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Biocatalytic and chemical investigations in the synthesis of sucrose analogues

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Abstract—Herein, we report about the synthesis of sucrose analogues, obtained by two different approaches: a chemical and an enzymatic. The one step synthesis of the sucrose analogues with the exo-fructosyltransferase (EC 2.4.1.162) from *Bacillus subtilis* NCIMB 11871, which transfers the fructosyl residue of the substrate sucrose to the monosaccharide acceptors galactose, mannose, xylose and fucose, has been developed. Effects in the fructosylation by variation of the positions of the hydroxyl-groups in glycopyranoside acceptors have been studied in respect to their acceptor properties. In contrast, the chemical equivalent nonenzymatic organic synthesis of galacto-sucrose and manno-sucrose has been achieved including six synthetic steps.

1. Introduction

Oligosaccharides such as galacto-oligosaccharides, xylo-oligosaccharides and lactosucrose have been produced in industrial scale[1] and developed as bulking sugar substitutes that have beneficial health effects.[2] For example, the sucrose analogue sucralose has been examined for its usefulness as noncariogenic sweeting agent. It is 600 times sweeter than sucrose and inhibits certain oral bacterial species including *mutans streptococci* (MS).[3] More recently, these compounds have been demonstrated to exhibit immunomodulatory effects on systemic immune response. Thus, the life sciences industry has an increasing demand in oligosaccharides, because these biomolecules have potential application as therapeutics.[4] Some studies have concluded that fucose and mannose appeared to be the most effective of the essential sugars when it came to slowing the growth of cancer cells.[5] Fucose studies are also showing, that it plays a significant role in many diseases, including cancer and its spread and neuron transmission in the brain.[6]

However, the degree of molecular diversity that can be generated from glycosidic linkage assembly is enormous and the synthesis of specific glycosidic linkages is difficult, as carbohydrates are highly functionalized with hydroxyl groups of similar reactivity.[7] To obtain relatively simple oligosaccharides, a wide range of selective protecting-group strategies has to be planed in synthetic routes.[8] In nature, there are hundreds of different enzymes involved in the synthesis of oligosaccharides. We are recently interested in the synthesis of oligosaccharides by enzymes called non Leloir-glycosyltransferases, which utilize the substrate sucrose.[9] The binding energy of substrates, preserved in sucrose analogues, is used in further/subsequent synthesis, as synthons. In our studies, we present the chemical and enzymatic synthesis of the galactose, xylose, mannose and fucose analogues of sucrose.

2. Results and discussion

2.1. Synthetic approach

The chemistry of sucrose is limited due to the eight hydroxyl groups of similar reactivity. Thus, regioselective protection is difficult.[10] For the synthesis, we started a synthetic classical approach and a parallel enzymatic route. Chemical synthesis of sucrose analogues has been studied by Lichtentaler et al.[11] According to their previous work, we got access to the sucrose analogue β-D-fructofuranosyl-α-D-mannopyranoside, which was obtained in 26% overall yield, respectively.

Inspired by this work, a new route for the synthesis of β-D-fructofuranosyl-α-D-galactopyranoside (Gal-Fru) **6** was investigated (Scheme 1). Thus, isopropylidenation of commercially and cheap available sucrose **1** using 2,2′-dimethoxypropane (DMP) afforded 4,6-mono-*O*-isopropylidenesucrose **2** in

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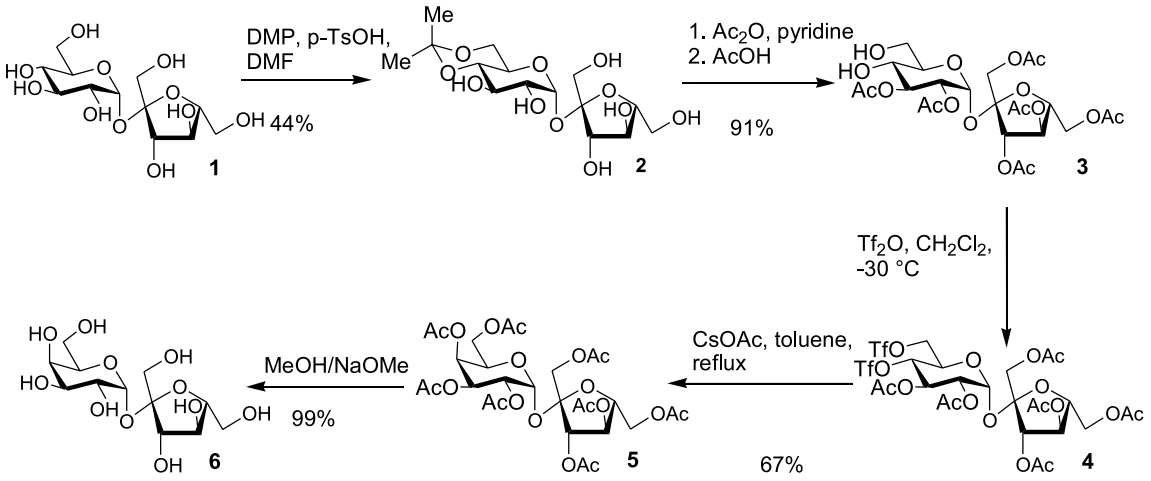


Scheme 1.

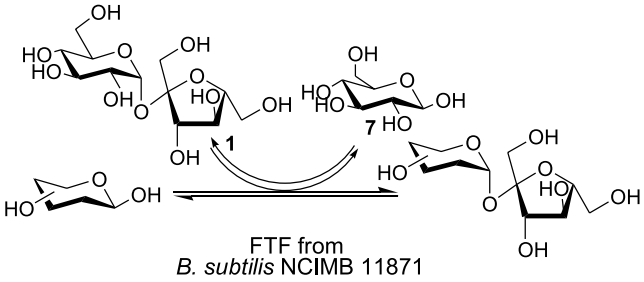
44% yield.[12] Peracetylation, followed by deacetylation using acetic acid, gave 1,3,4,6-tetra-*O*-acetyl-β-D-fructofuranosyl 2,3-di-*O*-acetyl-α-D-glucopyranoside **3** in excellent yield. The free diol was converted in the corresponding ditriflate **4**, which was highly unstable. Thus, refluxing **4** in toluene with caesium acetate gave 1′,2,3,3′,4,4′,6,6′-octa-*O*-acetyl-β-D-fructofuranosyl-α-D-galactopyranoside **5**, which upon deactetylation afforded Gal-Fru **6** in 66% overall yield.

2.2. Enzymatic synthesis

The sucrose analogue synthesis is a time-consuming process, due to the protective group manipulations and the isolation of the intermediates, which decreases overall efficiency. Recently, Römer et al. reported on the synthesis of the sucrose analogue β-D-fructofuranosyl-α-D-xylopyranoside **12** from the donor substrate UDP-α-D-xylose and D-fructose as acceptor by a recombinant sucrose synthase (SuSy1) from potato, respectively.[13] In contrast, in our studies we used an enzyme for a transfructosylation process, which does not require sugar nucleotides, as do all glycosyltransferases of the Leloir pathway, with respect for industrial purposes.

The FTF produced by *Bacillus subtilis* NCIMB 11871[14,15] was tested for its ability to synthesize sucrose analogues by fructosyltransfer from sucrose in the presence of glycopyranosides as in acceptors (Scheme 2). In the presence of D-galactose **8** (400 g/L) and sucrose **1** (400 g/L) the FTF formed the disaccharide Gal-Fru **6**. Optimization of the media and temperature revealed, that the yield of the desired Gal-Fru **6** was maximized at 54%, because an equilibrium is formed,[9] which relies on two transfer reactions: the transfer of the fructosyl residue from sucrose **1** to the acceptor D-galactose **8**, and the reverse reaction the transfer of the fructosyl residue from Gal-Fru **6** to the D-glucose **7**. We also observed the hydrolysis of Gal-Fru **6**. Consequently, the acceptor spectrum for the transfructosylation reaction was expanded. In contrast to D-galactose **8** the acceptor D-mannose **9** demonstrated to be a weak acceptor. The reason should be addressed to its axial position of the hydroxyl group at C-2. Only a maximum yield of 25 g/L manno-sucrose **10** was observed even by variation of the reaction conditions. In addition, the formation of xylosyl-sucrose **12** using D-xylose **11** as acceptor was observed in maximum concentrations of 226 g/L, respectively. The results indicate that the hydroxyl groups of D-glycopyranosides in position 4 and 6 are not crucial for the transfructosylation, in contrast to the position 2. Very recently, Kalovidouris et al. demonstrated that Fuc-α-(1–2)-Gal carbohydrates are capable of modulating neuronal outgrowth and morphology.[16]

This observation prompted us to investigate the acceptor properties of L-fucose **13**. Surprisingly in our studies, the L-fucose **13** was also fructosylated by the enzyme in a concentration of 54 g/L^{-1} (Fig. 1). Because the



Scheme 2.

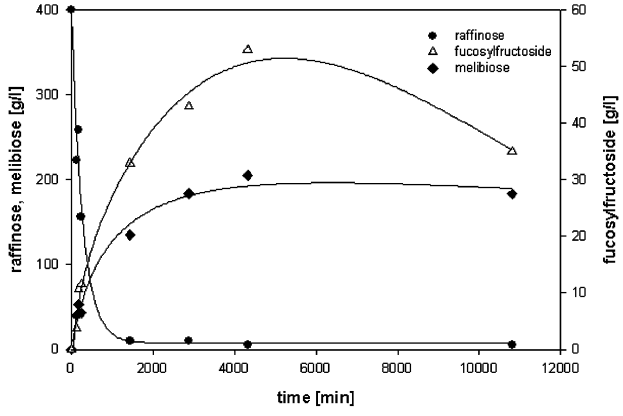
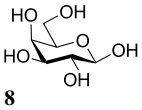
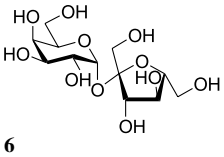
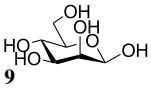
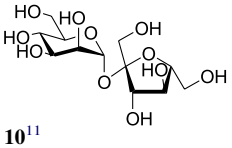
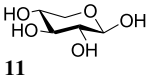
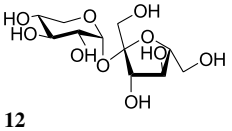
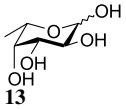
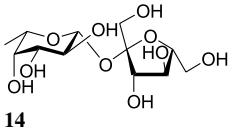


Figure 1.

Table 1. Biocatalytic and chemical synthesis of sucrose analogues

Donor	Product	Organic synthesis	Biocatalysis	
		Yield (%) (synthetic steps)	Yield (%) (enzymatic steps)	Product concentration ($g\,l^{-1}$)
 **8**	 **6**	27 (6)	54 (1)[a]	256
 **9**	 **10**[11]	26 (6)[11]	4 (1)[a]	25
 **11**	 **12**	—	62 (1)[a]	226
 **13**	 **14**	—	21 (1)[b]	53

[a] Yields are calculated from sucrose.
[b] Yields are calculated from raffinose.

fructosylated fucose **14** (Fuc-Fru) had nearly the same polarity as glucose the separation was difficult. Thus, raffinose was used as main substrate for this acceptor, which does not produce glucose, but instead melibiose (Fig. 1). Structural evidence for all sucrose analogues were confirmed by the ^{1}H and ^{13}C NMR spectroscopy. The elucidation of the sucrose analogue structures (galacto-sucrose, xylo-sucrose and fuco-sucrose) were possible only by the combination of all the data acquired from the ^{1}H, ^{13}C and 2D NMR spectra. The doublets at δ_H 5.40 ppm (galacto-sucrose) and 5.36 ppm (xylo-sucrose) exhibited the expected anomeric coupling constants $J_{1,2}$ of 3.9 and 3.6 Hz, characteristic for the anomeric protons of an α-(1-2)-glycosidic linkage. According to ^{1}H NMR spectra we observed, that Fuc-Fru **14** has a β-(1-2)-glycosidic linkage. It is assumed that the L-configuration ($^{1}C_4$) of fucose effects a different orientation of the acceptor in the active side of the FTF. The 2-H resonance of the fucose residue at δ_H 3.45 had $J_{2,1}=8.04$ Hz. In the 2D NMR spectra correlations were observed between H-1 and H-3 of the fructose residue in the ^{1}H, ^{1}H NOESY spectrum, indicating that the fructosyl residue has a β-furanosidic conformation and is bound to fucose through a β-2,1 linkage. The main peaks in the ^{1}H NMR spectrum were assigned using 2D-COSY spectroscopy. It was possible to measure most of the coupling constants. The values observed for the couplings of proton H-3 ($J_{3,2}=9.9$ Hz, $J_{3,4}=3.6$ Hz) showed a fucopyranose residue. The complete interpretation of the ^{13}C spectrum was performed using 2D $^{1}H/^{13}C$ correlation spectroscopy (HMBC, HMQC). Therefore, it can be concluded that the transfructosylation product is a β-D-Fructofuranosyl-β-L-fucopyranoside **14** (Table 1).

In conclusion, we have demonstrated, that a levansucrase from *B. subtilis* NCIMB 11871 is a remarkable catalyst for the synthesis of sucrose analogues. For the production of the oligosaccharide Gal-Fru **6** and further analogues we were able to replace a six step synthetic route (yield 26%) by using this enzyme. The biocatalyst takes just one step and is able to produce a wide repertoire of oligosaccharides, indicating the power of enzymes in oligosaccharide synthesis. Downstream processing for the isolation has been developed.[9]

The application of this biocatalyst in the oligosacchariode synthesis represents an opportunity for the development of industrial chemical and pharmaceutical processes. In addition, sucrose analogues like Gal-Fru, Man-Fru, Xyl-Fru and Fuc-Fru present interesting oligosaccharides, which will be tested in future for biological activity, prebiotic effects and as sweeteners. The structural similarities of the sugars to sucrose may endow them with an ability to inhibit the cariogenicity of sucrose.

3. Experimental

3.1. General

All reactions requiring anhydrous conditions were conducted in flame- or oven-dried apparatus under an atmosphere of Ar. Syringes and needles for the transfer of reagents were dried at 140 °C and allowed to cool in a desiccator over P_2O_5 before use. CH_2Cl_2, toluene and DMF were distilled from CaH_2 under Ar. External reaction temperatures are reported unless stated otherwise. Reactions were monitored by TLC using commercially available plates, precoated with a 0.25 mm layer of silica containing a fluorescent indicator (Merck) and compounds were sprayed with anisaldehyde reagent followed by heating. Organic

layers were dried over $MgSO_4$ unless stated otherwise. Column chromatography was carried out on Kieselgel 60 (40–63 μm). Petroleum ether refers to the fraction with bp 40–60 °C. ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ and D_2O unless stated otherwise using a Bruker AM-400 instrument, operating at 400 MHz for ^1H and at 100 MHz for ^{13}C. Chemical shifts are reported relative to $CHCl_3$ [δ_H 7.26, δ_C (central of triplet) 77.0] or CH_3OH [δ_H 3.35, δ_C (central of septet) 49.0]. Melting points were determined on a Melt-Temp 2 microscope. Electrospray-ionization mass spectra (ESIMS) were recorded with a Finnigan MAT 8340 on samples suspended in CH_3OH. IR spectra in pressed KBr discs were recorded on a Bio-Rad FTS-25 spectrometer. Optical rotation values were measured with a Dr. Kernchen sucromat polarimeter.

The enzymatic reactions were analyzed by high-performance liquid chromatography (HPLC). HPLC was performed with a RCM Monosaccharide Ca^{2+} column (300×7.8 mm, Phenomenex®, Germany) operated at 80 °C and an Ion Chromatograph (IC) (Metrohm, Germany) with refractive index detector (ERC-7512, Erma, Germany), using a refractive index detector and an eluent of bidistilled water at 0.8 ml min^{-1}.

Standard solutions were prepared in the range of 0.1–10 g l^{-1}. The monosaccharides D-fructose, D-galactose, D-glucose, D-xylose, L-fucose, the disaccharide sucrose, melibiose, the trisaccharides raffinose, 1-kestose and the tetrasaccharide nystose were used as external standards for peak identification and quantification. The relative standard deviation of this system is of approx. 3%.

The aliquots from enzymatic reactions were also analyzed using TLC. The solvent system ethylacetate/isopropanol/water in a ratio of 6/3/1 (v/v/v) (rt) was used as mobile phase.

The reaction samples were applied on silica thin-layer plates (TLC aluminium sheets 20×20 cm, silica gel 60 F_{254} with concentrating zone 20×2.5 cm—MERCK, Germany) after appropriate dilution (final concentration between 0.05 and 1 g l^{-1}).

The carbohydrates were separated by using four ascents (4×90 min). Spots were detected by dipping the plates into the detecting reagent (0.3% (w/v) of *N*-(1-naphtyl)-ethylenediamine (Fluka, Germany) and 5% (v/v) concentrated sulfuric acid in methanol using a CAMAG Chromatogram Immersion Device III (speed 2, time 4) (MERCK, Germany), followed by heating in an oven at 120 °C for 15 min. The sugars were visualized as dark spots on a pale pink background. The quantitative determination of the sugars was performed by scanning densitometry (50–2000 ng) using a Bio-Rad Imaging Densitometer utilizing Quantity One® Software (Version 4.2).

3.2. Chemical synthesis of Gal-Fru

3.2.1. 4,6-Mono-*O*-isopropylidensucrose 2. To a stirred solution of sucrose **1** (4.00 g, 11.7 mmol) in DMF (20 ml) was added 2,2-dimethoxypropane (15.0 ml, 122.4 mmol) and catalytic amounts of *para*-toluenesulfonic acid monohydrate (25 mg) at rt. After 2 h the reaction mixture was neutralized with triethylamine and concentrated. Purification by column chromatography (9:1 $CHCl_3$/MeOH, R_f 0.20) gave the title compound as a white solid (2.0 g, 5.2 mmol, 44%).

^1H and ^{13}C NMR spectra data are in accordance with lit.[12]

3.2.2. 1′,2,3,3′,4′,6′-Hexa-*O*-acetylsucrose 3. To a stirred solution of 4,6-mono-*O*-isopropylidensucrose **2** (1.50 g, 3.9 mmol) in pyridine (10 ml) was added acetic anhydride (3.2 ml, 33.3 mmol) at rt. After 12 h methanol (1 ml) was added and evaporated. The residue was added acetic acid (60%, 15 ml). The mixture was stirred at 80 °C for 15 min and concentrated. Purification by column chromatography (1:2 cyclohexane/EtOAc) gave the title compound (2.11 g, 3.5 mmol, 91%) as a colourless oil.

$[\alpha]_D$ +55.0 (*c* 1.0, $CHCl_3$), lit.[17] $[\alpha]_D$ +57.5 (*c* 1.0, $CHCl_3$); R_f 0.20 (1:2 cyclohexane/EtOAc); ^1H NMR (400 MHz, $CDCl_3$) δ 5.63–5.64 (d, $J_{1,2}=3.6$ Hz, 1H, H-1), 5.44–5.46 (d, $J_{3',4'}=6.0$ Hz, 1H, H-3′), 5.37–5.40 (t, $J_{3',4'}=J_{4',5'}=6.0$ Hz, 1H, H-4′), 5.32–5.37 (t, $J_{3,4}=J_{3,2}=9.9$ Hz, 1H, H-3), 4.76–4.79 (dd, $J_{2,1}=3.6$ Hz, $J_{2,3}=9.9$ Hz, 1H, H-2), 4.26–4.30 (dd, $J_{5',6'a}=3.6$ Hz, $J_{5',4'}=8.0$ Hz, 1H, H-5′), 4.11–4.17 (m, 2H, H-1_a′, H-1_b′), 4.20–4.25 (m, 1H, H-6_b′), 4.01 (m, 1H, H-5), 3.89–3.93 (dd, $J_{6a,5}=3.0$ Hz, $J_{6b,a}=8.9$ Hz, 1H, H-6a), 3.80–3.85 (dd, $J_{5',6b'}=4.9$ Hz, $J_{6a',b'}=7.2$ Hz, 1H, H-6b′), 2.10–2.18 (m, 18H, 6OAc), 3.67 (t, $J_{4,3}=J_{4,5}=9.9$ Hz, 1H, H-4). ESIMS: *m*/*z* 617.0 100% [M+Na^+].

3.2.3. 1′,2,3,3′,4,4′,6,6′-Octa-*O*-acetyl-β-D-fructofuranosyl-α-D-galactopyranoside 5. To a stirred solution of 1′,2,3,3′,4′,6′-hexa-*O*-acetylsucrose **3** (1.00 g, 1.68 mmol) in CH_2Cl_2 (50 ml) was added on molecular sieves (4 Å) pyridine (560 μl, 6.9 mmol), followed by trifluoromethanesulfonic anhydride (860 μl, 7.0 mmol) at −30 °C. After 12 h the reaction was quenched by the addition of sat. aqueous $NaHCO_3$ (100 ml). The layers were separated, and the aqueous layer was extracted with CH_2Cl_2 (2×50 ml). The combined organic layers were dried ($MgSO_4$) and filtered. The filtrate was concentrated to afford the triflate **4** (1.31 g, 1.53 mmol, 91%) as a yellow oil, which was used without further purification in the next step.

To a stirred solution of the triflate **4** (1.31 g, 1.53 mmol) in toluene (100 ml) on molecular sieve (4 Å) was added cesium acetate (1.50 g, 7.81 mmol) and tetrabutylammonium acetate (1.50 g, 5.0 mmol) at rt. The suspension was heated at reflux for 2 h. After cooling at rt H_2O (100 ml) was added. The layers were separated, and the aqueous layer was extracted with DCM (3×50 ml). The combined organic layers were washed with brine (1×50 ml), dried ($MgSO_4$), filtered and concentrated. Purification by column chromatography (4:1 diethyl ether/petroleum ether) gave the title compound as a foamy solid (767 mg, 1.13 mmol, 67% overall).

R_f 0.20 (4:1 diethyl ether/petroleum ether); ^1H NMR (400 MHz, $CDCl_3$) δ 5.73–5.72 (d, $J_{1,2}=3.7$ Hz, 1H, 1-H), 5.50–5.48 (d, $J_{3',4'}=6.6$ Hz, 1H, 3′-H), 5.48–5.40 (dd, $J_{5,4}=0.9$ Hz, $J_{5,6}=6.4$ Hz, 1H, 5-H), 5.40–5.36 (t, $J_{3',4'}=J_{4',3'}=6.6$ Hz, 4-H), 5.36–5.32 (dd, $J_{3,2}=11.0$ Hz, $J_{3,4}=3.3$ Hz, 1H,

3-H), 5.18–5.14 (dd, $J_{2,3}=11.0$ Hz, $J_{2,1}=3.7$ Hz, 1H, 2-H), 4.51–4.48 (t, $J=6.60$ Hz, 1H, 5′-H), 4.35–4.05 (m, 7H, 1′-H_2, 4-H, 6′-H_2, 6-H_2). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.46, 170.34, 170.10, 169.93, 169.87, 169.72 (7×*C*OCH$_3$), 103.58 (C-2′), 90.41 (C-1), 78.72 (C-5′), 75.46 (C-3′), 74.63 (C-4′), 68.00 (C-5), 67.47, 67.41, 67.12 (C-2, C-3, C-4), 63.90 (C-1′), 63.08 (C-6′), 61.69 (C-6), 20.67, 20.62, 20.57, 20.54 (CO*C*H$_3$).

3.2.4. β-D-Fructofuranosyl-α-D-galactopyranoside (Gal-Fru) 6. To a stirred solution of **5** (100 mg, 147 μmol) in MeOH (5 ml) was added NaOMe (200 μl of a 5 M solution in MeOH, 1 mmol) dropwise at rt. After 10 min the solution was neutralized to pH 7 with amberlite IR-120 H^+, filtered and concentrated. Purification by column chromatography (6:1 CH_3CN/H_2O) gave the title compound (50.0 mg, 99%) as a white solid.

White solid, mp: 160 °C, lit[18] mp: 174–177 °C; $[\alpha]_D$ +81.2 (*c* 1.0, H_2O), lit.[19] $[\alpha]_D$ +79.0 (*c* 1.0, H_2O); R_f 0.42 (6:3:1 EtOAc/isopropanol/H_2O, 3 ascends); IR (cm^{-1}): 3428, 1132, 1087, 1049, 1017; ^{1}H NMR (400 MHz, D_2O) δ 5.40–5.39 (d, $J_{1,2}=3.9$ Hz, 1H, 1-H), 4.18–4.15 (d, $J_{3',4'}=8.7$ Hz, 1H, 3′-H), 4.11–4.07 (dt, $J_{5,4}=0.9$ Hz, $J_{5,6}=6.4$ Hz, 1H, 5-H), 4.04–4.00 (t, 1H, $J_{3',4'}=J_{4',3'}=8.7$ Hz, 4′-H), 3.99–3.98 (dd, $J_{4,5}=0.9$ Hz, $J_{4,3}=3.20$ Hz, 1H, 4-H), 3.89–3.86 (dd, $J_{3,2}=10.5$ Hz, $J_{4,3}=3.20$ Hz, 1H, 3-H), 3.85–3.76 (m, 3H, 2′-H, 5′-H, 6′-H_2), 3.70–3.68 (t, $J=6.4$ Hz, 2H, 6-H_2), 3.64 (s, 2H, 1′-H_2). ^{13}C NMR (100 MHz, D_2O) δ 106.2 (C-1′), 94.87 (C-1), 83.86 (C-5′), 79.12 (C-3′), 76.72 (C-4′), 73.99 (C-5), 71.70 (C-3), 71.67 (C-4), 70.55 (C-2), 64.93 (C-6′), 64.08 (C-1′), 63.44 (C-6). ESIMS: *m/z* 365.0 100% [M+Na^+].

3.3. Enzymatic synthesis of sucrose analogues

3.3.1. General description of the fructosylation reaction. For the cultivation of *B. subtilis* NCIMB 11871 a liquid mineral salt medium containing 2.5% sucrose (w/v) was prepared. The mineral salt medium contained (in mg/100 ml): KH_2PO_4-136; $Na_2HPO_4\cdot 2H_2O$-267; $(NH_4)_2SO_4$-60; $MgSO_4\cdot 7H_2O$-20; $CaCl_2\cdot 2H_2O$-1; $FeSO_4\cdot 7H_2O$-0.5; $MnSO_4\cdot H_2O$-0.18 and $Na_2MoO_4\cdot 2H_2O$-0.25. Shaken culture was incubated at 30 °C and 150 rpm for 48 h.

When reaching the stationary phase, the cells were separated by centrifugation at 5000×*g* for 15 min at 4 °C (SORVAL® Centrifuge, USA) and then discarded. The supernatant obtained was analyzed undiluted, as crude enzyme solution for the characterisation but also as concentrated solution (ultrafiltration).

To a reaction mixture containing 40% (w/v) sucrose as substrate and 40% (w/v) glycopyranoside as acceptor in 5.0 ml phosphate buffer (pH 6) was added the equivalent volume of FTF supernatant (25 mU FTF per 5.0 ml supernatant). The sucrose analogue formation was investigated by discontinuous analysis of aliquots from the reaction mixture at suitable time intervals up to 48 h.

The enzyme was inactivated by boiling the samples in a water-bath for 10 min. After cooling, the inactivated samples were filtered through a 0.22 μm nitrocellulose membrane filter (Millipore, Germany) and analyzed, after appropriate dilution. Analysis of the samples was carried out using several chromatographic systems.

3.3.2. Preparative chromatography. Prior to preparative chromatographic separation, the sucrose analogue solution was subjected to an enzymatic treatment with a wild type glycosyltransferase (Gtf) from *Streptococcus oralis* cloned in *Escherichia coli*, kindly provided by Dr. Hofer (GBF mbH, Germany). By this step, sucrose was converted into dextran and fructose, which can be separated easily by chromatography. The pH of the crude product solution was adjusted to 5.4 and the reaction was started by adding 1 U Gtf ml^{-1} solution at 30 °C. After 2 h, the reaction was stopped by heat denaturation.

Separation of sucrose analogues from the reaction mixture was carried out with the PCR 6 in Na^+ form (300–330 μm, Purolite, France), packed in a 2 m glass column (∅=3.9 cm) (Borosilicat 3.3, QVF, Germany) and thermostated at 70 °C.

Fifteen millilitre of Gtf (from *S. oralis*) reaction mixture with a total sugar concentration of maximal 400 g l^{-1} was subjected on the column and eluted with a flow rate of 4 ml min^{-1} distilled water. Equal fractions of 12 ml were collected after measurement by differential refractometry.

3.3.3. β-D-Fructofuranosyl-α-D-mannopyranoside (Man-Fru) 10. $[\alpha]_D$ +18.2 (*c* 1.0, H_2O), lit.[11] $[\alpha]_D$ +19.1 (*c* 1.2, H_2O); R_f 0.40 (6:3:1 EtOAc/isopropanol/H_2O, 3 ascends); ^{1}H NMR (400 MHz, D_2O) δ 5.30–5.29 (d, $J_{1,2}=1.9$ Hz, 1H, 1-H), 4.14–4.12 (d, $J_{3',4'}=8.7$ Hz, 1H, 3′-H), 4.02–3.99 (t, $J_{4',3'}=J_{4',5'}=8.7$ Hz, 1H, 4′-H), 3.86–3.67 (m, 9H, 2-H, 3-H, 4-H, 5-H, 6-H_2, 5′-H, 6′-H_2), 3.61 (s, 1′-H_2). ^{13}C NMR (100 MHz, D_2O) δ 106.55 (C-2′), 96.18 (C-1), 83.93 (C-5′), 78.58 (C-3′), 76.51 (C-4′), 75.91, 73.68, 72.70 (C-2, C-3, C-5), 69.02 (C-4), 64.98 (C-6′), 63.55 (C-1′), 63.21 (C-6). ESIMS: *m/z* 365.0 100% [M+Na^+].

3.3.4. β-D-Fructofuranosyl-α-D-xylopyranoside (Xyl-Fru) 12. White solid, mp 120 °C; $[\alpha]_D$ +59.5 (*c* 1.1, H_2O), lit.[20] $[\alpha]_D$ +62 (*c* 1.0, H_2O); R_f 0.46 (6:3:1 EtOAc/isopropanol/H_2O, 2 ascends); IR (cm^{-1}): 3412, 1121, 1046; ^{1}H NMR (400 MHz, D_2O) δ 5.30–5.29 (d, $J_{1,2}=3.6$ Hz, 1H, 1-H), 4.17–4.15 (d, $J_{3',4'}=8.9$ Hz, 1H, 3′-H), 4.07–4.02 (t, $J_{4',3'}=J_{4',5'}=8.9$ Hz, 1H, 4′-H), 3.85–3.81 (dt, $J_{5',4'}=8.9$ Hz, $J_{5',6'}=2.8$ Hz, 1H, 5′-H), 3.78–3.74 (2d, $J_{6a',5'}=J_{6b',5'}=2.8$ Hz, 2H, 6_a′-H, 6_b′-H), 3.68–3.60 (m, 2H, 3-H, 5-H), 3.60 (s, 2H, 1′-H_2), 3.56–3.54 (m, 1H, 4-H), 3.50–3.46 (dd, $J_{2,3}=9.9$ Hz, $J_{2,1}=3.6$ Hz, 1H, 2-H). ^{13}C NMR (100 MHz, D_2O) δ 106.35 (C-2′), 94.97 (C-1), 84.01 (C-5′), 78.83 (C-3′), 76.26 (C-4′), 75.40 (C-3), 73.67 (C-2), 71.82 (C-4), 64.45 (C-6′), 64.38 (C-5), 63.48 (C-1′). ESIMS: *m/z* 335.0 100%, [M+ Na^+].

3.3.5. β-D-Fructofuranosyl-β-L-fucopyranoside (Fuc-Fru) 14. White solid, mp 120 °C; $[\alpha]_D$ −18.8 (*c* 0.6, H_2O); R_f 0.42 (6:3:1 EtOAc/isopropanol/H_2O, 2 ascends); IR (cm^{-1}): 3440, 1117, 1046, 1012; ^{1}H NMR (400 MHz, D_2O) δ 4.74–4.71 (d, $J_{1,2}=8.0$ Hz, 1-H), 4.20–4.16 (m, 1H, 4′-H), 4.18–4.16 (d, $J_{3',4'}=7.8$ Hz, 1H, 3′-H), 3.87–3.84 (m, 1H, 5′-H), 3.82–3.77 (m, 2H, 6_a′-H, 5-H), 3.73–3.70 (m, 2H,

$6_b'$-H, 4′-H), 3.68–3.65 (d, $J_{1a'}=12.6$ Hz, 1H, $1'_a$-H), 3.64–3.60 (dd, $J_{3,2}=9.9$ Hz, $J_{3,4}=3.6$ Hz, 1H, 3-H), 3.60–3.57 (d, $J_{1'b}=12.6$ Hz, 1H, $1'_b$-H), 3.48–3.43 (d, $J_{2,1}=8.0$ Hz, $J_{2,3}=9.9$ Hz, 1H, 2-H), 1.21–1.20 (d, $J_{6,5}=6.6$ Hz, 3H, 6-H_3). ^{13}C NMR (100 MHz, D_2O) δ 106.60 (C-2′), 98.28 (C-1), 84.17 (C-5′), 78.65 (C-3′), 75.21 (C-4′), 75.13 (C-3), 73.79 (C-5), 73.69 (C-4), 72.74 (C-2), 63.51 (C-1′, C-6′), 17.98 (C-6). ESIMS: *m/z* 349.0 100%, [M + Na^+].

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