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Synthesis of sucrose analogues and the mechanism of action of *B. subtilis* fructosyltransferase (levansucrase)

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Abstract— In the present study, we have coupled detailed acceptor and donor substrate studies of the fructosyltransferase (FTF, levansucrase) (EC 2.4.1.162) from *B. subtilis* NCIMB 11871, with a structural model of the substrate enzyme complex in order to investigate in detail the roles of the active site amino acids in the catalytic action of the enzyme and the scope and limitation of substrates. Therefore we have isolated the *ftf* gene, expressed in *Escherichia coli*, yielding a levansucrase. Consequently, detailed acceptor property effects in the fructosylation by systematic variation of glycoside acceptors with respect to the positions (2, 3, 4 and 6) of the hydroxyl groups from equatorial to axial have been studied for preparative scale production of new oligosaccharides. Such investigations provided mechanistic insights of the FTF reaction. The configuration and the presence of the C-2 and C-3 hydroxyl groups of the glucopyranoside derivatives either as substrates or acceptors have been identified to be rate-limiting for the trans-fructosylation process. The rates are rationalized on the basis of the coordination of D-glycopyranoside residues in $^4C_1$ configuration with a network of amino acids by Arg360, Tyr411, Glu342, Trp85, Asp247 and Arg246 stabilisation both acceptors and substrates. In addition we also describe the first FTF reaction which catalyzes the β-1,2-β fructosyl transfer to 2-OH of L-sugars (L-glucose, L-rhamnose, L-galactose, L-fucose, L-xylose) presumably in a $^1C_4$ conformation. In those conformation the L-glycopyranosides are stabilized by the same hydrogen network. Structures of the acceptor products were determined by NMR-and mass spectrometry analysis.

Keywords: fructosyltransferase, sucrose analogues, biocatalysis, fructooligosaccharides, reaction mechanism

1. Introduction

The enormous structural diversity of the carbohydrates in form of oligosaccharides on cell surfaces allows them for example to regulate cell growth, differentiation, and interaction with other cells, bacteria and viruses.[1] Thus, oligosaccharides are involved in numerous diseases including cancer and bacterial and viral infections.[2,3] Fructooligosaccharides stimulate the immune system, resulting in a decrease of cancer cells.[4] They are produced by fructosyltransferases (FTF), which, according to the Carbohydrate-Active EnZYme database (CAZy), belong to the glycoside hydrolase family 68 (GH68).[5] These retaining enzymes synthesize from sucrose either inulin,[6] composed of β-1,2-linked fructose residues (inulosucrase, EC 2.4.1.9) or levan,[7] composed of β-2,6 linked fructose residues (levansucrase, EC 2.4.10). Initial kinetic studies suggested that the reaction catalyzed by FTF proceeds by a ping-pong mechanism, where an unknown aspartate from the enzyme acts as nucleophile to form a stabilized fructosyl-enzyme complex.[8] The FTF catalytic activity is most likely effected by a conserved active site Asp-Glu-Asp.[9] These amino acids have been recently identified by X-ray crystallography as Asp86, Glu342 and Asp247 in the levansucrases from *B. subtilis*[10] and *Gluconacetobacter diazotrophicus* SRT4[11]. The sucrose-bound complex structure[10] suggests that Asp86 and Glu342 form the pair of essential catalytic side chains, while Asp247 forms strong hydrogen bonding interactions with the C-3' and C-4' hydroxyls of the fructosyl unit. Based on sequence alignments of *Lactobacillus reuteri* 121 Lev and Inu as well as *B. subtilis* levansucrase SacB site-directed mutagenesis studies of the three identified catalytic residues were carried out and supported the hypothesis that the Asp86 residue acts as nucleophile.[12] In the crystalline sucrose-enzyme complex (PDB code 1PT2),[10] the furanose ring adopts an envelope shape with a more pronounced amplitude of deformation between $^{3}T_{4}$ and E_{4}. Glu342 as general acid/base catalyst presumably protonates the glycosidic oxygen of the substrate and forms an oxonium ion with the favoured envelope shape conformation E_{4} (scheme 1). The retaining mechanism further proceeds

through the formation of a covalent glycosyl-enzyme intermediate as demonstrated with these enzymes and a nucleophilic attack of the acceptor substrate (scheme 1).[9,13]

"Place Scheme 1 here"

However, the function of Glu342 as general acid/base catalyst has not been analyzed further and is still unclear, as Glu342 is part of a complex network of interactions that includes Arg246, Tyr411 and Arg360 as well as Glu262 and Glu340 and multiple hydrogen bonding contacts with the substrate. Those amino acid residues are believed to be crucial for the activation step of sucrose and further possible substrates.[10]

The intimate relationship between transglycosylation reaction and catalytic mechanism of the FTF prompted us to analyze the catalytic mechanism of FTF (levansucrase) and its acceptor specificity for the production of new sucrose analogues and oligosaccharides, which also will be used for the identification of new glycosyltransferase specificities. Additionally, we modeled structural complexes of FTF with sucrose analogue substrates. These structural complexes were constructed by molecular docking of substrates into FTF crystal structure.

2. Results and Discussion

2.1 Expression of recombinant FTF from *B. subtilis* NCIMB 11871

The FTF from *B. subtilis* NCIMB 11871[14] migrated as a 50 kDa protein band in SDS-PAGE. The protein was blotted from a SDS-gel to a PVDF-membrane for analysis of the N-terminal amino acid sequence. The data from these peptides were used to locate the ORF coding for FTF in the *B. subtilis* NCIMB 11871.

The NH_2-terminal amino acid sequence of the FTF was found to be as follows: KETNQKP, which is identical with the amino acid sequence (30-36) of *sacB*[10] and corresponds to the first amino acids of the mature protein after cleavage of the signal sequence.

Based on this sequence, primers were synthesized for the PCR amplification of full-length *ftf*. Sequence analysis of FTF from *B. subtilis* NCIMB 11871 revealed 99% identity to the DNA

sequence of *sacB* from *B. subtilis*. In comparison to "wild type" *sacB, ftf* from *B. subtilis* NCIMB 11871 carries 14 nucleotide changes distributed all over the gene sequence. All changes are silent mutations thus leading to 100% identity on the amino acid level with levansucrase *sacB*.

E. coli BL21 (DE3) cells harbouring plasmid p24FTF11871 were grown after induction with 0.5 mM isopropyl-β-thio-D-galactoside (IPTG) for 24 h. The crude extract of these cells was subjected to high-speed centrifugation and FTF activity was located both in the supernatant fraction and in the pellet.

However, the majority of the activity in the pellet could be released into the soluble fraction upon repeated sonication. The enzymatic properties of recombinant FTF were identical to the native enzyme from the *B. subtilis* NCIMB 11871 extract.

In initial kinetic studies the synthesis of β-D-fructofuranosyl-α-D-galactopyranoside (**2**, D-Gal-Fru) was achieved in 61% conversion of sucrose and D-galactose (**1**) to D-Gal-Fru (**2**) yielding a quasi-equilibrium.

The glycopyranosides (D-mannose, 2-deoxy-D-glucose, D-galactose, 6-deoxy-D-galactose (D-fucose), D-xylose, L-glucose, L-rhamnose, L-galactose, L-fucose, L-xylose) and disaccharides (maltose, lactose, isomaltose, cellobiose and melibiose) have been successfully explored as FTF acceptors.

2.2 Acceptor studies of Positions 4 and 6

D-xylose (**3**) was a good acceptor to form β-D-fructofuranosyl-α-D-xylopyranoside (**4**) in a maximum yield of 56% and a space time yield (sty) of 24.0 $gl^{-1}h^{-1}$ (table 1, figure 1). Even the combination of the configurational changes of the glucopyranoside in C-4 and C-6 in the example of 6-deoxy-D-galactose (D-fucose, **5**), when a CH_3 group replaces the hydroxymethyl group and an axial O-4 exists, yielded in the corresponding β-D-fructofuranosyl-α-D-fucopyranoside (**6**) in 62% (33.8 $gl^{-1}h^{-1}$, table 1), respectively.

"Place Table 1 here"

The acceptor studies of C-6 were expanded to disaccharides, where D-glucose is connected over C-6'-hydroxyl with alpha configurated D-glucose (isomaltose, **7**) or alternatively D-galactose (melibiose, **11**). The product formation of α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside (**8)** (53% yield; 18.1 $gl^{-1}h^{-1}$) was as good as for the corresponding raffinose (**12)** (45% yield; 18.2 $gl^{-1}h^{-1}$). The acceptor maltose (**9**), where D-glucose is connected over C-4'-hydroxyl with alpha D-glucose, yielded the trisaccharide erlose (**10**) in 45% yield (15.3 $gl^{-1}h^{-1}$) (table 2). D-cellobiose (**13**) and lactose (**15**) were suggested to come in conflict with Arg246 (3.3Å) and Glu340 (2.7Å) due to their beta-connected D-glycopyranosides with C-4'. These effects have not been observed, the acceptor products β-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside (**14**) (18.0 $gl^{-1}h^{-1}$) and β-D-galactopyranosyl-(1→4)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside (**16**) (21.9 $gl^{-1}h^{-1}$) were formed in 30% and 34% yield.

"Place Table 2 here"

"Place Figure 1 here"

The products were separated either by thin-layer chromatography (TLC) and quantitated by scanning densitometry or HPLC.

2.3 Acceptor studies of Positions 2 and 3

To determine the effects of the configuration at C-2, we compared mannose (**17**), 2-deoxy-D-glucose (**19**) and sophorose (**25**). The axial O-2 present in D-mannose (**17**) effected a dramatic drop of the production of β-D-fructofuranosyl-α-D-mannopyranoside (D-Man-Fru, **18**)[15] with 0.3% yield (0.1 $gl^{-1}h^{-1}$). Also 2-deoxy-D-glucose (**19**) showed very low acceptor reaction (0.4%; 0.3 $gl^{-1}h^{-1}$) under the conditions described (table 3). To get deeper insights into the mechanisms of action of the fructosyltransferase acceptors, the sucrose analogue allosucrose (**22)** was synthesized by catalytic hydrogenation of 3-ketosucrose (**24)** according to previous studies.[16] Because the transfructosylation reactions have been shown to be reversible, the transfer of the fructosyl residue

from allosucrose (**22)** to the acceptor substrate D-glucose should yield in the formation of sucrose and D-allose. Surprisingly allosucrose (**22)** did not react with D-glucose to the product sucrose as sucrose was not observed. In contrast 3-ketosucrose (**24)** was an acceptable donor substrate for the FTF and formed with the acceptor substrate D-glucose the disaccharide sucrose (5.6% yield; 2.2 $gl^{-1}h^{-1}$) in low yields. However, allosucrose itself was a very inefficient donor substrate indicated by the slow formation small amounts of the D-allose, fructose and levan (<0.1%) compared to sucrose as substrate.

The disaccharide 2-O-β-D-glucopyranosyl-α-D-glucose (sophorose, **25**) was no acceptor.

"Place Table 3 here"

2.4 Acceptor studies with L-sugars

Interestingly we observed by using L-glucose (**26**) as acceptor the formation of β-D-fructofuranosyl-β-L-glucopyranoside (**27**) (L-Glc-Fru; 10.7% yield), a sucrose analogue with a β-(1-2)-glycosidic linkage (table 4).[17] Those β-(1-2)-glycosidic linkages are found in nature as nuleotide activated L-sugars, i.e. uridine diphosphate (UDP)-β-L-arabinose and the GDP-β-L derivative of fucose (Fuc) and Cytidine monophosphate activated sialic acid (β-CMP-NeuAc). We also observed the fructosylation of L-rhamnose (**28**) (<0.05 $gl^{-1}h^{-1}$), L-galactose (**30**, 9.5%) and L-fucose (**32**, 3.7%). L-xylose (**34**) proved to be the best acceptor yielding two acceptor products: **35** with the expected β-(1-2)-glycosidic linkage (16.7%) and **36,** a reducing Xyl-Fru (26.9%), respectively. The structure of L-Rha-Fru (**29**) was just verified by mass spectroscopy, not by NMR, because of very low yields of the acceptor product. Very low yields might be due to the axial orientation of the C-2-hydroxyl.

"Place Table 4 here"

2.5 Conformation of acceptor products (sucrose analogues)

Structural evidence for all sucrose analogues and oligosaccharides were confirmed by the ^{1}H NMR and ^{13}C NMR spectroscopy except L-Rha-Fru (**29**). The elucidation of the sucrose analogue structures were possible only by the combination of all the data acquired from the ^{1}H, ^{13}C and 2D-NMR spectra (tables 5, 6). The anomeric coupling constants $J_{1,2}$ (3.60- 3.98 Hz) and $J_{2,3}$ (9.90-10.61 Hz) of D-Gal-Fru (**2**), D-Xyl-Fru (**4**) and D-Fuc-Fru (**6**) are characteristic for an α-(1-2)-glycosidic linkage and a $^{4}C_{1}$ conformation for the D-glycopyranosyl residues. Also allosucrose (**22**) adopts a $^{4}C_{1}$ conformation indicated by $J_{1,2}$ (4.03 Hz) and $J_{2,3}$ (3.70 Hz). In addition the trisaccharides synthesized by the acceptors isomaltose (**7**), melibiose (**11**), maltose (**9**), cellobiose (**13**) and lactose (**15**) have coupling constants of $J_{1',2'}$ 3.84-3.90 Hz and $J_{2',3'}$ 9.85-10.02 Hz. Our NOESY experiments of the products of these compounds evidence the $^{4}C_{1}$ conformation. The L-glycopyranoside acceptor products (**27**, **31**, **33**, **35**) have a β-(1-2)-glycosidic linkage and adopt a $^{1}C_{4}$ conformation indicated by their coupling constants $J_{1,2}$ (8 Hz) and $J_{2,3}$ (9-10 Hz).

"Place Tables 5,6 here"

2.6 Docking of sucrose analogues

To further investigate the precise mechanism of ordered-substrate binding to FTF we carried out in silico docking experiments with AUTODOCK.[18] The use of AUTODOCK was validated by testing the ability of the program to dock the substrate sucrose into the x-ray structure of FTF (PDB ID code 1OYG)[11] and by docking D-Gal-Fru (**2**) into the x-ray structure of the FTF. For sucrose and D-Gal-Fru (**2**), 100 docked structures, i.e., 100 runs, were generated using the Lamarckian Genetic Algorithm. The lowest docked energies, together with the number of structures in each cluster, were ranked in order of increasing energy. The conformation with the lowest docked energy for sucrose (-11.58 kcal/mol) and D-Gal-Fru (-12.32 kcal/mol) gave the best match with the experimental data (Figure 2).

"Place Figure 2 here"

The predicted binding mode of sucrose, found practically identical in the 10 lowest energy dockings, correlates well with the results of the x-ray structure of the sucrose bound inactive FTF variant Glu342Ala (PDB ID code 1PT2)[10]. The Asp86 carboxylate is 3.1 Å away from the fructosyl anomeric carbon (C-2'), the glycosidic oxygen is 2.8 Å away from Glu342. Hence, it can be assumed that these complexes are most favourable and most probable. The calculations show for D-Gal-Fru (**2**) an identical binding mode for the 7 lowest energy dockings D-Gal-Fru (**2**) seems to be the slightly better substrate, indicated by the lower docking energy, which is in agreement with the kinetic studies. Calculations with D-Man-Fru (**18**) show only 3 of the 10 lowest energy dockings in an orientation similar to sucrose with docking energies slightly lower than for allosucrose (**22**). The influence of the hydrogen bond to the 2-hydroxyl group is demonstrated by the even poorer docking energies found for 2-deoxy-D-Glc-Fru (20), where only 2 of the 10 lowest energy dockings are in an orientation similar to sucrose. These observations correspond to the kinetic studies and indicate that Glu342 is not able to coordinate the axial C-2 hydroxyl in the D-mannose (**18**) anymore. This precoordination of the glutamate residue has been demonstrated to be important as transition state stabilizer and general acid/base for an efficient catalysis for glycosidases.[19] In those enzymes the glycopyranoside residue is transferred and catalysis often proceeds via a disortion of the glycopyranoside from the ground state $^4C_1$ to a ^4E, $^1S_3$ or $^{1,4}B$ conformer. In our studies the glycopyranoside residues are used as acceptors or in sucrose analogues as leaving group, a conformational change of the glycopyranoside residue seems not be nescessary. Thus, Glu342 protonation of the glycosidic bond is assumed to accelerate the reaction essentially and the Glu340 to direct and stabilize the glycopyranoside residue as acceptor or substrate. The hydroxyl group at C-3 is particularly important as it determines the binding orientation of Arg360 and Glu340 in the sucrose bound FTF X-ray structure.[10] Our results suggest that Glu342 orientation is also effected by the variation of C-3. Docking calculations with allosucrose (**22)** (axial orientation of C-3 hydroxyl group) show only 4 of the 10 lowest energy dockings in an orientation similar to sucrose and with

poorer docking energy (-9.8 kcal/mol). In this orientation the axial position of the hydroxyl-group in position 3 interferes with the Glu342 side chain (figure 3a), forming an abnormally small distance (2.5 Å) to Glu342. As a result Glu342 is unable to protonate the glycosidic oxygen either due to an increased distance to the glycosidic oxygen when the short distance to the hydroxyl group is relaxed or due to a redistribution of the Glu342 proton away from the glycosidic oxygen. In a second favoured orientation allosucrose adopts a "reverse" orientation (figure 3a, grey figure), where the glycosyl unit occupies approximately the position of the fructosyl moiety in the sucrose complex. In this orientation, however, Glu342 is too far from the glycosidic oxygen (4.9 Å) and no catalytic activity is expected.

"Place Figure 3 here"

It is assumed that the L-configuration of glucose effects a different orientation of the acceptor in the active side of the transferase. A $^{1}C_{4}$ conformation of L-glucose is much more favoured than a $^{4}C_{1}$ due to steric reasons. In this orientation an axial configured C-1-hydroxyl group of $^{1}C_{4}$ L-glucose could not attack the oxycarbenium-cation of the fructopyranoside to form L-Glc-Fru (**27**). Docking experiments showed that the equatorial configured C-1 hydroxyl group of $^{1}C_{4}$ glucose can be stabilized by Glu342 and Glu340, which provides the right orientation for the β-(1-2)-glycosidic linkage formation and *syn* protonation of Glu342 in view from the β-D-fructofuranosyl-residue (figure 4). The docking calculations showed for these substrates a varying number of different conformations and orientations among the lowest energy dockings, including a second preferred orientation (figure 4) in which Trp163 has a ring stacking interaction with the glycopyranosides (L-Glc, L-Gal, L-Xyl, L-Fuc). Due to the large distance of Glu342 to the glycosidic oxygen these orientations seem unfavourable for catalytic activity (figures 4,5). Assuming an equilibrium between the inactive and active orientations would be consistent with the enzymes decreased catalytic efficiency towards the L-glycopyranoside acceptor products versus sucrose as substrate.

"Place Figures 4, 5 here"

Conclusion

The increasing demand from the consumer for alternative sweetners and additional important physiological properties on the immune response of FOS and OS prompted us to investigate the possible large scale production of sucrose analogues and subsequent reaction products. Thus, various acceptor reactions were evaluated for their formation catalyzed by the FTF from *B. subtilis* NCIMB 11871. With these studies we were able to obtain further information about the transfructosylation mechanism. From the kinetic and docking studies it can be assumed that the enzyme possesses only one acceptor site which is identical with the substrate site. The fixation of the different D-glycopyranosides and L-glycopyranosides is thermodynamically controlled.

"Place Table 7 here"

The D-glycopyranoside D-Gal-Fru (**2**) is an even better substrate compared to sucrose because of more important binding forces contributed by Arg360, Tyr411, Glu342, Trp85, Asp247 and Arg246 (table 7). In contrast allosucrose (**22**) is a very inefficient substrate for the FTF, although it has "only" an axial orientation of its 3-OH. This experimental observation is in accordance with the docking experiments as the binding mode in the productive orientation is not favourable for catalysis as the hydrogen bridges between Arg360 and 3-OH and 2-OH are missing, which are assumed to be necessary for a fixed orientation. The L-configured glycopyranosides adobe in a $^{1}C_{4}$ conformation a superb fit for an unnatural β-1,2-β fructosyl transfer. In this conformation the L-glycopyranosides are stabilized by the same hydrogen network. The calculated binding energies of L-glycopyranosides compared to sucrose are in accordance with the observed kinetic data, resulting in the lower yields. L-glycopyranosides are missing hydrogen bonds with Glu340 and especially L-Gal-Fru (**31**) is missing in addition a hydrogen bond with Arg360. As Arg360 stabilizes the 3-OH in all glycopyranosides except L-Gal, L-Gal is able to compensate this by an additional hydrogen bond between Tyr 429 and 6-OH necessary for the fixation of L-galactose and a catalytic favourable orientation.

In conclusion these studies can be used for efficient production of oligosaccharides. It also contributes to investigate novel substrates and variants of this class of enzyme leading in further novel products for pharmaceutical and functional food applications.

3. Experimental

3.1 General methods

^{1}H and ^{13}C NMR spectra were recorded in D_2O unless using a Bruker AM-400 instrument, operating at 400 MHz for ^{1}H and at 100 MHz for ^{13}C. 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 x 7.8 mm, Phenomenex®, Germany) operated at 80°C and an Ion Chromatograph (IC) (Metrohm, Germany) using a refractive index detector (ERC-7512, Erma, Germany) and an eluent of bidistilled water at 0.5 ml min^{-1}.

Standard solutions were prepared in the range of 0.1 and 10 gl^{-1}. The monosaccharides D-fructose, D-galactose, D-glucose, the disaccharides sucrose, D-melibiose, the trisaccharides D-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 analysed using TLC. The solvent system ethylacetate/isopropanol/water in a ratio of 6/3/1 (v/v/v) (room temperature) 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 (3 × 60 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 3, time 1) (MERCK, Germany), followed by heating in an oven at 120°C for 15 min. The sugars were visualised as dark spots. The quantitative determination of the sugars was performed by scanning densitometry (50-2000 ng) using a Bio-Rad Imaging Densitometer utilising Quantity One® Software (Version 4.2).

3.2 Bacterial strains and plasmids

E. coli strain DH5α was used for cloning experiments and amplification of plasmid DNA. *E.coli* strain BL21 (DE3) (Novagen, Madison, WI, USA) was used for expression of the recombinant *ftf* gene. Plasmid pET24a+ (Novagen, Madison, WI, USA) was used for cloning of *ftf*.

3.3 DNA techniques

Plasmid DNA was isolated from *E. coli* by alkaline lysis.[20] DNA purification, restriction enzyme digestion, ligation, analysis on agarose gels and transformation were carried out following standard methods.[21]

Nucleotide sequencing of *ftf* gene inserted in pET24a+ expression plasmid was performed by Sequiserve (Vaterstetten, Germany) following the application of automated DNA sequencing method based on dideoxy chain termination method,[22] using primers of T7 promoter and terminator.

3.4 Construction of an ftf-his6 fusion plasmid

Genomic DNA was isolated from *Bacillus subtilis* NCIMB 11871 and DNA amplification performed by PCR using designed primers specific for the 5' and 3' end of *B. subtilis sacB*. The synthetic oligonucleotides (purchased from Metabion international AG, Germany) had the following sequences: 5'-GATATAAACATATGAACATCAAAAAGTTTGCA-3' for the forward

primer and 5'-CCAACTCGAGTTTGTTAACGTTTAATTG-3' for the reverse primer with restriction sites for *NdeI* and *XhoI* (underlined). A 1.4 kb fragment was amplified by PCR using TITANIUM *taq*-polymerase (Clonetech Laboratories, Palo Alto, CA, USA) and subsequently cloned into hexahistidyl tag vector pET24a+ generating plasmid p24FTF11871.

The entire *ftf-his6* gene fusion was sequenced to confirm the fidelity of the amplification.

3.5 Protein expression

E. coli BL21(DE3) cells containing p24FTF11871 were grown in Luria-Bertani medium at 37°C with 50 μg/ml canamycin to an OD_{580} of 0.7, where the expression of FTF was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Induced cells were cultured for 24 h at 30°C, harvested by centrifugation and resuspended in 60mM Na_2HPO_4/NaH_2PO_4 (pH 6.0). Cells were disrupted by sonication, followed by centrifugation.

3.6 Enzymatic synthesis of sucrose analogues

The general reaction mixture contained 0.6 M sucrose as substrate and 1.2 M glycopyranoside as acceptor in phosphate buffer (pH 6.0), this included an 1:4 ratio volume of recombinant FTF protein extract (12.2 U/ml FTF activity in 0.6 M sucrose, messured by glucose-release). The sucrose analogue formation was investigated by discontinuous analysis of aliquots from the reaction mixture at suitable time intervals up to 24 h.

The enzyme was inactivated by boiling the samples in a water-bath for ten min. After cooling, the inactivated samples were filtered through a 0.22 μm nitrocellulose membrane filter (Millipore, Germany) and analysed by HPLC and TLC.[16]

3.7 Preparative chromatography

The purification of the oligosaccharides was carried out as previously described.[2,16]

3.8 Automated Docking Procedure

The program AUTODOCK was used for the automated docking of ligands to the levansucrase structure, Protein Data Bank id 1OYG. Affinity maps were calculated on a 0.375 Å raster for a cubic 257 point grid. In each case, 100 docking runs were calculated using the Genetic Algorithm with a maximum of 2500,000 energy evaluations, otherwise default parameters were used. Structures for the ligand molecules were prepared using Sybyl 7.1 (Tripos Inc.) for energy minimization and calculation of Gasteiger charges.

3.9 β-D-Fructofuranosyl-α-D-fucopyranoside (D-Fuc-Fru) (6)

White solid, mp 235 °C; R_f = 0.66 (6:3:1 EtOAc-Isopropanol-H_2O, 3 ascends); $[\alpha]_D$ = +73.7° (c = 0.8, H_2O); IR (cm^{-1}): 3441, 1139, 1112; ^1H NMR (400 MHz, D_2O) δ 5.31-5.30 (d, J_{12} = 3.98 Hz, 1H, 1-H), 4.21-4.16 (m, 1H, 5-H), 4.16-4.14 (d, $J_{3'4'}$ = 8.61 Hz, 1H, 3'-H), 4.03- 3.99 (t, 1H, $J_{3'4'}$ = $J_{4'3'}$ = 8.61 Hz, 4'-H), 3.90-3.87 (dd, J_{34} = 3.35, J_{32} = 10.61 Hz, 1H, 3-H), 3.84-3.82 (m, 1H, 5'-H), 3.78-3.75 (m, 1H, 4-H), 3.73-3.70 (m, 3H, 2-H, 6'-H_2), 3.62 (s, 2H, 1'-H_2), 1.18-1.16 (d, J_{65} = 6.59 Hz, 3H, CH_3).^{13}C NMR (100 MHz, D_2O) δ 106.12 (C-1'), 95.01 (C-1), 83.89 (C-5'), 79.10 (C-3'), 76.58 (C-4'), 74.39 (C-4), 71.78 (C-3), 70.28 (C-2), 69.92 (C-5), 64.77 (C-6'), 63.75 (C-1'), 13.00 (C-6). ESIMS: *m/z* 349.0 100%, [M + Na^+].

3.10 α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside (isomaltose-Fru) (8)

White solid, mp 135 °C; R_f = 0.19 (6:3:1 EtOAc-Isopropanol-H_2O, 3 ascends); $[\alpha]_D$ = +82.3° (c = 0.8, H_2O); IR (cm^{-1}): 3412, 1151, 1048; ^1H NMR (400 MHz, D_2O) δ 5.40-5.39 (d, $J_{1'2'}$ = 3.90 Hz, 1H, 1'-H), 4.93-4.92 (d, $J_{1''2''}$ = 3.90 Hz, 1H, 1''-H), 4.18-4.17 (d, J_{34} = 8.52 Hz, 1H, 3-H), 4.03-3.99 (t, J_{43} = J_{45} = 8.52, 1H, 4-H), 4.04-3.97 (m, 2H, 5'-H, 6'-H_a), 3.88-3.79 (m, 3H, 5-H, 6''-H_a, 6-H_a), 3.76-3.72 (m, 2H, 3''-H, 6-H_b), 3.71-3.61 (m, 3H, 3'-H, 6''-H_b, 6'-H_b), 3.63 (s, 2H, 1-H_2), 3.55-3.48 (m, 4H, H-2'',H-5'', H-2', H-4'), 3.42-3.37 (t, $J_{4''3''}$ = $J_{4''5''}$ = 9.32, 1H, 4''-H). ^{13}C NMR

(100 MHz, D_2O) δ 106.39 (C-2), 100.81 (C-1''), 94.70 (C-1'), 83.88 (C-5), 78.94 (C-3), 76.58 (C-4), 75.63 (C-3''), 75.34 (C-3'), 74.46 (C-2''), 74.11 (C-5''), 73.89 (C-5'), 73.54 (C-2'), 72.12 (C-4''), 71.94 (C-4'), 68.21 (C-6'), 65.03 (C-6), 64.01 (C-1), 63.07 (C-6''). ESIMS: *m/z* 527.0 100%, $[M + Na^+]$.

3.11 α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside (maltose-Fru) (10)

White solid, mp 105 °C; R_f = 0.20 (6:3:1 EtOAc-Isopropanol-H_2O, 3 ascends); $[\alpha]_D$ = +76.7° (c = 1.1, H_2O); IR (cm^{-1}): 3423, 1148, 1050, 992; ^{1}H NMR (400 MHz, D_2O) δ 5.37-5.36 (d, $J_{1',2'}$, $J_{1'',2''}$ = 3.84 Hz, 2H, 1'-H, 1''-H), 4.17-4.15 (d, J_{34} = 8.60 Hz, 1H, 3-H), 4.01-3.97 (t, $J_{43} = J_{45}$ = 8.60, 1H, 4-H), 4.00-3.96 (t, $J_{3',2'} = J_{3',4'}$ = 9.91 Hz, 1H, 3'-H), 3.94-3.91 (td, $J_{5',4'}$ = 10.00, $J_{5',6'}$ = 2.94 Hz, 1H, 5'-H), 3.86-3.82 (m, 1H, 5-H), 3.82-3.78 (m, 3H, 6'-H_2, 6''-H_a), 3.78-3.70 (m, 3H, 6''-H_b, 6-H_2), 3.70-3.61 (m, 3H, 4'-H, 3''-H, 5''-H), 3.70-3.61 (m, 3H, 4'-H, 3''-H, 5''-H), 3.62 (s, 2H, 1-H_2), 3.56-3.53 (dd, $J_{2'',3''}$ = 9.91, $J_{2'',1''}$ = 3.38 Hz, 1H, 2''-H), 3.55-3.52 (dd, $J_{2',3'}$ = 9.91, $J_{2',1'}$ = 3.38 Hz, 1H, 2'-H), 3.40-3.35 (t, $J_{4'',3''} = J_{4'',5''}$ = 9.23, 1H, 4''-H). ^{13}C NMR (100 MHz, D_2O) δ 106.21 (C-2), 102.36 (C-1''), 94.53 (C-1'), 83.95 (C-5), 79.37 (C-4'), 79.06 (C-3), 75.57 (C-3'), 75.49 (C-3''), 75.28 (C-5''), 74.34 (C-2''), 73.56 (C-5'), 73.39 (C-2'), 71.91 (C-4''), 64.92 (C-6), 63.92 (C-1), 63.07 (C-6''), 62.74 (C-6'). ESIMS: *m/z* 527.0 100%, $[M + Na^+]$.

3.12 α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside (raffinose) (12)

White solid, mp 130 °C; R_f = 0.24 (6:3:1 EtOAc-Isopropanol-H_2O, 3 ascends); $[\alpha]_D$ = +83.4° (c = 0.8, H_2O); IR (cm^{-1}): 3426, 1160, 1053; ^{1}H NMR (400 MHz, D_2O) δ 5.37-5.36 (d, $J_{1',2'}$ = 3.86 Hz, 1H, 1'-H,), 4.95-4.97 (d, $J_{1'',2''}$ = 3.68 Hz, 1H, 1''-H,), 4.18-4.16 (d, J_{34} = 8.80 Hz, 1H, 3-H), 4.03-3.99 (t, $J_{43} = J_{45}$ = 8.80 Hz, 1H, 4-H), 4.03-3.94 (m, 3H, 4'-H, 4''-H, 6''-H_a), 3.94-3.89 (m, 1H, 5'-H), 3.86-3.83 (dd, $J_{3'',2''}$ =10.48 Hz, $J_{3'',4''}$ = 3.44 Hz 1H, 3''-H), 3.88-3.82 (m, 1H, 5-H), 3.80-3.73

(m, 3H, 2''-H, 6-H_2), 3.72-3.68 (m, 3H, 3'-H, 6'-H_2), 3.66-3.62 (m, 1H, 6''-H_b), 3.62 (s, 2H, 1-H_2), 3.54-3.50 (dd, $J_{2',3'}$ = 9.85 Hz, $J_{2',1'}$ = 3.86 Hz, 1H, 2'-H), 3.52-3.46 (m, 1H, 5''-H). ^{13}C NMR (100 MHz, D_2O) δ 106.38 (C-2), 101.06 (C-1''), 94.69 (C-1'), 83.92 (C-5), 78.94 (C-3), 76.58 (C-4), 75.24 (C-3'), 73.98 (C-4'), 73.60 (C-5'), 73.54 (C-2'), 72.03 (C-5''), 71.99 (C-3''), 71.79 (C-4''), 71.06 (C-2''), 68.49 (C-6''), 65.05 (C-6), 63.99 (C-1), 63.99 (C-6'). ESIMS: *m/z* 527.0 100%, [M + Na^+].

3.13 β-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside (cellobiose-Fru) (14)

White solid, mp 195 °C; R_f = 0.37 (6:3:1 EtOAc-Isopropanol-H_2O, 3 ascends); $[\alpha]_D$ = +35.0° (c = 1.1, H_2O); IR (cm^{-1}): 3412, 1068, 996; ^{1}H NMR (400 MHz, D_2O) δ 5.35-5.34 (d, $J_{1',2'}$ = 3.79 Hz, 1H, 1'-H), 4.48-4.46 (d, $J_{1'',2''}$ = 7.83 Hz, 1H, 1''-H), 4.16-4.14 (d, J_{34} = 8.56 Hz, 1H, 3-H), 3.99-3.95 (t, J_{43} = J_{45} = 8.56, 1H, 4-H), 3.94-3.90 (td, $J_{5',4'}$ = 9.85 Hz, $J_{5',6'}$ = 2.53 Hz, 1H, 5'-H), 3.87-3.79 (m, 5H, 5-H, 3'-H, 6'-H_2, 6_a''-H), 3.76-3.73 (m, 2H, 6-H_2), 3.70-3.63 (m, 2H, 4'-H, 6_b''-H), 3.61 (s, 2H, 1-H_2), 3.48-3.36 (m, 3H, 3''-H, 4''-H, 5''-H). ^{13}C NMR (100 MHz, D_2O) δ 106.27 (C-2), 105.11 (C-1''), 94.46 (C-1'), 83.95 (C-5), 80.93 (C-4'), 78.97 (C-3), 78.56 (C-5''), 78.08 (C-3''), 76.58 (C-4), 75.77 (C-2''), 73.69 (C-3'), 73.64 (C-5'), 73.38 (C-2'), 72.05 (C-4''), 64.93 (C-6), 63.89 (C-1), 63.16 (C-6''), 62.07 (C-6'). ESIMS: *m/z* 527.0 100%, [M + Na^+].

3.14 β-D-galactopyranosyl-(1→4)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside (Lac-Fru) (16)

White solid, mp 195 °C; R_f = 0.20 (6:3:1 EtOAc-Isopropanol-H_2O, 3 ascends); $[\alpha]_D$ = +27.1° (c = 0.7, H_2O); IR (cm^{-1}): 3418, 1062, 991; ^{1}H NMR (400 MHz, D_2O) δ 5.34-5.33 (d, $J_{1',2'}$ = 3.84 Hz, 1H, 1'-H), 4.41-4.40 (d, $J_{1'',2''}$ = 7.80 Hz, 1H, 1''-H), 4.16-4.14 (d, J_{34} = 8.77 Hz, 1H, 3-H), 3.99-3.95 (t, J_{43} = J_{45} = 8.58, 1H, 4-H), 3.93-3.89 (td, $J_{5',4'}$ = 9.90, $J_{5',6'}$ = 2.66 Hz, 1H, 5'-H), 3.88-3.82 (m, 1H, 3'-H), 3.87-3.80 (m, 4H, 5-H, 4''-H, 6'-H_2), 3.75 (d, J_{65} = 3.11 Hz, 2H, 6-H_2), 3.74-3.70

(m, 1H, 5’’-H), 3.70 (d, J_{65} = 3.11, 2H, 6-H_2), 3.66-3.59 (m, 2H, 4’-H, 3’’-H), 3.61 (s, 2H, 1-H_2), 3.56-3.52 (dd, $J_{2',3'}$ = 10.02, $J_{2',1'}$ = 3.85 Hz, 1H, 2’-H), 3.51-3.46 (dd, $J_{2'',3''}$ = 9.95, $J_{2'',1''}$ = 7.80 Hz, 1H, 2’’-H). ^{13}C NMR (100 MHz, D_2O) δ 106.29 (C-2), 105.49 (C-1’’), 94.48 (C-1’), 83.96 (C-5), 80.66 (C-4’), 78.98 (C-3), 77.94 (C-5’’), 76.59 (C-4), 75.11 (C-3’’), 73.78 (C-3’), 73.64 (C-5’), 73.57 (C-2’’), 73.32 (C-2’), 71.14 (C-4’’), 64.96 (C-6), 63.87 (C-1), 63.60 (C-6’’), 62.11 (C-6’). ESIMS: *m/z* 527.0 100%, [M + Na^+].

3.15 β-D-Fructofuranosyl-β-L-glucopyranoside (L-Glc-Fru) (27)

White solid, mp 190 °C; R_f = 0.28 (6:3:1 EtOAc-Isopropanol-H_2O, 3 ascends); $[\alpha]_D$ = -11.0° (c = 0.7, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 4.87-4.85 (d, $J_{1,2}$ = 8.0 Hz, 1-H), 4.21- 4.18 (d, $J_{3',4'}$ = 8.7 Hz, 1H, 3’-H), 4.17-4.13 (t, J = 8.7 Hz, 1H, 4’-H), 3.89-3.83 (m, 1H, 5’-H), 3.80-3.78 (m, 2H, 6-H_2), 3.78- 3.71 (m, 1H, 6’-H_b), 3.71-3.68 (d, $J_{1'b}$ = 12.5 Hz, 1H, 1’$_b$-H), 3.64-3.60 (d, $J_{1a'}$ = 12.5 Hz, 1H, 1’$_a$-H), 3.52–3.47 (m, 2H, 3-H, 5-H), 3.47-3.44 (m, 1H, 6’-H_a), 3.41 (m, 1H, 4-H), 3.31-3.26 (dd, $J_{2,1}$ = 8.0, $J_{2,3}$ = 9.1 Hz, 1H, 2-H).^{13}C NMR (100 MHz, D_2O) δ 106.39 (C-2’), 97.56 (C-1), 83.85 (C-5’), 78.25 (C-5, C-3’), 77.66 (C-3), 75.34 (C-4’), 74.98 (C-2), 71.35 (C-4), 63.65 (C-6), 63.24 (C-1’), 62.41 (C-6’). ESIMS: *m/z* 365.0 100%, [M + Na^+].

3.16 β-D-Fructofuranosyl-β-L-galactopyranoside (L-Gal-Fru) (31)

White solid, mp 150 °C; R_f = 0.24 (6:3:1 EtOAc-Isopropanol-H_2O, 3 ascends); $[\alpha]_D$ = -21.6° (c = 0.2, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 4.56-4.54 (d, $J_{1,2}$ = 8.1 Hz, 1-H), 4.17- 4.14 (d, $J_{3',4'}$ = 8.6 Hz, 1H, 3’-H), 4.12-4.08 (t, J = 8.6 Hz, 1H, 4’-H), 3.93-3.92 (d, J = 3.5 Hz, 1H, 4-H), 3.87-3.83 (m, 1H, 5’-H), 3.77-3.70 (m, 7H, 5-H, 6-H_2, 1’-H_2, 6’-H_2), 3.64-3.61 (m, 1H, 3-H), 3.49-3.44 (dd, $J_{2,1}$ = 8.1, $J_{2,3}$ 10.1 Hz, 1H, 2-H).^{13}C NMR (100 MHz, D_2O) δ 105.93 (C-2’), 98.67 (C-1), 83.45 (C-5’), 79.03 (C-3’), 76.85 (C-4’), 74.94 (C-5), 74.09 (C-2), 73.93 (C-3), 71.07 (C-4), 64.63 (C-6’), 62.59 (C-6), 62.42 (C-1’). ESIMS: *m/z* 365.0 100%, [M + Na^+].

3.17 β-D-Fructofuranosyl-β-L-xylopyranoside (L-Xyl-Fru) (35)

White solid, mp 210 °C; R_f = 0.41 (6:3:1 EtOAc-Isopropanol-H_2O, 3 ascends); $[\alpha]_D$ = -11.6° (c = 0.6, H_2O); ^{1}H NMR (400 MHz, D_2O) δ 4.79-4.77 (d, $J_{1,2}$ = 8.1 Hz, 1-H), 4.21- 4.19 (d, $J_{3',4'}$ = 8.8 Hz, 1H, 3'-H), 4.14-4.09 (t, J = 8.8 Hz, 1H, 4'-H), 3.96-3.91 (dd, J = 11.6, 5.6 Hz 1H, 5-Ha), 3.90-3.87 (m, 1H, 5'-H), 3.84-3.70 (m, 2H, 6'-H_2), 3.70-3.60 (m, 3H, 4-H, 1'-H_2), 3.46-3.43 (t, J = 9.4 Hz, 1H, 3-H), 3.36–3.30 (d, J = 11.6, 10.6 Hz, 1H, 5-H_b), 3.31-3.27 (dd, $J_{2,1}$ = 8.1, $J_{2,3}$ 9.4 Hz, 1H, 2-H).^{13}C NMR (100 MHz, D_2O) δ 106.72 (C-2'), 98.74 (C-1), 84.24 (C-5'), 78.47 (C-3'), 78.14 (C-3), 75.62 (C-4'), 75.27 (C-2), 71.67 (C-4), 67.76 (C-5), 63.95 (C-6'), 63.50 (C-1'). ESIMS: *m/z* 335.0 100%, [M + Na^+].

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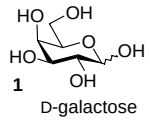
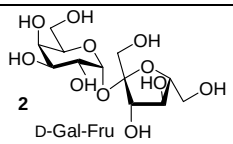
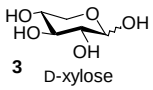
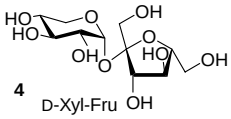
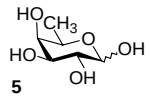
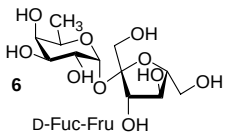
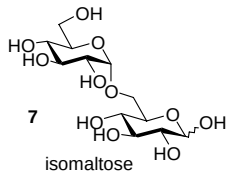
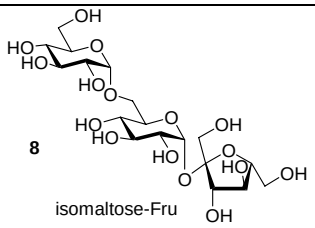
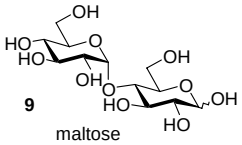
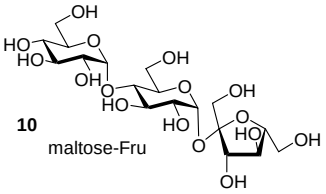
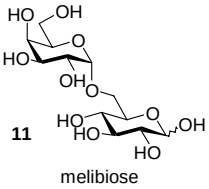
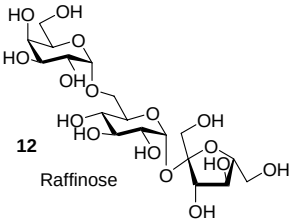
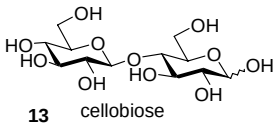
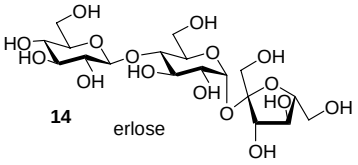
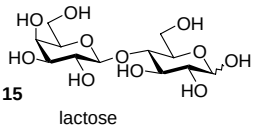
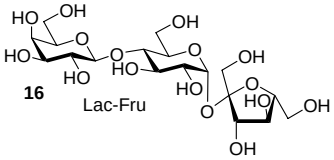
Table 1. Enzymatic synthesis of sucrose analogues.

Acceptor	Product	yield at optimum/time[a] [$gl^{-1}h^{-1}$]	yield at optimum[a,b] [%]
 **1** D-galactose	 **2** D-Gal-Fru	23.5	61
 **3** D-xylose	 **4** D-Xyl-Fru	24.0	56
 **5** D-fucose	 **6** D-Fuc-Fru	33.8	62

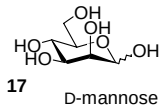
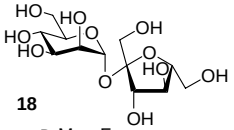
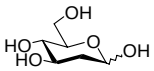
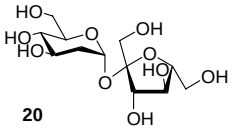
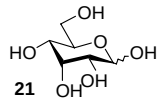
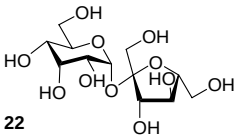
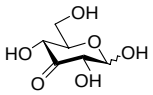
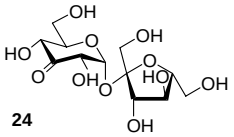
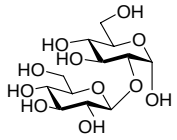
[a] yields are determined at the peak product concentration.
[b] yields are calculated from the donor sucrose.

Table 2. Enzymatic synthesis of fructosylated trisaccharides.

Acceptor	Product	yield at optimum/time $[gl^{-1}h^{-1}]^{a}$	yield at optimum $[\%]^{a,b}$
 **7** isomaltose	 **8** isomaltose-Fru	18.1	53
 **9** maltose	 **10** maltose-Fru	15.3	45
 **11** melibiose	 **12** Raffinose	18.2	45
 **13** cellobiose	 **14** erlose	18.0	30
 **15** lactose	 **16** Lac-Fru	21.9	34

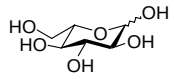
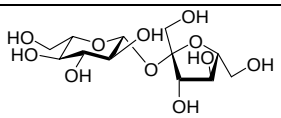
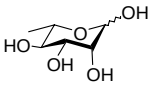
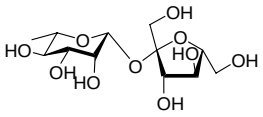
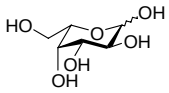
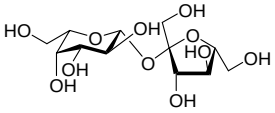
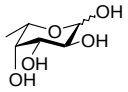
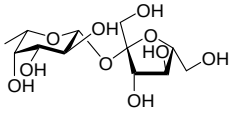
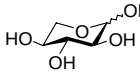
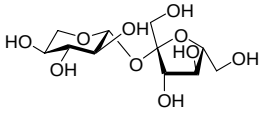
[a] yields are determined at the peak product concentration.
[b] yields are calculated from the donor sucrose.

Table 3. Acceptor reactions with mannose, 2 deoxyglucose, allo-sucrose and 3-keto-sucrose and sophorose.

Acceptor	Product	yield at optimum/time $[gl^{-1}h^{-1}]^a$	yield at optimum $[\%]^{a,b}$
 **17** D-mannose	 **18** D-Man-Fru	0.1	0.3
 **19** 2-Deoxy-D-glucose	 **20** 2-Deoxy-D-Glc-Fru	0.3	0.4
 **21** D-allose	 **22** allosucrose	<0.01	<0.01
 **23** 3-Ketoglucose	 **24** 3-Ketosucrose	2.2	5.6
 **25** sophorose	-	-	-

[a] yields are determined at the peak product concentration.
[b] yields are calculated from the donor sucrose.

Table 4. Acceptor reactions with L-sugars with the substrate raffinose.

Acceptor	Product	initial rate for transfructosylation [$gl^{-1}h^{-1}$]	yield at optimum/time[a] [$gl^{-1}h^{-1}$]	yield at optimum[a,b] [%]
 **26** L-glucose	 **27** L-Glc-Fru	7.1	4.9	10.7
 **28** L-rhamnose	 **29** L-Rha-Fru	0.1	<0.05	<0.1
 **30** L-galactose	 **31** L-Gal-Fru	6.8	4.5	9.5
 **32** L-fucose	 **33** L-Fuc-Fru	0.2	1.2	3.7 (21.0)[17]
 **34** L-xylose	 **35** L-Xyl-Fru	73.0	8.7	16.7
	36[c)]	41.8	14.1	26.9

[a] yields are determined at the peak product concentration.
[b] yields are calculated from the donor raffinose.
[c] structure of acceptor product 36 was not determined.

Table 5. Coupling constants of sucrose analogues (D-acceptor products) show a $^4C_1$ conformation.

Compound	coupling constants (Hz)		
	J_{12}	J_{23}	J_{34}
2	3.90	10.50	3.20
4	3.60	9.90	m
6	3.98	10.61	3.35
8	3.90 3.90 ($J_{1'',2''}$)	m	m 8.52 ($J_{1'',2''}$)
10	3.84 3.84 ($J_{1'',2''}$)	9.91 9.91 ($J_{2'',3''}$)	9.91 9.23 ($J_{3'',4''}$)
12	3.86 3.68 ($J_{1'',2''}$)	9.85 10.48 ($J_{2'',3''}$)	m 3.44 ($J_{3'',4''}$)
14	3.79 7.83 ($J_{1'',2''}$)	m m	m m
16	3.84 7.80 ($J_{1'',2''}$)	10.02 9.95 ($J_{2'',3''}$)	m m
18	1.9	m	m
22	4.03	3.70	m
24	4.55	1.50 ($J_{2',4'}$)[a]	9.81 ($J_{4',5'}$)

[a]long-range coupling, W arrangement of H-2' and H-4' in $^4C_1$ conformation.

Table 6. Coupling constants of sucrose analogues (L-acceptor products) show a $^1C_4$ conformation.

Compound	coupling constants (Hz)		
	J_{12}	J_{23}	J_{34}
27	8.0	9.1	m
31	8.1	10.1	m
33	8.0	9.9	3.6
35	8.1	9.4	9.4

Table 8. Substrate-protein contacts in the substrate-bound complexes.

Amino acids of FTF-substrate complex	**sucrose**	**D-Gal-Fru (2)**	**D-Man-Fru (18)**	**2-Deoxy-D-Glc-Fru (20)**	**allosucrose (22)**	**L-Glc-Fru (27)**	**L-Gal-Fru (31)**	**L-Fuc-Fru (33)**	**L-Xyl-Fru (35)**
Asp86 (distances in Å)	1'-OH 2.86 C-2' 3.1	C-2' 3.1	C-2' 3.3 1'-OH 2.86	C-2' 3.7 1'-OH 2.60	C-2' 3.4	C-2' 3.0 1'-OH 2.71	C-2' 3.1 1'-OH 2.85	C-2' 3.1 1'-OH 2.88	C-2' 3.4 1'-OH 2.77
Glu340 (distances in Å)	4-OH 2.45, 3-OH 2.83	3-OH 2.84	4-OH 2.33, 3-OH 2.68	3-OH 2.56	3-OH 3.21	-	-	-	-
Arg360 (distances in Å)	3-OH 3.14, 2-OH 3.23	3-OH 3.03, 2-OH 3.04	3-OH 2.71, 2-OH 2.69	-	-	3-OH 2.92	-	3-OH 3.03	3-OH 2.94
Tyr411 (distances in Å)	2-OH 2.93	2-OH 2.67	3-OH 3.27	-	2-OH 3.01	2-OH 2.82	-	2-OH 2.95	2-OH 2.94
Glu342 (distances in Å)	1-O 2.75, 2-OH 3.00, 3'-OH 2.75	1-O 2.55, 2-OH 2.93, 1'-OH 2.63, 3'-OH 2.95	1-O 2.96	3'-OH 2.97	1-O 3.11, 3-OH 2.52, 1'-OH 2.90, 3'-OH 2.62	1-O 2.77, 2-OH 2.50, 3'-OH 3.29	1-O 2.71, 2-OH 2.57, 3'-OH 2.84	1-O 3.04, 2-OH 2.60, 3'-OH 3.05	1-O 3.10, 2-OH 2.54, 3'-OH 3.09
Trp85 (distances in Å)	6'-OH 3.09	6'-OH 3.14	6'-OH 2.97	6'-OH 3.00	6'-OH 2.85	6'-OH 3.02	6'-OH 3.14	6'-OH 3.10	6'-OH 3.20
Asp247 (distances in Å)	4'-OH 2.76, 3'-OH 2.85	4'-OH 2.84, 3'-OH 2.63		4'-OH 2.89 3'-OH 3.20	4'-OH 3.02	4'-OH 3.05, 3'-OH 2.57	4'-OH 2.94, 3'-OH 2.76	4'-OH 2.94, 3'-OH 2.79	4'-OH 2.93, 3'-OH 2.83
Arg246 (distances in Å)	3'-OH 2.80	3'-OH 3.09, 6-OH 2.86 + 2.75 (2 x NH)	2.71 3'-OH (NH)	3'-OH 2.62	3'-OH 3.08	3'-OH 3.23	3'-OH 2.77	3'-OH 2.83	3'-OH 2.86
Ser164 (distances in Å)	-	4'-OH 3.28	2 x 4'-OH 3.16 (OH), 3.10 (NH)	2 x 4'-OH 2.86 (OH), 2.84 (NH)	4'-OH 3.30, 3.31 (2xNH)	4'-OH 3.17, 2.99 (2xNH)	4'-OH 3.34	4'-OH 3.20, 2.94 (2xNH)	4'-OH 3.23, 2.94 (2xNH)
Tyr429 (distances in Å)	-	-	-	-	-	-	6-OH 2.84	-	-
Lys363 (distances in Å)	-	-	4-OH 3.01	-	-	-	-	-	-

Figure and scheme legends

Figure 1.

A: Formation of isomaltose-Fru, ● sucrose & isomaltose (scale left), ○ isomaltose-Fru (scale right); B: Formation of maltose-Fru (erlose), ● sucrose & maltose (scale left), ○ maltose-Fru(scale right); C: Formation of melibiose-Fru (raffinose), ● sucrose & melibiose (scale left), ○ melibiose-Fru (raffinose) (scale right); D: Formation of cellobiose-Fru, ● sucrose & cellobiose (scale left), ○ cellobiose-Fru (scale right); E: Formation of Lac-Fru, ● sucrose (scale left), ○ Lac-Fru (scale right). Δ D-Glc, ♦ D-Fru; side products are formed (oligosaccharides and levan), which are not quantified in this figure.

Figure 2. Lowest energy dockings of the substrates sucrose (left) and D-Gal-Fru (right) with FTF show identical orientation in the active site of the enzyme. Further conformations of D-Gal-Fru docking experiments are also superimposed (grey).

Figure 3. Docking calculations of a) allosucrose b) 2-deoxyglucose c) D-Man-Fru with FTF show the active orientation affected by the short distance to the glycosidic oxygen. Further conformations of docking experiments are also superimposed (grey).

Figure 4. Docking results of a) L-Glc-Fru, b) L-Gal-Fru, c) L-Fuc-Fru and d) L-Xyl-Fru with FTF showing putative active orientations for the substrates. Further conformations of docking experiments are also superimposed (grey).

Figure 5. Hydrogen network of a) L-Glc-Fru, b) L-Xyl-Fru with FTF.

Scheme 1. Possible Reaction Pathway of FTF. See the text for details. a) Sucrose coordinates in the active site of the enzyme. b) D-Glucose is released and a reactive oxocarbenium ion of the fructosyl-residue is formed and subsequently attacked by Asp86 of the FTF. c) Covalent fructosyl-enzyme complex, substituted by the acceptor to form a sucrose analogue. d) Coordination of the sucrose-analogue in the active site of the enzyme.

Figure 1.

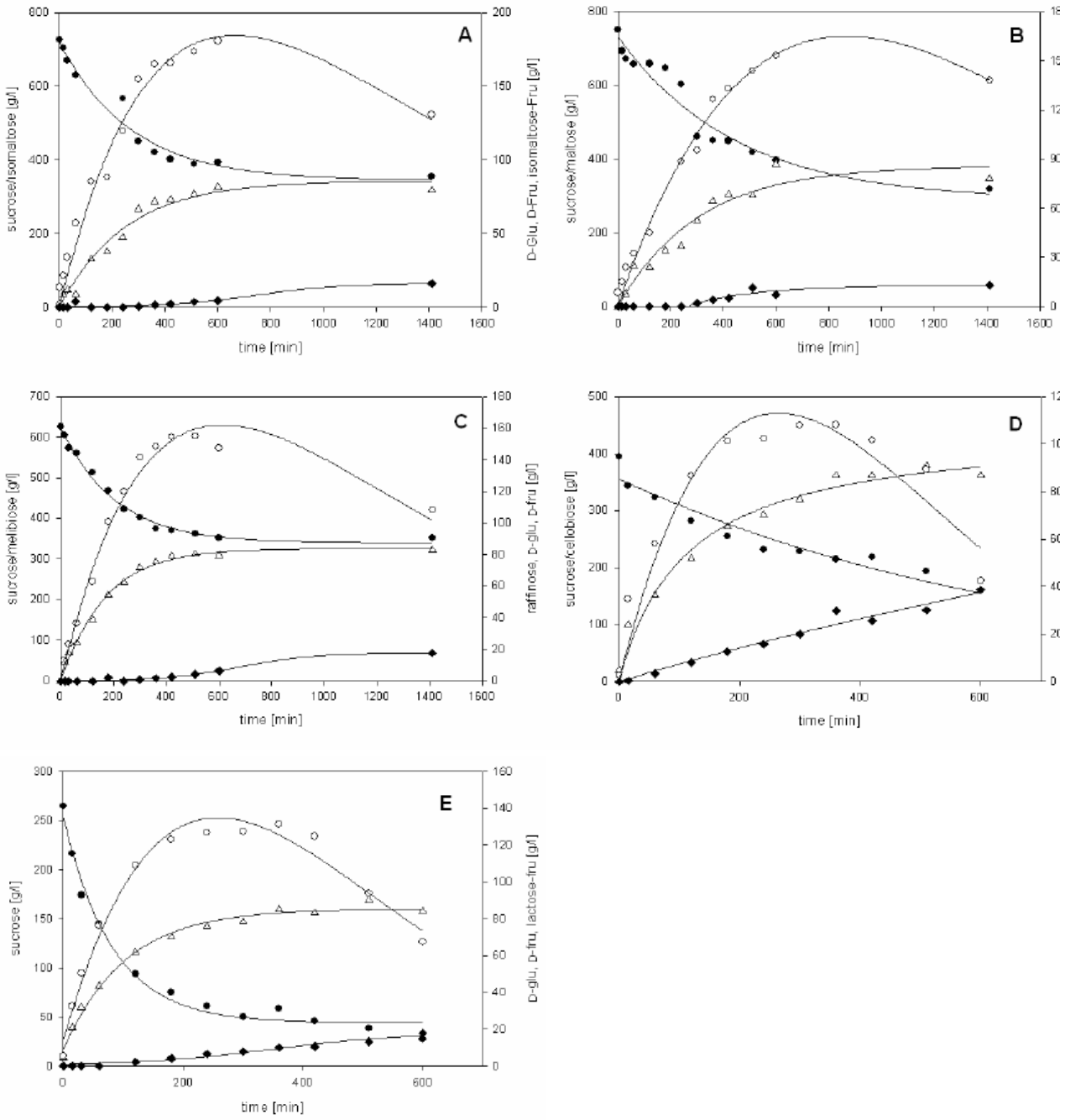

A
sucrose/isomaltose [g/l]
D-Glu, D-Fru, isomaltose-Fru [g/l]
time [min]
B
sucrose/maltose [g/l]
time [min]
C
sucrose/melibiose [g/l]
raffinose, D-glu, D-fru [g/l]
time [min]
D
sucrose/cellobiose [g/l]
time [min]
E
sucrose [g/l]
D-glu, D-fru, lactose-fru [g/l]
time [min]

Figure 2.

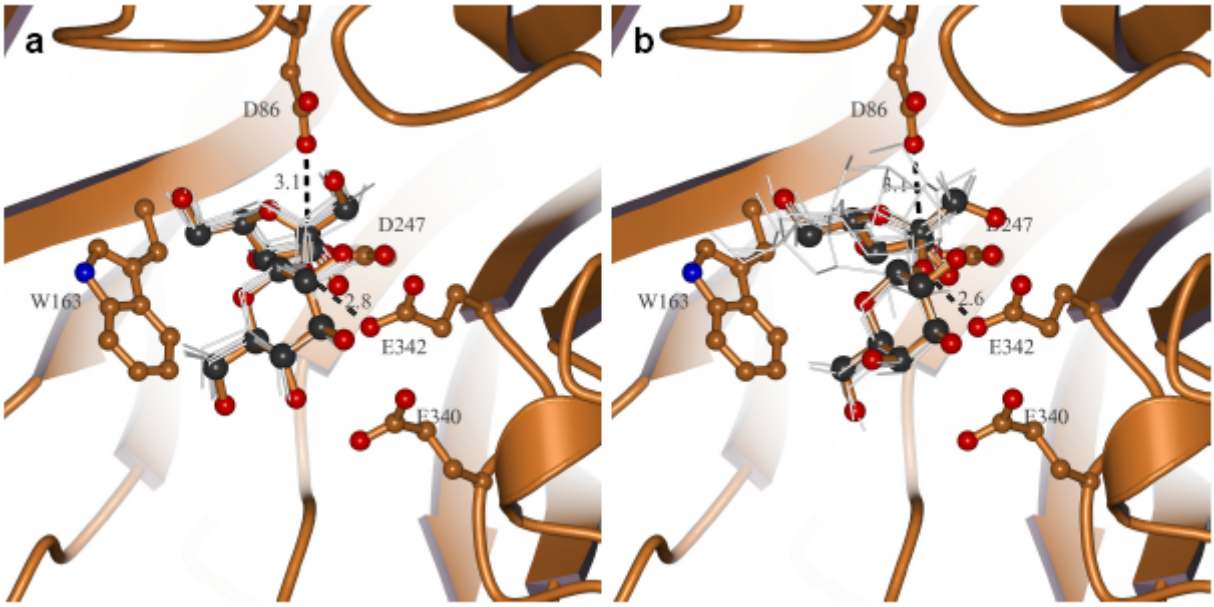


Figure 3.

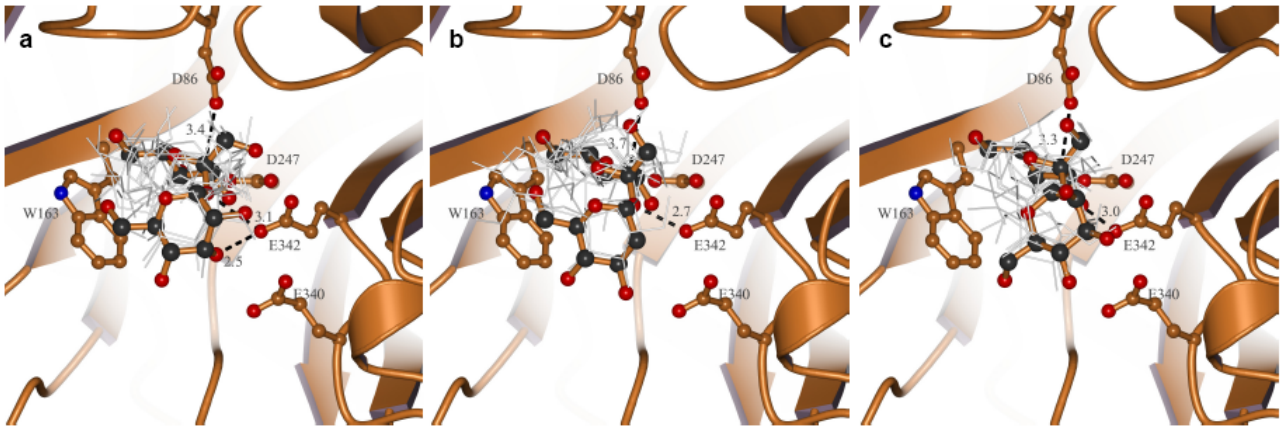

a
D86
3.4
D247
W163
3.1
E342
2.5
E340
b
D86
3.7
D247
W163
2.7
E342
E340
c
D86
3.3
D247
W163
3.0
E342
E340

Figure 4.

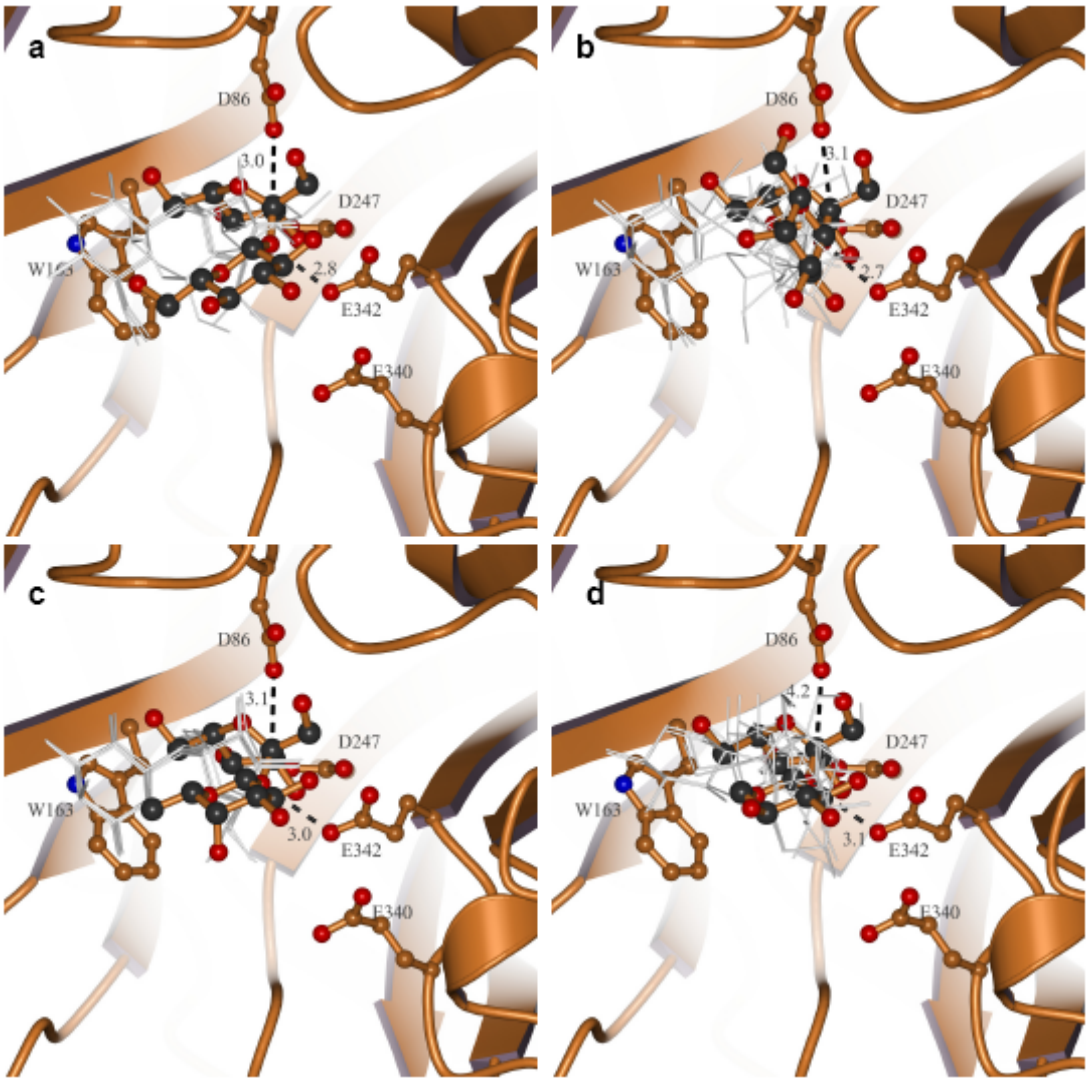
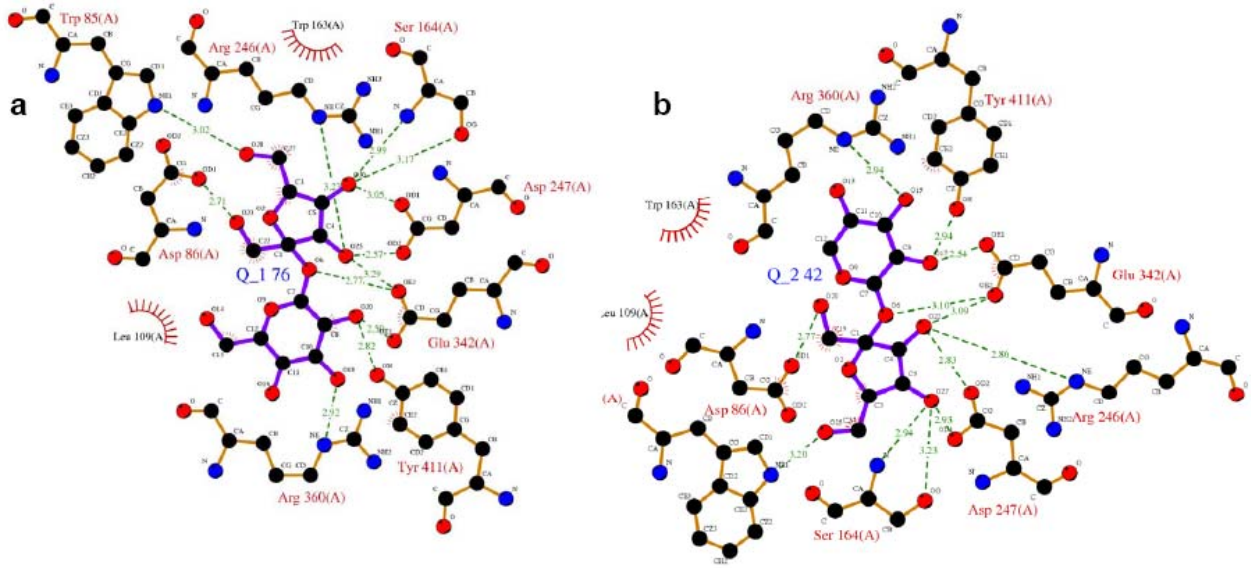
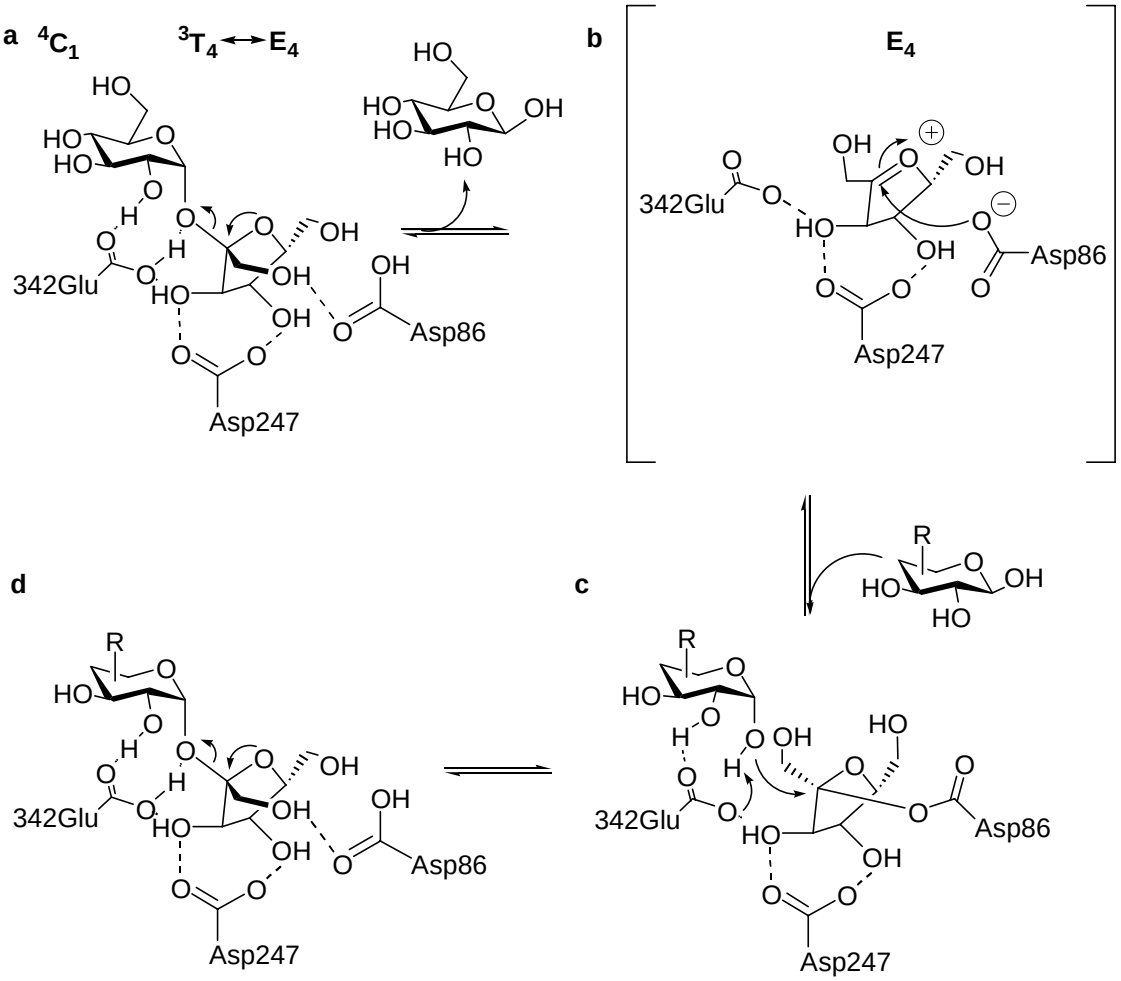

a
D86
3.0
D247
W163
2.8
E342
E340
b
D86
3.1
D247
W163
2.7
E342
E340
c
D86
3.1
D247
W163
3.0
E342
E340
d
D86
4.2
D247
W163
3.1
E342
E340

Figure 5.


a
Trp 85(A)
Arg 246(A)
Trp 163(A)
Ser 164(A)
Asp 247(A)
Asp 86(A)
Q_1 76
Leu 109(A)
Glu 342(A)
Tyr 411(A)
Arg 360(A)
b
Arg 360(A)
Tyr 411(A)
Trp 163(A)
Q_2 42
Glu 342(A)
Asp 86(A)
Arg 246(A)
Ser 164(A)
Asp 247(A)

Scheme 1.



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