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A simple synthesis of octyl 3,6-di-*O*-(α-D-mannopyranosyl)-β-D-mannopyranoside and its use as an acceptor for the assay of *N*-acetylglucosaminyltransferase-I activity

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A simple synthesis of octyl 3,6-di-*O*-(α-D-mannopyranosyl)-β-D-mannopyranoside is described. The key features of the synthetic scheme are the formation of the β-mannosidic linkage by 1-*O*-alkylation of 2,3,4,6-tetra-*O*-acetyl-α,β-D-mannopyranose with octyl iodide and glycosylation of unprotected octyl β-D-mannopyranoside using limiting acetobromomannose. The trisaccharide is shown to be an acceptor for *N*-acetylglucosaminyltransferase-I with a K_M of 585 μM.

Keywords: *N*-acetylglucosaminyltransferase-I, glycosyltransferase assay, core-trimannoside, oligosaccharide synthesis

N-Acetylglucosaminyltransferase-I (GnT-I, EC 2.4.1.101) is the key enzyme controlling the conversion of oligomannose asparagine-linked (N-linked) oligosaccharides to so-called hybrid or complex structures [1]. Several methods are available to assay the activity of this enzyme, all of which measure the rate of transfer of radiolabelled *N*-acetylglucosamine (GlcNAc) from the donor, UDP-GlcNAc, to a suitable acceptor oligosaccharide [2–4]. The natural acceptor for GnT-I is an N-linked $Man_5GlcNAc_2$ heptasaccharide [1], but it is known that the simple core trimannosides like **1**, where R is an alkyl group [5], also function well as specific acceptors. If the aglycone in this core trimannoside is sufficiently hydrophobic, as in the case of **1** where it is the 8-methoxycarbonyloctyl group, the assays for GnT-I activity become especially simple. The product of the enzymatic reaction is then the tetrasaccharide **2**, which binds quantitatively to reversed-phase C-18 cartridges from which it can be eluted directly into scintillation vials for quantification. Such assays, referred to as Sep-Pak assays [6], require less than 1 min for processing enzyme incubation mixtures. The ester at the end of the aglycone in **1** also allows for future covalent attachment to proteins and solid supports through this hydrophobic linking-arm [7].

The synthesis of trisaccharide **1** has been reported [8] and is typical of oligosaccharide synthesis in general. Approximately fifteen steps were required from commercial starting materials, and the synthesis required almost 2 months in the hands of an experienced individual. Many of these steps involved time-consuming and delicate protecting group manipulations to produce a mannose derivative with only the 3- and 6-OH groups free for subsequent glycosylation [8]. A key step in the synthesis was the difficult preparation of the β-mannose linkage by glycosylation using silver zeolite as insoluble promoter [8,9].

We required more acceptor to monitor the activity of GnT-I in the course of its purification and decided to prepare instead the octyl trimannoside **3**, which should serve equally well in the Sep-Pak assay, producing the radiolabelled octyl tetrasaccharide **4**. We furthermore decided to develop a simple and rapid synthesis of **3** which would not require any selective protection procedures or difficult glycosylation steps. In this way trisaccharide **3** might be prepared easily in laboratories less experienced in the specialist methodology of oligosaccharide synthesis, such as biochemical laboratories where the primary interest is not in the chemistry but in the enzyme activities themselves. We report here such a synthesis of **3** and its kinetic comparison with **1**, a known acceptor for GnT-I [6].

A key feature of the synthesis of **3** was the formation of the octyl β-D-mannopyranosidic linkage by alkylation of tetraacetate **6** [10] (readily prepared from the commercial pentaacetate **5** by reaction with hydrazine dimethylformamide (DMF) [11]) with octyl iodide in the presence of silver oxide. Despite the fact that the α:β ratio of reducing sugars was near 10:1 the reaction produced the α and β glycosides **7** (30%) and **8** (25%) in near equal amounts.

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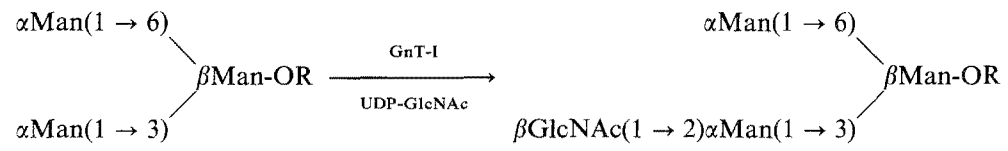
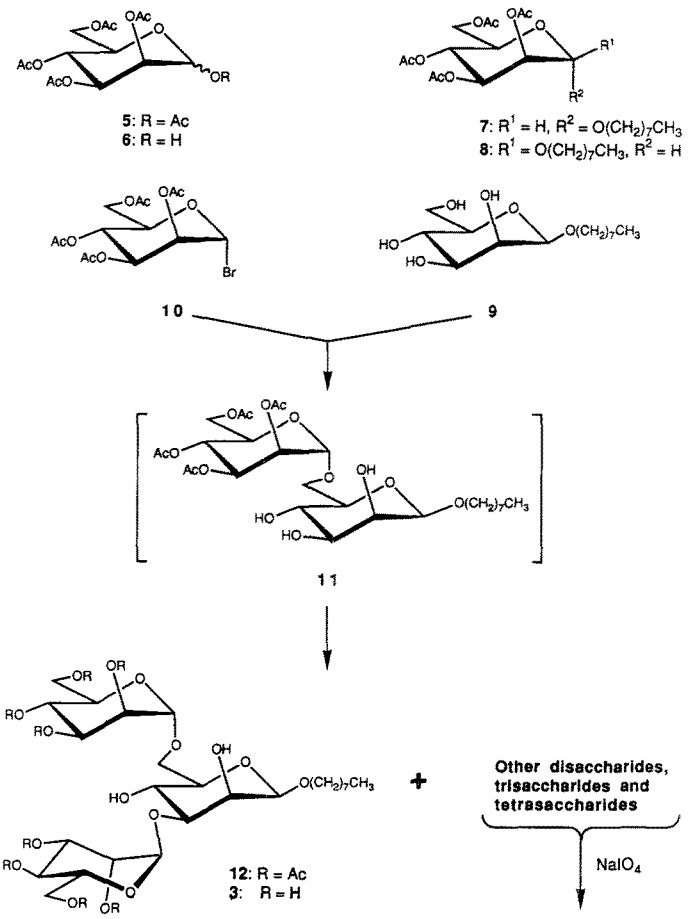


1 R = $O(CH_2)_8COOCH_3$
3 R = $O(CH_2)_7CH_3$

2 R = $O(CH_2)_8COOCH_3$
4 R = $O(CH_2)_7CH_3$



Anomers **7** and **8** were readily separated by chromatography on silica gel. Deacetylation of **8** then gave octyl β-D-mannopyranoside **9**.

Compound **9** was glycosylated, without protection of the hydroxyl groups, using 2.5 equivalents of acetobromomannose (**10**) and $HgBr_2/HgCN_2$ as promoters. As expected, a very complex mixture of oligomannosides was formed, but we reasoned that the isolation of the desired 3,6-linked trisaccharide from this mixture would be straightforward for the following reasons. Any di- and tetrasaccharides that might form in the reaction would likely have quite different mobilities than the mixture of corresponding trisaccharides. An initial rapid chromatographic purification was therefore expected to yield a fraction at least very rich in trisaccharides. The glycosylation of primary hydroxyl groups is generally accepted as being more rapid than that of more hindered secondary hydroxyl groups. It was therefore expected that the major products of the glycosylation would be derived from the 1,6-linked disaccharide **11**. The major trisaccharides formed should therefore be the 2,6-linked, 3,6-linked and 4,6-linked trimannosides. There was of course no reason to believe that these three trisaccharides should be easily separable by chromatography. This was not a concern, however, since any 2,6-linked and 4,6-linked trisaccharides which formed would have vicinal diols present, and could therefore be selectively destroyed by periodate oxidation, while the desired 3,6-linked trisaccharide would be unreactive towards this oxidizing agent.

The above expectations were borne out. In the initial chromatography step on silica gel (hexane:ethyl acetate, 1:2 by vol) the mixture of oligosaccharide products with R_Fs in the range 0.2–0.3, which contained the desired trisaccharide **12** along with other unidentified products, were collected and subjected to periodate oxidation for 24 hr. Other products with R_Fs near 0.05 and 0.50, presumably di- and tetrasaccharides, respectively, were discarded without characterization. The crude oxidation product was *O*-deacetylated using sodium methoxide, which also degraded the oxidized oligosaccharides. All products still containing the hydrophobic octyl aglycone were then isolated on a Sep-Pak C-18 cartridge. The material obtained at this stage showed only two major products with R_Fs 0.25 and 0.13 on silica gel using dichloromethane:methanol:water, (60:35:6) as the solvent. These products were easily separated by chromatography on Iatrobeads yielding the 3,6-linked trimannoside **3** in 17% yield based on **9**. The proton chemical shifts and coupling constants, as well as the carbon chemical shifts, for trisaccharide **8** were identical to those of the fully characterized 8-methoxycarbonyloctyl trisaccharide **1** [8] prepared by an unambiguous procedure, thereby confirming the structural assignment.

Figure 1 shows the ^{1}H-NMR spectrum of **3** obtained by the above procedure. Careful examination of the anomeric region of this spectrum showed that other oligosaccharides were also present and varied between 2% and 5% of the total material. The identify of these contaminants was not established but they did not interfere with the assay for GnT-I activity in crude rabbit liver extracts. Figure 2 shows the double reciprocal plot of **3** used as a substrate for GnT-I. Michaelis Menten kinetics are followed and acceptor **3** was found to have a K_M of 585 ± 33 μM with

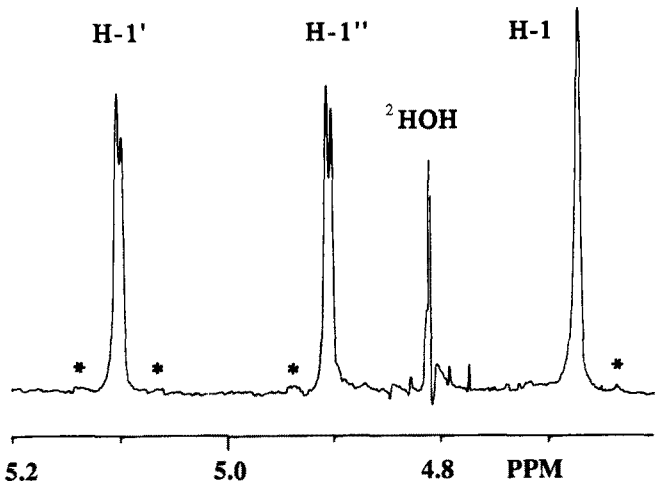


Figure 1. Partial 360 MHz ^{1}H-NMR spectrum of **3** in ^2H_2O. The asterisks (*) indicate probable oligosaccharide contaminants. The spectral artifacts near 4.8 ppm result from the suppression of the residual ^{2}HOH signal by presaturation.

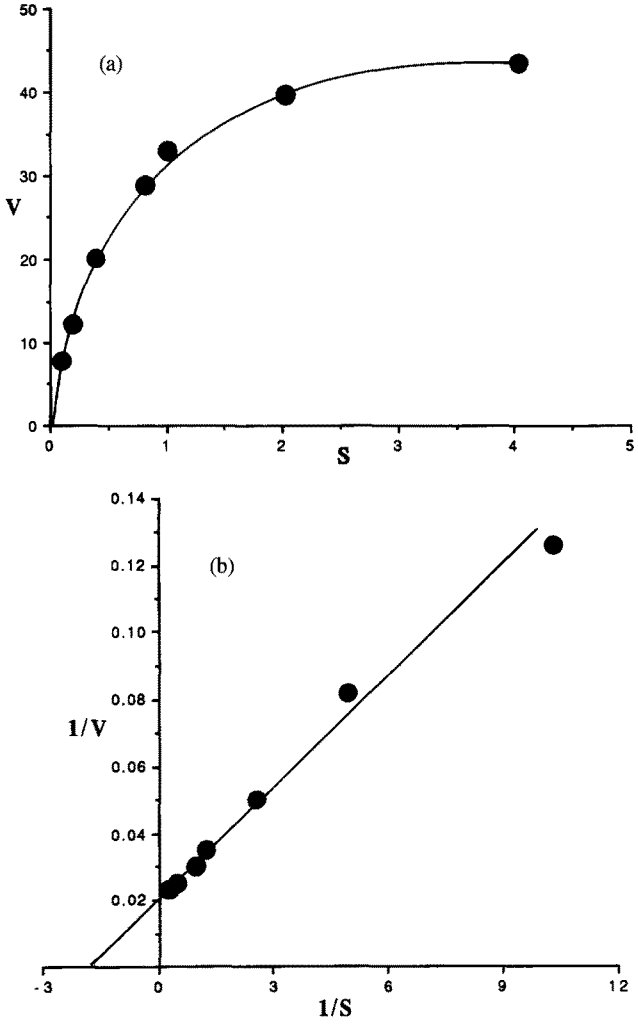


Figure 2. (a) Plot of the rate (V, pmol/min) of transfer of *N*-acetylglucosamine from UDP-GlcNAc as a function of the concentration (S, mM) of acceptor **3** catalyzed by GnT-I in crude rabbit liver extracts. (b) Double reciprocal plots of the data in (a).

$V_{max} = 50 \pm 1$ pmol/min in the assay tubes. Under identical assay conditions with this extract, the 8-methoxycarbonyloctyl acceptor **1** had $K_M = 650 \pm 25$ μM and $V_{max} = 54 \pm 1$ pmol/min (data not shown). Compound **1** was previously reported to have a K_M of 590 ± 70 μM for GnT-I from this same source [6]. Acceptors **1** and **3** are therefore seen to possess very similar kinetic characteristics and may, for all practical purposes, be used interchangeably in the assay of GnT-I activity.

In summary, a very simple and direct synthesis of trisaccharide **3** has been achieved which obviated the formation of the β-mannosidic linkage by glycosylation as well as the need for selective protection prior to glycosylation. Starting from mannone pentaacetate, the entire procedure can easily be accomplished in just over a week as opposed to about two months for the conventional multistep synthesis. Compound **3** thus obtained was furthermore shown to be an efficient acceptor for GnT-I using the Sep-Pak assay, and the material produced (38 mg) is sufficient for over 3000 of these assays.

Materials and methods

Optical rotations were measured with a Perkin-Elmer 241 polarimeter at $22 \pm 2°$. TLC was performed on silica gel 60-F_{254} (Merck) with detection by quenching of fluorescence and/or by charring with sulfuric acid. Unless otherwise noted, column chromatography was performed on Silica Gel 60 (Merck, 40–63 μm). Iatrobead refers to a beaded silica gel 6RS-8060 manufactured by Iatron Laboratories (Tokyo). Sep-Pak C-18 sample-preparation cartridges were from Waters Associates. UDP-6-[^{3}H]GlcNAc (specific activity 18.9 Ci/mmol) was from New England Nuclear, and liquid scintillation counting was performed with a Beckman LS-5000 instrument using quench correction [6]. ^{1}H-NMR spectra were recorded at 300 or 360 MHz (Bruker spectrometers) from solutions in C^2HCl_3 (internal Me_4Si) or ^2H_2O (internal acetone, δ 2.225), ^{13}C-NMR spectra were recorded at 75.5 MHz from solutions in C^2HCl_3 (internal Me_4Si) or ^2H_2O (external 1% 1,4-dioxane in ^2H_2O, δ 67.4). Only partial NMR data are reported, the other data were in accord with the proposed structures. The chemical shifts and coupling constants (as observed splittings) for ^{1}H resonances are reported as though they were first order. Organic solutions were dried (Na_2SO_4) prior to concentration at $\leqslant 40$ °C (bath)/12 mm Hg. The microanalyses were carried out by the Analytical Services Laboratory of this Department. The following solvent systems were used: A, ethyl acetate:hexane, 2:5 by vol; B, ethyl acetate:hexane, 2:3 by vol; C, ethyl acetate:hexane, 2:1 by vol; D, dichloromethane:methanol, 9:1 by vol; E, dichloromethane:methanol:water, 60:35:6 by vol. Protein concentrations were estimated using the Bio-Rad protein assay kit as described by Bradford [12], with bovine serum albumin as the standard.

Chemical synthesis

*Octyl 2,3,4,6-tetra-O-acetyl-α- and β-D-mannopyranoside (**7α** and **8β**)*: A mixture of **6** (3.66 g; 10.52 mmol) and silver oxide (12.20 g; 52.61 mmol) was dried *in vacuo* over P_2O_5 overnight, then dry toluene (25 ml) was added followed by pulverized 4 Å molecular sieves (12 g). This mixture was stirred at 0 °C for 1 h. Octyl iodide (9.5 ml; 52.61 mmol) was then added and, after 1 h at 0 °C, the reaction mixture was stirred at room temperature for 66 h. The mixture was diluted with dichloromethane and filtered through celite; the solid was washed with dichloromethane (10 × 25 ml). All dichloromethane washings were combined and evaporated. The resulting syrup was purified by chromatography using solvent A as eluent. The earlier fractions provided the α-anomer **7** (1.45 g; 30%) as a syrup (R_F 0.37, solvent B), $[\alpha]_D$ + 44.3° (*c* 2.0, chloroform).

NMR data: ^1H (C^2HCl_3): δ 4.81 (d, 1 H, $J_{1,2}$ = 1.5 Hz, H-1), 2.16, 2.11, 2.05, 2.00 (s, 3 H each, $COCH_3$), 0.89 (t, 3 H, *J* = 6.5 Hz, CH_3). ^{13}C (C^2HCl_3): δ 170.48, 169.95, 169.75, 169.63 ($COCH_3$), 97.50 (C-1), 13.98 (CH_3).

Analytical data: calculated for $C_{22}H_{36}O_{10}$: C, 57.38; H, 7.88. Found: C, 57.42; H, 8.08).

The later fractions afforded the β-anomer **8** (1.21 g, 25%) as a syrup (R_F 0.31, solvent B) $[\alpha]_D$ −38.3° (*c* 1.1, chloroform).

NMR data: ^1H (C^2HCl_3): δ 4.63 (d, 1 H, $J_{1,2}$ < 1.0 Hz, H-1), 2.19, 2.09, 2.04, 1.99 (s, 3 H each, $COCH_3$), 0.88 (t, 3 H, *J* = 6.5 Hz, CH_3); ^{13}C-NMR (C^2HCl_3): δ 170.68, 170.35, 170.04, 169.57 ($COCH_3$), 98.74 (C-1), 14.07 (CH_3).

Analytical data: calculated for $C_{22}H_{36}O_{10}$: C, 57.38; H, 7.88. Found: C, 57.17; H, 8.10.

*Octyl β-D-mannopyranoside (**9**)*: Compound **8** (1.19 g; 2.58 mmol) was dissolved in dry methanol (5 ml) containing sodium methoxide (≈0.01 N). After 4 h the reaction mixture was neutralized with Amberlite IR-120(H^+) resin, filtered and evaporated to afford **9** as white solid (747 mg, 99%) (R_F 0.21, solvent D), which was not further characterized.

*Octyl 3,6-di-O-(α-D-mannopyranosyl)-β-D-mannopyranoside (**3**)*: To a solution of **9** (105 mg; 0.36 mmol) in dry acetonitrile (3 ml) containing powdered molecular sieves (500 mg) were added, sequentially at 35 °C under nitrogen atmosphere, mercuric bromide (323 mg; 0.90 mmol), mercuric cyanide (227 mg; 0.90 mmol) and a solution of acetobromomannose (**10**) (369 mg; 0.90 mmol) in dry acetonitrile (2 ml). The reaction mixture was stirred at the above temperature for 20 min and then filtered through celite. Evaporation of the solvent gave a residue, which was extracted three times with dichloromethane. The extracts were combined and washed successively with saturated aqueous potassium chloride, saturated aqueous sodium bicarbonate, and water. The organic layer was dried, filtered, and evaporated to dryness. The resulting syrup showed six spots on TLC. Column chromatography was done on Iatrobeads (2 × 24 cm) using solvent C as eluent. All fractions containing the compounds having R_F between 0.18 and 0.31 were combined. Evaporation of the solvent provided a syrup (150 mg), which was dissolved in methanol (5 ml) and treated with an aqueous solution of sodium periodate (250 mg). After stirring for 24 h at room temperature, the reaction mixture was diluted with water (100 ml) and extracted with dichloromethane (2 × 75 ml). The organic layer was again washed with water (200 ml). Evaporation of the solvent provided a residue which was de-*O*-acetylated as described for the preparation of **9** to afford a white solid. This was dissolved in water (30 ml) and divided into six portions each of which was loaded onto a Sep-Pak C_{18} cartridge. The cartridges were washed with water (3 × 10 ml), followed by methanol (3 × 10 ml). The methanol eluates were combined and concentrated to dryness to provide a solid which showed two major spots (R_F 0.25 and R_F 0.13, solvent E) on TLC. This mixture was purified by chromatography on Iatrobeads (2 × 24 cm, solvent E) to provide trisaccharide **3** (38 mg; 17% based on compound **9**).

NMR data: ^1H (^2H_2O): δ 5.105 (d, 1 H, $J_{1',2'}$ = 1.5 Hz, H-1'), 4.909 (d, 1 H, $J_{1'',2''}$ = 1.5 Hz, H-1''), 4.677 (br s, 1 H, H-1), 4.137 (d, 1 H, $J_{2,3}$ = 3.0 Hz, H-2), 4.067 (dd, 1 H, $J_{2',3'}$ = 3.0 Hz, H-2'), 3.992 (dd, 1 H, $J_{2'',3''}$ = 3.0 Hz, H-2''), 0.863 (t, 3 H, *J* = 6.5 Hz, CH_3); ^{13}C (^2H_2O): δ 103.14, 100.55, 100.21 (C-1', C-1'', C-1), 14.20 (CH_3).

Signals are designated as follows: βMan, non-primed; αMan(1–3), single-primed; αMan(1–6), double-primed.

Enzyme assays

Extracts of rabbit liver (0.75% Triton X-100 in 50 mM sodium cacodylate, 10 mM $MnCl_2$, pH 6.5) were prepared as previously described [6]. Micro-assays for GnT-I activity were also performed as previously described. Briefly, microfuge tubes charged with 50 µl rabbit liver extract containing 69 µg of protein, between 4.9 and 200 nmol of acceptor (**1** or **3**), 250,000 dpm UDP-[^{3}H]GlcNAc and 15 nmol UDP-GlcNAc were incubated for 40 min at 37 °C. The mixture was then diluted with water to 5 ml and passed through a Sep-Pak cartridge which was washed with water (20 ml). Radiolabelled product was eluted with methanol (2 × 5 ml) directly into scintillation vials for counting. The kinetic parameters K_M and V_{max} were evaluated using a computer program based on the statistical method of Wilkinson [13].

Acknowledgement

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