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Identification of new acceptor specificities of Glycosyltransferase R via the aid of substrate microarrays

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Abstract

Finding possibilities to construct sugar-motifs, transfer them to targets of biological relevance and rapid identification of glycosylation events are important goals for glycobiology and a field of raising interest. Here we applied an enzyme microarray screening system for the identification of new acceptor specificities of the glycosyltransferase R (GTFR) from *Streptococcus oralis* (E.C. 2.4.1.5), which was able to display the synthesis of sugar-motifs in short time and with low amounts of substrate. These observations led to the development of a convenient α-glycosylation by the non-Leloir glycosyltransferase GTFR with sucrose as substrate and different alcohols and amino acid derivatives as acceptors for the synthesis of glycoether and glycosylated amino acids, which were not observed by familiar GTFs with high sequence homology.

Introduction

In most biological systems, sugar motifs are an important part of cellular interaction processes. Every cell is coated by a layer of glycoproteins, glycolipids and glycobinding proteins, which play major roles in recognition events between cells and proteins, or other cells, viruses or bacteria. The understanding of those interactions and the synthesis of biological relevant carbohydrate structures[1] are major challenges of modern glycobiology which could lead to a deeper inside to cellular events and the development of biological active drugs like anti-cancer or anti-inflammatory ones.[2,3]

Glycosyltransferases

Conventional approaches for the synthesis of oligosaccharides, glycolipids, glycopeptides and glycoconjugates may not be the solution, especially when considering the practicality of large-scale synthesis. Reactions catalyzed by Leloir type glycosyltransferases exhibit high stereo- and regiocontrol, however both enzymes and the sugar nucleotides are expensive, and not always accessible. Thus, development of simple and convenient methods for oligosaccharide synthesis is an emerging field. A convenient pathway for the synthesis of oligo- (OS) and polysaccharides (PS) is based on sucrose as the substrate using non-Leloir type glucosyl- (GTF) and fructosyltransferases (FTF)[4]. Glucosyltransferases (E.C. 2.4.1.5; glucansucrases) are extracellular enzymes from oral *Streptococci*, *Leuconostoc mesenteroides*, and certain *Lactobacilli* that are capable to transfer glucose from sucrose to different acceptors. Two different reactions can be distinguished: a) hydrolysis, that means transfer to water as acceptor, and b) glucosyl transfer, which can be subdivided in 1) polymerisation, when a glucan-chain is formed, and 2) acceptor reactions, when other acceptors, i.e.

oligosaccharides, are used. Their products include dextrans of different structural specificity (α-1,6-bound glucose (Glc) backbone, with α-1,2, α-1,3 side chains)[5,6], mutan (α-1,3 bound Glc units)[7], alternan (α-1,6 and α-1,3 bound Glc units)[8,9] and amylose (α-1,4 bound Glc units)[10].

Enzymes of the GTF type (glucansucrases[11]), such as dextransucrases[12], alternansucrases[13] and amylosucrases[14], exhibit high sequence and structural similarities with a highly conserved catalytic core region of about 900 amino acids that is a cyclically permuted version of the $(\beta/\alpha)_8$ barrel[15], classified in family GH70[16] and found in the amylase superfamily[17] (GH13).

The GTFR[18] from *streptococus oralis* synthesizes from sucrose **1** dominantly dextran **3** (86%) (scheme 1). Glucose **4**, being the product of the acceptor reaction of water, was also found in small amounts 7%. Two acceptor products from fructose **2** were always present in small proportions; leucrose **5** (O-α-D-Glucopyranosyl-(1→5)-D-glucopyranoside, 6%) and isomaltulose **6** (O-α-D-Glucopyranosyl-(1→6)-D-fructofuranoside) approx. 1%, which is in accordance with the Dextransucrase from *L. mesenteroides* B-512F.[19] Here we demonstrate new acceptor reactions of the GTFR from *streptococus oralis* on enzyme microarrays for glycosylation of immobilized OS, the synthesis of glycoether and glycopeptides.

((scheme 1))

Results and Discussion

While immobilization, detection and transformation processes of DNA/RNA, proteins and peptides are well published, only a few approaches for immobilizing sugars or constructing sugar-arrays are known.[20] Fukui *et al.* arrayed neoglycolipids on nitrocellulose membranes,[21] while Wang *et al.* demonstrated that carbohydrates can also be spotted directly on nitrocellulose-coated glass slides without covalent conjugation.[22] Adams *et al.* recently coupled different synthesized mannosyl oligosaccharides, i.e. triantennary *N*-linked mannoside $(Man)_9(GlcNAc)_2$ and evaluated their biological properties on microarrays.[23] Most of the published work focussed on the immobilization of already synthesized carbohydrate structures and identification of sugar-binding or recognizing proteins, including lectins and antibodies. Jobron *et al.* reported the measuring of glycosyltransferase activity using acceptors immobilized on membrane.[24]

We aimed at identification of new glycosylation events and pathways for the synthesis of oligosaccharides, glycolipids and glycopeptides on enzyme microarrays by the variation of enzymes, acceptors and substrates.

Immobilization of sugars at NH-presenting microtiterplates

According to Fazio *et al.* we used the cycloaddition between azides and alkynes to immobilize lactose and maltose to microtiter-plates.[25] We started with 6-Azidohexyl-4-*O*-α-D-glucopyranosyl-β-D-glucopyranoside (**10**), which was prepared from β-maltose octaacetate (**7**) in 28% yield (Scheme 2). The thermal 1,3-dipolar copper(I)-catalyzed cycloaddition between **10** and propynoic acid afforded the regiospecific triazole 1-[-4-*O*-α-D-glucopyranosyl-β-D-glucopyranosyl)-hexyl]-1*H*-[1,2,3]triazole-4-carboxylic acid (**11**) in 45% yield. **11** was coupled in different concentrations (0.2 to 10 mM) to amino

functionalized microtiter plates (covalink, Nunc, approx. 0.5 nmol binding sites per well) with standard conditions using *N*-hydroxysuccinimide (NHS) and 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC). Each well was blocked with 0.5% bovine serum albumin (BSA) before immobilized maltose was measured by binding of the fluorescein (FITC) labeled lectin concanavalin A (ConA)(figure 1). ConA is a glucose/mannose specific lectin,[26] so it showed a clear maltose-concentration dependend signal (figure 1). The signal strength reaches a plateau at 10 mM incubation concentration, implicating that all 0.5 nmol binding sites were used for coupling, so we chosed a corresponding concentration (11 mM) for further experiments.

((Scheme 2))

((Figure 1))

Enzymatic glycosylation on microtiter plates

To evaluate the scope of the GTFR[18], we used our arrays to measure a series of different acceptors, including immobilized maltose, lactose and a primary aliphatic alcohol.

((Scheme 3))

Maltose is known to be an excellent acceptor for glycosyltransferases of the glucansucrase type, like dextransucrase,[27] in contrast to primary alcohols which were also chosen as acceptors. However, the glycosylation of alcohols with dextransucrase has not been reported so far. Prior to reactions the wells were blocked with 0.5% polyvinylpyrrolidone K30 (PVPK30) to inhibit sticking of dextran formed to the surface. Reactions were initiated by adding a solution of the enzyme in phosphate buffer containing sucrose (73 mM) in each microtiter well. Successful glycosylation was detected with FITC-labeled ConA after

blocking (figure 2). One array was measured without acceptors to provide the reference rates for the non specific binding of the polysaccharide which is formed in solution (scheme 1) as natural product (negative controls). Reactions were followed over ten hours by removing the reaction mixture at different time points. After incubation of FITC-labeled ConA, the fluorescence was measured using the corresponding microtiter plate reader instrument. The signal strength rised for maltose as an acceptor, which showed a much higher signal than maltose without enzymatic reaction and an empty well without acceptor in the range of 2-10h. Goldstein *et al.* described an two fold increase of ConA binding specifity to the trisaccharide panose (*O*-α-D-Glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→4)-α,β-D-glucopyranoside) compared to maltose[28], so we interpret the rise of the signal as a glycosylation of maltose. In contrast immobilized lactose was not glycosylated. The signal was even lower than that of the negative control, presumably due to less unspecific binding of ConA. For dextransucrase of *Leuconostoc mesenteroides* B-512F (DSRS) it is known that the acceptor product of lactose forms a Glu-α-(1,2)-Glu bond.[29] If the transfer of GTFR is homolog, the glycosylation is assumed to be blocked due to steric reasons by the linker, what would declare the absence of the glycosylation signal of lactose on the array (figure 2).

((Figure 2))

Interestingly, the immobilized alcohol showed the highest signal, indicating glycosylation of this acceptor.
However, the recorded signals could not be converted into product concentrations by using calibration curves, or for the calculation of reaction rates, because the signal intensity is a function of binding affinity between ConA and the oligosaccharides on the microtiter plate.

With the results of the array, further experiments were done to characterize the GTFR.

Acceptor reactions and sequence alignment with familiar GTFs

GTFs found in *Leuconostoc* sp., *Streptococcus* sp. and *Lactobacillus* sp. belong to family 70 of glycoside hydrolase enzymes (GH70). They share four highly conserved catalytic domains, a *N*-terminal signal peptide is followed by a *N*-terminal variable region, the catalytic core and the *C*-terminal region with a putative glucan-binding domain. Until now, no X-ray structure of a GTF could be produced, but modelling revealed homologies to enzymes[14a] of amylase superfamily[17] (GH13), so that a putative structure is available.

Mutagenesis experiments which were done with enzymes of family GH70 lead to the identification of three amino acids which are essential for catalytic activity and corrospond to known sequences of family GH13[15,30]. In sequence alignment (figure 3) we identified those three amino acids in GTFR: D516 (putative catalytic nucleophile), E554 (putative acid/base catalyst) and D627 (putative transition state stabilizer). This identification of the active site is important for further modelling and mutation experiments in order to change the donor specificity.

((Scheme 4))

Other important regions whith relevance for acceptor-specificity were identified in the dextransucrase DSRS, so Kralj *et al.* reported that the three amino acids following D662 (DSRS) are important for the structure of the acceptor product. Mutation of this motive "NNS" of reuteransucrase GTFA to the motive "SEV" of DSRS changed the enzyme function for glucan production from reuteran (α-1→4) to dextran (α-1→6)[31]. The "SEV" motive of DSRS can be also found in GTFR,

implicating a dextran (α-1→6) formation. However, this motive doesn't determine an enzyme to dextran-formation and (α-1→6) transfer, because GTFB[32] and GTFI[33] both show the "SEV" motive, but synthesise mutan (α-1→3). The sequence of the GTFR shows a very high homology to GTFG (90%) and DSRS (45%), differences can be found mostly in the variable region. It is not known which kind of glucan is formed by GTFR, also the polymer is partly soluble, which should be dextran with low side chain density, huge amounts of insoluble polymers are formed as well.

((Figure 3))

As a consequence from the array results and the sequence alignments studies of acceptor reactions were performed in solution.

Our first acceptor studies with maltose (**12**) identified the GTFR forming *O*-α-D-Glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→4)-α,β-D-glucopyranoside (**13,** panose) and the further glycosylated product *O*-α-D-Glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→4)-α, β-D-glucopyranoside (**14**) in 15% and 11% yield, respectively, with dextran (**3**) and glucose (**4**) formed as by-products. The α-(1,6)-linked acceptor products (scheme 5) are characteristic in their ^{1}H-NMR spectra (figure 4), where the doublets at δ_H 4.95 ppm (**13, 14**) exhibited the expected anomeric coupling constants $J_{1'',2''}$ and $J_{1''',2'''}$ of 3.5, are characteristic for the anomeric protons of an α-(1-6)-glycosidic linkage. The results of the product formation with a α-(1-6)-glycosidic linkage correspond with the observation of Krajl *et al.*[27], where the sequence homology of their defined acceptor binding sequence motive exists between 629-631 (SEV).

((Scheme 5))

((Figure 4))

Glycosylation of primary alcohols

The identification of the glycosylation of the immobilized primary alcohol promped us to investigate different alcohols as acceptors in solution to form glycoether. Activity and stability of dextransucrase DSRS in the presence of organic solvents including methanol has previously studied, but no glycosylation of methanol was observed.[34] The stability of the GTFR was tested with different concentrations of methanol (**15**) and 1-propanol (**19**) from 0 to 80% (figure 5).

((Figure 5))

Methanol (**15**) was chosen in a 9:1 water:methanol mixture as acceptor and sucrose (**1**) as substrate. The reaction was followed by TLC and the formation of methyl-α-D-glucopyranoside (**16**) observed in 13% yield, respectively (figure 6).

((Figure 6))

We found that a broad range of different primary alcohols are exclusively alpha glycosylated by the GTFR to form ethyl-α-D-glucopyranoside (**18**) (4.2% yield), propyl-α-D-glucopyranoside (**20**) (1.4%) and butyl-α-D-glucopyranoside (**22**) (1.3%). Surprisingly also chloroderivates, like 2-chloroethanol (**23**, 4.8%), 4-chlorobutanol (**25**, 0.6%) and 6-chlorohexanol (**27**, 0.1%) were glycosylated (figure 7, table 1). The yields went down rapidly with growing chain length. This can be explained by the lower solubility of longer alcohols in water and by the rapid inactivation of the enzyme in presence of extended chain alcohols. The main product formed was dextran(**3**)(spot at R_f = 0 in figure 7), leucrose (**5**) being formed as a side product. We

also observed that the rate of sucrose reaction decreases as the amount of alcohol increases. Comparison of GTFR and DSRS demonstrated that DSRS is not able to catalyze the acceptor reaction with aliphatic alcohols, only methanol (**15**) was detected to be glycosylated in low yields (<0.1%) (figure 8, table 1). This shows a major difference between the two enzymes, stating that the known "SEV" motive which seems to be important for the acceptor-reaction and is shared by both enzymes couldn't be used to determine the acceptor-spectrum.

((Figure 7))

((Table 1))

((Figure 8))

Glycosylation of amino acid-derivatives

More than half of all the proteins in the human body have carbohydrate molecules attached. Posttranslational modification is widely used by nature to modify the folding, stability, activity, and interactions of proteins, playing a central role in cell signaling, development, immunology, and many other important biological processes. The only way to obtain glycopeptides and glycoproteins of defined structure in sufficient amounts is through chemical and enzymatic synthesis. The synthesis of glycopeptides and glycoproteins from readily available components is therefore an important goal. Lui *et al.* recently described a method for preparing glycoprotein mimetics that contain an unnatural amino acid which can be glycosylated.[35] These products mimic naturally glycosylated proteins in order to have an easy way to examine the effect of glycosylation on the structure or function of proteins and to develop glycosylated therapeutics, such as certain antibodies, cytokines, erythropoietin, and other medicines.[36] We recently reported about the microwave assisted synthesis of *O*-linked β-glycosylated amino acids. But even with

simple glycosyl donors the exclusive formation of α-glycosidic linkage to Ser or Thr is difficult, often proceeding in low yields giving a mixture of the α- and β-anomers.[37,38] Thus the enzymatic synthesis is an attractive alternative to the total chemical synthesis. Usually preformed glycosyl–amino acid building blocks are applied in solution- or solid-phase strategies for the synthesis of glycopeptides. In previous studies Cantacuzene *et al.* have demonstrated that β-galactosidase from *E. coli* catalyzes the synthesis of galactopyranosyl-L-serine derivatives from lactose.[39] As a consequence of the alcohol acceptor studies, *N*-(tert-butoxycarbonyl)-D-serine methyl ester (**29**), *N*-(tert-butoxycarbonyl)-L-serine methyl ester (**31**) and *N*-(tert-butoxycarbonyl)-L-threonine methyl ester (**33**) were investigated as acceptors for the transglycosylation (scheme 6, figure 9, table 2).

((scheme 6))

Surprisingly the GTFR discriminates between D and L configuration. While the glycosylation from sucrose (**1**) (290 mM) was observed with *N*-(tert-butoxycarbonyl)-D-serine methyl ester (**29,** 117 mM) in 16% (7.2 g/L) forming *N*-tert-butoxycarbonyl-3-O-α-D-glycopyranosyl-D-serine methyl ester (**30**), the acceptor reaction with *N*-(tert-butoxycarbonyl)-L-serine methyl ester (**31,** 117 mM) and *N*-(tert-butoxycarbonyl)-L-threonine methyl ester (**33,** 117 mM) were performed in 1.5% (0.7g/L) and <0.1% yield (figure 8, table 2). The DSRS did not glycosylate *N*-(tert-butoxycarbonyl)-D-serine methyl ester (**29**) in significant amounts.

((Figure 9))

((Table 2))

Conclusion

At the moment carbohydrate research is mainly driven by studies of the biological role of carbohydrates, called glycomics. These high complex and diverse biopolymers are present in nature for example as glycolipids and glycopeptides. Their rich structural diversity allows them to have very specific and selective interactions with other molecules, but the chemical synthesis of specific glycosidic linkages is difficult, as carbohydrates are highly functionalized with hydroxyl groups of similar reactivity. One of the challenges is to generate synthetic and enzymatic tools for the design of pure and defined carbohydrate structures. Thus, it is necessary to combine different synthetic investigations with glycobiology. Here we demonstrate with a few examples that the enzymatic synthesis of different carbohydrate structures can be performed on arrays and lead to the identification of new properties of glycosyltranserases, which were not observed previously and can not be concluded by sequence alignment. We also demonstrated the convenient α-glycosylation by GTFR with sucrose and different alcohols and amino acid derivatives for the synthesis of glycoether and glycosylated amino acids, which are not easy to obtain by chemical or enzymatic synthetic methods. These products can be used for solid phase synthesis to generate glycopeptides. Studies on the enzymatic synthesis of glycopeptides are ongoing. The study does also indicate that further substrates like sucrose analogues can be applied to this array. Our aim is to extend this synthetic pathway to the transfer of other sugars than Glc, i.e. to galactose (Gal), fucose (Fuc), mannose (Man) etc. For this purpose sucrose analogues, β-D-Fructofuranosyl-α-D-galactopyranoside, β-D-Fructofuranosyl-α-D-mannopyranoside, β-D-Fructofuranosyl-β-L-fucopyranoside (Gal-Fru, Man-Fru, Fuc-Fru), have been synthesised.[4a] New variants of the GTFR will be screened with sucrose-analogues on the

presented acceptors array format using ConA (glucose, mannose) and other lectins.

Experimental Section

Materials and analytical methods:

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). For preparative size exclusion chromatography, samples were fractionated in water with a biogel P-2 column (fine) (40 cm x 3 cm). Petroleum ether refers to the fraction with bp 40–60 °C. ^{1}H 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 ^{1}H 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.

Enzymatic reactions were analyzed using TLC and HPLC according to previous protocols.[4] For preparative TLC chromatograpy, the samples were spotted to a TLC plate (20x20 cm, silica gel 60 F_{254}, 1mm, MERCK, Germany) and separated in the solvent system ethylacetate/isopropanol/water in a ratio of 6:3:1 (v/v/v) or acetonitrile/water (v/v) (8:2). Part of the plate was developed and isolated by scratching.

Synthesis of acceptors for arrays:

6-Chlorohexyl-*O*-2,3,6-tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl)-β-D- glucopyranoside (8)

To a stirred solution of maltose octaacetate **7** (24.00 g, 35.0 mmol) and chlorohexanol (5.80 g, 42.5 mmol) in dichloromethane (200 mL) on molecular sieve 4Å was added $BF_3 \cdot OEt_2$ (48% solution in OEt_2, 52.3g, 177 mmol) in 30min at 0°C dropwise. The suspension was stirred at rt for 18h, followed by the addition of saturated sodium bicarbonate solution (50 mL). The layers were separated, and the aqueous layer was extracted with CH_2Cl_2 (3 x 100 mL). The combined organic layers were washed with brine (1 x 50 mL), dried (Na_2SO_4), filtered and concentrated. Purification by column chromatography (50:1 CH_2Cl_2/MeOH) gave the title compound **8** as a white solid (11.20 g, 42%).

mp: 93°C; $[\alpha]_D$ +62.5 (c 1.0, $CHCl_3$); R_f 0.33 (diethyl ether/petroleum ether 4:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ=5.41 (d, *J*=4.0 Hz, 1H; H-1''), 5.39-5.34 (dd, *J*=10.1, 10.4 Hz, 1H; H-3''), 5.27-5.23 (t, *J*=8.8 Hz, 1H; H-3'), 5.08-5.02 (t, *J*=10.1 Hz, 1H; H-4''), 4.88-4.84 (dd, *J*=4.0, 10.4 Hz, 1H; H-2''), 4.83-4.79 (dd, *J*=9.6, 8.1 Hz, 1H; H-2'), 4.52 (d, *J*=8.1 Hz, 1H; H-1'), 4.50-4.46 (dd, *J*=3.0, 12.1 Hz, 1H; H-6_b''), 4.27-4.21 (m, 2H; H-5'', H-4'), 4.08-3.95 (m, 3H; 1-H_2, 6_a''-H), 3.88-3.82 (m, 1H; 6_b'-H), 3.70-3.65 (m, 1H; H-5'), 3.57-3.45 (m, 1H; 6_a'-H), 3.54-3.51 (t, *J*=6.6 Hz, 2H; 6-H_2), 2.14, 2.10, 2.04, 2.03, 2.02, 2.00 (6s, 21H; -CH_3), 1.81-1.54 (m, 4H; 2-H_2, 5-H_2), 1.49-1.30 (m, 4H; 3-H_2, 4-H_2). ^{13}C NMR (100 MHz, $CDCl_3$) δ=170.49, 170.48, 170.42, 170.21, 169.91, 169.54, 169.37 (7 ester CO), 100.26 (C-1'), 95.49 (C-1''), 75.41 (C-3'), 72.76 (C-4'), 72.19 (C-5'), 72.07 (C-2'), 69.97 (C-2''), 69.84 (C-1), 69.32 (C-3''), 68.45 (C-5''), 68.02 (C-4''), 64.31 (C-1), 62.84 (C-6'), 61.49 (C-6''), 44.89 (C-6), 32.44, 32.41, 29.18, 26.45 (C-2, C-3, C-4, C-5), 20.88, 20.80, 20.64, 20.60, 20.56,

20.53 (7 C*C*H_3). ESI-MS: *m/z*: calcd for $C_{32}H_{47}ClO_{18}$ 777.2 $[M+Na]^+$, found 777.1 $[M+Na]^+$.

6-Azidohexyl-*O*-2,3,6-tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl)-β-D- glucopyranoside (9)

To a stirred solution of the chloride **8** (11.20 g, 14.8 mmol) in DMF (300 mL) was added sodium azide (7.10 g, 109 mmol) at rt. The suspension was heated at 60° C for 24h. After cooling at rt $CHCl_3$ (300 mL) and H_2O (300 mL) was added. The layers were separated, and the aqueous layer was extracted with CH_2Cl_2 (3 x 100 mL). The combined organic layers were washed with brine (1 X 50 mL), dried (Na_2SO_4), filtered and concentrated. Purification by column chromatography (50:1 CH_2Cl_2/MeOH) gave the title compound **9** as a white solid (8.10 g, 72%).

mp: 78°C; $[\alpha]_D$ +56.5 (c 1.0, $CHCl_3$); R_f 0.32(diethyl ether/petroleum ether 4:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ=5.35 (d, *J*=4.0 Hz; 1H, H-1''), 5.32-5.27 (dd, *J*=9.9, 10.5 Hz, 1H; H-3''), 5.21-5.16 (t, *J*=9.2 Hz, 1H; H-3'), 5.01-4.96 (t, *J*=9.9 Hz, 1H; H-4''), 4.81-4.77 (dd, *J*=4.0, 10.5, 1H; H-2''), 4.76-4.72 (t, *J*=7.9 Hz, 1H; H-2'), 4.44 (d, *J*=7.9 Hz, 1H; H-1'), 4.43-4.39 (dd, *J*=2.8, 12.2 Hz, 1H; H-6_b''), 4.20-4.14 (m, 2H; H-5'', H-4'), 4.07-3.89 (m, 3H; 1-H_2, H-6_a''), 3.81-3.74 (m, 1H; H-6_b'), 3.62-3.58 (m, 1H; H-5'), 3.44-3.38 (m, 1H; H-6_a'), 3.20-3.17 (t, *J*=6.8 Hz, 2H; H_2-6), 2.07, 2.03, 1.98, 1.96, 1.95, 1.93 (7s, 21H; -CH_3), 1.53-1.50 (m, 4H; 2-H_2, 5-H_2), 1.34-1.17 (m, 4H; 3-H_2, 4-H_2). ^{13}C NMR (100 MHz, $CDCl_3$) δ=170.45, 170.43, 170.37, 170.17, 169.87, 169.49, 169.33 (7 ester CO), 100.22 (C-1'), 95.46 (C-1''), 75.36 (C-3'), 72.73 (C-4'), 72.15 (C-5'), 72.05 (C-2'), 69.93 (C-2''), 69.80 (C-1), 69.28 (C-3''), 68.41 (C-5''), 67.98 (C-4''), 64.25 (C-1), 62.80 (C-6'), 61.46 (C-6''), 51.26 (C-6), 29.16, 28.69, 26.30, 25.33 (C-2, C-3, C-4, C-5), 20.84, 20.76, 20.60, 20.56, 20.52, 20.49 (7 C*C*H_3). ESI-MS *m/z*: calcd for $C_{32}H_{47}N_3O_{18}$: 784.2 $[M+Na]^+$, found 784.2 $[M+Na]^+$.

6-Azidohexyl-4-*O*-α-D-glucopyranosyl-β-D-glucopyranoside (10)

To a stirred solution of **9** (3.81 g, 5.0 mmol) in MeOH (40 mL) was added NaOMe (3 mL of a 5 M solution in MeOH, 15 mmol) dropwise at rt. After 5 min the solution was neutralized to pH 7 with amberlite IR-120 H^+, filtered and concentrated. Purification by column chromatography (6:3:1 EtOAc-Isopropanol-methanol) gave the title compound **10** (2.2 g, 92 %) as a colorless oil.

R_f = 0.62 (EtOAc/Isopropanol/MeOH 6:3:1, 3 ascends); $[\alpha]_D$ = +59.0 (c = 1.1, H_2O); ^{1}H NMR (400 MHz, D_2O) δ=5.17 (d, J=3.8 Hz, 1H; 1''-H), 4.29-4.27 (d, J=7.8 Hz, 1H; 1'-H), 3.98-3.80 (m, 4H; 1-H_a, 6'-H_a, 6''-H_2), 3.69-3.60 (m, 3H; 3'-H, 6'-H_b, 3''-H), 3.57-3.48 (m, 2H; 1-H_b, 4'-H), 3.48-3.45 (dd, J=9.6, 3.8 Hz, 1H; 2''-H), 3.40-3.36 (ddd, J=9.6, 4.4, 2.0 Hz, 1H; 5'-H), 3.32-3.28 (m, 1H; 4'-H), 3.31-3.27 (t, J=6.8 Hz, 2H; 6-H_2), 3.26-3.21 (dd, J=9.3, 7.8 Hz, 1H; 2'-H), 1.65-1.55 (m, 4H; 2-H_2, 5-H_2), 1.43-1.36 (m, 4H; 3-H_2, 4-H_2). ^{13}C NMR (100 MHz, D_2O) δ=104.19 (C-1'), 102.72 (C-1''), 81.11 (C-4'), 77.87 (C-5''), 77.79 (C-3'), 76.48 (C-5'), 75.00 (C-3''), 74.66 (C-2'), 74.03 (C-2''), 71.42 (C-4''), 70.72 (C-1), 62.65 (C-6'), 62.15 (C-6''), 52.35 (C-6), 30.53 (C-2), 29.77 (C-5), 27.53 (C-4), 26.55 (C-3). ESI-MS *m/z*:, calcd for $C_{18}H_{33}N_3O_{11}$: 490.2 $[M+Na]^+$, found 490.1 $[M+Na]^+$.

1-[-4-*O*-α-D-glucopyranosyl-β-D-glucopyranosyl)-hexyl]-1*H*-[1,2,3]triazole-4-carboxylic acid (11)

10 (1.00 g, 2.0 mmol) and the propynoic acid (444 mg, 6.0 mmol) were dissolved in MeOH (30 mL) and traces of CuI (10 mg) added. The solution was heated at 60 °C for 6h and concentrated. Purification by column chromatography (6:3:1 EtOAc-Isopropanol-methanol) gave the title compound **11** (650 mg, 45 %) as a white solid.

mp 52°C; R_f = 0.07 (EtOAc/Isopropanol/MeOH 6:3:1, 3 ascends); $[\alpha]_D$ = +12.5 (c = 1.1, H_2O); ^{1}H NMR (400 MHz, D_2O) δ=8.50 (sb, 1H; 5-H), 5.32 (d, J=3.9 Hz, 1H; 1’’’-H), 4.43-4.40 (t, J=6.8 Hz, 2H; 6’-H), 4.36-4.34 (d, J=8.0 Hz, 1H; 1’’-H), 3.96-3.80 (m, 10H; 1’-H_2, 6’’-H_2, 5’’-H, 4’’-H, 3’’-H, 6’’’-H_2, 3’’’-H, 2’’’-H), 3.37-3.32 (t, J=9.5 Hz, 1H; 4’’-H), 3.21-3.19 (dd, J=9.2, 8.0 Hz, 1H; 2’’-H), 1.87-1.81 (m, 2H; 5-H_2), 1.55-1.39 (m, 2H; 2-H_2), 1.34-1.15 (m, 4H; 3-H_2, 4-H_2). ^{13}C NMR (100 MHz, D_2O) δ=178.94 (COOH), 144.90 (C-4), 131.98 (C-5), 104.64 (C-1’’), 102.24 (C-1’’’), 79.57 (C-4’’), 76.85 (C-5’’’), 77.11, 75.58, 75.44, 75.27, 74.27 (C-5’’, C-3’’, C-2’’, C-3’’’, C-2’’’), 72.81 (C-1’), 71.92 (C-4’’’), 64.16 (C-6’’), 63.09 (C-6’’’), 53.45 (C-6’), 33.62, 31.69 (C-2’, C-5’), 27.84, 26.96 (C-4’, C-3’). ESI-MS *m/z*:, calcd for $C_{21}H_{35}N_3O_{13}$: 560.2 $[M+Na]^+$, found 560.0 $[M+Na]^+$.

Acceptor reactions with GTFR

Fermentation of GTFR

Strains of *Escherichia coli* TG1 harbouring the vector pTH-275 (Fujiwara et al.)[18] for the expression of the *gtfr* gene encoding the glycosyltransferase R from *Streptococcus oralis* were used for expression, which was performed as follow. Cryoculture (10 µl) was given to LB-media (10 mL) that contained ampicilline (LB-Amp, 100 mgL^{-1}). This preculture was incubated 24 h at 37°C.

Preculture (2 mL) was transferred to LB-Amp (500 mL) and the culture was incubated 24h at 37°C

The cells were collected by centrifugation, washed and suspended in Soerensen buffer (20 mL, 100 mM Na_2HPO_4, 735 mM KH_2PO_4, 17 mM phosphate, final solution pH 7.0). Cells were disrupted by sonication (50% of 4 minutes at 120 W).

Microarray experiments

Coupling of acceptors to microtiter plates

A solution of **11** (or Lactose-analogue) (11 mM in H_2O, 100 µL) or 12-hydroxydodecanic acid (11 mM in DMSO, 100 µl) was added per well in Covalink NH plates. The reaction was started by adding of a NHS solution (13 mM in DMSO, 50 µl) and of an EDC solution (37 mM in DMSO, 50 µl) and shaken 12h at rt. Afterwards, the solvent was removed and the wells were washed with water.

Kinetics of GTFR with immobilized maltose and immobilized hydroxydodecanic acid

Covalinkwells were blocked with 0.5% PVP K30 in water overnight at 4°C prior reaction. After washing with H_2O (2 x 100 µl), sucrose (73 mM, 100 µl) in Soerensen buffer (pH 7.0) was added in each well with immobilized acceptor. The reaction was started by adding GTFR (34 UL^{-1}, 100 µl) in Soerensen buffer. The wells were incubated at a shaker at 37°C and stopped at different time points by removing the liquid with a pipette, followed by a washing step with qH_2O (200 µL). After the last reaction was stopped, the wells were washed three times with 200 µl qH_2O, followed by intensive washing with warm water.

Lectin-staining of sugars in microtiterplates

Blocking buffer (200 µL, PBS (50 mM), containing 0.5% BSA, 1 mM $MnCl_2$ and 1 mM $CaCl_2$) was added to the acceptor-binding 96-well plate, and the plate was incubated for 1h at rt.
Wells were then incubated with FITC-labeled ConA (200 µL, 20 μgmL^{-1}, CalBioChem) in blocking buffer for 1h, followed by

washing with blocking buffer (3x200 µl). The fluorescence was read at 485 nm extinction and 535 nm exication.

Enzymatic Glycosylation in solution

Enzymatic synthesis of *O*-α-D-Glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→4)-α, β-D-glucopyranoside (13)

To a reaction mixture containing sucrose (292 mM) (**1**) as substrate and maltose (292 mM) (**12**) as acceptor in Soerensen buffer was added the GTFR (110 UL^{-1}). The product formation was investigated by discontinuous analysis of aliquots from the reaction mixture at suitable time intervals up to 24h. The product was isolated by size exclusion chromatography.

R_f = 0.20 (acetonitrile/water 8:2, 2 ascends);

β-anomer:

^{1}H NMR (400 MHz, D_2O) δ=5.39 (d, J=4.0 Hz, 1H; 1'-H), 4.95 (d, J=3.5 Hz, 1H; 1''-H), 4.65-4.63 (d, J=7.8 Hz, 1H; 1-H), 3.99-3.91 (m, 2H; 6_a-H, 6_a'-H), 3.89-3.78 (m, 1H; 6_a''-H), 3.78-3.71 (m, 3H; 3-H, 6_b-H, 6_b''-H), 3.71- 3.65 (m, 1H; 6_b'-H,), 3.71-3.65 (m, 3H; 3'-H, 2''-H, 3''-H), 3.65-3.58 (m, 3H; 4-H, 5-H, 5''-H), 3.57, 3.53 (dd, J=9.85, 4.0 Hz, 1H; 2'-H), 3.57-3.53 (m, 1H; 5'-H), 3.51-3.46 (t, J=9.1 Hz, 1H; 4''-H), 3.44-3.40 (t, J=9.6 Hz, 1H; 4'-H), 3.29-3.25 (dd, J=9.4, 7.8, 1H; 2-H).
^{13}C NMR (100 MHz, D_2O) δ=102.42 (C-1'), 100.71 (C-1''), 98.40 (C-1), 79.80 (C-4), 78.80 (C-3), 77.20 (C-5), 76.61 (C-2), 75.71 (C-3', C-3''), 74.44 (C-2''), 74.25 (C-5''), 74.10 (C-5'), 73.95 (C-2'), 72.17 (C-4''), 72.04 (C-4'), 68.55 (C-6'), 63.12 (C-6''), 63.45 (C-6).

α-anomer:

^{1}H NMR (400 MHz, D_2O) δ=5.39 (d, J=4.0 Hz, 1H; 1'-H), 5.22 (d, J=3.8 Hz, 1H; 1-H), 4.95 (d, J=3.5 Hz, 1H; 1''-H), 3.99-3.91 (m, 3H; 2-H, 3-H, 6_a'-H), 3.89-3.78 (m, 2H; 6-H_2, 6_a''-H), 3.78-3.71 (m, 1H; 6_b''-H), 3.71- 3.65 (m, 1H; 6_b'-H), 3.71-3.65

(m, 4H; 5-H, 3′-H, 2′′-H, 3′′-H), 3.65-3.58 (m, 2H; 4-H, 5′′-H), 3.57, 3.53 (dd, *J*=9.85, 4.0 Hz, 1H; 2′-H), 3.57-3.53 (m, 1H; 5′-H), 3.51-3.46 (t, *J*=9.1 Hz, 1H; 4′′-H), 3.44-3.40 (t, *J*=9.6 Hz, 1H; 4′-H). ^{13}C NMR (100 MHz, D_2O) δ=102.33 (C-1′), 100.71 (C-1′′), 94.53 (C-1), 80.03 (C-4), 77.78 (C-5), 75.81 (C-3), 75.71 (C-3′, C-3′′), 74.44 (C-2′′), 74.25 (C-5′′), 74.10 (C-5′), 73.91 (C-2′), 72.59 (C-2), 72.17 (C-4′′), 72.04 (C-4′), 68.55 (C-6′), 63.12 (C-6′′), 63.31 (C-6).
ESI-MS *m/z*:, calcd for $C_{18}H_{32}O_{16}$: 527.2 $[M+Na]^+$, found 527.2 $[M+Na]^+$.

_O_-α-D-Glucopyranosyl-(1→6)-_O_-α-D-glucopyranosyl-(1→6)-_O_-α-D-glucopyranosyl-(1→4)-α, β-D-glucopyranoside (14)

R_f = 0.10 (acetonitrile/water 8:2, 2 ascends);

β-anomer:

^{1}H NMR (400 MHz, D_2O) δ=5.39 (d, J=3.5 Hz, 1H; 1′-H), 4.95 (2d, J=3.5, 3.5 Hz, 2H; 1′′-H, 1′′′H), 4.64-4.62 (d, *J*=7.8 Hz, 1H; 1-H), 4.00-3.79 (m, 9H; 3-H, 6-H_2, 6′-H_2, 6′′-H_2, 6′′′-H_2), 3.71- 3.65 (m, 6H; 4-H, 3′-H, 2′′-H, 3′′-H, 2′′′-H, 3′′′-H), 3.64-3.60 (t, *J*=8.8 Hz, 1H; 5-H), 3.57-3.53 (m, 4H; 2′-H, 5′-H, 5′′-H, 5′′′-H), 3.52-3.47 (m, 2H; 4′′-H, 4′′′-H), 3.43-3.39 (t, *J*=9.4 Hz, 1H; 4′-H), 3.28-3.24 (dd, J=9.4, 7.8 Hz, 1H; 2-H).

α-anomer:

^{1}H NMR (400 MHz, D_2O) δ=5.39 (d, *J*=3.5 Hz, 1H; 1′-H), 5.21 (d, *J*=3.8 Hz, 1H; 1-H), 4.95 (2d, *J*=3.5, 3.5 Hz, 2H; 1′′-H, 1′′′H), 4.00-3.79 (m, 10H; 2-H, 3-H, 6-H_2, 6′-H_2, 6′′-H_2, 6′′′-H_2), 3.71- 3.65 (m, 7H; 4-H,5-H, 3′-H, 2′′-H, 3′′-H, 2′′′-H, 3′′′-H), 3.57-3.53 (m, 4H; 2′-H, 5′-H, 5′′-H, 5′′′-H), 3.52-3.47 (m, 2H; 4′′-H, 4′′′-H), 3.43-3.39 (t, *J*=9.4 Hz, 1H; 4′-
ESI-MS *m/z*:, calcd for $C_{24}H_{42}O_{21}$: 689.2 $[M+Na]^+$, found 689.2 $[M+Na]^+$.

Glycosylation of primary alcohols in solution and isolation of acceptor products

Sucrose (29 mM) and an appropriate amount of alcohol were treated with GTFR (500 UL^{-1}) in Soerensen buffer (pH 7) and reacted until all sucrose was consumed. For methanol and ethanol, 10% of alcohol was used. For higher alcohols, the amount of alcohol was reduced to 5% and the amount of GTFR (up to 5 kUL^{-1}) increased due to the inactivation of the enzyme. The products were isolated by size exclusion chromatography.

Methyl-α-D-glucopyranoside (16)

R_f = 0.28 (EtOAc/Isopropanol/H_2O 6:3:1); ^{1}H NMR (400 MHz, D_2O) δ=4.78 (d, J=3.8 Hz, 1H; 1-H), 3.87-3.84 (dd, J=12.3, 2.3 Hz, 1H; 6b-H), 3.76-3.72 (dd, J=12.3, 5.5 Hz, 1H; 6a-H), 3.67-3.58 (m, 2H; 5-H, 4-H), 3.56-3.52 (dd, J= 9.8, 3.8 Hz, 1H; 2-H), 3.40 (s, 3H; OCH_3), 3.40-3.36(m, 1H; 3-H). ^{13}C NMR (100 MHz, D_2O) δ=101.90 (C-1), 75.75, 74.21 (C-2, C-3, C-4, C-5), 63.24 (C-6), 57.67 (OCH_3).
ESI-MS m/z:, calcd for $C_7H_{14}O_6$: 217.1 $[M+Na]^+$, found 217.1 $[M+Na]^+$.

Ethyl-α-D-glucopyranoside (18)

R_f = 0.36 (EtOAc/Isopropanol/H_2O 6:3:1); ^{1}H NMR (400 MHz, D_2O) δ=4.91 (d, $J_{1,2}$ 3.8 Hz, 1H; 1-H), 3.86-3.83 (dd, J=12.4, 2.5 Hz, 1H; 6b-H), 3.80-3.74 (m, 1H; 6a-H), 3.72-3.66 (m, 2H; 5-H, 4-H), 3.60-3.55 (q, J=7.1 Hz, 2H; CH_3CH_2O), 3.55-3.52 (dd, J=9.9, 3.8 Hz, 1H; 2-H),), 3.41-3.36(dd, J=9.9, 9.1 Hz, 1H; 3-H), 1.23-1.20 (t, J=7.1 Hz, 3H; CH_3CH_2O).
ESI-MS m/z:, calcd for $C_8H_{16}O_6$: 231.1 $[M+Na]^+$, found 231.0 $[M+Na]^+$.

2-Chloro-ethyl-α-D-glucopyranoside (24)

R_f = 0.37 (EtOAc/Isopropanol/H_2O 6:3:1); ^1H NMR (400 MHz, D_2O) δ=4.99 (d, $J_{1,2}$ 3.8 Hz, 1H; 1-H), 3.88-3.68 (m, 8H; 4-H, 5-H, 6-H_2, 1'-H_2, 2'-H_2), 3.59-3.56 (dd, J=9.9, 3.8 Hz, 1H; 2-H),), 3.44-3.39(dd, J=9.9, 9.1 Hz, 1H; 3-H).

ESI-MS m/z:, calcd for $C_8H_{15}ClO_6$: 265.0 $[M+Na]^+$, found 265.0 $[M+Na]^+$.

Glycosylation of amino acids

Sucrose (290 mM) and *N*-tert-butoxycarbonyl-D-serine methyl ester (**29**), *N*-tert-butoxycarbonyl-L-serine methyl ester (**31**), *N*-tert-butoxycarbonyl-L-threonine methyl ester (**33**) (117 mM) were treated with GTFR (1000 UL^{-1}) in Soerensen buffer and reacted until all sucrose was consumed.

***N*-tert-butoxycarbonyl-3-*O*-α-D-glycopyranosyl-D-serine methyl ester (30)**

R_f = 0.55 (EtOAc/Isopropanol/H_2O 6:3:1); $[\alpha]_D$ = 20° (c = 1.1, H_2O); ^1H NMR (400 MHz, D_2O) δ=4.91 (d, J=3.8 Hz, 1H; 1-H), 4.53-4.51 (t, J=3.3 Hz, 1H; 2-H), 3.89-3.78 (m, 4H; 4-H, 5-H, 6-H_2), 3.81 (d, J=3.3 Hz, 2H; 3-H2), 3.76 (s, 3H, OCH_3), 3.57-3.54 (dd, J=3.8, 9.9 Hz, 1H; 1-H), 3.44-3,39 (dd, J=9.9, 8.1 Hz, 1H; 1-H), 1.45 (s, 12H).

ESI-MS m/z:, calcd for $C_{15}H_{27}NO_{10}$: 404.2 $[M+Na]^+$, found 404.2 $[M+Na]^+$.

Acknowledgement

Financial support from the Sonderforschungsbereich 578 "From Gene to Product" is gratefully acknowledged.

Tables, schemes, figures

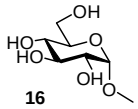
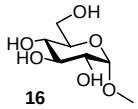
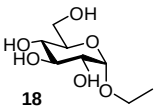
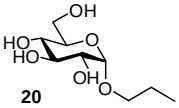
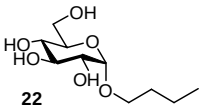
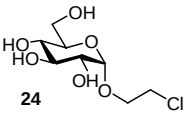
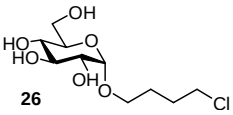
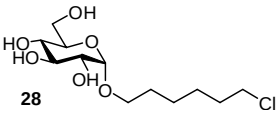
Acceptor	Product	Yield [%][a]	Enzyme	Product concentration (gL^{-1})
15 CH_3OH	 **16**	13.0	GTFR	0.74
15 CH_3OH	 **16**	<0.1	DSRS	n.d.
17 OH	 **18**	4.2	GTFR	0.25
19 OH	 **20**	1.4	GTFR	0.1
21 OH	 **22**	1.3	GTFR	0.1
23 Cl OH	 **24**	4.8	GTFR	0.3
25 Cl OH	 **26**	0.6	GTFR	0.04
27 Cl OH	 **28**	0.1	GTFR	0.01

Table 1: Glycosylation of primary alcohols catalyzed by GTFR (17.4 kUL^{-1}), alcohol (5-10%), sucrose (10 gL^{-1}).

[a] yields are calculated from sucrose.

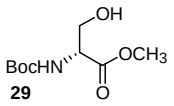
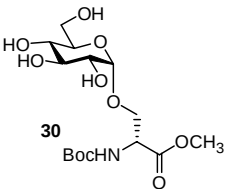
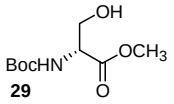
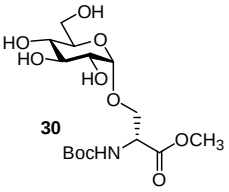
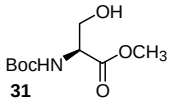
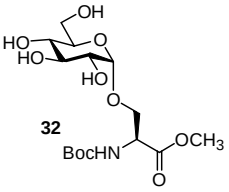
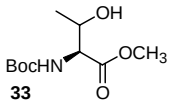
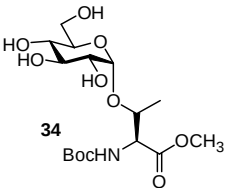
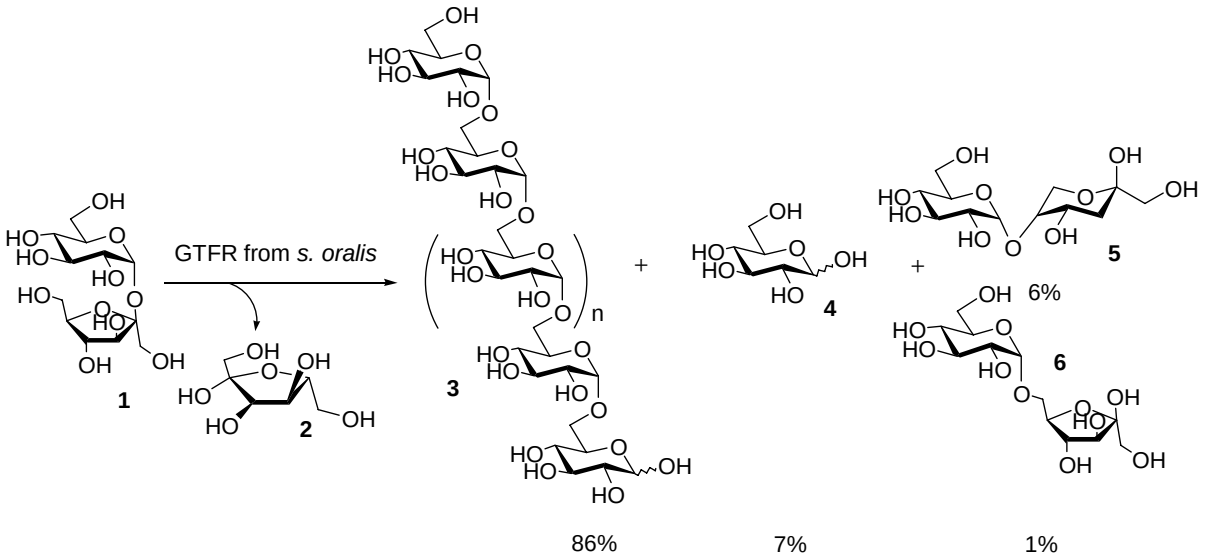
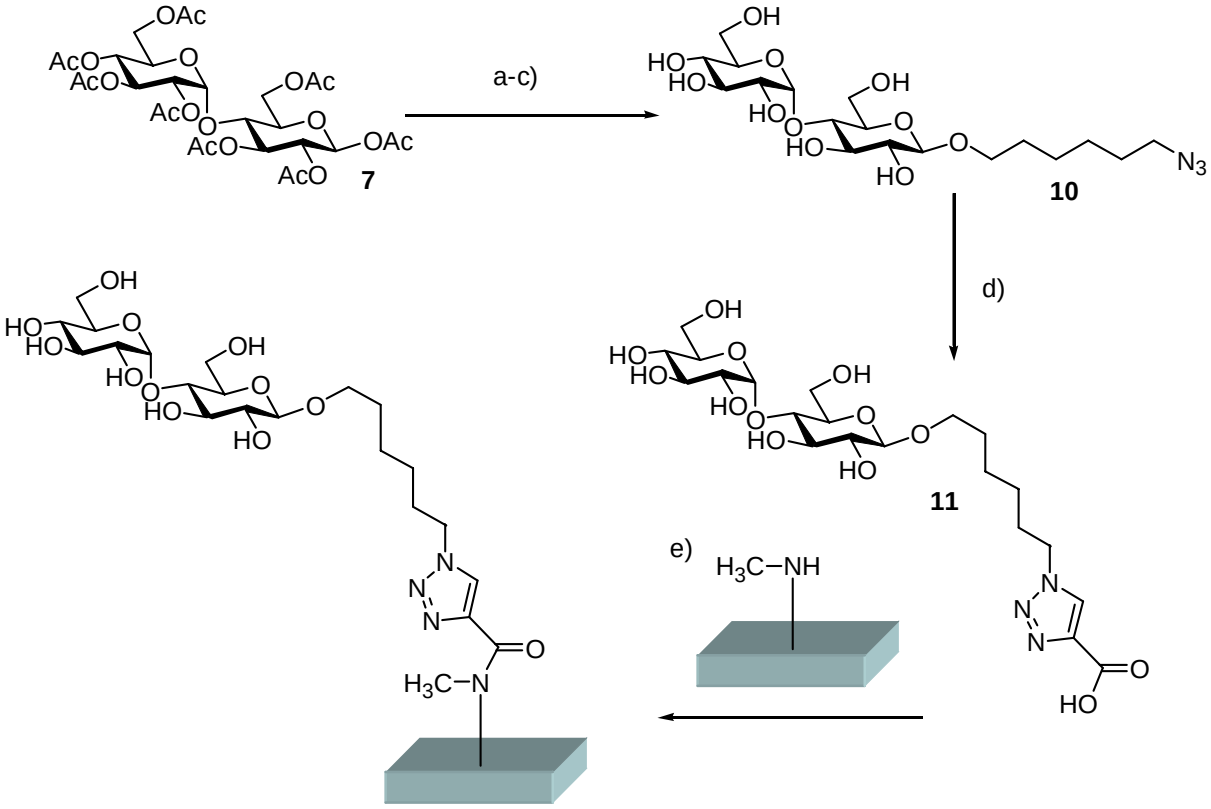
Acceptor	Product	Yield [%][a]	Enzyme	Product concentration (gL^{-1})
 **29**	 **30**	16.1	GTFR	7.2
 **29**	 **30**	<0.1	DSRS	n.d.
 **31**	 **32**	1.5	GTFR	0.74
 **33**	 **34**	<0.1	GTFR	n.d

Table 2: Glycosylation of amino acid derivatives.

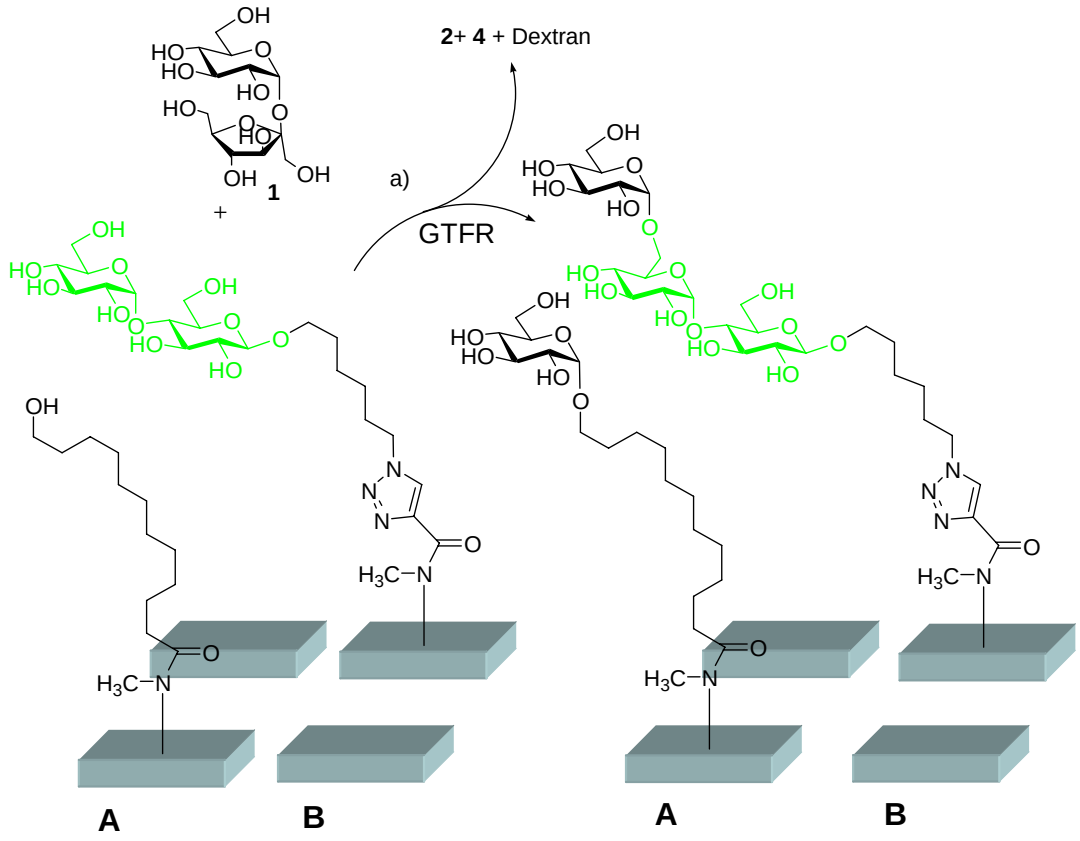
[a] yields are calculated from the acceptors.



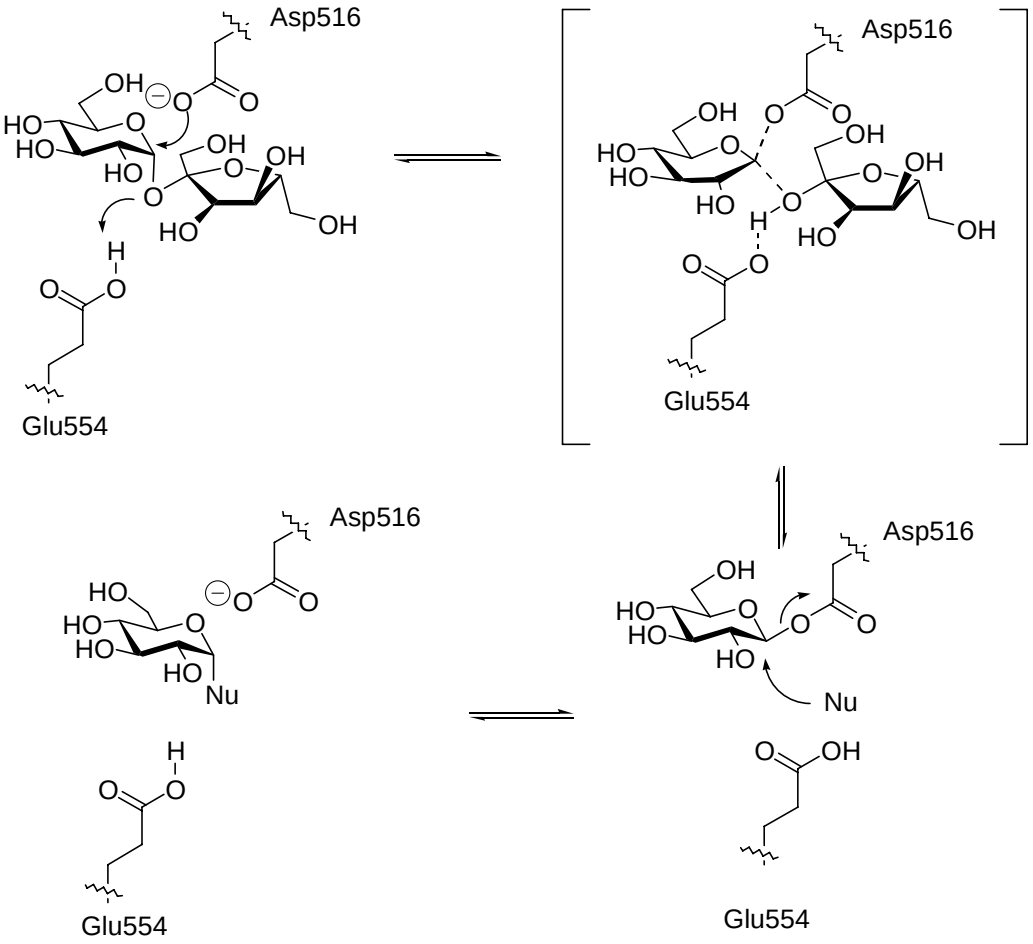
Scheme 1: The product formed from sucrose **1** (25 gL^{-1}) by GTFR (110 UL^{-1}) is dominantly dextran **3**. Glucose **4,** leucrose **5** and isomaltulose **6** are by-products.



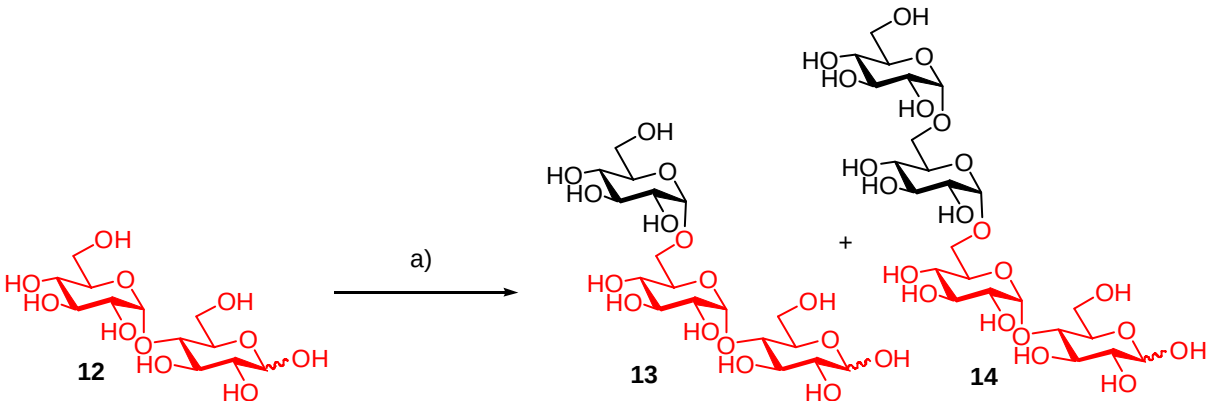
Scheme 2: Synthesis and immobilization of maltose derivate **11**: a) 6-chlorohexanol, $BF_3 \cdot OEt_2$, CH_2Cl_2, 0°C, 30 min; rt, 18h, 42%; b) NaN_3, DMF, 60°C, 24h, 72%; c) MeOH/MeONa, rt, 5 min, 92%; d) propionic acid, CuI, MeOH, reflux, 6h, 45%; e) NHC, EDC, H_2O, 12h, rt.



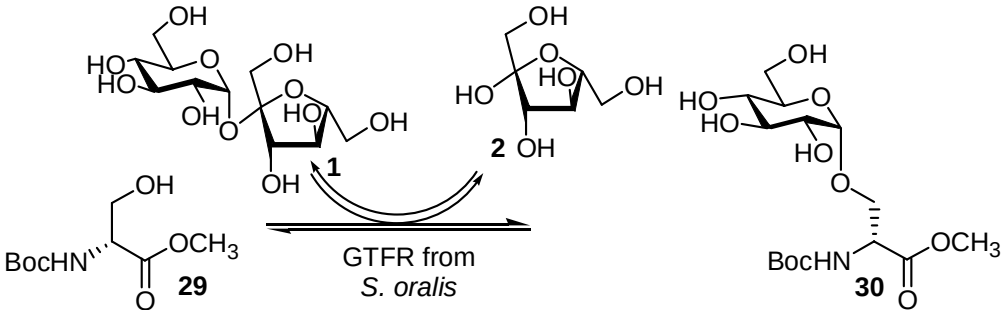
Scheme 3: Enzymatic glycosylation of different acceptors on arrays a) sucrose (73 mM), Soerenson buffer, GTFR (17 UL^{-1}).



Scheme 4: Proposed mechanism of the GTFR: Double displacement provides the retention of the configuration.



Scheme 5: Formation of panose (**13**): a) sucrose (292mM) (**1**), maltose (292mM) (**12**), GTFR (110 UL^{-1}), 12h, 15% (**13**), 11% (**14**).



scheme 6: Formation of **30:** a) sucrose (292 mM) (**1**), **29**(117 mM), GTFR (1.0 kUL^{-1}), 24h.

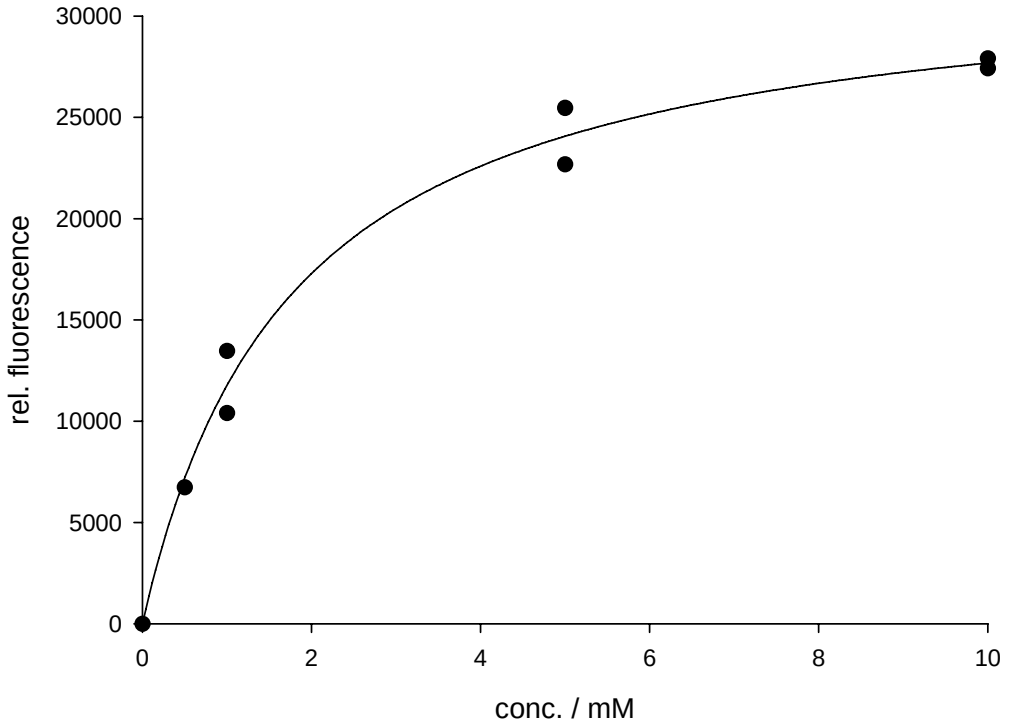


Figure 1: Fluorescence of FITC-labeled ConA as a function of maltose concentration used for immobilization.

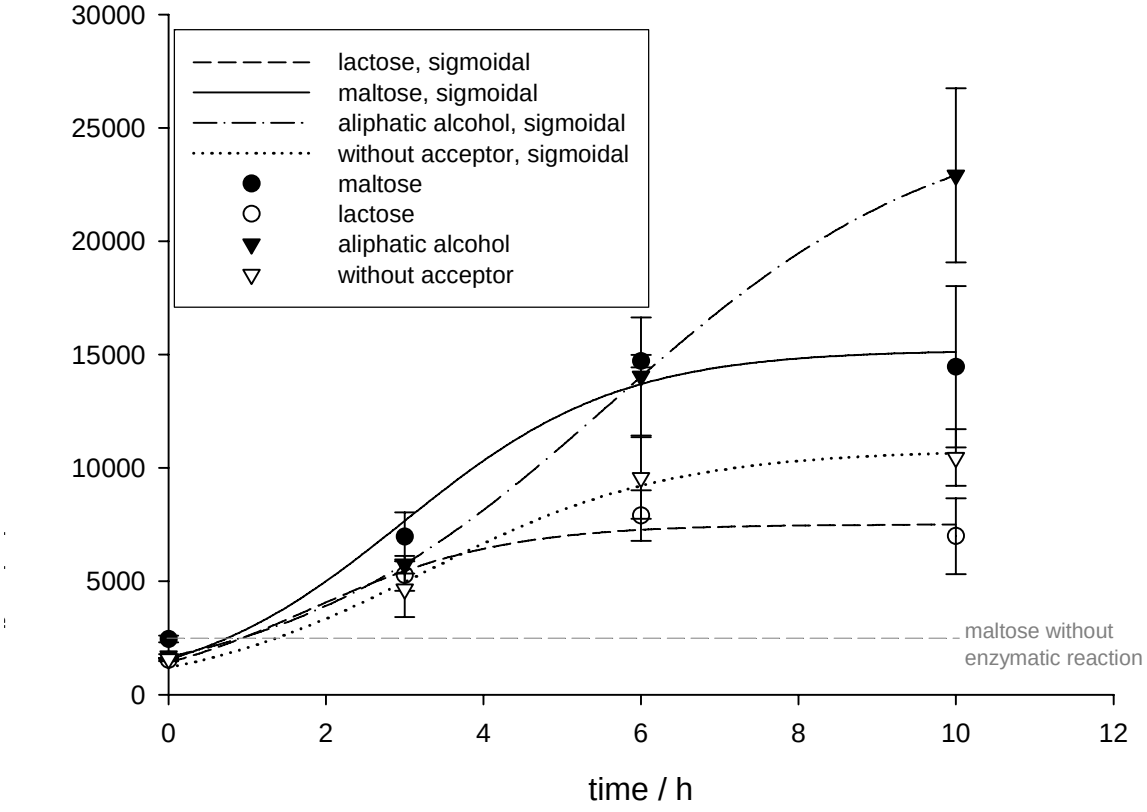


Figure 2: The microtiter plate arrays were monitored with glucose specific lectin ConA, identifying novel acceptor products derived from sucrose with the GTFR. Acceptor: approx. 0.5 nmol, sucrose: 7.3 µmol, GTFR: 3.4 mU.

```
GTFR          508   ANFDGVRVDAVDNVNADLLQI 549 HLSILEAWSDNDPDY 620 YIFVRAHDSEVQTVI
DSRS   (1-6)  543   ANFDGIRVDAVDNVDADLLQI 584 HLSILEDWSHNDPLY 655 YSFVRAHDSEVQTVI
GTFG          510   ANFDGVRVDAVDNVNADLLQI 551 HLSILEAWSDNDPDY 622 YIFVRAHDSEVQTVI
GTFJ   (1-3)  463   ANFDGIRVDAVDNVDADMLQL 504 HISVLEAWSLNDNHY 605 YVFIRAHDNNVQDII
GTFI   (1-3)  445   ANFDSIRVDAVDNVDADLLQI 486 HLSILEAWSDNDTPY 557 YSFIRAHDSEVQDLI
GTFB   (1-3)  443   ANFDSIRVDAVDNVDADLLQI 484 HLSILEAWSDNDTPY 555 YSFIRAHDSEVQDLI
GTFA   (1-4) 1016   ANFDSVRVDAPDNIDADLMNI1056 HINILEDWNHADPEY1126 YSFVRAHDNNSQDQI
GTFASEV(1-6)                                              1126 YSFVRAHDSEVQDQI
```

Figure 3: Sequence alignment of familiar GTFR in the catalytic domain. (GTFR: *S. oralis* ATCC 10557[18], DSRS: *Ln. mesenteroides* NRRL B-512F, GTFG: *S.gordonii* U12643[18], GTFJ: *S.salivarius* ATCC 25975[40], GTFI: *S. downei* Mfe28[29], GTFB: *S.mutans* GS5[28], GTFA: *Lb.reuteri* 121[27], GTFASEV: *Lb.reuteri* 121: mutated[27])

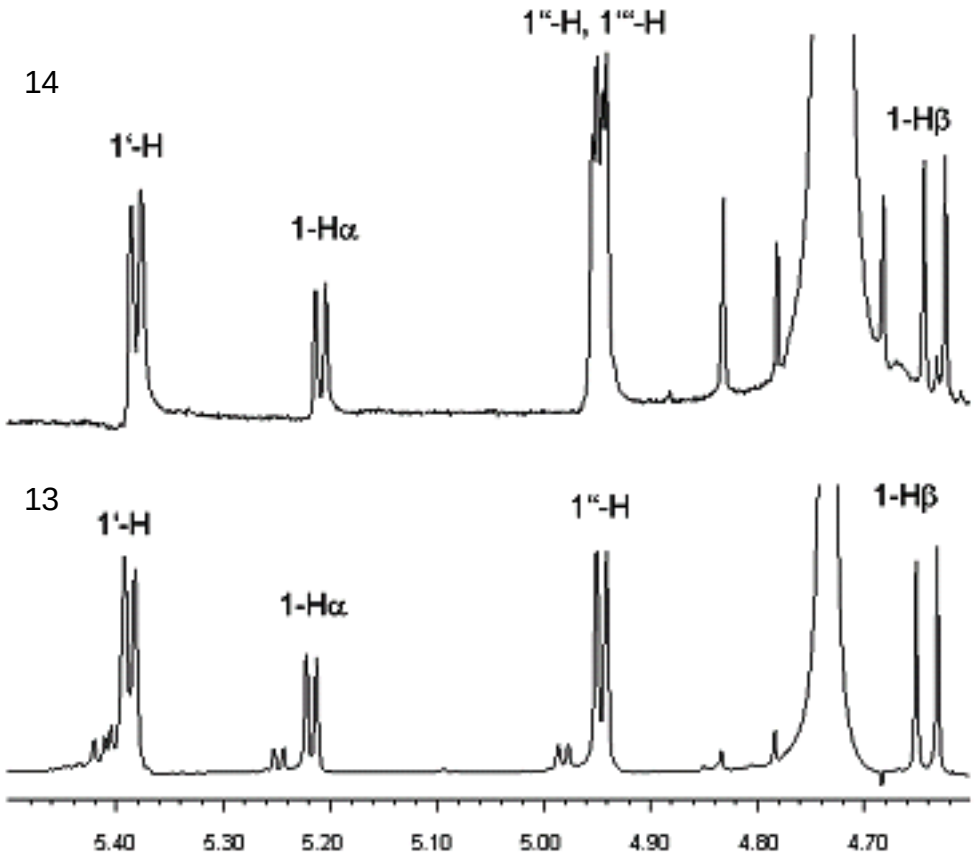


Figure 4: Comparison of ^{1}H-NMR spectra of (**13**) and (**14**).

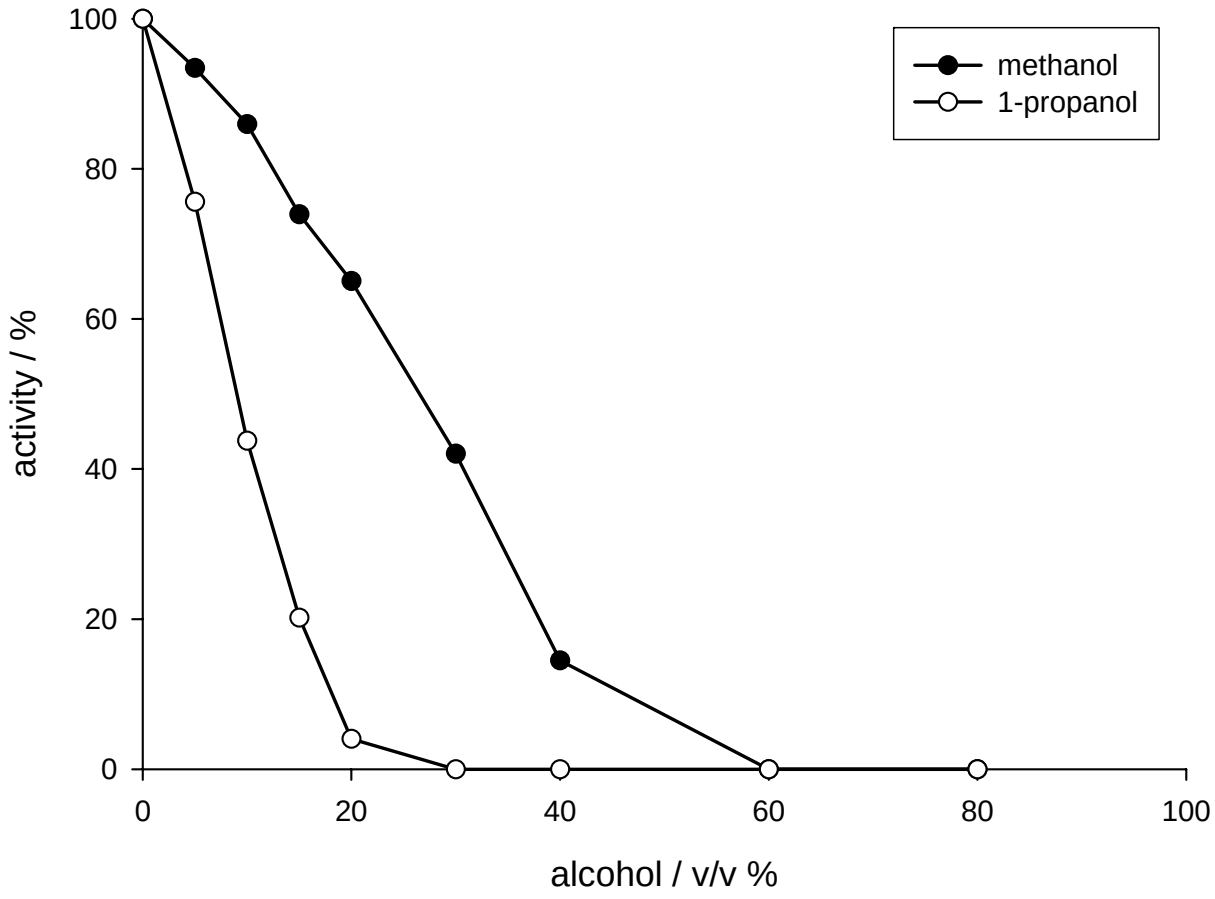


Figure 5: Activity of GTFR with different concentrations of alcohol.

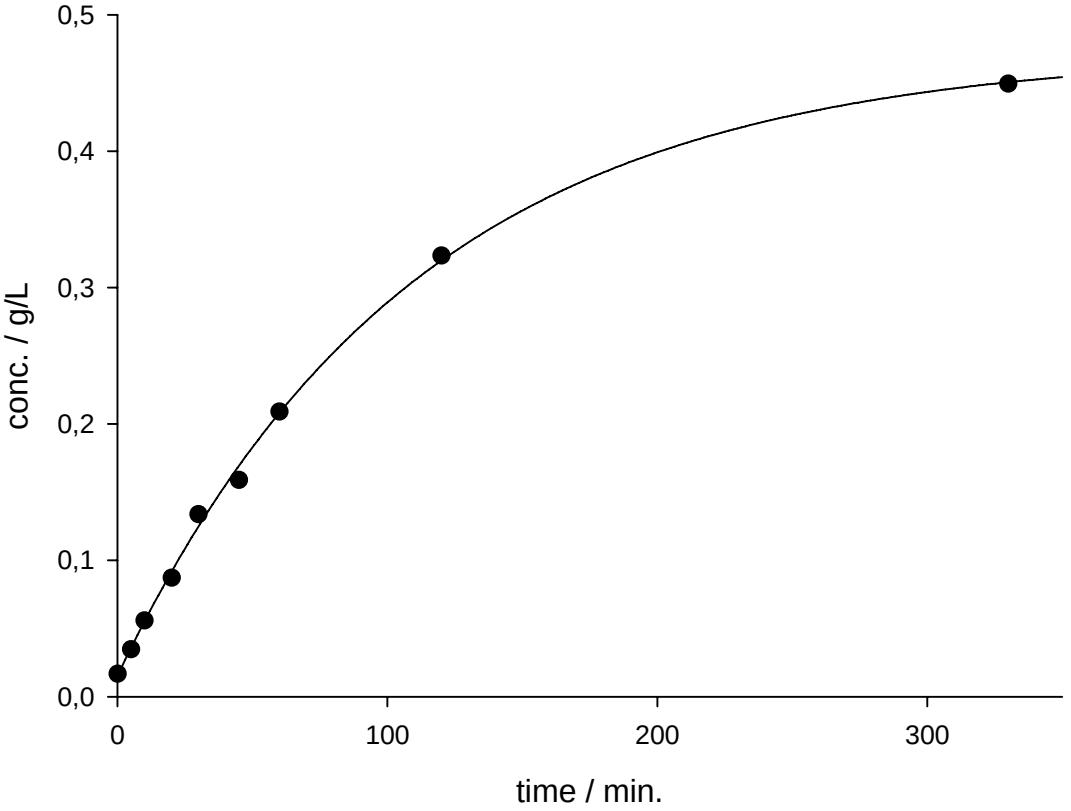


Figure 6: Kinetics of the formation of methyl-α-D-glucopyranoside (**12**).

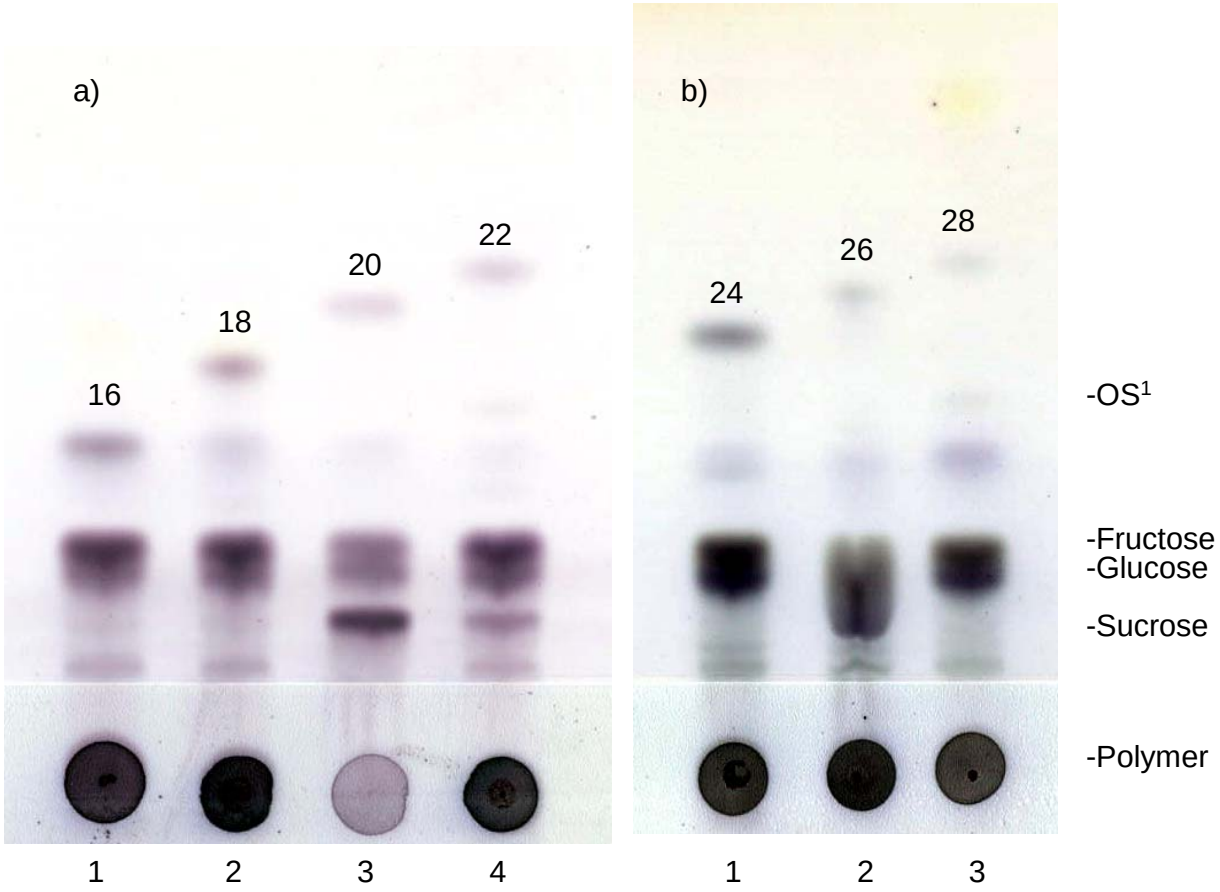


Figure 7: Glycosylation products formed after 24h by GTFR (17.4 kUL^{-1}) with different acceptors (5-10%) and sucrose (**1**) (29 mM) a) lane 1: methanol (**15**), lane 2: ethanol (**17**), lane 3: 1-propanol (**19**), lane 4: 1-butanol (**21**); b) lane 1: 2-chloroethanol (**23**), lane 2: 4-chlorobutanol (**25**), lane 3: 6-chlorohexanol (**27**).

[1)] Further work is going on in order to identify other spots.

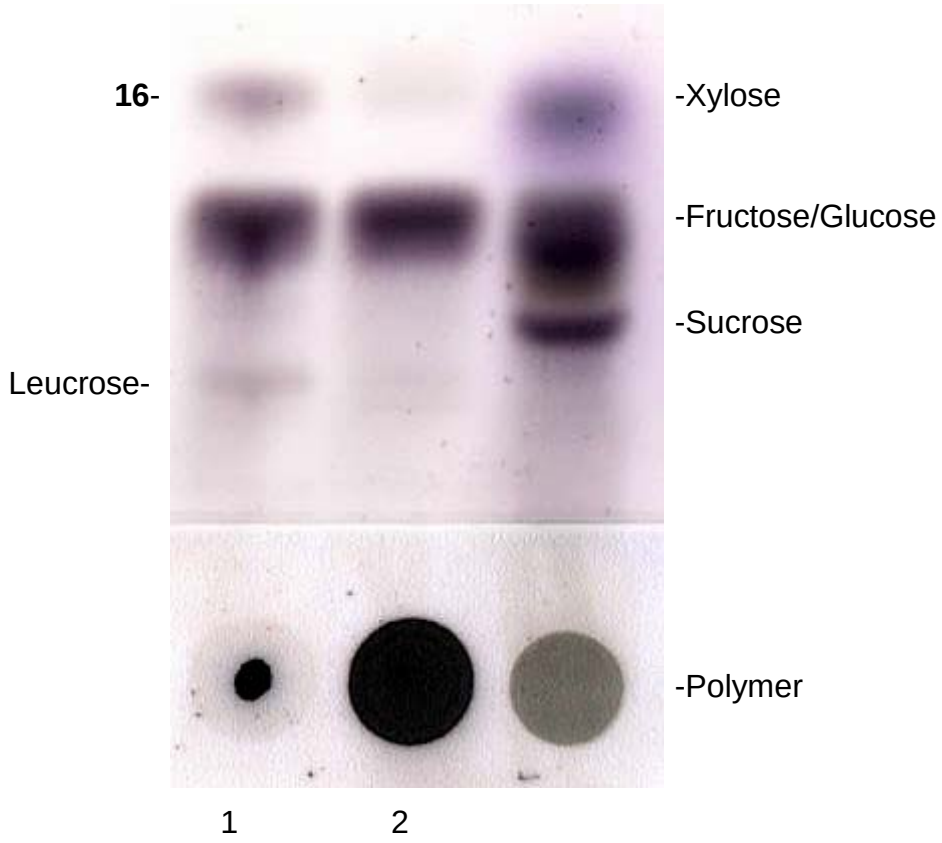


Figure 8: Comparison of GTFR and DSRS for the synthesis of methyl-α-D-glucopyranoside (**16**) lane 1 GTFR, lane 2 DSRS.

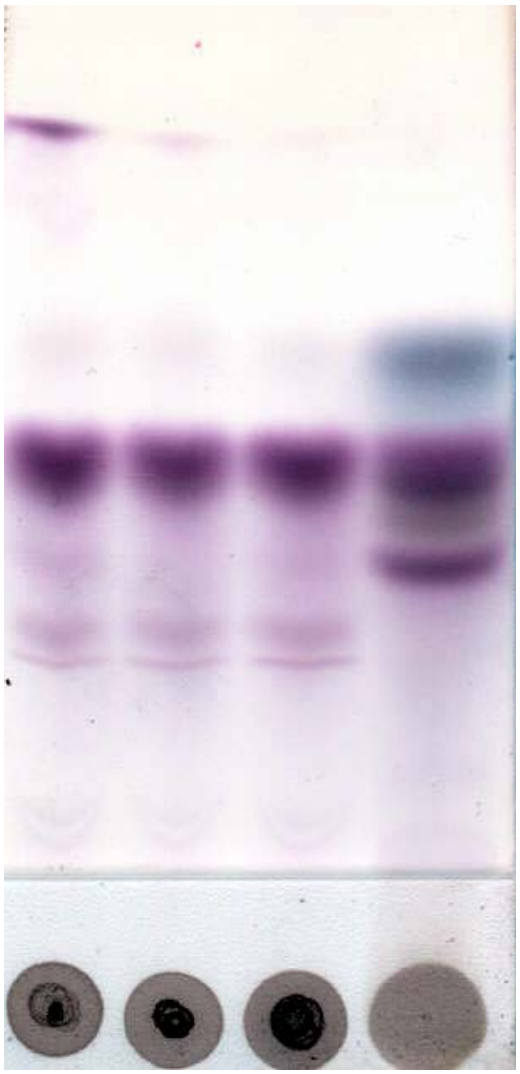


Figure 9: Glycosylation of amino acid derivatives (117 mM) with sucrose (**1**, 290 mM) by GTFR (1.0 kUL^{-1}): lane 1: *N*-(tert-butoxycarbonyl)-D-serine methyl ester (**30**), lane 2: *N*-(tert-butoxycarbonyl)-L-serine methyl ester (**32**), lane 3: *N*-(tert-butoxycarbonyl)-L-threonine methyl ester (**34**).

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