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Synthesis of maltooligosyl fructofuranosides catalyzed by immobilized cyclodextrin glucosyltransferase using starch as donor

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Abstract—Cyclodextrin glucosyltransferase (CGTase) from *Thermoanaerobacter* sp. was covalently immobilized on Eupergit C and used for the synthesis of maltooligosyl fructofuranosides employing soluble starch as donor and sucrose as acceptor. Using a weight ratio starch–sucrose of 1:2, the conversion of starch into acceptor products catalyzed by soluble and immobilized CGTases was higher than 80% in 48 h. Under these conditions, the reaction was selective for the formation of maltosyl fructofuranoside.

1. Introduction

Cyclodextrin glucosyltransferase (CGTase, EC 2.4.1.19) is a bacterial enzyme belonging to the α-amylase family (or glycoside hydrolase family 13)[1] that catalyzes four reactions.[2,3] Cyclization is an intramolecular transglycosylation in which the CGTase converts starch into cyclodextrins (CDs). Coupling is an intermolecular transglycosylation between a CD molecule and a linear oligosaccharide. Disproportionation is an intermolecular transglycosylation between two carbohydrates. In addition, CGTase displays a weak starch hydrolysis activity (where water acts as acceptor).

Either via coupling or disproportionation, CGTases are able to perform the so-called acceptor reaction allowing the synthesis of products of industrial interest. The main requirement for strong acceptor properties is the presence of a D-glucopyranose structure (chair form) with equatorial hydroxyl groups at C-2, C-3 and C-4.[4] Different carbohydrates (such as xylose, sucrose, glucose, maltose and lactose), glycosides, sugar alcohols, vitamins and flavonoids, have been successfully used as CGTase acceptors.[5] The resulting transglycosylated products usually exhibit improved functionality compared with the parent acceptor molecules, e.g. increased water solubility or stability, bifidogenic properties, etc.[6]

In this context, when sucrose is employed as acceptor, maltosyl fructofuranoside ([α-D-Glu-(1→4)-α-D-Glu-(1→2)-β-D-Fru) and higher polymerization products are obtained.[7] A mixture of maltooligosyl fructofuranosides ('maltooligosyl fructose') with a degree of polymerization of 3–7 is currently being commercialized as coupling sugar, and is obtained using starch as donor and sucrose as acceptor, in a process catalyzed by CGTase.[8] Coupling sugar, a non-cariogenic sweetener, is also used as a viscosity modifier, being more stable than other reducing sugar mixtures.[7]

Thermoanaerobacter sp. (a thermophilic anaerobic bacterium) produces a highly thermostable CGTase that displays a high disproportionation activity.[9–11] This enables the enzyme to be used in several acceptor reactions.[12]

In this work, we have studied the synthesis of maltooligosyl fructose employing soluble starch as donor and sucrose as acceptor, catalyzed by CGTase from *Thermoanaerobacter* sp. immobilized on Eupergit C. The reaction rates using the soluble and immobilized CGTases were compared. The main reaction products were isolated by preparative column chromatography, and characterized using NMR and MS analysis.

Keywords: Cyclodextrin glucanotransferase; Glycosyltransferases; Transglycosylation; *Thermoanaerobacter* sp; Coupling sugar; Glucosyl sucrose.
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2. Results and discussion

CGTase is able to use starch[12] or cyclodextrins[13] as glucose donor in transglycosylation processes. Starch is a cheap, abundant and renewable agroproduct, therefore it is preferred to expensive cyclodextrins for industrial purposes. CGTases do not require sugar nucleotides (as all the glycosyltransferases of the Leloir pathway) nor sugar–phosphates (as most glycosyltransferases of the non-Leloir pathway) as donors, since it uses the free energy of cleavage of the readily available starch.[3]

For these acceptor reactions, the immobilization of glucosyltransferases may allow for their stabilization as well as enabling the separation of the biocatalyst. Different approaches have been applied to the immobilization of CGTases, based on adsorption or covalent coupling.[14] We have recently reported CGTase immobilization by covalent binding to aminated silica, activated Sepharose 4B and epoxy-activated acrylic polymers such as Eupergit C.[15] The latter support represents one of the best candidates for CGTase immobilization displaying a half life at 95 °C five times higher that that of the soluble enzyme, as was demonstrated elsewhere.[15b]

The acceptor reaction with soluble starch (Paselli SA2, with an average degree of polymerization of 50) as donor and sucrose as acceptor was studied with soluble and CGTase immobilized on Eupergit C. This enzyme has a higher affinity for disaccharides as acceptors compared with monosaccharides.[3,6,16] When sucrose is employed as acceptor, maltosyl fructose (**1**) is initially formed (Scheme 1). Maltosyl fructose also acts as acceptor, yielding maltotriosyl fructose (**2**). A series of maltooligosyl fructosides is subsequently formed.

The effect of the weight ratio starch–sucrose (from 2:1 to 1:2) on product distribution, using 10% (w/v) soluble starch and variable amounts of sucrose, was tested (Table 1). The maximum conversion to oligosaccharides, regardless of the donor/acceptor ratio, was achieved at 48 h. There are no commercially available standards of maltosyl fructofuranosides. Thus, the main reaction products were isolated by column chromatography (purities higher than 95%) and characterized using NMR and MALDI-TOF mass spectrometry. Maltosyl fructose (**1**), maltotriosyl fructose (**2**) and maltotetraosyl fructose (**3**) were confirmed to be the major products. Spectral data correlated well with those reported for maltosyl fructose by Munksgaard,[17] and for maltotriosyl and maltotetraosyl fructose by Monthieu.[13] However, small amounts of maltooligosyl fructosides with a degree of polymerization up to 7–8 were also observed by TLC and HPLC (Fig. 1).

In the absence of sucrose, the consumption of starch led to the formation of CDs.[12] The presence of co-substrate molecules such as sucrose that are strong acceptors (and, consequently, strong promoters of the formation of linear transfer products) inhibited CD formation. As shown in Table 1, in the presence of the same weight of starch and sucrose, the formation of CDs was almost negligible. Reaction selectivity can be modulated by varying the donor/acceptor ratio. Thus, using a starch/sucrose ratio 2:1 (w/w) the yield of maltooligosyl fructose was 72% and minor amounts of CDs (7–10%) were formed. Under the above conditions, the selectivity to maltosyl fructose was quite low. In contrast, using a starch/sucrose ratio of 1:2 (w/w) the yield of oligosaccharides was 80%, and the formation of maltosyl fructose was enhanced (60%).

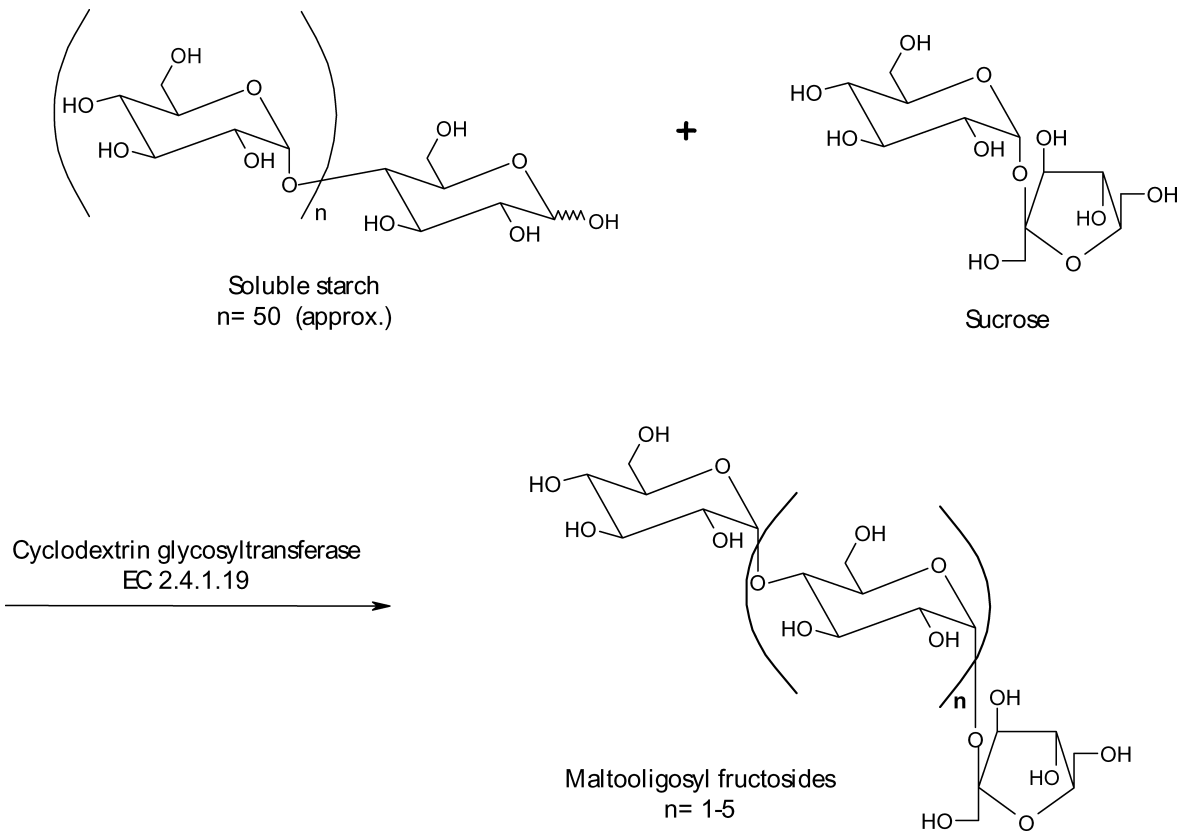
No significant differences were found concerning yield and product distribution when analysing the effect of enzyme immobilization on the reaction (Table 1). However, the reaction rate was higher with the immobilized CGTase (Fig. 2). The initial rate of maltosyl fructose formation with the Eupergit C-immobilized CGTase (14.6 g/l h) was about 3-fold higher as compared with the soluble enzyme (5.6 g/l h). This could be due to improved thermostability of CGTase immobilized on Eupergit C compared to the soluble enzyme.[15]

In conclusion, CGTase immobilized on Eupergit C is able to use starch as donor and sucrose as acceptor, resulting in excellent yields of maltooligosyl fructose (92%). The starch/sucrose ratio is of great importance for inhibiting cyclodextrin synthesis and for modulating the selectivity of the process. Two industrial processes for the CGTase-catalyzed synthesis of coupling sugar have been patented using the enzyme from *Bacillus macerans*[8] and *Thermoanaerobacter*.[18] Our Eupergit-CGTase can become an attractive alternative to the soluble enzyme for these processes. In addition, new applications may emerge from employing distinct acceptors in reactions catalyzed by CGTase from *Thermoanaerobacter* sp.

Table 1. Maltooligosyl fructosides and cyclodextrin production using different starch/sucrose (w/w) ratios catalyzed by soluble and immobilized CGTase. Conditions: 10% (w/v) soluble starch, 60 °C, 10 mM sodium citrate buffer (pH 5.5) containing 0.15 mM $CaCl_2$, 48 h reaction. The conversion of starch into maltooligosyl fructose and cyclodextrins is also presented

Ratio starch/sucrose (w/w)	Enzyme	Products formed (g/l)[a]						Starch conversion (%)[b]	
		1	**2**	**3**	αCD	β-CD	γCD	Maltooligosyl-fructose	Cyclodextrins
2:1	Soluble	28	21	11	3.3	6.8	—	72	10.1
	Immobilized	35	26	13	2	5.2	—	72	7.2
1:1	Soluble	51	35	15	0.6	2.5	—	78	3.1
	Immobilized	51	34	14	—	—	—	79	—
1:2	Soluble	88	48	15	—	—	—	83	—
	Immobilized	95	50	16	—	—	—	92	—

[a] Nomenclature of the different maltooligosyl fructosides: **1**, maltosyl fructose; **2**, maltotriosyl fructose; **3**, maltotetraosyl fructose.
[b] Based on starch consumption.



Scheme 1. Acceptor reaction using soluble starch as donor and sucrose as acceptor catalyzed by *Thermoanaerobacter* CGTase. Nomenclature used for maltoolygosyl fructofuranosides: maltosyl fructose (**1**) where n=1; maltotriosyl fructose (**2**) where n=2; maltotetraosyl fructose (**3**) where n=3. Reaction conditions given in Section 3.

3. Experimental

3.1. General

CGTase from *Thermoanaerobacter* sp. was kindly provided by Novo Nordisk (Toruzyme 3.0 L, batch ACN00019), and purified as described.[15] Cyclodextrins (α-, β- and γ-) were purchased from Sigma. Potato soluble starch (Paselli SA2) was kindly donated by Avebe (Foxhol, The Netherlands).

NMR spectra were recorded on a Varian UNITY spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C) at 30 °C for solutions in D_2O. The identity of the protons and carbons of the different units were obtained through 2D- homo- (DQCOSY and TOCSY) and hetero- (HMQC and HMBC) experiments. Chemical shifts are referred to the residual signal of HOD at 4.71 ppm for ^{1}H NMR, and that of acetone as external reference at 31.07 ppm for ^{13}C NMR. Mass spectral analyses were obtained on a a Bruker (Bremen,

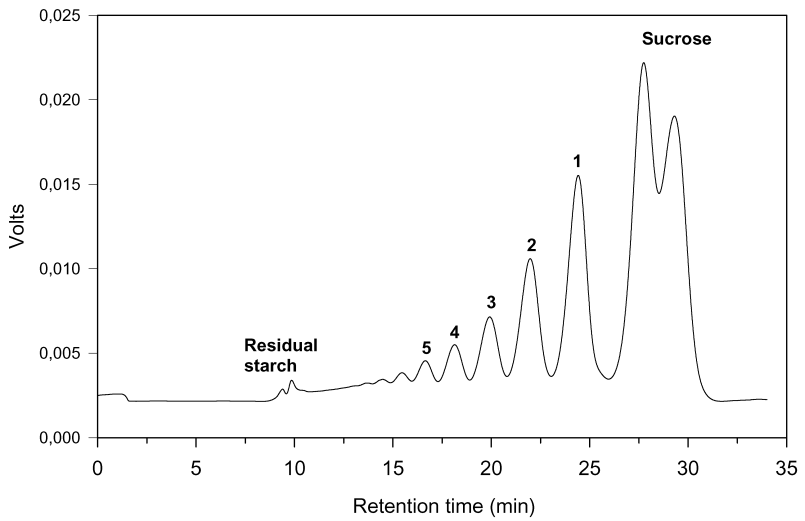


Figure 1. HPLC chromatogram of the reaction mixture at 48 h using soluble starch as donor and sucrose as acceptor catalyzed by CGTase immobilized on Eupergit C. Conditions: 10% soluble starch (w/v), 20% (w/v) sucrose, 10 mM sodium citrate buffer (pH 5.5) containing 0.15 mM $CaCl_2$, 60 °C. The sucrose peak appeared split in two peaks. **1** to **5** represents the homologous series of acceptor products where **1** is maltosyl fructose.

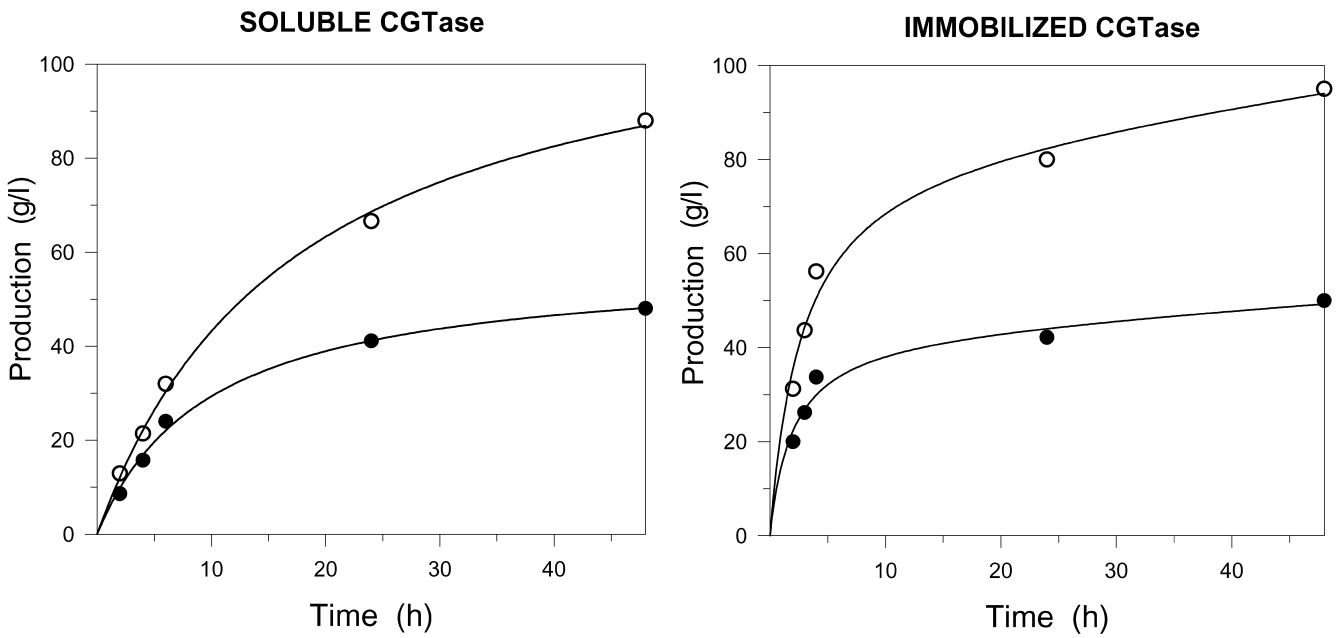


Figure 2. Maltosyl fructose (○) and maltotriosyl fructose (●) production employing a starch:sucrose (w/w) ratio of 1:2 catalyzed by soluble *Thermoanaerobacter* CGTase and immobilized CGTase on Eupergit C. Reaction conditions described in Section 3.

Germany) Reflex III MALDI-TOF mass spectrometer equipped with an ion source with visualization optics and a N_2 laser (337 nm). Mass spectra were recorded using a dihydroxybenzoic acid matrix and methanol as eluent.

Cyclization assays. The production of cyclodextrins was detected spectrophotometrically via the formation of inclusion complexes with several organic compounds. Paselli SA2 (partially hydrolyzed potato starch with an average degree of polymerization of 50) was used as substrate at final concentrations of 5% (w/v) for β- and γ-CD, and 2% (w/v) for α-CD. α-CD was determined at 490 nm on the basis of its ability to form a stable, colorless inclusion complex with methyl orange.[19] β-CD was determined at 552 nm on the basis of its ability to form a stable, colorless inclusion complex with phenolphthalein.[20] γ-CD was determined measuring the color increase at 630 nm due to the formation of an inclusion complex with bromocresol green.[21] The CGTase activity was measured at 85 °C by incubating the enzyme (0.03–0.4 U/ml) in the presence of soluble starch in 10 mM sodium citrate buffer (pH 5.5) containing 0.15 mM $CaCl_2$. The formation of the corresponding CD was followed for 5 min. One unit of activity was defined as that catalyzing the production of 1 μmol of CD per min under the above conditions.

The progress of the acceptor reaction was analyzed by high-performance liquid chromatography (HPLC). 1.7 β-CD units of soluble (140 U/mg protein) or immobilized CGTase (147 U/g support) were incubated at 60 °C with 10% (w/v) soluble starch in 10 mM sodium citrate buffer (pH 5.5) containing 0.15 mM $CaCl_2$ in the presence of different amounts of sucrose -studied ratio starch–sucrose (w/w): 2:1, 1:1 and 1:2. The final volume was 10 ml. At different times, aliquots were taken and mixed with the same volume of 0.4 M NaOH to quench the reaction. Samples were centrifuged during 15 min at 8500*g*. HPLC was performed using a ternary pump 9012 (Varian) and two Aminex HPX-42A columns (300×7.8 mm, Bio-Rad) connected in series. Two deashing cartridges (30×4.6 mm, Bio-Rad) were used prior to the column. Water was used as mobile phase (0.7 ml/min). The column temperature was maintained at 85 °C using a Timberline oven. A refractive-index detector 9040 (Varian) was employed. Integration was carried out using the Varian Star 4.0 software.

Thin-layer chromatography (TLC) was carried out on aminated silica gel plates (Lichroprep-NH_2, 25–40 μm, Merck) using water/butanol/ethanol 2:3:5 (v:v.v) as eluent.[22] Spots were detected by immersion of plates into a mixture formed by 45 ml of 1 g urea dissolved in 85% H_3PO_4 aqueous, 48 ml butanol and 50 ml ethanol,[23] drying and heating at 120 °C for 5 min.

Immobilization on Eupergit C was carried out at 25 °C with gentle orbital shaking (150 rpm) in 1.2 M potassium phosphate buffer pH 7.0. A 1:100 (w/w) enzyme–support ratio was employed. After incubation for 72 h, the beads were collected by vacuum filtration using a porous glass filter. The beads were rinsed thoroughly on the same filter with approximately 3×20 ml of diluted potassium phosphate and 2×20 ml of 10 mM sodium citrate buffer, pH 5.5.[15]

3.2. Synthesis and purification of maltooligosyl fructofuranosides

1.7 β-CD units of immobilized CGTase (147 U/g support) were incubated at 60 °C with 10% (w/v) soluble starch in 10 mM sodium citrate buffer (pH 5.5) containing 0.15 mM $CaCl_2$ in the presence of 20% (w/v) sucrose. The final volume was 10 ml. The reaction was maintained for 48 h. The immobilized CGTase and the remaining starch were removed by centrifugation. The aqueous supernatant was evaporated azeotropically with methanol under reduced pressure. After evaporation, the resulting residue was

fractionated by column chromatography using Lichroprep-NH_2 silica gel (25–40 μm, Merck) as adsorbent and an adsorbent–sample ratio of approx. 30:1 (w/w). The reaction products were eluted with a mixture H_2O–ethanol–1-butanol 2:5:3 (v/v/v). The order of elution was maltosyl fructose (**1**), maltotriosyl fructose (**2**), maltotetraosyl fructose (**3**) and higher polymerization products, as shown by TLC and HPLC. Solvents were removed under vacuum from collected fractions of the respective oligosaccharides and characterized.

3.2.1. Maltosyl fructose (1) [α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside]. ^{1}H NMR (δ, ppm): 5.40 (d, 1H, H-1′, $J_{1'2'}$=3.7 Hz), 5.39 (d, 1H, H-1″, $J_{1''2''}$=3.7 Hz), 4.20 (d, 1H, H-3, $J_{3,4}$=8.7 Hz), 4.02 (m, 2H, H-4+H-3′), 3.98 (m, 1H, H-5′), 3.88 (m, 1H, H-5), 3.88–3.76 (m, 6H, H-6a+H-6b+H-6′a+6′b+H-6″a+6″b), 3.76–3.64 (m, 2H, H-3″+H-5″), 3.69 (m, 1H, H-4′), 3.67 (s, 2H, H-1a+H-1b), 3.58 (m, 2H, H-2′+H-2″), 3.40 (t, 1H, H-4″, $J_{3,4}$=$J_{4,5}$=9.2 Hz); ^{13}C NMR (δ, ppm): 104.5 (C-2), 100.7 (C-1″), 92.3 (C-1′), 82.2 (C-5), 77.6 (C-4′), 77.3 (C-3), 74.9 (C-4), 73.9+73.8 (C-3′+C-3″), 73.6 (C-5″), 72.6 (C-2″), 71.8 (C-5′), 71.7 (C-2′), 70.2 (C-4″), 63.2 (C-6), 62.2 (C-1), 61.3 (C-6″), 61.0 (C-6′). MS (MALDI-TOF): calcd for $C_{18}H_{32}O_{16}Na$ 527.3, found 527.1; calcd for $C_{18}H_{32}O_{16}K$ 543.4, found 543.1.

3.2.2. Maltotriosyl fructose (2) [α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside]. ^{1}H NMR (δ, ppm): 5.38 (d, 1H, H-1′, $J_{1'2'}$=3.7 Hz), 5.36+5.35 (2d, H-1″+H-1‴, J=3.7 Hz), 4.18 (d, 1H, H-3, $J_{3,4}$=8.6 Hz), 4.05 (t, 1H, H-4, $J_{4,5}$=8.6 Hz), 4.00 (dd, 1H, H-3′, J=8.9+10 Hz), 3.94 (m, 1H, H-5′), 3.86 (m,1H,H-5), 3.86–3.70 (m, 8H, H-6a+6b+H-6′a+6′b+H-6″a+6″b+H-6‴a+6‴b), 3.68 (m, 2H, H-5″+H-5‴), 3.64 (s, 2H, H-1a+H-1b), 3.70–3.60 (m, 2H, H-4′+H-4″), 3.60–3.50 (m, 3H, H-2′+H-2″+H-2‴), 3.38 (t, 1H, H-4‴, J=9.6+9.2 Hz); ^{13}C NMR (δ, ppm): 104.6 (C-2), 100.7+100.6 (C-1″+C-1‴), 92.9 (C-1′), 82.3 (C-5), 77.9+77.7 (C-4′+C-4″), 77.5 (C-3), 75.0 (C-4), 74.3+73.9+73.8+73.7 (C-3′+C-3″+C-3‴+C-5″), 72.7+72.5+72.2 (C-2′+C-2″+C-2″), 71.9 (C-5′), 71.8 (C-5‴), 70.3 (C-4‴), 63.3 (C-6), 62.3 (C-1), 61.5 (2)+61.1 (C-6′+C-6″+C-6‴). MS (MALDI-TOF): calcd for $C_{24}H_{42}O_{21}Na$ 689.3, found 689.2; calcd for $C_{24}H_{42}O_{21}K$ 705.4, found 705.2.

3.2.3. Maltotetraosyl fructose (3) [α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside]. ^{1}H NMR (δ, ppm): 5.40–5.38 (m, 4H, H-1′+H-1″+H-1‴+H-1^{IV}), 4.20 (d, 1H, H-3, $J_{3,4}$=8.7 Hz), 4.03 (m, 2H, H-4+H-3′), 3.96 (m, 3H, H-5+H-5′+H-3^{IV}), 3.86–3.70 (m, 10H, H-6a+6b+H-6′a+6′b+H-6″a+6″b+H-6‴a+6‴b+H-6^{IV}a+6^{IV}b), 3.67 (s, 2H, H-1a+H-1b), 3.65–3.55 (m, 4H, H-2′+H-2″+H-2‴+H-2^{IV}), 3.42 (t, 1H, H-4^{IV}, J=9.5+9.7 Hz); ^{13}C NMR (δ, ppm): 104.5 (C-2), 100.6+100.4+100.3 (C-1″+C-1‴+C-1^{IV}), 92.8 (C-1′), 82.2 (C-5), 77.8+77.4 (2) (C-4′+C-4″+C-4‴), 76.8 (C-3), 74.9 (C-4), 74.2 (2)+73.8+73.7+73.6 (C-3′+C-3″+C-3‴+C-3^{IV}), 72.6+72.4 (2)+72.1+71.8+71.7 (2) (C-2′+C-2″+C-2‴+C-2^{IV}+C-5′+C-5″+C-5‴), 70.2 (C-4^{IV}), 63.2 (C-6), 62.3 (C-1), 61.4 (3)+61.0 (C-6′+C-6″+C-6‴+C-6^{IV}). MS (MALDI-TOF): calcd for $C_{30}H_{52}O_{26}Na$ 851.4, found 851.3; calcd for $C_{30}H_{52}O_{26}K$ 867.5, found 867.2.

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