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Enzyme-catalysed synthesis of galactosylated 1D- and 1L-*chiro*-inositol, 1D-pinitol, *myo*-inositol and selected derivatives using the β-galactosidase from the thermophile *Thermoanaerobacter* sp. strain TP6-B1

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Abstract—The products from the enzymatic β-D-galactopyranosylation of 1D-*chiro*-inositol, 1D-pinitol, 1D-3-*O*-allyl-4-*O*-methyl-*chiro*-inositol, 1D-3,4-di-*O*-methyl-*chiro*-inositol, 1L-*chiro*-inositol and *myo*-inositol in combined yields ranging from 46% to 64% have been obtained using the β-galactosidase isolated from an anaerobic extreme thermophile, *Thermoanaerobacter* sp. strain TP6-B1 and *p*-nitrophenyl β-D-galactopyranoside as the donor. Analysis of the products from these reactions reveals information about the acceptor preferences of the enzyme.

Keywords: Glycosidases; Thermostable enzymes; Inositol oligosaccharides; Glycosylations; *Thermoanaerobacter* sp.

1. Introduction

Oligosaccharides containing 1D-*chiro*-inositol (**1**) and its 3-*O*-methyl ether 1D-pinitol (**2**) have previously been isolated from the seeds of leguminous plants such as buckwheat[1–3] and jojoba beans.[4] They are thought to play a role in protecting the organelles and proteins of the seeds during desiccation and storage.[3,5] In addition, disaccharides consisting of **1**, **2** or *myo*-inositol coupled to a sugar such as galactosamine are the focus of attention for their putative role as second messengers of insulin in vivo.[6–14] It has been proposed that these disaccharides are released from glycosyl phosphatidyl inositols anchored to the outer surface of insulin sensitive cells, in response to insulin-receptor binding[10,11,13–15] and a lack of these second messengers has been implicated in the etiology of Type 2 diabetes in humans.[7] Recently, Larner and co-workers reported the first full characterisation of a putative insulin mediator from insulin sensitive tissue consisting of galactosamine β-linked to the 4-position of **2**.[14] However, most previous efforts at identifying the mediator largely focused on synthesising candidate structures and assessing their insulin mimetic ability.[12,16–18]

Recently, considerable interest has focussed on the use of glycoside hydrolase enzymes (glycosidases) to synthesise di- and oligo-saccharides in only one step.[19–24] The particular characteristics of the glycosidase employed determines, which transglycosylation products, if any, are formed and the products formed are a function of the enzyme's acceptor and donor preferences as well as its glycosylation regio- and stereoselectivity. However, these selectivities need not be regarded as limitations to the use of glycosidases in oligosaccharide synthesis. Instead, the approach can be thought of as

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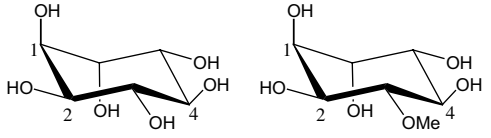
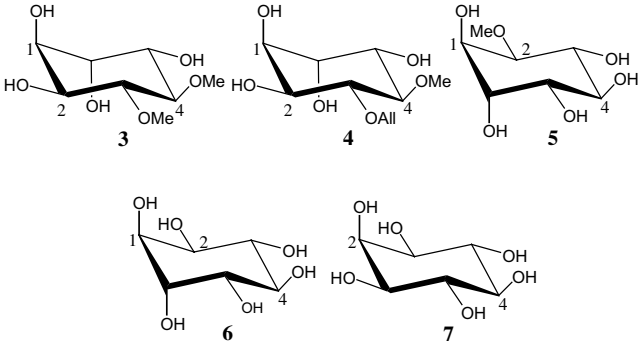
being directed and biomimetic, because the regioselectivity encountered is more likely to be of biological significance than a corresponding random chemical approach.



1 **2**

We have previously reported the galactosylation of 1D-*chiro*-inositol **1** and 1D-pinitol **2**, using the β-galactosidase from *Bacillus circulans*,[19] the first report of the enzymatic glycosylation of inositols. An analysis of the products obtained from these glycosylations allowed us to gain some insight into the hydroxyl group configurations key to the recognition of the acceptor as a substrate in the active site of the enzyme.

A thermostable β-galactosidase isolated from an anaerobic extreme thermomophile, *Thermoanaerobacter* sp. strain TP6-B1 (henceforth referred to as TP6-B1) from a New Zealand geothermal source,[25] displayed a preference for hydrolysing β-linked galactopyranosides such as *o*- and *p*-nitrophenyl β-D-galactopyranoside and lactose as opposed to α-linked galactopyranosides and glucopyranosides.[25] As part of an investigation into the transglycosylation activity of various glycosidases, we report the results of the TP6-B1 β-galactosidase-catalysed galactosylations of a number of inositols: 1D-*chiro*-inositol **1**, 1D-pinitol **2**, 1D-3,4-di-*O*-methyl-*chiro*-inositol **3**, 1D-3-*O*-allyl-4-*O*-methyl-*chiro*-inositol **4**, 1L-quebrachitol **5**, 1L-*chiro*-inositol **6** and *myo*-inositol **7**. In conjunction with NMR analysis, GC–MS analysis was used to characterise and/or confirm the identity of the glycosylation products. These results are described, along with the synthetic routes to a series of mono-*O*-acetyl-penta-*O*-methylated *chiro*- and *myo*-inositol standards required for the GC–MS identification of the individual product components. In addition, the acceptor preferences of the enzyme have been deduced on the basis of the products formed.



3 **4** **5** **6** **7**

2. Results and discussion

2.1. Galactosylation of selected inositols using TP6-B1 β-galactosidase

The galactosylation products obtained from the selected inositol acceptors **1**–**7**, employing the β-galactosidase from TP6-B1 are summarised in Table 1, and the positions on the substrates where galactosylation has occurred are depicted in Figure 1. All reactions were conducted using a moderate excess of the inositol acceptor and the yields reported are based on the limiting reagent, namely *p*-nitrophenyl β-D-galactopyranoside.

In contrast to the single disaccharide reaction product observed for the galactosylation of 1D-*chiro*-inositol **1** by the β-galactosidase from *B. circulans*,[19] two disaccharide products were obtained in a 10:3 ratio from the galactosylation of 1D-*chiro*-inositol **1** using the β-galactosidase from TP6-B1, in a combined yield of 46%. An analysis of the product mixture by ^{1}H and ^{13}C NMR spectroscopy revealed that the resonances of the major component were identical to those of the disaccharide product observed from the enzymatic galactosylation of **1** using the β-galactosidase from *B. circulans*, namely 1D-1-*O*-(β-D-galactopyranosyl)-*chiro*-inositol **8**.[19] The

Table 1. Summary of galactosylation products obtained using TP6-B1 β-galactosidase with *p*-nitrophenyl β-D-galactopyranoside as the donor

Acceptor	Products	Isolated yield (%)[a]
1D-*chiro*-Inositol **1**	β-D-Gal*p*-(1 → 1)-DCI **8** and β-D-Gal*p*-(1 → 3)-DCI **9**	46[b]
1D-Pinitol **2**	β-D-Gal*p*-(1 → 1)-4-OMe-DCI **10** and β-D-Gal*p*-(1 → 1)-3-OMe-DCI **11**	50[b]
1D-3,4-Di-*O*-methyl-*chiro*-inositol **3**	β-D-Gal*p*-(1 → 1)-3,4-di-OMe-DCI **12**	52
1D-3-*O*-Allyl-4-*O*-methyl-*chiro*-inositol **4**	β-D-Gal*p*-(1 → 1)-4-*O*-All-3-OMe-DCI **13** and β-D-Gal*p*-(1 → 1)-3-*O*-All-4-OMe-DCI **14**	60[b]
1L-Quebrachitol **5**	n.r.	n.r.
1L-*chiro*-Inositol **6**	β-D-Gal*p*-(1 → 3)-LCI **15** and β-D-Gal*p*- (1 → 1)-LCI **16**	64[b]
myo-Inositol **7**	β-D-Gal*p*-(1 → 2)-*myo*-inositol **17** and β-D-Gal*p*-(1 → 4/6)-*myo*-inositol **18** and β- D-Gal*p*-(1 → 5)-*myo*-inositol **19** and β-D-Gal*p*-(1 → 1/3)-*myo*-inositol **20**	63[b]

n.r. No reaction.

[a] Yields based on limiting reagent, that is *p*-nitrophenyl β-D-galactopyranoside.

[b] Combined yield.

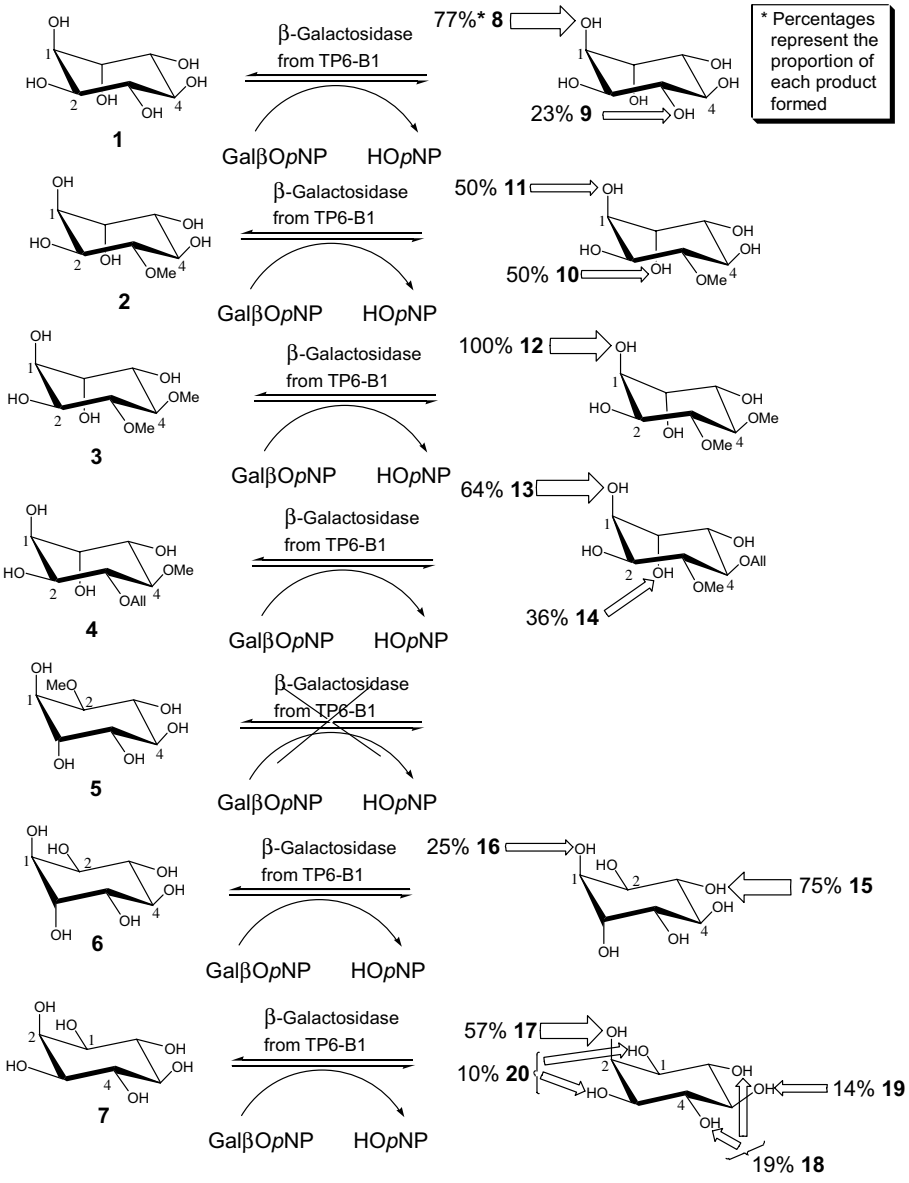


Figure 1. Products from galactosylation of various inositols catalysed by TP6-B1 β-galactosidase.

second, minor, component was shown by ^{1}H and ^{13}C NMR analysis to be a disaccharide with a β-linkage to 1D-*chiro*-inositol at a different position to that observed for compound **8**. However, it was not possible to define the attachment point on the inositol due to the complex nature of the NMR spectra. The structure of the minor component was shown to be 1D-3-*O*-(β-D-galactopyranosyl)-*chiro*-inositol **9** by methylation analysis of the product mixture (vide infra).

A 1:1 mixture of two products was obtained from the galactosylation of 1D-pinitol **2** using the β-galactosidase from TP6-B1, in a combined yield of 50%. Previously, we reported that the galactosylation of 1D-pinitol **2** by the β-galactosidase from *B. circulans* gave only one disaccharide product, **10**, whereby galactosylation occurred on the axial position remote from the methyl group.[19] NMR spectroscopic analysis of the product mixture indicated that one of the disaccharide products was that observed previously, namely 1D-1-*O*-(β-D-galactopyranosyl)-4-*O*-methyl-*chiro*-inositol **10**.[19] A detailed analysis of the unassigned NMR correlations in the 2D-NMR spectra of the product mixture revealed that the second product was 1D-1-*O*-(β-D-galactopyranosyl)-3-*O*-methyl-*chiro*-inositol **11**. It is apparent that disaccharide **10** is the result of galactosylation of **2** at the axial hydroxyl position remote from that of the methyl group of **2**, that is OH-6, whereas **11** is the result of galactosylation at the other axial hydroxyl of **2**, OH-1, two positions removed from that of the methyl group.

For the β-galactosidase from TP6-B1, the presence of the methyl group in **2** does not appear to have a direct influence on the direction of galactosylation towards either of the two axial hydroxyl groups at positions OH-1 and OH-6 of **2** as both regioisomers **10** and **11** were observed in equal amounts as reaction products. This result is in contrast with that obtained for the *B. circulans* β-galactosidase whereby galactosylation occurred only at the OH-6 of **2** to give disaccharide **10**, that is only at the position remote from that of the methyl group (Fig. 2).

It is worth noting that the numbering of the products **10** and **11** follows the IUPAC rules applicable to the naming of inositols: the highest priority is given to the galactosyl-substituent attached to the inositol sugar, with lower priority given to the methyl group. This results in an apparent change in the position of the methyl group of **10** (OH-4) relative to those of **2** and **11** (OH-3), as the galactosyl substituent is defined in both compounds **10** and **11** as being at the 1-position. Closer inspection reveals that the galactosyl substituent is attached at the axial hydroxyl remote to that of the methyl group in **10** (numbered as the 6-position of **2**), and in **11** it is attached at the other axial hydroxyl (numbered as the 1-position of **2**), and the methyl group has not changed position.

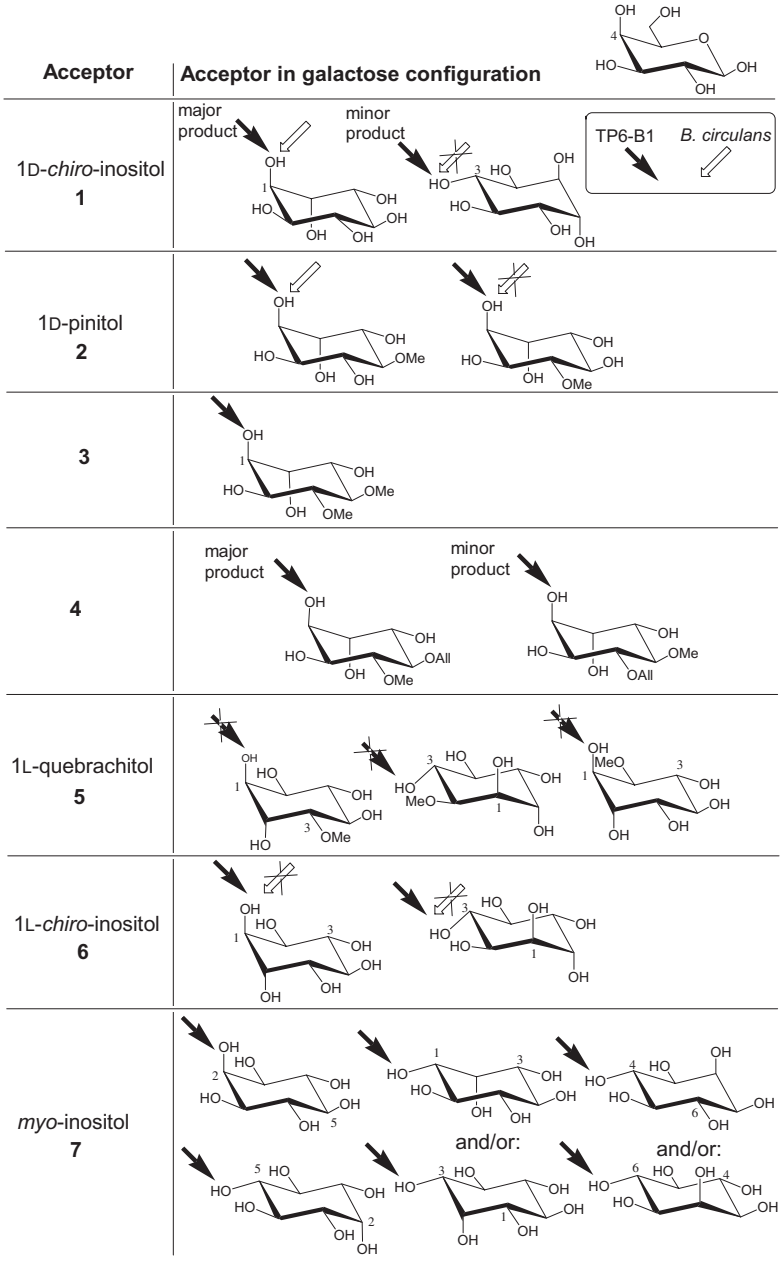
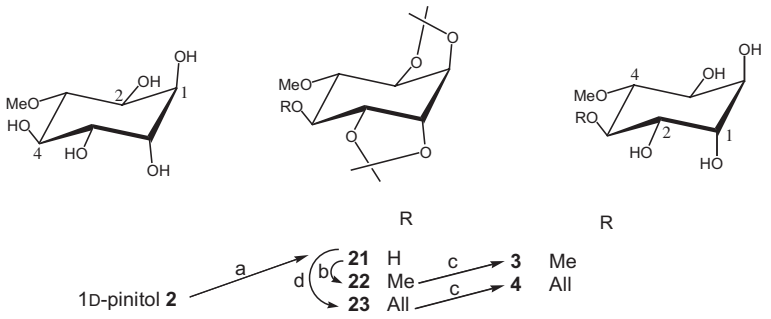


Figure 2. Galactosylation patterns for *B. circulans* and TP6-B1 β-galactosidases with various inositols.



Scheme 1. Synthesis of inositol derivatives **3** and **4**. Reagents and conditions: (a) cat. *p*TSA, Me_2CO, dimethyoxypropane, DMF; (b) NaH, MeI, DMF; (c) TFA–H_2O (1:1); (d) NaH, AllBr, DMF.

In order to investigate the influence of additional groups on 1D-pinitol **2** with regard to the galactosylation pattern observed, two inositol derivatives (**3** and **4**) were synthesised (Scheme 1). The allyl group was chosen because of its increased size relative to the methyl group and its potential for further synthetic utility. The C_2-symmetric inositol 1D-3,4-di-*O*-methyl-*chiro*-inositol **3** with methyl groups at the OH-3 and OH-4 positions of **1**, and the mono-allyl derivative **4**, were synthesised from a common intermediate, pinitol diacetonide **21**, by performing standard methylation and allylation reactions, respectively. Removal of the acetonide groups under acid conditions yielded the desired derivatives **3**[26] and **4**, respectively.

Galactosylation of the C_2-symmetrical dimethylated inositol derivative **3** using the β-galactosidase from TP6-B1 yielded only one product, in an isolated yield of 52%. Analysis of the NMR spectra obtained for this product revealed the galactose was attached at either of the equivalent OH-1 or OH-6 positions of **3**, to give the β-linked disaccharide **12**. The β-galactosidase from TP6-B1 therefore displays an apparent tolerance for methyl groups at both the OH-3 and OH-4 positions of 1D-*chiro*-inositol **1**, as seen by the occurrence of galactosylation at either of the two equivalent axial hydroxyl groups of **3**, namely OH-1 or OH-6.

Galactosylation of the mono-allylated inositol derivative **4** by the TP6-B1 β-galactosidase resulted in two products in a 16:9 ratio (as determined by ^1H NMR spectroscopy), isolated in a combined yield of 60%. For the major component, analysis of the NMR data obtained from the product mixture revealed that the galactose was β-linked to the axial hydroxyl of **4** that is remote from the allyl group to give disaccharide **13**. A corresponding analysis of the unassigned correlations in the NMR spectra of the product mixture showed that the minor product was the result of β-galactosylation at the axial hydroxyl of **4** remote from the methyl group to give disaccharide **14** (Fig. 1).

The presence of the allyl group in **4** influenced the product distribution of the reaction catalysed by the TP6-B1 β-galactosidase such that a 1.8-fold preference for galactosylation towards the hydroxyl position remote from that of the allyl group was observed. If the inositol derivatives are depicted in a 'β-D-galactose configuration' so that the structures have the same configuration as seen at C-1–C-4 of β-D-galactose (Fig. 2) it can be seen that the TP6-B1 β-galactosidase has a higher degree of tolerance for larger groups at the position, which corresponds to the C-2 of galactose than the *B. circulans* β-galactosidase, which displays a strong preference for a hydroxyl group at this position.

Galactosylation of 1L-*chiro*-inositol **6** using the β-galactosidase from TP6-B1 gave two separable products in a 3:1 ratio with a combined yield of 64%. The major component was shown by an analysis of the 1D- and 2D-NMR data from the mixture to contain a galactose moiety β-linked at the OH-3 of **6** that is 1L-3-*O*-(β-D-galactopyranosyl)-*chiro*-inositol **15**. Similarly, a detailed analysis of the 2D-NMR spectra of the minor reaction product revealed that 1L-1-*O*-(β-D-galactopyranosyl)-*chiro*-inositol **16** was the disaccharide formed, that is a galactose β-linked to the OH-1 position of **6**. In contrast, 1L-quebrachitol **5**, the 2-*O*-methyl ether of **6**, was not glycosylated by the β-galactosidase from TP6-B1.

Four inseparable products were obtained from the galactosylation of *myo*-inositol **7** using the β-galactosidase from TP6-B1, in a combined yield of 63%. Analysis of the product mixture by GC–MS was required to determine the linkage patterns on the *myo*-inositol (vide infra).

2.2. Preparation of inositol standards for GC–MS analysis

To identify the disaccharide products that were obtained as mixtures of inseparable components that could not be established by NMR analysis alone, and to confirm the identities of those products for which the NMR data were sufficient to enable a full characterisation, methylation analyses employing GC–MS were undertaken on the enzymatic products (Fig. 3). In order to identify the derivatised inositol components by GC–MS, it was first necessary to synthesise the set of penta-*O*-methylated

mono-*O*-acetyl standards for *chiro*-inositol and *myo*-inositol viz. compounds **24**–**30**, as shown in Schemes 2–5. Because of the C_2-symmetry inherent in the *chiro*-inositol molecule, only three pentamethylated derivatives need to be synthesised to obtain the full set of *chiro*-inositol standards, viz. the 1- (or 6-), the 2- (or 5-) and the 3- (or 4-) *O*-acetyl-penta-*O*-methyl-*chiro*-inositols. For *myo*-inositol, a *meso* compound, acetylations at the 2- or the 5-positions also give rise to *meso*-compounds. However, for acetylations at either of the enantiotopic 1- or 3-hydroxyl groups, the possibility of enantiomers arises; acetylation of the 1-hydroxyl produces the 1D-enantiomer, whereas acetylation of the 3-hydroxyl gives the 1L-enantiomer. A similar situation exists for the enantiotopic 4- and 6-hydroxyl groups. However, for the sake of simplicity we decided to produce only the four *myo*-inositol derivatives **25**, **27**, **28** and **29** arising from acetylation at the 1-, 2-, 4- and 5-hydroxyls of *myo*-inositol, respectively. It is worth noting that the four penta-*O*-methylated *myo*-inositols **41**, **51**, **56** and **61** have been reported previously,[27] however the syntheses of these compounds involved, as the first step, the separation of three *myo*-inositol dicyclohexylidene adducts and this approach resulted in the generation of the 1/3- and 4/6-*myo*-inositol penta-*O*-methyl derivatives as racemates. More recently, the individual enantiomeric 1D- and 1L-1-*O*-acetyl-penta-*O*-methyl-*myo*-inositols and the corresponding 1D- and 1L-4-*O*-acetyl-enantiomers were reported.[28,29] The 1D- and 1L-4-*O*-acetyl-penta-*O*-methyl-*myo*-inositols were obtained from a chiral resolution of L-(+)-*O*-acetylmandelate dicyclohexylidene *myo*-inositol derivatives and the 1D- and 1L-1-*O*-acetyl-penta-*O*-methyl-*myo*-inositol enantiomers were obtained from a chiral resolution of the isopropyl 2,3-di-*O*-benzoyl-L-(+)-tartrate dicyclohexylidene *myo*-inositol derivatives.

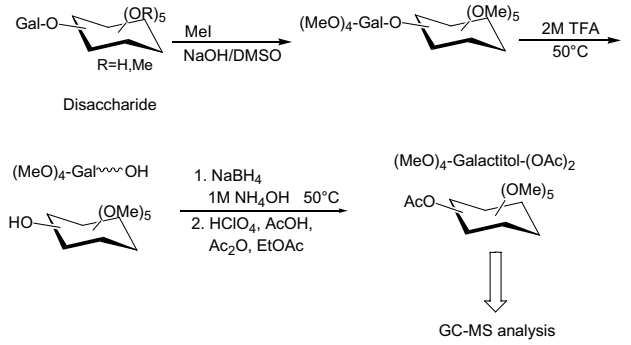
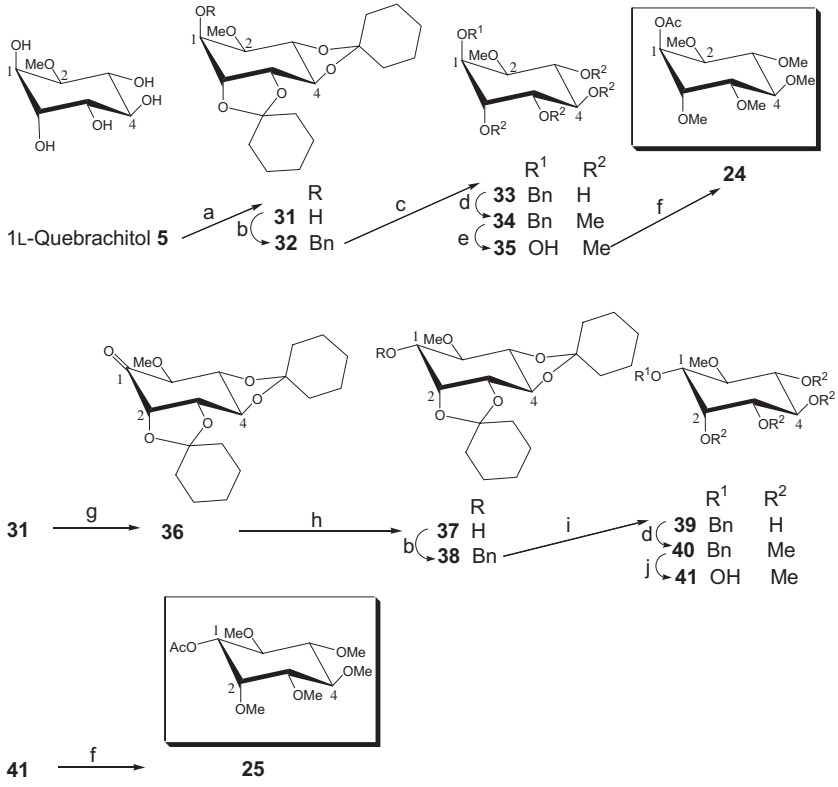
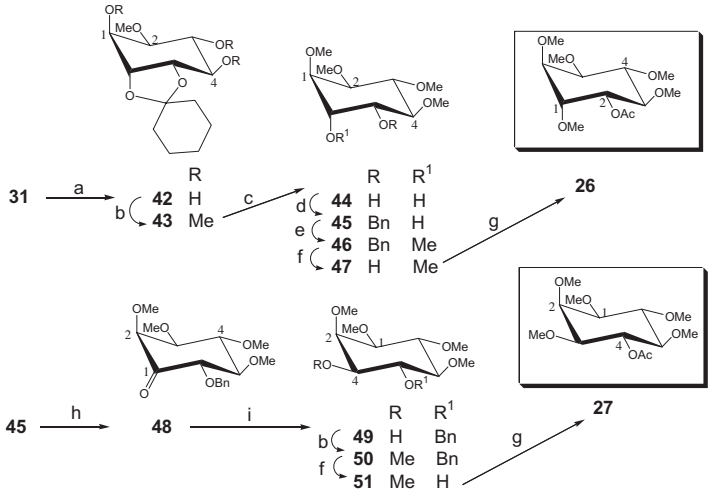


Figure 3. The derivatisation method for methylation analysis.

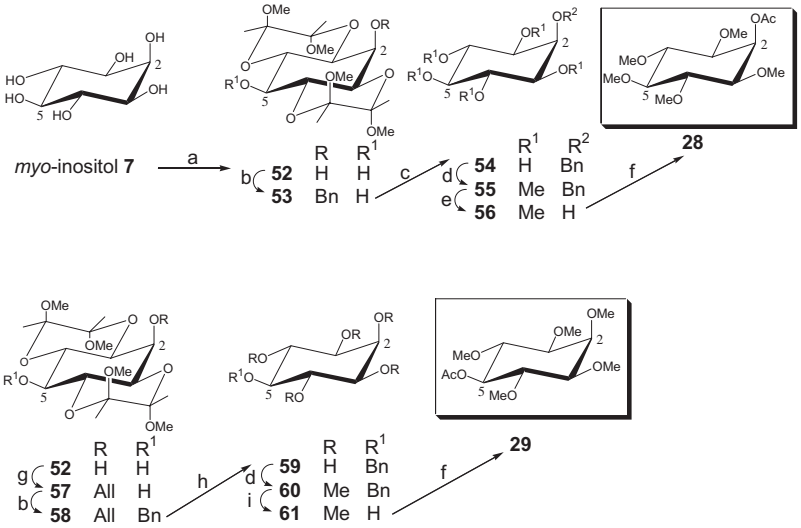
The standard compounds were prepared as shown from 1L-quebrachitol **5** (**24**, **25**, **26**, **27**) in Schemes 2 and



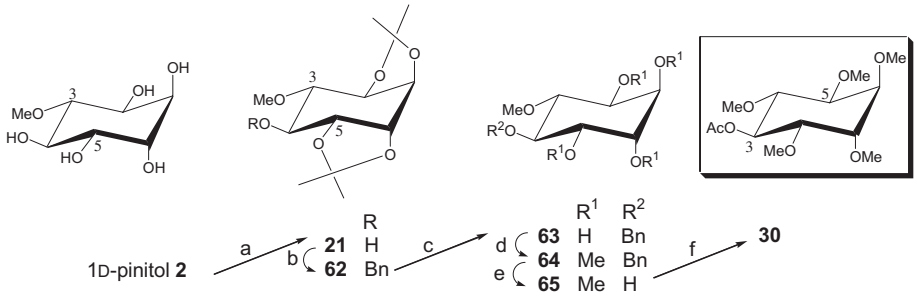
Scheme 2. Syntheses of the methylation analysis standards 1-OAc-*chiro*-$(OMe)_5$ (**24**) and 1-OAc-*myo*-$(OMe)_5$ (**25**). Reagents and conditions: (a) cat. H_2SO_4, cyclohexanone, benzene, DMF; (b) NaH, BnBr, DMF; (c) TFA–H_2O (1:1); (d) NaH, MeI, DMF; (e) Pd/C, H_2, EtOH, AcOH; (f) Ac_2O, py; (g) $RuCl_3$, $NaIO_4$, K_2CO_3, MeCN, DCM, H_2O; (h) $NaBH_4$, H_2O, THF; (i) AcOH–H_2O (4:1); (j) Pd/C, H_2, TFA, EtOH.



Scheme 3. Syntheses of the methylation analysis standards 2-OAc-*chiro*-$(OMe)_5$ (**26**) and 4-OAc-*myo*-$(OMe)_5$ (**27**). Reagents and conditions: (a) H_2O–AcOH (1:6); (b) NaH, MeI, DMF; (c) TFA–H_2O (1:1); (d) $(Bu_3Sn)_2O$, Bu_4NBr, BnBr, toluene, reflux; (e) NaH, MeI, DMF; (f) Pd/C, H_2, TFA, EtOH; (g) Ac_2O, py; (h) $RuCl_3$, $NaIO_4$, K_2CO_3, MeCN, DCM, H_2O; (i) $NaBH_4$, H_2O, THF.



Scheme 4. Syntheses of the methylation analysis standards 2-OAc-*myo*-$(OMe)_5$ (**28**) and 5-OAc-*myo*-$(OMe)_5$ (**29**). Reagents and conditions: (a) 2,3-butadione, $CH(OMe)_3$, CSA, MeOH; (b) NaH, BnBr, DMF; (c) TFA–H_2O (1:1); (d) NaH, MeI, DMF; (e) Pd/C, H_2, TFA, H_2O; (F) Ac_2O, py; (g) NaH, AllBr, DMF; (h) Pd/C, *p*TSA, H_2O, MeOH; (i) Pd/C, H_2, TFA, EtOH.



Scheme 5. Synthesis of the methylation analysis standard 3-OAc-*chiro*-$(OMe)_5$ (**30**). Reagents and conditions: (a) cat. *p*TSA, Me_2CO, dimethoxypropane, DMF; (b) NaH, BnBr, DMF; (c) TFA–H_2O (1:1); (d) NaH, MeI, DMF; (e) Pd/C, H_2, AcOH, EtOH; (f) Ac_2O, py.

3, *myo*-inositol **7** (**28**, **29**) in Scheme 4, and 1D-pinitol **2** (**30**) in Scheme 5.

In the future the synthesis of the full set of six possible mono-*O*-acetyl penta-*O*-methyl *myo*-inositols in conjunction with analysis by chiral GC–MS could be used to reveal additional information regarding the stereopreferences of the enzyme.

2.3. Methylation analysis of the enzyme products using GC–MS

The *chiro*- and separately the *myo*-series of standards gave rise to chromatographic peaks that were well resolved by GC–MS (Table 2). The derivatives generated from the various enzyme-catalysed glycosylation products by methylation analysis (methylation, reductive hydrolysis, acetylation (Fig. 3)) were analysed by GC–MS and the retention times were compared with those of the standards to reveal the identities of the individual component products. As an illustration of the power of this method, Figure 4 shows the GC–MS trace of the *chiro*-inositol standards and of derivatives obtained from the 1D-*chiro*-inositol monogalactoside product mixture. It can be seen that the major product is 1-*O*-linked to galactose and the minor component is 3-*O*-linked to galactose. The galactosyl moiety is derivatised to tetra-*O*-methyl galactitol diacetate. Employing this method, the identities of the components in the reaction mixtures were successfully established and this information is given in Table 1.

For the galactosylation of *myo*-inositol **7**, the GC trace obtained for the derivatised product mixture revealed four separate inositol-related peaks with retention times corresponding to the four *myo*-inositol standards. The major component corresponded to galactosylation at the 2-hydroxyl group of **7** to give 2-*O*-(β-D-galactopyranosyl)-*myo*-inositol (**17**), while the third largest peak corresponded to galactosylation at the other *meso*-position of **7**, the 5-position, to give 5-*O*-(β-D-galactopyranosyl)-*myo*-inositol (**19**). The smallest peak was attributed to galactosylation at either the 1- or the 3-hydroxyl groups of **7** to give 1D- or 1L-1-*O*-(β-D-galactopyranosyl)-*myo*-inositol (**20**), respectively, but without chiral GC analysis it was not possible to distinguish whether only one, or both disaccharides were produced. The second largest peak corresponding to galactosylation at the 4- or 6-hydroxyl groups of **7** to give 1D- or 1L-4-*O*-(β-D-galactopyranosyl)-*myo*-inositol (**18**), respectively, could also be attributed to the presence of one or both of the disaccharide products.

Table 2. Relative GC–MS retention times for methylation analysis standards

Methylation analysis standard[a]	Relative R_t[b]
1-OAc-*chiro*-(OMe)$_5$ **24**	0.538
2-OAc-*chiro*-(OMe)$_5$ **26**	0.608
3-OAc-*chiro*-(OMe)$_5$ **30**	0.641
2-OAc-*myo*-(OMe)$_5$ **28**	0.539
1-OAc-*myo*-(OMe)$_5$ **25**	0.581
5-OAc-*myo*-(OMe)$_5$ **29**	0.658
4-OAc-*myo*-(OMe)$_5$ **27**	0.668

[a] Abbreviated compound names used, for example, '1-OAc-*chiro*-(OMe)$_5$' is shorthand for 1L-1-*O*-acetyl-2,3,4,5,6-penta-*O*-methyl-*chiro*-inositol.

[b] Relative to hexa-*O*-acetyl-*myo*-inositol = 1.00.

2.4. Analysis of enzyme specificity

The TP6-B1 β-galactosidase is reported to hydrolyse lactose as well as β-galactosides such as *p*-nitrophenyl β-D-galactopyranoside[25] therefore it is likely that galactose and glucose can be accommodated into the acceptor site of the enzyme for reactions occurring in the transglycosylation direction. An assessment of the acceptor preferences of the enzyme, as detailed in Figure 2, revealed that TP6-B1 is relatively nonselective with regard to substitution of the inositol acceptor in comparison with the results obtained with the *B. circulans* β-galactosidase.[19] The structures in Figure 2 are drawn as if the enzyme were forming a β-(1 → 4)-linked disaccharide. The enzyme galactosylated hydroxyl groups in an equatorial (i.e., *gluco*-) or an axial (i.e., *galacto*-) configuration at the 4-position.

It is apparent that the key determinant for transglycosylation to occur is the configurations of the 2- and 3-positions, namely the requirement that either C-2 or C-3 possess an equatorial hydroxyl substituent for galactosylation to occur at C-4 (galactose numbering). Galactosylation of the inositol acceptor was achieved with a wide range of inositol substitution patterns and configurations, for example, for the 2-position (galactose numbering) the enzyme generally accommodated equatorial hydroxyl, methoxyl and allyl groups. The exception to this is seen for 1L-quebrachitol **5** where a methoxyl group is in the C-2 position and no glycosylation occurs. An axial hydroxyl group at C-2 was also accommodated for some '*gluco*-type' systems (i.e., an equatorial 4-OH) such as 1L-*chiro*-inositol **6** but not for others, for example, 1L-quebrachitol **5**, which has an equatorial methoxyl group at C-3 (galactose numbering). For the 3-, 4- and 5-positions (galactose numbering) the enzyme tolerated the presence of axial or equatorial hydroxyl groups. However, it did not galactosylate at the 4-position if there were methoxyl or methylene hydroxy (CH_2OH) groups present at the 5-position (since the TP6-B1 enzyme did not appear to galactosylate galactose) or a methoxyl group at the 3-position (as is the case with 1L-quebrachitol **5**). The information described above is summarised in Figure 5.

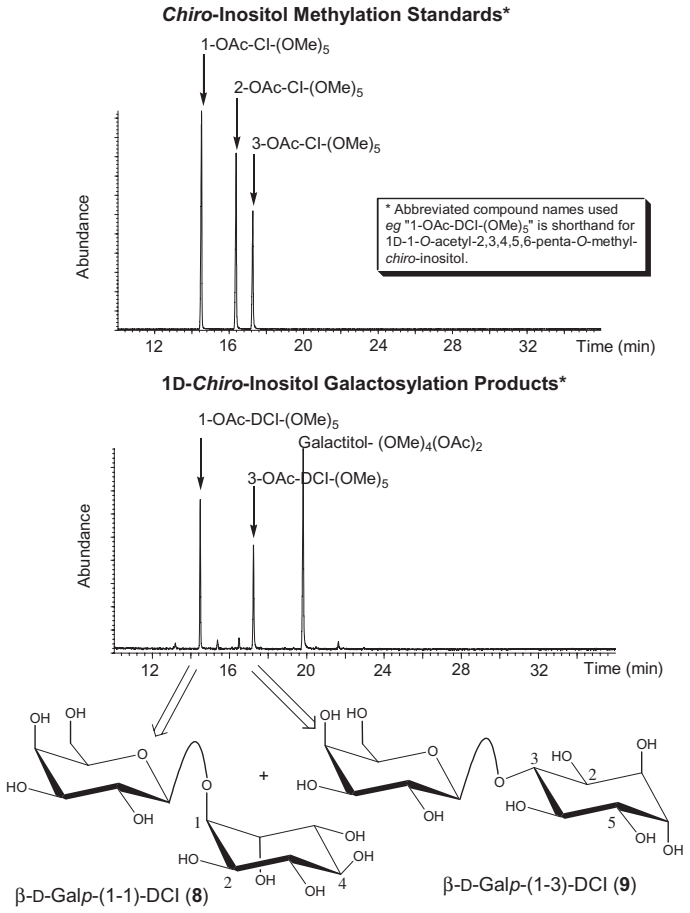


Figure 4. GC–MS analysis of 1D-*chiro*-inositol (**1**) reaction products and standards.

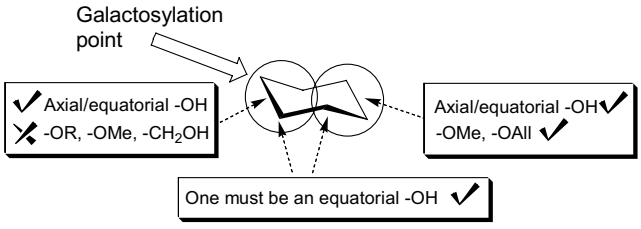


Figure 5. Summary of the acceptor specificity of TP6-B1.

3. Summary

The β-galactosidase isolated from the extreme anaerobic thermophile, *Thermoanaerobacter* sp. strain TP6-B1 galactosylated 1D-*chiro*-inositol **1**, 1D-pinitol **2**, 1L-*chiro*-inositol **6**, *myo*-inositol **7** and two related 1D-*chiro*-inositol derivatives, **3** and **4**. In contrast, it did not galactosylate 1L-quebrachitol **5**. The products from the galactosylation reactions were identified and an analysis of the products revealed that the enzyme displayed a greater tolerance towards substitution of the inositol acceptor than was observed previously for the β-galactosidase from *B. circulans*. Specifically, TP6-B1 requires an equatorial hydroxyl group at either C-2 or C-3 for glycosylation to occur at C-4 (galactose numbering) of the acceptor moiety.

4. Experimental

4.1. General methods

NMR spectra were acquired either on a Bruker Advance NMR Spectrometer operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C nuclei or on a Varian Unity 500 NMR Spectrometer operating at 500 MHz for ^{1}H and 126 MHz for ^{13}C nuclei, and chemical shifts are listed in ppm. NMR experiments performed in $CDCl_3$ are referenced to Me_4Si (0 ppm), those in D_2O to external acetone (δ 218.1 ppm (C=O) and δ 33.2 ppm (Me) for ^{13}C and δ 2.22 ppm for ^{1}H) and those in CD_3OD to external MeOH (δ 49.0 ppm for ^{13}C and δ 3.30 ppm (Me) for ^{1}H). Chemical shift assignments were made on the basis of ^{13}C-DEPT, DQF HH-COSY, HC-COSY or HMQC, HMBC, ROESY and HMQC-TOCSY spectral

analysis as required. Melting points were determined using a Reichert hot stage microscope apparatus. All reagents used were AR grade and solvents used were AR grade or were distilled, unless otherwise stated. Flash chromatography (FC) was performed using Scharlau silica gel 60 (0.04–0.06 mm, 230–400 mesh ASTM). Purification of the enzyme reaction products was achieved using a Bio-Rad Bio-Gel P-2 (45–90 μm) column (1100×25 mm) equipped with a peristaltic pump and a fraction collector. Optical rotations were acquired with a Perkin Elmer 241 Polarimeter, and mass spectra were obtained on either a VG70-250S Double Focusing Magnetic Sector Mass Spectrometer or a MARINER Biospectrometer for electrospray mass spectrometry results. GC–MS was performed by GC separation on a Hewlett Packard (HP) Ultra 2 capillary column (50 m×0.2 mm i.d., 0.33 μm film thickness) with the oven programmed from 75 °C (held for 1 min) to 130 °C at a rate of 40 °C/min then at a rate of 4 °C/min to 250 °C followed by detection using a HP 5970 MSD. Yields for enzymatic reactions are reported relative to the amount of *p*-nitrophenyl galactopyranoside as the donor. Pinitol was supplied by New Zealand Pharmaceuticals Ltd. The β-galactosidase from *Thermoanaerobacter* sp. strain TP6-B1 was supplied by Prof. Roy Daniel, Waikato University, Hamilton, New Zealand with an activity of 5500 MU/mg.

4.2. Synthesis of substrates

4.2.1. 1D-3-*O*-Allyl-1,2:5,6-di-*O*-isopropylidene-4-*O*-methyl-*chiro*-inositol (23). NaH (0.32 g of 60% w/w in oil, 8.0 mmol) was added to a stirred solution of **21**[26] (2.0 g, 7.3 mmol) in DMF (20 mL) under Ar at 0 °C. After stirring at room temperature for 15 min allyl bromide (0.69 mL, 8.0 mmol) was added dropwise at 0 °C, the reaction was allowed to warm to room temperature and stirring was continued overnight. EtOH (2 mL) was added, the solvents removed and FC performed on the residue (EtOAc–hexanes 1:3) gave the *title compound* as a colourless oil (2.04 g, 88%); $[\alpha]_D^{20}$ −18.5 (*c* 1.2, $CHCl_3$); ^{1}H NMR (300 MHz, CD_3OD): δ 5.95 (m, 1H, CH=), 5.32 (dd, 1H, *J* 1.4, 17.3 Hz, CH_{trans}=), 5.17 (d, 1H, *J* 10.5 Hz, CH_{cis}=), 4.30–4.17 (m, 6H, H-1–H-6), 3.60 (s, 3H, OCH_3), 3.42 (m, 2H, CH_2), 1.51 (s, 3H, CH_3), 1.50 (s, 3H, CH_3), 1.35 (s, 6H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 135.4 (CH=), 117.1 (CH_2=), 109.9 ($C(CH_3)_2$), 82.0, 79.8, 79.6, 79.2, 77.9, 76.9 (C-1–C-6), 72.8 (CH_2), 60.4 (OCH_3), 28.1 (CH_3), 25.6 (CH_3); HR FAB$^+$-MS (NBA/CH_2Cl_2): Calcd for $C_{16}H_{26}O_6$ (M+H$^+$) *m*/*z* 315.1808. Found: 315.1798; Anal. Calcd for $C_{16}H_{26}O_6$: C, 61.13; H, 8.34. Found: C, 61.27; H, 8.08.

4.2.2. 1D-3-*O*-Allyl-4-*O*-methyl-*chiro*-inositol (4). A solution of **23** (1.90 g, 6.1 mmol) in trifluoroacetic acid: H_2O (1:1, 20 mL) was kept at room temperature for 2.5 h. Removal of the solvents followed by FC performed on the residue (EtOAc) gave the *title compound* as a white foam (1.35 g, 95%); $[\alpha]_D^{20}$ +46.9 (*c* 1.0, MeOH); ^{1}H NMR (300 MHz, CD_3OD): δ 6.02 (m, 1H, CH=), 5.28 (d, 1H, *J* 17.3 Hz, CH_{trans}=), 5.14 (d, 1H, *J* 10.4 Hz, CH_{cis}=), 4.30 (m, 2H, CH_2), 3.88 (d, 2H, *J* 2.4 Hz, H-1, H-6), 3.76 (dt, 2H, *J* 10.2, 2.5, H-2, H-5), 3.60 (s, 3H, CH_3), 3.42 (t, 1H, *J* 9.3 Hz, H-3 or H-4), 3.31 (t, 1H, *J* 9.4 Hz, H-3 or H-4); ^{13}C NMR (75 MHz, $CDCl_3$): δ 137.6 (CH=), 116.8 (CH_2=), 85.4, 83.4 (C-3, C-4), 75.4 (CH_2), 74.1, 74.0 (C-1, C-6), 72.8, 72.6 (C-2, C-5), 61.6 (CH_3); HR ES-MS: Calcd for $C_{10}H_{19}O_6$ (M+H$^+$) *m*/*z* 235.1176. Found 235.1183.

4.3. TP6-B1 β-galactosidase-catalysed reactions

4.3.1. Galactosylation of 1D-*chiro*-inositol (1). *p*-Nitrophenyl β-D-galactopyranoside (555 mg, 1.8 mmol) and 1D-*chiro*-inositol **1** (1.99 g, 11.1 mmol, 6.2 equiv) were incubated in 20 mL phosphate buffer (pH 7.0, 0.1 M NaH_2PO_4) with the β-galactosidase from TP6-B1 (900 mg, 4950 MU) at 37 °C. After 3 days the enzyme was denaturated by heating at 95 °C for 10 min. The solution was extracted with EtOAc to remove *p*-nitrophenol and lyophilised. The residue was applied to a mixed-bed ion exchange column (Ionac resin NM-60) eluting with H_2O and the eluent lyophilised. The solid was re-dissolved in H_2O and applied to a Bio-Gel P-2 column eluting with Millipore H_2O and the disaccharide-containing fractions (by TLC) were lyophilised to give a mixture of 1D-1-*O*-(β-D-galactopyranosyl)-*chiro*-inositol (**8**)[19] and 1D-3-*O*-(β-D-galactopyranosyl)-*chiro*-inositol (**9**) in the ratio of 10:3. Yield: 292 mg, 46%; Major product, 1D-1-*O*-(β-D-galactopyranosyl)-*chiro*-inositol (**8**): ^{1}H and ^{13}C NMR data were identical with those previously obtained.[19] The minor product, 1D-3-*O*-(β-D-galactopyranosyl)-*chiro*-inositol **9**, was identified by methylation analysis using GC–MS. Partial NMR data was obtained for **9**: ^{1}H NMR (300 MHz, CD_3OD): δ 4.55 (d, 1H, *J* 7.8 Hz, H-1′), 3.85 (obsc., 1H, H-4′), 3.70 (obsc., 1H, H-3), 3.62 (obsc., 1H, H-3′), 3.54 (obsc., 1H, H-2′); ^{13}C NMR (75 MHz, $CDCl_3$): δ 104.2 (C-1′), 83.3 (C-3), 61.8 (C-6′); For the mixture, LR ES-MS (isobaric mixture of **8** and **9**): Calcd for $C_{12}H_{22}O_{11}$ (M+H$^+$) *m*/*z* 343. Found 343.

4.3.2. Galactosylation of 1D-pinitol (2). *p*-Nitrophenyl β-D-galactopyranoside (23.0 mg, 0.076 mmol) and 1D-pinitol **2** (91 mg, 0.47 mmol, 6.2 equiv) were incubated in 1 mL phosphate buffer (pH 7.0, 0.1 M NaH_2PO_4) with the β-galactosidase from TP6-B1 (20 mg, 110 MU) at 37 °C. After 3 days the reaction was processed as for the galactosylation of **1** above to give a 1:1 mixture of two disaccharides, 1D-1-*O*-(β-D-galactopyranosyl)-4-*O*-methyl-*chiro*-inositol (**10**) and 1D-1-*O*-(β-D-galactopyr-

anosyl)-3-*O*-methyl-*chiro*-inositol (**11**) (13.5 mg, 50%). ^{1}H and ^{13}C NMR data for one of the products, **10**, were identical with those obtained previously.[19] For the second product, **11**, the following NMR data were obtained; ^{1}H NMR (500 MHz, D_2O): δ 4.51 (d, 1H, *J* 5.1 Hz, H-1′), 4.30 (dd, 1H, *J* 3.9, 3.8 Hz, H-6), 4.03 (dd, 1H, *J* 3.7, 3.7 Hz, H-1), 3.91 (obsc., 1H, H-4′), 3.87 (obsc., 1H, H-2), 3.81 (obsc., 1H, H-5), 3.74 (obsc., 2H, H6′/H6′), 3.68 (obsc., 1H, H-5′), 3.63 (obsc., 1H, H-4), 3.62 (obsc., 1H, H-3′), 3.69 (s, 3H, OCH_3), 3.54 (obsc., 1H, H-2′), 3.40 (dd, 1H, *J* 9.7, 9.7 Hz, H-3); ^{13}C NMR (126 MHz, D_2O): δ 106.0 (C-1′), 83.9 (C-3), 82.7 (C-1), 76.3 (C-5′), 73.6 (C-3′), 73.2 (C-4), 72.3 (C-2′), 71.5 (C-6), 71.5 (C-5), 70.9 (C-2), 69.6 (C-4′), 62.1 (C-6′), 60.8 (OCH_3); For the mixture, HR ES-MS (isobaric mixture of **10** and **11**): Calcd for $C_{13}H_{28}NO_{11}$ ($M+H^+$) *m*/*z* 374.1657. Found: 374.1659.

4.3.3. Galactosylation of 1D-3,4-di-*O*-methyl-*chiro*-inositol (3). *p*-Nitrophenyl β-D-galactopyranoside (25.5 mg, 0.085 mmol) and 1D-3,4-di-*O*-methyl-*chiro*-inositol **3**[26] (99 mg, 0.42 mmol, 5.0 equiv) were incubated in 1 mL phosphate buffer (pH 7.0, 0.1 M NaH_2PO_4) with the β-galactosidase from TP6-B1 (20 mg, 110 MU) at 37 °C. After 3 days the reaction was processed as for the galactosylation of **1** above to give 1D-1-*O*-(β-D-galactopyranosyl)-3,4-di-*O*-methyl-*chiro*-inositol (**12**) (17.6 mg, 52%); $[\alpha]_D^{20}$ +32 (*c* 1.0, H_2O); ^{1}H NMR (500 MHz, D_2O): δ 4.47 (d, 1H, *J* 7.8 Hz, H-1′), 4.26 (dd, 1H, *J* 3.4, 3.4 Hz, H-6), 3.99 (dd, 1H, *J* 3.6, 3.6 Hz, H-1), 3.88 (obsc., 1H, H-4′), 3.86 (obsc., 1H, H-2), 3.84 (dd, 1H, *J* 3.3, 9.7 Hz, H-5), 3.75 (dd, 1H, *J* 7.8, 8.5 Hz, H-6′), 3.70 (dd, 1H, *J* 3.6, 7.8 Hz, H-6′), 3.65 (obsc., 1H, H-5′), 3.62 (dd, 1H, *J* 3.3, 9.8 Hz, H-3′), 3.57 (s, 3H, 3-OCH_3), 3.57 (s, 3H, 4-OCH_3), 3.52 (dd, 1H, *J* 7.8, 9.8 Hz, H-2′), 3.46 (dd, 1H, *J* 9.7, 9.7 Hz, H-3), 3.35 (dd, 1H, *J* 9.7, 9.7 Hz, H-4); ^{13}C NMR (126 MHz, D_2O): δ 105.3 (C-1′), 82.9 (C-3), 82.8 (C-4), 81.7 (C-1), 75.6 (C-5′), 72.9 (C-3′), 71.6 (C-2′), 70.8 (C-6′), 70.3 (C-5), 70.3 (C-2), 68.9 (C-4′), 61.4 (C-6′), 60.19, 60.15 (4-OCH_3 and 3-OCH_3); HR ES-MS: Calcd for $C_{14}H_{26}O_{11}Na$ ($M+Na^+$) *m*/*z* 393.1367. Found 393.1388.

4.3.4. Galactosylation of 1D-3-*O*-allyl-4-*O*-methyl-*chiro*-inositol (4). *p*-Nitrophenyl β-D-galactopyranoside (28.6 mg, 0.095 mmol) and 1D-3-*O*-allyl-4-*O*-methyl-*chiro*-inositol **4** (99 mg, 0.48 mmol, 5.0 equiv) were incubated in 1 mL phosphate buffer (pH 7.0, 0.1 M NaH_2PO_4) with the β-galactosidase from TP6-B1 (20 mg, 110 MU) at 37 °C. After 3 days the reaction was processed as for the galactosylation of **1** above to give a mixture of two disaccharides in a 16:9 ratio (21.0 mg, 60%); Major product, 1D-4-*O*-allyl-1-*O*-(β-D-galactopyranosyl)-3-*O*-methyl-*chiro*-inositol (**13**): ^{1}H NMR (500 MHz, D_2O): δ 6.00 (m, 1H, CH=), 5.34 (dd, 1H, *J* 1.2, 17.1 Hz, CH_{trans}=), 5.24 (d, 1H, *J* 10.3 Hz, CH_{cis}=), 4.47 (d, 1H, *J* 7.8 Hz, H-1′), 4.28 (m, 2H, CH_2), 4.26 (obsc., 1H, H-6), 4.00 (obsc., 1H, H-1), 3.88 (obsc., 1H, H-4′), 3.86 (obsc., 1H, H-2), 3.84 (obsc., 1H, H-5), 3.72 (m, 2H, H-6′/H-6′), 3.65 (obsc., 1H, H-5′), 3.62 (obsc., 1H, H-3′), 3.60 (s, 3H, OCH_3), 3.52 (obsc., 1H, H-2′), 3.49 (obsc., 1H, H-4), 3.42 (dd, 1H, *J* 9.5, 9.5 Hz, H-3); ^{13}C NMR (126 MHz, D_2O): δ 134.7 (CH=), 119.0 (CH_2=), 105.3 (C-1′), 83.2 (C-3), 81.7 (C-1), 81.1 (C-4), 75.6 (C-5′), 74.4 (CH_2), 73.0 (C-3′), 71.6 (C-6), 71.6 (C-2′), 70.6, 70.4 (C-2, C-5), 68.9 (C-4′), 61.4 (C-6′), 60.6 (OCH_3); Minor product, 1D-3-*O*-allyl-1-*O*-(β-D-galactopyranosyl)-4-*O*-methyl-*chiro*-inositol (**14**): ^{1}H NMR (500 MHz, D_2O): δ 6.00 (m, 1H, CH=), 5.34 (dd, 1H, *J* 1.2, 17.1 Hz, CH_{trans}=), 5.24 (d, 1H, *J* 10.3 Hz, CH_{cis}=), 4.47 (d, 1H, *J* 7.8 Hz, H-1′), 4.28 (m, 2H, CH_2), 4.26 (obsc., 1H, H-6), 4.00 (obsc., 1H, H-1), 3.88 (obsc., 1H, H-4′), 3.88 (obsc., 1H, H-2), 3.84 (obsc., 1H, H-5), 3.72 (m, 2H, H-6′/H-6′), 3.65 (obsc., 1H, H-5′), 3.62 (obsc., 1H, H-3′), 3.60 (s, 3H, OCH_3), 3.56 (obsc., 1H, H-3), 3.52 (obsc., 1H, H-2′), 3.35 (dd, 1H, *J* 9.5, 9.5 Hz, H-4); ^{13}C NMR (126 MHz, D_2O): δ 134.7 (CH=), 119.0 (CH_2=), 105.3 (C-1′), 83.1 (C-4), 82.0 (C-1), 81.3 (C-3), 75.6 (C-5′), 74.5 (CH_2), 73.0 (C-3′), 71.6 (C-2′), 70.8 (C-6), 70.6, 70.4 (C-2, C-5), 68.9 (C-4′), 61.4 (C-6′), 60.6 (OCH_3); For the mixture, HR ES-MS (isobaric mixture of **13** and **14**): Calcd for $C_{16}H_{28}O_{11}Na$ ($M+Na^+$) *m*/*z* 397.1737. Found 397.1704.

4.3.5. Galactosylation of 1L-*chiro*-inositol (6). *p*-Nitrophenyl β-D-galactopyranoside (111 mg, 0.37 mmol) and 1L-*chiro*-inositol **6** (398 mg, 2.2 mmol, 6.0 equiv) were incubated in 4 mL phosphate buffer (pH 7.0, 0.1 M NaH_2PO_4) with the β-galactosidase from TP6-B1 (80 mg, 440 MU) at 37 °C. After 3 days the enzyme was denaturated by heating at 95 °C for 10 min. The solution was extracted with EtOAc to remove *p*-nitrophenol and lyophilised. The residue was redissolved in H_2O and passed through Amberlyst-A21 resin, lyophilised, and then re-dissolved in Millipore H_2O, applied to a Bio-Gel P-2 column and eluted with Millipore H_2O and lyophilised. The residue was redissolved in Millipore H_2O and re-applied to a Bio-Gel P-2 column to give 1L-3-*O*-(β-D-galactopyranosyl)-*chiro* -inositol (**15**) (61 mg, 49%) and a minor fraction that contained mainly 1L-1-*O*-(β-D-galactopyranosyl)-*chiro*-inositol (**16**) (14 mg, 11%); Major product, 1L-3-*O*-(β-D-galactopyranosyl)-*chiro*-inositol (**15**): $[\alpha]_D^{20}$ −27 (*c* 1.1, H_2 O); ^{1}H NMR (500 MHz, D_2O): δ 4.62 (dd, 1H, *J* 1.0, 7.9 Hz, H-1′), 4.04 (dd, 1H, *J* 2.7, 2.7 Hz, H-1 or H-6), 4.00 (dd, 1H, *J* 2.7, 2.7 Hz, H-1 or H-6), 3.89 (d, 1H, *J* 3.4 Hz, H-4′), 3.80 (obsc., 1H, H-2 or H-5), 3.78 (obsc., 1H, H-3), 3.76 (obsc., 1H, H-2 or H-5), 3.76 (obsc., 1H, H-4), 3.76 (obsc., 1H, H-6′), 3.72 (obsc., 1H, H-6′), 3.70 (obsc., 1H, H-5′), 3.66 (ddd, 1H, *J* 1.0, 3.4, 9.9 Hz, H-3′), 3.57 (ddd, 1H, *J* 1.0, 7.9, 9.9 Hz, H-2′); ^{13}C NMR (126 MHz, D_2O): δ 104.0 (C-1′), 82.9 (C-3), 75.8 (C-5′), 73.0 (C-3′), 72.7 (C-4),

71.7 (C-1 or C-6), 71.6 (C1 or C-6), 70.6 (C-2 or C-5), 69.5 (C-2 or C-5), 69.1 (C-4′), 61.6 (C-2′), 61.6 (C-6′); HR ES-MS: Calcd for $C_{12}H_{22}O_{11}Na$ ($M+Na^+$) m/z 365.1054. Found 365.1060. Minor product, 1L-1-*O*-(β-D-galactopyranosyl)-*chiro*-inositol (**16**): δ 4.42 (d, 1H, J 7.8 Hz, H-1′), 4.14 (dd, 1H, J 3.5, 3.5 Hz, H-1), 4.12 (dd, 1H, J 3.5, 3.5 Hz, H-6), 3.90 (d, 1H, J 3.4 Hz, H-4′), 3.78 (obsc., 1H, H-5), 4.75 (obsc., 1H, H-2), 3.74 (m, 2H, H-6′/H-6′), 3.67 (dd, 1H, J 3.9, 8.3 Hz, H-5′), 3.62 (dd, 1H, J 3.4, 10.0 Hz, H-3′), 3.57 (obsc., 1H, H-3), 3.57 (obsc., 1H, H-4), 3.49 (dd, 1H, J 7.8 Hz, 10.0, H-2′); ^{13}C NMR (126 MHz, D_2O): δ 102.3 (C-1′), 79.1 (C-1), 75.6 (C-5′), 73.5 (C-3 or C-4), 73.1 (C-3 or C-4), 73.1 (C-3′), 70.9 (C-2′), 70.8 (C-5), 70.1 (C-2), 69.6 (C-6), 69.1 (C-4′), 61.6 (C-6′); HR ES-MS: Calcd for $C_{12}H_{22}O_{11}Na$ ($M+Na^+$) m/z 365.1054. Found 365.1064.

4.3.6. Galactosylation of *myo*-inositol (7). *p*-Nitrophenyl β-D-galactopyranoside (111 mg, 0.37 mmol) and *myo*-inositol **7** (398 mg, 2.2 mmol, 6.0 equiv) were incubated in 4 mL phosphate buffer (pH 7.0, 0.1 M NaH_2PO_4) with the β-galactosidase from TP6-B1 (80 mg, 440 MU) at 37 °C. After 3 days the enzyme was denaturated by heating at 95 °C for 10 min. The solution was extracted with EtOAc to remove *p*-nitrophenol and lyophilised. The residue was redissolved in H $_2$O and passed through a mixed bed ion exchange column (Ionaic resin NM-60), lyophilised, re-dissolved in Millipore H_2O and applied to a Bio-Gel P-2 column and eluted with Millipore H_2O to give a mixture of disaccharides (80 mg, 63%). GC–MS showed the mixture to contain 2-*O*-(β-D-galactopyranosyl)-*myo*-inositol (**17**), 1D- and/or 1L-4-*O*-(β-D-galactopyranosyl)-*myo*-inositol (**18**), 5-*O*-(β-D-galactopyranosyl)-*myo*-inositol (**19**) and 1D- and/or 1L-1-*O*-(β-D-galactopyranosyl)-*myo*-inositol (**20**) in a ratio of 12:4:3:2.

4.4. Derivatisation of enzyme products for GC–MS analysis

All derivatisation reactions were performed in 5 mL Teflon-lined screw-cap tubes. To each disaccharide sample (500 μg) was added DMSO (200 μL) and MeI (50 μL), followed by a slurry of powdered NaOH in DMSO (4 pellets of NaOH to 2 mL DMSO; 200 μL). The suspension was sonicated for 30 min, MeI (50 μL) was added then the suspension was sonicated for a further 30 min. The reaction was quenched with H_2O (4 mL), extracted with CH_2Cl_2 (2 mL), then the extract washed with H_2O (2 × 1 mL) and dried at 30 °C under a stream of dry air. The samples were hydrolysed in TFA (2 M, 0.25 mL, 1 h, 120 °C). The sample was then evaporated at 40 °C under a stream of dry air followed by the addition and evaporation of toluene (1 mL). Reduction was performed with $NaBH_4$ (15 mg/mL in aqueous 1 M NH_4OH, 0.25 mL, 1 h, 40 °C). The reaction was quenched with acetone (0.5 mL) and the sample was evaporated to dryness. Perchloric acid-catalysed acetylation was undertaken using the method of Falshaw and Furneaux,[30] except that the organic layer was washed with water (4 mL), Na_2CO_3 (0.5 M, 4 mL) then water (4 mL). The partially methylated and acetylated derivatives were analysed by GC–MS.

4.5. Synthesis of GC–MS standards

4.5.1. Standard benzylation procedure. NaH (60% w/w in oil, 0.6 mol equiv per OH) was added under Ar to the compound in DMF at 0 °C and the reaction mixture was allowed to warm to rt. BnBr (0.55 mol equiv per OH) was added and, after stirring for 1 h at rt, the mixture was re-cooled (0 °C) and the NaH and BnBr additions were repeated. After stirring overnight at rt, EtOH was added to quench and the mixture was poured into H_2O and extracted with $CHCl_3$ (3×). The combined organic extracts were dried ($MgSO_4$), filtered and the solvents removed in vacuo to give the crude product.

4.5.2. Standard acetylation procedure. The compound dissolved in equal volumes of pyridine (excess) and Ac_2O (excess) was stirred overnight at rt under Ar, then diluted with CH_2Cl_2, washed with H_2O (2×), which was back-extracted with CH_2Cl_2. The combined organic extracts were dried ($MgSO_4$), filtered and the solvents removed in vacuo to give the crude product.

4.5.3. Standard methylation procedure. The compound was dissolved in DMF and NaH (60% w/w in oil, 0.55 mol equiv per OH) was added at 0 °C followed by MeI (0.55 mol equiv per OH) under Ar. The mixture was stirred at rt for 1 h before cooling to 0 °C and repeating the NaH and MeI additions. After stirring overnight at rt under Ar, the reaction was quenched with EtOH, and concentrated to give the crude product.

4.5.4. Standard hydrogenolysis procedure. The compound in EtOH and acetic acid (6:1 v/v EtOH–AcOH) was hydrogenolysed for 48 h at rt under an atm pressure of H_2 over 10% Pd/C catalyst (ca. 1:5 w/w catalyst:starting material). After filtration through Celite to remove the catalyst, the solvents were removed in vacuo and toluene was added and removed to remove traces of AcOH, yielding the crude product.

4.5.5. Standard $RuCl_3$ catalysed oxidation procedure. Catalytic $RuCl_3$ (0.02 mol equiv), K_2CO_3 (1.2 mol equiv) and $NaIO_4$ (2.5 mol equiv) were added to the compound in a mixture of CH_2Cl_2, MeCN and H_2O (1:1:1 v/v/v) and stirred vigorously overnight at rt. After quenching with propan-2-ol, the mixture was poured through a pad of Celite, washing with CH_2Cl_2. The aqueous portion was washed with CH_2Cl_2, the combined organic extracts

were washed with brine, dried ($MgSO_4$), filtered and the solvents removed in vacuo to give the crude product.

4.5.6. Standard $NaBH_4$ reduction procedure. To an ice-cooled solution of the compound in THF and H_2O (5:1 v/v) was added $NaBH_4$ (1.5 mol equiv) and the solution was brought to rt and stirred for 3 h. The reaction mixture was diluted with Et_2O, washed with H_2O and the aqueous layer was backwashed with Et_2O and the combined organic extracts were washed with brine, dried ($MgSO_4$), filtered and concentrated to give the crude product.

4.5.7. 1L-1-*O*-Acetyl-2,3,4,5,6-penta-*O*-methyl-*chiro*-inositol (24). The standard benzylation method applied to **31**[31] (1.08 g, 3.0 mmol) gave **32** (310 mg, 0.7 mmol). Compound **32** (290 mg, 0.6 mmol) was stirred in trifluoroacetic acid (5 mL) and H_2O (5 mL) overnight at rt. The solvents were removed to give a syrup (250 mg) containing **33**, which was methylated using the standard conditions and FC (1:4 EtOAc–hexanes) performed on the crude product gave **34** (50 mg, 0.15 mmol) as an oil. Compound **34** (50 mg, 0.15 mmol) was hydrogenolysed, yielding crude **35** (40 mg). Crude **35** (40 mg) was acetylated and the crude residue obtained was subjected to FC (1:2 EtOAc–hexanes) to give the *title compound* **24** (27 mg, 0.09 mmol, 4% from **31**) as an oil; $[\alpha]_D^{23}$ −41.0 (*c* 1.3, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$): δ 5.45 (dd, 1H, *J* 3.7, 3.7 Hz, H-1), 3.66 (s, 3H, OCH_3-3), 3.61 (s, 3H, OCH_3-4), 3.51, 3.50 (s, 3H, OCH_3-2 and OCH_3-5), 3.43 (s, 3H, OCH_3-6), 3.37 (dd, 1H, *J* 3.4, 8.7 Hz, H-2), 3.31–3.24 (m, 4H, H3-H6), 2.10 (s, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.3 (C=O), 83.6 (C-3), 83.5 (C-4), 81.7 (C-5), 79.7 (C-2), 76.2 (C-6), 66.9 (C-1), 61.4 (OCH_3-3), 61.3 (OCH_3-4), 59.6, 59.2 (OCH_3-5 and OCH_3-2), 58.8 (OCH_3-6), 21.4 (CH_3); HR FAB$^+$-MS (Cs^+ in glycerol/MeOH): Calcd for $C_{13}H_{25}O_7$ ($M+H^+$) *m*/*z* 293.1600. Found 293.1612.

4.5.8. 1L-1-*O*-Acetyl-2,3,4,5,6-penta-*O*-methyl-*myo*-inositol (25). $RuCl_3$ catalysed oxidation of **31**[31] (1.01 g, 2.8 mmol) gave **36** (710 mg, 2.0 mmol). $NaBH_4$ reduction of **36** (710 mg, 2.0 mmol) gave crude **37** (500 mg, 1.4 mmol) as an oil. Benzylation of **37** (500 mg, 1.4 mmol) gave crude **38** (500 mg) as an oil. Crude **38** (500 mg) in acetic acid (20 mL) and H_2O (5 mL) was stirred at rt for 48 h. The reaction mixture was washed with CH_2Cl_2 and the organic extract was washed with H_2O. The solvents were removed from the combined aqueous extracts and then toluene was added and the solvents removed to give crude **39** (300 mg). Crude **39** (300 mg, 1.1 mmol) was permethylated and FC (1:2 EtOAc–hexanes) performed on the residue obtained gave **40** (130 mg, 0.46 mmol) as a colourless oil. Compound **40** (130 mg, 0.46 mmol) was hydrogenolysed, yielding crude **41** (200 mg). Crude compound **41** (200 mg) was acetylated and FC (1:1 EtOAc–hexanes) performed on the resulting residue gave the *title compound* **25** (50 mg, 0.17 mmol, 6% from **31**) as a colourless oil; $[\alpha]_D^{23}$ +4.7 (*c* 0.9, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$): δ 4.62 (dd, 1H, *J* 2.5, 7.8 Hz, H-1), 3.78 (dd, 1H, *J* 2.5, 2.5 Hz, H-2), 3.61 (s, 3H, OCH_3-5), 3.60 (s, 3H, OCH_3-4), 3.55 (s, 3H, OCH_3-6), 3.54 (s, 3H, OCH_3-2), 3.52 (m, 1H, H-6), 3.47 (s, 3H, OCH_3-3), 3.43 (d, 1H, *J* 9.5 Hz, H-4), 3.06 (dd, *J* 2.5, 9.5 Hz, H-3), 3.00 (dd, 1H, *J* 9.1, 9.1 Hz, H-5), 2.14 (s, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.9 (C=O), 85.6 (C-5), 83.2 (C-4), 82.4 (C-3), 81.2 (C-6), 77.2 (C-2), 74.4 (C-1), 61.6 (OCH_3-2), 61.3 (OCH_3-4 and OCH_3-5), 61.2 (OCH_3-6), 58.9 (OCH_3-3), 21.4 (CH_3); HR FAB$^+$-MS (CS^+ in glycerol/MeOH): Calcd for $C_{13}H_{25}O_7$ ($M+H^+$) *m*/*z* 293.1600. Found 293.1607.

4.5.9. 1L-2-*O*-Acetyl-1,3,4,5,6-penta-*O*-methyl-*chiro*-inositol (26). Compound **31**[31] (1.5 g, 4.2 mmol) was stirred in acetic acid (7.5 mL) and H_2O (1.5 mL) for 4 h at rt. After concentration, the reaction mixture was treated with toluene (2×20 mL) and the solvents removed in vacuo to give **42** (1.15 g, 4.2 mmol, quant.) as a white solid. Methylation of **42** (1.15 g, 4.2 mmol) gave a residue that was subjected to FC (1:2 EtOAc–hexanes) to give **43** (1.39 g, 4.3 mmol) as an oil. Compound **43** (1.39 g, 4.3 mmol) in trifluoroacetic acid (7 mL) and H_2O (35 mL) was stirred overnight at rt. The reaction mixture was washed with hexanes (30 mL), concentrated to an oil and toluene was added (30 mL) and the solvents were removed in vacuo, the process being repeated with MeCN (30 mL), to give **44** (0.96 g, 4.1 mmol) as an oil. Compound **44** (390 mg, 1.7 mmol) was refluxed with bis(tributyltin) oxide (841 μL, 1.7 mmol) in toluene (20 mL) under Dean Stark conditions for 1.5 h. The reaction mixture was ice-cooled then BnBr (436 mg, 2.6 mmol) and tetrabutylammonium bromide (219 mg, 0.9 mmol) were added and the mixture was refluxed overnight under Ar. After cooling the solvents were removed in vacuo and FC performed on the residue (1:2 EtOAc–hexanes) gave **45** (320 mg, 1.0 mmol). Methylation of **45** (150 mg, 0.46 mmol) gave a concentrate, which was subjected to FC (1:2 EtOAc–hexanes) to give **46** along with traces of the tin reagent (190 mg) as an oil. Crude **46** (190 mg) was subjected to hydrogenolysis to give crude **47** (199 mg) as a yellow oil that was used directly in the next step. Crude **47** (190 mg) was acetylated and FC performed on the residue obtained (1:2 EtOAc–hexanes) gave the *title compound* **26** (127 mg, 0.43 mmol, 57% from **31**) as an oil; $[\alpha]_D^{23}$ −52.4 (*c* 0.7, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$): δ 5.00 (dd, 1H, *J* 3.0, 7.0 Hz, H-2), 3.78 (dd, 1H, *J* 3.0, 3.9 Hz, H-1), 3.70 (dd, 1H, *J* 2.6, 4.0 Hz, H-6), 3.61 (s, 3H, OCH_3-4), 3.54 (s, 3H, OCH_3-3), 3.51 (s, 3H, OCH_3-5), 3.47 (s, 3H, OCH_3-6), 3.44 (s, 3H, OCH_3-1), 3.43 (m, 2H, H-4 and H-3), 3.37 (m, 1H, H-5), 2.13 (s, 3H, CH_3); ^{13}C NMR

(75 MHz, $CDCl_3$): δ 169.5 (C=O), 82.1 (C-4), 80.1 (C-3), 79.8 (C-5), 75.1 (C-1), 74.8 (C-6), 72.5 (C-2), 60.0 (OCH_3-4), 59.7 (OCH_3-3), 58.5 (OCH_3-1), 57.8 (OCH_3-5), 57.7 (OCH_3-6), 20.2 (CH_3); HR FAB^+-MS (Cs^+ in glycerol/MeOH): Calcd for $C_{13}H_{25}O_7$ ($M+H^+$) m/z 293.1600. Found 293.1609.

4.5.10. 1D-4-*O*-Acetyl-1,2,3,5,6-penta-*O*-methyl-*myo*-inositol (27). $RuCl_3$ catalysed oxidation of **45** (183 mg, 0.56 mmol) gave crude **48** (162 mg, 0.50 mmol). Crude **48** (162 mg, 0.5 mmol) was reduced with $NaBH_4$ to give **49** (122 mg, 0.37 mmol) as colourless crystals. Methylation of **49** (122 mg, 0.37 mmol) gave **50** (63 mg, 0.19 mmol), which was obtained as white crystals. Hydrogenolysis of **50** (63 mg, 0.19 mmol) gave crude **51** (65 mg) as a brown oil. Acetylation of **51** (65 mg) gave crude **27** and the crude material was then subjected to FC (1:1 EtOAc–hexanes). The resulting solid was extracted with CH_2Cl_2 several times and the solvents were removed from the combined washings to give the *title compound* **27** (46 mg, 0.16 mmol, 29% from **45**) as a pale yellow oil; $[\alpha]_D^{23}$ +1.1 (*c* 1.2, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$): δ 5.24 (dd, 1H, *J* 9.0, 9.0 Hz, H-4), 3.82 (dd, 1H, *J* 2.3, 2.3 Hz, H-2), 3.53 (s, 3H, OCH_3-6), 3.52 (s, 3H, OCH_3-2), 3.48 (dd, 1H, *J* 9.6, 9.6 Hz, H-6), 3.44 (s, 3H, OCH_3-1), 3.42 (s, 3H, OCH_3-5), 3.34 (s, 3H, OCH_3-3), 2.99 (dd, 1H, *J* 9.6, 9.6 Hz, H-5), 2.98 (obsc., 1H, H-3), 2.93 (dd, 1H, *J* 2.3, 9.6 Hz, H-1), 2.03 (s, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.0 (C=O), 82.1 (C-5), 81.5 (C-6), 81.3 (C-1), 79.7 (C-3), 73.9 (C-2), 71.9 (C-4), 60.0 (OCH_3-2), 59.9 (OCH_3-6), 59.0 (OCH_3-5), 57.5, 57.2 (OCH_3-1 and OCH_3-3), 20.1 (CH_3); HR FAB^+-MS (Cs^+ in glycerol/MeOH): Calcd for $C_{13}H_{25}O_7$ ($M+H^+$) m/z 293.1600. Found 293.1610.

4.5.11. 2-*O*-Acetyl-1,3,4,5,6-penta-*O*-methyl-*myo*-inositol (28). Bis-butane-diyl acetal **52** was prepared according to the literature.[32] Benzylation of **52** and FC (1:1 EtOAc–hexanes) performed on the concentrated reaction mixture gave **53** (180 mg, 0.36 mmol) as a white solid. Compound **53** (180 mg, 0.36 mmol) was stirred in trifluoroacetic acid (5 mL) and H_2O (5 mL) at rt overnight. The reaction solution was washed with hexanes (30 mL) and concentrated to an oil, toluene (30 mL) was added and evaporated, followed by MeCN (30 mL) to give crude **54** (100 mg) as a solid. Permethylation of crude **54** (100 mg) gave **55** (40 mg, 0.12 mmol) as a crystalline solid. Hydrogenolysis of **55** (40 mg, 0.12 mmol) gave crude **56** (50 mg) as a yellow oil. Acetylation and FC performed on the crude product (1:1 EtOAc–hexanes) gave the *title compound* **28** (28 mg, 0.10 mmol) as a white solid; ^1H NMR (300 MHz, $CDCl_3$): δ 5.69 (dd, 1H, *J* 2.8, 2.8 Hz, H-2), 3.66 (s, 3H, OCH_3-5), 3.60 (s, 6H, OCH_3-4 and OCH_3-6), 3.42 (s, 6H, OCH_3-1 and OCH_3-3), 3.34 (dd, 2H, *J* 9.2, 9.2 Hz, H-4 and H-6), 3.04 (dd, 2H, *J* 2.8, 9.2 Hz, H-1 and H-3), 3.00 (dd, 1H, *J* 9.2, 9.2 Hz, H-5), 2.12 (s, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.6 (C=O), 85.5 (C-5), 83.2 (C-4 and C-6), 80.6 (C-1 and C-3), 66.1 (C-2), 61.5 (OCH_3-5), 61.4 (OCH_3-6 and OCH_3-4), 58.4 (OCH_3-1 and OCH_3-3), 21.3 (CH_3); HR FAB^+-MS (Cs^+ in glycerol/MeOH): Calcd for $C_{13}H_{25}O_7$ ($M+H^+$) m/z 293.1600. Found 293.1607.

4.5.12. 5-*O*-Acetyl-1,2,3,4,6-penta-*O*-methyl-*myo*-inositol (29). NaH (37 mg, 60% w/w in oil, 0.92 mmol) and allyl bromide (80 μL, 0.92 mmol) were added to a solution of **52**[32] (311 mg, 0.76 mmol) in DMF (10 mL) at 0 °C and the reaction was stirred overnight at rt. EtOH (5 mL) was added to quench the reaction, the reaction mixture was concentrated and FC performed on the concentrate (1:1 EtOAc–hexanes) to give **57** (50 mg, 0.11 mmol) as a white solid. Benzylation of **57** (50 mg, 0.11 mmol) gave crude **58** (80 mg). *p*-Toluenesulfonic acid (30 mg, 0.15 mmol) and 10% Pd/C (20 mg) were added to a solution of crude **58** (80 mg) in MeOH (4.5 mL) and H_2O (1 mL) and the solution was refluxed for 6 h. After filtration though Celite the solvents were removed from the filtrate to give crude **59** (40 mg). Standard permethylation conditions were employed in the synthesis of **60** from crude **59** (40 mg). The residue obtained was subjected to FC (1:1 EtOAc–hexanes) to give **60** (10 mg, 0.03 mmol) as a white solid. Hydrogenolysis gave crude **61** (5 mg) as a white solid. Acetylation of crude **61** (10 mg) gave a residue that was subjected to FC (1:1 EtOAc–hexanes) to give the *title compound* **29** (4 mg, 0.01 mmol, 1% from **52**) as a colourless solid; ^1H NMR (300 MHz, $CDCl_3$): δ 4.84 (dd, 1H, *J* 9.7, 9.7 Hz, H-5), 3.86 (dd, 1H, *J* 2.4, 2.4 Hz, H-2), 3.62 (s, 3H, OCH_3-2), 3.52 (dd, 2H, *J* 9.7, 9.7 Hz, H-4 and H-6), 3.50 (s, 6H, OCH_3-1 and OCH_3-3), 3.40 (s, 6H, OCH_3-4 and OCH_3-6), 3.06 (dd, 2H, *J* 9.7, 2.4 Hz, H-1 and H-3), 2.12 (s, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.7 (C=O), 82.6 (C-1 and C-3), 81.0 (C-4 and C-6), 76.2 (C-2), 74.8 (C-5), 61.7 (OCH_3-2), 61.0 (OCH_3-4 and OCH_3-6), 59.1 (OCH_3-1 and OCH_3-3), 21.5 (CH_3); HR FAB^+-MS (Cs^+ in glycerol/MeOH): Calcd for $C_{13}H_{25}O_7$ ($M+H^+$) m/z 293.1600. Found 293.1586.

4.5.13. 1D-3-*O*-Acetyl-1,2,4,5,6-penta-*O*-methyl-*chiro*-inositol (30). Benzylation of **21** (2.0 g, 7.3 mmol) gave a residue that was subjected to FC (1:30 EtOAc–hexanes) to give **62** (2.4 g, 6.7 mmol). Compound **62** (2.03 g, 5.6 mmol) was kept in trifluoroacetic acid (10 mL) and H_2O (10 mL) at rt for 6 h. The solvents were removed in vacuo and co-evaporated with toluene and the residue was subjected to FC (EtOAc) to give **63** (1.2 g, 4.6 mmol) as a white solid. Permethylation of **63** (600 mg, 2.1 mmol) gave a crude product, which was subjected to FC (1:3 EtOAc–hexanes) to give **64** (560 mg, 1.6 mmol) as an oil. Hydrogenolysis of **64** (560 mg, 1.6 mmol) gave **65** (ca. 700 mg) as an orange oil. Acetylation gave the

crude product, which was subjected to FC (1:2 EtOAc–hexanes), redissolved in CH_2Cl_2 and passed through a Whatman filter cartridge (0.2 μm) to give the *title compound* **30** (290 mg, 0.99 mmol, 36% from **21**) as a colourless oil; $[\alpha]_D^{23}$ +43.5 (*c* 1.0, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$): δ 5.19 (dd, 1H, *J* 9.4, 9.4 Hz, H-3), 3.78 (dd, 1H, *J* 2.9, 4.0 Hz, H-1 or H-6), 3.73 (dd, *J* 2.9, 4.0 Hz, H-6 or H-1), 3.51, 3.50, 3.49, 3.48, 3.41 (OCH_3-6, OCH_3-5, OCH_3-4, OCH_3-2 and OCH_3-1), 3.78–3.46 (m, 3H, H-4, H-5 and H-2), 2.10 (s, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.3 (C=O), 81.4 (C-4), 81.2 (C-5 or C-2), 79.9 (C-2 or C-5), 76.6 (C-6 or C-1), 75.4 (C-1 or C-6), 73.1 (C-3), 60.4, 59.1, 58.8 (OCH_3-4, OCH_3-2 and OCH_3-5), 59.9, 59.6 (OCH_3-1 and OCH_3-6), 21.4 (CH_3); HR FAB$^+$-MS (Cs^+ in glycerol/MeOH): Calcd for $C_{13}H_{25}O_7$ ($M+H^+$) *m*/*z* 293.1600. Found 293.1595.

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