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Synthesis and characterisation of novel chromogenic substrates for human pancreatic α-amylase

Iben Damager,[a,†] Shin Numao,[a,†,‡] Hongming Chen,[a] Gary D. Brayer[b] and Stephen G. Withers[a,b,*]

[a]*Department of Chemistry, University of British Columbia, 2036 Main Mall, Vancouver, BC, Canada V6T 1Z1*
[b]*Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada V6T 1Z3*

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Abstract—Derivatives of maltose and maltotriose were chemically synthesised as substrates for human pancreatic α-amylases and subjected to kinetic analysis. Rates measured were shown to reflect both hydrolysis and transglycosylation reactions. 4-*O*-Methylated derivatives of these substrates underwent only hydrolysis, thereby simplifying kinetic analyses. These modified substrates may be used for the detection and kinetic analysis of α-amylases, and are useful in rapidly screening for novel α-amylase inhibitors and for subsequent kinetic characterisation.

Keywords: Human pancreatic α-amylase; Glycosidase; Chromogenic substrates; α-Amylase inhibitors

1. Introduction

α-Amylase (α-1,4-glucan-4-glucanohydrolase; EC 3.2.1.1) is an endoglucosidase that catalyses the hydrolysis of α-(1→4) linkages within glucose polymers such as amylose and amylopectin. As these polymers are major dietary sources of energy for many organisms, this class of enzymes is important in various aspects of biology. Physiologically, it has been shown that the activity of human pancreatic α-amylase (HPA) in the small intestine correlates to post-prandial blood glucose levels,[1,2] a fact important in the treatment of diabetes.[3] As such, novel α-amylase inhibitors are sought after by the pharmaceutical industry. In order to facilitate the identification of such inhibitors, a method of quickly and accurately quantitating HPA activity in the presence of inhibitors is essential for screening of libraries of compounds. In addition, serum amylase levels are used as clinical markers of pancreatic damage,[4] thus amylase assays are valuable in that application also.

The large molecular weight and heterogeneity of the natural starch substrates makes them unsuitable for detailed kinetic and inhibition studies. In order to get around these problems, and furthermore to facilitate detection of enzymatic activity, many smaller, defined substrates have been synthesised. While each has its advantages, most of these substrates require coupling enzymes or have competing side reactions in addition to hydrolysis. Of particular concern is the occurrence of transglycosylation reactions. These properties make these synthetic substrates problematic in their use for the kinetic analysis of α-amylase inhibitors.

Abbreviations: CNP-G3, 2-chloro-4-nitrophenyl α-maltotrioside; DABCO, diazabicyclo[2.2.2]octane; DNFB, 2,4-dinitrofluorobenzene; DNP, 2,4-dinitrophenol; DNP-G1, 2,4-dinitrophenyl α-glucoside; DNP-G2, 2,4-dinitrophenyl α-maltoside; DNP-G3, 2,4-dinitrophenyl α-maltotrioside; DNP-G4, 2,4-dinitrophenyl α-maltotetraoside; DNP-G6, 2,4-dinitrophenyl α-maltohexaoside; ELSD, evaporative light scattering detector; G2F, α-maltosyl fluoride; G3F, α-maltotriosyl fluoride; HPA, human pancreatic α-amylase; MeDNP-G2, 2,4-dinitrophenyl 4′-*O*-methyl-α-maltoside; MeG2F, 4′-*O*-methyl-α-maltosyl fluoride; PNP-G2, *p*-nitrophenyl α-maltoside.

* Corresponding author. Tel.: +1-604-822-3402; fax: +1-604-822-8867; e-mail: withers@chem.ubc.ca
† These authors contributed equally to this work.
‡ Present address: Institut für Mikrobiologie, ETH Zürich, CH-8092 Zürich, Switzerland.

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In this paper, we present the synthesis and characterisation of some novel substrates that avoid these problems while still allowing for the straightforward detection of α-amylase activity. These substrates are therefore useful in the rapid screening of libraries of compounds to identify α-amylase inhibitors as well as in detailed kinetic analyses.

2. Results and discussion

The simplest and most reliably monitored substrates for glycosidases are probably the aryl glycosides. Cleavage of these substrates at the aryl glycoside linkage results in the release of a coloured phenolate product, which is readily monitored spectrophotometrically. Among the most useful of such substrates are the 2,4-dinitrophenyl glycosides. The particular value of 2,4-dinitrophenyl glycosides lies in the low pK_a value (3.96) of the aglycone phenol. This has two beneficial consequences. One is that the phenol remains substantially ionised, thus yellow, at low pH values considerably facilitating the spectrophotometric assay of enzymes with low pH optima. The second advantage is that these are highly activated substrates with relatively high k_{cat} values. In general, the glycosylation step in the catalytic mechanisms of retaining glycosidases such as α-amylases, formation of the glycosyl–enzyme intermediate, is accelerated relative to the deglycosylation step for such substrates.[5] This can be of great value in mechanistic studies. However, until very recently[6] the α-anomers of the 2,4-dinitrophenyl glucosides were only available as their protected derivatives. All former attempts to deprotect these molecules had led to decomposition.

Another problem that can occur during kinetic analysis is that of transglycosylation reactions in addition to the normal hydrolysis events. This is particularly confusing if the second step, deglycosylation, is rate-limiting since the rate measured will additionally depend upon the contribution of this transglycosylation process. Even if the glycosylation step is rate-limiting, this remains problematic, since the formation of elongated products provides competitive substrates that affect rates measured.

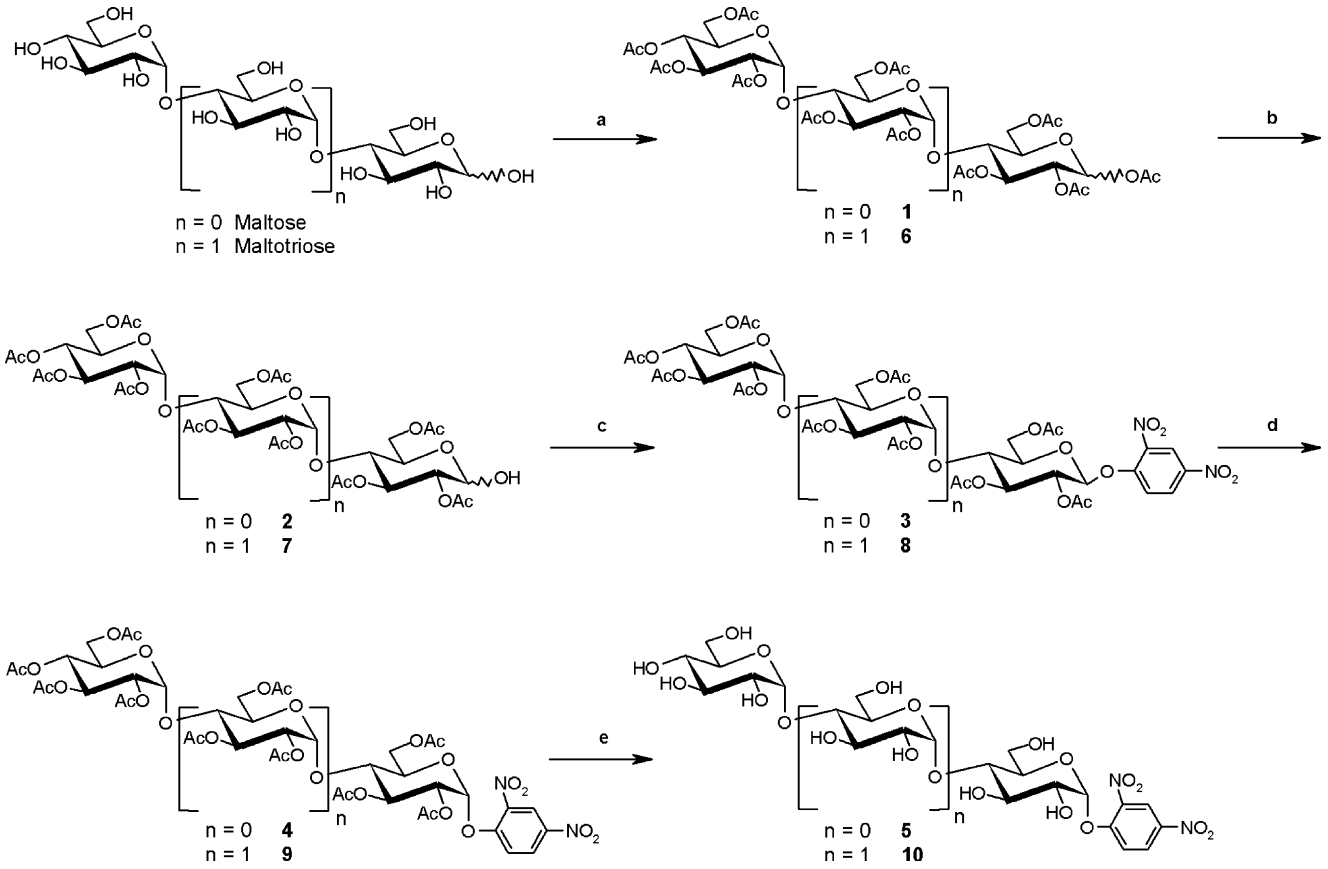
Transglycosylation reactions can be eliminated if the nonreducing terminal 4-hydroxyl is modified to prevent addition. The simplest method for this, with minimal steric implications, involves methylation of the hydroxyl group. Through use of relatively short (di- and trisaccharide) substrates with excellent leaving groups, in many cases the preferred cleavage mode is that in which the bond to the unnatural aglycone is cleaved, greatly facilitating both reaction monitoring and kinetic analysis. Indeed, longer substrates would be disadvantages since inter-sugar cleavage modes could then become feasible, generating mixtures of products with no direct release of chromophore.

In this manuscript, we describe the synthesis and kinetic analysis of the α-dinitrophenyl glycosides of maltose and maltotriose with human pancreatic α-amylase, as well as 4-*O*-methyl ether derivatives of dinitrophenyl maltoside and maltosyl fluoride, the latter being another excellent amylase substrate as pioneered by Hehre and co-workers.[7,8]

2.1. Synthesis of 2,4-dinitrophenyl α-maltoside (5) and 2,4-dinitrophenyl α-maltotrioside (10)

The synthetic strategy to obtain the desired dinitrophenyl compounds **5** and **10** from maltose and maltotriose, respectively, is outlined in Scheme 1. The initial step for synthesis of 2,4-dinitrophenyl α-maltoside (**5**) was acetylation of maltose in a standard procedure[9] using anhydrous sodium acetate and acetic anhydride to give the per-*O*-acetylated maltose (**1**) in quantitative yield. The acetate group at the anomeric centre was selectively removed from **1** using hydrazine·acetate in DMF to give 2,2′,3,3′,4′,6,6′-hepta-*O*-acetyl-maltose (**2**) in 70% yield.[10] 2,4-Dinitrophenyl 2,2′,3,3′,4′,6,6′-hepta-*O*-acetyl-α,β-maltoside (**3**) was obtained by treating **2** with 2,4-dinitrofluorobenzene (DNFB) and diazabicyclo[2.2.2]octane (DABCO) in DMF.[11] This nucleophilic aromatic substitution resulted in primarily the per-*O*-acetylated 2,4-dinitrophenyl β-maltoside **3** (α/β-ratio; 1:9) in 63% yield. The corresponding α-anomer, 2,4-dinitrophenyl 2,2′,3,3′,4′,6,6′-hepta-*O*-acetyl-α-maltoside (**4**), was obtained by base-catalysed anomerisation by treating **3** with K_2CO_3 in DMF.[12] The equilibrium mixture of the α- and β-anomers was obtained in a ratio of 3:1. This ratio is similar to that previously reported for the anomerisation of per-*O*-acetylated 2,4-dinitrophenyl α-glucoside.[12] The α-anomer was crystallised to give **4** in 71% yield. Deacetylation of **4** was achieved by treatment with 5% HCl in methanol as reported for 2,4-dinitrophenyl α-glucoside[6] to give the desired 2,4-dinitrophenyl α-maltoside (**5**) in 20% yield.

The synthesis of **10** was achieved in a similar manner to that used for synthesis of **5** (Scheme 1). Maltotriose was per-*O*-acetylated according to literature procedure using anhyd sodium acetate and acetic anhydride,[9] resulting in compound **6** as an α,β-anomeric mixture (α/β-ratio; 1:4) in quantitative yield. The anomeric acetate group of **6** was selectively removed using hydrazine·acetate in DMF,[10] resulting in 2,2′,2″,3,3′,3″,4″,6,6′,6″-deca-*O*-acetyl-maltotriose (**7**) in 96% yield. Introduction of the 2,4-dinitrophenyl group at the anomeric centre by treating **7** with DNFB and DABCO in DMF yielded the peracetylated 2,4-dinitrophenyl β-maltotrioside (**8**) in 70% yield.[11] The corresponding α-anomer **9** was obtained by base-catalysed anomerisation of compound **8** (α,β-ratio; 3:1) using K_2CO_3 in DMF.[12]



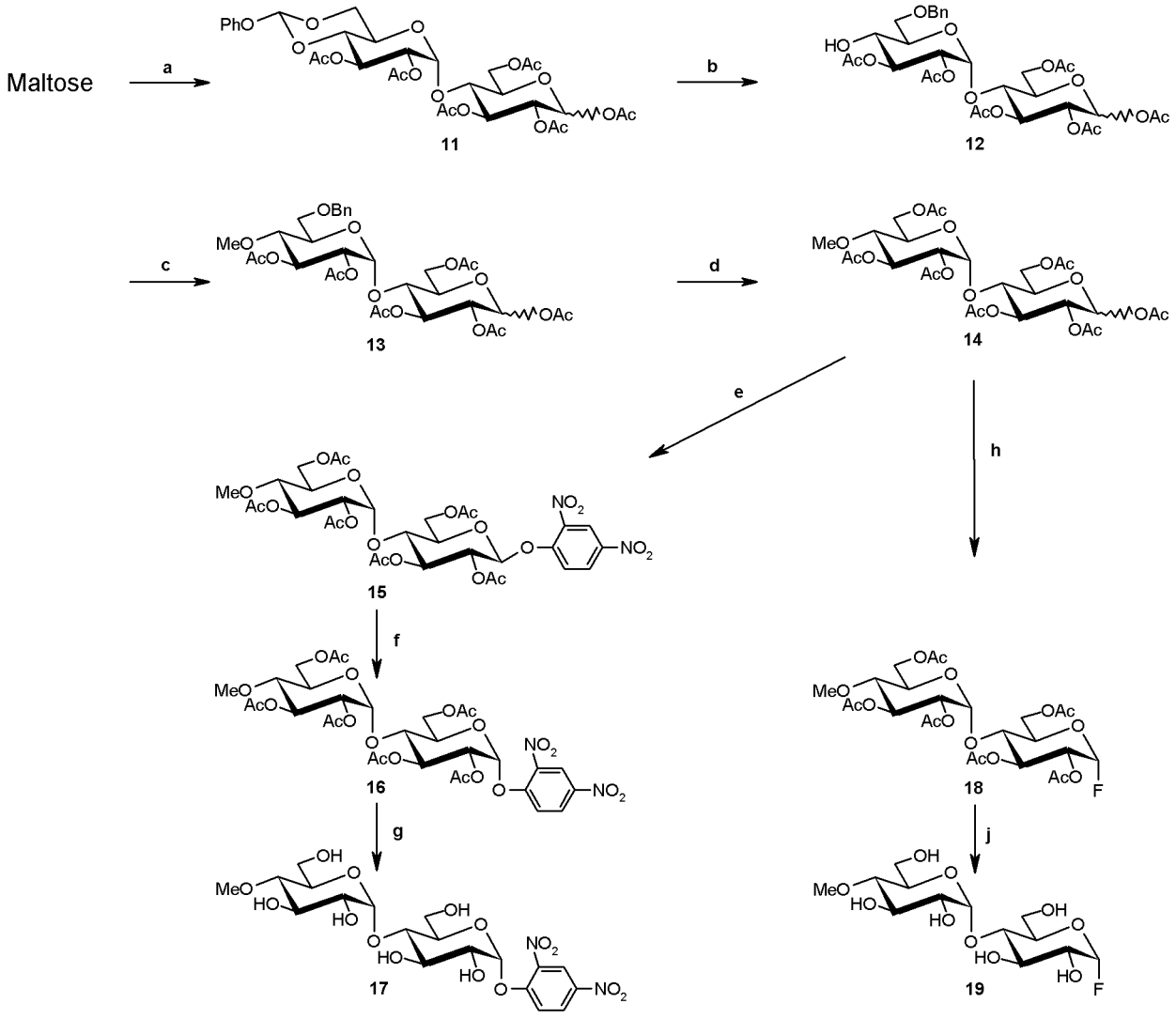
Scheme 1. Synthesis of 2,4-dinitrophenyl α-maltoside and 2,4-dinitrophenyl α-maltotrioside. Reagents and conditions: (a) CH_3COONa, Ac_2O; (b) NH_2NH_2·acetate, DMF; (c) DNFB, DABCO, DMF; (d) K_2CO_3, DMF; (e) 5% AcCl, MeOH.

The α-anomer, 2,4-dinitrophenyl 2,2′,2″,3,3′,3″,4″,6,6′, 6″-deca-*O*-acetyl-α-maltotrioside (**9**), was obtained in 70% yield. Deprotection of **9** was achieved by treatment with 5% HCl in methanol,[6] resulting in the desired product 2,4-dinitrophenyl α-maltotrioside (**10**, DNP-G3) in 42% yield.

2.2. Synthesis of 2,4-dinitrophenyl 4′-*O*-methyl-α-maltoside (17) and 4′-*O*-methyl-α-maltosyl fluoride (19)

In order to access the 4′-hydroxyl group of maltose, maltose was first benzylidenated using α,α-dibromotoluene in pyridine and subsequently in situ acetylated using acetic anhydride in pyridine, yielding the per-*O*-acetylated benzylidene maltose derivative **11** in 65% yield (Scheme 2).[13–15] Regioselective ring opening of the 4′,6′-dioxolane ring was achieved using sodium cyanoborohydride and HCl in diethyl ether,[16] resulting in compound **12** with a free hydroxyl group at the 4′-position, and the 6′-position protected as a benzyl ether. Methylation of the free hydroxyl group of **12** was accomplished using trimethylsilyl diazomethane under acidic conditions. ^{1}H NMR analysis of this compound showed that the 4′-hydrogen was shifted to high field and the appearance of a singlet peak at 3.32 ppm (three protons) indicated the presence of the methoxy group at the 4′-position as verified by 2D NMR analysis. Despite use of excess reagent, only partial reaction (⩽50% as seen by TLC) was observed. However, the starting material could easily be separated from the product and then re-reacted under the same condition to further increase the yield. The 6′-benzyl ether was replaced with an acetate group by hydrogenation with palladium on charcoal followed by acetylation using pyridine and acetic anhydride resulting in the 4′-methylated per-*O*-acetylated maltose derivative **14** in 94% yield. Selective deprotection of the anomeric acetate followed by coupling of the DNP via nucleophilic aromatic substitution resulted in the per-*O*-acetylated 4′-*O*-methyl-β-DNP-maltoside **15** in 87% yield over two steps.[11] Anomerisation of this compound resulted in an α/β-mixture from which the α-product (**16**) was selectively crystallised in 63% yield. Deprotection of compound **16** by treatment with 5% HCl in methanol[12] resulted in 2,4-dinitrophenyl 4′-*O*-methyl-α-maltoside (**17**, MeDNP-G2) in 60% yield.[6]

The blocked glycosyl fluoride substrate 4′-*O*-methyl-α-maltosyl fluoride (MeG2F) was easily obtained in two steps from compound **14**. Thus treatment of **14** with HF-pyridine resulted in the pure α-anomer **18** in 76%



Scheme 2. Synthesis of 2,4-dinitrophenyl 4′-*O*-methyl-α-maltoside and 4′-*O*-methyl-α-maltosyl fluoride. Reagents and conditions: (a) i. α,α-dibromotoluene, pyridine, ii. Ac_2O; (b) $NaBH_3CN$, THF, HCl–Et_2O; (c) TMS–CH=N=N, $BF_3{\cdot}Et_2O$, CH_2Cl_2; (d) i. Pd/H_2, EtOAc, ii. Ac_2O, pyridine; (e) i. $NH_2NH_2{\cdot}$acetate, DMF, ii. DNFB, DABCO, DMF; (f) K_2CO_3, DMF; (g) 5% AcCl, MeOH; (h) HF/pyridine; (j) NH_3, MeOH.

yield.[17] Deprotection with ammonia in methanol gave 4′-*O*-methyl–maltosyl fluoride **19** in 73% yield.

2.3. Kinetic analysis of DNP-G2 and DNP-G3 as HPA substrates

Testing of DNP-G2 and DNP-G3 as substrates for HPA was accomplished by monitoring the change in absorbance at 400 nm of the reaction mixture upon incubation of HPA with the substrate. The background rate of hydrolysis for both compounds in the absence of enzyme was negligible over the timeframe of the assay (20 min). Enzyme-catalysed hydrolysis rates were constant over the first several minutes and the initial rates, with respect to substrate concentration, fit well to the Michaelis–Menten equation. From this, the kinetic parameters for these substrates were determined (Table 1).

Table 1. A comparison of the kinetic parameters for hydrolysis of synthetic substrates by HPA

Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	Reference
G2F	150	2.1	71	8
G3F	280	0.3	933	8
MeG2F (**19**)	1237	2.4	515	This work
PNP-G2	0.04	4.0	0.01	20
DNP-G2 (**5**)	0.4	3.6	0.11	This work
DNP-G3 (**10**)	3.0	1.5	2.00	This work
MeDNP-G2 (**17**)	2.7	5.5	0.49	This work
CNP-G3	1.9	3.6	0.53	This work

Average errors in kinetic parameters: K_m (±6–9%) and k_{cat} (±5–7%).

Comparison of the K_m value for DNP-G2 with that of *p*-nitrophenyl α-maltoside (PNP-G2) or maltosyl fluoride (G2F) revealed that there is no significant difference in the apparent binding of these substrates, consistent

with the fact that both are disaccharide derivatives. The main difference between these substrates was seen in their k_{cat} values, that for G2F being almost a thousand fold greater than that for DNP-G2, which itself was 10-fold greater than that for PNP-G2. Interestingly a somewhat different pattern of kinetic behaviour was seen for the trisaccharide substrate. The k_{cat} value for G3F was only 100-fold greater than that for DNP-G3, but in this case the K_m value was 5-fold lower. This is probably due to the deglycosylation step becoming at least partially rate-limiting for G3F, thereby resulting in intermediate accumulation and consequently, a reduction in K_m value. Consistent with this finding, the k_{cat} value for DNP-G3 was 7.5-fold higher than for DNP-G2—a significantly greater difference than the 2-fold spread between G3F and G2F.

2.4. Analysis of product formation during HPA-catalysed cleavage of DNP-G2 and DNP-G3

To determine the mode by which HPA was catalysing the release of DNP, the products formed from the HPA-catalysed reaction of DNP-G2 were determined by HPLC attached in series to both a UV detector and an evaporative light scattering detector (ELSD). After 20 min, the predominant products formed were maltose (G2), maltotriose (G3), DNP, DNP-G1 and DNP-G4 (Fig. 1). Since DNP-glycosides produce a much smaller response than the free oligosaccharides in the ELSD, they were not readily detected in such chromatograms—though UV detection clearly revealed their presence. The DNP-G4 is a possible intermediate species, which would explain the formation of all other observed products and is itself the product of a transglycosylation of DNP-G2 onto another molecule of DNP-G2 with the release of DNP. This product can be hydrolysed in three different ways: (1) by formation of maltotetraose and DNP, (2) by formation of maltotriose and DNP-G1, and finally, (3) by formation of DNP-G2 and maltose. Formation of maltose of course also occurs by direct hydrolysis of the DNP group from DNP-G2. From these data either route is plausible and more detailed studies are required in order to determine the detailed mode of DNP release.

Product analysis performed on the HPA-catalysed cleavage of DNP-G3 revealed that, after 20 min of reaction, several products including DNP, maltotriose, DNP-G4 and DNP-G6 had been formed (data not shown). The observation of substantial quantities of DNP-G6 suggested that a major cleavage mode involves an initial transglycosylation, followed by hydrolysis in the various modes, much as had been seen with G3F previously.[8] The cleavage modes of both DNP-G2 and DNP-G3 are gratifyingly similar to those seen previously for maltosyl fluoride and maltotriosyl fluoride.[18]

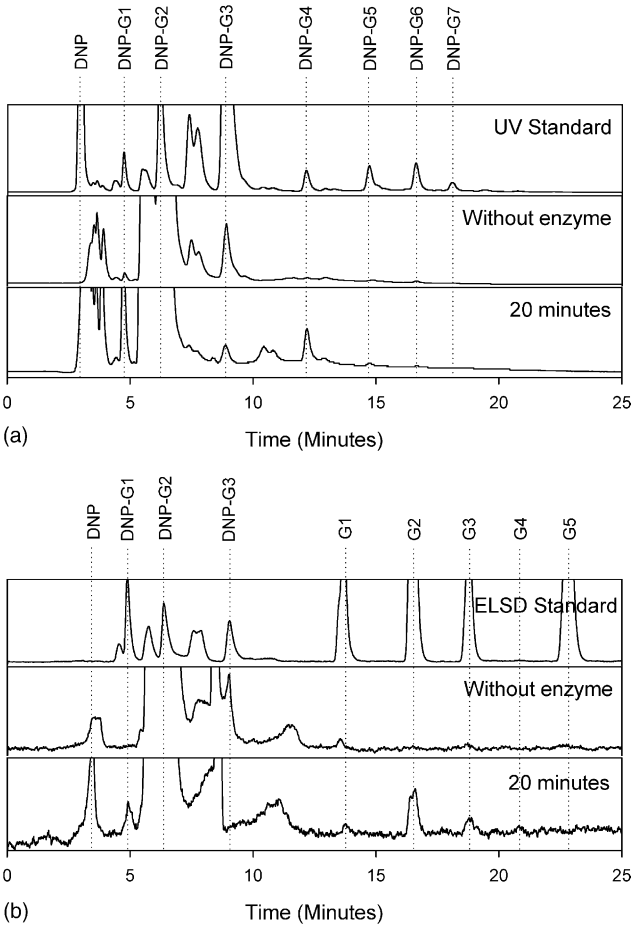


Figure 1. HPLC analysis of the reaction products of the HPA-catalysed reaction of **5** after 20 min. The eluate was analysed using (a) UV detector, and (b) ELSD detector. Conditions: Tosoh Amide-80 column, 0.7 mL/min (CH_3CN/H_2O 75:25 → 55:45 in 10 min followed by hold at this concentration).

2.5. Analysis of the hydrolysis of MeDNP-G2 and MeG2F by HPA

Analysis of the cleavage of 2,4-dinitrophenyl 4′-*O*-methyl-α-maltoside (MeDNP-G2) by HPA was monitored by HPLC attached in series to both a UV detector and an ELSD. This revealed only two products; DNP and 4′-*O*-methyl-maltose both of which arise from direct hydrolysis. Similar results were obtained with 4′-*O*-methyl-α-maltosyl fluoride (MeG2F) with only the hydrolysis product, 4′-*O*-methyl-maltose, being detected, and no transglycosylation.

Kinetic analysis of the methylated substrates was performed exactly as for their parent versions, Michaelis–Menten behaviour being observed in each case. Methylation of the 4′ hydroxyl has very little effect on K_m values, suggesting that ground state binding interactions are very similar. Interestingly, the major effect is on k_{cat}, which increased 7–8-fold when comparing the k_{cat} value for each of the methylated substrates to that of its parent compound. Since an ether oxygen at the 4′

position is kinetically preferred, transition state binding of the ether oxygen must be more favourable than is binding of an alcohol at that position. In this regard, the methylated derivatives are more like the natural substrates, since neither has a free hydroxyl group at the 4′ position. A similar conclusion could be drawn from the earlier finding that 4′-deoxy-maltosyl fluoride has a k_{cat} value almost 4-fold higher than that of G2F.[18] The consequence of this is that, not only are these blocked substrates advantageous because of the reduced complexity of their kinetic behaviour, but also their higher k_{cat} values result in more sensitive substrates.

2.6. Conclusion

2,4-Dinitrophenyl maltoside, 2,4-dinitrophenyl maltotrioside, 2,4-dinitrophenyl 4′-*O*-methyl maltoside and 4′-*O*-methyl maltosyl fluoride were synthesised, characterised and used in kinetic studies of human pancreatic α-amylase. Whereas the kinetic analysis of the two former compounds is complicated by transglycosylation, the two 4′-*O*-methyl protected maltoside derivatives have no side reactions. 2,4-Dinitrophenyl 4′-*O*-methyl maltoside is a chromogenic compound that can be used for detection of α-amylases, can be readily used for rapid screening of α-amylase inhibitors, and is particularly valuable for detailed kinetic analysis.

3. Experimental

3.1. General methods

All chemicals and buffer salts were obtained from Sigma/Aldrich Canada unless otherwise indicated. Recombinant HPA was purified according to literature procedures.[19] Melting points were determined using a Laboratory Devices Mel-TempII melting point apparatus and are uncorrected. All reactions were monitored by TLC on aluminium sheets coated with SilicaGel $60F_{254}$ (0.2 mm thickness, E. Merck, Darmstadt, Germany) and the components present were detected by charring with 10% H_2SO_4 in MeOH. Column chromatography was carried out using Silica Gel 60 (particle size 0.040–0.063 mm, 230–400 mesh ASTM, E. Merck). Solvent extracts were dried with anhyd $MgSO_4$ unless otherwise specified. The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AV 300 or 400 spectrometer. In D_2O, dioxane was used as internal reference (δ_H(dioxane) = 3.75; δ_C(dioxane) = 67.4). In $CDCl_3$, the δ_H-values are relative to internal Me_4Si and the δ_C-values are referenced to the solvent ($\delta_C(CDCl_3) = 77.0$). Elemental analysis was carried out at the University of British Columbia microanalytical laboratory. The MALDI-TOF spectra were collected using a Voyager-DE-STR (Applied Biosystems) system in reflectron mode with an acceleration voltage of 20 kV. One high-resolution mass spectrum was carried out using Kratos Concept 11 HQ, ionisation method; LS/MS (in glycerol). The remaining electrospray ionisation mass spectra were recorded using a MDS-SCIEX API QSTAR Pulsar *i* mass spectrometer (Sciex, Thornhill, ON).

3.1.1. 2,2′,3,3′,4′,6,6′-Hepta-*O*-acetyl-maltose (2). To a soln of **1** (3.24 g, 4.77 mmol) in DMF (40 mL), hydrazine·acetate (0.5 g, 5.32 mmol) was added and the reaction mixture was stirred at 50 °C for 20 min and then allowed to proceed for 2 h at room temperature. The soln was concentrated under diminished pressure, co-evaporated with toluene (2 × 30 mL) and the residue was dissolved in EtOAc (50 mL). The organic phase was successively washed with satd aq $NaHCO_3$ (2 × 30 mL), water until neutral pH, and brine (30 mL), and then dried. The solvent was evaporated and the residue was purified on Silica Gel using diethyl ether–*n*-pentane (8:2) as eluent. Crystals of **2** (α,β-mixture (1:1), 2.14 g, 70%) were obtained from diethyl ether–*n*-pentane and used directly for the next step without any further characterisation than elemental analysis: Anal. Calcd for $C_{26}H_{36}O_{18}$ (636.56): C, 49.06; H, 5.70. Found: C, 48.70; H, 5.50.

3.1.2. 2,4-Dinitrophenyl 2,2′,3,3′,4′,6,6′-hepta-*O*-acetyl-α,β-maltoside (3). To a soln of **2** (1.79 g, 2.81 mmol) in dry DMF (30 mL), DNFB (0.4 g, 3.15 mmol) and DABCO (0.36 g, 3.15 mmol) were added and the reaction mixture was stirred at room temperature overnight in the dark. The DMF was then evaporated under diminished pressure and the residue was dissolved in EtOAc (30 mL). The soln was washed with satd aq $NaHCO_3$, water and brine, and then dried. After evaporation of solvent, the product was purified on Silica gel using diethyl ether–*n*-pentane (8:2 and 1:0) as eluent to give an α,β mixture (1:9) of **3** (1.63 g, 63%) as a yellow foam. This mixture was used directly for the next step without further purification. ^{1}H NMR data for the β isomer: ($CDCl_3$, 300 MHz): δ 8.69 (d, 1H, Ar–H), 8.44 (dd, 1H, Ar–H), 7.41 (dd, 1H, Ar–H), 5.38 (d, 1H, $J_{1',2'}$ 3.9 Hz, H-1′), 5.36 (dd, 1H, $J_{2',3'}$ 10.4 Hz, $J_{3',4'}$ 9.6 Hz, H-3′), 5.36 (d, 1H, $J_{1,2}$ 6.6 Hz, H-1), 5.27 (dd, 1H, H-3), 5.13 (dd, 1H, $J_{2,3}$ 7.7 Hz, H-2), 5.05 (dd, 1H, H-4′), 4.85 (dd, 1H, $J_{2',3'}$ 10.4 Hz, H-2′), 4.55 (dd, 1H, $J_{5',6a'}$ 3.1 Hz, $J_{6a',6b'}$ 12.7 Hz, H-6a′), 4.16–4.28 (m, 2H, H-6b′, H-6a), 4.01–4.16 (m, 2H, H-4, H-5), 3.91–4.05 (m, 2H, H-6b, H-5′), 1.94, 1.95, 1.98, 2.00, 2.00, 2.04, 2.05 (7s, 21H, 7COCH_3). ^{13}C NMR: ($CDCl_3$, 75 MHz): δ 170.3, 170.5, 170.4, 170.1, 170.0, 169.8, 169.3 (7C=O), 153.5, 142.3, 140.3, 128.6, 121.4, 118.6 (6Ar–C), 98.7 (C-1), 95.9 (C-1′), 74.3, 73.0, 72.3, 71.0, 70.1, 69.2, 68.7, 68.0 (C-2, C-3, C-4, C-5, C-2′, C-3′, C-4′, C-5′), 62.3, 61.5 (C-6, C-6′), 20.8, 20.6, 20.6, 20.6, 20.6, 20.6, 20.5 (7COCH_3). EISMS: Calcd for $C_{38}H_{36}O_{22}N_2$+Na^+: 825.7. Found:

825.2. Calcd for $C_{38}H_{36}O_{22}N_2+K^+$: 841.8. Found: 841.2. Anal. Calcd for $C_{38}H_{36}O_{22}N_2$ (802.66): C, 47.89; H, 4.77; N, 3.49. Found: C, 48.10; H, 4.76; N, 3.72.

3.1.3. 2,4-Dinitrophenyl 2,2′,3,3′,4′,6,6′-hepta-*O*-acetyl-α-maltoside (4). To a soln of **3** (1.43 g, 1.78 mmol) in dry DMF (30 mL), anhyd K_2CO_3 (0.8 g, 5.79 mmol) was added and the reaction mixture stirred at room temperature overnight in the dark. The DMF was then evaporated under diminished pressure and the residue was dissolved in EtOAc (30 mL). The soln was washed with satd aq $NaHCO_3$ (2×15 mL), water (15 mL) and brine (15 mL), then dried and the solvent was evaporated under reduced pressure to yield **4** as a yellow foam. Crystals of **4** were obtained from EtOAc–petroleum ether (1.0 g, 71%) as the pure α-anomer. Mp 152–154 °C. ^{1}H NMR data ($CDCl_3$, 300 MHz): δ 8.75 (d, 1H, Ar–H), 8.42 (dd, 1H, Ar–H), 7.48 (dd, 1H, Ar–H), 5.87 (d, 1H, $J_{1,2}$ 3.6 Hz, H-1), 5.69 (dd, 1H, $J_{2,3}$ 10.0 Hz, $J_{3,4}$ 8.2 Hz, H-3), 5.43 (d, 1H, $J_{1',2'}$ 4.0 Hz, H-1′), 5.33 (dd, 1H, $J_{2',3'}$ 10.5 Hz, $J_{3',4'}$ 9.9 Hz, H-3′), 5.04 (dd, 1H, H-4′), 4.92 (dd, 1H, H-2), 4.84 (dd, 1H, H-2′), 4.46 (dd, 1H, $J_{5,6a}$ 1.7 Hz, $J_{6a,6b}$ 12.0 Hz, H-6a), 4.18–4.29 (m, 2H, H-6b, H-6b′), 4.01–4.16 (m, 3H, H-5, H-6a′, H-4), 3.91 (m, 1H, H-5′), 2.10, 2.10, 2.09, 2.05, 2.05, 1.99, 1.98 (7s, 21H, 7COCH_3). ^{13}C NMR: ($CDCl_3$, 75 MHz): δ 170.7, 170.5, 170.5, 170.1, 169.8, 169.4, 169.3 (7C═O), 153.5, 142.1, 140.3, 128.8, 121.7, 117.9 (6Ar–C), 96.1 (C-1), 95.6 (C-1′), 71.9, 71.5, 70.3, 70.2, 69.9, 69.2, 68.6, 68.0 (C-2, C-3, C-4, C-5, C-2′, C-3′, C-4′, C-5′), 62.0, 61.4 (C-6, C-6′), 20.8, 20.6, 20.6, 20.6, 20.6, 20.6, 20.5 (7CO*C*H_3). EISMS: Calcd for $C_{38}H_{36}O_{22}N_2+Na^+$: 825.7. Found: 825.2. Calcd for $C_{38}H_{36}O_{22}N_2+K^+$: 841.8. Found: 841.1. Anal. Calcd for $C_{38}H_{36}O_{22}N_2$ (802.66): C, 47.89; H, 4.77; N, 3.49. Found: C, 48.11; H, 4.78; N, 3.63.

3.1.4. 2,4-Dinitrophenyl α-maltoside (5). A suspension of **4** (0.34 g, 0.42 mmol) in MeOH (6 mL) was cooled to 0 °C and acetyl chloride (0.25 mL) was added dropwise. The reaction mixture was stirred at 4 °C for 48 h, then the solvent was evaporated under reduced pressure and the residue purified by column chromatography (EtOAc–MeOH–AcOH; 17:2:1). Compound **5** (100 mg, 20%) was precipitated from MeOH–diethyl ether as a light yellow powder. ^{1}H NMR data (MeOD, 300 MHz): δ 8.75 (d, 1H, Ar–H), 8.46 (dd, 1H, Ar–H), 7.68 (dd, 1H, Ar–H), 5.90 (d, 1H, $J_{1,2}$ 3.5 Hz, H-1), 5.13 (d, 1H, $J_{1',2'}$ 3.8 Hz, H-1′), 3.38–4.13 (m, 12H, H-2, H-3, H-4, H-5, H-6a, H-6b, H-2′, H-3′, H-4′, H-5′, H-6a′, H-6b′). ^{13}C NMR (CD_3OD, 75 MHz): δ 155.4, 142.5, 140.3, 131.3, 123.3, 118.8 (6Ar–C), 101.0 (C-1′), 98.8 (C-1), 80.5, 77.8, 74.5, 74.0, 73.7, 73.2, 73.1, 72.0 (C-2, C-3, C-4, C-5, C-2′, C-3′, C-4′, C-5′), 61.6, 61.5 (C-6, C-6′). Found: C, 42.70; H, 4.82; N, 5.14. EISMS: Calcd for $C_{18}H_{24}O_{15}N_2+Na^+$: 531.4. Found: 531.1. Anal. Calcd for $C_{18}H_{24}O_{15}N_2$ (508.39): C, 42.53; H, 4.76; N, 5.51.

3.1.5. 2,2′,2″,3,3′,3″,4″,6,6′,6″-Deca-*O*-acetyl-maltotriose (7). A soln of compound **6** (8.0 g, 8.3 mmol) in DMF (50 mL) was heated to 50 °C and hydrazine·acetate (1.0 g, 10.87) was added. The reaction mixture was lowered to room temperature and stirred for 2.5 h. The DMF was evaporated under diminished pressure and the residue was dissolved in $CHCl_3$ (50 mL). The soln was washed with satd aq $NaHCO_3$ (2×30 mL), water (30 mL) and brine, and then dried. The crude product was purified by column chromatography using petroleum ether–EtOAc as eluent (3:1) to give an α,β-mixture of **7** (1:1) as a white foam in 96% yield (7.35 g) and used directly for the next step without any further characterisation than EISMS: Calcd for $C_{38}H_{52}O_{26}+Na^+$: 947.8. Found: 947.5.

3.1.6. 2,4-Dinitrophenyl 2,2′,2″,3,3′,3″,4″,6,6′,6″-deca-*O*-acetyl-β-maltotrioside (8). To a soln of **7** (7.0 g, 7.58 mmol) in dry DMF (100 mL), DNFB (2.05 g, 11.01 mmol) and DABCO (3.41 g, 30.45 mmol) were added, and the reaction mixture was stirred at room temperature for 20 h in the dark. The DMF was evaporated under diminished pressure and the residue was dissolved in EtOAc (300 mL). The soln was washed with satd aq $NaHCO_3$ (3×150 mL), water (150 mL) and brine, and then over Na_2SO_4. The solvent was evaporated and the product purified by column chromatography using petroleum ether–EtOAc (3:2 and 1:1) as eluent to give **8** in 70% yield (5.8 g) as a white solid. ^{1}H NMR data ($CDCl_3$, 400 MHz): δ 8.68 (d, 1H, Ar–H), 8.43 (dd, 1H, Ar–H), 7.46 (d, 1H, Ar–H), 5.39 (d, 1H, $J_{1'',2''}$ 4.1 Hz, H-1″), 5.38 (m, 2H, H-3, H-3′), 5.33 (t, 1H, $J_{2'',3''} = J_{3'',4''}$ 10.5 Hz, H-3″), 5.28 (d, 1H, $J_{1,2}$ 7.7 Hz, H-1), 5.27 (d, 1H, $J_{1',2'}$ 3.7 Hz, H-1′), 5.10 (dd, 1H, H-2), 5.04 (t, 1H, $J_{3'',4''}$ 9.8 Hz, H-4″), 4.83 (dd, 1H, H-2″), 4.72 (dd, 1H, H-2′), 4.54 (dd, 1H, $J_{5,6a}$ 2.7 Hz, $J_{6a,6b}$ 12.3 Hz, H-6a), 4.44 (dd, 1H, $J_{5',6a'}$ 1.6 Hz, $J_{6a',6b'}$ 12.5 Hz, H-6a′), 4.28 (dd, 1H, $J_{5,6b}$ 4.4 Hz, H-6b), 4.02 (dd, 1H, $J_{5',6b'}$ 3.5 Hz, H-6b′), 3.97–4.06 (m, 2H, H-5, H-5′), 3.87–3.97 (m, 5H, H-4, H-4′, H-6a″, H-6b″, H-5″), 2.12, 2.09, 2.07, 2.05, 2.02, 2.01, 1.98, 1.98, 1.97, 1.97 (s, 30H, 10COCH_3). ^{13}C NMR data ($CDCl_3$, 100 MHz): δ 170.5, 170.5, 170.5, 170.3, 170.2, 170.0, 169.9, 169.8, 169.4, 169.4 (6C═O), 155.5, 142.2, 140.1, 128.7, 121.4, 118.5 (Ar–C), 98.5, 96.1, 95.7 (C-1, C-1′, C-1″), 74.0, 73.2, 72.9, 72.3, 71.7, 70.8, 70.5, 70.0, 69.3, 69.0, 68.5, 67.9 (C-2, C-3, C-4, C-5, C-2′, C-3′, C-4′, C-5′, C-2″, C-3″, C-4″, C-5″), 62.4, 62.3, 61.3 (C-6, C-6′, C-6″), 20.9, 20.7, 20.7, 20.7, 20.6, 20.6, 20.5, 20.5, 20.5, 20.5 (10CO*C*H_3). EISMS: Calcd for $C_{44}H_{54}O_{30}+Na^+$: 1113.9. Found: 1113.4.

3.1.7. 2,4-Dinitrophenyl 2,2′,2″,3,3′,3″,4″,6,6′,6″-deca-*O*-acetyl-α-maltotrioside (9). To a soln of compound **8** (5.0 g, 4.59 mmol) in DMF (15 mL), anhyd K_2CO_3 (1.8 g, 12.75 mmol) was added and the reaction mixture was stirred for 3 days in the dark. The DMF was

evaporated under diminished pressure and the residue was dissolved in $CHCl_3$ (200 mL). The solution was washed with satd aq $NaHCO_3$ (2×100 mL), water (2×100 mL) and then dried over Na_2SO_4. The solvent was evaporated under reduced pressure and compound **9** was crystallised from EtOAc and cyclohexane. The α- and β-isomers in the mother liquor were separated by column chromatography (EtOAc–petroleum ether; 1:3) resulting in a 70% yield of **9** (3.48 g). Mp 167–168 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): 8.74 (d, 1H, Ar–H), 8.43 (dd, 1H, Ar–H), 7.52 (d, 1H, Ar–H), 5.84 (d, 1H, $J_{1,2}$ 3.7 Hz, H-1), 5.66 (dd, 1H, $J_{3,4}$ 8.7, $J_{2,3}$ 9.8 Hz, H-3), 5.38 (d, 1H, $J_{1',2'}$ 4.1 Hz, H-1′), 5.35 (t, 1H, $J_{2',3'}$ 10.3 Hz, $J_{3',4'}$ 9.9 Hz, H-3′), 5.30 (t, 1H, $J_{2'',3''}$ 10.5 Hz, $J_{3'',4''}$ 9.8 Hz, H-3″), 5.30 (d, 1H, $J_{1',2'}$ 4.2 Hz, H-1″), 5.03 (t, 1H, H-4″), 4.91 (dd, 1H, H-2), 4.82 (dd, 1H, H-2″), 4.73 (dd, 1H, H-2′), 4.47 (br d, 2H, $J_{6a,6b} = J_{6a'6b'}$ 12.4 Hz, H-6a, H-6a′), 4.28 (dd, 1H, $J_{5',6b'}$ 3.9 Hz, H-6b′), 4.11 (m, 2H, H-6b, H-5′), 4.04 (m, 2H, H-4, H-5), 4.02 (dd, 1H, $J_{5'',6b''}$ 2.2, $J_{6a'',6b''}$ 12.4 Hz, H-6b″), 3.93 (m, 2H, H-6a″, H-4′), 3.86 (m, 1H, H-5″), 2.14, 2.14, 2.07, 2.04, 2.04, 2.02, 2.01, 1.99, 1.98, 1.96 (10s, 30H, 10COCH_3). ^{13}C NMR:($CDCl_3$, 100 MHz): δ 170.8, 170.6, 170.5, 170.5, 170.3, 170.2, 169.8, 169.6, 169.4, 169.2 (10C=O), 153.6, 142.0, 140.3, 128.8, 121.7, 118.1 (Ar–C), 96.2, 95.7, 95.6 (C-1, C-1′, C-1″), 74.0, 73.2, 72.9, 72.3, 71.7, 70.8, 70.5, 70.0, 69.3, 69.0, 68.5, 67.9 (C-2, C-3, C-4, C-5, C-2′, C-3′, C-4′, C-5′, C-2″, C-3″, C-4″, C-5″), 62.4, 62.3, 61.3 (C-6, C-6′, C-6″), 20.8, 20.8, 20.8, 20.8, 20.6, 20.6, 20.6, 20.6, 20.6, 20.5 (10COCH_3). EISMS: Calcd for $C_{44}H_{54}O_{30}$+Na^+: 1113.9. Found: 1113.4.

3.1.8. 2,4-Dinitrophenyl α-maltotrioside (10). A soln of compound **9** (860 g, 0.79 mmol) in MeOH (50 mL) was cooled to 0 °C and acetyl chloride (3.2 mL) was added slowly while stirring. The reaction mixture was left stirring at 4 °C for 4 days. The solvent was evaporated under reduced pressure and the solid was dissolved in MeOH and precipitated from diethyl ether. The precipitate was filtered off, washed with diethyl ether and purified by column chromatography ($CHCl_3$–MeOH 3:1) to afford compound **10** in 42% yield (220 mg). ^{1}H NMR data (CD_3OD, 400 MHz): δ 8.76 (d, 1H, Ar–H), 8.48 (dd, 1H, Ar–H), 7.70 (d, 1H, Ar–H), 5.95 (d, 1H, $J_{1,2}$ 3.5 Hz, H-1), 5.19 (d, 1H, $J_{1'',2''}$ 3.8 Hz, H-1″), 5.16 (d, 1H, $J_{1',2'}$ 3.8 Hz, H-1′), 4.20–3.20 (m, 18H, skeleton protons). ^{13}C NMR: (CD_3OD, 100 MHz): δ 155.5, 142.5, 141.1, 129.9, 122.5, 118.6 (6Ar–C), 102.8, 102.8 (C-1′, C-1″), 99.8 (C-1), 81.2, 81.0, 75.1, 74.9, 74.8, 74.7, 74.3, 74.3, 73.9, 73.4, 72.5, 71.6 (C-2, C-3, C-4, C-5, C-2′, C-3′, C-4′, C-5′, C-2″, C-3″, C-4″, C-5″), 62.7, 62.2, 61.6 (C-6, C-6′, C-6″). ESIHRMS: Calcd for $C_{24}H_{34}O_{20}N_2$: 670.2. Found: 670.2.

3.1.9. 1,2,2′,3,3′,6-Hexa-*O*-acetyl-6′-*O*-benzyl-4′-*O*-methyl-maltose (13). A soln of compound **12**[13–16] (0.45 g, 0.66 mmol) in dry CH_2Cl_2 (5 mL) was cooled to 0 °C and $BF_3{\cdot}Et_2O$ (200 μL, 1.57 mmol) and trimethylsilyl diazomethane (0.8 mL, 1.57 mmol) were slowly added. The reaction mixture was left stirring at 0 °C for 2 h. The mixture was diluted with EtOAc (5 mL) and successively washed with satd aq $NaHCO_3$ (2×3 mL), water (3 mL) and brine (3 mL), and then dried. The solvent was evaporated under reduced pressure and the residue was chromatographed on Silica Gel using diethyl ether–*n*-pentane (8:2) to give **13** as a white solid in 50% yield (238 mg; α,β-mixture 3:7). ^{1}H NMR data ($CDCl_3$, 300 MHz): δ 7.29 (5H, Ar–H), 6.18 (d, 0.3H, $J_{1,2}$ 3.8 Hz, H-1α), 5.69 (d, 0.7H, $J_{1,2}$ 8.1 Hz, H-1β), 5.36 (d, 0.3H, $J_{1',2'}$ 4.1 Hz, H-1′α), 5.33 (d, 0.7H, $J_{1',2'}$ 4.0 Hz, H-1′β), 5.28 (t, 1H, $J_{2',3'} = J_{3',4'}$ 10.5 Hz, H-3′), 5.25 (t, 1H, $J_{2,3} = J_{3,4}$ 9.2 Hz, H-3), 4.92 (t, 1H, H-2), 4.75 (dd, 1H, H-2′), 4.55 (q, 2H, PhCH_2), 4.37 (dd, 1H, $J_{5,6a}$ 2.3 Hz, $J_{6a,6b}$ 12.3 Hz, H-6a), 4.10 (dd, 1H, $J_{5,6b}$ 4.3 Hz, H-6b), 3.97 (t, 1H, H-4), 3.77 (m, 1H, H-5), 3.53–3.70 (m, 3H, H-6′a, H-6′b, H-5′), 3.46 (t, 1H, H-4′), 3.32 (s, 3H, OCH_3), 2.06, 2.03, 2.00, 1.98, 1.97, 1.96 (6s, 18H, 6COCH_3). ^{13}C NMR: ($CDCl_3$, 75 MHz): δ 170.7, 170.3, 169.8, 169.8, 169.8, 169.0 (6C=O), 139.1–129.3 (Ar–C), 96.0 (C-1′), 95.2 (C-1β), 88.9 (C-1α), 76.8, 76.6, 73.6, 72.4, 71.4, 70.5, 70.2, 69.8, 67.6 (C-2, C-3, C-4, C-5, C-2′, C-3′, C-4′, C-5′, CH_2), 62.5, 62.4 (C-6, C-6′), 60.1 (OC_3), 21.0, 20.9, 20.9, 20.6, 20.6, 20.4 (6COC_3). MALDI-TOF; Calcd for $C_{32}H_{42}O_{17}$: 722.7. Found: 721.4. Anal. Calcd for $C_{32}H_{42}O_{17}$ (722.70): C, 55.01; H, 6.06. Found: C, 55.04; H, 6.11.

3.1.10. 1,2,2′,3,3′,6,6′-Hepta-*O*-acetyl-4′-*O*-methyl-maltose (14). To a soln of **13** (145 mg, 0.21 mmol) in EtOAc (3.5 mL) was added 10% Pd/C (200 mg) and the reaction mixture was stirred under hydrogen (1 atm) for 5 h. The catalyst was removed by filtration through a layer of Silica Gel and the filtrate was concentrated to dryness. The residue was chromatographed on Silica Gel with diethyl ether and *n*-pentane (8:2 and 1:0) as eluent to give 1,2,2′,3,3′,6-hexa-*O*-acetyl-4′-*O*-methyl-maltose as a transparent syrup, which was dissolved in pyridine (3 mL). The soln was cooled to 0 °C and Ac_2O (3 mL) was added, then warmed to room temperature and stirred for 1 h. The solvent was evaporated under diminished pressure and the residue was dissolved in EtOAc (5 mL), then washed with satd aq $NaHCO_3$ (2×3 mL), HCl (1 M, 3 mL), water (3 mL) and brine (3 mL), and then dried. The solvent was evaporated under reduced pressure to give an α,β-mixture (7:3) of **14** as a white foam in 94% yield (127 mg). ^{1}H NMR data ($CDCl_3$, 300 MHz): δ 6.18 (d, 0.7H, $J_{1,2}$ 3.7 Hz, H-1α), 5.67 (d, 0.3H, $J_{1,2}$ 8.1 Hz, H-1β), 5.45 (dd, 0.7H, $J_{2,3}$ 9.1 Hz, $J_{3,4}$ 9.7 Hz, H-3α), 5.15–5.35 (m, 3H, H-1′α, H-1′β, H-3′, H-3β), 4.90 (t, 1H, H-2), 4.70 (dd, 1H, $J_{1',2'}$ 4.1 Hz, $J_{1',2'}$ 10.5 Hz, H-2′), 4.38 (dd, 1H, $J_{5,6a}$ 2.4 Hz, $J_{6a,6b}$ 12.3 Hz, H-6a), 4.26 (dd, 1H, $J_{5',6a'}$ 2.0 Hz, H-6a′),

4.00–4.21 (m, 3H, H-6b, H-5, H-6′b), 3.95 (m, 1H, $J_{3,4}$ 9.1 Hz, H-4), 3.76 (m, 1H, H-5′), 3.36 (s, 3H, OCH_3), 3.26 (t, 1H, $J_{3',4'}$ 9.7 Hz, H-4′), 2.07, 2.07, 2.02, 2.02, 1.98, 1.95, 1.95 (7s, 21H, 7COCH_3). ^{13}C NMR: ($CDCl_3$, 75 MHz): For α-isomer: δ 170.7, 170.4, 170.3, 169.9, 169.9, 169.5, 168.8 (7C=O), 95.8 (C-1′), 88.7 (C-1α), 77.2, 72.3, 72.2, 71.1, 70.3, 70.1, 69.7, 69.7 (C-2, C-3, C-4, C-5, C-2′, C-3′, C-4′, C-5′), 62.4, 62.1 (C-6, C-6′), 60.1 (OCH_3), 20.8, 20.7, 20.7, 20.6, 20.6, 20.5, 20.2 (7COCH_3). For β-isomer: 91.2 (C-1β). MALDI-TOF; Calcd for $C_{27}H_{38}O_{18}$+Na: 673.6. Found: 673.5. $C_{27}H_{38}O_{18}$+K: 689.7. Found: 689.4. Anal. Calcd for $C_{27}H_{38}O_{18}$ (650.59): C, 49.85; H, 5.89. Found: C, 49.88; H, 5.91.

3.1.11. 2,4-Dinitrophenyl-2,2′,3,3′,6,6′-hexa-*O*-acetyl-4′-*O*-methyl-β-maltoside (15). A soln of compound **14** (0.58 g, 0.89 mmol) in DMF (6 mL) was heated to 50 °C and hydrazine·acetate (92 mg, 0.98 mmol) was added. The reaction mixture was cooled to room temperature and stirred for 2 h. The DMF was evaporated under diminished pressure and the residue was dissolved in $CHCl_3$ (5 mL), then washed with satd aq $NaHCO_3$ (2×3 mL), H_2O (3 mL) and brine (3 mL), and then dried. The solvent was evaporated under diminished pressure to afford 2,2′,3,3′,6,6′-hexa-*O*-acetyl-4′-*O*-methyl-maltose as a white foam, which was dissolved in dry DMF (5 mL). DNFB (0.18 g, 0.97 mmol) and DABCO (100 mg, 0.89 mmol) were added and the reaction mixture was stirred overnight in the dark. The DMF was then evaporated under diminished pressure and the residue was dissolved in $CHCl_3$ (5 mL), washed with satd aq $NaHCO_3$ (2×3 mL), water (3 mL) and brine (3 mL), and then dried. The solvent was evaporated under reduced pressure and the residue was chromatographed on Silica Gel with petroleum ether–EtOAc as eluent (1:1) to give **15** as a white solid in 87% yield (604 mg). ^{1}H NMR data ($CDCl_3$, 300 MHz): δ 8.68 (d, 1H, H–Ar), 8.42 (dd, 1H, H–Ar), 7.45 (d, 1H, H–Ar), 5.34 (dd, 1H, $J_{2',3'}$ 10.7 Hz, $J_{3',4'}$ 9.7 Hz, H-3′), 5.33 (d, 1H, $J_{1,2}$ 6.8 Hz, H-1), 5.33 (d, 1H, $J_{1',2'}$ 4.0 Hz, H-1′), 5.27 (t, 1H, $J_{2,3}$ 8.1 Hz, H-3), 5.12 (dd, 1H, H-2), 4.76 (dd, 1H, H-2′), 4.53 (dd, 1H, $J_{5,6a}$ 2.7 Hz, $J_{6a,6b}$ 12.3 Hz, H-6a), 4.32 (dd, 1H, $J_{5',6a'}$ 2.2 Hz, $J_{6a',6b'}$ 12.3 Hz, H-6a′), 4.24 (dd, 1H, $J_{5',6b'}$ 4.2 Hz, H-6b′), 4.20 (dd, 1H, $J_{5,6b}$ 4.9 Hz, H-6b), 4.10 (t, 1H, H-4), 4.06 (m, 1H, H-5), 3.81 (m, 1H, H-5′), 3.41 (s, 3H, OCH_3), 3.30 (t, 1H, H-4′), 2.11, 2.09, 2.07, 2.05, 2.04, 2.03 (6s, 18H, 6COCH_3). ^{13}C NMR: ($CDCl_3$, 75 MHz): δ 170.5, 170.4, 170.1, 170.0, 169.8, 169.3 (6C=O), 153.5, 142.3, 140.3, 128.6, 121.4, 118.6 (6Ar–C), 98.7 (C-1), 95.9 (C-1′), 74.3, 73.0, 72.3, 71.0, 70.1, 69.2, 68.7, 68.0 (C-2, C-3, C-4, C-5, C-2′, C-3′, C-4′, C-5′), 62.3, 61.5 (C-6, C-6′), 60.1 (OCH_3), 20.8, 20.6, 20.6, 20.6, 20.6, 20.5 (6COCH_3). EISMS: Calcd for $C_{31}H_{38}O_{21}N_2$+NH_3: 791.7. Found: 792.1. Calcd for $C_{31}H_{38}O_{21}N_2$+Na: 797.6. Found: 797.2. Calcd for $C_{31}H_{38}O_{21}N_2$+K: 813.7. Found: 813.1. Anal. Calcd for $C_{31}H_{38}O_{21}N_2$ (774.64): C, 48.07; H, 4.94; N, 3.62. Found: C, 47.97; H, 4.62; N, 4.02.

3.1.12. 2,4-Dinitrophenyl 2,2′,3,3′,6,6′-hexa-*O*-acetyl-4′-*O*-methyl-α-maltoside (16). To a soln of compound **15** (600 mg, 0.78 mmol) in DMF (15 mL), anhyd K_2CO_3 (552 mg, 4.00 mmol) was added and the reaction mixture was stirred overnight in the dark. The DMF was evaporated under diminished pressure and the residue was dissolved in $CHCl_3$ (15 mL), washed with satd aq $NaHCO_3$ (2×10 mL), water (10 mL) and brine (10 mL), and then dried. The solvent was evaporated under reduced pressure and **16** was crystallised from EtOAc–petroleum ether in 63% (375 mg) as the pure α-anomer. ^{1}H NMR data ($CDCl_3$, 300 MHz): δ 8.71 (d, 1H, H–Ar), 8.42 (dd, 1H, H–Ar), 7.50 (d, 1H, H–Ar), 5.84 (d, 1H, $J_{1,2}$ 3.6 Hz, H-1), 5.67 (dd, 1H, $J_{2,3}$ 9.7 Hz, $J_{3,4}$ 8.3 Hz, H-3), 5.36 (d, 1H, $J_{1',2'}$ 4.0 Hz, H-1′), 5.32 (dd, 1H, $J_{2',3'}$ 10.4 Hz, $J_{3',4'}$ 9.5 Hz, H-3′), 4.90 (dd, 1H, H-2), 4.76 (dd, 1H, H-2′), 4.45 (dd, 1H, $J_{5,6a}$ 2.0 Hz, $J_{6a,6b}$ 12.2 Hz, H-6a), 4.32 (dd, 1H, $J_{5',6a'}$ 2.2 Hz, $J_{6a',6b'}$ 12.2 Hz, H-6a′), 4.22 (dd, 1H, $J_{5',6b'}$ 3.9 Hz, H-6b′), 4.20 (dd, 1H, $J_{5,6b}$ 4.0 Hz, H-6b), 4.10 (m, 1H, H-5), 4.06 (t, 1H, H-4), 3.77 (m, 1H, H-5′), 3.40 (s, 3H, OCH_3), 3.30 (t, 1H, H-4′), 2.12, 2.10, 2.08, 2.06, 2.05, 2.03 (6s, 18H, 6COCH_3). ^{13}C NMR: ($CDCl_3$, 75 MHz): δ 170.7, 170.4, 170.4, 170.4, 169.8, 169.8 (6C=O), 153.6, 142.0, 140.3, 128.6, 121.7, 118.1 (6Ar–C), 96.2 (C-1), 95.6 (C-1′), 71.7, 71.5, 71.0, 70.3, 70.2, 70.2, 70.2, 69.8 (C-2, C-3, C-4, C-5, C-2′, C-3′, C-4′, C-5′), 62.2, 62.0 (C-6, C-6′), 60.1 (OCH_3), 20.8, 20.8, 20.8, 20.8, 20.6, 20.4 (6COCH_3). EISMS: Calcd for $C_{31}H_{38}O_{21}N_2$+NH_3: 791.7. Found: 792.1. Calcd for $C_{31}H_{38}O_{21}N_2$+Na: 797.6. Found: 797.1. Calcd for $C_{31}H_{38}O_{21}N_2$+K: 813.7. Found: 813.1. Anal. Calcd for $C_{31}H_{38}O_{21}N_2$ (774.64): C, 48.07; H, 4.94; N, 3.62. Found: C, 47.97; H, 4.62; N, 4.02.

3.1.13. 2,4-Dinitrophenyl 4′-*O*-methyl-α-maltoside (17). A soln of compound **16** (199 mg, 0.26 mmol) in MeOH (10 mL) was cooled to 0 °C and acetyl chloride (0.5 mL) was added slowly while stirring. The reaction mixture was left stirring at 4 °C for 48 h, then the solvent was evaporated under reduced pressure and the residue was chromatographed on Silica Gel using EtOAc–MeOH–AcOH (17:2:1) as eluent. The remaining solid was precipitated from MeOH–diethyl ether to give **17** in 60% yield (80 mg). ^{1}H NMR data (CD_3OD, 300 MHz): δ 8.72 (d, 1H, *J* 2.8 Hz, H–Ar), 8.43 (dd, 1H, *J* 2.8 Hz, *J* 9.4 Hz, H–Ar), 7.65 (d, 1H, *J* 9.4 Hz, H–Ar), 5.88 (d, 1H, $J_{1,2}$ 3.5 Hz, H-1), 5.12 (d, 1H, $J_{1',2'}$ 3.8 Hz, H-1′), 4.09–3.40 (m, 11H, H-2, H-3, H-4, H-5, H-6a, H-6b, H-2′, H-3′, H-5′, H-6a′, H-6b′), 3.25 (s, 3H, OCH_3), 3.01 (t, 1H, $J_{3,4}$ 9.2 Hz, H-4′). ^{13}C NMR (CD_3OD, 75 MHz): δ 155.4, 142.5, 140.3, 131.3, 123.3, 118.8 (6Ar–C), 101.0 (C-1′), 98.8 (C-1), 80.5, 77.8, 74.5, 74.0, 73.7, 73.2, 73.1,

72.0 (C-2, C-3, C-4, C-5, C-2′, C-3′, C-4′, C-5′), 61.7, 61.6, 61.5 (C-6, C-6′, OCH$_3$). EISMS: Calcd for $C_{19}H_{26}O_{15}N_2$+NH_3: 539.2. Found: 540.1. Calcd for $C_{19}H_{26}O_{15}N_2$+Na: 545.2. Found: 545.1. Calcd for $C_{19}H_{26}O_{15}N_2$+K: 561.3. Found: 561.0. Anal. Calcd for $C_{19}H_{26}O_{15}N_2$ (522.19): C, 43.68; H, 5.02; N, 5.36. Found: C, 43.28; H, 5.42; N, 4.96.

3.1.14. 2,2′,3,3′,6,6′-Hexa-*O*-acetyl-4′-*O*-methyl-α-maltosyl fluoride (18). Compound **14** (115 mg, 0.18 mmol) was placed in a plastic vial and HF-pyridine (3 mL) was added at −78 °C and stirred under N_2 for 2 h. The reaction mixture was then kept at 0 °C and stirred for an additional 5 h, diluted with EtOAc and washed successively with satd aq $NaHCO_3$ (2×10 mL), water (10 mL), brine (10 mL) and dried. The solvent was evaporated and the residue was purified on Silica Gel using diethyl ether–*n*-pentane (8:2) as eluent to give **18** in 76% (82 mg) as a white solid. ^{1}H NMR data ($CDCl_3$, 300 MHz): δ 5.63 (dd, 1H, $J_{1,2}$ 2.8 Hz, $J_{1,F}$ 53.0 Hz, H-1), 5.53 (dd, 1H, $J_{2,3}$ 10.2 Hz, $J_{3,4}$ 9.3 Hz, H-3), 5.33 (d, 1H, $J_{1',2'}$ 4.0 Hz, H-1′), 5.31 (t, 1H, H-3′), 4.84 (dd, 1H, H-2), 4.75 (dd, 1H, $J_{1',2'}$ 4.0 Hz, $J_{2',3'}$ 10.5 Hz, H-2′), 4.50 (dd, 1H, $J_{5,6a}$ 1.4 Hz, $J_{6a,6b}$ 12.2 Hz, H-6a), 4.32 (dd, 1H, $J_{5',6b'}$ 2.3 Hz, H-6a′), 4.10–4.27 (m, 3H, H-6b, H-5, H-6′b), 4.02 (t, 1H, $J_{3,4}$ 9.3 Hz, H-4), 3.77 (m, 1H, H-5′), 3.40 (s, 3H, OCH_3), 3.32 (t, 1H, $J_{3',4'}$ 9.7 Hz, H-4′), 2.13, 2.11, 2.06, 2.05, 2.04, 2.00 (6s, 18H, 6COCH_3). ^{19}F NMR data ($CDCl_3$, 141 MHz): δ −150.2 (dd, $J_{1,F}$ 53.0 Hz, $J_{2,F}$ 26.0 Hz, F-1). ^{13}C NMR: ($CDCl_3$, 75 MHz): δ 170.9, 170.5, 170.3, 170.1, 169.6, 169.5 (6C=O), 103.7 (C-1), 95.8 (C-1′), 71.8, 71.7, 71.1, 70.8, 70.4, 70.4, 69.7 (C-3, C-4, C-5, C-2′, C-3′, C-4′, C-5′),70.3 (C-2), 62.2, 62.1 (C-6, C-6′), 60.1 (OC$_3$), 20.8, 20.8, 20.7, 20.7, 20.6, 20.5 (6COC$_3$). MALDI-TOF; Calcd for $C_{25}H_{35}O_{16}F$+Na: 633.6. Found: 633.6. $C_{25}H_{35}O_{16}F$+K: 649.6. Found: 649.8.

3.1.15. 4′-*O*-Methyl-α-maltosyl fluoride (19). A soln of compound **18** (70 mg, 1.16 mmol) in MeOH (5 mL) was cooled to 0 °C. NH_3 was bubbled through the soln for 10 min and the reaction mixture was stirred at room temperature overnight. The solvent was then evaporated and the residue was purified on Silica Gel using EtOAc–MeOH–H_2O (7:2:1) as eluent to give **19** in 73% yield (30 mg) as a white solid. ^{1}H NMR data (D_2O, 300 MHz): δ 5.57 (dd, 1H, $J_{1,2}$ 2.5 Hz, $J_{1,F}$ 52.9 Hz, H-1), 5.30 (d, 1H, $J_{1',2'}$ 3.8 Hz, H-1′), 3.43–3.95 (m, 11H, H-2, H-3, H-4, H-5, H-6a, H-6b, H-2′, H-3′, H-5′, H-6′a, H-6′b), 3.24 (s, 3H, OCH_3), 3.11 (t, 1H, $J_{3',4'}$ 9.6 Hz, H-4′). ^{19}F NMR data (D_2O, 141 MHz): δ −150.3 (dd, $J_{1,F}$ 52.9 Hz, $J_{2,F}$ 26.0 Hz, F-1). ^{13}C NMR: (D_2O, 75 MHz): δ 107.4 (C-1), 100.0 (C-1′), 76.0, 73.1, 73.1, 73.0, 72.9, 72.0, 69.5 (C-3, C-4, C-5, C-2′, C-3′, C-4′, C-5′), 71.1 (C-2), 62.2, 62.1 (C-6, C-6′), 60.1 (OCH$_3$). MALDI-TOFMS; Calcd for $C_{13}H_{23}O_{10}F$+Na: 381.3. Found: 381.1.

3.2. Enzymatic assay

All studies were carried out at 30 °C in 50 mM sodium phosphate buffer, pH 6.9 containing 100 mM NaCl. Quantitation of the hydrolysis of **5**, **10** and **17** by HPA (varying the substrate concentration between 0 and 15 mM) was performed by following the increase in the absorbance at 400 nm upon the addition of enzyme (final enzyme concentration; 0.5 μM for DNP-G2 and 50 nM for DNP-G3 and MeDNP-G2) using a Varian CARY 4000 spectrophotometer attached to a temperature control unit. The rates were calculated using an extinction coefficient of $\Delta\varepsilon = 10{,}900\ M^{-1}\,cm^{-1}$ (pH 6.9). Enzyme concentrations were calculated using an extinction coefficient of $\varepsilon = 2.6\ mg\,C\,m^{-1}\,mL^{-1}$. The hydrolysis of **19** (0–4 mM) by HPA was monitored by following the increase in fluoride concentration after addition of enzyme (final concentration; 12 nM) using an ORION 96-04 combination fluoride electrode interfaced to a personal computer running the program LoggerPro (Vernier Software, Oregon). The kinetic parameters for all substrates were estimated by direct fit of the data to the Michaelis–Menten equation using the program GraFit 4.0.14.

3.3. Product analysis

All product analyses were performed on a Waters 600 HPLC system equipped with a Waters 2487 UV detector and a Sedex 75 Evaporative Light Scattering detector (ELSD). DNP-G2 (1 mM) was incubated for 20 min with HPA at 30 °C in 50 mM sodium phosphate buffer, pH 6.9 containing 100 mM NaCl. The protein was first removed from the sample using a 10 kDa molecular weight cutoff centrifugal device (Millipore) and the filtrate was diluted to 25% (v/v) with acetonitrile. Approximately 30 μL of sample was loaded onto a Tosoh Amide-80 (4.6 mm×25 cm) column. The products were then eluted at 0.7 mL/min with an acetonitrile–water (75:25–55:45) gradient for 10 min followed by MeCN–water (55:45) elution.

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References

1. Jenkins, D. J.; Taylor, R. H.; Goff, D. V.; Fielden, H.; Misiewicz, J. J.; Sarson, D. L.; Bloom, S. R.; Alberti, K. G. *Diabetes* **1981**, *30*, 951–954.

2. Rachmani, R.; Bar-Dayan, Y.; Ronen, Z.; Levi, Z.; Slavachevsky, I.; Ravid, M. *Diabetes Obes. Metab.* **2004**, *6*, 63–68.
3. Bailey, C. J. *Chem. Ind.* **1998**, 53–57.
4. Fernstad, R.; Skoldefors, H.; Pousette, A.; Groth, C. G.; Tyden, G.; Ost, L.; Lindholm, A.; Carlstrom, K. *Pancreas* **1989**, *4*, 44–52.
5. Williams, S. J.; Withers, S. G. *Carbohydr. Res.* **2000**, *327*, 27–46.
6. Lee, S. S.; Yu, S.; Withers, S. G. *Biochemistry* **2003**, *42*, 13081–13090.
7. Genghof, D. S.; Brewer, C. F.; Hehre, E. J. *Carbohydr. Res.* **1978**, *61*, 291–299.
8. Rydberg, E. H.; Li, C.; Maurus, R.; Overall, C. M.; Brayer, G. D.; Withers, S. G. *Biochemistry* **2002**, *41*, 4492–4502.
9. Kartha, K. P. R.; Jennings, H. J. *J. Carbohydr. Chem.* **1990**, *9*, 777.
10. Excoffier, G.; Gagnaire, D.; Utille, J. P. *Carbohydr. Res.* **1975**, *39*, 368–373.
11. Koerners, H. J.; De Kok, A. J.; Romers, C.; Van Boom, J. H. *Recl. Trav. Chim. Pays-Bas* **1980**, *99*, 355–362.
12. Berven, L. A.; Dolphin, D. H.; Withers, S. G. *J. Am. Chem. Soc.* **1988**, *110*, 4864–4866.
13. Garegg, P. J.; Maron, L.; Swahn, C.-G. *Acta Chem. Scand.* **1972**, *26*, 518.
14. Garegg, P. J.; Maron, L.; Swahn, C.-G. *Acta Chem. Scand.* **1972**, *26*, 3895.
15. DeBelder, A. N. *Adv. Carbohydr. Chem. Biochem.* **1977**, *34*, 179.
16. Garegg, P. J.; Hultberg, H. *Carbohydr. Res.* **1981**, *93*, C10.
17. Hayashi, M.; Hashimoto, S.; Noyori, R. *Chem. Lett.* **1984**, *10*, 1747–1750.
18. Brayer, G. D.; Sidhu, S.; Maurus, R.; Rydberg, E. H.; Braun, C.; Wang, Y.; Nguyen, N. T.; Overall, C. O.; Withers, S. G. *Biochemistry* **2000**, *39*, 4778–4791.
19. Rydberg, E. H.; Sidhu, G.; Vo, H. C.; Hewitt, J.; Cote, H. C. C.; Wang, Y.; Numao, S.; MacGillivary, R. T. A.; Overall, C. M.; Brayer, G. D.; Withers, S. G. *Protein Sci.* **1999**, *8*, 635–643.
20. Iskhikawa, K.; Matsui, I.; Honda, K.; Nakatani, H. *Biochem. Biophys. Res. Commun.* **1992**, *183*, 286–291.