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## Environmentally benign glycosylation of aryl pyranosides and aryl/alkyl furanosides demonstrating the versatility of thermostable CGTase from *Thermoanaerobacterium* sp.<sup>†</sup>

Alizé Pennec,<sup>a,b</sup> Laurent Legentil,<sup>a,b</sup> Luis Herrera-Estrella,<sup>c</sup> Vincent Ferrières,<sup>a,b</sup> Anne-Laure Chauvin<sup>\*c</sup> and Caroline Nugier-Chauvin<sup>a,b</sup>

An extensive study on the specificity of transglycosylation and disproportionation of *Thermoanaerobacterium* sp. cyclodextrin glucosyltransferases against aryl glucopyranosides or furanosides is reported. While a mixture of maltoside and isomaltoside was obtained respectively using *p*-nitrophenyl glucopyranoside as an acceptor, only one regioisomer, namely *p*-nitrophenyl  $\alpha$ -D-Glcp-(1,3)- $\alpha$ -L-Araf, was isolated using *p*-nitrophenyl arabinofuranoside as an acceptor. Interestingly, similar outcomes were found when using *p*-nitrophenyl galactofuranoside. Furthermore, activation by microwave irradiation resulted in faster reaction times and higher yields and led to glucosidic oligosaccharides with up to 70% conversion. The influence of the anomeric and C-4 configurations of the glycosidic acceptors on the transglycosylation, previously stated for the CGTase family, was not observed and unconventional substrate specificity towards alkyl furanosides was highlighted.

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## Introduction

Glycoconjugates incorporating rare furanosides are nowadays gaining momentum for detergent applications and also as new therapeutics. Indeed, while alkyl C-glycofuranosides were sought for their amphiphilic properties,<sup>1</sup> oligomers of L-arabinofuranose were described as potential immunomodulators as they stimulate the secretion of various cytokines by macrophages.<sup>2</sup> Furthermore, alkyl derivatives like octyl  $\beta$ -D-galactofuranoside have exhibited mycobacteriostatic properties<sup>3–5</sup> and are detrimental for the survival of *Leishmania donovani* and infection of macrophages by the parasite.<sup>6</sup> Recently, we described the access to such monosaccharides from renewable resources thanks to one-pot biocatalyzed furanosylation of various alcohols in the presence of an arabinofuranosidase of the GH 51 family.<sup>7</sup> Such a strategy used furanosyl donors for transglycosylation. However, the transglycosylation on furano-

side acceptors and in particular the selective formation of alkyl glucopyranosyl-furanoside conjugates by enzymatic means have still to be developed. Such a scaffold can be found in the exopolysaccharide expressed by *Lactobacillus rhamnosus*<sup>8</sup> and some strains of *Streptococcus thermophilus*<sup>9</sup> or as a disaccharide unit grafted onto some natural flavonoids.<sup>10</sup> Such architectures found applications in the food industry as thickening agents for example.

Intermolecular transglycosylation catalyzed by cost-effective catalysts is a key process for the synthesis of high value carbohydrate-based substrates from donors extracted from biomass. Within the available catalysts, cyclodextrin glucosyltransferase enzymes (CGTase, E.C. 2.4.1.19) are starch degrading enzymes belonging to the important family of *endo*-glycosyl transferases.<sup>11</sup> Further hydrolysing starch [ $\alpha$ -1,4-hydrolysis], the enzyme also catalyzes linear oligosaccharide disproportionation as well as cyclic and linear dextrin coupling, depending on the bacterial source involved (Fig. 1).<sup>12</sup>

Substrate specificity of CGTases has been extensively studied and demonstrated the efficiency of CGTases to transglycosylate several glucosyl units onto analogs of glucopyranoside.<sup>13</sup> Additionally, it was highlighted that derivatization of the acceptor at C-1 did not exhibit significant differences in activity whereas changing the configuration of the C-2 and C-4 hydroxyl groups resulted in serious loss of the CGTase efficiency.<sup>14</sup> Nakamura *et al.* showed also by analogy with  $\alpha$ -amylase that a *p*NP group on the anomeric position of the

<sup>a</sup>Ecole Nationale Supérieure de Chimie de Rennes, CNRS, UMR 6226, 11 allée de Beaulieu, CS 50837, 35708 Rennes Cedex 7, France.

E-mail: laurent.legentil@ensc-rennes.fr; Tel: +33 223 238 140

<sup>b</sup>Université européenne de Bretagne,

<sup>c</sup>Laboratorio Nacional de Genómica para la Biodiversidad (Langebio), CINVESTAV-IPN, Km 9.6 Lib. Nte, Carr. Irapuato – León, CP36821, Irapuato, México. Fax: +52 462 607 8276; Tel: +52 462 166 3000

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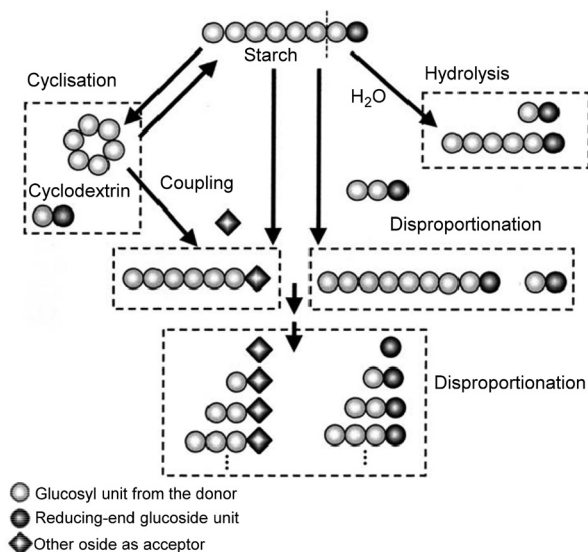


Fig. 1 Reactions catalyzed by cyclodextrin glucosyltransferases.

acceptor is not detrimental to the catalysis.<sup>15</sup> Finally, transglycosylation on dodecyl maltoside was recently performed and allowed the access to alkyl oligoglucosides with non-ionic surfactant properties.<sup>16</sup> Such results demonstrated the high versatility of the CGTase family towards various acceptors. Nevertheless its potential for glucosylation of alkyl furanosides has still to be evaluated.

Thus, in this study the intermolecular transglycosylation and disproportionation reactions of the CGTase of *Thermoanaerobacterium* sp. (EC 2.4.1.19) or Toruzyme® 3.0 L, against pyranosidic and furanosidic acceptors, were compared. In addition, conventional and microwave-assisted reaction (MAR) conditions were evaluated with both substrate types. To achieve this goal, readily available, low cost and bearing a chromogenic anomeric group compounds, *p*-nitrophenyl  $\alpha/\beta$ -D-glucopyranoside (*p*NP  $\alpha$ -Glc **1** and *p*NP  $\beta$ -Glc **2**) and furanoside targets, *p*-nitrophenyl  $\alpha$ -L-arabinofuranoside (*p*NP  $\alpha$ -Araf **3**) and *p*-nitrophenyl  $\beta$ -D-galactofuranoside (*p*NP  $\beta$ -Galf **4**), were

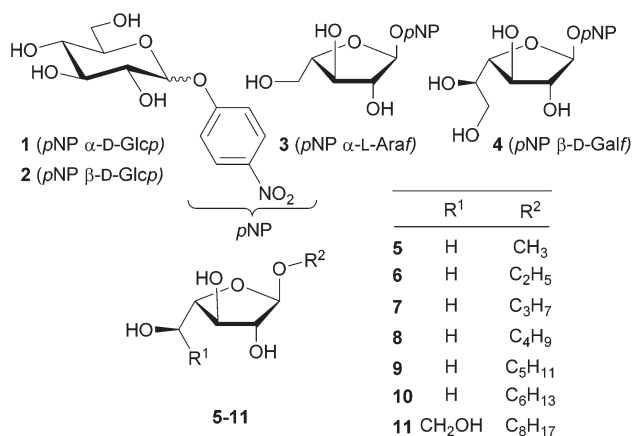


Fig. 2 *p*-Nitrophenyl and alkyl glycosides used as acceptors.

considered (Fig. 2). The method was eventually extended to alkyl furanosides **5** to **11** as acceptors owing to their potential as therapeutics and surfactants.

## Results and discussion

To evaluate the scope of glucosyl transfer, the reaction was first performed with UV-active pyranosides and furanosides **1** to **4** (Fig. 2). Recently, Yoon and Robyt demonstrated that the acceptor/donor ratio influences the degree of polymerization (DP) of the oligoglucanes generated and that a ratio higher than 3 favoured transglycosylation/disproportionation reaction over cyclization.<sup>17</sup> Consequently, all acceptors **1** to **4** and  $\beta$ -cyclodextrin, in a ratio of 7/2, were incubated for 72 hours in 50 mM phosphate buffer pH 6 at 50 °C in the presence of Toruzyme® 3.0 L (0.05 mg mL<sup>-1</sup>). These conditions were described as optimum for CGTase glucosylation.<sup>18</sup>

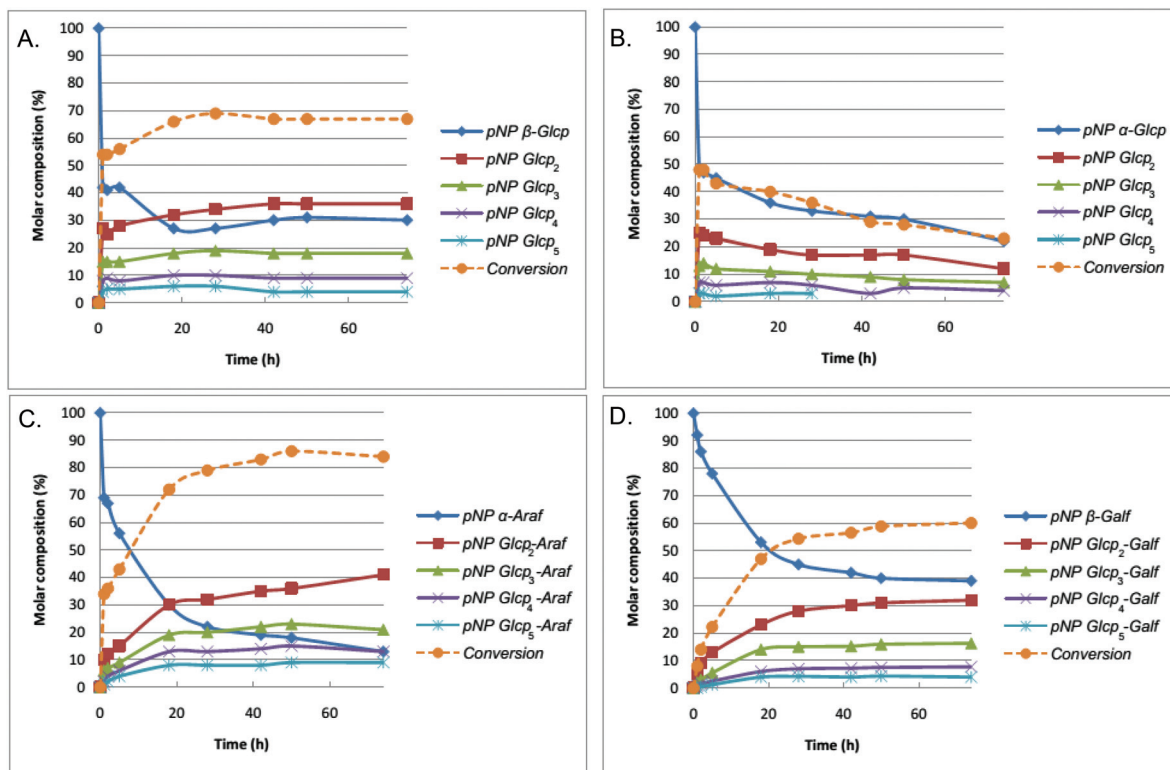
After 72 hours, the reaction mixture was analyzed by liquid chromatography coupled to mass spectrometry (LC-MS). For each substrate, the chromatogram showed a succession of peaks with decreasing intensities (see ESI†). The peaks with the shortest retention times correspond to the remaining starting *p*NP glycosides according to mass analysis (peak at 3.80 min for **1**, 3.64 min for **2**, 3.38 min for **3** and 3.22 min for **4**). The MS spectra also indicated a mass difference of 162 between each major consecutive peak which is characteristic of a molecule of glucose minus one molecule of water. This confirmed the successive transfer of one glucosyl unit to the *p*NP glycoside backbone to form *p*NP Glc<sub>*n*</sub>-glycoside. Furthermore, with compound **1**, an additional signal at *m/z* 139 corresponding to the *p*-nitrophenol group was present. It is most likely due to the hydrolysis of compound **1** or its use as a donor by the CGTase. Indeed **1** had the same  $\alpha$ -configuration as cyclodextrin or malto-oligosaccharides, both substrates of CGTase.<sup>19</sup>

Integration of relevant peaks in the chromatograms led to estimation of the percentage of the remaining starting acceptors, the oligosaccharides formed and the subsequent hydrolysis (Table 1). Overall transglycosylation reached 67, 89 and 59% with acceptors **2**, **3** and **4**, respectively (entries 2 to 4), but only 23% with **1** (entry 1). These data corroborated the significant hydrolysis of the  $\alpha$ -D-glycosidic bond (55%) by the CGTase. Nevertheless, oligomers with a degree of polymerization up to 10 could be detected in the reaction mixture whatever the nature of the substrate. The di- and trisaccharides were the main products from the reaction, corresponding to the transfer of one or two glucosyl entities from the cyclodextrin. It highlighted the prevalence of transglycosylation over disproportionation.

To confirm these initial results, the kinetic profiles of the enzymatic reactions with each substrate were performed (Fig. 3). As expected, the plots showed a decrease in the amount of acceptor over time. The pyranosidic substrates were rapidly consumed after only 2 hours whereas less than 40% of *p*NP Araf **3** and 25% of *p*NP Galf **4** were glycosylated after 5 h

**Table 1** Conversion yield of the reaction between cyclodextrin and *p*NP  $\alpha/\beta$ -glycoside in the presence of CGTase from *Thermoanaerobacterium* sp.

Entry	Acceptors	Remaining acceptor (%)	Hydrolysis (%)	<i>trans</i> -Glycosylation (%)	% of conversion			
					<i>p</i> NP Glcp-glycoside	<i>p</i> NP Glcp <sub>2</sub> -glycoside	<i>p</i> NP Glcp <sub>3</sub> -glycoside	<i>p</i> NP Glcp <sub>4</sub> -glycoside
1	<i>p</i> NP $\alpha$ -D-Glcp (1)	22	55	23	10	7	6	Trace
2	<i>p</i> NP $\beta$ -D-Glcp (2)	28	0	67	36	18	9	4
3	<i>p</i> NP $\alpha$ -D-Araf (3)	11	0	89	41	21	13	9
4	<i>p</i> NP $\beta$ -D-Galf (4)	40	1	59	32	16	7	4

**Fig. 3** Kinetic profile of the reaction catalyzed by CGTase between cyclodextrin and 1 (A), 2 (B) 3 (C) and 4 (D).

indicating a slower reaction rate for the furanosidic vs. pyranosidic acceptors. When 1 was used as an acceptor, the transglycosylation rate reached a maximum after just 2 h, and then the amount of all resulting oligosaccharides decreased over time. Again, this was proof that the *p*NP  $\alpha$ -D-Glcp and the resulting *p*NP oligosides were acceptors and donors of the CGTase. With 2, 3 and 4 as acceptors, the amount of all reaction products reached a steady state after 30 h and the global conversion remained stable. Therefore, the resulting *p*NP glycosides were not consumed during the course of the reaction and thus are not themselves donors for the CGTase. Furthermore such a plateau is characteristic of an acceptor/donor ratio below 3 where cyclization of linear maltosides back to cyclodextrin is favoured over transglycosylation.<sup>17</sup> The furanosides 3 and 4 demonstrated a good response to transglycosylation, and the sugar cyclic size did not affect the reaction yield. This result confirmed the large reaction scope of the CGTase towards pento- and hexofuranosidic acceptors.

The main products of all reactions were isolated for identification. In the chromatograms of the reaction on *p*NP  $\beta$ -Glcp 2, the presence of a minor peak at 7.43 minutes next to the major peak at 6.64 minutes was pointed out. Both peaks had a mass of 463 corresponding to a disaccharide (*p*NP  $\beta$ -Glcp<sub>2</sub>). After isolation and NMR analysis, it was established that the peak at 6.64 min coincided with the *p*NP  $\beta$ -maltoside 12 (anomeric proton at 5.21 and 5.09 ppm and anomeric carbons at 102.9 and 102.7 ppm in the <sup>1</sup>H and <sup>13</sup>C NMR spectra, respectively)<sup>20,21</sup> and the peak at 7.43 minutes resulted from the transglycosylation of a glucose unit in an  $\alpha$ -1,6-fashion producing the *p*NP  $\beta$ -isomaltoside 13 (Fig. 4) as confirmed by the peaks at 5.05 and 4.80 ppm, and at 101.7 and 100.7 ppm in the <sup>1</sup>H and <sup>13</sup>C NMR spectra, respectively.<sup>22</sup> The formation of the isomaltoside was also noticeable in the HPLC chromatogram of the reaction on *p*NP  $\alpha$ -Glcp 1 but to a lesser extent. As for the *p*NP arabinoside acceptor 3, fractions containing *p*NP Glcp-Araf and *p*NP Glcp<sub>2</sub>-Araf were isolated after reaction on a

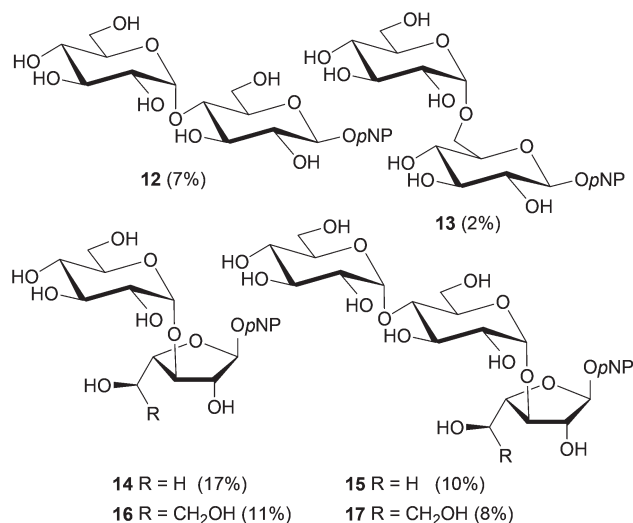


Fig. 4 Isolated products and subsequent yields.

preparative scale. While the chromatogram also showed an additional peak at the base of the major one, both isolated fractions contained only one regioisomer. The isolated disaccharide presented an anomeric proton at 5.71 ppm associated with a carbon at 107.7 ppm. The coupling constant  $J_{H1,H2}$  reaches 1.2 Hz, a typical value for an anomeric proton of a furanoside in the 1,2-*trans* configuration. The second anomeric position showed a proton at 4.96 ppm and a carbon at 101.8 ppm representative of a pyranosidic anomeric position with a  $J_{H1,H2}$  of 4 Hz characteristic of a glycopyranosidic bond in the 1,2-*cis* configuration. 2D-<sup>1</sup>H NMR HMBC experiments exhibited also a long range correlation between H<sub>p</sub>-1 and C<sub>r</sub>-3 indicating a (1,3)-glycosidic bond (see ESI†). These data pointed unambiguously towards *p*NP α-D-Glcp-(1,3)-α-L-Araf 14 as the sole disaccharide isolated from the action of CGTase on 3. Similarly, characterisation of the fraction corresponding to *p*NP Glcp<sub>2</sub>-Araf allowed the identification of the trisaccharide *p*NP α-D-Glcp-(1,4)-α-D-Glcp-(1,3)-α-L-Araf 15 as the unique constituent of this fraction. Isolated yields reach 17% for the disaccharide and 10% for the trisaccharide. Those values were slightly higher than the ones obtained after the action of the CGTase on 2. Similarly, *p*NP α-D-Glcp-(1,3)-β-D-Galf 16 and *p*NP

α-D-Glcp-(1,4)-α-D-Glcp-(1,3)-β-D-Galf 17 were identified as the major products of the glucosylation of *p*NP Galf (Fig. 4). Here the yields were rather low and other regioisomers [*p*NP α-D-Glcp-(1,6)-β-D-Galf principally] were detected. In conclusion, the CGTase action on furanosides was slower than on pyranosides but the regioselectivity for α-(1,3) ligation was higher in particular with *p*NP Araf. However, substitution at C-5 of the furanose led to a drop in the transglycosylation yield and the regioselectivity.

In order to further optimize the action of the CGTase onto the substrates, microwave activation was attempted to (i) accelerate the reaction and (ii) improve the transglycosylation yields.<sup>23</sup> Such a protocol should not degrade the enzyme as CGTase of *Thermoanaerobacterium* sp. has the highest thermostability within the CGTase family.<sup>24,25</sup> The initial investigation of the microwave radiation (MAR) activation was performed on *p*NP α-Glcp 1, and later extended to the other glycosides in order to investigate the effect of the anomeric configuration and the ring size (Table 2). Firstly, the microwave assisted reaction, by drastically reducing the hydrolysis of *p*NP α-Glcp 1 otherwise observed, increased the transglycosylation yield from 23% up to 76% with a reaction time of 10 seconds instead of 48 hours (entries 1 and 2). Secondly, the amount of products resulting from the transfer of 1, 2 or 3 glucosyl residues to 1 was almost tripled (29, 24 and 15% compared to 10, 7 and 6%, respectively). Furthermore, disproportionation reaction was also affected as the ratio disaccharides/tri- and tetrasaccharides decreased under MAR conditions. In addition, by increasing the MAR power from 500 W (entry 2) to 1575 W (entry 4), no notable variation in the transglycosylation yield was detected. The CGTase of *Thermoanaerobacterium* sp. was denatured above 1575 W microwave power as only starting materials were detected. Finally, by raising the reaction time from 10 seconds to 30 seconds, the transglycosylation yields dropped from 74% to 52% (entry 3). The same conditions were applied with the other *p*NP glycosides. With the β-anomer 2, the transglycosylation yield was slightly improved and again the tri- and tetrasaccharide amounts were higher (entries 5 to 7). Surprisingly no by-products resulting from the transglycosylation of a glucosyl unit in an α-1,6-fashion were observed. As for the *p*NP furanosides 3 and 4, no improvement in terms of conversion and selectivity was observed. Those findings estab-

Table 2 Microwave-power effect on the transglycosylation yields of *p*NP glycosides with the CGTase, over a 10 s period

Entry	Acceptor	Microwave power (W)	<i>trans</i> -Glycosylation (%)	Hydrolysis (%)	Conversion (%)			
					<i>p</i> NP Glcp <sub>2</sub>	<i>p</i> NP Glcp <sub>3</sub>	<i>p</i> NP Glcp <sub>4</sub>	<i>p</i> NP Glcp <sub>5</sub>
1	<i>p</i> NP α-Glcp 1	—	23	55	10	7	6	Trace
2		500	74	0	29	24	15	6
3		500 <sup>a</sup>	52	0	nd <sup>b</sup>	nd <sup>b</sup>	nd <sup>b</sup>	nd <sup>b</sup>
4		1575	78	0	28	25	17	8
5	<i>p</i> NP β-Glcp 2	—	67	0	36	18	9	4
6		500	80	0	30	24	17	9
7		1575	78	0	28	24	17	9

<sup>a</sup> Reaction performed over a 30 second period. <sup>b</sup> nd: not determined.



**Table 3** Extension of the glucosylation catalyzed by CGTase to alkyl furanosides

Reaction scheme: Alkyl furanoside (5-11) +  $\alpha$ -D-Glucose  $\xrightarrow[\text{Phosphate Buffer 50 mM, pH 6}]{\beta\text{-Cyclodextrin Toruzyme® 3.0 L}}$  Oligosaccharide (18-31)

Products:  $n = 0$  18-24;  $n = 1$  25-31

Entry	Series	Alkyl furanoside	R <sup>1</sup>	R <sup>2</sup>	Conversion (%)	Products (yield, %)	
						<i>n</i> = 0	<i>n</i> = 1
1	$\alpha$ -D-Araf	5	H	CH <sub>3</sub>	68	18 (14)	25 (11)
2		6	H	CH <sub>3</sub> CH <sub>2</sub>	62	19 (25)	26 (14)
3		7	H	CH <sub>3</sub> (CH <sub>2</sub> ) <sub>2</sub>	73	20 (21)	27 (13)
4		8	H	CH <sub>3</sub> (CH <sub>2</sub> ) <sub>3</sub>	82	21 (19)	28 (12)
5		9	H	CH <sub>3</sub> (CH <sub>2</sub> ) <sub>4</sub>	88	22 (19)	29 (11)
6	$\beta$ -D-Galf	10	H	CH <sub>3</sub> (CH <sub>2</sub> ) <sub>5</sub>	58	23 (25)	30 (10)
7		11	CH <sub>2</sub> OH	CH <sub>3</sub> (CH <sub>2</sub> ) <sub>7</sub>	16	24 (5)	31 (3)

lished that microwave-assisted enzymatic reaction did not modify the selectivity and the substrate specificity of the CGTase *Therm*. It enabled the successful synthesis of both targets *p*NP glucosides in shorter time and higher yields and thus would provide a practical synthetic method for the glucosylation of specific maltoside derivatives. It also demonstrated the importance of microwave activation in reducing the presence of 1,6-linked byproducts. In addition, this process due to the sole use of aqueous solvents and no hazardous substances can be considered environmentally friendly.

Finally, the replacement of anomeric nitrophenoxo by an alkoxy chain on Araf and Galf could allow the biocatalyzed synthesis of relevant alkyl oligosaccharides with both pyranose and furanose rings. It would provide an attractive alternative to the chemical synthesis of the  $\alpha$ -D-Glcp-(1,3)-furanoside bond often tricky to obtain selectively.<sup>26</sup> Alkyl arabinofuranosides, having a chain length varying from methyl to hexyl for the arabinofuranoside and octyl for the galactofuranoside, were thus considered as acceptors (Table 3). They were readily obtained following a known procedure by glycosylation of arabinan in the presence of an arabinofuranosidase and a suitable alcohol.<sup>7</sup> Alkyl furanosides and cyclodextrin were incubated in the presence of Toruzyme® 3.0 L for 72 hours according to the protocol previously established. The resulting crude mixture was analyzed by UPLC-MS spectrometry.  $[M - H]^-$  masses corresponding to the different products of transglycosylation were deduced from the mass spectrum allowing the neat visualization of glycoconjugates with up to 4 residues of glucose on the alkyl furanoside (see ESI†). However, a certain amount of the starting material remained but no hydrolysis could be observed. So the tested alkyl furanosides were confirmed as acceptors of the CGTase even if they did not bear an aromatic ring at the anomeric position. Preparative scale-up was implemented in order to

isolate and identify the different glucosylation products (Table 3). Only the di- and trisaccharides were characterized thanks to NMR experiments. With alkyl arabinofuranosides 11–16, similar isolated yields than with *p*NP substrates were obtained. The di- and tri-saccharides represented an average of 30% of the oligosaccharides and were thus the major products of the reaction. The nature of the glycosidic bond was elucidated thanks to HMBC experiments. As previously stated, the  $\alpha$ -(1,3) bond between the first glucosyl unit and the furanosyl residue was favoured. In contrast, the reaction with octyl Galf 17 as an acceptor yielded a lower amount of di- and trisaccharides confirming the lower affinity of the galactofuranoside for the enzyme. Anyway, in both cases, in only two steps and without protecting group manipulation, oligosaccharides incorporating an  $\alpha$ -D-Glcp-(1,3)-furanoside link could be obtained. Such a pattern could be found for example as part of the exopolysaccharide expressed by some strains of *Streptococcus pneumoniae*.<sup>27</sup> or associated with triterpenoidal saponin extracted from *Meryta denhamii* seem.<sup>28</sup> This last compound showed some antihyperglycemic effect and hence demonstrated the therapeutic potential of those scaffolds.

## Conclusions

In conclusion, this paper reported an extensive study of the versatility of CGTase *Therm*. against acceptors having either a pyranosyl or a furanosyl conformation. Our methodology enabled the enzymatic transglycosylation reaction of a glucosyl unit onto a range of carbohydrate acceptors using readily available donors and biocatalysts, in water and under microwave irradiation, following an environmentally benign procedure. It demonstrated that the enzyme conserved its catalytic activity independently of the substrate form. Additionally, the appli-

cation of microwave irradiation showed a positive effect on yields, reaction time and byproduct formation without denaturing the enzyme when *p*NP pyranoside was used as a substrate. Interestingly our methodology enabled the enzymatic transglycosylation reaction of a glucosyl unit onto a range of alkyl furanoside acceptors to selectively obtain alkyl  $\alpha$ -Glc $\beta$ -(1,3)-Ara $\beta$  or Gal $\beta$ . Such scaffolds need yet to be evaluated for potential surfactant properties or as bacteriostatics.

## Experimental section

### Materials

All reagents were purchased from commercial sources and were used without further purification unless noted. *Thermoanaerobacter* sp. CGTase (Toruzyme® 3.0 L) was a gift from Novozymes A/S (Bagvaerd, Denmark).

### Kinetics of the enzymatic reaction with *p*NP glycosides

The synthesis ability of the CGTases from *Thermoanaerobacter* sp. was previously investigated and the reaction with the enzyme was optimized to efficiently produce the primary coupling product. Typically, CGTase (10  $\mu$ L, 2.34 mg mL<sup>-1</sup>) was added into 1.5 mL microtubes containing  $\beta$ -cyclodextrin (100 mg, 0.1 mM) and various carbohydrates (0.2 mM) in 50 mM phosphate buffer, pH 6.0 (1 mL) at 50 °C. The reaction mixtures were incubated in a thermomixer (Eppendorf) for 72 h. 50  $\mu$ L samples were withdrawn at different intervals and diluted with 950  $\mu$ L of acetonitrile–water (50 : 50) followed by a filtration before chromatographic analysis.

### HPLC analysis

Chromatographic analysis was carried out on an Agilent 1200 HPLC system (Agilent Technologies, Mexico), equipped with the ChemStation software, a micro-vacuum degasser G1322A, a quaternary pump G1311A, an autosampler G1329A, a thermostatic column compartment G1316A, and a diode array detector G1315B. An analytical Alltech® Prevail™ carbohydrate ES column (Analytical, 4.6  $\times$  250 mm, 5  $\mu$ m, Grace) was used for chromatographic separation at 35 °C. Using acetonitrile (mobile phase A) and purified and distilled water (mobile phase B) at a flow rate of 1 mL min<sup>-1</sup>, HPLC analysis started with 20% B followed by a gradient to 50% B over 40 min, then by a gradient step for 1 min back to 20% B, before isocratic elution with starting conditions within 30 min. The injection volume of each sample was 10  $\mu$ L. Simultaneous monitoring was performed at 300, 368 and 400 nm.

### Transglycosylation reactions with a glycosidic acceptor on a preparative scale

Reaction conditions were implemented as previously described in a 1 mL final volume using a molar ratio glycoside/ $\beta$ -cyclodextrin 3/1 and CGTase (10  $\mu$ L, 2.34 mg mL<sup>-1</sup>). The resulting mixture was incubated at 50 °C for 72 h. The reaction mixture was lyophilized and the residue was purified by silica gel flash chromatography using an eluent gradient AcOEt–(AcOH–H<sub>2</sub>O,

1 : 1) from 90 : 5 to 60 : 40. Elution of the products was monitored by UV detection and by TLC. Fractions corresponding to individual products were collected, evaporated and lyophilized.

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