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Alternansucrase acceptor reactions with D-tagatose and L-glucose

Gregory L. Côté,[a,*] Christopher A. Dunlap,[b] Michael Appell[c] and Frank A. Momany[c]

[a]*Bioproducts and Biocatalysis Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, United States Department of Agriculture†, 1815 North University Street, Peoria, IL 61604, USA*
[b]*Crop Bioprotection, National Center for Agricultural Utilization Research, Agricultural Research Service, United States Department of Agriculture†, 1815 North University Street, Peoria, IL 61604, USA*
[c]*Plant Polymer Research Units, National Center for Agricultural Utilization Research, Agricultural Research Service, United States Department of Agriculture†, 1815 North University Street, Peoria, IL 61604, USA*

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Abstract—Alternansucrase (EC 2.4.1.140) is a D-glucansucrase that synthesizes an alternating α-(1→3), (1→6)-linked D-glucan from sucrose. It also synthesizes oligosaccharides via D-glucopyranosyl transfer to various acceptor sugars. Two of the more efficient monosaccharide acceptors are D-tagatose and L-glucose. In the presence of D-tagatose, alternansucrase produced the disaccharide α-D-glucopyranosyl-(1→1)-β-D-tagatopyranose via glucosyl transfer. This disaccharide is analogous to trehalulose. We were unable to isolate a disaccharide product from L-glucose, but the trisaccharide α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→4)-L-glucose was isolated and identified. This is analogous to panose, one of the structural units of pullulan, in which the reducing-end D-glucose residue has been replaced by its L-enantiomer. The putative L-glucose disaccharide product, produced by glucoamylase hydrolysis of the trisaccharide, was found to be an acceptor for alternansucrase. The disaccharide, α-D-glucopyranosyl-(1→4)-L-glucose, was a better acceptor than maltose, previously the best known acceptor for alternansucrase. A structure comparison of α-D-glucopyranosyl-(1→4)-L-glucose and maltose was performed through computer modeling to identify common features, which may be important in acceptor affinity by alternansucrase.
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1. Introduction

Glucansucrases are D-glucosyltransferases that synthesize α-D-glucans from sucrose. They are of interest not only for their ability to synthesize unique polysaccharides, but also for their ability to synthesize oligosaccharides via acceptor reaction. Alternansucrase (EC 2.4.1.140), which synthesizes an alternating α-(1→3), (1→6)-linked D-glucan[1] is especially interesting in this regard. Not only do its acceptor products differ in structure from those synthesized by *Leuconostoc mesenteroides* NRRL B-512F dextransucrase,[2,3] but they are also produced somewhat more efficiently.[3] These oligosaccharide acceptor products may be useful as prebiotics.[4]

In this study, we describe the structures of alternansucrase acceptor products arising from D-tagatose and from L-glucose. Our previous work has shown that both of these compounds are among the more efficient monosaccharide acceptors for alternansucrase.[4] D-Tagatose is of interest due to its recent approval as an alternative sweetener in foods[5] and its similarity to D-fructose, from which it differs only by inversion of the 4-OH group. As it becomes more readily available, it may represent a useful material for the synthesis of functional oligosaccharides.

* Corresponding author. Tel.: +1 309 681 6319; fax: +1 309 681 6040; e-mail: cotegl@ncaur.usda.gov

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2. Experimental

2.1. Materials

L-Glucose, sucrose and *Rhizopus* sp. glucoamylase were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Glucoamylase was dialyzed against water prior to use in order to remove low-MW contaminants. D-Tagatose was a gift from Arla Foods Ingredients (Union, NJ, USA).

2.2. Acceptor reaction conditions

Acceptor reactions were carried out essentially as previously described,[2,4] using alternansucrase from *Leuconostoc mesenteroides* NRRL B-21297. Dialyzed and concentrated cell-free culture fluid was used as alternansucrase without further purification, as this strain produces only alternansucrase with no detectable levels of dextransucrase.[6] Variations in the concentrations of sucrose and acceptor affect the distribution of acceptor products, but do not influence their structures.[7]

2.3. Analytical and preparative methods

Oligosaccharide products were isolated from reaction mixtures by ion-exchange and gel-permeation chromatography. Mixtures were initially chromatographed over Dowex 99CA/320 ion-exchange resin (Ca^{2+} form) in water. This successfully removed fructose and leucrose from the other components and partially resolved lower-DP (degree of polymerization) oligosaccharides. Appropriate fractions were then chromatographed over Bio-Gel P-2 (Bio-Rad, Hercules, CA, USA) to isolate oligosaccharides based on DP. Analytical thin-layer chromatography was performed on Whatman K5 silica gel plates, using multiple ascents in 4:1 acetonitrile–water as previously described.[2,8] Quantitative densitometry was performed on scanned images of the TLC plates by use of Un-Scan-It gel software (Silk Scientific, Orem, UT, USA). Structures were determined by methylation/GC–MS and NMR spectroscopy.[9] ^{1}H, ^{13}C, COSY, HMQC, and HMBC NMR spectra were recorded for each sample on a Bruker 400 MHz instrument in D_2O at 27 °C. Peak assignments for NMR spectra were made based on the 2D NMR experiments, and the glycosidic linkages were confirmed by HMBC long-range coupling experiments.

2.4. Molecular modeling

All calculations reported here were carried out on Parallel Quantum Solutions hardware and software (v3.1, PQS Ab Initio Program Package, Parallel Quantum Solutions, 2013 Green Acres, Suite E, Fayetteville, AR 72703). Geometry optimizations were carried out at the B3LYP/6-31+G* and B3LYP/6-311++G** levels as previously described with mentioned modifications.[10] Geometry optimization was considered satisfactory if the energy differences between cycles was less than 1×10^{-6} Hartree and a gradient of 3×10^{-4} a.u. All results reported here are at the B3LYP/6-311++G** level. Zero-point vibrational energy, enthalpy, and entropy were calculated using an analytical hessian program with the default threshold. Models were initially built and displayed using Hyperchem Professional Release 7.5 for Windows on a Dell 8200 PC (HyperChem 7.5, Hypercube, Inc., 1115 NW 4th Street, Gainesville, FL 32601).

3. Results and discussion

3.1. D-Tagatose

Alternansucrase reaction mixtures with sucrose and D-tagatose were first chromatographed over Dowex 99CA/320 to remove fructose. Leucrose was separated from the disaccharide acceptor product by two passages over Bio-Gel P-2; apparently their hydrodynamic radii differ sufficiently to enable this separation.

Upon borohydride reduction and subsequent methylation, the disaccharide product yielded only 2,3,4,6-tetra-*O*-methylhexitol derivatives, which were not resolved by gas–liquid chromatography. This suggested a 1,1-linked disaccharide. The structure was determined to be α-D-glucopyranosyl-(1→1)-β-D-tagatopyranose (structure 1) by NMR spectroscopy. Peak assignments appear in Table 1. It is noteworthy that the NMR spectrum reveals only a single anomeric form of this disaccharide in aqueous solution. A similar phenomenon has been noted for the α-D-glucopyranosyl D-fructose disaccharides trehalulose[11] and leucrose, in which the D-fructosyl moiety occurs predominantly in the β-pyranosyl form.

In the presence of sucrose and D-tagatose, alternansucrase catalyzes three competing reactions. The first is

Table 1. NMR peak assignments for disaccharide acceptor product **1** from D-tagatose

	H-1a	H-1b C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6a C-6	H-6b
β-D-Tag*p*	3.32	3.81 69.17	— 97.82	3.75 66.16	3.76 70.79	3.8 70.05	3.5 62.25	3.68
α-D-Glc*p*		4.87 98.32	3.43 71.42	3.63 72.94	3.31 69.39	3.59 71.84	3.61 60.37	3.74

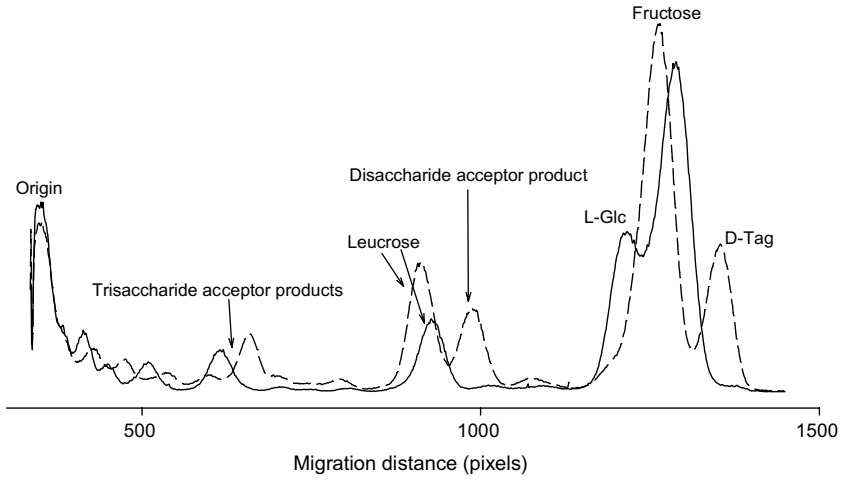

Figure 1. TLC densitogram of D-tagatose (- - - -) and L-glucose (——) acceptor reaction mixtures. Initial monosaccharide acceptor concn was 0.22 M, initial sucrose concn was 0.47 M, and enzyme concn was 0.2 U/mL in 50 mM pH 5.4 NaOAc buffer with 0.02% NaN_3, carried out at 20 °C for 48 h.

polymerization of the D-glucosyl moiety of sucrose to yield alternan.[1] The second and third are the acceptor reactions consisting of D-glucopyranosyl transfer to either D-tagatose or to the D-fructose liberated from sucrose. The latter reaction yields leucrose as the primary product.[2] It is well established that the relative amounts of alternan, leucrose, and other acceptor products depend on the concentrations of sucrose and acceptor. To measure these parameters for the D-tagatose reaction, acceptor reactions were carried out at various substrate concentrations and product yields were measured by TLC densitometry. Figure 1 shows a typical TLC densitogram of both a D-tagatose and an L-glucose acceptor reaction mixture. Results of the quantitative analyses appear in Figures 2 and 3. At sufficiently high concentrations of acceptor, up to 94% of the glucosyl portion of sucrose was incorporated into oligosaccharide acceptor products. Within the concentration range tested here, the best ratio of disaccharide product to leucrose occurred at a sucrose concentration of approximately 0.2 M and a D-tagatose concentration of 1.25 M. It may be assumed that at lower sucrose concentrations and higher acceptor concentrations, the product/leucrose ratio would be even more favorable, as the lower sucrose concentrations would yield lower fructose concentrations. However, since D-tagatose is more expensive than sucrose, it would be best to optimize concentrations to maximize yields from D-tagatose, rather than from sucrose, while still minimizing leucrose yields.

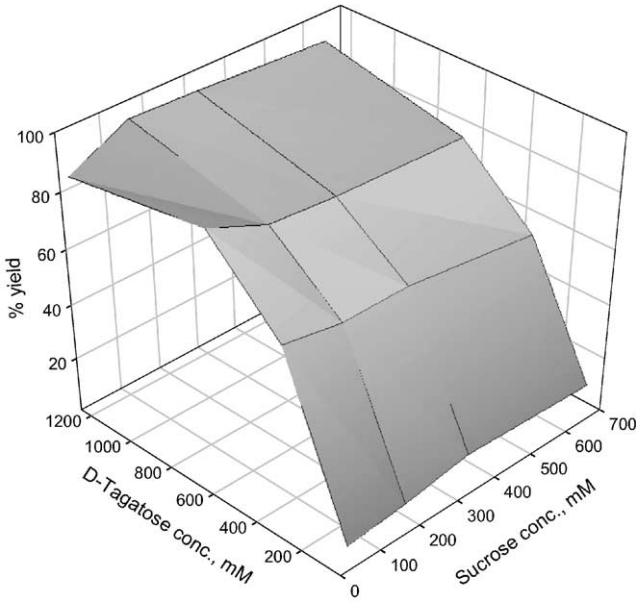

Figure 2. Percent conversion of sucrose to oligosaccharides as a function of sucrose and D-tagatose concentrations.

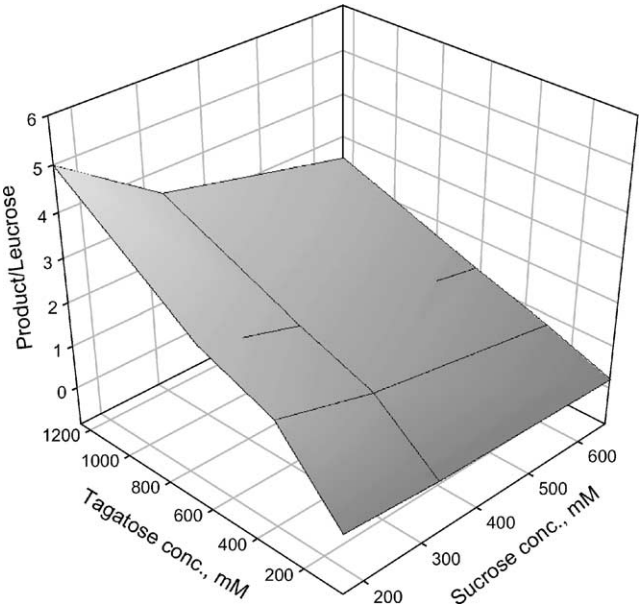

Figure 3. Ratio of disaccharide **1** to leucrose in product mixture as a function of sucrose and D-tagatose concentrations.

This would most likely occur at both high sucrose and high acceptor concentrations (Figs. 2 and 3).

Other, higher-DP acceptor products were also formed, but were not isolated. It is presumed that these arise from subsequent D-glucopyranosyl transfers to the D-glucopyranosyl moiety of the disaccharide product, and so their structures should be analogous to those previously described.[1,4]

3.2. L-Glucose

Thin-layer chromatographic analysis of alternansucrase acceptor reactions invariably showed only a single disaccharide product was formed in measurable quantities at all stages of the reaction that we were able to sample (e.g., Fig. 1). Isolation of this disaccharide showed it to be leucrose.

However, significant yields of a trisaccharide and lesser amounts of higher-DP products were formed, and the trisaccharide was readily isolated by Bio-Gel P-2 chromatography. Methylation analysis and GC–MS revealed equimolar amounts of 2,3,4,6-tetra-*O*-methylglucose, 2,3,4-tri-*O*-methylglucose and 2,3,6-tri-*O*-methylglucose, indicating a trisaccharide with a (1→6)- and a (1→4)-linkage. Analysis of the NMR spectra showed the compound to be α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→4)-L-glucose (structure 2, Table 2).

It is difficult to understand why no disaccharide acceptor product was observed in the L-glucose reaction mixtures. One hypothesis is that the disaccharide, α-D-glucopyranosyl-(1→4)-L-glucose, is initially formed, but is such a good acceptor that it immediately gives rise to the trisaccharide. To test this hypothesis, an aqueous solution of trisaccharide **2** was treated with an excess of dialyzed *Rhizopus* sp. glucoamylase in water at room temperature until no trisaccharide remained, as judged by TLC (reaction time = 5 days). The products were glucose and an unknown disaccharide. The latter was isolated from this mixture by Bio-

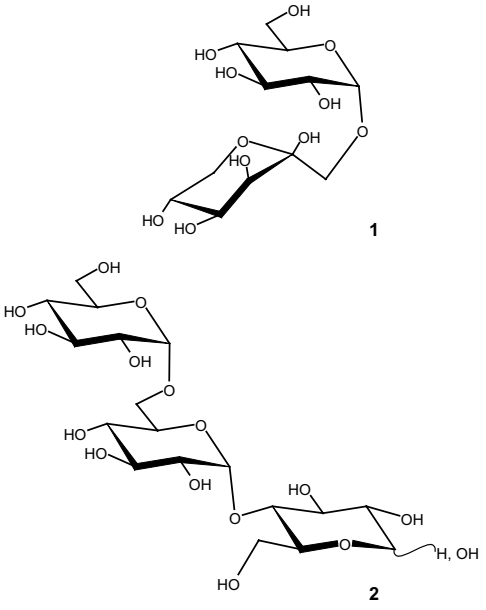

Table 2. NMR peak assignments for acceptor products from L-glucose

	H-1 C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6a C-6	H6b
Disaccharide[a]							
α-L-Glc*p*	5.15	3.48	3.90	3.50	3.53	3.77	3.91
	91.84	71.50	70.50	77.65	71.50	60.10	
α-D-Glc*p*-(1→4)	4.92	3.49	3.61	3.45	3.95	3.72	3.97
	99.42	71.50	72.84	69.25	71.80	60.10	
β-L-Glc*p*	4.56	3.22	3.54	3.53	3.53	3.79	3.92
	95.76	74.50	74.50	77.53	75.18	60.10	
α-D-Glc*p*-(1→4)	4.92	3.49	3.61	3.45	3.95	3.72	3.97
	99.46	71.50	72.84	69.25	71.80	60.10	
Trisaccharide **2**							
α-L-Glc*p*	5.19	3.52	3.77	3.53	3.92	3.80	3.90
	91.86	71.52	71.42	77.63	70.57	59.93	
α-D-Glc*p*-(1→4)	4.98	3.55	3.63	3.55	4.17	3.64	3.99
	99.46	71.36	73.08	69.4	70.65	65.43	
α-D-Glc*p*-(1→6)	4.92	3.53	3.72	3.38	3.70	3.62	3.75
	98.07	71.59	73.10	69.55	71.85	60.50	
β-L-Glc*p*	4.61	3.22	3.58	3.53	3.54	3.82	3.92
	95.76	74.55	74.52	77.48	75.20	60.04	
α-D-Glc*p*-(1→4)	4.98	3.55	3.63	3.55	4.17	3.64	3.99
	99.46	71.36	73.08	69.37	70.65	65.47	
α-D-Glc*p*-(1→6)	4.92	3.53	3.72	3.38	3.70	3.62	3.75
	98.07	71.59	73.10	69.55	71.85	60.50	

[a] Disaccharide derived from glucoamylase-digested trisaccharide.

Gel P-2 chromatography. Methylation and NMR analysis proved the disaccharide to be α-D-glucopyranosyl-(1→4)-L-glucose (Table 2). When this disaccharide was tested as an acceptor for alternansucrase, TLC revealed that it did indeed act as an excellent acceptor. Qualitative comparison with maltose, the best known acceptor for alternansucrase, showed that the disaccharide, α-D-glucopyranosyl-(1→4)-L-glucose, was consumed slightly more rapidly than maltose under identical conditions and concentrations. Densitometric analysis of the reaction mixtures after seven hours indicated that the reaction mixture using α-D-glucopyranosyl-(1→4)-L-glucose as the acceptor contained approximately 10% less disaccharide acceptor, 10% more oligosaccharide product, and an equal amount of D-fructose, compared to the maltose acceptor reaction. This was based on a single experiment, and more extensive quantitative studies remain to be done. However, it can be concluded that α-D-glucopyranosyl-(1→4)-L-glucose is at least as good an acceptor as maltose, and possibly better.

3.3. Structural comparison of disaccharide acceptors

The geometry of the active site of alternansucrase is not known; however, maltose and α-D-glucopyranosyl-(1→4)-L-glucose have been shown to be strong acceptors for alternansucrase.[4] A structural comparison has been performed on maltose and α-D-glucopyranosyl-(1→4)-L-glucose in an attempt to gain insight into molecular recognition of these molecules at the alternansucrase active site.

Conformational preferences of β-maltose have been previously studied,[12] and the lowest energy form of β-maltose has been identified, Figure 4A. This maltose conformer was used for a structural comparison study in vacuo. α-D-Glucopyranosyl-(1→4)-L-glucose was geometry optimized from a conformation similar to that of maltose. The maltose structure, 4A, is energetically favored over α-D-glucopyranosyl-(1→4)-L-glucose, 4B, in overall energy, corrected energy, and free energy (see Table 3).

Glycosidic torsion angles are of considerable importance in the resulting spatial arrangement of disaccharides

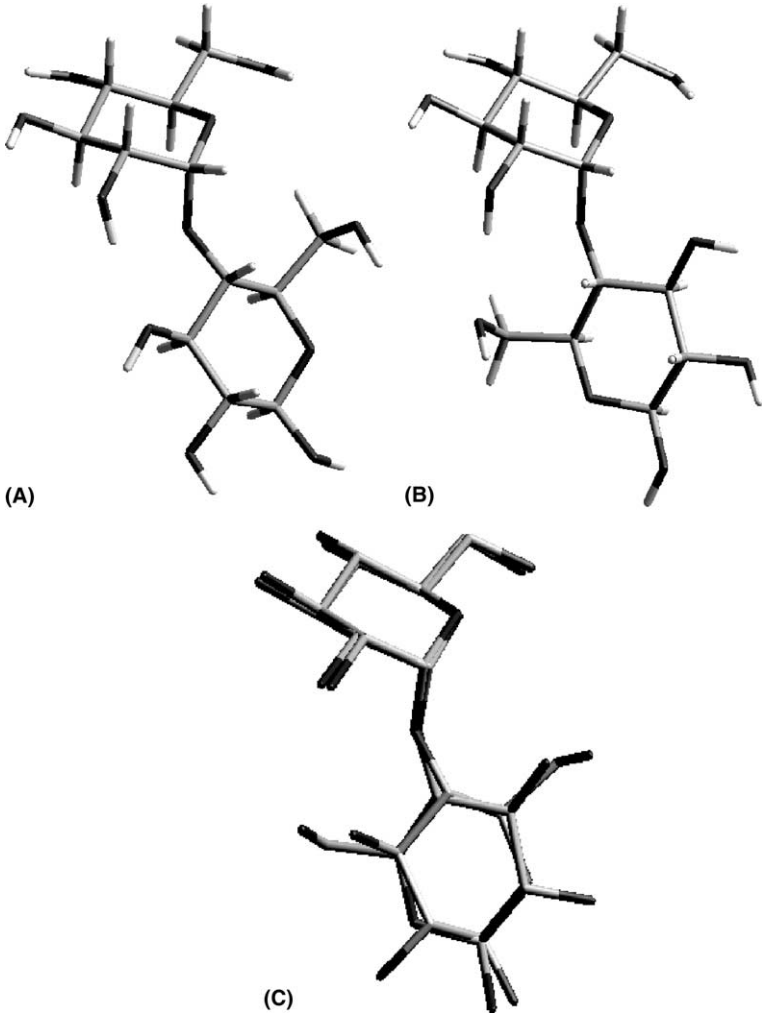

Figure 4. Geometry optimized structures at the B3LYP/6-311++G** level of theory for maltose, A, and α-D-Glc*p*-(1→4)-L-Glc*p*, B. Alignment of A with B is shown below as C.

Table 3. Selected energies, bond lengths, and bond angles for **4a** and **4b** at the B3LYP/6-11++G** level of theory

	Maltose A (Fig. 4)	α-D-Glc*p*-(1→4)-L-Glc*p* B (Fig. 4)
Energy	−814,727.013	−814,724.743
ΔE	0	2.270
ZPVE	232.064	232.195
$\Delta E_{corrected}$	0	2.401
Enthalpy (*H*)	246.064	247.602
Entropy (*S*) (cal/mol K)	155.997	162.004
ΔG^0_{298}	0	1.139
Dihedral angles		
ϕ_H	−4.68	14.43
ψ_H	10.67	43.06
O-6–C-6–C-5–O-5	58.97	60.49
O-6′–C-6′–C-5′–O-5′	−62.03	75.69
HO-2–O-2–C-2–C-3	87.36	69.10
HO-2′–O-2′–C-2–′C-3′	−176.74	174.09
Bond lengths		
C-1–O-1	1.424	1.434
C-1′–O-1′	1.395	1.394
C-5–C-6	1.522	1.521
C-5′–C-6′	1.523	1.522
HO-6–O-6	0.964	0.965
HO-6′–O-6′	0.964	0.964
HO-2–O-2	0.973	0.973
HO-2′–O-2′	0.964	0.964
Hydrogen-bond lengths		
HO-4···HO-3	2.421	2.429
HO-3···HO-2	2.430	2.429
HO-2···HO-3′	1.960	
HO-2···HO-6′		1.960
HO-3′···HO-2′	2.408	2.411
HO-2′···HO-1′	2.559	2.570
Bond angles		
C-1–O1–C-4′	119.33	118.04
O-1–C-1–O-5	111.38	109.60
O-1′–C-1′–O-5′	108.86	108.91
H-1–C-1–O-5	105.76	106.11
H-1′–C-1′–O-5′	109.24	108.89
C-1–O-5–C-5	115.82	115.69
C-1′–O-5′–C-5′	114.13	112.30
HO-2–O-2–C-2	107.27	111.38
HO-2′–O-2′–C-2′	108.40	108.39
HO-4–O-4–C-4	107.21	107.28

Energies are in kcal/mol unless noted otherwise. Bond lengths are given in Å.

and have been under extensive computational and experimental investigation. The structure 4A is similar to that reported for maltose by neutron diffraction and X-ray crystal structures.[12] The (1→4) glycosidic linkage of 4A contains torsion angles of $\phi_H = -4.69°$ and $\psi_H = 10.67°$. During geometry optimization, α-D-glucopyranosyl-(1→4)-L-glucose went through a transition without barrier to the optimized structure 4B with $\phi = 14.43°$ and $\psi = 43.06°$. This change in glycosidic torsion angles of the 4B structure accommodated remarkable similarity between hydrogen-bond lengths of these disaccharides.

Structures 4A and 4B have their hydroxymethyl rotamers in the *gt* orientation for the nonreducible ring and the gg orientation for the reducible ring. A notable feature of 4A is the HO-4···HO-3···HO-2···HO-3′···HO-2′···HO-1′ cooperative hydrogen-bond network with a 1.960 Å HO-2···HO-3′ hydrogen bond. Interestingly, the structure 4B has the HO-2 of the first glycosidic residue forming a 1.960 Å hydrogen bond with the hydroxymethyl substituent of the reducible ring. In contrast, structure 4B has the less extensive HO-4···HO-3···HO-2···HO-6′ network and a HO-3′···HO-2′···HO-1 network. The synergistic effect of the cooperative hydrogen-bond network in structure 4A can be seen in energy and the entropic terms, where 4A is lower in entropy and free energy.

The alignment of 4B to 4A shows remarkable similarity between the two structures. Both structures have their hydroxyl substituents in similar position through the disaccharide, with the largest discrepancy being 1.009–1.185 Å for the O-6′ to O-3′ comparison. The geometries of structures 4A and 4B can reasonably achieve excellent fit to the same binding site, apparently explaining why 4B is such a good acceptor for alternansucrase, and why none of this disaccharide could be isolated from acceptor reaction mixtures that had proceeded to completion.

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