



# Acceptor products of alternansucrase with gentiobiose. Production of novel oligosaccharides for food and feed and elimination of bitterness<sup>☆</sup>

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## ABSTRACT

In the presence of suitable acceptor molecules, dextranase makes a homologous series of oligosaccharides in which the isomers differ by a single glucosyl unit, whereas alternansucrase synthesizes one trisaccharide, two tetrasaccharides, etc. Previously, we showed that alternansucrase only forms certain isomers of DP > 4 from maltose in measurable amounts, and that these oligosaccharides belong to the oligoaltaran series rather than the oligodextran series. We now demonstrate that the acceptor products from gentiobiose, also formed in good yields (nearly 90% in unoptimized reactions), follow a pattern similar to those formed from maltose. The initial product is a single trisaccharide,  $\alpha$ -D-Glcp-(1 $\rightarrow$ 6)- $\beta$ -D-Glcp-(1 $\rightarrow$ 6)-D-Glc. Two tetrasaccharides were formed in approximately equal quantities:  $\alpha$ -D-Glcp-(1 $\rightarrow$ 3)- $\alpha$ -D-Glcp-(1 $\rightarrow$ 6)- $\beta$ -D-Glcp-(1 $\rightarrow$ 6)-D-Glc and  $\alpha$ -D-Glcp-(1 $\rightarrow$ 6)- $\alpha$ -D-Glcp-(1 $\rightarrow$ 6)- $\beta$ -D-Glcp-(1 $\rightarrow$ 6)-D-Glc. Just one pentasaccharide was isolated from the reaction mixture,  $\alpha$ -D-Glcp-(1 $\rightarrow$ 6)- $\alpha$ -D-Glcp-(1 $\rightarrow$ 3)- $\alpha$ -D-Glcp-(1 $\rightarrow$ 6)- $\beta$ -D-Glcp-(1 $\rightarrow$ 6)-D-Glc. Our hypothesis that the enzyme is incapable of forming two consecutive  $\alpha$ -(1 $\rightarrow$ 3) linkages, and does not form products with more than two consecutive  $\alpha$ -(1 $\rightarrow$ 6) linkages, apparently applies to other acceptors as well as to maltose. The glucosylation of gentiobiose reduces or eliminates its bitter taste.

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## 1. Introduction

Dextranase (EC 2.4.1.5) and alternansucrase (EC 2.4.1.140) are bacterial enzymes that transfer glucosyl units from sucrose into long-chain polymers of glucose.<sup>1</sup> Glucosyl transfer can also occur when acceptor molecules, particularly carbohydrates, are added to a sucrose digest, resulting in the formation of glucose oligosaccharides of varying molecular sizes (degree of polymerization, DP).<sup>2</sup> Commercial dextran, produced by *Leuconostoc mesenteroides* NRRL B-512F, contains approximately 95%  $\alpha$ -(1 $\rightarrow$ 6) linkages, whereas altaran contains alternating  $\alpha$ -(1 $\rightarrow$ 3) and  $\alpha$ -(1 $\rightarrow$ 6) linkages.<sup>3</sup> A major difference between NRRL B-512F dextranase and alternansucrase is that the dextranase acceptor products consist of a homologous series of oligosaccharides in which the isomers differ by a single  $\alpha$ -(1 $\rightarrow$ 6)-linked D-glucosyl unit, whereas alternansucrase synthesizes one DP 3 product, two DP 4 products, etc.<sup>2</sup> For the example of maltose as the acceptor, if one considers only the linear, unbranched possibilities for alternansucrase, the hypothetical number of potential products increases exponentially

as a function of DP. Experimental evidence indicates that far fewer products are actually formed. It was shown previously that the enzyme does not synthesize an  $\alpha$ -(1 $\rightarrow$ 3) linkage by acceptor reactions unless an  $\alpha$ -(1 $\rightarrow$ 6) linkage is present, and that the enzyme is incapable of forming two consecutive  $\alpha$ -(1 $\rightarrow$ 3) linkages.<sup>2</sup> Currently, alternansucrase acceptor products are under consideration as ingredients for food and feed uses.<sup>4–6</sup> It is useful to know the structures of the acceptor products in order to understand the structure–function relationships of these novel ingredients.

## 2. Experimental

### 2.1. Carbohydrates

Sucrose and gentiobiose were reagent-grade from Sigma–Aldrich Corp. (St. Louis, MO). Isomaltose and isomaltotriose were prepared by dextranase hydrolysis of commercial-grade dextran<sup>7</sup> and isolated by Bio-Gel P-2 chromatography.

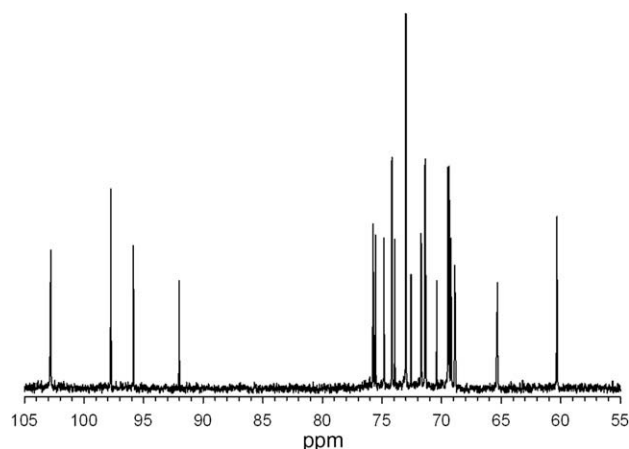
### 2.2. Enzymes

Alternansucrase was prepared from *L. mesenteroides* NRRL B-21297 as previously described.<sup>4</sup> In brief, the cell-free culture fluid of sucrose-grown bacteria was concentrated, dialyzed against 20 mM pH 5.4 sodium acetate buffer, and used without further

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**Figure 1.** Proton-decoupled  $^{13}\text{C}$  NMR spectrum of gentiobiose trisaccharide acceptor product. Complete peak assignment was not done; anomeric peak assignments are as follows: 92.0 ppm, C1 $\alpha$ , reducing end glucose unit; 95.9 ppm, C1 $\beta$ , reducing end glucose unit; 97.8 ppm, C1, nonreducing terminal glucopyranosyl unit, in an  $\alpha$ -(1 $\rightarrow$ 6) linkage; 102.8 and 102.9 ppm, partially resolved, C1, middle glucopyranosyl unit, in a  $\beta$ -(1 $\rightarrow$ 6) linkage.

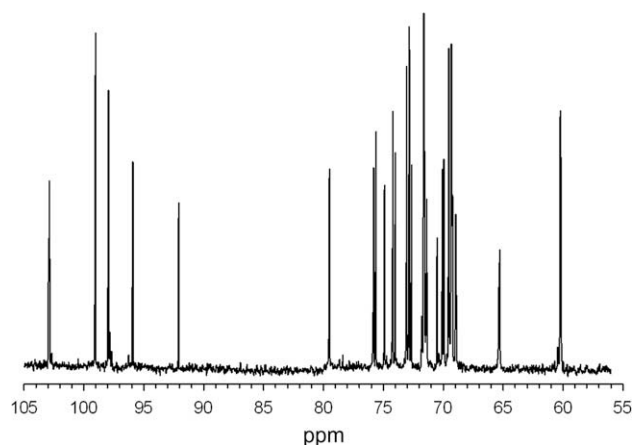
purification. *Arthrobacter globiformis* NRRL B-4425 isomaltodextranase was prepared according to the method described by Okada et al.<sup>8</sup> Unless otherwise stated, enzyme reactions were carried out at room temperature (21–24 °C) in 20 mM pH 5.4 sodium acetate buffer containing 0.01% sodium azide.

### 2.3. Preparation of oligosaccharides

Acceptor reactions were carried out as previously described.<sup>4</sup> After the sucrose was consumed, as ascertained by TLC, the reaction mixtures were chromatographed over a 7.5  $\times$  150 cm column of Bio-Gel P-2 (fine mesh) with water as the eluent. Fractions of approximately 20 mL were collected, and oligosaccharides in the eluate, separated by size according to DP, were monitored by TLC. Appropriate fractions were combined and freeze dried. The amount of material applied to the column varied, but was typically on the order of 1–5 g total solids. For separation of isomeric oligosaccharides of the same size, preparative HPLC was carried out using a Waters Breeze system with a refractive index detector. A Phenomenex Synergi 10- $\mu\text{m}$  Hydro-RP 80 column (21.2  $\times$  250 mm) was eluted at room temperature with water.

### 2.4. Analytical methods

Carbohydrate content was determined by the phenol–sulfuric acid method.<sup>9</sup> Thin-layer chromatography was performed on Whatman K5 silica gel plates as previously described.<sup>2,4</sup> For the resolution of oligosaccharides between DP 1 and 3, four ascents in 4:1 acetonitrile–H<sub>2</sub>O was the usual method of choice. For higher DP oligosaccharides, a single ascent in 4:10:14:11 ethyl acetate–1-propanol–acetonitrile–water was used. Detection was carried out by the method of Bounias.<sup>10</sup> Developed TLC plates were scanned using a desktop scanner in reflectance mode, and densitometry was carried out on the images using Un-Scan-It Gel version 6.1 (Silk Scientific, Orem, UT). Molecular weights were measured using a Bruker Daltonics Omnisflex MALDI-TOF mass spectrometer. Aqueous solutions of oligosaccharides were mixed with an equal volume of saturated 2,5-dihydroxybenzoic acid solution in acetonitrile, allowed to dry on the probe, and were subjected to MALDI-TOF mass spectrometry. Methylation analysis was carried out using the method of Ciucanu and Kerek<sup>11</sup> in which methylation was accomplished with finely powdered NaOH in DMSO, and hydrolysis was accomplished using 2 M trifluoroacetic acid. The

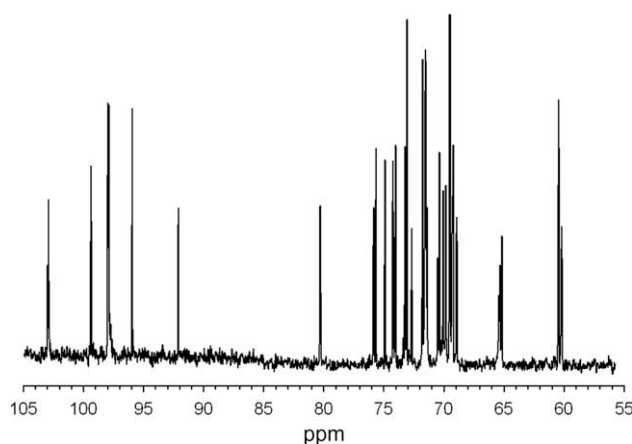


**Figure 2.** Proton-decoupled  $^{13}\text{C}$  NMR spectrum of gentiobiose tetrasaccharide acceptor product **4A**. Complete peak assignment was not done; anomeric peak assignments are as follows: 92.0 ppm, C1 $\alpha$ , reducing end glucose unit; 95.9 ppm, C1 $\beta$ , reducing end glucose unit; 97.9 ppm, C1, nonreducing glucopyranosyl unit, in an  $\alpha$ -(1 $\rightarrow$ 6) linkage; 99.1 ppm, C1 of nonreducing terminal glucopyranosyl unit in an  $\alpha$ -(1 $\rightarrow$ 3) linkage; 102.9 and 103.0 ppm, partially resolved C1, nonreducing glucopyranosyl unit, in a  $\beta$ -(1 $\rightarrow$ 6) linkage. In addition, the peak at 79.5 ppm represents C-3 in an  $\alpha$ -(1 $\rightarrow$ 3) linkage.

permethylated derivatives were analyzed by capillary GC–MS as the peracetylated aldononitrile (PAAN) derivatives.<sup>12</sup> For NMR spectroscopy, individual samples of oligosaccharides (~15 mg) were dissolved in D<sub>2</sub>O (0.6 mL). All NMR experiments were performed on a Bruker Avance 500 spectrometer equipped with a 5-mm broadband inverse probe with z-gradient.

### 3. Results

TLC and HPLC analysis of each individual fraction from the gel-filtration column demonstrated the presence of a single trisaccharide, two tetrasaccharides in roughly equal proportions, and a single pentasaccharide. Higher oligosaccharides were also observed on TLC, but in such low amounts that no attempt was made to isolate them. In a typical reaction, using 167 mM sucrose and 83 mM gentiobiose, yields based on gentiobiose were 73% trisaccharide,



**Figure 3.** Proton-decoupled  $^{13}\text{C}$  NMR spectrum of gentiobiose pentasaccharide acceptor product. Complete peak assignment was not done; anomeric peak assignments are as follows: 92.1 ppm, C1 $\alpha$ , reducing end glucose unit; 95.9 ppm, C1 $\beta$ , reducing end glucose unit; 97.9 and 98.0 ppm, partially resolved C1, two nonreducing glucopyranosyl units, in an  $\alpha$ -(1 $\rightarrow$ 6) linkages; 99.3 ppm, nonreducing glucopyranosyl unit in an  $\alpha$ -(1 $\rightarrow$ 3) linkage; 102.9 and 103.0 ppm, partially resolved C1, nonreducing glucopyranosyl unit, in a  $\beta$ -(1 $\rightarrow$ 6) linkage. In addition, the peak at 79.5 ppm represents C-3 in an  $\alpha$ -(1 $\rightarrow$ 3) linkage.

11% tetrasaccharide, and 5% pentasaccharide. The remainder was unreacted gentiobiose and higher DP oligosaccharides. Under the given conditions, higher DP oligosaccharide yields were scarcely detected on TLC. Unreacted gentiobiose was not separated from leucrose, so the amount remaining unreacted was not determined. By difference, it is estimated to be less than 11%.

### 3.1. Trisaccharide

Only a single trisaccharide was isolated in significant quantities. Its mobility on TLC was similar to that of isomaltotriose, but HPLC resolved it from isomaltotriose. Methylation analysis yielded 2,3,4,6-tetra-*O*-methyl glucose PAAN and 2,3,4-tri-*O*-methyl glucose PAAN in a 1:2 molar ratio. NMR results (Fig. 1) indicate an  $\alpha$ -(1→6) linkage and a  $\beta$ -(1→6) linkage as well as a single reducing anomeric C-1. The compound was resistant to hydrolysis by isomaltodextranase. As this trisaccharide arises via glucosylation of gentiobiose, the only possible structure consistent with these data would be of gentiobiose bearing an  $\alpha$ -D-glucopyranosyl unit at the

free 6-position. Taken together, these results show that the structure of the trisaccharide **3** must be  $\alpha$ -D-Glcp-(1→6)- $\beta$ -D-Glcp-(1→6)-D-Glc.

### 3.2. Tetrasaccharides

An approximately equimolar mixture of two tetrasaccharide products was isolated by gel-filtration chromatography. Saccharide **4A** migrated ahead of **4B** on TLC. Treatment of the entire tetrasaccharide mixture with isomaltodextranase<sup>13</sup> converted **4B** completely to a disaccharide fraction indistinguishable from isomaltose and gentiobiose on TLC, whereas **4A** remained intact. The disaccharide products resulting from hydrolysis of **4B** were separated from **4A** by Bio-Gel P-2 chromatography. This disaccharide fraction was partially resolved by HPLC into isomaltose and gentiobiose. Methylation of the disaccharide fraction gave 2,3,4,6-tetra-*O*-methyl glucose PAAN and 2,3,4-tri-*O*-methyl glucose PAAN in an equimolar ratio. Methylation analysis of **4A** gave 2,3,4,6-tetra-*O*-methyl glucose PAAN, 2,4,6-tri-*O*-methyl glucose

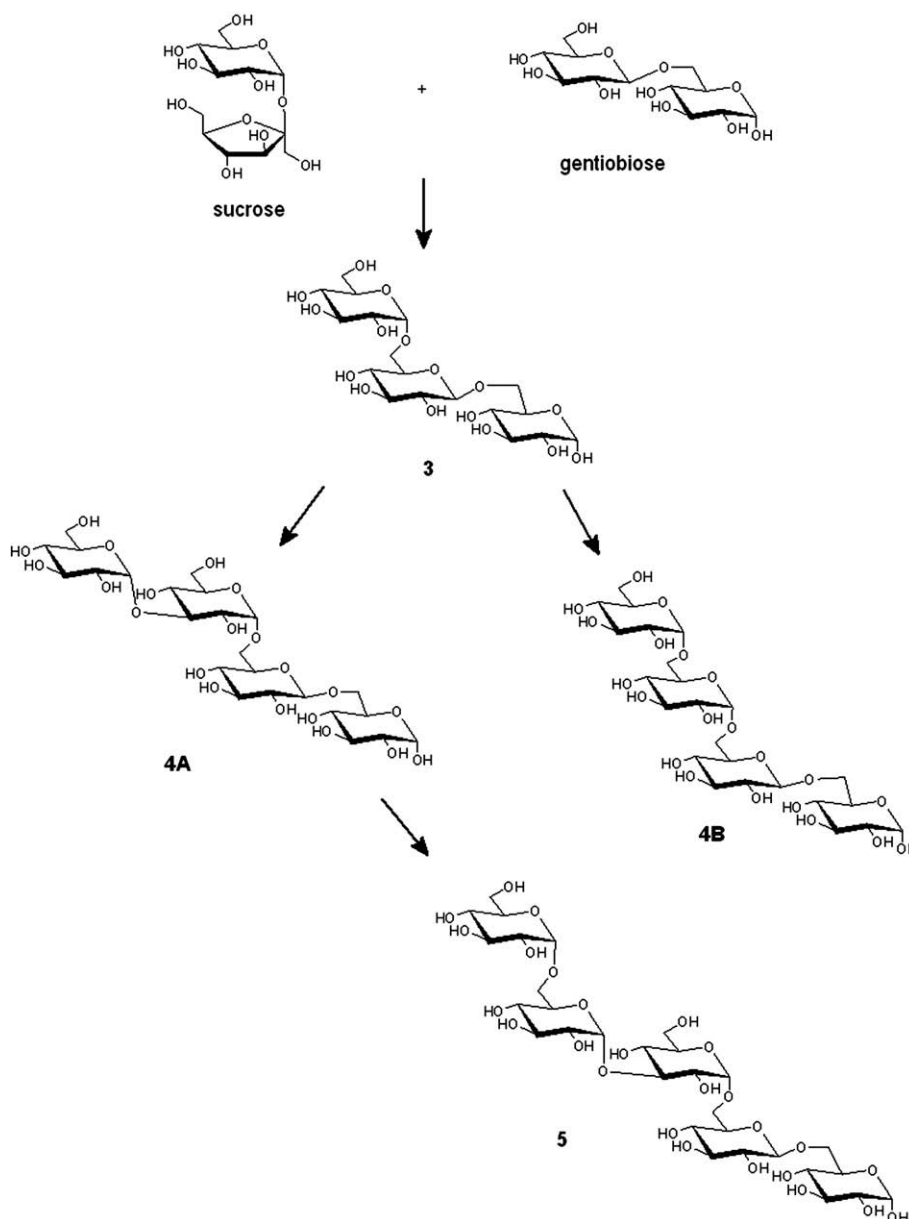


Figure 4. Alternansucrase-catalyzed acceptor reactions with gentiobiose.

PAAN, and 2,3,4-tri-*O*-methyl glucose PAAN in a 1:1:2 molar ratio.  $^{13}\text{C}$  NMR spectroscopy of **4A** (Fig. 2) shows an  $\alpha$ -(1 $\rightarrow$ 3) linkage in addition to the  $\alpha$ -(1 $\rightarrow$ 6) and  $\beta$ -(1 $\rightarrow$ 6) linkages present in the trisaccharide. As both products arise via glucosylation of the trisaccharide **3**, the only structures possible that are consistent with these results are shown as **4A** and **4B**. The data indicate that **4A** must be  $\alpha$ -D-Glcp-(1 $\rightarrow$ 3)- $\alpha$ -D-Glcp-(1 $\rightarrow$ 6)- $\beta$ -D-Glcp-(1 $\rightarrow$ 6)-D-Glc, and **4B** must be  $\alpha$ -D-Glcp-(1 $\rightarrow$ 6)- $\alpha$ -D-Glcp-(1 $\rightarrow$ 6)- $\beta$ -D-Glcp-(1 $\rightarrow$ 6)-D-Glc. One unusual phenomenon noted during preparative HPLC separation was that, of the two tetrasaccharide products, **4A** gave a single peak upon elution from the C-18 column, but **4B** was resolved into its  $\alpha$  and  $\beta$  anomers. Products **3** and **5** also gave single peaks. It is not unusual for anomers to be separated on a C-18 column, but why some of our isomers were thus separated and some were not is unknown.

### 3.3. Pentasaccharide

A single pentasaccharide was isolated by gel-filtration chromatography and judged to be pure by TLC and HPLC. This tetrasaccharide must arise via glucosylation of either **4A** or **4B**. Methylation analysis yielded 2,3,4,6-tetra-*O*-methyl glucose PAAN, 2,4,6-tri-*O*-methyl glucose PAAN, and 2,3,4-tri-*O*-methyl glucose PAAN in a 1:1:3 molar ratio. The  $^{13}\text{C}$  NMR spectrum shown in Figure 3, plus DEPT NMR (not shown), shows another  $\alpha$ -(1 $\rightarrow$ 6) linkage in addition to those shown in the spectrum for **4A**. These data alone would be sufficient to conclude its structure that is shown in Figure 4. Hydrolysis by isomaltodextranase<sup>13</sup> converted it to isomaltose and the trisaccharide product **3**, thus confirming structure **5**. Its structure was, therefore, determined to be  $\alpha$ -D-Glcp-(1 $\rightarrow$ 6)- $\alpha$ -D-Glcp-(1 $\rightarrow$ 3)- $\alpha$ -D-Glcp-(1 $\rightarrow$ 6)- $\beta$ -D-Glcp-(1 $\rightarrow$ 6)-D-Glc.

## 4. Discussion

In a survey of alternansucrase acceptor reactions, we found that gentiobiose was better as an acceptor than isomaltose, and nearly as good as maltose, the best acceptor.<sup>4</sup> This was surprising, as the other  $\alpha$ -linked glucodisaccharides were better acceptors than their respective  $\beta$ -linked isomers.<sup>4</sup> Subsequently, we demonstrated that alternansucrase synthesized acceptor products from maltose in a controlled and predictable manner.<sup>2,14,15</sup> According to those findings, the enzyme transfers a glucosyl unit to position O-6 at the nonreducing end of the maltose acceptor. The resulting trisaccharide then undergoes glucosylation at either O-3 or O-6 of the nonreducing end. Some of the rules we observed were that no sequential  $\alpha$ -(1 $\rightarrow$ 3) linkages were formed, and that no more than two sequential  $\alpha$ -(1 $\rightarrow$ 6) linkages were formed. Other evidence suggested that the specificity was under kinetic control.<sup>15</sup> That is to say, product distribution depended on relative rates of formation of acceptor products from each immediate precursor, rather than on an absolute structural specificity. The results described here, using the  $\beta$ -linked disaccharide gentiobiose, show that this selectivity extends to gentiobiose. As far as we know, only two other  $\beta$ -linked D-glucopyranosides have been studied in any detail as acceptors for alternansucrase. Our previous work showed that methyl  $\beta$ -D-glucopyranoside was a less effective acceptor than methyl  $\alpha$ -D-glucopyranoside, although the products were analogous.<sup>2,4,16</sup> However, Argüello-Morales et al.<sup>17</sup> found that cellobiose was glucosylated both at position O-2 of the reducing unit and at O-6 of the nonreducing unit, thus differing dramatically from the maltose acceptor products.<sup>14</sup> It should be pointed out that gentiobiose can also serve as an acceptor for NRRL B-512F dextranase,<sup>18</sup> and that the initial trisaccharide product is the same as compound **3** described here. To the best of our knowledge, the higher DP products from dextranase acceptor reactions with

gentiobiose have not yet been isolated and identified. By analogy with maltose and isomaltose, there should be a linear series containing only (1 $\rightarrow$ 6)-linked glucosyl units, although this hypothesis remains to be proven.

Although gentiobiose and gentiooligosaccharides have shown some promise as potential prebiotics,<sup>19</sup> the bitter aftertaste imparted by gentiobiose is one impediment to their adoption as food ingredients. We had hypothesized that glucosylation of gentiobiose by alternansucrase may eliminate this bitterness, and modify the prebiotic profile of gentiobiose.<sup>4</sup> This author can now attest that, whereas small amounts of solid gentiooligosaccharides placed on the tongue possess an unpleasant bitter aftertaste, similar amounts of the solid trisaccharide acceptor product described herein are bland or very slightly sweet, with no bitter aftertaste. Our prior results showed that alternansucrase acceptor products from gentiobiose were generally better prebiotics in vitro than gentiooligosaccharides themselves.<sup>6</sup> Their digestibility is not known, but similar maltose-derived alternansucrase acceptor products are absorbed by humans and slowly converted to blood glucose. This property has been exploited in their use as a low-glycemic sweetener.<sup>20</sup> The improved prebiotic profile, lack of bitterness, and the relatively high yields (approaching 90% in unoptimized reactions) suggest promise for the use of these oligosaccharides as food or feed ingredients.<sup>21</sup>

The methylation results, isomaltodextranase hydrolysis products, and  $^{13}\text{C}$  NMR data presented herein, taken in combination with the previous knowledge that each acceptor product must arise via glucosylation of the next-lower DP oligosaccharide, provide proof of the structures shown. This paper and others describing the structures of alternansucrase acceptor products show the structural nature of the entire disaccharide, and not just the linkage of the terminal sugar that determines the acceptor specificity of alternansucrase. Structural studies such as these kinetic and binding studies and molecular modeling must all be used collaboratively to better understand the mode of action of glucansucrases.

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