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Structural characterization of the maltose acceptor-products synthesized by *Leuconostoc mesenteroides* NRRL B-1299 dextransucrase

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Abstract

The glucooligosaccharides (GOS), produced by *Leuconostoc mesenteroides* NRRL B-1299 dextransucrase through an acceptor reaction with maltose and sucrose, were purified by reverse phase chromatography. Logarithmic plots of retention time vs. dp of the GOS gave three parallel lines suggesting the existence of at least three families of homologous molecules. The structure (^{13}C and ^{1}H NMR spectroscopy) and reactivity of the purified molecules of the three families were investigated. All the products bear a maltose residue at the reducing end. The GOS in the first family (named OD) contained additional glucosyl residues all α-(1 → 6) linked. The smallest molecule in this first series was panose or α-D-glucopyranosyl-(1 → 6)-D-maltose (dp 3). All the OD molecules were shown to be good acceptors for dextransucrase in the presence of sucrose. The second family, named R, was composed of linear GOS containing α-(1 → 6)-linked glucosyl residues and a terminal α-(1 → 2)-linked residue at the non-reducing end of the molecule; the smallest molecule in this family was α-D-glucopyranosyl-(1 → 2)-D-panose (dp 4). The third family, R′, was formed of GOS containing additional residues linked through α-(1 → 6) linkages that constitute the linear chain, and an α-(1 → 2)-branched residue located on the penultimate element of the chain, near the non-reducing end. The smallest molecule in this series is α-D-glucopyranosyl-(1 → 6)-[α-D-glucopyranosyl-(1 → 2)]-α-D-glucopyranosyl-(1 → 6)-D-panose, dp 6. R and R′ GOS are very poor acceptors for *L. mesenteroides* NRRL B-1299 dextransucrase. This study makes it possible to suggest a rather simple reaction scheme,

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where molecules R_i, R'_i and OD_i of the same dp all result from the glucosylation of the same GOS: OD_{i-1}. © 1998 Elsevier Science Ltd.

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1. Introduction

Dextransucrase is a glucosyltransferase (EC 2.4.1.5) which catalyses the synthesis of dextran from sucrose according to the equation [1]:

n sucrose → dextran + *n* fructose.

To be given the name dextran, the glucosyl polymer must contain at least 50% of contiguous α-(1 → 6) linkages, forming the linear chain of the molecule [2]. The nature and the frequency of the other linkages present are highly dependent on the glucosyltransferase-producing strain [3]. In this way, the most widely studied polymer, *Leuconostoc mesenteroides* NRRL B-512F dextran, contains about 95% of α-(1 → 6) linkages and only 5% of α-(1 → 3) branched linkages [3], while *L. mesenteroides* NRRL B-1299 dextran is composed of 65% α-(1 → 6) linkages, 30% α-(1 → 2) linkages and 5% α-(1 → 3) branched linkages [4–6].

The mechanism of dextran synthesis has been widely studied with *L. mesenteroides* NRRL B-512F dextransucrase [1,7–9]. It involves a glucosyl-enzyme intermediate [10] and polymerization occurs by an insertion mechanism at the reducing end of the molecule [7,11,12]. These studies also showed that in the presence of an auxiliary sugar, called an acceptor, the enzyme no longer synthesizes only dextran but mainly catalyses the formation of oligosaccharides by transferring glucosyl residues to the non-reducing end of acceptors [13,14]. With some acceptors, transfer can also occur at the reducing end, but to a lesser extent [15]. Several sugars have been analyzed with regard to their acceptor efficiency [13,16]. D-Fructose is a weak acceptor and gives only a single acceptor-product, leucrose [17]. But in the case of efficient acceptors such as maltose, a series of homologous molecules is obtained. The average degree of polymerization of the glucooligosaccharides (GOS) produced, as well as the amount of dextran formed, can be increased by increasing the sucrose/maltose concentration ratio [18,19]. Concerning the structure of the acceptor-products, various *L. mesenteroides* glucosyltransferases were shown, during the acceptor reaction, to display a specificity close to that observed in high molecular weight glucan synthesis [18–20]. As a consequence, the GOS obtained from the action of B-512F dextransucrase on maltose and sucrose are composed of a maltose residue located at the reducing end and additional glucosyl residues all α-(1 → 6) linked [14,19]. In 1992, Paul et al. [21] patented a method for obtaining α-(1 → 2) GOS by the action of B-1299 dextransucrase in the presence of maltose and sucrose. The molecules were shown to have prebiotic properties [22] and are now produced on an industrial scale, thus extending the industrial importance of dextransucrase. However, the structure of the molecules has never been adequately described. The only identified GOS is R_4, a molecule with dp 4, which structure was determined by ^{13}C NMR to be α-D-glucopyranosyl-(1 → 2)-D-panose [23]. It was given the name R because it is Resistant to hydrolysis by glucoamylase.

At the present time, the reaction yields are calculated by means of the resistance of the products to glucoamylase hydrolysis, which is assumed to be related to their α-(1 → 2)-linkage content [23]. To further optimize α-(1 → 2) GOS synthesis, it seemed necessary to identify the exact structure of the various products. Moreover, these results are essential to understand the acceptor reaction mechanism involved in the formation of the α-(1 → 2) linkage. In this study, various products of the acceptor reaction catalyzed by B-1299 dextransucrase in the presence of maltose and sucrose were purified in order to determine their molecular weight and structure by mass spectrometry, ^1H and ^{13}C-NMR spectroscopy. Finally, the reactivity of each family of molecules will be discussed and a biosynthetic scheme proposed.

2. Results

HPLC analysis of the oligosaccharides.—As shown in Fig. 1, the HPLC method used (high-dp molecule analysis) enabled the separation of products with a dp ranging from 4 to 10, but did not allow separation of fructose, maltose, sucrose and panose. The homologous GOS produced by B-512F dextransucrase were eluted in order of increasing dp. They are linear GOS composed of α-(1 → 6) linkages and a maltose residue at the reducing end. They were named OD_i, for oligodextrans, with dp *i* (Fig. 1A).

Fig. 1B shows the variety of GOS produced by B-1299 dextransucrase compared to those synthesized by the B-512F enzyme. Comparison of chromatograms 1A and 1B made it possible to determine the common peaks, e.g., those having the same retention time. They corresponded to the GOS synthesized by B-1299 dextransucrase that have the same structure as those synthesized by B-512F dextransucrase. These peaks are indicated in Fig. 1B by reference OD_i. The other peaks, corresponding to GOS specifically synthesized by the NRRL B-1299 dextransucrase, were numbered 1 to 10. Peak no. 1 corresponded to GOS R_4, i.e., α-D-glucopyranosyl-(1 → 2)-D-panose [23]. GOS 2, 3, and 4 were purified by preparative HPLC. Their molecular weights were respectively 828, 990 and 990 g as shown by mass spectrometry. This meant that GOS 2 had a dp of 5, and GOS 3 and 4 had a dp of 6. It thus appeared in the chromatogram (Fig. 1B) that R_4 (dp 4) is eluted between OD_3 and OD_4, while GOS 2 (dp 5) is eluted between OD_4 and OD_5, and GOS 3 and GOS 4, both having a dp of 6, are eluted between OD_5 and OD_6. For GOS 5, 6 and beyond, it was considered that all the products eluted in peaks located between those of OD_i and OD_{i+1} had a dp equal to $i + 1$. That is to say that GOS 5 and 6 were assumed to have a dp of 7, GOS 7 and 8 a dp of 8.

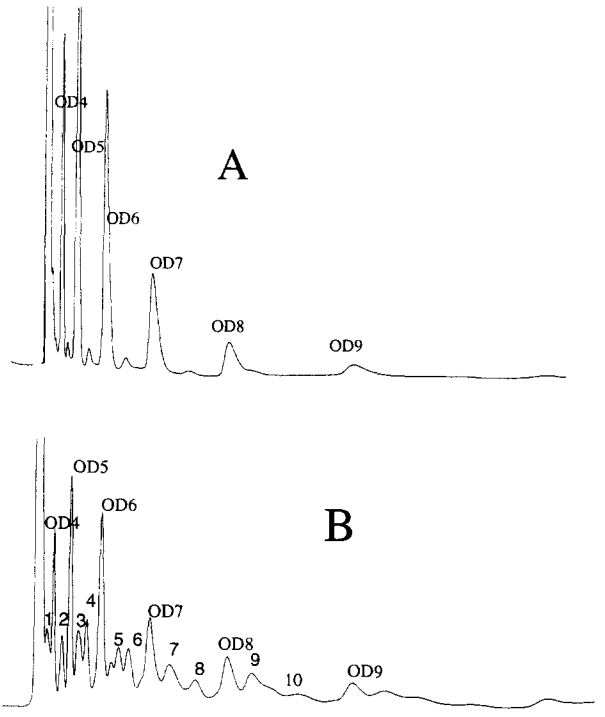

Fig. 1. HPLC analysis (high-dp analysis) of the GOS produced by *L. mesenteroides* dextransucrases in the presence of maltose and sucrose. (A) GOS synthesized by *L. mesenteroides* NRRL B-512F dextransucrase from maltose and sucrose. The oligosaccharides were named OD_i (for oligodextran), i being the degree of polymerization of the molecule. (B) GOS synthesized by *L. mesenteroides* NRRL B-1299 dextransucrase from maltose and sucrose. Numbers have been attributed to the main peaks found only in the NRRL B-1299 GOS preparation (GOS 1 = α-D-glucopyranosyl-(1 → 2)-D-panose).

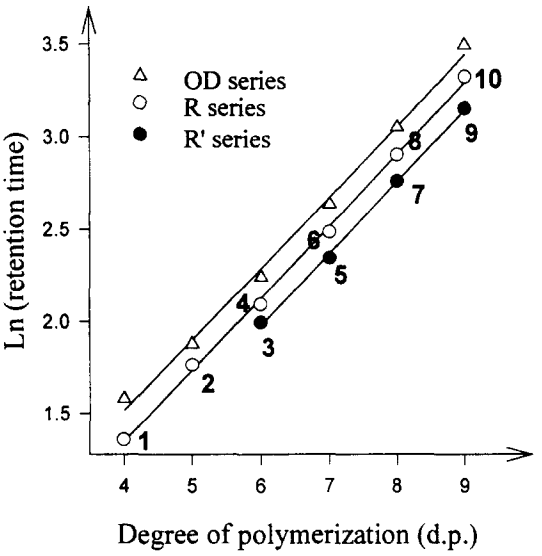

Fig. 2. Retention time analysis of the GOS synthesized by *L. mesenteroides* NRRL B-1299 dextransucrase. (High-dp HPLC analysis). Numbers have been attributed to the main peaks found only in the NRRL B-1299 GOS preparation (see Fig. 1B).

Plots of Ln (retention time) versus dp of the GOS are presented in Fig. 2. The first plot (triangles) corresponds to the OD series, consisting of all the GOS synthesized by both the B-512F and the B-1299 dextransucrases. Two other lines, parallel to that of OD, suggested the existence of at least three series of homologous molecules in the B-1299 GOS preparation. The second line (open circles) passed through points corresponding to peaks found only in the NRRL B-1299 chromatograms and especially through the point corresponding to Peak 1. Therefore the series might contain products homologous to R_4 and was given the name R. As a result, Peak 2 would correspond to R_5, Peak 4 to R_6 and so on. The third series was given the name R', since it differed from the R series (different plot, dark circles), but it also contained GOS specifically synthesized by B-1299 dextransucrase.

NMR spectroscopy.—GOS 2 (R_5). The proton spectrum of GOS 2 (Fig. 3A) showed six doublets in the region 4.5 to 5.3 ppm integrating for five protons, which indicated a pentasaccharide structure, in accor-

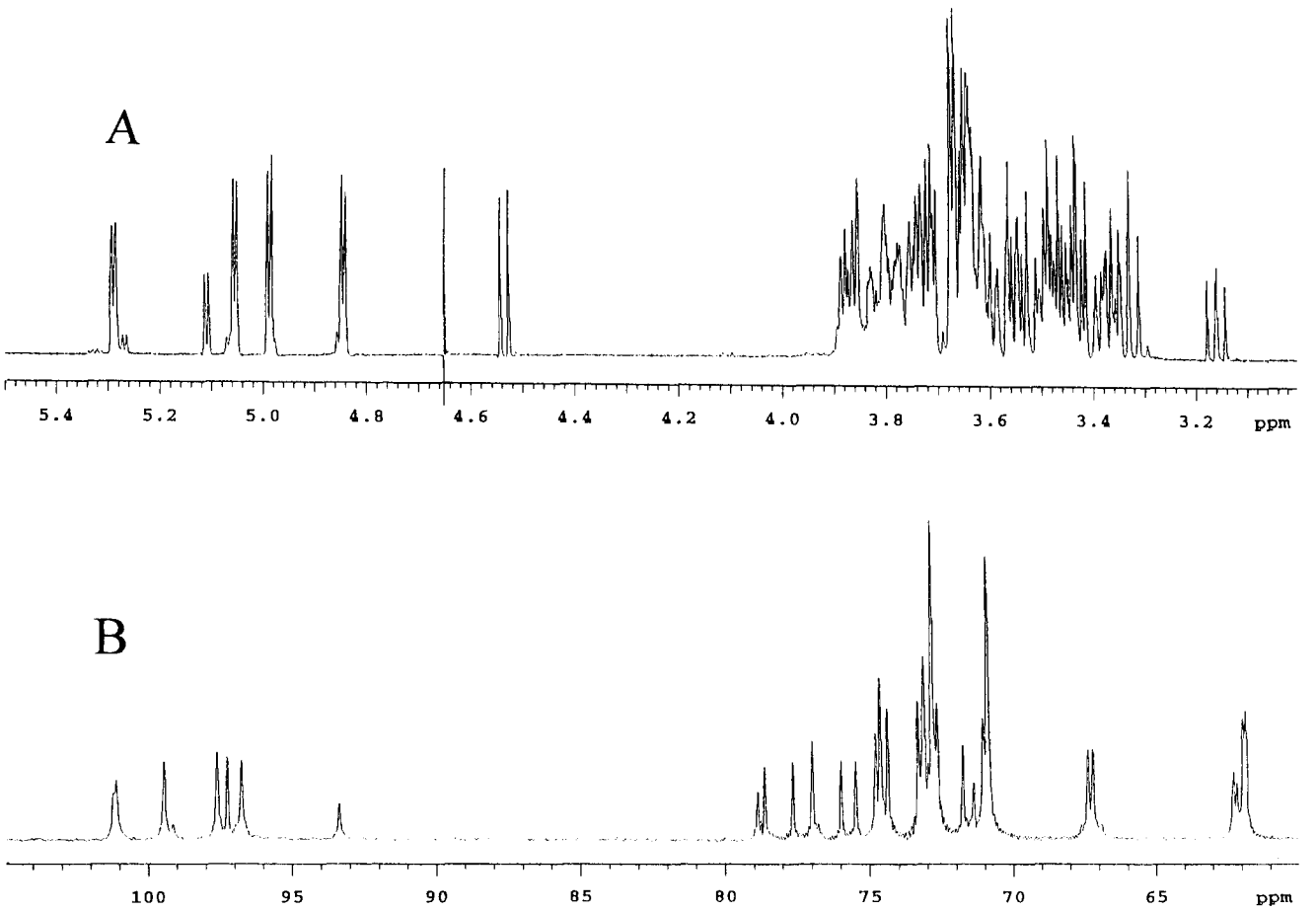

Fig. 3. NMR spectra of GOS 2 (R^5): (A) ^1H 500 MHz. (B) ^{13}C at 75.468 MHz.

dance with the mass spectroscopy results. All the doublets exhibited weak $J_{1,2}$ coupling constants (3.5 Hz) characteristic of α-linked glucopyranose units, except for H-$1a_\beta$ (8 Hz).

Two signals were affected by the mutarotation equilibrium and corresponded to the reducing unit (H-1_α, 5.11 and H-1_β, 4.54 ppm). Another proton was slightly affected by the mutarotation, indicating that it must be assigned to the Glc^{II} unit α-(1 → 4)-linked (H-$1^{II}_{\alpha,\beta}$ at 5.29 and 5.285 ppm). Doublets at 4.84 and 4.99 ppm were assigned to glucose units respectively α-(1 → 6) (H-1^{III}) and α-(1 → 2) (H-1^{V}) linked. Finally, the signal at 5.06 ppm was assigned to an α-(1 → 6)-linked glucose unit glucosylated at O-2 (H-1^{IV}). 2D COSY and 1D TOCSY NMR experiments were performed to assign all the proton signals and the data are reported in Table 1.

Comparison of the ^1H NMR data of H-2^{III} (3.47 ppm) and H-3^{III} (3.61 ppm), respectively with H-2^{IV} (3.55 ppm) and H-3^{IV} (3.72 ppm), showed significant downfield shifts in accordance with the 2-glucosylation of Glc^{IV} unit. Moreover, the protons H-6a of Glc^{IV} and Glc^{V} units resonated at 3.72 and 3.74 ppm respectively, whereas protons H-6a of Glc^{II} and Glc^{III} units were shifted downfield at 3.87 ppm, indicating that their O-6 positions were glucosylated. The sequence of GOS 2 was unambiguously determined from an HMBC experiment. In the 2D ^{13}C–^1H HMBC spectrum (Fig. 4), intraresidual two- and three-bond ^{13}C,^1H couplings were observed, in addition to interresidual three-bond connectivities over the glycosidic linkages. The Glc^{IV} (1 → 2) Glc^{V} linkage was confirmed by strong cross-peaks between Glc^{V} C-1 and Glc^{IV} H-2, and Glc^{IV} C-2 and Glc^{V} H-1 in the HMBC spectrum. Both the (1 → 6) linkages for Glc^{IV} (1 → 6) Glc^{III} and Glc^{III} (1 → 6) Glc^{II} were confirmed by two long-range couplings: C-1^{IV}, H-$6a^{III}$ and C-6^{III}, H-1^{IV} (Glc^{IV} → Glc^{III} sequence); C-1^{III}, H-$6a^{II}$ and C-6^{II}, H-1^{III} (Glc^{III} → Glc^{II} sequence). Finally, the strong connectivities C-1^{II}, H-$4\alpha,\beta^1$ and C-$4\alpha,\beta^1$, H-1^{II} corroborated the (1 → 4) linkage between Glc^{II} and Glc^{I} units.

The ^{13}C NMR spectrum of GOS 2 (Fig. 3B) confirmed the results of the ^1H analysis. It presented

seven signals in the anomeric region (93 to 102 ppm), the signal of the reducing end unit being affected by the α,β mutarotational equilibrium, as well as the signal of the Glc^{II} unit. All the ^{13}C signals were assigned by performing a 2D HMQC experiment (Table 1). Therefore R_5 was identified as α-D-glucopyranosyl-(1 → 2)-α-D-glucopyranosyl-(1 → 6)-D-panose. Its linear structure was thus homologous to that of GOS R_4 previously described [23].

GOS 3 (R'_6). Particular attention was given to GOS 3 which belongs to a series of GOS isolated for the first time in this study among the acceptor-products synthesized by B-1299 dextransucrase in the presence of maltose. The results are summarized in Table 1.

The proton spectrum of GOS 3 showed seven doublets in the region 4.5 to 5.3 ppm integrating for six protons, which indicated a hexasaccharide structure in accordance with the mass spectroscopy results. All the doublets exhibited weak $J_{1,2}$ coupling constants (3.5 Hz) characteristic of α-linked glucopyranose units, except for H-1a_β (8 Hz).

Two signals were affected by the mutarotation equilibrium and corresponded to the reducing unit (H-1^I_α, 5.11 and H-1^I_β, 4.54 ppm). Another proton was slightly affected by the mutarotation, indicating that it must be assigned to the Glc^{II} unit directly linked to the Glc^I unit (H-$1^{II}_{\alpha,\beta}$ at 5.29 and 5.285 ppm). These doublets (H-$1^{II}_{\alpha,\beta}$), at 5.29 ppm, corresponded to an α-(1 → 4)-linked glucose unit. Protons of Glc^{III} and Glc^{VI} units of this molecule had almost the same chemical shifts and are not distinguishable. They were assigned to α-(1 → 6) glucose units. The doublets at 4.98 ppm were assigned to an α-(1 → 2)-linked glucose unit (H-1^V). Finally, the signal at 5.07 ppm corresponded to an α-(1 → 6)-linked glucose unit glucosylated at O-2 (H-1^{IV}).

Table 1
^1H and ^{13}C NMR data for GOS 2 and GOS 3

Compound	Residue	Chemical shift[a]							Assignment of C-1
		H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	
		C-1	C-2	C-3	C-4	C-5	C-6		
GOS 2	α $Glcp^I$	5.11	3.45	3.85	3.53	3.83	3.75	3.70	C-1α
		93.38	72.74	74.60	78.89	71.43	62.15		
	β-$Glcp^I$	4.54	3.16	3.65	3.53	3.49	3.8	3.66	C-1β
		97.28	75.42	77.62	78.65	76.03	62.29		
	α $Glcp^{II}$	5.29, 5.285	3.48	3.57	3.38	3.81	3.87	3.62	C-1 → 4
		101.14	73.07	74.58	70.88	72.69	67.44		
		101.17							
	α $Glcp^{III}$	4.84	3.47	3.61	3.43	3.76	3.87	3.66	C-1 → 6
		99.48	72.82	74.75	70.88	71.81	67.27		
	α $Glcp^{IV}$	5.06	3.55	3.72	3.36	3.79	3.74	3.66	C-1 → 6
		96.78	77.02	73.16	70.88	72.82	61.93[b]		
	α $Glcp^V$	4.99	3.43	3.66	3.33	3.79	3.72	3.66	C-1 → 2
		97.64	72.82	74.33	71.05	73.35	61.83[b]		
GOS 3	α $Glcp^I$	5.11	3.45	3.84	3.52	3.82	3.75	3.70	C-1α
		93.30	72.88	74.53	79.06	71.43	62.15		
	β-$Glcp^I$	4.54	3.16	3.64	3.52	3.49	3.81	3.66	C-1β
		97.27	75.38	77.51	78.80	76.00	62.29		
	α $Glcp^{II}$	5.29	3.48	3.57	3.38	3.81	3.87	3.62	C-1 → 4
		101.15	73.10	74.53	70.96	72.65	67.43		
		101.26							
	α $Glcp^{III}$	4.845	3.46	3.61	3.42	3.76	3.88	3.66	C-1 → 6
		99.45	72.90[c]	74.71	71.13[d]	71.55	67.27		
	α $Glcp^{IV}$	5.07	3.57	3.71	3.46	3.82	3.86	3.65	C-1 → 6
		96.75	77.03	73.05	71.00	71.69	67.00		
	α $Glcp^V$	4.98	3.43	3.66	3.34	3.79	3.72	3.66	C-1 → 2
		97.69	72.77	74.32	70.86	73.33	61.87[e]		
	α $Glcp^{VI}$	4.853	3.46	3.61	3.42	3.76	3.73	3.66	C-1 → 6
		99.20	72.88[c]	73.24	70.91[d]	72.77	61.97[e]		

[a]In ppm relative to the signal of internal acetone at 2.1 ppm (^1H) in deuterium oxide at 25 °C or at 31.5 ppm (^{13}C) in deuterium oxide at 60 °C.
[b,c,d,e]Carbons can be interchangeable.

Comparison of the ^{1}H NMR data for H-2^{III} (3.46 ppm) and H-3^{III} (3.61 ppm) with H-2^{IV} (3.57 ppm) and H-3^{IV} (3.71 ppm) showed significant downfield shifts in accordance with the 2-glucosylation of Glc^{IV}. Moreover, protons H-6a of Glc^{V} and Glc^{VI} units resonated at 3.72 and 3.73 ppm, while protons H-6a of units Glc^{II} (3.87 ppm), Glc^{III} (3.88 ppm) and Glc^{IV} (3.86 ppm) units were shifted downfield, indicating that their O-6 positions were glucosylated.

The sequence of GOS 3 was unambiguously determined from a 2D HMBC experiment. In the HMBC spectrum the Glc^{VI} (1 → 6) Glc^{IV} linkage was confirmed by a strong cross-peaks between C-1 of Glc^{VI} and H-6a of Glc^{IV}, or C-6 of Glc^{IV} and H-1 of Glc^{VI}. Strong cross-peaks, as already observed in the case of GOS 2, confirmed linkages: Glc^{V} (1 → 2) Glc^{IV}, Glc^{IV} (1 → 6) Glc^{III}, Glc^{III} (1 → 6) Glc^{II} and Glc^{II} (1 → 4) Glc^{I}.

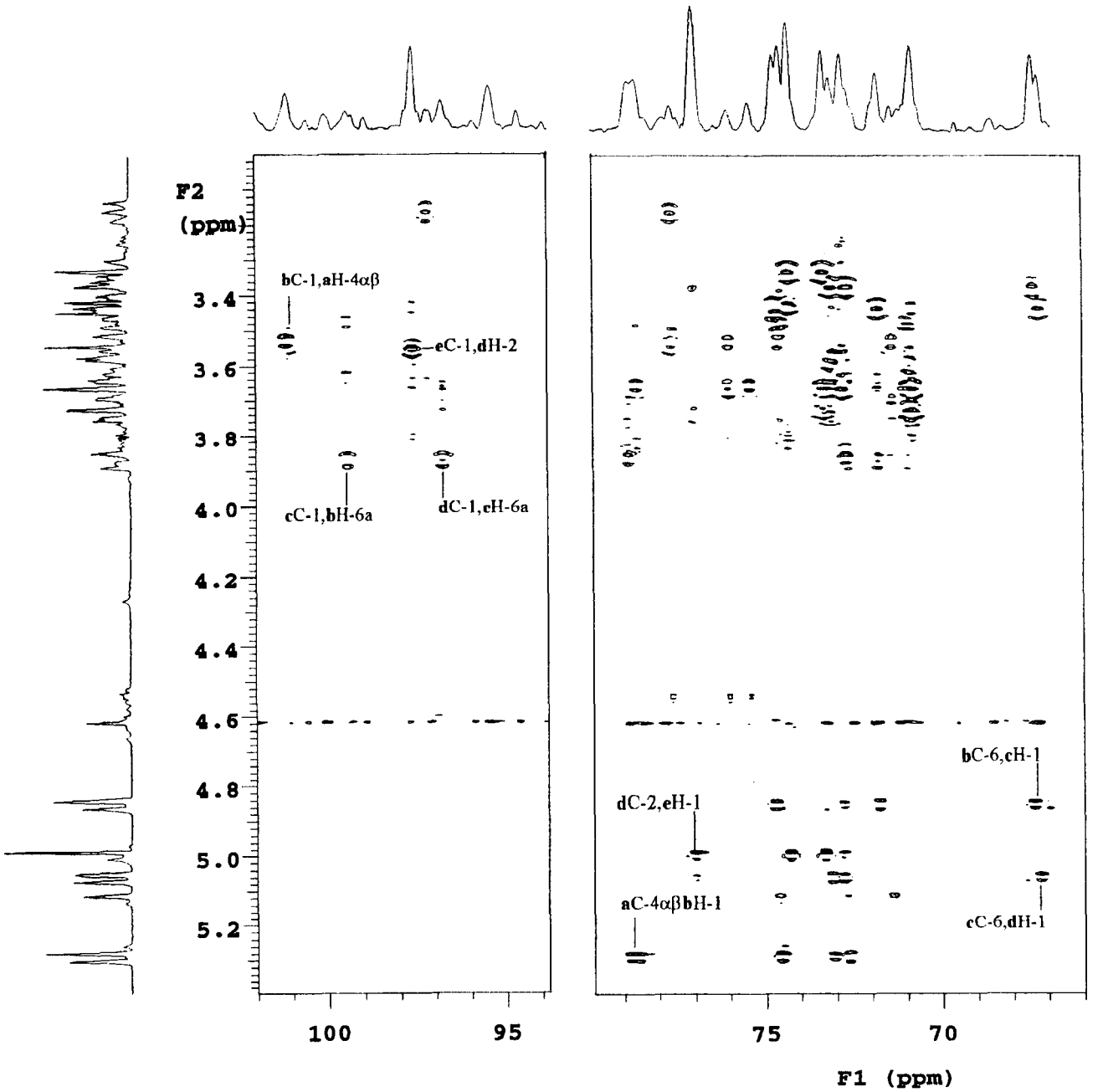

Fig. 4. 400 MHz HMBC spectrum of GOS 2 (R_5) recorded in deuterium oxide at 60 °C. The code C-6, H-1 indicates a long-range coupling between C-6 of Glc^{III} unit and H-1 of Glc^{IV} unit.

The ^{13}C NMR spectrum of GOS 3 was in agreement with the results of the ^{1}H NMR analysis. It presented seven signals in the anomeric region (92 to 102 ppm), the signal of the reducing end unit being affected by the mutarotation equilibrium. The ^{13}C signals were assigned from a 2D HMQC experiment

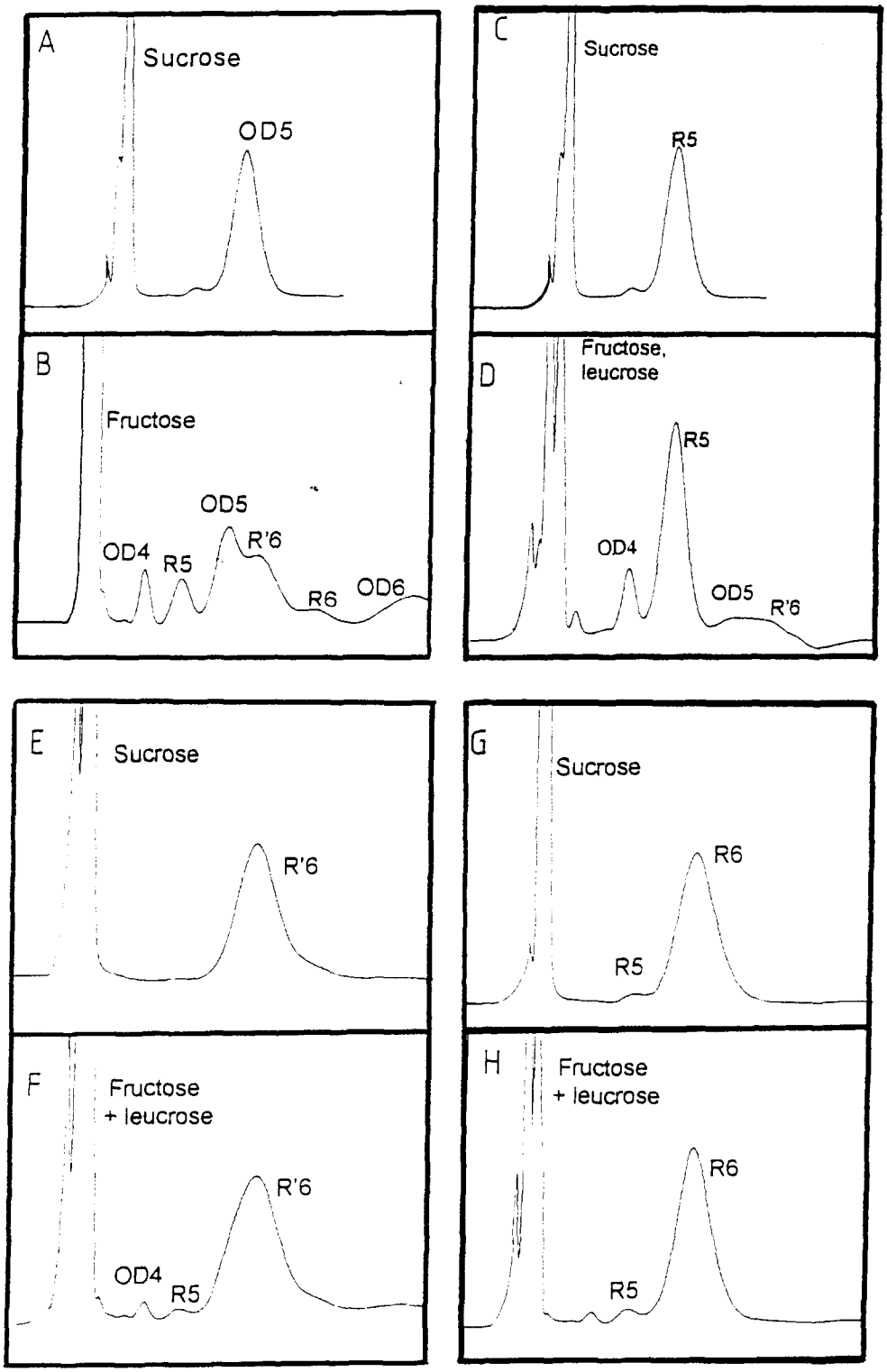

Fig. 5. Reactivity of various GOS in the presence of sucrose and *L. mesenteroides* NRRL B-1299 dextransucrase. Chromatograms of initial medium in the presence of OD_5 (A), R_5 (C), R'_6 (E) and R_6 (G). Chromatograms of final medium in the presence of OD_5 (B), R_5 (D), R'_6 (F) and R_6 (H). Leucrose is the acceptor-product obtained from D-fructose. (Note: with routine HPLC analysis oligosaccharides of dp higher than 7 are not detectable).

(Table 1). GOS 3 is consequently the branched hexasaccharide α-D-glucopyranosyl-(1 → 6)-[α-D-glucopyranosyl-(1 → 2)]-α-D-glucopyranosyl-(1 → 6)-D-panose (R'_6).

Enzymatic reactivity of the products.—Acceptor reactions were carried out using purified GOS as acceptors in the presence of sucrose (sucrose to acceptor concentration g/L ratio = 1) and B-1299 dextransucrase. According to [13,14,16], if the GOS introduced in the reaction medium is an acceptor, then the B-1299 dextransucrase will catalyze the transfer of glucopyranosyl residues to its non-reducing end to give GOS of higher dp. This reaction occurs at the expense of dextran synthesis [16]. Conversely, if the GOS used is not an acceptor, then no GOS synthesis will occur, but dextransucrase will convert sucrose to dextran and leucrose (transfer to fructopyranose).

Under the conditions used for testing GOS reactivity, B-1299 dextransucrase efficiently catalyzed glucopyranosyl transfer to maltose, panose or OD_5 molecules. The three series of products isolated after the acceptor reaction with maltose (see Fig. 1) were also encountered after reaction with panose or OD_5. The yields of each type of GOS were slightly modified depending on the dp of the acceptor. Fig. 5A and 5B present the chromatograms obtained before and after acceptor reaction with OD_5. Most of the acceptor products (95%) had a dp higher than 5, but the presence of OD_4 and R_5 (which represented about 5% of the GOS final concentration) suggested the occurrence of some partial hydrolysis or disproportionation reactions [24,25].

GOS of the R and R′ series were also used as acceptors for B-1299 dextransucrase in the presence of sucrose. The conversion of such molecules was very limited and most of the sucrose (more than 90%) was converted into dextran and leucrose. Most of the acceptor products, since they had lower dp values than the introduced acceptor, must therefore be the result of disproportionation of the acceptor (Fig. 6D, F, and H), showing that GOS of the R and R′ series were poor acceptors for B-1299 dextransucrase.

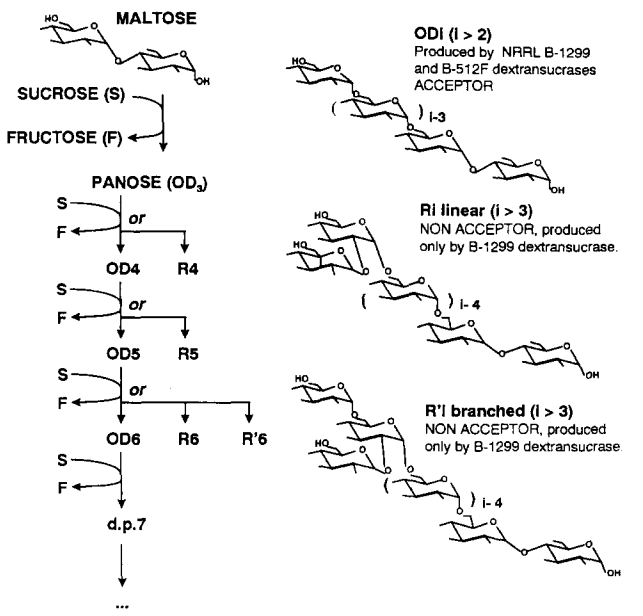

Fig. 6. Recapitulative scheme of the acceptor reactions catalyzed by *L. mesenteroides* NRRL B-1299, in the presence of maltose and sucrose, and general structure of the acceptor-products. The acceptor reaction on fructose was omitted.

3. Discussion

In the presence of sucrose and maltose, B-1299 soluble dextransucrase catalyzed the synthesis of a great variety of GOS. Linear plots of Ln (retention time) vs. dp had already been reported by Remaud-Simeon et al. [23] and Taylor et al. [26] but this is the first report concerning the occurrence of parallel lines for different series of homologous products synthesized by a single enzyme. This means that two structural parameters govern the chromatographic behavior during the analysis of B-1299 GOS by reverse chromatography. The first parameter is apparently linked to the molecular size of the molecule and whatever the series, GOS are always eluted in order of increasing dp. The occurrence of a linear plot (Fig. 2) means that the retention time is an exponential function of the dp of the molecule. The column enables a second level of separation, owing to the structure of the GOS. Three lines are obtained, corresponding to three general structures of GOS among the B-1299 acceptor-products. The parallel nature of the lines means on one hand that the three series have no common product. On the other hand, it suggests that the exponential function that defines the first parameter of separation is the same for the three groups isolated.

All GOS present a maltose residue at their reducing end and a varying number of additional α-(1 → 6)-linked glucopyranosyl residues. Also, the GOS may or may not possess a single α-(1 → 2) linkage, depending on the series to which they belong (second

level of separation). Some are linear, others branched, as in the case of the acceptor products synthesized by *L. mesenteroides* NRRL B-742 dextransucrase [18]. The α-(1 → 6) molecules of the OD series can be easily recognized since they are similar to the GOS produced by B-512F dextransucrase under the same conditions. They bear additional glucopyranosyl residues all linked through α-(1 → 6) linkages, the smallest molecule of this group being panose (OD_3). The α-(1 → 2) terminated GOS of the R series are linear, the α-(1 → 2) linkage being located at the non-reducing end. The smallest molecule of this family is R_4, whose structure was determined as α-D-glucopyranosyl-(1 → 2)-D-panose by Remaud-Simeon et al. [23]. From the present study, the structure of R_5 was found to be α-D-glucopyranosyl-(1 → 2)-α-D-glucopyranosyl-(1 → 6)-D-panose. Therefore, the general structure of the R_i molecules should be as in Fig. 6. This structure implies that R_{i+1} cannot be an acceptor-product of R_i. The α-(1 → 2) branched GOS of the R′ series, isolated for the first time in this study, are composed of an α-(1 → 6) linear chain and a single glucosyl residue bound to the main chain through an α-(1 → 2) linkage. In the case of R'_6, which is the smallest molecule in the series, the branch point is located on the penultimate residue of the linear chain, near the non-reducing end. Thus, the systematic name of R'_6 should be (α-D-glucopyranosyl-(1 → 6)-[α-D-glucopyranosyl-(1 → 2)]-α-D-glucopyranosyl-(1 → 6)-D-panose. For the R′ series, the α-(1 → 2) branching point was assumed to be always located on the penultimate glucopyranosyl residue in the linear chain (Fig. 6). As in the case of the R series, the general R′ structure implies that R'_{i+1} cannot be the acceptor product of R'_i.

Maltose and all the α-(1 → 6) GOS of the OD series are efficient acceptors B-1299 dextransucrase. On the other hand, both linear and branched α-(1 → 2) GOS undergo only limited disproportionation reactions. This is the first report concerning this kind of glucosyl transfer catalyzed by B-1299 dextransucrase. Apart from these particular transglucosylation reactions, R and R′ GOS are unable to give acceptor-products when used as sole acceptors in the presence of sucrose and B-1299 dextransucrase. The assumption that they will be even worse acceptors when used in the presence of efficient acceptors such as maltose, panose and other products of the OD series can then be made. Consequently, the reaction scheme of the acceptor reaction on maltose catalyzed by B-1299 dextransucrase must be that presented in Fig. 5: maltose reacts with sucrose to give a single product, panose. Transfer to maltose never results in an α-(1 → 2) linkage. Panose can react with sucrose to give either R_4 or OD_4. R_4 is not an acceptor for dextransucrase (M. Remaud, unpublished data) and the reaction stops. But OD_4 can react with sucrose to give either R_5 or OD_5. Only OD_5 reacts with sucrose to give either OD_6, R_6 or R'_6. Oligosaccharides R_6 and R'_6 are poor acceptors and only OD_6 can react with sucrose to give products with dp 7.

This reaction scheme implies that the structural difference between different molecules of the same dp is located at the non-reducing end of the molecule since they all come from the same GOS: OD_{i-1}. This is in accordance with the NMR and HPLC results. Moreover, the glucosyl transfer occurs either to C-2 or C-6 of the glucosyl residue located at the non-reducing end of the molecule, or sometimes to C-2 of the penultimate non-reducing-end glucopyranosyl residue, depending on (1) the acceptor dp and (2) the acceptor position in the acceptor site. Transfer to C-2 of other glucosyl residues may also occur, but as a very minor reaction, leading to undetectable amounts of products. Very little information is available concerning the way by which acceptors bind to dextransucrase. From this study, it appears that the presence of an α-(1 → 2) linkage at the non-reducing end prevents the GOS from effectively binding with the B-1299 enzyme to enable the acceptor reaction.

To explain the formation of the GOS that bear linkages other than α-(1 → 6), Fu and Robyt [15] proposed that asymmetric acceptors (maltodextrins) could bind to the acceptor site of B-512F dextransucrase in "two different ways, with the non-reducing end or the reducing end bound at the position where glucose can be transferred". This assumption is not acceptable in the case of the B-1299 dextransucrase, since transfer always occurs on a glucosyl residue located at the non-reducing end of the acceptor molecule. Nevertheless, it is possible that, for OD GOS of dp 5 or more, both the ultimate and the penultimate glucosyl residue at the non-reducing end can bind this enzymatic site where glucosyl transfer can occur. This 'sliding' [15] of the acceptor into the catalytic site could not occur in the case of small acceptors (dp 2, 3 and 4).

The last point for discussion deals with the position of the carbon where the glucosyl transfer takes place. Bhattacharjee and Mayer [27] showed that when the C-6 of the non-reducing end of maltose was modified, the next glucose unit was transferred to C-2 by *Streptococcus sanguis* ATCC 10558 dextransucrase. They concluded that when C-6 was substituted,

the acceptor molecule combined with the enzyme in other modes to give linkages other than α-(1 → 6). It is, therefore, possible that, under certain reaction conditions, *L. mesenteroides* NRRL B-1299 dextransucrase itself inhibits access to C-6 at the non-reducing end of the OD molecules (but not to C-6 of maltose), leading to the formation of an α-(1 → 2) linkage. A close study of the probability of α-(1 → 2) linkage formation (branched or linear) is now necessary before making any assumptions about the enzymatic mechanisms involved. Furthermore, site-directed mutagenesis of dextransucrase may give important information about the amino-acids in the catalytic site concerned.

4. Experimental

Enzyme production and concentration.—*L. mesenteroides* NRRL B-512F and B-1299 were supplied by the NRRL (Peoria, USA). The cells were stored in 18% glycerol at −18 °C. Erlenmeyer flask cultures were grown in a rotary shaker at 27 °C, 200 RPM with standard medium (40 g/L sucrose; 20 g/L yeast extract (Biomérieux); 20 g/L potassium hydrogen phosphate; 0.2 g/L $MgSO_4 \cdot 7H_2O$; 0.01 g/L manganese sulfate monohydrate; 0.01 g/L NaCl; 0.02 g/L $CaCl_2$ and 0.01 g/L ferrous sulfate heptahydrate), with an initial pH of 6.9. After 7 h of cultivation, the culture broth was centrifuged at 7000 × *g*, +4 °C, for 30 min. The soluble dextransucrase was then concentrated from the supernatant by means of aqueous two-phase partition between dextran and polyethyleneglycol (PEG Mw = 1500), which also enabled all the other sucrase activities to be removed from the culture supernatant [28]. The dextran phase was harvested by centrifugation at 7000 × *g*, +4 °C, for 30 min, and dissolved in sodium acetate buffer (20 mM, pH 5.4) to ensure a standard dextransucrase activity of 4 U/mL. The enzyme activity was 0.1 and 0.05 U/mg of dextran for the NRRL B-1299 and B-512F dextransucrase preparations respectively. Dextransucrase activity (1 U) is defined as the amount of enzyme that catalyses the formation of 1 μmol of fructose per min at 30 °C in 20 mM sodium acetate buffer, pH 5.4, with 100 g/L sucrose and 0.05 g/L $CaCl_2$. Fructose assay was carried out by the dinitrosalicylic method [29]. The absence of contaminating activities (levansucrase, sucrose-phosphorylase, invertase) and the dextran content of the preparations were checked as described by Dols et al. [28].

Oligosaccharide synthesis.—Oligosaccharide synthesis was performed at 25 °C, in 20 mM sodium acetate buffer, pH 5.4, with 0.05 g/L $CaCl_2$, 100 g/L maltose, and 400 g/L sucrose. After 36 h reaction, corresponding to sucrose exhaustion, the reaction was stopped by heating for 5 min at 90 °C, and the medium was centrifuged for 5 min at 15,000 × *g*, room temperature, to eliminate the insoluble dextran.

HPLC analysis.—Reverse phase chromatography (C18 column, Ultrasep 100, 6 μm, 5 × 300 mm, Bischoff Chromatography) was carried out using a Hewlett Packard 1050 series system consisting of a pump, an injector and an HP 1047A refractometer. For routine analysis, the eluant (ultrapure water) had a constant flow rate of 0.5 mL/min. Samples were diluted in order to ensure a total sugar concentration lower than 6 g/L. Each sample was analyzed within 30 min at room temperature. For high-dp molecule analysis, the eluant (99:1 ultrapure water–MeOH) had a constant flow rate of 0.6 mL/min. Samples were diluted in order to ensure a total sugar concentration lower than 6 g/L. Each sample was analyzed within 1 h at 45 °C. For preparative reverse phase chromatography (C18 column, Ultrasep 100, 6 μm, 10 × 300 mm, Bischoff Chromatography) at 45 °C, a system consisting of a pump (Bischoff Chromatography), a refractometer (Bischoff 2250) and a Rheodyne® injection valve (0.5 mL) was used. The eluant (ultrapure water) had a constant flow rate of 1 mL/min. Samples were diluted in order to ensure a total sugar concentration lower than 10 g/L. The oligosaccharides were harvested with a Pharmacia F100 fraction collector, and all the fractions having more than 95% purity for GOS 2 (or 3, 4 or OD_5) were freeze-dried.

Mass spectrometry.—Matrix-assisted laser desorption ionization time of flight mass spectra, (MALDI-TOF), were recorded on a TofSpec instrument (Micromass, Manchester, UK) fitted with a single-stage reflectron and a 337-nm pulsed nitrogen laser. The accelerating voltage was 18 kV. The targets, which consisted of flat stainless-steel plates, were loaded with 1 μL of the matrix solution (7.5 mg of 2,5-dihydroxybenzoic acid in 2:1 acetonitrile–water (1 mL), containing 0.1% TFA) and 1 μL of aq sample soln (15 μg/μL) and allowed to dry. Measurements were performed with positive detection mode.

NMR spectroscopy.—^{1}H spectra were recorded on a Varian unit Plus 500 spectrometer equipped with an ultrashim system (operating frequency, 499.836 MHz). Samples were studied as solns in deuterium oxide (5 mg in 0.75 mL of solvent) at 25 °C in 5 mm

o.d. tubes without spinning (internal acetone 2.1 ppm relative to Me_4Si). ^1H spectra were recorded using 90° pulses, 3300 Hz spectral width, 12,480 data points, 1.891 s acquisition time; 32 scans were accumulated. 1D TOCSY Experiments were recorded using a soft pulse sequence with an eburp 1-256 shape (186 ms) and (10 to 80 ms) mixing time.

The heteronuclear multiple quantum coherence spectroscopy (HMQC) and heteronuclear multiple bond correlation spectroscopy (HMBC) experiments were performed on a Varian unit Plus 400 spectrometer (proton frequency of 399.958 MHz, and ^{13}C frequency of 100.135 MHz, using the VARIAN standard pulse sequences. The delay time for the detection of long-range ^{13}C–^1H couplings was set to 120 ms in HMBC experiments.

Oligosaccharide reactivity.—Commercial preparations of pure maltose or panose (Sigma) or the purified molecules 2 (R_5), 3 (R'_6), 4 (R_6) and OD_5 (10 g/L) were assayed for their acceptor efficiency in the presence of 1 U/mL B-1299 dextransucrase, 10 g/L sucrose, 20 mM sodium acetate buffer, pH 5.4, 0.05 g/L $CaCl_2$, at 25 °C. Initial and final (after sucrose exhaustion, 12 h of reaction) samples were heated for 5 min at 90 °C to stop the reaction, before routine HPLC analysis.

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