Carbohydrate Research 360 (2012) 93–101

Contents lists available at SciVerse ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Regioselective glucosylation of inositols catalyzed by *Thermoanaerobacter* sp. CGTase

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ARTICLE INFO

Article history:
Received 28 May 2012
Received in revised form 2 August 2012
Accepted 4 August 2012
Available online 14 August 2012

Keywords:
CGTase
Glucoamylase
Inositols
α-D-Glucosylation
Regiopecificity
Configuration

ABSTRACT

Monoglucosylated products of L-*chiro*-, D-*chiro*-, *muco*-, and *allo*-inositol were synthesized by regioselective α-D-glucosylation with cyclodextrin glucosyl transferase from *Thermoanaerobacter* sp. after hydrolysis of by products with *Aspergillus niger* glucoamylase. While the reactions carried out with D-*chiro*-, *muco*-, and *allo*-inositol resulted in the regioselective formation of monoglucosylated products, two products were obtained in the reaction with L-*chiro*-inositol. Through the structural characterization of the glucosylated inositols here we demonstrated that the selectivity observed in the glucosylation of several inositols by *Thermoanaerobacter* sp. CGTase, is analogous to the specificity observed for the glucosylation of β-D-glucopyranose and equivalent glucosides.

1. Introduction

Inositols (cyclohexanehexols) are sugar-like molecules with significant chemical and biological properties. Among all possible inositol stereoisomers, *myo*-inositol is the most prominent form, widely occurring in nature. Other naturally occurring isomers are *scyllo*-, *neo*-, D-*chiro*-, and *muco*-inositol.[1] The remaining three, *epi*-, *allo*-, L-*chiro*-, and *cis*-inositol are considered unnatural synthetic inositol isomers.[2,3] It has been reported that all inositol stereoisomers may be prepared from *myo*-inositol by inversion of the configuration (epimerization) of one or two of its hydroxyl groups.[4]

In recent years, evidence of the physiological role of inositols and their glycosylated derivatives in cell functions has considerably increased the interests in these compounds for pharmaceutical applications. Indeed, it has been reported that glycosylphosphatidylinositols (GPI) contribute to activate innate immune cells or to exert partial insulin-mimetic activity on glucose and lipid metabolism in insulin-sensitive cells (adipocytes, cardiomyocytes and diaphragms).[5,6] Moreover, phosphatidylinositol mannosides (PIMs), the components of mycobacterial cell walls, have demonstrated to be immunomodulators.[7] More recently, two series of new *myo*-inositol-derived glycolipid analogues were isolated from leaves of the Mexican medicinal plant *Solanum lanceolatum*, which demonstrated in vivo important anti-inflammatory activity.[8]

Although, most of the glycosylated forms of inositols are naturally present in the vegetal kingdom, the low content in plants has limited their isolation and commercial application.[9] In order to cope with the lack of a convenient source of inositols, several chemical routes have been described for their synthesis. However, due to the similar reactivity of the multiple inositol hydroxyl groups, the *regio*- and *stereo*-selective glycosylations of these compounds remain a challenge in organic chemistry.[3] During the last two decades, biocatalysis has become an interesting alternative in organic synthesis due to the *chemo*-, *regio*-, and *stereo*-selectivities of enzymes and their ability to carry out reactions in mild conditions.[10–17]

Cyclodextrin glucosyl transferases (CGTase) (EC 2.4.1.19) are hexosyltransferase enzymes, produced extracellularly by a wide variety of bacteria, particularly *Bacillus*. The distinctive feature of these enzymes is the formation of cyclodextrins from starch through intra-molecular transglycosylation of α-D-(1→4)-linked D-glucose oligosaccharides, resulting in cyclization[18] Additionally, these enzymes also catalyze intermolecular transglycosylations involving disproportionation and hydrolysis of the amylose

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http://dx.doi.org/10.1016/j.carres.2012.08.002

chains.[19] An extension of these activities is the transfer of glucose to a wide variety of acceptors, such as sugars, sugar alcohols, glycosides, flavonoids, and aglycones, which have been successfully subjected to glucosylation by CGTase.[20]

In a previous work, we reported the enzymatic regioselective synthesis of α-D-glucopyranosyl-(1→4)-4D-*myo*-inositol (**2**) and α-D-glucopyranosyl-(1→1)-1D-*myo*-inositol (**3**) with anti-inflammatory activity, using the CGTase from *Thermoanaerobacter* sp.[21] The regioselectivity was explained as a consequence of the general amylase preference of positions C-4 and βC-1 in glucose for transglucosylation.[22] In order to extend the scope of the application of this enzymatic glucosylation-hydrolysis strategy, we then focused on the regioselective enzymatic synthesis of glucosyl derivatives of various inositols, such as D-pinitol, L-*chiro*-inositol, D-*chiro*-inositol, *muco*-inositol, and *allo*-inositol, with excellent regioselectivities. Particularly, we focused on the influence of hydroxyl configuration on enzyme specificity.

2. Results and discussion

2.1. Enzymatic glucosylation of L-*chiro*-, D-*chiro*-, *muco*-, and *allo*-inositols

An enzymatic process was designed in order to glucosylate five different inositol stereoisomers: D-pinitol, L-*chiro*-inositol, D-*chiro*-inositol, *muco*-inositol, and *allo*-inositol. Transglucosylation reactions were carried out with β-cyclodextrin (β-CD) as glucosyl donor and CGTase from *Thermoanaerobacter* sp. as biocatalyst.[21] The glucosylation reaction was followed by the selective hydrolysis of the poly-glucosylated products with *Aspergillus niger* glucoamylase (GA). The products of all reactions were analyzed by TLC (Supplementary data) and high-performance liquid chromatography (HPLC), as described in Section 4. The HPLC profile of products obtained in the presence of acceptors after CGTase reactions is illustrated in Figure 1, including the profile of CGTase products in the absence of acceptors (Fig. 1a). All acceptor reaction products obtained from the glycosylation of the five inositol acceptors were compared after 24 h of reaction (Fig. 1b–g). In addition, when β-CD is used as a single substrate (Fig. 1a), only maltooligosaccharides ranging from glucose (Gl) to maltohexose (G6) were observed.

With the exception of D-pinitol, all inositol were able to act as acceptors of glucosyl residues transferred by CGTase. Several of the observed glucosylation products may correspond to poly-glucosylated inositols, as deduced from their retention times.

Afterward, the hydrolysis of maltooligosaccharides and poly-glucosylated inositols (Fig. 1) to glucose and single glucosylated products was carried out with GA. The HPLC profile after hydrolysis for each inositol is illustrated in Figure 2, where it may be observed that in reactions using L-*chiro*-inositol (Fig. 2c), D-*chiro*-inositol (Fig. 2d), *muco*-inositol (Fig. 2f), and *allo*-inositol (Fig. 2g) as acceptor substrate, different peaks corresponding to enzymatic products were observed (**10**–**11**, **14**, **19**, and **24**). These products were purified by preparative HPLC and analyzed by 1D and 2D NMR for structural characterization. In order to confirm the identity of the products, benzoylation of the monoglucosylated inositol products was carried out. As already stated, no glycosylated products were observed by TLC and HPLC analysis, in reactions where D-pinitol was used as acceptor, as illustrated in Figures 1e and 2e.

According to these preliminary results, α-D-glucopyranosyl-(1→2)-2L-*chiro*-inositol (**10**) and α-D-glucopyranosyl-(1→5)-5L-*chiro*-inositol (**11**) were synthesized from L-*chiro*-inositol (Scheme 1); α-D-glucopyranosyl-(1→3)-3D-*chiro*-inositol (**14**) and α-D-glucopyranosyl-(1″→4′)-α-D-glucopyranosyl-(1′→3)-3D-*chiro*-inositol (**15**) were derived from the transglucosylation of D-*chiro*-inositol (Scheme 2); α-D-glucopyranosyl-(1→1)-*muco*-inositol (**19**) was obtained from the glucosylation of *muco*-inositol (Scheme 3); and α-D-glucopyranosyl-(1→5)-*allo*-inositol (**24**) and α-D-glucopyranosyl-(1″→4′)-α-D-glucopyranosyl-(1→5)-*allo*-inositol (**25**) derived from the glucosylation of *allo*-inositol (Scheme 4).

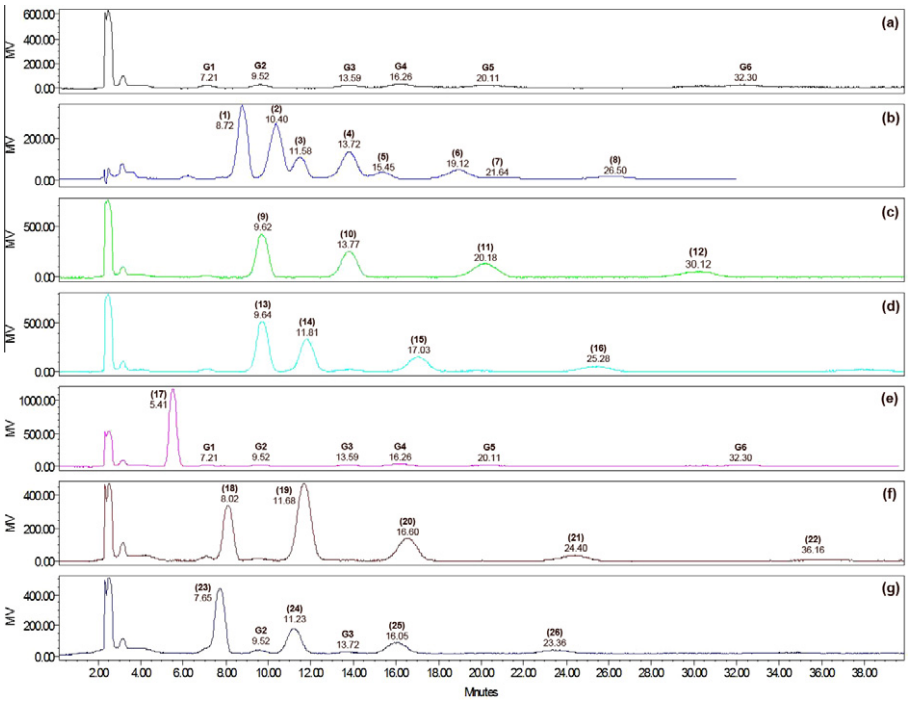


Figure 1. HPLC product profile obtained in CGTase glucosylation reactions of various inositols using β-CD as glucosyl donor (a) Reaction control in the absence of inositol (b) *myo*-inositol (**1**), (c) L-*chiro*-inositol (**9**), (d) D-*chiro*-inositol (**13**), (e) D-pinitol (**17**), (f) *muco*-inositol (**18**), and (g) *allo*-inositol (**23**). Products are obtained after 24 h of reaction Retention times (min) are shown for each peak.

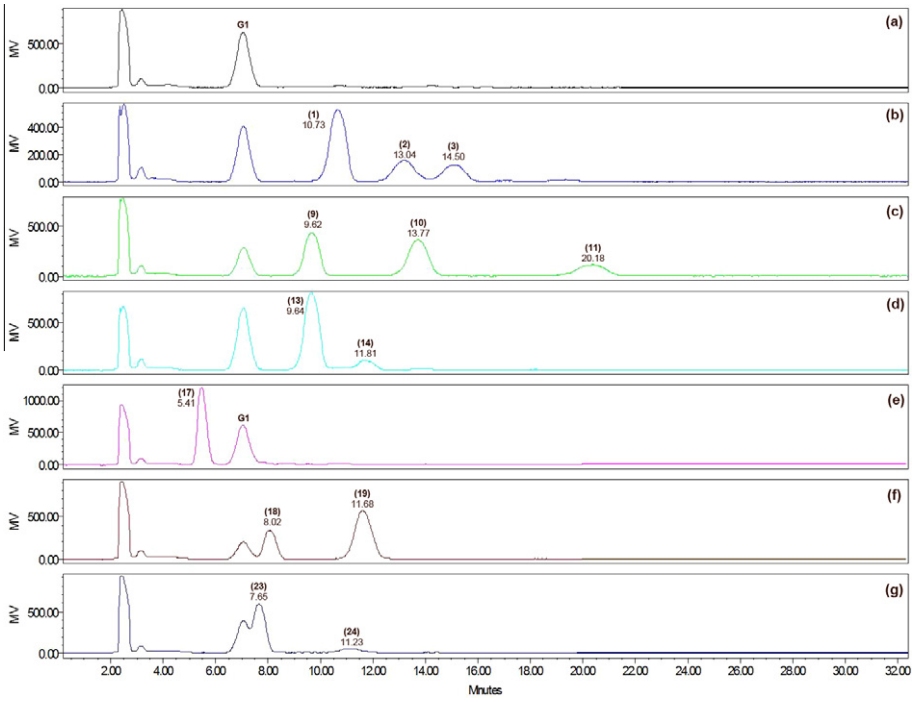
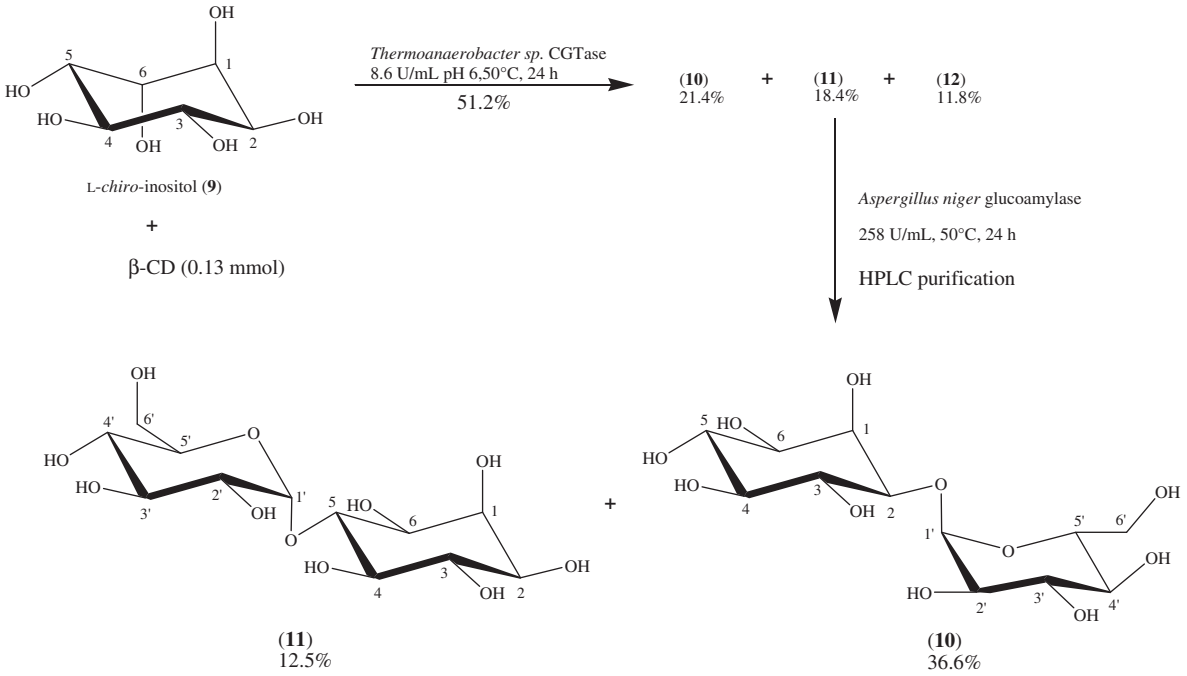


Figure 2. HPLC product profile obtained after GA hydrolysis of the transglucosylation products using inositols as acceptors as described in Figure 1. (a) Control in the absence of inositols, (b) *myo*-inositol,[21] (c) L-*chiro*-inositol, (d) D-*chiro*-inositol, (e) D-pinitol, (f) *muco*-inositol and (g) *allo*-inositol. Products are obtained after 24 h of reaction. Retention times (min) are shown at the top of each peak.



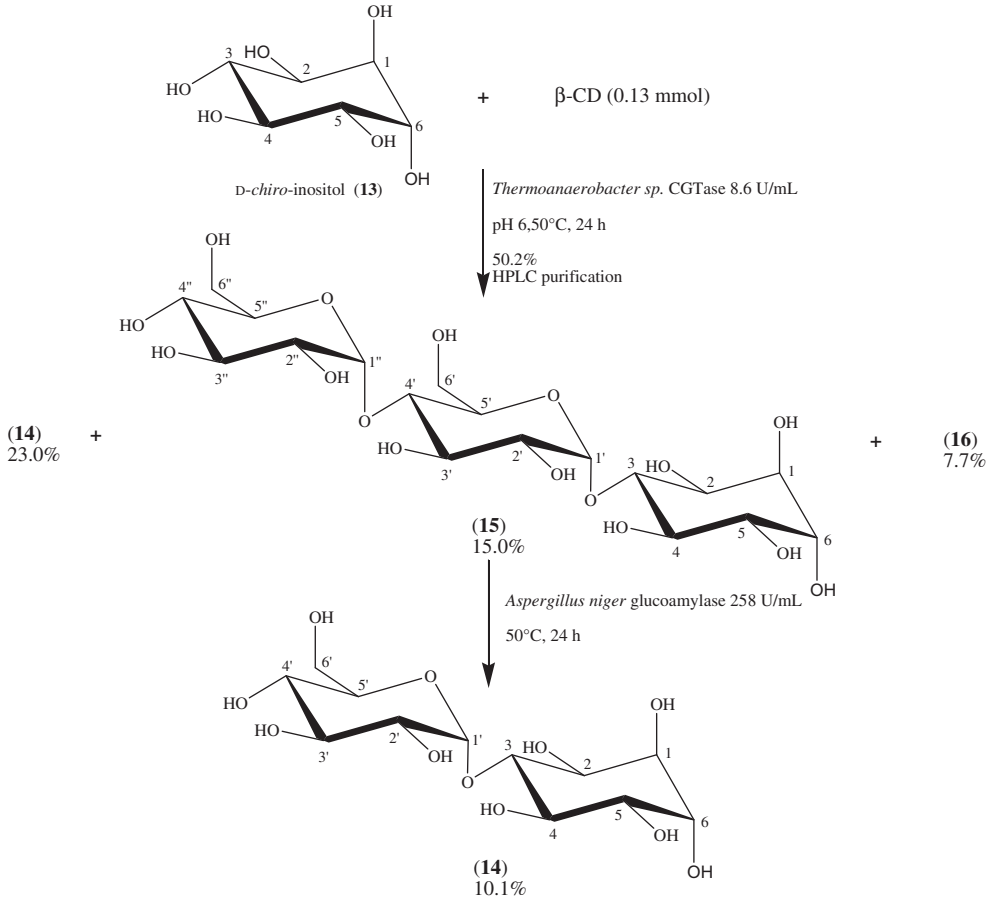
Scheme 1. Synthetic strategy of L-*chiro*-inositol glucosylation, using β-cyclodextrin as glucosyl donor and CGTase from *Thermoanaerobacter* sp., followed by the selective hydrolysis of the poly-glucosylated products with *A. niger* glucoamylase.

2.2. Structure characterization of the transfer products

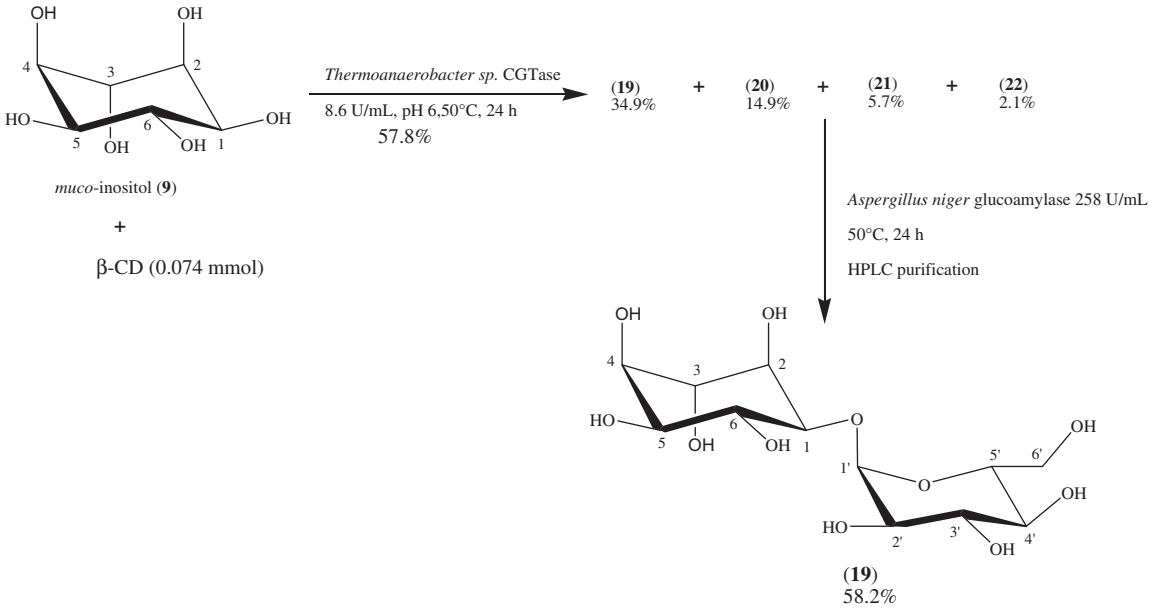
The structures of the transfer products, were elucidated on the basis of analyses of their spectroscopic data (^{1}H NMR, ^{13}C NMR, ^{1}H–1H COSY, HMQC, HMBC, and HRFABMS), as well as of their per-benzoylated derivatives.

The molecular ions of monoglucosylated products **10**, **11**, and **19** were obtained by HRFAB mass spectrometry analysis in the positive mode, while those of compounds **14**, **15**, and **24**, were obtained in the negative mode, and in general, accounted fine for their molecular formula (see Section 4).

In the ^{1}H NMR spectra of monoglucosylated products **10**, **11**, and **14**, only one anomeric proton in the α orientation was observed at δ 5.07, 5.11, and 5.38, and HSQC showed their attachment to the anomeric carbons at δ_C 101.4, 101.5, and 99.6, respectively.

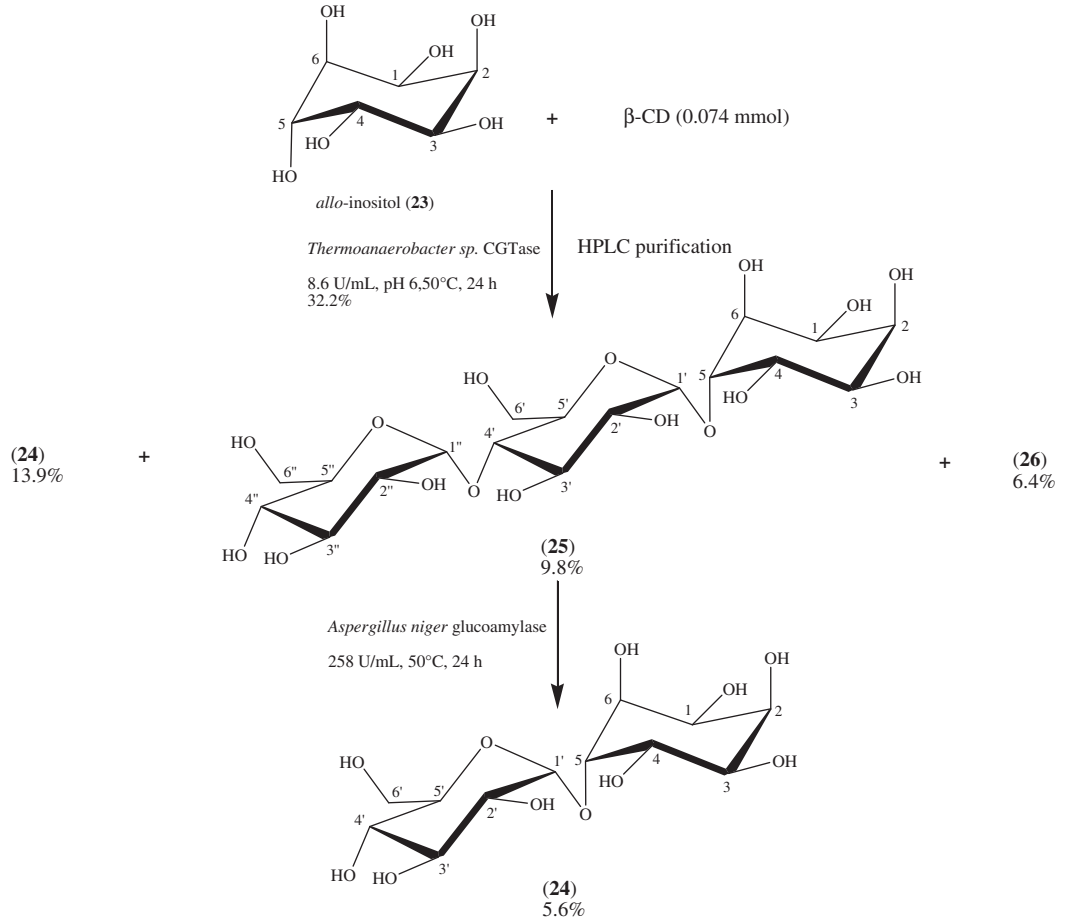


Scheme 2. Synthetic strategy using D-*chiro*-inositol, β-cyclodextrin as glucosyl donor and CGTase from *Thermoanaerobacter* sp., followed by the selective hydrolysis of the poly-glucosylated products with *Aspergillus niger* glucoamylase.



Scheme 3. Synthetic strategy using *muco*-inositol, β-cyclodextrin as glucosyl donor and CGTase from *Thermoanaerobacter* sp., followed by the selective hydrolysis of the poly-glucosylated products with *Aspergillus niger* glucoamylase.

The characteristically downfield anomeric carbons and anomeric hydrogens of the D-glucopyranosyl residue in each compound, served as the starting point for the analysis of the ^{1}H–1H COSY and NOESY experiments for connectivities within the spin system. The two diasterotopic H-6′ hydrogens on the glucosyl residue were also useful markers, clearly identifiable by a doublet with double



Scheme 4. Synthetic strategy using *allo*-inositol, β-cyclodextrin as glucosyl donor and CGTase from *Thermoanaerobacter* sp., followed by the selective hydrolysis of the poly-glucosylated products with *Aspergillus niger* glucoamylase.

intensity at δ 3.65 and at 3.72 in compound **10**; at δ 3.75 and 3.69 in compound **11**; and in δ 3.91 and 3.88, in **14**, in their ^{1}H NMR spectrum, and attached to the most upfield carbon at δ 61.33, 61.46, and δ 60.63, respectively in the ^{13}C spectrum. Once the ^{1}H signals of the glucopyranose residue have been assigned, the six remaining ^{1}H resonances, were assigned to each cyclitol ring, and connectivities were delineated from ^{1}H–^{1}H COSY and TOCSY experiments with the characteristic signal of the equatorial proton of each cyclitol as the starting point. The H-1eq and H-6eq signals of the L-*chiro*-inositol residue in compound **10** were observed at δ 4.06 (t, 1H, $J_{1,6}$ = 3.5 Hz, $J_{1,2}$ = 3.5 Hz), and δ 3.87 (dd, 1H, $J_{1,6}$ = 3.5 Hz, $J_{5,6}$ = 4.0 Hz), respectively; in compound **11**, these protons appeared at δ 3.71 (dd, 1H, $J_{1,6}$ = 2.5 Hz, $J_{1,2}$ = 3.0 Hz), and 4.08 (d, 1H, $J_{1,6}$ = 1.5 Hz, $J_{5,6}$ = 1.5 Hz), respectively. In compound **14**, the equatorial H-1 and H-6 signals of the D-*chiro*-inositol residue appeared at δ 4.08 (dd, 1H, $J_{1,6}$ = 2.8 Hz, $J_{1,2}$ = 4.0 Hz), and 4.09 (dd, 1H, $J_{1,6}$ = 3.2 Hz, $J_{5,6}$ = 4.0 Hz), respectively.

Through the HSQC experiment, the proton attached to the most downfield carbon in each cyclitol ring was determined (**10**: δ_C 79.87, **11**: δ_C 73.73, and **14**: δ_C 81.23),[23] and correspond to the point of linkage to glucose. This was confirmed by the interactions observed between the anomeric hydrogen and this proton in each compounds in the NOESY experiment (**10**: δ_H 5.07→3.66, **11**: δ_H 5.11→3.72, and **14**: δ_H 5.38→3.78). Coupling constants observed for these hydrogens and those observed for the adjacent ones, confirmed assignment of each proton. In addition, in the heteronuclear multiple bond coherence (HMBC) spectrum, the following ^{3}J correlations were observed: C-2 (δ 79.87) of L-*chiro*-inositol and the H-1′ at δ 5.07 (d, J = 4.0 Hz) of the glucose residue in compound **10**, C-5 (δ 73.73) of L-*chiro*-inositol and the H-1′ at δ 5.11 (d, J = 4.0 Hz) of the glucose residue in compound **11**, and C-3 (δ 81.23) of D-*chiro*-inositol and the H-1′ at δ 5.38 (d, J = 4.0 Hz) of the glucose residue in compound **14**. It is noteworthy that glucoside **14** is an analogue of fagopyritol A1 (α-D-galactopyranosyl-(1→3)-3D-*chiro*-inositol), a putative insulin mediator, which is useful in the treatment of non-insulin dependent *diabetes mellitus*.[24,25]

The ^{1}H NMR of **15** showed two anomeric signals with the same relative intensity: H-1′ (d, J = 3.5 Hz, δ 5.35) and H-1″ (d, J = 3.5 Hz, δ 5.43). ^{13}C NMR spectra of **15** in D_2O solution also showed two anomeric signals with the same relative intensity. The peaks at δ 99.52 (C-1″) and 99.83 (C-1′) and two methylene carbons at δ 60.40 and 60.34 indicated the presence of two pyranoside type residues. The anomeric proton H-1″ showed NOE to H-4′ (dd, J = 9.1 Hz, J = 9.8 Hz, δ 3.71), while H-1′ of this ring is in close proximity to H-3 (dd, J = 8.4 Hz, J = 9.8 Hz, δ 3.73) of the D-*chiro*-inositol. These positions were unambiguously identified by using a gHMBC experiment. According to these results, the structure for the pseudotrisaccharide may be established as α-D-glucopyranosyl-(1″→4′)-α-D-glucopyranosyl-(1′→3)-3D-*chiro*-inositol (**15**).

Due to the slow interconversion of the two *meso* chair forms of *muco*-, and *allo*-inositols,[26] broad unresolved ^{1}H NMR in D_2O were obtained for their transfer products **19** and **24**, respectively. However, six well-defined signals for the inositol moiety were obtained in the ^{1} H NMR of the perbenzoylated product **19a** and in the ^{1}H NMR spectra of **24** ran in Py-d_5, indicating unsymmetrical compounds. Full assignments of the proton and carbon resonances on

19, **19a**, and **24** were thus supported from the 2D NMR data. On the basis of long-range correlation between C-1 (δ 75.59) of the *muco*-inositol and H-1′ of glucose at δ 5.72, it was established the structure of compound **19** as α-D-glucopyranosyl-(1→1)-*muco*-inositol. Similarly, the long-range correlation between H-1′ of the glucose moiety and C-5 or C-4 (δ 82.4) of the *allo*-inositol in the gHMBC spectrum indicated that the glucose residue is α-linked to OH-5 of *allo*-inositol. Therefore, compound **24** corresponds to α-D-glucopyranosyl-(1→5)-*allo*-inositol.

The ^{1}H NMR of **25** showed two anomeric signals with the same relative intensity: H-1′ (d, J = 4.0 Hz, δ 5.18) and H-1″ (d, J = 4.0 Hz, δ 5.45). The analysis of glucoamylase reaction and the specificity of CGTase's, allowed us to conclude that the two glucopyranose rings were connected via α-(1→4) type linkage like compound **15** and that one glucose is also α-linked to C-5 of the cyclitol residue. According to these results, the primary structure proposed for the diglucosylated *allo*-inositol is α-D-glucopyranosyl-(1″→4′)-α-D-glucopyranosyl-(1→5)-*allo*-inositol (**25**).

2.3. Analysis of regiospecificity of CGTase from *Thermoanaerobacter* sp.

The acceptor specificity of CGTase from *Thermoanaerobacter* sp. in transglucosylation reactions was explored using six different inositols as acceptors. In a previous work, we had already reported that this enzyme recognizes D-*myo*-inositol as acceptor transferring glucose residues to the 4-OH and 1-OH hydroxyl groups to yield stereoselectively α-D-glucopyranosyl-(1→4)-4D-*myo*-inositol (**2**) and α-D-glucopyranosyl-(1→1)-1D-*myo*-inositol (**3**), respectively, in a 73:27 ratio.[21] In this work, the ability of the CGTase from *Thermoanaerobacter* sp. to transfer glucose residues to four additional inositol acceptors: L-*chiro*-inositol, D-*chiro*-inositol, *muco*-inositol, and *allo*-inositol was demonstrated; it was also found that no glucosylation products are formed when D-pinitol is used as acceptor. It is noteworthy that these four substrates bear structural similarity with D-glucopyranose, which is a natural acceptor for CGTase (Fig. 3). Indeed, the transfer of α-(1→4)-glucans to the 4′-OH of D-glucose is part of the intrinsic mechanism of CGTases activity, while the transfer of α-(1→1)-glucans to the 1′-OH of β-D-glucose has also been reported.[20,21] Observations derived from the structural analysis of D-glucose derivatives obtained in different transglycosylation reactions with CGTase, suggest that the configuration of the D-glucose 2′-OH, 3′-OH, and 4′-OH groups is the key structural feature defining specificity.[27,28] Moreover, other enzymes from the α-amylase family are also able to transfer glucose over the β-anomer of the 1′-OH of D-glucose.[22] If inositols are depicted in a similar configuration as β-D-glucose, it may be observed that some of the studied inositol hydroxyls have equivalent configurations to that of 1′-OH and 4′-OH groups of β-D-glucose in its $^4C_1$ conformation (Fig. 3) and therefore fulfill the *Thermoanaerobacter* sp. CGTase *regio*- and *stereo*-selectivity requirements. As expected, the experimental results showed that inositols are glucosylated by *Thermoanaerobacter* sp. CGTase on equatorial hydroxyls equivalent to 1′-OH and/or 4′-OH of β-D-glucose. This is the case of *myo*-inositol, L-*chiro*-inositol, D-*chiro*-inositol, and *muco*-inositol. The glycosylated positions in L-*chiro*-inositol were the 2-OH and 5-OH groups in a 76:24 ratio, the minor product being α-D-glucopyranosyl-(1→5)-5L-*chiro*-inositol (**11**). When the configuration of L-*chiro*-inositol is compared to that of β-D-glucopyranose, we find that the 2-OH, 3-OH, 4-OH, and 5-OH groups of L-*chiro*-inositol correspond to the 1′-OH, 2′-OH, 3′-OH, and 4′-OH groups of a β-D-glucopyranose. It is reasonable to conclude that the 5-OH

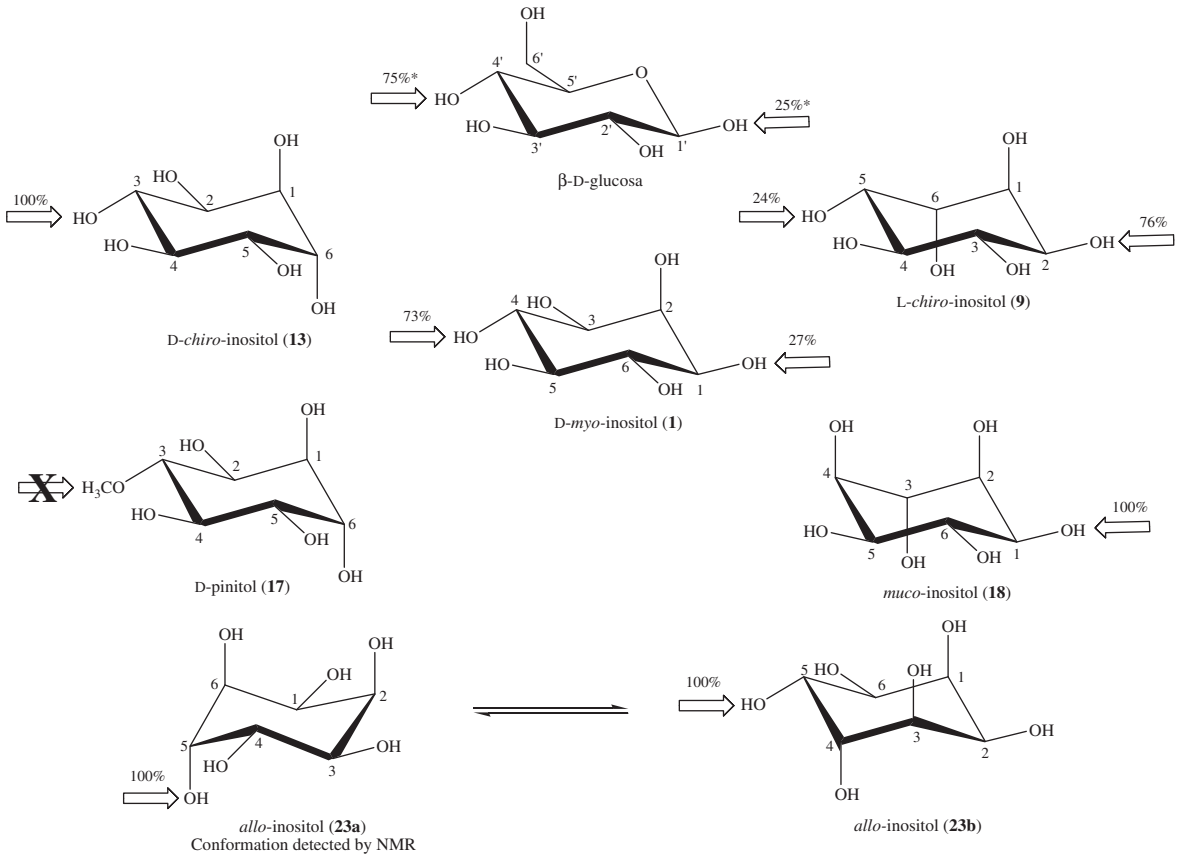


Figure 3. Products from glucosylation of various inositols catalyzed by CGTase from *Thermoanaerobacter* sp. Percentages represent the proportion of each product formed. *Products of glycosylation catalyzed by *Thermotoga maritima* α-amylase.[22]

group of L-*chiro*-inositol, which corresponds to the 4′-OH group of D-glucopyranose, is a favorable acceptor site for the transglycosylation of CGTase, according to the described specificity. However, the main glycosylation took place in the 2-OH group, which corresponds to 1′-OH- of β-D-glucopyranose, and D-*myo*-inositol. In fact, it is important to consider that if the OH groups at 2- and 5-positions of L-*chiro*-inositol were superimposed to the corresponding 1′- and 4′-OH groups of β-D-glucose, the oxygen in the ring is replaced by an axial –CH–OH. In the case of *myo*-inositol as acceptor, the enzyme showed a better selectivity to the 4-OH group of D-*myo*-inositol resulting in monoglycosylated products in a 73:3% ratio.[21] Thus, the different orientation of the 6-OH group (axial) of L-*chiro*-inositol with respect to the 3-OH group (equatorial) of D-*myo*-inositol may affect the transglycosylation regioselectivity of the enzyme.

Interestingly, when D-*chiro*-inositol was essayed as acceptor, the α-D-glucose residue was transferred only to the 3-OHeq, but not to the 6-OHax. The 3-OHeq is analogous to the 4′-OH of D-glucopyranose and D-*myo*-inositol, while the 6-OHax of D-*chiro*-inositol is not equivalent to the 1′-OH of β-D-glucopyranose. D-*chiro*-Inositol has a conformation in which 3-OH, 4-OH, and 5-OH groups are all equatorial like in D-glucopyranose. Thus, α-D-glucopyranosyl-(1→3)-3D-*chiro*-inositol (**14**) is the only glucosylated product of CGTase on D-*chiro*-inositol. The presence of the 6-OHax group in D-*chiro*-inositol influenced the reaction specificity in such a way that glucosylation was observed only in the 3-OH, in contrast to glucosylation in 2-OH and 5-OH observed in L-*chiro*-inositol.

On the other hand, although the 3-OH in D-pinitol has the same orientation of the 4′-OH of D-glucopyranose, the presence of a methyl group in the 3-OH did not allow glucosylation.

In the case of *muco*-inositol, there are two possible glycosylation sites defined by its symmetry plane. The spectroscopic data indicated that the α-D-glucose residue was attached to one of the two enantiotopic 1-OH or 5-OH positions of *muco*-inositol. In this case, the exact position to which the glucose residue was attached could not be determined by the NMR data alone. However, from *Thermoanaerobacter* sp. CGTase specificity preferences for the 1-OH and 4-OH positions in D-*myo*-inositol, it was deduced that the glucose residue was attached to the 1-OH group of *muco*-inositol. It is interesting to note that among the four inositols evaluated, *muco*-inositol resulted as the better acceptor (57.8% yield).

From the NMR spectra of monoglucosylated *allo*-inositol (**24**), it was established that glucosylation occurred in the 5-OHax group to give α-D-glucopyranosyl-(1→5)-*allo*-inositol (**24**). It is important to consider that *allo*-inositol is in fact a *meso*-compound, which means that the molecule interconverts the two stereoisomeric chair forms (23a and 23b, Fig. 3) and averages H-1 and H-4, H-3 and H-2, and H-5 and H-6. Therefore, when the *allo*-inositol interconverts to the chair 23b, this 5-OH group is in an equatorial position favoring the glucosylation on this hydroxyl. Indeed, in this chair conformation the 5-OHeq corresponds to the 4′-OH group of D-glucopyranose.

It is worth mentioning that glycosylations in non-interconvertible axial hydroxyl groups have been favorably performed only when using β-galactosidases as catalysts.[13,15]

It is known that CGTase catalyzes not only the cyclization reaction forming cyclodextrin from starch but also the transglycosylation reaction in the presence of suitable acceptors such as carbohydrate derivatives and hydroxylated compounds. Pyranoses with similar configuration as glucopyranose are efficient glucosyl acceptors for CGTases, the transglycosylation reaction occurring at analogue positions of the 4′-OH and/or 1′-OH of D-glucose.[28,29] Moreover, glucosylation products of D-fructose using a CGTase from *Bacillus stearothermophilus* were determined at the 3-OH and 1-OH groups, the main product (turanose) resulted from the glucosylation of D-fructose at the 3-OH group.[30] On the contrary, D-galactose, D-ribose, D-mannose, D-arabinose, and D-fructose have resulted as poor acceptors for these enzymes.[28,31]

3. Conclusions

A practical enzymatic method for the regioselective glycosylation of five inositols is reported. It is proposed that regioselectivity of CGTase from *Thermoanaerobacter* sp. is defined by the hydroxyl configuration of inositols. Indeed, it was demonstrated that inositols bearing the stereochemical structure corresponding to 1′-OH and/or 4′-OH of β-D-glucose, including *myo*-inositol, D-*chiro*-inositol, L-*chiro*-inositol, *muco*-inositol, and *allo*-inositol, are recognized as acceptor for the synthesis of glucosyl-inositols. The reactions carried out with L-*chiro*-inositol, D-*chiro*-inositol, *muco*-inositol, and *allo*-inositol resulted in the regiospecific formation of glucosylated products. Although, the 3-OH in D-pinitol has the same stereochemical structure of the 4′-OH of D-glucopyranose, the presence of a methyl group in the 3-OH did not allow glucosylation. It was established that the combined enzymatic transglucosylation-hydrolysis strategy here described results in a useful tool for the simple and rapid access to monoglycosylated inositols.

4. Experimental

4.1. General

NMR spectra were acquired either on Varian Unity NMR Spectrometers operating at 400 MHz, 500 MHz, and 700 MHz for ^{1}H and 100 MHz, 126 MHz, and 175 MHz for ^{13}C nuclei, respectively. Chemical shifts are listed in parts per million (ppm), referenced to D_2O or pyridine-d_5 and were made on the basis of ^{1}H–1H gCOSY, ^{1}H–1H TOCSY, NOESY, gHSQC, and gHMBC spectral analyses as required. NMR experiments performed in $CDCl_3$ are referenced to Me_4Si (0 ppm). HRFABMS spectra in a matrix of *m*-nitrobenzyl alcohol or glycerol were recorded on a JEOL JMX-AX 505 HA mass spectrometer. Melting points were determined using a Fisher Johns apparatus and were not corrected. All reagents and solvents used were of analytical grade. Optical rotations were acquired with a Perkin–Elmer 241MC polarimeter (10 cm, 1 mL cell) at the sodium D line. The benzoylation reactions were made in a CEM microwave apparatus.

Formation of oligoglucosyl-inositols and monoglucosyl-inositols was monitored by high performance liquid chromatography (HPLC) using a Waters-Millipore chromatograph with a refraction index detector (Waters 410) equipped with an automatic injector model 717 plus. The measurement conditions were as follows: Column Prevail Carbohydrate ES 5μ 4.6 mm × 250 mm; eluent, CH_3CN–H_2O (68–32 v/v); flow rate, 1.0 mL/min; column temperature, 32 °C; RI detector (Waters).

CGTase from *Thermoanaerobacter* sp. (Toruzyme® 3.0 L, a liquid enzyme preparation) was a kind gift of Novozymes (México). D-*chiro*-Inositol, L-*chiro*-inositol, *muco*-inositol, *allo*-inositol, D-pinitol, and D-glucose, were obtained from Sigma Aldrich, Inc. (MO, USA). β-Cyclodextrin was obtained from American Maize-Products Company. Glucoamylase from *Aspergillus niger* used for digestion of oligoglucosyl-inositols was obtained by ANZECO®.

4.2. Transglucosylation of inositols with CGTase from *Thermoanaerobacter* sp. followed by glucoamylase digestion

A solution containing 150 mg (0.83 mmol) of inositol (D-*chiro*-inositol or L-*chiro*-inositol) or D-pinitol (150 mg, 0.77 mmol) and 150 mg of β-CD (0.13 mmol) was incubated with 8.6 units/mL of CGTase from *Thermoanaerobacter* sp. in 1 mL of phosphate buffer (pH 6.0, 50 mM Na_2HPO_4) at 50 °C for 24 h. *muco*- and *allo*-Inositols

(84 mg each, 0.46 mmol) were incubated in the same conditions using 84 mg (0.074 mmol) of β-cyclodextrin. The reaction mixtures were immersed in boiling water for 10 min to inactivate the enzyme and were stored at −18 °C until subsequent analyses by TLC and HPLC and compared to blank experiments obtained with the enzyme in the absence of inositol.

The products obtained after transglucosylation reactions from inositols were subjected to the action of *A. niger* glucoamylase. Reactions were incubated at 50 °C for 24 h with 258 U/mL of the enzyme, which was directly added to the CGTase reaction medium. Samples were taken and analyzed by TLC and HPLC. The reaction was stopped by boiling for 10 min and was stored at −18 °C until subsequent high-performance chromatographic separation.

4.2.1. α-D-Glucopyranosyl-(1→2)-2L-chiro-inositol (10)

White amorphous powder (36.6% yield); mp 158-160 °C (decomposed); $[\alpha]_D^{20}$ +72.90 (*c* 0.12, H_2O); ^1H NMR (500 MHz, D_2O): δ = 5.07 (d, 1H, $J_{1',2'}$ = 4.0 Hz, H-1′), 4.06 (t, 1H, $J_{1,6}$ = 3.5 Hz, $J_{1,2}$ = 3.5 Hz, H-1), 3.87 (dd, 1H, $J_{1,6}$ = 3.5 Hz, $J_{5,6}$ = 4.0 Hz, H-6), 3.72 (dd, 1H, $J_{5',6b'}$ = 2.0 Hz, $J_{6a',6b'}$ = 12.0 Hz, H-6b′), 3.69 (t, 1H, $J_{3,4}$ = 10.0 Hz, $J_{4,5}$ = 10.0 Hz, H-4), 3.66 (m, 1H, H-2), 3.65 (t, 1H, $J_{2',3'}$ = 10.0 Hz, $J_{3',4'}$ = 10.0 Hz, H-3′), 3.65 (dd, 1H, $J_{5',\ 6a'}$ = 2.0 Hz, $J_{6a',6b'}$ = 12.0 Hz, H-6a′), 3.64 (m, 1H, H-5′), 3.63 (m, 1H, H-5), 3.49 (dd, 1H, $J_{2,3}$ = 9.0 Hz, $J_{3,4}$ = 9.5 Hz, H-3), 3.42 (dd, 1H, $J_{1',2'}$ = 4.0 Hz, $J_{2',3'}$ = 9.75 Hz, H-2′), 3.29 (t, 1H, $J_{3',4'}$ = 10.0 Hz, $J_{4',5'}$ = 10.0 Hz H-4′); ^{13}C NMR (125 MHz, D_2O): δ = 101.47 (C-1′), 79.87 (C-2), 73.63 (C-5), 73.38 (C-3), 73.10 (C-4), 72.96 (C-3′), 72.56 (C-2′), 72.43 (C-6), 72.09 (C-1), 70.97 (C-5′), 70.34 (C-4′), 61.33 (C-6′); HRFABMS (positive mode), calcd for $C_{12}H_{22}O_{11}Na$: 365.2889, found: 365.1074.

4.2.2. α-D-Glucopyranosyl-(1→5)-5L-chiro-inositol (11)

White amorphous powder (12.5% yield); mp 165–167 °C (decomposed); $[\alpha]_D^{20}$ +71.80 (*c* 0.24, H_2O); ^1H NMR (500 MHz, D_2O): δ = 5.11 (d, 1H, $J_{1',2'}$ = 4.0 Hz, H-1′), 4.08 (d, 1H, $J_{1,6}$ = 1.5 Hz, $J_{5,6}$ = 1.5 Hz, H-6), 3.75 (dd, 1H, $J_{5',6a'}$ = 2.0 Hz, $J_{6a',6b'}$ = 12.0 Hz, H-6a′), 3.72 (dd, 1H, $J_{5,4}$ = 9.5 Hz, $J_{5,6}$ = 3.0 Hz, H-5), 3.72 (dd, 1H, $J_{2',3'}$ = 9.5 Hz, $J_{3',4'}$ = 10.0 Hz, H-3′), 3.71 (t, 1H, $J_{3,4}$ = 9.5 Hz, $J_{4,5}$ = 9.5 Hz, H-4), 3.71 (dd, 1H, $J_{1,6}$ = 2.5 Hz, $J_{1,2}$ = 3.0 Hz, H-1), 3.69 (dd, 1H, $J_{5',6b'}$ = 2.0 Hz, $J_{6a',6b'}$ = 12.0, H-6b′), 3.69 (dd, 1H, $J_{1,2}$ = 2.5 Hz, $J_{2,3}$ = 10.0 Hz, H-2), 3.68 (dd, 1H, $J_{2,3}$ = 9.0 Hz, $J_{3,4}$ = 10.0 Hz, H-3), 3.66 (m, 1H, H-5′), 3.46 (dd, 1H, $J_{1',2'}$ = 4.0 Hz, $J_{2',3'}$ = 10.0 Hz, H-2′), 3.32 (t, 1H, $J_{3',4'}$ = 9.5 Hz, $J_{4',5'}$ = 9.5 Hz H-4′); ^{13}C NMR (125 MHz, D_2O): δ = 101.53 (C-1′), 73.73 (C-5), 73.73 (C-1, C-2, C-3 and C-5′), 73.22 (C-4), 72.96 (C-3′), 72.64 (C-2′), 72.17 (C-6), 70.46 (C-4′), 61.46 (C-6′); HRFABMS (positive mode), calcd for $C_{12}H_{22}O_{11}Na$: 365.2889, found: 365.1078.

4.2.3. α-D-Glucopyranosyl-(1→3)-3D-chiro-inositol (14)

Purified before hydrolysis with glucoamylase. White amorphous powder (23.0% yield); mp 145–148 °C (decomposed); $[\alpha]_D^{20}$ +53.42 (*c* 0.1, H_2O); ^1H NMR (400 MHz, D_2O): δ = 5.38 (d, 1H, $J_{1',2'}$ = 4.0 Hz, H-1′), 4.09 (dd, 1H, $J_{1,6}$ = 3.2 Hz, $J_{5,6}$ = 4.0 Hz, H-6), 4.08 (ddd, 1H, $J_{5',6a'}$ = 2.8 Hz, $J_{5',6b'}$ = 4.8 Hz, $J_{4',5'}$ = 10.1 Hz, H-5′), 4.08 (dd, 1H, $J_{1,6}$ = 2.8 Hz, $J_{1,2}$ = 4.0 Hz, H-1), 3.91 (dd, 1H, $J_{5',6b'}$ = 2.2 Hz, $J_{6a',6b'}$ = 12.2 Hz, H-6b′), 3.90 (dd, 1H, $J_{3,4}$ = 10.0 Hz, $J_{4,5}$ = 10.4 Hz, H-4), 3.89 (dd, 1H, $J_{1,2}$ = 2.8 Hz, $J_{2,3}$ = 9.9 Hz, H-2), 3.88 (dd, 1H, $J_{5',6a'}$ = 3.2 Hz, $J_{6a',6b'}$ = 13.6 Hz, H-6$^{a\prime}$), 3.80 (dd, 1H, $J_{2',3'}$ = 9.6 Hz, $J_{3',4'}$ = 10.0 Hz, H-3′), 3.78 (t, 1H, $J_{2,3}$ = 9.6 Hz, $J_{3,4}$ = 9.6 Hz, H-3), 3.78 (dd, 1H, $J_{5,4}$ = 2.4 Hz, $J_{5,6}$ = 9.6 Hz H-5), 3.64 (dd, 1H, $J_{1',2'}$ = 4.0 Hz, $J_{2',3'}$ = 9.6 Hz, H-2′), 3.50 (t, 1H, $J_{3',4'}$ = 9.6 Hz, $J_{4',5'}$ = 9.6 Hz H-4′); ^{13}C NMR (100 MHz, D_2O): δ = 99.61 (C-1′), 81.23 (C-3), 73.16 (C-3′ and C-4), 72.14 (C-1), 72.08 (C-6), 71.99 (C-5′), 71.52 (C-5), 70.47 (C-2), 69.65 (C-2′), 69.23 (C-4′), 60.63 (C-6′); HRFABMS (negative mode), calcd for $C_{12}H_{21}O_{11}Na$: 341.1078, found: 341.1063.

4.2.4. α-D-Glucopyranosyl-(1″→4′)-α-D-glucopyranosyl-(1′→3)-3D-chiro-inositol (15)

White amorphous powder (15.0% yield); mp 177–180 °C (decomposed); $[\alpha]_D^{20}$ +103.11 (*c* 0.15, H_2O); ^1H NMR (700 MHz, D_2O): δ = 5.43 (d, 1H, $J_{1'',2''}$ = 3.5 Hz, H-1″), 5.35 (d, 1H, $J_{1',2'}$ = 3.5 Hz, H-1′), 4.15 (ddd, 1H, $J_{5',6a'}$ = 2.3 Hz, $J_{5',6b'}$ = 4.4 Hz, $J_{4',5'}$ = 9.98 Hz, H-5′), 4.05 (t, 1H, $J_{1,6}$ = 3.5 Hz, $J_{5,6}$ = 3.5 Hz, H-6), 4.04 (dd, 1H, $J_{2',3'}$ = 8.4 Hz, $J_{3',4'}$ = 10.5 Hz, H-3′), δ = 4.03 (dd, 1H, $J_{1,6}$ = 2.8 Hz, $J_{1,2}$ = 4.2 Hz, H-1), 3.88 (ddd, 1H, $J_{5'',6a''}$ = 2.8 Hz, $J_{5'',6b''}$ = 7.88 Hz, $J_{4'',5''}$ = 11.02 Hz, H-5″), 3.87 (dd, 1H, $J_{5',6a'}$ = 2.8 Hz, $J_{6a',6b'}$ = 11.2 Hz, H-6$^{a\prime}$), 3.86 (m, 1H, H-6a″), 3.81 (dd, 1H, $J_{3,4}$ = 9.1 Hz, $J_{4,5}$ = 10.5 Hz, H-4), 3.806 (dd, 1H, $J_{4,5}$ = 3.5 Hz, $J_{5,6}$ = 10.15 Hz H-5), 3.80 (dd, 1H, $J_{5',6b'}$, = 2.45 Hz, $J_{6a',6b'}$ = 10.85 Hz, H-6b′), 3.75 (dd, 1H, $J_{1,2}$ = 2.5 Hz, $J_{2,3}$ = 9.5 Hz, H-2), 3.73 (dd, 1H, $J_{2,3}$ = 8.4 Hz, $J_{3,4}$ = 9.8 Hz, H-3), 3.71 (dd, 1H, $J_{3',4'}$ = 9.1 Hz, $J_{4',5'}$ = 9.8 Hz H-4′), 3.68 (t, 1H, $J_{2'',3''}$ = 9.8 Hz, $J_{3'',4''}$ = 9.8 Hz, H-3″), 3.63 (dd, 1H, $J_{1',2'}$ = 3.85 Hz, $J_{2',3'}$ = 10.15 Hz, H-2′), 3.60 (dd, 1H, $J_{1'',2''}$ = 4.2 Hz, $J_{2'',3''}$ = 9.8 Hz, H-2″), 3.43 (t, 1H, $J_{4'',3''}$ = 9.8 Hz, $J_{4'',5''}$ = 9.8 Hz H-4″); ^{13}C NMR (175 MHz, D_2O): δ = 99.83 (C-1′), 99.52 (C-1″), 80.75 (C-3), 76.66 (C-4′), 73.32 (C-6), 72.95 (C-4), 72.81 (C-3″), 72.58 (C-2), 71.83 (C-3′), 71.67 (C-2″), 71.52 (C-2′), 71.27 (C-1), 70.38 (C-5′), 70.21 (C-5), 69.27 (C-4″), 68.95 (C-5″), 60.40 (C-6″), 60.34 (C-6′); HRFABMS (negative mode), calcd for $C_{18}H_{32}O_{16}$: 503.4381 found: 503.4381.

4.2.5. α-D-Glucopyranosyl-(1→1)-muco-inositol (19)

White amorphous powder (58.2% yield); mp 158–160 °C (decomposed); $[\alpha]_D^{20}$ +117.63 (*c* 0.11, H_2O); ^1H NMR (500 MHz, D_2O): δ = 5.03 (d, 1H, $J_{1',2'}$ = 3.5 Hz, H-1′), 4.02 (dd, 1H, $J_{1,2}$ = 5.5 Hz, $J_{2,3}$ = 6.5 Hz, H-2 or H-4), 3.87 (m, 2H, H-3 and H-4), 3.82 (m, 1H, H-1 or H-5), 3.78 (m, 1H, H-5′), 3.77 (m, 2H, H-5 and H-6), 3.71 (dd, 1H, $J_{5',6b'}$, = 2.0 Hz, $J_{6a',6b'}$ = 12.5 Hz, H-6b′), 3.63 (dd, 1H, $J_{5',6a'}$, = 2.0 Hz, $J_{6a',6b'}$ = 12.0 Hz, H-6a′), 3.62 (dd, 1H, $J_{2',3'}$ = 9.5 Hz, $J_{3',4'}$ = 10.0 Hz, H-3′), 3.42 (dd, 1H, $J_{1',2'}$ = 3.5 Hz, $J_{2',3'}$ = 9.75 Hz, H-2′), 3.29 (t, 1H, $J_{3',4'}$ = 9.5 Hz, $J_{4',5'}$ = 9.5 Hz, H-4′); ^{13}C NMR (125 MHz, D_2O): δ = 99.27 (C-1′), 79.62 (C-1 or C-5), 73.77 (C-3′), 72.94 (C-5′), 72.94 (C-5 or C-1 and C-6), 72.34 (C-2′ and C-3), 70.89 (C-4 or C-2), 70.21 (C-4′), 69.02 (C-2 or C-4), 61.27 (C-6′); HRFABMS (positive mode), calcd for $C_{12}H_{22}O_{11}Na$: 365.2889, found: 365.1074.

4.2.6. α-D-Glucopyranosyl-(1→5)-allo-inositol (24)

Purified before hydrolysis with glucoamylase. White amorphous powder (13.8% yield); mp 156–159 °C (decomposed); $[\alpha]_D^{20}$ +57.58 (*c* 0.14, H_2O); ^1H NMR (400 MHz, pyridine-d_5): δ = 5.71 (d, 1H, $J_{1',2'}$ = 4.0 Hz, H-1′), 5.11 (dd, 1H, $J_{2',4'}$ = 3.0 Hz, $J_{3',4'}$ = 9.0 Hz, H-4), 4.93 (dd, 1H, $J_{2',4'}$ = 2.6 Hz, $J_{3',4'}$ = 7.8 Hz, H-3), 4.82 (m, 3H, H-2, H-5′), 4.72 (br s, H-1), 4.62 (m, 1H, H-5), 4.61 (t, 1H, $J_{2',3'}$ = 9.2 Hz, $J_{3',4'}$ = 9.2 Hz, H-3′), 4.54 (br s, 1H, H-6), 4.43 (dd, 1H, $J_{5',6b'}$ = 2.0 Hz, $J_{6a',6b'}$ = 11.6 Hz, H-6b′), 4.36 (dd, 1H, $J_{5',6a'}$ = 5.0 Hz, $J_{6a',6b'}$ = 11.8 Hz, H-6a′), 4.25 (dd, 1H, $J_{2',4'}$ = 9.2 Hz, $J_{3',4'}$ = 9.6 Hz, H-4′), 4.18 (dd, 1H, $J_{1',2'}$ = 4.0 Hz, $J_{2',3'}$ = 9.60 Hz, H-2′); ^{13}C NMR (175 MHz, pyridine-d_5): δ = 103.52 (C-1′), 81.18 (C-3), 75.80 (C-1, C-5 and C-3′), 75.25 (C-2), 74.36 (C-6, C-5′ and C-2′), 72.10 (C-4′), 70.59 (C-4), 62.89 (C-6′); HRFABMS (negative mode), calcd for $C_{12}H_{21}O_{11}$: 341.1078, found: 341.1059.

4.3. General benzoylation procedure

Benzoyl chloride was added dropwise to a 10 mL glass microwave reaction vessel at room temperature containing a stirred solution of **10**, **11**, **14**, or **19** in pyridine. The reaction vessel was sealed with a cap and then placed into the microwave cavity. The microwave was programed to heat the reaction mixture to the desired temperature. After the reaction was completed, the vessel was cooled below 50 °C using a flow of compressed air. The

reaction was quenched with ice (5 mL), washed with water, 1 M hydrochloric acid, dried ($NaSO_4$), filtered, and extracted with CH_2Cl_2 (3 × 25 mL) and concentrated. The product was purified by column chromatography (hexane–ethyl acetate, 90:10 → 50:50) on silica gel to give the corresponding perbenzoylated derivatives **10a**, **11a**, **14a**, and **19a**.

4.3.1. α-D-(2,3,4,6-Tetra-*O*-benzoyl)-glucopyranosyl-(1→2)-2L-(1,3,4,5,6-penta-*O*-benzoyl)-chiro-inositol (10a)

Prepared from **10** (0.01 g, 0.033 mmol), benzoyl chloride (0.2 mL, 1.72 mmol) and pyridine (0.5 mL). The reaction was heated at 90 °C with 50 W and 13 psi for 1 h to afford compound **10a** (in 74.05% yield) as a white solid after purification; mp 115–118 °C; $[\alpha]_D^{20}$ −18.26 (*c* 0.55, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$), and ^{13}C NMR (100 MHz, $CDCl_3$): See Supplementary data; HRFABMS (positive mode), calcd for $C_{75}H_{60}O_{20}$: 1280.2590, found: 1280.3903.

4.3.2. α-D-(2,3,4,6-Tetra-*O*-benzoyl)-glucopyranosyl-(1→5)-5L-(1,2,3,4,6-penta-*O*-benzoyl)-chiro-inositol (11a)

Prepared from **11** (0.0112 g, 0.0370 mmol), benzoyl chloride (0.2 mL, 1.72 mmol) and pyridine (0.5 mL). The reaction was heated at 90 °C with 50 W and 13 psi for 1 h to afford compound **11a** (in 45% yield) as a white solid after purification; mp 92–95 °C; $[\alpha]_D^{20}$ +3.63 (*c* 0.43, $CHCl_3$); ^1H NMR (700 MHz, $CDCl_3$), and ^{13}C NMR (175 MHz, $CDCl_3$): See Supplementary data; HRFABMS (positive mode), calcd for $C_{75}H_{59}O_{20}$: 1302.3497, found: 1302.3644.

4.3.3. α-D-(2,3,4,6-Tetra-*O*-benzoyl)-glucopyranosyl-(1→3)-3D-(1,2,4,5,6-penta-*O*-benzoyl)-D-chiro-inositol (14a)

Prepared from **14** (0.0103 g, 0.0334 mmol), benzoyl chloride (0.2 mL, 1.72 mmol) and pyridine (0.5 mL). The reaction was heated at 90 °C with 50 W and 13 psi for 1 h to afford compound **14a** (in 83.65% yield) as a white solid after purification; mp 97–100 °C; $[\alpha]_D^{20}$ +107.66 (*c* 1.08, $CHCl_3$); ^1H NMR (700 MHz, $CDCl_3$), and ^{13}C NMR (175 MHz, $CDCl_3$): See Supplementary data; HRFABMS (positive mode), calcd for $C_{75}H_{59}O_{20}$: 1302.3497, found: 1302.3706.

4.3.4. α-D-(2,3,4,6-Tetra-*O*-benzoyl)-glucopyranosyl-(1→1)-(2,3,4,5,6-penta-*O*-benzoyl)-muco-inositol (19a)

Prepared from **19** (0.0091 g, 0.030 mmol), benzoyl chloride (0.20 mL, 1.72 mmol) and pyridine (0.5 mL). The reaction was heated at 90 °C with 50 W and 13 psi for 1 h to afford compound **19a** (in 86.47% yield) as a white solid after purification; mp 123–125 °C; $[\alpha]_D^{20}$ +53.85 (*c* 0.53, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$), and ^{13}C NMR (100 MHz, $CDCl_3$): See Supplementary data; HRFABMS (positive mode) calcd for $C_{75}H_{60}O_{20}Na$: 1302.2408, found: 1302.3461.

4.4. Thin-layer chromatography

TLC was done on silica gel 60 (Merck) pre-coated plates with a solvent system (v/v) of *n*-butanol–ethanol–water (3:5:2) (four ascends). The spots of products were detected by spraying with an alcoholic solution of α-naphthol and sulfuric acid, followed by heating at 120 °C for 10 min.

4.5. Purification of glucosyl-inositols

After glucoamylase digestion treatment of the reaction products obtained with CGTase, compounds **10, 11**, **14**, **15**, **19**, **24**, and **25** were separated by preparative HPLC. The compounds **14**, **15**, **24**, and **25** were purified before treatment with glucoamylase. The HPLC conditions were as follows: Column μBondapak™ NH_2 10 μm, 125 Å, 7.8 × 300 mm (Waters); eluent CH_3CN–H_2O (80–20 v/v); flow rate, 7.0 mL/min; column temperature, 35 °C; RI detector (Waters).

Acknowledgments

The authors wish to acknowledge PAPIIT (project: IN226706-3) and CONACyT (projects: 56431, CB 82851 81637) for the financial support. We also thank F. González from IBt-UNAM for technical assistance in HPLC analysis, as well as Victoria Labastida and Gabriela Vargas from CIQ-UAEM, for mass spectroscopy and NMR analysis respectively. Alfonso Miranda Molina thanks CONACYT for fellowship 189120 and DGAPA-UNAM for a postdoctoral fellowship.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carres.2012.08.002.

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