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Synthesis of 4-methylumbelliferyl α-D-mannopyranosyl-(1→6)-β-D-mannopyranoside and development of a coupled fluorescent assay for GH125 *exo*-α-1,6-mannosidases

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ABSTRACT

Certain bacterial pathogens possess a repertoire of carbohydrate processing enzymes that process host N-linked glycans and many of these enzymes are required for full virulence of harmful human pathogens such as *Clostridium perfringens* and *Streptococcus pneumoniae*. One bacterial carbohydrate processing enzyme that has been studied is the pneumococcal virulence factor SpGH125 from *S. pneumoniae* and its homologue, CpGH125, from *C. perfringens*. These *exo*-α-1,6-mannosidases from glycoside hydrolase family 125 show poor activity toward aryl α-mannopyranosides. To circumvent this problem, we describe a convenient synthesis of the fluorogenic disaccharide substrate 4-methylumbelliferone α-D-mannopyranosyl-(1→6)-β-D-mannopyranoside. We show this substrate can be used in a coupled fluorescent assay by using β-mannosidases from either *Cellulomonas fimi* or *Helix pomatia* as the coupling enzyme. We find that this disaccharide substrate is processed much more efficiently than aryl α-mannopyranosides by CpGH125, most likely because inclusion of the second mannose residue makes this substrate more like the natural host glycan substrates of this enzyme, which enables it to bind better. Using this sensitive coupled assay, the detailed characterization of these metal-independent *exo*-α-mannosidases GH125 enzymes should be possible, as should screening chemical libraries for inhibitors of these virulence factors.

1. Introduction

Certain bacterial pathogens possess a repertoire of carbohydrate processing enzymes that process host N-linked glycans and many of these enzymes are required for full virulence of harmful human pathogens such as *Clostridium perfringens* and *Streptococcus pneumoniae*.[1,2] Several proposals regarding the functional role of these enzymes in virulence have been advanced including processed monosaccharides serving as a carbon source for growth and biofilm formation, the exposure of cell surface receptors for adherence, and modulation of the host immune response.[2] Although the ability to process glycans is emerging as being critically important in pathogenesis, knowledge of the biochemical properties of these bacterial carbohydrate processing enzymes that cleave human glycans is rather limited.

One such bacterial carbohydrate processing enzyme that has been studied is the pneumococcal virulence factor SpGH125 from *S. pneumoniae* and its homologue, CpGH125, from *Clostridium perfringens*.[3,4] These enzymes have been recently characterized as highly specific *exo*-α-1,6-mannosidases[3] that comprise GH125 (for a discussion of the sequence based classification system of glycoside hydrolases see the carbohydrate active enzymes (CAZy) database[5] at http://www.cazy.org/). This new family of glycoside hydrolases, unlike divalent metal-dependent α-mannosidases from GH families 38, 47, and 92, use a metal-independent inverting catalytic mechanism.[3]

These *exo*-α-1,6-mannosidases show poor activity against even highly activated monosaccharide substrates such as 2,4-dinitrophenyl α-D-mannopyranoside (**1**) and natural substrates containing α-D-mannopyranosyl-(1→6)-mannopyranose units (Fig. 1) have no convenient fluorogenic reporter group.[3] Structural studies, however, have revealed the mannose unit binding to the +1 subsite of a well defined active site, forming multiple carbohydrate protein interactions. The specific and extensive interactions of these enzymes, their high specificity for the α1,6-mannobiose linkage, coupled with mannopyranosides bearing even a very good 2,4-dinitrophenol leaving group being poorly processed, suggests that

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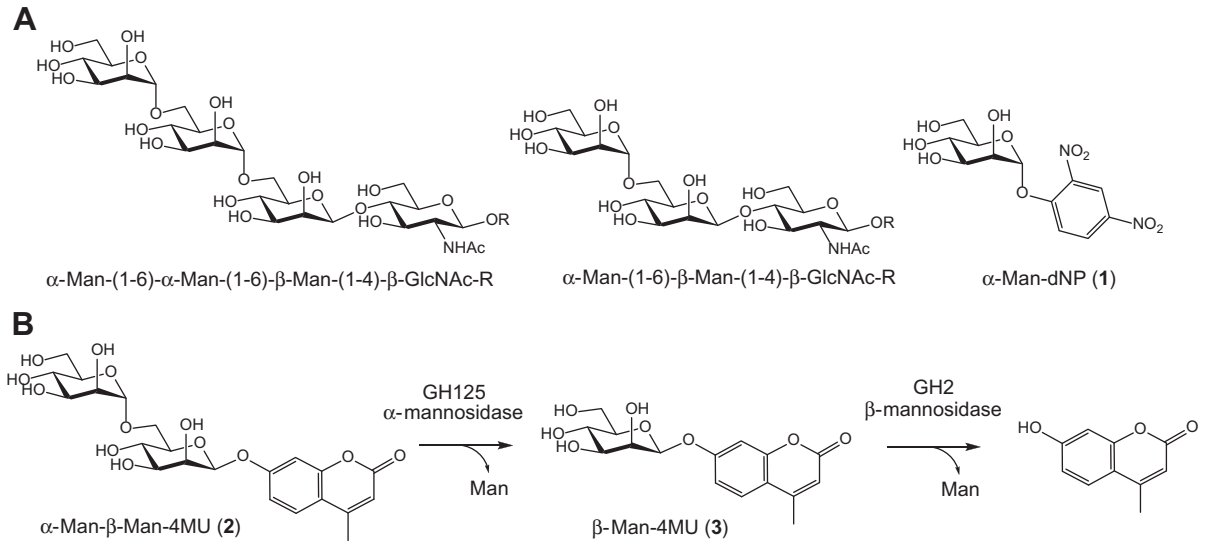


Figure 1. Structures of natural substrates of GH125 enzymes and the coupled assay system. (A) Natural oligosaccharide substrates and a poor previously identified chromogenic substrate (**1**) for GH125 enzymes. (B) The proposed coupled assay system using 4-methylumbelliferone α-D-mannopyranosyl-(1→6)-β-D-mannopyranoside (α-Man-β-Man-4MU, **2**). This substrate is hydrolyzed by a GH125 enzyme to generate 4-methylumbelliferone β-D-mannopyranoside (β-Man-4MU, **3**), which is a substrate that can be cleaved by a GH2 β-mannosidase to liberate the 4-methylumbelliferone fluorophore.

the mannose residue binding to the +1 subsite of these enzymes facilitates catalysis.[3] Given that GH125 *C. perfringens* and *S. pneumoniae* enzymes seem not to have a defined +2 subsite, we felt that we could install a fluorogenic leaving group in the β-configuration pendent to the mannose residue binding in the +1 subsite. To address this question and develop a convenient activity assay that could be used to screen for inhibitors of GH125 enzymes we describe the synthesis of a fluorogenic disaccharide substrate. 4-Methylumbelliferone α-D-mannopyranosyl-(1→6)-β-D-mannopyranoside (α-Man-β-Man-4MU, **2**) should be a better mimic of the natural host glycan substrates as compared to α-Man-dNP (**1**) and we therefore expect that GH125 enzymes should efficiently cleave the α1,6-glycosidic linkage to liberate 4-methylumbelliferone β-D-mannopyranoside (Fig. 1). A coupled assay could therefore be developed by including a β-mannosidase in the reaction to hydrolyze the liberated 4-methylumbelliferone β-D-mannopyranoside and release 4-methylumbelliferone, which can be detected conveniently (Fig. 1). Here we detail the synthesis of α-man-β-man-4MU and the implementation of this sensitive coupled assay for GH125 *exo*-α-1,6-mannosidases.

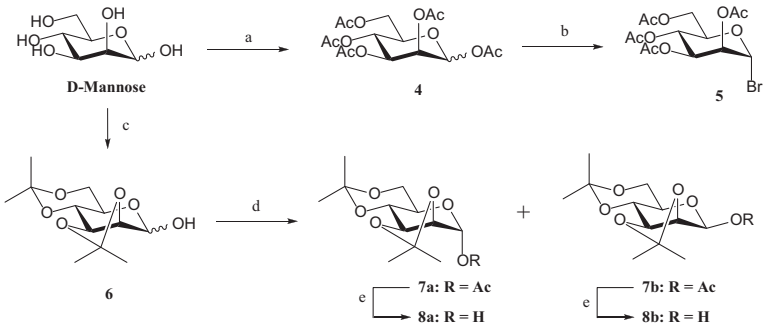
2. Results and discussion

2.1. Chemical synthesis of 4-methylumbelliferyl α-D-mannopyranosyl-(1→6)-β-D-mannopyranoside (α-Man-β-Man-4MU, 2)

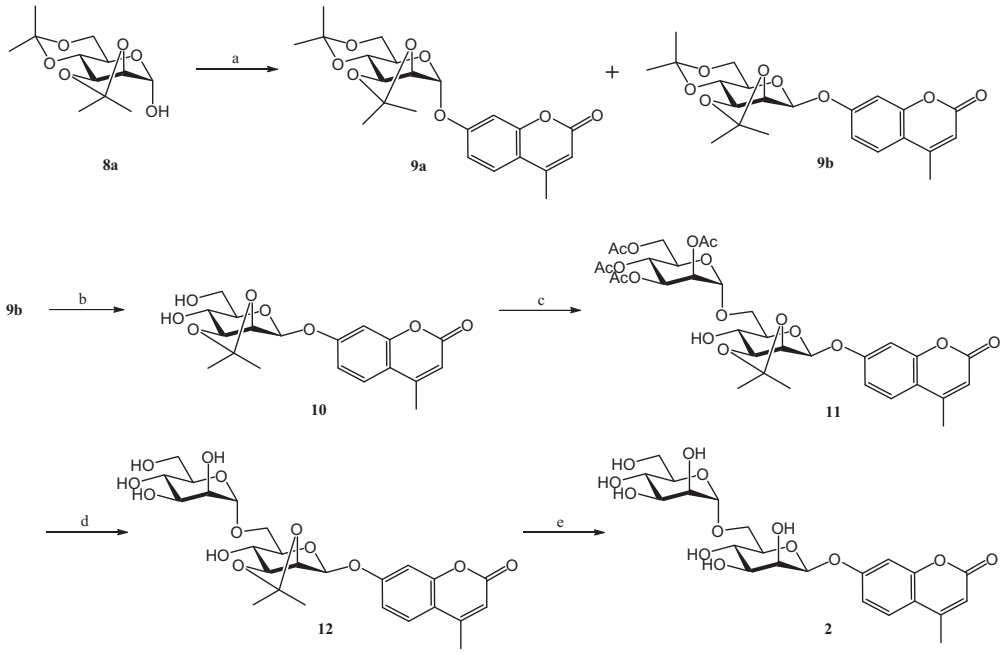
As a first step we set out to synthesize α-Man-β-Man-4MU (**2**), which we speculated would be a better substrate than simple aryl α-D-mannopyranosides for GH125 α-mannosidases. The synthesis of 4-nitrophenyl α-D-mannopyranosyl-(1→6)-β-D-mannopyranoside has been reported[6] but we felt that a fluorogenic substrate would be more desirable to have for a high sensitivity assay. Nevertheless, the route developed by Khan and coworkers involving Koenigs–Knorr glycosylation to form the intersaccharide linkage appeared to be efficient and so we used a related approach differing primarily in the preparation of the β-mannoside acceptor. Selective protection of the 2–4 and 6 positions was achieved by formation of 2,3:4,6-di-*O*-isopropylidene-D-mannopyranose (**6**) as previously described by Gelas and Horton using 2-methoxypropene with catalytic *p*-toluenesulfonic acid[7] with minor modifications (Scheme 1). We elected to separate the anomers by *O*-acetylation (**7a** and **b**) followed by silica gel chromatography and subsequent de-*O*-acetylation to yield the pure anomers (**8a** and **b**). With the pure α-hemiacetal in hand we used the Mitsunobu approach developed by Garegg for the formation of aryl β-mannosides[8] and generalized by Cocinero et al.[9] Coupling of 2,3:4,6-di-*O*-isopropylidene-D-mannopyranose (**8a**) with 4-methylumbelliferone in the presence of diisopropyl azodicarboxylate (DIAD) and triphenylphosphine furnished an anomeric mixture (5:1 in favour of the desired β-anomer) of 4-methylumbelliferyl 2,3:4,6-di-*O*-isopropylidene-D-mannopyranoside (**9a** and **b**), from which the desired β-anomer (**9b**) was conveniently obtained by crystallization in high yield (Scheme 2). Selective removal of the 4,6-*O*-isopropylidene afforded 4-methylumbelliferyl 2,3-*O*-isopropylidene-β-D-mannopyranoside (**10**), which was selectively glycosylated using $HgCN_2$ as the catalyst and per-*O*-acetyl-mannopyranosyl bromide as the donor following the general approach reported by Khan et al.[6] Zemplen de-O-acetylation followed by hydrolytic cleavage of the 2,3-isopropylidene protecting group afforded the target fluorogenic substrate α-Man-β-Man-4MU (**2**) (Scheme 2).

2.2. Evaluation of 4-methylumbelliferyl α-D-mannopyranosyl-(1→6)-β-D-mannopyranoside (α-Man-β-Man-4MU, 2 as a substrate for CpGH125 exo-α-mannosidase

The coupled assay we envisioned depended on a GH125 *exo*-α-mannosidase acting to liberate methylumbelliferyl β-D-mannopyranoside (β-Man-4MU), which could then be processed by a β-mannosidase. We therefore considered various β-mannosidases and felt that the *Cellulomonas fimi* β-mannosidase known as CfMan2A[10], which was shown by Zechel et al. as being highly active against Man-4MU,[11] would be a good candidate coupling enzyme. CfMan2A is a retaining glycoside hydrolase that uses nucleophilic and acid–base catalysis, having a pH optimum of 7.5[11] which is compatible with the activity of CpGH125. We first evaluated the activity of CfMan2A toward β-Man-4MU (Fig. 2) and found the kinetic constants (Table 1) to be similar to those reported in the literature.[11] We next evaluated CfMan2A as a coupling enzyme with CpGH125. We kept CpGH125 at a constant concentration of 1 nM and set out to establish an adequate concentration of CfMan2A that



Scheme 1. Synthesis of the mannose donor (**5**) and acceptor precursor (**8a**). Reagents and conditions: (a) Ac_2O, pyridine, rt; (b) 33% HBr–HOAc, rt, 84% over two steps; (c) 2-methoxypropene, *p*-TsOH·H_2O, DMF, 0 °C to rt, overnight, 70%; (d) Ac_2O, pyridine, rt, 100% (44% for **7a**, 56% for **7b**); (e) NaOMe, MeOH, rt, 97% for **8a**, 95% for **8b**.



Scheme 2. Synthesis of α-Man-β-Man-4MU. Reagents and conditions: (a) 4-methylumbelliferone, PPh_3, DIAD, toluene, rt, 85% (14% for **9a**, 71% for **9b**); (b) 80% aq. HOAc, rt; (c) donor **5**, $HgCN_2$, MeCN, 4 Å MS, rt, 64% (for two steps); (d) NaOMe, MeOH, rt, 60%; (g) TFA, $CHCl_3/H_2O$ (100:1), rt, 88%; (e) 80% aq HOAc, 60–70 °C, 3 h, 80%.

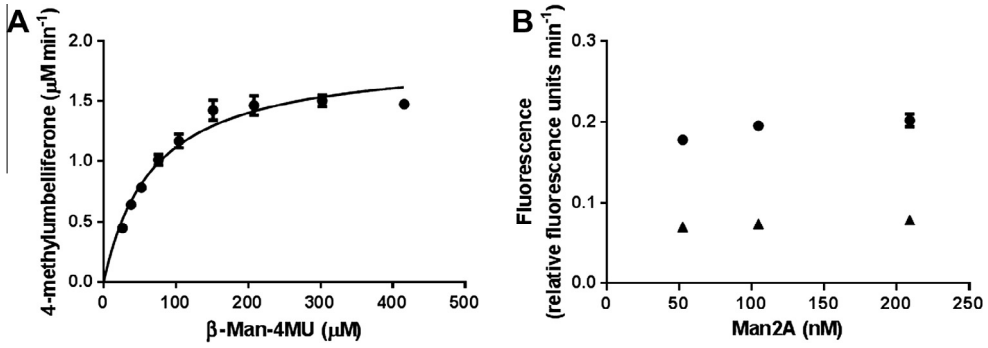


Figure 2. Validation of CfMan2A activity toward 4-methylumbelliferyl β-D-mannopyranoside (β-Man-4MU) and determining the required CfMan2A concentration for the coupled assay. (A) Michaelis–Menten kinetics for the activity of CfMan2A (1 nM) toward the monosaccharide substrate β-Man-4MU (**3**). (B) CfMan2A concentrations of greater than 100 nM are sufficient to ensure no dependence on CfMan2A concentration for the assay of CpGH125 (1 nM) activity toward the disaccharide substrate α-Man-β-Man-MU (**2**). Symbols represent the assay performed using α-Man-β-Man-MU concentrations of either 180 μM (circles) or 60 μM (triangles). Error bars represent the standard deviation of triplicate measurements.

could be used to ensure that CpGH125 is rate limiting when monitoring the production of 4-methylumbelliferone.

We found that the reaction velocities did not change significantly when concentrations of β-mannosidase of 100 nM or greater were used (Fig. 2). We therefore decided to use a concentration of 140 nM for all subsequent coupled assays. Surprisingly, during control experiments we observed that CfMan2A showed low activity toward α-Man-β-Man-4MU (**2**). We therefore evaluated

Table 1
Kinetic parameters of enzymes employed in the assay

Enzyme	Substrate	k_{cat} (min-1)	K_m (μM)	k_{cat}/K_m (min^{-1} μM^{-1})
CfMan2A[a]	β-Man-4MU (**3**)	1880 ± 5	68 ± 6	17 ± 2
CpGH125[b]	α-Man-β-Man-4MU (**2**)	294 ± 6	380 ± 20	0.77 ± 0.05
CpGH125[c]	α-Man-dNP (**1**)	ND	ND	$1.80 \pm 0.006 \times 10^{-3}$

[a] Conditions: 1 nM CfMan2A.
[b] Conditions: 1 nM CpGH125, 140 nM CfMan2A.
[c] Conditions: 9050 nM CpGH125.

CfMan2A activity against 2,4-ditrophenyl α-D-mannopyranoside (αMan-dNP, **1**) to check for potential α-mannosidase contamination but observed no activity against this substrate (data not shown), which suggests that CfMan2A has low *endo*-β-mannosidase activity. Fortunately, however, the level of this *endo*-mannosidase activity was very low; when using the same concentration of CfMan2A as in the coupled enzyme assays (140 nM), the rates of CfMan2A catalyzed hydrolysis of α-Man-β-Man-4MU (**2**) were less than ~1% the rate observed in the presence of CpGH125 (1 nM) during the coupled assay. CfMan2A therefore processes α-Man-β-Man-4MU (**2**) over 11,000-fold more slowly than CpGH125. This contribution to substrate turnover can be considered minor for the purposes of these coupled assays and indicates CfMan2A is useful for routine coupled assays of CpGH125 using this disaccharide substrate. Given the known structure of CfMan2A,[12] any CfMan2A-catalyzed hydrolysis of α-Man-β-Man-4MU (**2**) is somewhat surprising given that the 6-hydroxyl group in the Michaelis complex of CfMan2A shows this group is constrained within the enzyme active site. This 6-hydroxyl group, however, points toward the solvent and it is possible some rearrangement in the enzyme active site might enable it to tolerate a second mannose residue appended to this group. We verified CfMan2A activity against α-Man-β-Man-4MU (**2**) using capillary electrophoresis (see the Supplementary data). Given that CfMan2A appears to show some low *endo*-β-mannosidase activity, we realized that this assay may not be useful in all circumstances. For this reason we also evaluated a commercial β-mannosidase that is prepared from *Helix pomatia* (Sigma). This β-mannosidase showed no detectable activity against α-Man-β-Man-4MU (**2**) and was also successfully used in the coupled assay system with the details provided in the methods section (data not shown).

Given the assay using CfMan2A was sufficiently robust to evaluate CpGH125 enzyme activity, we carried out a kinetic evaluation of CpGH125 against α-Man-dNP (**1**) as well as using this coupled system with α-Man-β-Man-4MU (**2**) (Fig. 3). We find that CpGH125 processes α-Man-β-Man-4MU (**2**) 400-fold more efficiently than α-Man-dNP (**1**) (Table 1) despite α-Man-dNP (**1**) having a much more activated leaving group. Notably, when using α-Man-dNP (**1**) as a substrate for CpGH125, saturation kinetics were not observed (Fig. 3), enabling us to determine only the second-order rate constant for this substrate (Table 1). These data suggest that binding of α-Man-dNP (**1**) to the enzyme is quite poor. In contrast, when using α-Man-β-Man-4MU (**1**), we observed saturation kinetics and a K_m value of 380 μM which, though K_m is a kinetic parameter, suggests this substrate is bound better by CpGH125. This finding is consistent with structural studies of CpGH125, which shows there is a +1 subsite that forms extensive contacts with a bound mannose residue.[3]

3. Conclusions

For GH125 enzymes, as exemplified using CpGH125, we find that α-Man-β-Man-4MU (**2**) is a far superior substrate as compared to α-Man-dNP (**1**). The coupled assay using CfMan2A as the coupling β-mannosidase shows high sensitivity and enables us to observe traditional Michaelis-Menten kinetics for CpGH125. The results show α-Man-β-Man-4MU (**2**) saturates CpGH125 and is processed with a k_{cat}/K_m value that is 400-fold higher than that observed for dNP-αMan. We find that Man2A from *C. fimi* shows low *endo*-β-mannosidase activity, which may limit its utility in this coupling system for certain applications. In those cases, the *H. pomatia* β-mannosidase is likely to prove useful because this enzyme shows no detectable *endo*-β-mannosidase activity toward this disaccharide substrate. With this sensitive coupling system available, a more detailed characterization of these interesting metal-independent GH125 *exo*-α-mannosidases should be possible and screening chemical libraries for inhibitors of these virulence factors should be feasible. Such inhibitors could prove to eventually be valuable research tools[13] for studying the roles of these enzymes in the host-pathogen interaction and may open a route to the generation of new antibiotics to combat pathogens such as *S. pneumoniae*.

4. Materials and methods

4.1. General

Unless otherwise indicated, reagents and starting materials were purchased from Sigma–Aldrich, TCI America, or Alfa Aesar and were used without purification. ^1H and ^{13}C NMR spectra were obtained on a BrukerAV600 (600 MHz for ^1H and 150.8 MHz for ^{13}C) spectrometer. Except where indicated, deuterated chloroform ($CDCl_3$) was used as the solvent with $CHCl_3$ (δ_H 7.26) or $CDCl_3$ (δ_C 77.0) being used as internal standards. NMR spectra run in D_2O used internal CH_3OH (δ_H 3.34, δ_C 49.0) as the standard. High

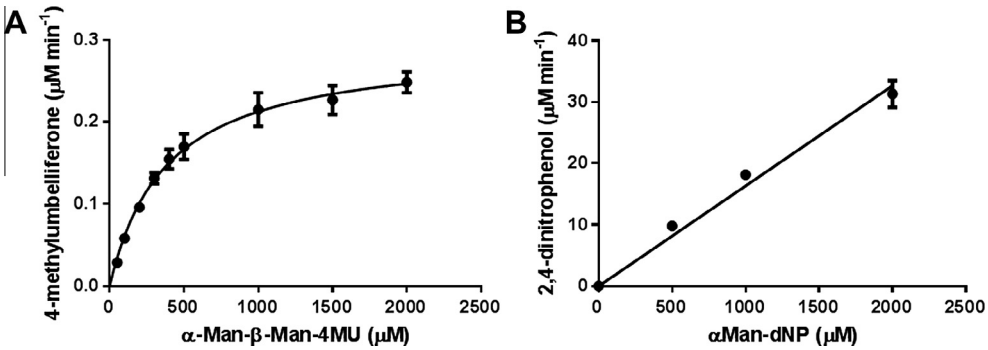


Figure 3. CpGH125 activity toward disaccharide and monosaccharide substrates. (A) Michaelis-Menten kinetics for the activity of CpGH125 (1 nM) toward the disaccharide substrate α-Man-β-Man-4MU (**2**) using the coupled assay in the presence of CfMan2A (140 nM). (B) Activity of CpGH125 (9050 nM) toward the monosaccharide substrate α-Man-dNP. Error bars represent the standard deviation of triplicate measurements.

resolution mass spectra were recorded using a Bruker micrOTOF-II LC–mass spectrometer. Flash chromatography was performed on BDH silica gel with the specified solvents. Thin-layer chromatography (TLC) was effected on Merck silica gel 60 F_{254} aluminum-backed plates that were stained by heating (⩽200 °C) with 5% sulfuric acid in EtOH. Percentage yields for chemical reactions as described are quoted only for those compounds that were purified by recrystallization or by column chromatography, and the purity was assessed by ^{1}H NMR spectroscopy. All solvents were purchased from Sigma–Aldrich, Caledon, or Anachemia and all solvents except DMF and MeCN were distilled before use and dried according to the methods of Burfield and Smithers.[14] CpGH125 and SpGH125 were expressed as previously described.[3] *C. fimi* Man2A was a kind gift from Professor S. G. Withers (University of British Columbia).

4.2. Chemical synthesis

4.2.1. 2,3,4,6-*tetra*-*O*-Acetyl-α-D-mannopyranosyl bromide (5)[6]

D-Mannose (10 g, 55.5 mmol) was dissolved in dry pyridine (50 mL), and Ac_2O (50 mL) was added at 0 °C, the resulting reaction mixture was stirred at rt overnight. The solvent was concentrated in vacuo, the residue was co-evaporated with toluene (4 × 20 mL), and then dried under vacuum to afford the title compound **4** (21.7 g crude), which was used directly in the next step without purification. Per-*O*-acetate **4** (1.84 g, 4.71 mmol) was dissolved in 33% HBr–HOAc (15 mL), the resulting solution was stirred at rt for 5 h, after which EtOAc (100 mL) was added and the reaction mixture, and poured into ice-cooled water, washed with cold water, cold sat. $NaHCO_3$, cold brine, and then dried ($MgSO_4$). After filtration the filtrate was dried in vacuo to afford the title compound **5** (1.62 g, 84% over two steps). The spectral data was in agreement with the literature.[6]

4.2.2. 2,3:4,6-di-*O*-Isopropylidene-D-mannopyranose (6)[7]

D-Mannose (3.6 g, 20 mmol) was dissolved in anhydrous DMF (12 mL) and cooled to 0 °C. *p*-TsOH·H_2O (19 mg, 1.0 mmol) and 2-methoxypropene (3.2 mL, 60 mL) were added to the resulting solution. The reaction mixture was stirred at 0 °C for 3 h, after which 2-methoxypropene (2.0 mL) was added by syringe. The mixture was allowed to warm to rt gradually over 14 h. The reaction mixture was neutralized by addition of Et_3N (0.20 mL) and the product partitioned between ether (40 mL) and water (40 mL). The aqueous phase was collected and extracted with ether (3 × 10 mL). The combined organic layers were washed with brine (20 mL) and dried using $MgSO_4$. After filtration and concentration in vacuo, the product was isolated from the resulting gum by crystallization using ethyl acetate/petrol to afford the desired product **6** (3.64 g, 70%) as a white solid containing a mixture of anomers.

4.2.3. 1-*O*-Acetyl-2,3:4,6-di-*O*-isopropylidene-α-D-mannopyranose (7a) and 1-*O*-acetyl-2,3:4,6-di-*O*-isopropylidene-β-D-mannopyranose (7b)[7]

Hemiacetals **6** (4.64 g, 17.8 mmol) were dissolved in dry pyridine (40 mL). To the resulting mixture was added Ac_2O (4.0 mL, 40 mmol) and the reaction mixture was stirred overnight at rt The solution was concentrated under high-vacuum and co-evaporated with toluene (4 × 10 mL) to afford a syrupy residue, which was dissolved in EtOAc. The desired the α-anomer, **7a** (2.40 g, 44%), was crystallised from EtOAc/petrol and the mother liquid afforded the β-anomer product **7b** (3.00 g, 56%).

4.2.3.1. Compound 7a. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 6.26 (s, 1H), 4.15 (dd, J = 7.6, 5.6 Hz, 1H), 4.10 (dd, J = 5.6, 0.8 Hz, 1H), 3.85 (dd, J = 10.8, 5.6 Hz, 1H), 3.74 (dd, J = 10.0, 8.0 Hz, 1H), 3.69 (t, J = 10.4 Hz, 1H), 3.55 (td, J = 10.4, 5.6 Hz, 1H), 2.07 (s, 3H), 1.51 (s, 3H), 1.47 (s, 3H), 1.38 (s, 3H), 1.31 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 168.50, 109.77, 99.63, 91.31, 75.11, 74.50, 72.07, 63.63, 61.68, 28.85, 27.95, 26.01, 20.81, 18.63.

4.2.3.2. Compound 7b. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 6.07 (s, 1H), 4.81 (dd, J = 6.0, 3.6 Hz, 1H), 4.65 (d, J = 6.0, 1H), 4.35 (dq, J = 10.0, 4.0 Hz, 1H), 4.05 (dd, J = 8.8, 6.0 Hz, 1H), 4.01-3.96 (m, 2H), 2.02 (s, 3H), 1.43 (s, 3H), 1.41 (s, 3H), 1.33 (s, 3H), 1.29 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 169.21, 113.09, 109.17, 100.65, 84.93, 82.10, 79.18, 72.76, 66.66, 26.85, 25.81, 25.01, 24.53, 20.92.

4.2.4. 2,3:4,6-di-*O*-Isopropylidene-α-D-mannopyranose (8a), and 2,3:4,6-di-*O*-isopropylidene-β-D-mannopyranose (8b)[7]

Compound **7a** (950 mg, 3.14 mmol) was dissolved in MeOH (15 mL) and NaOMe was added to adjust the pH of the solution to above 10, after which the reaction was stirred at rt for 2 h. The reaction was judged complete by TLC and then neutralized using Amberlite RH-120 (H^+) resin, filtered and concentrated under vacuum. The residue was dried under high vacuum to afford the title compound **8a** (796 mg, 97%).

Compound **7b** was subjected to the same procedure (1.40 g, 4.63 mmol) to afford the title compound **8b** (1.14 g, 97%).

4.2.4.1. Compound 8a. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.40 (s, 1H), 4.19–4.15 (m, 2H), 3.85 (dd, J = 9.2, 4.0 Hz, 1H), 3.80–3.68 (m, 3H), 3.61 (br s, 1H), 2.07 (s, 3H), 1.53 (s, 3H), 1.50 (s, 3H), 1.41 (s, 3H), 1.34 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 M Hz): δ 109.44, 99.76, 92.64, 76.19, 74.58, 72.67, 62.02, 61.43, 28.90, 28.09, 26.09, 18.75.

4.2.4.2. Compound 8a. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.35 (s, 1H), 4.78 (dd, J = 6.0, 3.6 Hz, 1H), 4.58 (d, J = 6.0, 1H), 4.40–4.34 (m, 1H), 4.15 (dd, J = 6.8, 4.8 Hz, 1H), 4.08–4.00 (m, 2H), 1.44 (s, 3H), 1.43 (s, 3H), 1.36 (s, 3H), 1.30 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 112.56, 109.05, 101.12, 85.44, 80.05, 79.56, 73.23, 66.42, 26.71, 25.76, 25.08, 24.38.

4.2.5. 4-Methylumbelliferone 2,3:4,6-di-*O*-isopropylidene-α-D-mannopyranoside (9a), and 4-methylumbelliferone 2,3:4,6-di-*O*-isopropylidene-β-D-mannopyranoside (9b)

Compound **8a** (796 mg, 3.06 mmol), 4-methylumbelliferone (809 mg, 4.59 mmol), and PPh_3 (1.20 g, 4.59 mmol) were dissolved in anhydrous toluene (40 mL). To the reaction mixture was added diisopropyl azodicarboxylate (DIAD, 0.90 mL, 4.59 mmol) and the resulting solution was stirred at rt overnight. The mixture was diluted with EtOAc (200 mL), washed with 1 N NaOH (200 mL), saturated $NaHCO_3$ (200 mL), and brine (200 mL), after which the organic layer was dried ($MgSO_4$). After filtration and removal of the solvent in vacuo, the residue was purified by gradient silica gel column chromatography (Hex:EtOAc, 3:1–2:1) to afford **9a** (180 mg crude) and **9b** (905 mg, 71%).

4.2.5.1. Compound 9a. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.51 (d, J = 8.8 Hz, 1H), 7.00 (J = 2.4 Hz, 1H), 6.94 (dd, J = 8.8, 2.8 Hz, 1H), 6.16 (d, J = 0.8 Hz, 1H), 5.81 (s, 1H), 4.40 (d, J = 6.0 Hz, 1H), 4.30 (dd, J = 8.0, 5.6 Hz, 1H), 3.81 (dd, J = 10.0, 8.0 Hz, 1H), 3.80-3.70 (m, 2H), 3.66–3.58 (m, 1H), 2.38 (d, J = 0.8 Hz, 3H), 1.58 (s, 3H), 1.51 (s, 3H), 1.40 (s, 3H), 1.38 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 160.96, 158.46, 154.82, 152.20, 125.65, 114.94, 113.23, 112.82, 109.94, 103.97, 99.80, 96.04, 75.66, 74.71, 72.27, 62.61, 61.67, 28.87, 28.05, 26.11, 18.69, 18.58.

4.2.5.2. Compound 9b. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.51 (d, J = 8.8 Hz, 1H), 7.00 (J = 2.4 Hz, 1H), 6.98 (dd, J = 8.8, 2.4 Hz, 1H), 6.17 (s, 1H), 5.55 (d, J = 3.2 Hz, 1H), 4.47 (dd, J = 6.8, 3.2 Hz, 1H),

4.40-4.30 (m, 2H), 3.93 (dd, *J* = 10.4, 4.8 Hz, 1H), 3.61 (t, *J* = 10.4 Hz, 1H), 3.51 (td, *J* = 10.4, 4.8 Hz, 1H), 2.40 (d, *J* = 1.2 Hz, 3H), 1.61 (s, 3H), 1.49 (s, 3H), 1.42 (s, 6H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 161.00, 159.54, 154.86, 152.27, 125.59, 114.94, 113.61, 112.76, 111.87, 104.22, 99.72, 96.02, 75.94, 73.25, 71.21, 66.12, 63.03, 28.91, 26.82, 25.62, 18.95, 18.63.

4.2.6. 4-Methylumbelliferone 2,3-*O*-isopropylidene-β-D-mannopyranoside (10)

Compound **9b** (130 mg, 0.311 mmol) was dissolved in an 80% aqueous HOAc solution (4.0 mL). The resulting solution was stirred at rt for 5 h and the mixure was then concentrated under high vacuum, co-evaporated with toluene (6 × 10 mL), after which the residue was dissolved in MeOH and co-evaporated with toluene (2 × 10 mL). The residue was dried under high vacuum to yield the glycoside **10** (115 mg, 98%), which was used directly in the next step.^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.47 (dd, *J* = 7.2, 2.4 Hz, 1H), 6.98-6.95 (m, 2H), 6.11 (d, *J* = 1.2 Hz, 1H), 5.54 (d, *J* = 2.8 Hz, 1H), 4.43 (dd, *J* = 7.2, 2.8 Hz, 1H), 4.35 (t, *J* = 2.8 Hz, 1H), 4.20 (dd, *J* = 6.0, 2.8 Hz, 1H), 3.85-3.74 (m, 2H), 3.61 (dt, *J* = 10.0, 4.0 Hz, 1H), 2.35 (d, *J* = 1.2 Hz, 3H), 1.56 (s, 3H), 1.41 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 161.21, 159.59, 154.68, 152.59, 125.68, 114.77, 113.48, 112.50, 111.30, 103.73, 95.66, 79.09, 75.46, 73.29, 68.41, 62.28, 26.90, 25.66, 18.56.

4.2.7. 4-Methylumbelliferyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→6)-2,3-*O*-isopropylidene-β-D-mannopyranoside (11) and 4-methylumbelliferyl α-D-mannopyranosyl-(1→6)-2,3-*O*-isopropylidene-β-D-mannopyranoside (12)

To the MeCN (2.5 mL) solution of donor **5** (256 mg, 0.622 mmol) was added 4 Å molecular sieves and $HgCN_2$ (78.6 mg, 0.311 mmol) and the mixture was stirred at rt for 10 min. A MeCN (2.5 mL) solution of compound **10b** (115 mg, 0.304 mmol) was then added to the reaction mixture and the solution was stirred at rt overnight. Solvent was then removed in vacuo, the residue was taken up in EtOAc (40 mL) and filtered. The solution was washed with water, 10% KI, saturated $NaHCO_3$, and brine. After drying ($MgSO_4$) and filtration the solvent was removed in vacuo to yield a gummy residue. Purification by gradient flash column silica chromatography (Hex:EtOAc, 1:1–1:1.5 to 100% EtOAc) afforded intermediate disaccharide **11** (141 mg of crude material). Disaccharide **11** (141 mg, 0.199 mmol) was dissolved in MeOH (6.0 mL), and NaOMe was added to adjust the pH of the reaction mixture to be **11** as estimated by litmus paper. The solution was stirred at rt for 3 h after which time Amberlite RH (H^+) 120 resin was added to neutralize the base. The mixture was filtered and concentrated in vacuo to afford a pale yellow gum. The desired product was purified from the residue by silica gel flash column chromatography (CH_2Cl_2:MeOH, 10:1) to afford disaccharide **12** (65.1 mg, 60%).^{1}H NMR (CD_3OD, 400 MHz): δ 7.71 (d, *J* = 8.8 Hz, 1H), 7.09 (d, *J* = 2.4 Hz, 1H), 7.06 (dd, *J* = 8.8, 2.4 Hz, 1H), 6.19 (d, *J* = 2.4 Hz, 1H), 5.64 (d, *J* = 2.8 Hz, 1H), 4.67 (d, *J* = 1.6 Hz, 1H), 4.49 (dd, *J* = 6.8, 2.4 Hz, 1H), 4.29 (t, *J* = 6.8 Hz, 1H), 3.98 (dd, *J* = 10.4, 7.2 Hz, 1H), 3.85 (dd, *J* = 10.4, 4.8 Hz, 1H), 3.74-3.65 (m, 4H), 3.63-3.53 (m, 2H), 3.45 (ddd, *J* = 9.6, 5.2, 2.4 Hz, 1H), 3.41 (dd, *J* = 3.2, 1.6 Hz, 1H), 2.46 (d, *J* = 1.2 Hz, 3H), 1.57 (s, 3H), 1.42 (s, 3H). ^{13}C NMR (CD_3OD, 100 MHz): δ 163.81, 161.64, 156.11, 155.79, 127.49, 116.00, 115.16, 112.79, 112.02, 104.46, 101.70, 97.06, 81.29, 76.19, 75.10, 74.40, 72.39, 71.98, 69.34, 68.43, 67.53, 62.72, 27.69, 26.20, 18.76. HR-MS: calcd for $C_{25}H_{33}O_{13}$ $(M+H)^+$, 541.1916; Found: 541.1931; Calcd for $C_{25}H_{32}NaO_{13}$ $(M+Na)^+$, 563.1735; Found: 563.1745.

4.2.8. 4-Methylumbelliferyl α-D-mannopyranosyl-(1→6)-β-D-mannopyranoside (α-Man-β-Man-4MU, 2)

Compound **12** (28.2 mg, 52.2 μmol) was dissolved in a mixed solution of TFA: H_2O:$CHCl_3$ (2.5:1:100, 2.0 mL in total), and stirred at rt for 3.5 h, then the solvent was removed under vacuo, the residue was co-evaporated with toluene (4 × 5.0 mL), and then was applied on a silica gel column and purified by flash chromatography (CH_2Cl_2:MeOH, 2:1) to afford the title product **10** (23.0 mg, 88%). ^{1}H NMR (CD_3OD, 400 MHz): δ 7.71 (d, *J* = 6.8 Hz, 1H), 7.14 (d, *J* = 2.4 Hz, 1H), 7.07 (dd, *J* = 8.8, 2.4 Hz, 1H), 6.20 (d, *J* = 1.2 Hz, 1H), 5.28 (d, *J* = 0.8 Hz, 1H), 4.84 (hidden in water peak, 1H), 4.12 (d, *J* = 1.2 Hz, 1H), 3.96–3.83 (m, 4H), 3.70 (dd, *J* = 12.0, 2.4 Hz, 1H), 3.66–3.55 (m, 5H), 3.51 (ddd, *J* = 9.6, 5.2, 2.4 Hz, 1H), 2.45 (d, *J* = 1.2 Hz, 3H). ^{13}C NMR (CD_3OD, 100 MHz): δ 164.03, 161.75, 156.10, 155.85, 127.31, 116.12, 115.34, 112.82, 104.85, 101.64, 99.57, 77.38, 75.13, 74.47, 72.35, 72.34, 72.14, 68.56, 68.42, 67.79, 62.82, 18.74. HR-MS: calcd for $C_{22}H_{29}O_{13}$ $(M+H)^+$, 501.1603; Found: 501.1601; Calcd for $C_{22}H_{28}NaO_{13}$ $(M+Na)^+$, 523.1422; Found: 523.1419; Calcd for $C_{22}H_{28}KO_{13}$ $(M+Na)^+$, 539.1161; Found: 539.1143.

4.2.9. HPLC purification of 4-methylumbelliferone α-D-mannopyranosyl-(1→6)-β-D-mannopyranoside (α-Man-β-Man-4MU, 2)

α-Man-β-Man-4MU was purified using an Agilent Eclipse XDB-C18 reversed-phase semi-preparative (9.4 × 250 mm) column. The disaccharide was injected and eluted using a linear gradient of MeCN (4–20% over 30 min) at a flow rate of 2 mL/min. The retention time for the disaccharide under these conditions was typically about 19.5 min. For all assays, the α-Man-β-Man-4MU used was found to be ⩾95% pure by HPLC.

4.3. Enzyme assays

4.3.1. Assay of 4-methylumbelliferone β-D-mannopyranoside (β-Man-4MU, 3) as a substrate for *C. fimi* Man2A

Fluorescence assays were performed in 96-well black plates (Nunc). A stock of substrate was prepared in DMSO and diluted to obtain the desired substrate concentrations and to ensure a final DMSO concentration of 2% in all reactions. At this DMSO concentration, the enzyme activity was within 5% of the activity as compared to control assays conducted in the absence of DMSO. The total assay volume was 100 μL. The buffer used in all reactions was PBS (pH = 7.4) and the reaction was preincubated at 37 °C for 5 min prior to initiation of the reaction by the addition of CfMan2A to a final concentration of 1 nM. The production of 4-methylumbelliferone was monitored for 10 min by fluorescence spectroscopy using a fMax plate reader (Molecular Devices) equipped with a 355/485 nm filter (excitation/emission) pair. The reaction velocity was determined by a linear fit of the data using SoftMAX Pro software (Version 1.3.1). Relative fluorescence values were converted to 4-methylumbelliferone concentration using a standard curve generated in the same buffer and DMSO conditions with a correlation coefficient of 0.9999.

4.3.2. Assay of 2,4-dinitrophenyl α-D-mannopyranoside (α-ManDNP, 1) as a substrate for *C. fimi* Man2A

Assays were performed as described above with the following variations. 96-well transparent polystyrene plates (Nunc) were used and absorbance values were read continuously at 415 nm on a fMax plate reader (Molecular Devices) over 2 h at 37 °C. Substrate concentrations between 0.25 and 2.45 mM were assayed using a concentration of 7 μM CfMan2A.

4.3.3. Assay of 2,4-dinitrophenyl α-D-mannopyranoside (α-ManDNP, 1) as a substrate for CpGH125

Assays were performed in 96-well transparent polystyrene plates (Nunc). Absorbance values at 415 nm were monitored using a fMax plate reader over three minutes. All reactions were performed at 37 °C and were carried out in PBS buffer (pH 7.4) with

a total assay solution volume of 100 μL. Absorbance values were converted to dNP concentrations using a path length of 0.326 cm and an extinction coefficient of 12,890 M^{-1} cm^{-1},[10].

4.3.4. Coupled assay of 4-methylumbelliferone α-D-mannopyranosyl-(1→6)-β-D-mannopyranoside (α-Man-β-Man-4MU, 2) using CpGH125 and CfMan2A

Fluorescence assays were performed in 96-well black plates (Nunc). A stock of α-Man-β-Man-4MU (***2***) was prepared in DMSO and diluted to obtain the desired substrate concentrations to ensure a final DMSO concentration of 2% in all reactions as described above. The total assay volume was 100 μL. The buffer used in all reactions was PBS (pH = 7.4) and CfMan2A was preincubated at 37 °C for 5 min prior to the initiation of the reaction by the addition of CpGH125. The final CfMan2A concentration in all assays was 140 nM and the final concentration of CpGH125 used in all assays was 1 nM. The production of 4-methylumbelliferone was monitored over 10 min by fluorescence spectroscopy using a fMax plate reader.

4.3.5. Coupled assay of 4-methylumbelliferone α-D-mannopyranosyl-(1→6)-β-D-mannopyranoside (α-Man-β-Man-4MU, 2) using CpGH125 and *H. pomatia* β-mannosidase

As an alternative approach, a β-mannosidase from *H. pomatia* (Sigma) was investigated as the coupling enzyme instead of CfMan2A. *H. pomatia* β-mannosidase, obtained as a suspension in 3 M ammonium sulphate buffer (pH = 4), was buffer exchanged into PBS (pH 7.4) prior to use by use of a 5 kDa MWCO filter apparatus from Millipore. First, fluorescence assays to test the activity of *H. pomatia* β-mannosidase were performed in 96-well black plates (Nunc) using a total assay volume of 100 μL. The buffer used in all reactions was PBS (pH = 5.95) containing 1% DMSO and varying concentrations of the substrate 4-methylumbelliferone β-D-mannopyranoside (β-Man-4MU, **3**). The mixture was preincubated at 37 °C for 5 min prior to initiation of the reaction by the addition of *H. pomatia* β-mannosidase to yield a final enzyme concentration of 100 nM. The production of 4-methylumbelliferone was monitored continuously for 10 minutes by fluorescence spectroscopy using a fMax plate reader (Molecular Devices) fitted with the 355 / 485 nm filter pair. The reaction velocity was determined by a linear fit of the data using SoftMAX Pro software (Molecular Devices). Relative fluorescence values were converted to 4-methylumbelliferone concentration using a standard curve (correlation coefficient of 0.9999) generated using the same buffer. Using these conditions we obtained the following kinetic parameters for the action of the commercial *H. pomatia* β-mannosidase preparation assuming all protein is active enzyme; K_m = 170 ± 9 μM, k_{cat} = 16.4 ± 0.3 min^{-1}, and k_{cat}/K_m = 9.6 ± 0.5 × 10^{-2} min^{-1} μM $^{-1}$. The k_{cat}/K_m value is some 200-fold less than that measured for CfMan2A. On the basis of this observation we determined the final protein concentration of the *H. pomatia* β-mannosidase preparation used in the coupled assay should be in the range of 0.5 to 1 μM when employing the same coupled assay conditions as described for CfMan2A and CpGH125.

4.3.6. Control reactions

In order to evaluate any background enzyme activities or spontaneous hydrolysis of substrates, fluorescence measurements were taken of the following control mixtures over a period of 10 min using a fMax plate reader equipped with a 355/485 nm filter pair. α-Man-β-Man-MU was incubated in PBS with CpGH125 alone, α-Man-β-Man-MU was incubated in PBS with CfMan2A alone, and α-Man-β-Man-MU was incubated in PBS with no enzyme present. Control reactions were performed for each experiment.

4.3.7. Capillary electrophoresis reactions

α-Man-β-Man-MU (1 mM) was incubated with CfMan2A for 7 h at 37 °C in PBS containing 1% DMSO, with fresh enzyme added every 2–3 h. The CfMan2A enzyme concentration was 7 μM at the beginning of the assay and 24 μM at the end of the reaction. The control sample contained 1 mM α-Man-β-Man-MU and buffer with no enzyme present. The control and reaction samples were frozen and later analyzed using micellar electrokinetic chromatography on a ProteomeLab PA800 (Beckman–Coulter) instrument. Electrophoresis was performed under normal polarity in a 60 cm (to detector) ×50 μm (internal diameter) fused silica capillary using a fixed applied voltage of 30 kV (resulting in a current of approximately 40 μA) in background electrolyte consisting of boric acid (50 mM), sodium dodecyl sulphate (50 mM) and γ-cyclodextrin (11 mM), pH 9.0. All buffer components were of the highest purity commercially available. The capillary was thermostated at 25 °C and samples were kept at 10 °C unless otherwise stated. Sample was injected at the anode by applying a pressure of 0.5 psi for 10 s and detected by measuring the absorbance at 280 nm at a rate of 4 Hz. A time course assay was performed in which the same sample was monitored by directly injecting reaction aliquots (~15 nL) into the capillary after 0.18, 1.5, 2.5, 3.5, 4.5, and 7 h. The initial CfMan2A concentration in these assays was 7 μM while the α-Man-β-Man-MU concentration was 500 μM. Additional CfMan2A was added to the mixture after 2 h (6.57 μM enzyme added; final enzyme concentration was 13.57 μM) and 4 h (6.44 μM enzyme added; final enzyme concentration was 20 μM). Although the final calculated enzyme concentration was 20 μM at the end of the assay (7 h), it is likely that the active enzyme was present at a lower concentration because of enzyme instability leading to loss of activity over the course of the experiment.

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Supplementary data

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