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Synthesis of α-D-Glc*p*-(1 → 2)-α-D-Glc*p*-(1 → 3)-α-D-Glc*p*-O-$(CH_2)_8COOCH_3$ for use in the assay of α-glucosidase I activity

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Abstract

The chemical synthesis of α-D-Glc*p*-(1 → 2)-α-D-Glc*p*-(1 → 3)-α-D-Glc*p*-O-$(CH_2)_8COOCH_3$ (**9**), a substrate specific for α-glucosidase I, is reported. This enzyme removes the terminal α-D-Glc*p* unit to produce α-D-Glc*p*-(1 → 3)-α-D-Glc*p*-O-$(CH_2)_8COOCH_3$ (**10**). This is the first synthetic substrate described for glucosidase I that allows kinetic evaluation of substrates and inhibitors of this enzyme. Tetramethylrhodamine was coupled to **9** through an ethylenediamine linker to produce a brilliant red derivative. Addition of this fluorescent dye did not affect enzyme binding to the substrate, as determined by a comparison of the K_m value (1.3 mM). The fluorescent label allows visual detection of 2–3 pmol of product by TLC. © 1996 Elsevier Science Ltd.

Keywords: α-Glucosidase I; Fluorescence-labelled oligosaccharide; Tetramethylhodamine; Glycosidase assay

1. Introduction

The synthesis of N-linked glycoproteins is a multistep process, initiated by the *en bloc* transfer of $Glc_3Man_9GlcNAc_2$ from dolichol pyrophosphate to an asparagine

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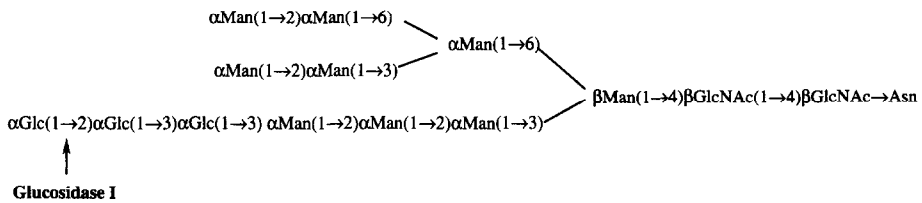


Fig. 1. The natural substrate of α-glucosidase I and the cleavage site for the enzyme. The sugars are D-pyranosides.

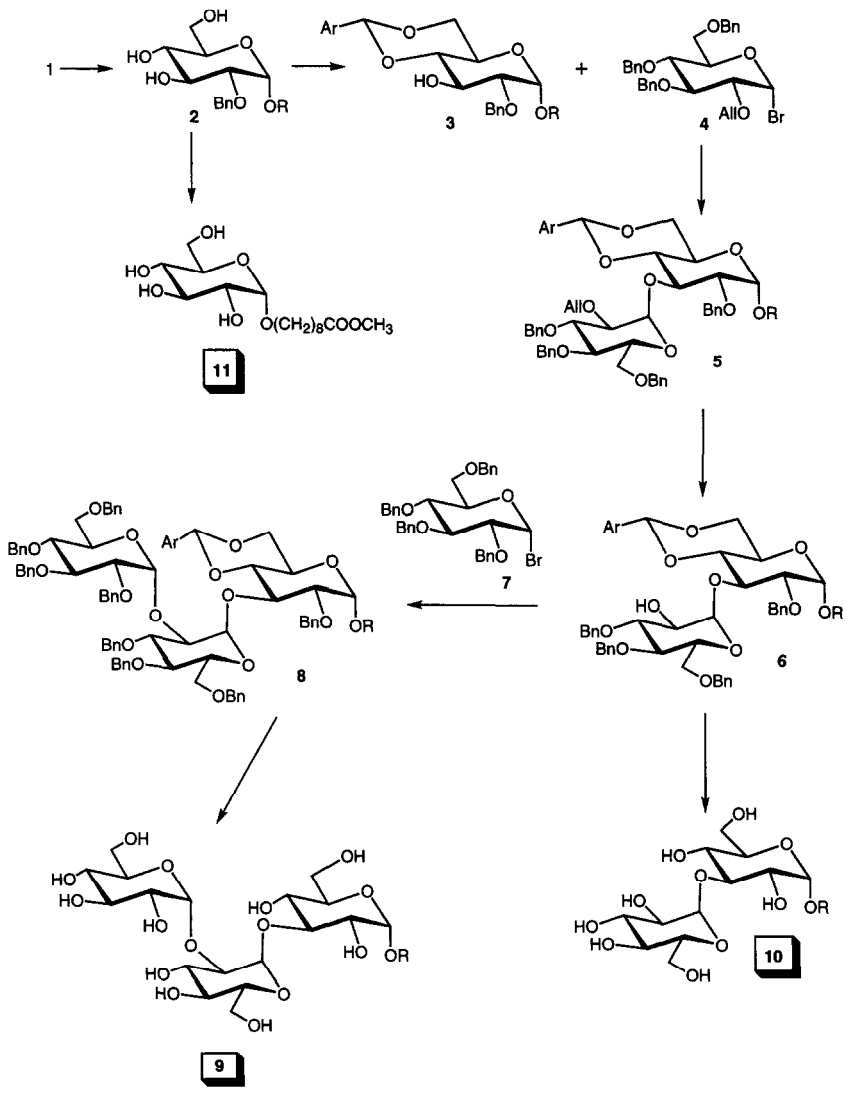
residue of a protein in the lumen of the endoplasmic reticulum [1]. Enzymatic processing of this common precursor structure begins with the cleavage of the terminal α-(1 → 2) glucose residue by α-glucosidase I [1] (Fig. 1). This allows sequential trimming of the remaining two α-(1 → 3)-D-glucose residues by glucosidase II, and hydrolysis of mannose residues by various mannosidases [2–4]. Additions are subsequently made to the carbohydrate structure by glycosyltransferases, resulting in the wide variety of complex and hybrid type glycosylation patterns found on proteins.

Glucosidase I represents a critical point in the regulation of glycoprotein synthesis, as further processing of the oligosaccharide is blocked until the terminal α-(1 → 2)-D-glucose residue is removed by this enzyme [5]. Therefore, monitoring the activity of glucosidase I, and the effect of inhibition of the enzyme, has provided information on the mechanism of glycoprotein formation and function in normal and cancerous cells [6]. Additionally, inhibitors of glucosidase I that block the processing of viral glycoproteins may be potential antiviral therapeutics [7]. In acquired immune deficiency syndrome (AIDS), prevention of the processing of the viral envelope glycoproteins, gp120 and gp41 by the glucosidase inhibitors, castanospermine and deoxynojirimycin, results in diminished infectivity of viruses and reduced syncytium formation [8].

Assays for glucosidase I have required the preparation and isolation of [^{14}C]- or [^{3}H]-glucose-labelled substrate, $Glc_3Man_9GlcNAc$, from microsomes or cell culture [9,10]. After incubation with the enzyme source, released radiolabelled glucose is separated from the substrate by a variety of techniques and quantitated [3,11,12]. Only limited kinetic information can be obtained using this natural radiolabelled substrate, however, as the amount and position of the incorporated label varies with each preparation.

We have recently reported on a spectrophotometric assay for glucosidase I activity using a synthetic trisaccharide substrate, α-D-Glc*p*-(1 → 2)-α-D-Glc*p*-(1 → 3)-α-D-Glc*p*-$O(CH_2)_8COOCH_3$ (**9**) [13]. Glucose released from the substrate was quantitated using the enzymes glucose oxidase and horseradish peroxidase, and the chromogenic compound, *o*-dianisidine. The assay could be carried out in a small volume (≤ 25 μL) and did not require separation of reducing glucose product from the trisaccharide. The utility of this assay for monitoring enzyme activity during isolation, and especially for use in kinetic and inhibition studies, was demonstrated [13].

[1] α-Glucosidase I as described in this paper most closely fits (at least a component of EC 3.2.1.106, which is mannosyl-oligosaccharide glucohydrolase.



All = Allyl; Ar = p-methoxyphenyl; Bn = benzyl; R = 8-methoxycarbonyloctyl

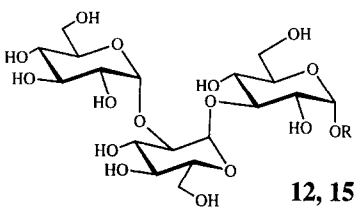
Scheme 1.

Here we report the synthesis of the trisaccharide **9**, along with products that would form on sequential removal of glucose units, namely, α-D-Glc*p*-(1 → 3)-α-D-Glc*p*-$O(CH_2)_8COOCH_3$ (**10**) and α-D-Glc*p*-$O(CH_2)_8COOCH_3$ (**11**). (See Scheme 1.) In addition, the preparation of the ethylenediamine monoamide of each saccharide, and the coupling of these with the *N*-hydroxysuccinamide ester of tetramethylrhodamine (TMR) is reported. Attachment of this fluorescent dye allows sensitive detection of enzyme activity by a variety of techniques, including thin-layer and high-performance liquid

chromatography, and capillary electrophoresis coupled with laser-induced fluorescence detection [14].

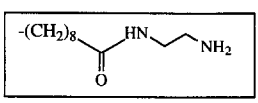
2. Results and discussion

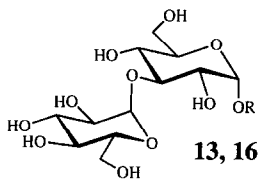
The key disaccharide alcohol **6** was prepared in three straightforward steps from the readily accessible 8-methoxycarbonyloctyl 2-*O*-benzyl-α-D-glucopyranoside (**2**) prepared from the saponification of 8-methoxycarbonyloctyl 3,4,6-tri-*O*-acetyl-2-*O*-benzyl-α-D-glucopyranoside (**1**) [15], which was converted into the *p*-methoxy-4,6-*O*-benzylidene derivative **3** by treatment with *p*-methoxybenzaldehyde dimethyl acetal in the presence of a catalytic amount of *p*-toluenesulfonic acid. Compound **3** was glycosylated with 2-*O*-allyl-3,4,6-tri-*O*-benzyl-α-D-glucopyranosyl bromide (**4**) under standard halide-ion catalyzed conditions [15] (tetraethylammonium bromide, DMF, molecular sieves) to provide the fully protected disaccharide **5** (90%). Deallylation involved isomerization of the 2-*O*-allyl ether of **5** by tris(triphenylphosphine)rhodium(I) chloride and 1,4-diazabicyclo-[2.2.2]-octane in 7:3:1 EtOH–benzene–H_2O, followed by hydrolysis of the vinyl ether to give the alcohol **6** (81%). Attempted a-glucosylation of **6** with bromide **7** [16] under standard halide-ion conditions gave only traces of **8** even under forcing conditions (5 equiv of **7** and a one-week incubation), so alternative conditions were developed.



12, 15

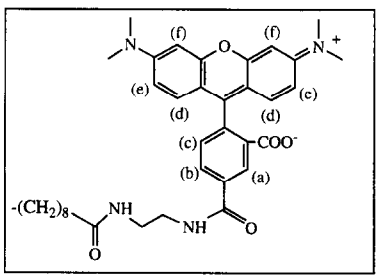
12, 13, 14 R =

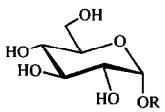




13, 16

15, 16, 17 R=





14, 17

The use of copper salts, especially $CuBr_2$, as glycosylation promoters has been reported using thioglycosides as the donor under a diverse set of conditions [17–22]. We found that the $CuBr_2$–DMF complex was an efficient promoter system using thioglycosides, as well as directly with per-*O*-benzylated glycosyl bromides such as **7**.

Thus reaction of alcohol **6** with glycosyl bromide **7**, using the $CuBr_2$–DMF complex as the promoter system, gave trisaccharide **8** (71%), which was obtained in pure form after chromatography on Iatrobeads. Hydrogenation (H_2, Pd/C) of **2**, **6** and **8** provided the required monosaccharide **11** (82%), disaccharide **10** (94%) and trisaccharide **9** (89%).

The 8-methoxycarbonyloctyl glycosides **9–11** were converted into their ethylenediamine monoamides **12–14** and subsequently derivatized by reaction with the commercial *N*-hydroxysuccinimide ester of *N*,*N*,*N*′,*N*′-tetramethylrhodamine (TMR) as previously described [23].

Previous work has already demonstrated the usefulness of **9** as a substrate for glucosidase I. It should be noted that assays for glucosidase I must be carried out in the absence of glucosidase II, which can act on the product of the glucosidase I reaction releasing additional reducing glucose. This would result in an overestimation of the glucosidase I activity. Alternatively, this overestimation of activity may aid in screening potential sources of glucosidase I, where low levels of activity may be amplified in situ.

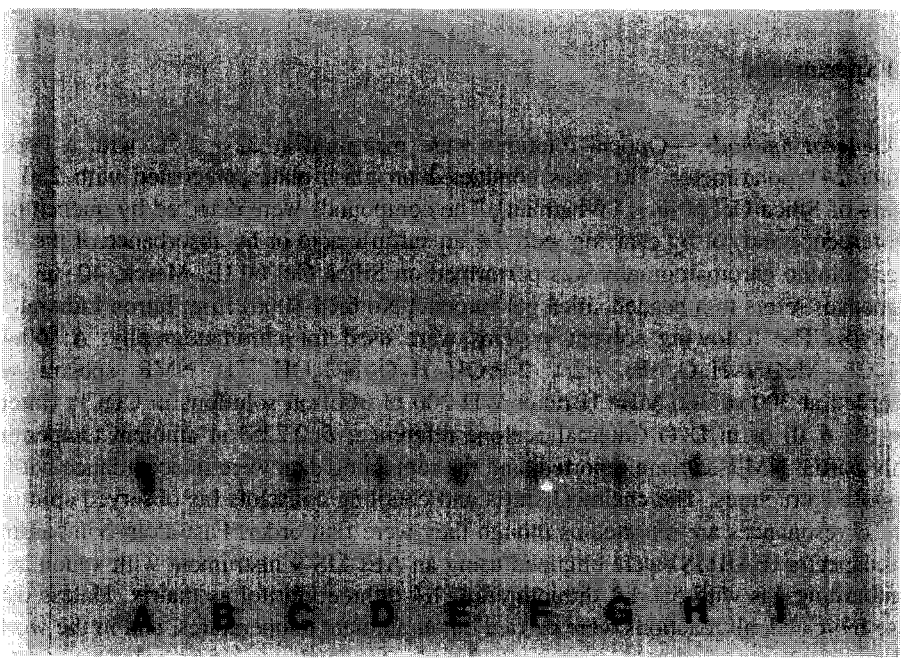


Fig. 2. Time course of TMR-trisaccharide (**15**) hydrolysis to TMR-disaccharide (**16**), catalyzed by soluble a-glucosidase I. Lanes A and B contain TMR-tri- and disaccharide standards, respectively. A reaction containing 0.025 mU of enzyme and 3.33 mM TMR-trisaccharide was incubated at 37 °C for 6 h. Aliquots of 0.25 μL were removed at 0 (C), 0.5 (D), 1.0 (E), 1.5 (F), 2.0 (G), 3.0 (H), and 6 (I) h and run on Silica Gel 60-F_{254} plates using solvent A (7:2:1, 2-PrOH–H_2O–NH_4OH).

Fig. 2 shows a silica gel TLC of the time course of the hydrolysis of the TMR-trisaccharide **15** to produce disaccharide **16** by soluble glucosidase I over a period of six h. Analysis of an aliquot of each sample, taken at various times, by capillary electrophoresis [24] indicated that the percentage hydrolysis increased from 5% after 30 min to approximately 50% after 6 h. The rate of enzymatic hydrolysis appeared to be constant during this time period. The K_m for trisaccharide **9** without the fluorescent label was found to be 1.28 mM for the soluble form of glucosidase I [13]. The K_m of the TMR-trisaccharide **15** was found to be 1.3 ($\pm$0.1) mM in the present work. This indicates that attachment of the fluorescent dye to the trisaccharide does not affect enzyme binding, while allowing rapid and sensitive visual detection of enzyme activity.

The visual detection limit for TMR-trisaccharide on a silica gel TLC plate was determined to be 2–3 pmols of material. The compound was detected after spotting 0.1–1.0 μL of a 10 pmol/μL aqueous solution of the TMR-trisaccharide on a plate, followed by elution with 7:2:1 2-PrOH–H_2O–NH_4OH. The sensitivity of detection could be further increased by using capillary electrophoresis separation of substrate and products, in conjunction with laser-induced fluorescence detection [24]. Using this analytical methodology with other oligosaccharides, 100 molecules of a TMR labelled compound were detected [14]. This level of detection sensitivity should allow the detection of very low levels of enzymatic activity, such as those found in single cells.

3. Experimental

General methods.—Optical rotations were measured at 22 $\pm$ 2 °C with a Perkin–Elmer 241 polarimeter. TLC was conducted on glass plates precoated with 250 μm layers of Silica Gel 60-F_{254} (Whatman). The compounds were detected by quenching of fluorescence and/or by charring with 5% aq sulfuric acid or by absorbance of the TMR dye. Column chromatography was performed on Silica Gel 60 (E. Merck, 40–63 μm). Iatrobeads refers to a beaded silica gel (product No 6RS-8060) from Iatron Laboratories (Tokyo). The following solvent systems were used for chromatography: **A**: 60:45:2 CH_2Cl_2–MeOH–H_2O; **B**: 7:2:1 2-PrOH–H_2O–NH_4OH. ^1H NMR spectra were recorded at 300 or 360 MHz (Bruker WH 300 or 360) on solutions in $CDCl_3$ (internal Me_4Si, δ 0) or in D_2O (internal acetone reference, δ 2.225) at ambient temperature. Only partial NMR data are reported, and the remaining data were in accordance with the proposed structures. The chemical shifts and coupling constants (as observed splittings) for ^1H resonances are reported as though they were first order. Fast-atom-bombardment mass spectra (FABMS) were obtained using an AEI MS-9 instrument with xenon as the bombarding gas with 5:1 1,4-dithiothreitol–1,4-dithioerythritol as matrix. Unless otherwise indicated, all reactions were carried out at ambient temperatures, and in the workup solutions inorganic solvents were washed with equal volumes of aq solutions. Organic solutions were generally dried (anhyd Na_2SO_4) prior to concentration on a rotary evaporator under the vacuum of a water aspirator with bath temperature of 40–50 °C. The microanalyses were performed on a Carlo Erba EA1108 analyzer and carried out by the Analytical Services Laboratory of this department.

Materials.—Millex-GV (0.22 μm) filter units were from Millipore (Mississauga, ON). Reversed-phase C_{18} Sep-Pak cartridges from Waters Associates (Mississauga, ON) were pre-equilibrated with 30 mL of MeOH and then washed with 30 mL of water before use. A Waters 501 pump, U6K injector, and 490E detector were used, with integration of chromatographic peaks using Dynamax HPLC Method Manager Version 1.2 (Rainin Instrument Co., Inc.). The 5-carboxytetramethylrhodamine succinimidyl ester (product # C-2211) was from Molecular Probes, Inc., Oregon. The capillary electrophoresis equipment and analysis methods have been previously described [14,24].

8-Methoxycarbonyloctyl 2-O-benzyl-4,6-O-p-methoxybenzylidene-α-D-glucopyranoside (**3**).—To a solution of 8-methoxycarbonyl 3,4,6-tri-*O*-acetyl-2-*O*-benzyl-α-D-glucopyranoside (**1**) (4.0 g, 6.92 mmol) in MeOH (20.0 mL) was added a catalytic amount of sodium methoxide in MeOH. The reaction mixture was stirred for 5 h at 22 °C and neutralized with IR 120 (H^+) ion-exchange resin. The mixture was filtered and evaporated to yield compound **2** (2.56 g, 5.81 mmol). Compound **2** was dissolved in acetonitrile (10 mL), then *p*-methoxybenzaldehyde dimethyl acetal (2.5 mL) and *p*-toluenesulfonic acid (250 mg) were added. The reaction mixture was stirred for 1 h at room temperature and neutralized with triethylamine (1.0 mL). The solvent was evaporated, and the residue was purified by chromatography on silica gel using 3:1 hexane–EtOAc as eluent to provide **3** (2.3 g, 71%) as a solid: $[\alpha]_D$ +45.2° (*c* 0.6, $CHCl_3$). ^1H NMR ($CDCl_3$): δ 5.29 (s, 1 H, $C_6H_5CHO_2$), 4.74 (d, 1 H, $J_{1,2}$ 3.8 Hz, H-1), 4.71 (ABq, 2 H, J_{gem} 12.0 Hz, $C_6H_5CH_2$), 3.79, 3.66 (s, 3 H each, OCH_3), 2.30 (t, 2 H, *J* 7.5 Hz, CH_2COO). Anal. Calcd for $C_{31}H_{42}O_9$: C, 66.65; H, 7.58. Found: C, 66.79; H, 7.50.

8-Methoxycarbonyloctyl 2-O-allyl-3,4,6-tri-O-benzyl-α-D-glucopyranosyl-(1 → 3)-2-O-benzyl-4,6-O-(p-methoxybenzylidene)-α-D-glucopyranoside (**5**).—To a mixture of compound **3** (2.1 g, 3.76 mmol) in dry dichloromethane (2 mL) containing tetraethylammonium bromide (1.1 g, 5.65 mmol), dry *N,N*-dimethylformamide (2.9 mL, 37.6 mmol) and molecular sieves (6.3 g), was added freshly prepared 2-*O*-allyl-3,4,6-tri-*O*-benzyl-α-D-glucopyranosyl bromide **4** (10.4 g, 18.8 mmol). The reaction mixture was left stirring at room temperature for 72 h. Excess glucosyl bromide was decomposed by adding MeOH (2 mL). The reaction mixture was filtered, evaporated, and purified by chromatography on silica gel using 3:1 hexane–EtOAc as eluent. Pure **5** (3.5 g, 90%) was obtained as a syrup: $[\alpha]_D$ +65.1° (*c* 0.3, $CHCl_3$). ^1H NMR ($CDCl_3$): δ 5.63 (m, 1 H, CH_2–CH=CH_2), 5.46 (s, 1 H, $C_6H_5CHO_2$), 4.85 (d, 1 H, $J_{1',2'}$ 3.8 Hz, H-1′), 4.78 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 3.80, 3.65 (s, 3 H each, OCH_3), 2.30 (t, 2 H, *J* 7.5 Hz, CH_2COO). Anal. Calcd for $C_{61}H_{74}O_{14}$: C, 71.04; H, 7.23. Found: C, 71.00; H, 7.15.

8-Methoxycarbonyloctyl 3,4,6-tri-O-benzyl-α-D-glucopyranosyl-(1 → 3)-2-O-benzyl-4,6-O-(p-methoxybenzylidene)-α-D-glucopyranoside (**6**).—A solution of compound **5** (3.2 g, 3.1 mmol), tris-triphenylphosphinerhodium(I) chloride (429.8 mg, 0.47 mmol), 1,4-diazabicyclo[2.2.2] octane (156.4 mg, 1.39 mmol) in 7:3:1 EtOH–benzene–H_2O (100 mL) was refluxed for 24 h. The solvent was evaporated, and the residue was dissolved in acetone (100 mL) containing a trace amount of mercuric oxide (10 mg). To this solution was added a solution of mercuric chloride (3.0 g) in 9:1 acetone–H_2O (50 mL), and the mixture was stirred at room temperature for 45 min. Following evaporation of the solvent, the residue was dissolved in dichloromethane (250 mL). The

dichloromethane solution was washed with 30% aq potassium bromide. The organic layer was dried (Na_2SO_4) and evaporated to give an oily residue that was purified by chromatography on silica gel using 2:1 hexane–EtOAc as eluent. Compound **6** was obtained as a white foam (2.5 g, 81%): $[\alpha]_D$ +44.9° (*c* 0.2, $CHCl_3$). ^1H NMR ($CDCl_3$): δ 5.48 (s, 1 H, $C_6H_5CHO_2$), 5.28 (d, 1 H, $J_{1',2'}$, 3.5 Hz, H-1'), 4.74 (d, 1 H, $J_{1,2}$ 3.8 Hz, H-1), 3.69, 3.66 (s, 3 H each, OCH_3), 2.73 (d, 1 H, *J* 9.5 Hz, OH, D_2O exchangeable), 2.30 (t, 2 H, *J* 7.5 Hz, CH_2COO). Anal. Calcd for $C_{58}H_{70}O_{14}$: C, 70.21; H, 7.11. Found: C, 70.33; H, 6.93.

*8-Methoxycarbonyloctyl 2,3,4,6-tetra-*O*-benzyl-α-*D*-glucopyranosyl-(1 → 2)-3,4,6-tri-*O*-benzyl-α-*D*-glucopyranosyl-(1 → 3)-2-*O*-benzyl-4,6-*O*-(*p*-methoxybenzylidene)-α-*D*-glucopyranoside* (**8**).—*N,N*-Dimethylformamide (140.6 μL, 1.82 mmol) and tetraethylammonium bromide (38.2 mg, 0.18 mmol) were added to a suspension of copper(II) bromide (243.4 mg, 1.1 mmol) and molecular sieves 4 Å (600 mg). After stirring the dark green mixture for 0.5 h at room temperature, solutions of compound **6** (120 mg, 0.12 mmol) in dichloromethane (1 mL) and of the glucosyl bromide **7** (365.4 mg, 0.61 mmol) in dichloromethane (1 mL) were added by syringe, dropwise, over a period of approximately 0.5 h. After stirring the reaction mixture for 36 h, collidine (100 μL) was added, and then the reaction was diluted with dichloromethane (25 mL). The solids were filtered and washed with dichloromethane (50 mL). The filtrate and washings were evaporated to a syrup that was purified by chromatography on Iatrobeads using 3:1 hexane–EtOAc as eluent. Pure **8** was obtained as a syrup (130 mg, 71%): $[\alpha]_D$ +75.6° (*c* 1.3, $CHCl_3$). ^1H NMR ($CDCl_3$): δ 5.68 (d, 1 H, $J_{1'',2''}$ 3.5 Hz, H-1''), 5.14 (s, 1 H, $C_6H_5CHO_2$), 4.77 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 3.65, 3.64 (s, 3 H each, 2 × OCH_3), 2.29 (t, 2 H *J* 7.5 Hz, CH_2COO). Anal. Calcd for $C_{92}H_{104}O_{19}$: C, 72.99; H, 6.90. Found: C, 73.23; H, 6.80.

*8-Methoxycarbonyloctyl α-*D*-glucopyranosyl-(1 → 2)-α-*D*-glucopyranosyl-(1 → 3)-α-*D*-glucopyranoside* (**9**).—Compound **8** (72 mg, 48 μmol) was dissolved in 95% EtOH (5 mL) containing 5% Pd/C (30 mg) and stirred under an H_2 atmosphere (1 atm) for 15 h, by which time TLC showed the disappearance of **8** to give one major spot that was devoid of UV-absorption on TLC. Removal of the catalyst by filtration, followed by evaporation and purification by passage through Bio-Gel P-2 (200–400 mesh, 50 × 2.5 cm) using 10% EtOH as eluent provided **9** (28.5 mg, 89%) as a white powder: $[\alpha]_D$ +155.0° (*c* 2.5, H_2O). ^1H NMR (D_2O): δ 5.54 (d, 1 H, $J_{1'',2''}$ 4.0 Hz, H-1''), 5.18 (d, 1 H $J_{1',2'}$ 4.0 Hz, H-1'), 4.91 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 3.69 (s, 3 H, OCH_3), 2.39 (t, 2 H, *J* 7.5 Hz, CH_2COO). FABMS of compound **9** *m/z* 697.1 (M + Na).

*8-Methoxycarbonyloctyl α-*D*-glucopyranosyl-(1 → 3)-α-*D*-glucopyranoside* (**10**).—A solution of compound **6** (65 mg, 66 mmol) in 95% EtOH (5 mL) was hydrogenated in the presence of 5% Pd/C (30 mg) at normal pressure overnight at room temperature. The catalyst was filtered on the pad of Celite and washed with EtOH, and the combined filtrate and washings were concentrated. Pure **10** (30.0 mg, 94%) was obtained after Bio-Gel P-2 (200–400 mesh, 50 × 2.5 cm) filtration using 10% EtOH as eluant: $[\alpha]_D$ +91° (*c* 0.15, H_2O). ^1H NMR (D_2O): δ 5.35 (d, 1 H, $J_{1',2'}$ 4.0 Hz, H-1'), 4.91 (d, 1 H, $J_{1,2}$ 34.0 Hz, H-1), 3.69 (s, 3 H, OCH_3), 2.39 (t, 2 H, *J* 7.5 Hz, CH_2COO). FABMS of compound **10** *m/z* 535.1 (M + Na).

8-Methoxycarbonyloctyl α-D-glucopyranoside (**11**).—Compound **2** (50 mg, 0.11 mmol) was hydrogenated in 95% EtOH (5 mL) using 5% Pd/C (25 mg) for 1 h at room temperature. The catalyst was filtered on the pad of Celite and washed with EtOH, and the combined filtrate and washings were concentrated. Pure **11** (32.5 mg, 82%) was obtained after Bio-Gel P-2 (200–400 mesh, 50 × 2.5 cm) filtration using 10% EtOH as eluent: $[\alpha]_D$ +99.5° (*c* 0.9, H_2O). ^1H NMR (D_2O): δ 4.96 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 3.75 (s, 3 H, OCH_3), 2.45 (t, 2 H, *J* 7.5 Hz, CH_2COO). FABMS of compound **11** *m/z* 373.1 (M + Na).

Standardized procedure for the preparation of ethylenediamine monoamides of methoxycarbonyl octyl tri-, di- and monosaccharides (**12**, **13**, and **14**).—The methyl ester of each saccharide (5 mg) was dissolved in neat anhydrous ethylenediamine (2 mL) and heated at 70 °C for 50 h in a screw-capped tube with a Teflon-lined cap. The tube was cooled in an ice bath, and water (5 mL) was added. The cooled solution was then passed through a C_{18} Sep-Pak cartridge that was then washed with water (30 mL). The aq flow-through was loaded onto a fresh Sep-Pak that was also washed with water (30 mL). The product was eluted from each Sep-Pak by washing with MeOH (30 mL), the eluates were combined, and the MeOH was evaporated. The residue was dissolved in water (10 mL) and re-isolated on a fresh Sep-Pak from which it was eluted with MeOH. The residue after evaporation was dissolved in water (8 mL) and passed through a 0.2 μM filter and lyophilized. Recoveries were in the range 80–90%.

FABMS of derivatized tri-, di- and mono-saccharides (**12**, **13**, and **14**) were *m/z* 725.55 (M + Na), 563.29 (M + Na), 378.24 (M + Na), respectively. ^1H NMR (D_2O) (**12**): δ 5.53 (d, 1 H, $J_{1'',2''}$ 3.5 Hz, H-1''), 5.17 (d, 1 H, $J_{1',2'}$ 3.7 Hz, H-1'), 4.90 (d, 1 H, $J_{1,2}$ 3.8 Hz, H-1), 2.25 (t, 2 H, *J* 7.3 Hz, $CH_2C(O)NH$), 2.89 (t, 2 H, *J* 6.2 Hz, CH_2NH_2) 3.34 (t, 2 H, *J* 6.3 Hz, -C(O)NHCH_2); (**13**): δ 5.34 (d, 1 H, $J_{1',2'}$ 3.7 Hz, H-1'), 4.90 (d, 1 H, $J_{1,2}$ 3.6 Hz, H-1), 2.25 (t, 2 H, *J* 7.2 Hz, $CH_2C(O)NH$), 2.85 (t, 2 H, *J* 6.1 Hz, CH_2NH_2) 3.32 (t, 2 H, *J* 6.2 Hz, -C(O)NHCH_2); (**14**): δ 4.90 (d, 1 H, $J_{1,2}$ 3.8 Hz, H-1), 2.25 (t, 2 H, *J* 7.3 Hz, $CH_2C(O)NH$), 2.83 (t, 2 H, *J* 6.2 Hz, CH_2NH_2) 3.31 (t, 2 H, *J* 6.2 Hz, -C(O)NHCH_2).

Standardized procedure for the fluorescence labelling of ethylenediamine-derivatized tri-, di- and mono-saccharides (**15**, **16**, and **17**).—Labelling of the amines was performed as described by the manufacturer. Briefly, the tetramethylrhodamine (TMR) *N*-hydroxysuccinimide ester (5 mg) was dissolved in DMF (0.25 mL) and added to the amine (3–4 mg) in 0.185 M $NaHCO_3$ (pH 8.5, 0.25 mL). After 4 h, the reaction mixture was diluted with water (4 mL) and applied to a column of DEAE-Sephadex A25 (Cl^- form, 0.8 × 5 cm), and water was passed through the column until the eluate was colourless (30–40 mL). The brilliant red product was isolated from the washes by C_{18} Sep-Pak adsorption as described above. The residue in MeOH was applied to a preparative silica gel plate that was developed using solvent B. The red band containing the product was scraped from the plate and extracted with MeOH (4 × 50 mL) by stirring for 10 min until the silica gel was colourless. The labelled compounds were then recovered on Sep-Pak as described above. Final purification was performed by passage through Bio-Gel P2 (2.5 × 40 cm) using 10% aq EtOH as solvent. Evaporation of the first red fraction, reisolation on a Sep-Pak, 0.2 μm filtration and lyophilization produced dark red fluffy powders which were stored at −20 °C protected from light.

Table 1
Selected ^{1}H NMR data for oligosaccharides **12–17** [a]

Proton	Compound					
	12	**13**	**14**	**15**	**16**	**17**
-CH_2C(O)NH-	2.25 (7.3)	2.25 (7.2)	2.25 (7.3)	2.33 (7.4)	2.31 (7.0)	2.35 (7.2)
-$CH_2$$NH_2$-	2.89 (6.2)	2.85 (6.1)	2.83 (6.2)	3.22	3.22	3.22
-C(O)NHCH_2-	3.34 (6.3)	3.32 (6.2)	3.31 (6.2)	3.22	3.22	3.22
H-1	4.90 (3.8)	4.90 (3.6)	4.90 (3.8)	4.81 (obscured)	4.81 (obscured)	4.81 (obscured)
H-1′	5.17 (3.7)	5.34 (3.7)		5.12 (3.9)	5.27 (3.9)	
H-1″	5.53 (3.5)			5.47 (3.6)		
TMR (a)				8.38 (1.8)	8.33 (1.5)	8.38 (1.5)
TMR (b)				8.14 (1.8,8.0)	8.09 (1.5, 8.0)	8.13 (1.5, 8.0)
TMR (c)				7.60 (8.0)	7.58 (8.0)	7.58 (8.0)
TMR (d)				7.26 (9.5, 2.6)	7.25 (1.5,9.5)	7.26 (9.5)
TMR (e)				6.94 (9.7)	6.93 (9.5, 2.0)	6.93 (9.5, 2.0)
TMR (f)				6.54	6.61 (1.5)	6.54 (2.0)

[a] Numbers in parentheses are couplings given in Hz. Chemical shifts are reported in ppm, referenced to internal acetone at 2.225 ppm. Assignment of protons for 5-carboxytetramethylrhodamine (a–f) as shown in the structures.

FABMS of derivatized tri-, di- and mono-saccharide (**15**, **16**, and **17**) were *m/z* 1137.33 (M + Na), 975.61 (M + Na), 813.73 (M + Na), respectively. Partial ^{1}H NMR data are shown in Table 1.

Glucosidase I.—The soluble form of α-glucosidase I was isolated as previously described from a dry culture of *Saccharomyces cerevisiae* [13]. The hydrolysis of TMR-trisaccharide by glucosidase I was followed by incubating 0.025 mU glucosidase I and 3.33 mM TMR-trisaccharide in a volume of 7.5 μL. At various time intervals, two 0.25-μL samples were removed from the reaction. One was spotted on a TLC plate and run in solvent B. The second was placed in 500 μL of 1.25 M Tris-HCl buffer, pH 7.6 to quench the reaction. The substrate and product were subsequently isolated using a C_{18} Sep-Pak cartridge, as described below, and separated and quantified using capillary electrophoresis [24]. Separations were carried out in a 42 cm × 10 μm o.d. fused-silica capillary and a running buffer composed of 10 mM each of Na_2HPO_4, borate, phenyl boronic acid, and sodium dodecyl sulfate at pH 9.3. The detection system was a postcolumn laser-induced fluorescence detector and a 1.0-mW He–Ne laser with a wavelength of 543.5 nm as the excitation source [24].

The K_m value of the TMR-trisaccharide for glucosidase I was estimated by incubating 0.038 mU of glucosidase I with 6.4, 12.8 and 33.3 nmol of TMR-trisaccharide in a 10 μL volume at 37 °C for 1 h. The reactions were quenched by addition of 100 μL of 1.25 M Tris-HCl, pH 7.6. Substrate and product were isolated from the reaction mixture by loading onto a C_{18} Sep-Pak cartridge. Buffer salts, glucose, and protein were washed from the Sep-Pak with 30 mL of water, and substrate and product were eluted with 2.5 mL of MeOH. Approximately 10 μL of the MeOH elution was injected onto a Nova-Pak C_{18} HPLC column, using 1:1 MeOH–H_2O as the solvent, at a flow rate of 0.9 mL/min and detection at 550 nm. Reaction rates were estimated by calculating the

percentage of trisaccharide hydrolysis, using an extinction coefficient for the trisaccharide and the disaccharide of $\varepsilon = 63000\ M^{-1}\ cm^{-1}$ (MeOH). The K_m value was estimated by fitting initial rate data to the Michaelis–Menton equation using nonlinear regression analysis employing the Marquardt–Levenberg algorithm (SigmaPlot, Jandel Scientific, 1991).

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References

[1] R. Kornfeld and S. Kornfeld, *Ann. Rev. Biochem.*, 54 (1985) 631–664.
[2] R.A. Ugalde, R.J. Staneloni, and L.E. Leloir, *Eur. J. Biochem.*, 113 (1980) 97–103.
[3] L.S. Grinna and P.W. Robbins, *J. Biol. Chem.*, 254, (1979) 8814–8818.
[4] K.W. Moreman, R.B. Trimble, and A. Herscovics, *Glycobiology*, 4 (1994) 113–125.
[5] Y.T. Pan, H. Hori, R. Saul, B.A. Sanford, R.J. Molyneux, and A.D. Elbein, *Biochemistry*, 22 (1983) 3975–3984.
[6] B. Saunier, R.D. Kilker Jr., J.S. Tkacz, A. Quaroni, and A. Herscovics, *J. Biol. Chem.*, 257 (1982) 14155–14161.
[7] E. Fenouillet, J.C. Gluckman, and I.M. Jones, *Trends Biochem. Sci.*, 19 (1994) 65–70.
[8] R.A. Gruters, J.J. Neefjes, M. Tersmette, R.E.Y. deGoede, A. Tulp, H.G. Huisman, F. Miedema, and H.L. Ploegh, *Nature*, 330 (1987) 74–77.
[9] R.D. Kilker Jr., B. Saunier, J.S. Tkacz, and A. Herscovics, *J. Biol. Chem.*, 256 (1981) 5299–5304.
[10] J.M. Michael and S. Kornfeld, *Arch. Biochem. Biophys.*, 169 (1980) 249–258.
[11] D.R.P. Tulsiani, S.C. Hubbard, P.W. Robbins, and O. Touster, *J. Biol. Chem.*, 257 (1982) 3669–3668.
[12] A. Herscovics, and S. Jelinek-Kelly, *Anal. Biochem.*, 166 (1987) 85–89.
[13] I. Neverova, C.H. Scaman, O.P. Srivastava, R. Szweda, I.K. Vijay, and M.M. Palcic, *Anal. Biochem.*, 222 (1994) 190–195.
[14] J.Y. Zhao, N.J. Dovichi, O. Hindsgaul, S. Gosselin, and M.M. Palcic, *Glycobiology*, 4 (1994) 239–242.
[15] G.O. Aspinall and M.K. Gurjar, *Carbohydr. Res.*, 143 (1985) 266–270.
[16] R.U. Lemieux, K.B. Hendriks, R.V. Stick, and K. James, *J. Am. Chem. Soc.*, 97 (1975) 4056–4062.
[17] P. Fugedi, P.J. Garegg, H. Lonn, and T. Norberg, *Glycoconjugate J.*, 4 (1987) 97–108.
[18] P. Kovac and L. Lerner, *Carbohydr. Res.*, 184 (1988) 87–112.
[19] S. Khan, R.K. Jain, and K.L. Matta, *Meeting Soc. Complex Carbohydr.*, Ann Arbor, MI, Nov. 8–11, 1989, Abstr. G160.
[20] K. Kioke, M. Sugimoto, S. Sato, Y. Ito, Y. Nakahara, and T. Ogawa, *Carbohydr. Res.*, 163 (1987) 189–208.
[21] S. Sato, M. Mori, Y. Ito, and T. Ogawa, *Carbohydr. Res.*, 155 (1986) C6–C10.
[22] Y. Nakahara, K. Sakai, N. Hong, and T. Ogawa, *Intl. Chem. Congress Pacific Basin Soc.*, Honolulu, Hawaii, 1989, Abstr. BIOS-0410.
[23] Y. Zhang, X. Le, N.J. Dovichi, C.A. Compston, M.M. Palcic, P. Diedrich, and O. Hindsgaul, *Anal. Biochem.*, 227 (1994) 368–376.
[24] X. Le, C.H. Scaman, Y. Zhang, J. Zhang, N.J. Dovichi, O. Hindsgaul, and M.M. Palcic, *J. Chromatogr. A.*, 716 (1995) 215–220.