t, 3 H, *J* 7.25 Hz, $SCH_2CH_3$), 1.94 (s, 3 H, $COCH_3$), 2.55–2.77 (m, 2 H, $SCH_2CH_3$), 3.25–3.88 (m, H), 4.39 (d, 1 H, *J* 8.13 Hz, H-1′), 4.46 (d, 1 H, *J* 9.88 Hz, H-1), 4.57 (d, 1 H, *J* 8.43 Hz, H-1″); $^{13}$C NMR ($D_2O$): δ 15.35, 23.10, 24.98, 53.45, 61.00, 61.42, 61.89, 68.55, 69.04, 71.58, 72.84, 73.72, 76.03, 76.23, 76.48, 79.11, 79.45, 85.10, 85.72 (C-1), 103.14 (C–), 103.26 (C–), 175.86 (C=O); HRMS: Found *m/z* 612.1932. $C_{22}H_{39}NNaO_{15}S$ requires 612.1932.

*Ethyl (2-acetamido-2-deoxy-β-D-galactopyranosyl)-(1 → 3)-(β-D-glucopyranosyl)-(1 → 6)-1-thio-β-D-glucopyranoside* **15** and ethyl (2-acetamido-2-deoxy-β-D-*galactopyranosyl)-(1 → 4)-(β-D-glucopyranosyl)-(1 → 6)-1-thio-β-D-glucopyranoside* **16**.—*p*-Nitrophenyl-*N*-acetyl-β-D-galactopyranoside **10** (0.1

g, 0.29 mmol) and **6** (0.65 g, 1.68 mmol) were suspended in citrate–phosphate buffer (0.05 M, pH 4.5, 2 mL). The mixture was heated at 45–50 °C for 2–3 min (water bath) and 30 °C for 5 min. *β-N*-Acetylhexosaminidase from *A. oryzae* (0.46 mg protein/mL, 68 U/mg protein, 35 μL) was added to the reaction mixture which was incubated at 30 °C for 22 h. The reaction was stopped by heating in a boiling water bath for 5 min. The mixture was purified first by carbon–Celite chromatography, with elution as above for trisaccharide **11**. The trisaccharide mixture was further purified by preparative HPLC (78:22, 10 mL/min) to give trisaccharides **13** (74 mg) and **14** (31 mg) in 61% overall yield. For trisaccharide **13**: $[\alpha]_D^{22}$ −7.46 (*c* 1.33, $H_2O$); $^1$H NMR ($D_2O$): δ 1.22 (t, 3 H, *J* 7.28 Hz, $SCH_2CH_3$), 1.98 (s, 3 H, $COCH_3$), 2.62–2.77 (m, 2 H, $SCH_2CH_3$), 3.21–3.46 (m, 6 H), 3.55–3.80 (m, 8 H), 3.82–3.90 (m, 3 H), 4.10–4.16 (m,1 H), 4.42 (d, 1 H, *J* 7.96 Hz, H-1′), 4.49 (d, 1 H, J 9.96 Hz, H-1), 4.61 (d, 1 H, *J* 8.60 Hz, H-1); $^{13}$C NMR ($D_2O$): δ 15.26, 22.92, 25.01, 53.33, 61.36, 61.73, 68.38, 68.97, 69.44, 69.98, 71.42, 72.46, 73.49, 75.85, 76.03, 77.75, 85.18 (C-3), 85.98 (C-1), 103.04 (C–), 103.42 (C–), 175.68 (C=O); HRMS: Found *m/z* 612.1895. $(M + Na)^+$ requires 612.1932. For trisaccharide **14**: $[\alpha]_D^{22}$ −1.62° (*c* 0.99, $H_2O$); $^1$H NMR ($D_2O$): δ 1.20 (t, 3 H, *J* 7.55 Hz, $SCH_2CH_3$), 1.99 (s, 3 H, $COCH_3$), 2.60–2.79 (m, 2 H, $SCH_2CH_3$), 3.23–3.30 (m, 2 H), 3.39–3.89 (m, 15 H), 4.12 (dd, 1 H, *J* 1.75 and 11.77 Hz, H), 4.42 (d, 1 H, *J* 8.13 Hz, H–), 4.45 (d, 1 H, *J* 7.83 Hz, H–), 4.48 (d, 1 H, *J* 9.60 Hz, H–); $^{13}$C NMR ($D_2O$): δ 15.18, 22.82, 25.01, 53.20, 60.75, 61.60, 68.22, 69.36, 69.90, 71.30, 72.77, 73.25, 75.02, 75.15, 75.98, 77.74, 79.35, 79.67, 85.91 (C-1), 102.40 (C–), 103.06 (C–), 175.41 (C=O); HRMS: Found *m/z* 612.1931. $C_{22}H_{39}NNaO_{15}S$ requires 612.1932.

*Ethyl (β-D-galactopyranosyl)-(1 → 6)-(β-D-galactopyranosyl)-(1 → 4)-1-thio-β-D-glucopyranoside* **18**.—*p*-Nitrophenyl *N*-acetyl-β-D-galactopyranoside **10** (0.1 g, 0.29 mmol) and ethyl 1-thio (β-D-galactopyranosyl)-(1 → 4)-β-D-glucopyranoside **17** (0.65 g, 1.68 mmol) were suspended in citrate–phosphate buffer (0.05 M, pH 4.5, 2 mL). The mixture was heated at 45–50 °C for 2–3 min (water bath) and 30 °C for 5 min. *β-N*-Acetylhexosaminidase from *A. oryzae* (0.46 mg protein/mL, 68 U/mg protein, 35 μL) was added to the reaction mixture which was incubated at 30 °C for 22 h. The reaction was stopped by heating in a boiling water bath for 5 min. The mixture was purified first by carbon–Celite chromatography eluted as above to give trisaccharide **18** (57 mg) and another compound (8 mg), which could not be identified. For trisaccharide **18**: $^1$H NMR ($D_2O$): δ 1.24 (t, 3 H, *J* 7.28 Hz, $SCH_2CH_3$), 2.64–2.76 (m, 2 H, $SCH_2CH_3$), 3.29–3.37 (m, 1 H), 3.47–3.80 (m, 12 H), 3.85–3.96 (m, 4 H), 4.01–4.05 (m, 1 H), 4.42 (d, 1 H, *J* 7.60 Hz, H-1′), 4.44 (d, 1 H, *J* 7.64 Hz, H-1′), 4.53 (d, 1 H, *J* 9.96 Hz, H-1); $^{13}$C NMR ($D_2O$): δ 15.19, 24.82, 60.94, 61.66, 69.13, 69.28, 69.71, 71.45, 71.50, 72.52, 73.09, 73.27, 74.66, 75.77, 79.14, 79.78, 85.53, 103.74, 104.00; HRMS: Found *m/z* 571.1646. $C_{20}H_{36}NaO_{15}S$ requires 571.1667.

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