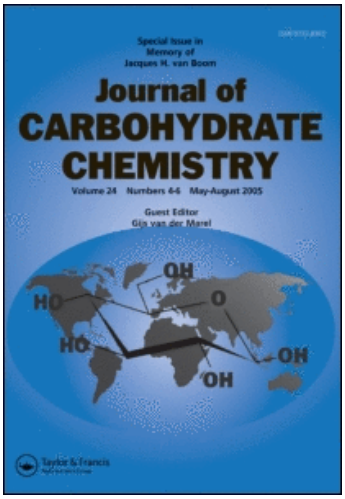


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# Characterization of α-(1→3) Branched Oligosaccharides Synthesized by Acceptor Reaction with the Extracellular Glucosyltransferases from *L. Mesenteroides* NRRL B-742

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# CHARACTERIZATION OF α-(1→3) BRANCHED OLIGOSACCHARIDES SYNTHESIZED BY ACCEPTOR REACTION WITH THE EXTRACELLULAR GLUCOSYLTRANSFERASES FROM *L. MESENTEROIDES* NRRL B-742

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## ABSTRACT

The glucosyltransferases from *L. mesenteroides* are known to catalyze the transfer of the D-glucosyl group of sucrose onto sugars, now commonly named acceptors. We investigated in the present work, the acceptor reaction catalyzed by the extracellular glucosyltransferases from *L. mesenteroides* NRRL B-742. The enzymes of the culture supernatant, purified by aqueous two-phase partition between dextran and polyethylene glycol solutions, were found to efficiently transfer the glucose moiety of sucrose onto maltose acceptor. By increasing the sucrose/maltose ratio (S/M), it was possible to catalyze the synthesis of oligosaccharides of increasing degree of polymerisation (d.p.). For an S/M ratio of 7, both linear oligosaccharides (only composed of α-(1→6) linkages and a maltose residue at the reducing end) and branched oligosaccharides were produced. A glucanase treatment permitted isolation of the branched products which were then analyzed by carbon 13 NMR spectroscopy. The chemical shifts arising from the purified glucanase-resistant oligosaccharides clearly

established that α-(1→3) linkages had been synthesized. A d.p. 6 oligosaccharide was found to be the smallest α-(1→3) branched oligosaccharide synthesized by acceptor reaction. The structure of this product which is in agreement with the enzyme hydrolysis, the NMR chemical shifts and spin-lattice relaxation data was determined as $6^3$-O-α-D-isomaltosyl [$3^3$-O-α-D-glucosyl]panose. The analysis of the oligosaccharides of smaller d.p., which resulted from the glucanase treatment, also demonstrated that the glucosyl group could be transferred at various O-3 position of the characterized oligosaccharides.

## INTRODUCTION

In 1952, Koepsell *et al.* [1] observed the synthesis of oligosaccharides, at the expense of dextran synthesis, after addition of small size sugars in the sucrose digest of the glucosyltransferase (E.C.2.4.1.5.) from *L. mesenteroides* NRRL B-512F. The enzyme was shown to transfer the D-glucosyl group of sucrose to mono- or disaccharides such as glucose, maltose or isomaltose which were called acceptors.[1] Since then, the so called "acceptor" reaction catalyzed by the glucosyltransferase from *L. mesenteroides* NRRL B-512F has been extensively described. The acceptor efficiency of a large variety of sugars (monosaccharides, disaccharides, dextran) and the structure of the synthesized products were determined.[2-14] Maltose and isomaltose were found to be the more efficient acceptors among the mono- and disaccharides.[1, 10,12] In both cases, the enzyme transfers the glucosyl group to the non-reducing end of the disaccharide by synthesis of an α-(1→6)-D-glucosidic bond. The acceptor reaction product may in turn be glucosylated and a series of linear oligosaccharides is produced. More recently, the dextransucrase from *L. mesenteroides* NRRL B-512F was demonstrated to transfer glucosyl residue either to the non-reducing end or to the reducing end of maltodextrin acceptors by synthesis of α-(1→6) linkage.[15] The enzyme was also shown to catalyze the synthesis of α-(1→3)-D-glucosidic bonds at branch position in the presence of a dextran acceptor.[14,16] The formation of α-(1→3)-D-linkages only occurs when dextran acceptor of an average molecular weight higher than 10,000 is used and always remains less favorable than the formation of α-(1→3)-D-linkages in the native dextran.[14]

In a survey of the dextrans produced by 96 bacterial strains, Jeanes *et al.* showed that the stucture of the dextran was highly dependent on the producing strains.[17] However, few studies on the acceptor reaction catalyzed by other strains of *L. mesenteroides* than *L. mesenteroides* NRRL B512F have been reported. The structure of the acceptor reaction product has rarely been demonstrated to be dependent on the glucosyltransferase producing strain. At this time, the alternansucrase, a glucosyltransferase produced by *L. mesenteroides* NRRL B-1355, is the only glucosyltransferase which has been shown to display a specificity in the acceptor reaction close to the one observed for high molecular weight glucan synthesis.[18,19] In the

presence of maltose or isomaltose, this enzyme synthesizes linear oligosaccharides which alternately contain α-(1→3)-D- and α-(1→6)-D-linkages like the alternan polymer synthesized in the absence of acceptor.

Among the various strains of *L. mesenteroides*, the strain NRRL B-742, isolated by Hucker and Pederson,[20] is also a rather unusual one. Like the B-1355 strain, it produces two different dextrans. The fraction L (the less soluble) contains 14 % of α-(1→4) branch linkages and the fraction S (the more soluble) contains 28 % of α-(1→3) branch linkages as well as 8 % of α-(1→4) linkages.[21-23] The glucosyltransferase which catalyzes the synthesis of the fraction L is the only one which has yet been isolated.[24] The glucosyltransferases from *L. mesenteroides* NRRL B-742 also effect acceptor reaction. A crude preparation was shown to catalyze the transfer of glucosyl group on galactose and lactulose giving products in a very low yield.[6,7] The structures of the products were the same as the structures of the products obtained with the B-512F dextransucrase. The dialyzed and concentrated glucosyltransferases from the culture supernatant of *L. mesenteroides* NRRL B-742 were also assayed in the presence of maltose.[24] Linear oligosaccharides identical to those produced by the B-512F dextransucrase were formed. The same study also reported that the glucosyltransferases transferred the glucosyl group from sucrose onto the B-742 fraction L and B-512F dextrans through the synthesis of α-(1→3)-D-glucosidic bonds at branch positions. However, the synthesis of α-(1→3) branch linkages has never been demonstrated with small size sugar acceptors.

In this work we have investigated the acceptor reaction in the presence of maltose catalyzed by a glucosyltransferase preparation from *L. mesenteroides* NRRL B-742. Oligosaccharides of increasing degree of polymerisation have been synthesized from maltose and sucrose. They have then been analyzed to find out if α-(1→3) or α-(1→4) branch linkages observed in the high molecular weight dextrans produced by this strain could also be synthesized by acceptor reaction with small size sugars. A glucanase treatment was applied to eliminate the linear oligosaccharides and facilitate the isolation of the branched products formed by acceptor reaction. The oligosaccharide structures were determined from $^{13}C$ NMR studies similar to those used to differentiate the various dextrans produced by the strains of *L. mesenteroides*[21,22] and to characterize oligosaccharides.[25,26]

## RESULTS

### Oligosaccharide synthesis

The acceptor reaction in the presence of maltose was first carried out using a sucrose/maltose (S/M) ratio of 2. As shown on **Figure 1**, the oligosaccharides

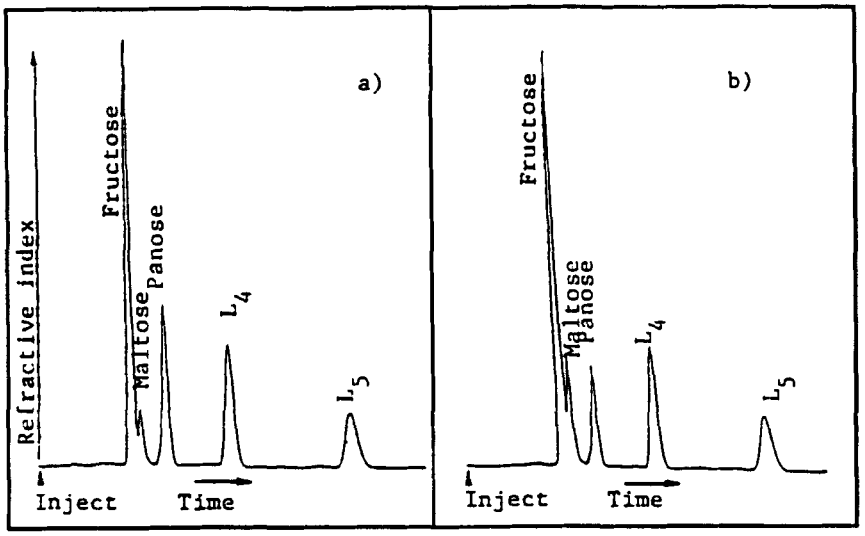


**FIG. 1. HPLC analysis of the oligosaccharides produced by the B-742 and the B-512F glucosyltransferase preparation**

*a) B-742 preparation b) B-512F dextransucrase.*

*The syntheses were carried out with an S/M ratio of 2. The products were separated on C18 Column using a water/methanol eluent (98/2, v/v) at 0.5 mL/min. The products noted $L_i$ represent linear oligosaccharides of d.p. i composed of α-(1→6) linkages and a maltose residue at the reducing end.*



synthesized by the B-742 glucosyltransferase preparation present the same retention time as those obtained with the B-512F glucosyltransferase (dextransucrase).

After elimination of high molecular weight dextran and fructose, the oligosaccharides were in both cases incubated with a mixture of endodextranase and glucoamylase. The oligosaccharides were hydrolyzed at the same rate in the two preparations. No trace of resistant oligosaccharides was detected after 15 hours of incubation. Glucose was the only product recovered. The B-742 preparation catalyzed under those conditions the synthesis of oligosaccharides which have the same structure as those obtained with the B-512F glucosyltransferase. They are linear oligosaccharides composed of α-(1→6) linkages and a maltose residue at the reducing end. The oligosaccharides which belong to this series have been noted $L_i$ (i corresponding to the degree of polymerisation (d.p.) of the oligosaccharide).

By increasing the S/M ratio of the acceptor reaction to 7, the synthesis of oligosaccharides of higher d.p. increased. In addition to the linear oligosaccharide series,

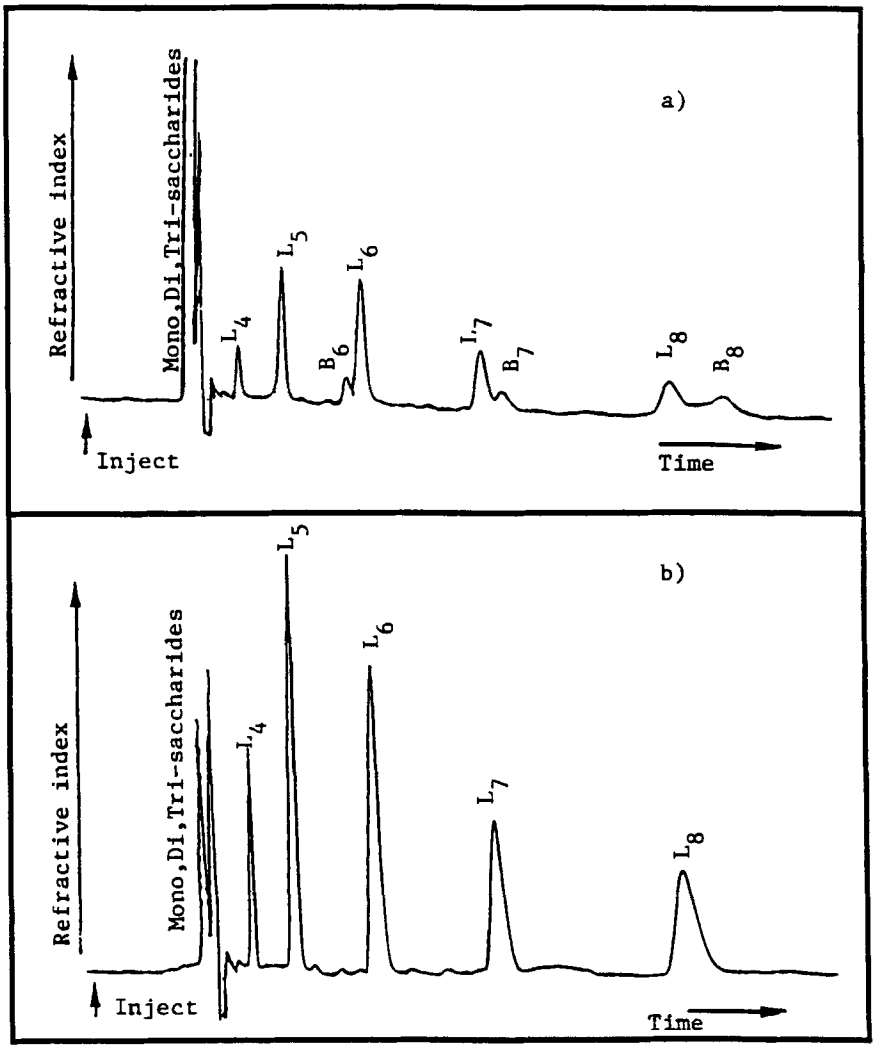


**FIG. 2. HPLC analysis of the oligosaccharides produced by the B-742 and the B-512F glucosyltransferase preparation**

*a) B-742 preparation b) B-512F dextransucrase.*

*The synthesis were carried out at S/M ratio of 7. The products were separated on C18 Column using a water / methanol eluent (98/2, v/v) at 0.5 mL/min. The products noted $L_i$ represent linear oligosaccharides of d.p. i composed of α-(1→6) linkages and a maltose residue at the reducing end.*

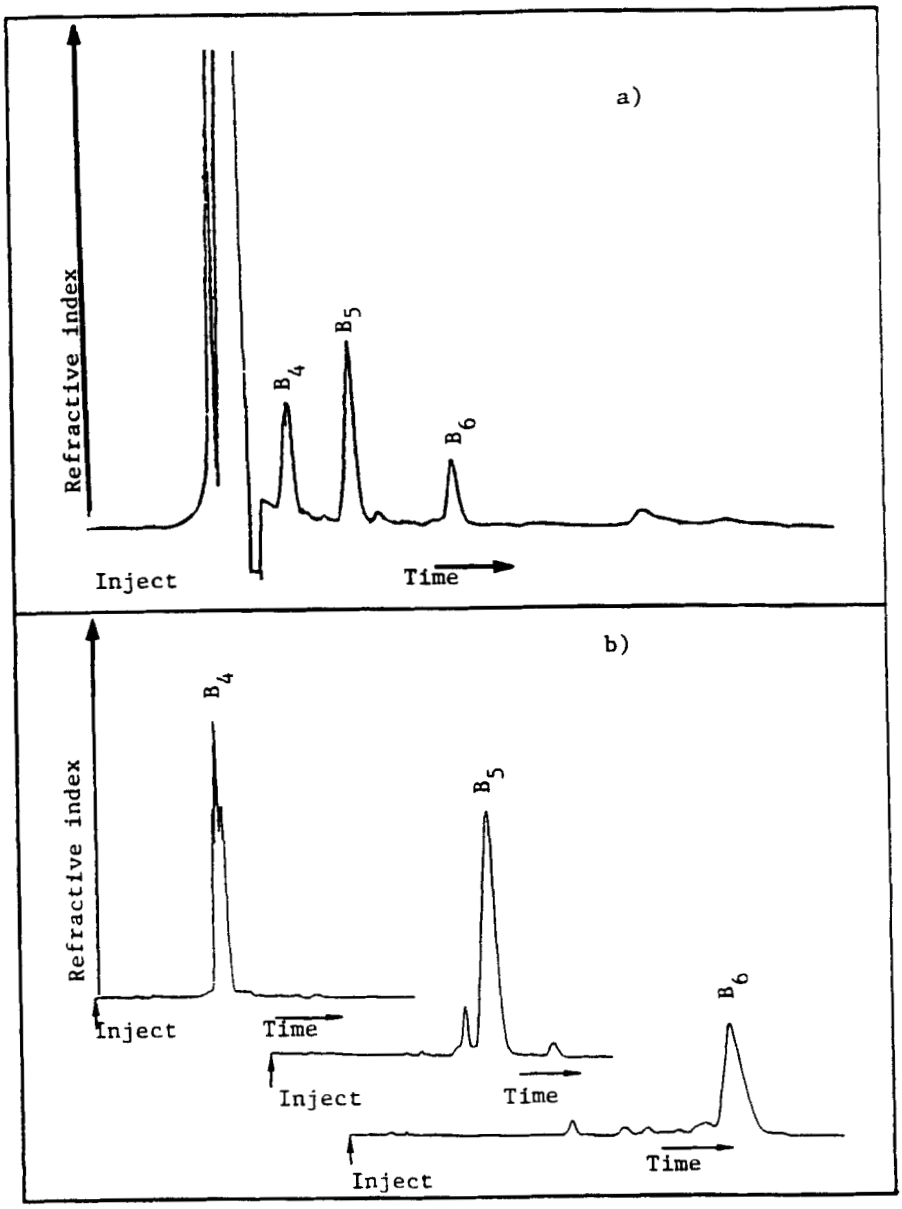


**FIG. 3. HPLC chromatograms of the glucanase resistant oligosaccharides before (a) and after (b) preparative chromatography**
*The separation was carried out using a C18 Partisil 10 ODS3 M40/50 column (WHATMAN).*

TABLE 1.
Oligosaccharide Yields before and after the Hydrolysis by the Glucanases

| Sucrose g/L | S/M (w/w) | Oligosaccharide yield* before hydrolysis, % | Oligosaccharide yield* after hydrolysis, % |
|---|---|---|---|
| 100 | 7 | 86 | 19 |

* Oligosaccharide yield: Oligosaccharide (g/L)/[0.474 Sucrose (g/L) + Maltose (g/L)]



the B-742 preparation produced oligosaccharides denoted $B_6$, $B_7$ and $B_8$, as shown on **Figure 2a**, which were not obtained with the B-512F dextransucrase (**Fig. 2b**). The acceptor reaction products, separated from high molecular weight dextran using ultrafiltration, were also incubated with the glucanases. The only product resulting from the hydrolysis of the oligosaccharides produced with the B-512F dextransucrase was shown to be glucose whereas glucanase-resistant oligosaccharides appeared on the chromatograms of the B-742 oligosaccharide hydrolyzate in addition to glucose (**Fig. 3a**). The oligosaccharides noted $B_4$ and $B_5$ were not observed in the preparation before the hydrolysis; they resulted from the hydrolysis of oligosaccharides of d.p. higher than 5. The oligosaccharide $B_6$ (which was present in the medium before the hydrolysis) resisted the action of the glucanases. The yields of the oligosaccharides before and after hydrolysis are presented in Table 1. After hydrolysis, the preparation of the resistant oligosaccharides has the following composition: $B_4$ 33% (w/w), $B_5$ 49 %(w/w), $B_6$ 18 % (w/w).

### Oligosaccharide analysis

The glucanase-resistant oligosaccharides were separated by preparative HPLC and separated in three different fractions as shown on **Figure 3b**. The chromatograms of the purified fraction $B_4$ show that this preparation contains a mixture of oligosaccharides of very close retention times that we could not separate using the preparative HPLC technique. Better separations were obtained for the oligosaccharides $B_5$ and $B_6$ which presented a purity of 87 and 95 % respectively (the purity calculations were based on the area of the peaks observed on the chromatogram of the oligosaccharides (**Fig. 3b**). Each independent preparation was then examined by $^{13}C$ NMR spectroscopy (**Fig. 4**). All the chemical shifts of oligosaccharides $B_6$, $B_5$, and $B_4$ are reported in Table 2.

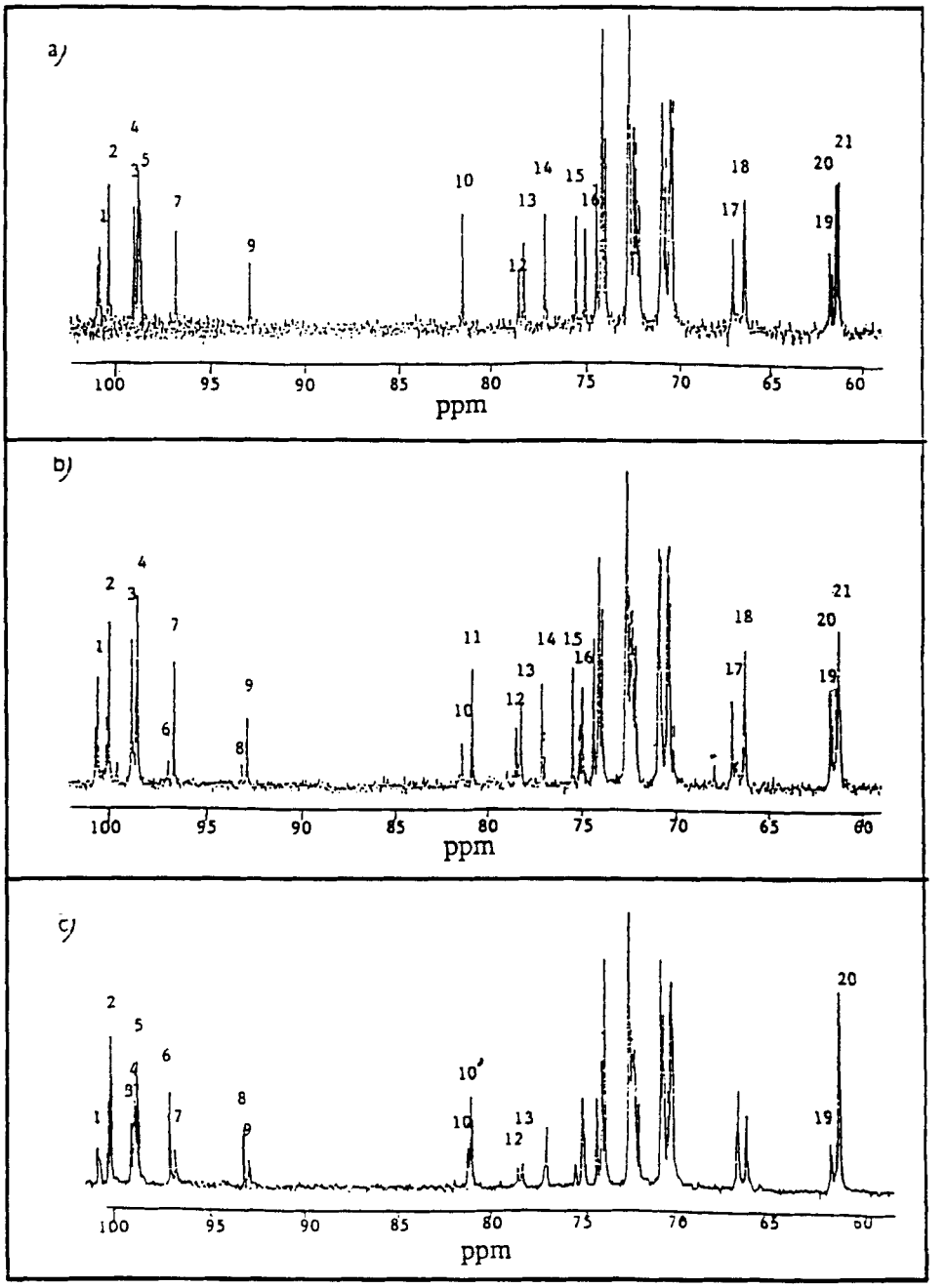


**FIG. 4. $^{13}C$ NMR Spectra of the glucanase-resistant oligosaccharides**
*(a) spectrum of hexasaccharide $B_6$ (b) spectrum of pentasaccharide $B_5$ (c) spectrum of fraction $B_4$.*

TABLE 2.

Chemical Schifts of the Oligosaccharides produced by Acceptor Reaction using the Glucosyltransferase from *L. mesenteroides* NRRL B-742

| Peak number | Assignment | Glucanase-resistant Oligosaccharides | | | (a) Maltose | (b) Panose |
|---|---|---|---|---|---|---|
| | | $B_6$ | $B_5$ | $B_4$ | | |
| 1 | C-1→4 | 100.7<br>100.6 | 100.7<br>100.6 | 100.78 | 100.7<br>100.6 | 100.7 |
| 2 | C-1→3 | 100.15 | 100.16 | 100.2 | | |
| 3 | C-1→6 | 98.87 | 98.9 | 99.1 | | 99.06 |
| 4 | C-1→6 | 98.65 | 98.6 | 98.9 | | |
| 5 | C-1→6 | 98.55 | | 98.79 | | |
| 6 | C-1β | | 97.01 | 97.1 | | |
| 7 | C-1β | 96.7 | 96.71 | 96.8 | 96.8 | 96.8 |
| 8 | C-1α | | 93.1 | 93.2 | | |
| 9 | C-1α | 92.8 | 92.8 | 92.9 | 92.9 | 92.9 |
| 10 | C-3→1 | 81.4 | 81.4 | 81.2 | | |
| 10' | C-3→1 | | | 81.03 | | |
| 11 | C-3→1 | | 80.8 | | | |
| 12 | C-4→1 | 78.5 | 78.5 | 78.6 | 78.2 | 78.6 |
| 13 | C-4→1 | 78.2 | 78.2 | 78.3 | 78 | 78.3 |
| 14 | C-3β (c) | 77.07 | 77.1 | 77.04 | 77.2 | 77.2 |
| 15 | C-5β (c) | 75.4 | 75.4 | 75.6 | 75.5 | 75.6 |
| 16 | C-2β (c) | 74.9 | 74.9 | 75.3 | 75.1 | 75.1 |
| 17 | C-6→1 | 66.9 | 66.9 | 66.9 | | 66.9 |
| 18 | C-6→1 | 66.25 | 66.3 | 66.4 | | |
| 19 | C-6 | 61.7 | 61.7 | 61.9 | | 61.9 |
| | C-6 | 61.6 | 61.6 | 61.8 | 61.8 | 61.8 |
| 20 | C-6 | 61.4 | 61.4 | 61.4 | 61.6 | 61.6 |
| 21 | C-6 | 61.3 | 61.2 | | | |

a. According to the assignments given in the literature[27-30]

b. According to the assignments of Usui *et al.*[28]

c. These chemical shifts correspond to the free carbon C-2β, C-3β, C-5β of the glucose residue (β anomer) located at the reducing end.

### $^{13}C$ NMR of the oligosaccharide $B_6$ (Fig. 4 a)

*Anomeric region*:

The anomeric carbons of the α-D-glucooligosaccharides usually resonate in the region from 92 ppm to 102 ppm. In the spectrum of $B_6$, no signal arises downfield of 100.7 ppm which indicates that the oligosaccharide is only composed of α-D-glucosidic linkages.

The two closely separated resonances (peak 1) at 100.6 and 100.7 ppm which integrate for one carbon and are also encountered in the spectrum of maltose, both correspond to a glucose residue linked to the reducing residue of the molecule by an α-(1→4) linkage. The splitting of the resonance shows that the residue is affected by the two configurations α and β of the molecule as in the case of maltose and further implies that the α-(1→4) linkage is located at the reducing end of $B_6$.

The signal at 100.15 ppm (peak 2) is characteristic of an α-(1→3)-linked residue. This resonance indicates that the glucanase-resistant oligosaccharide $B_6$ contains an α-D-(1→3) glucosidic bond which has been synthesized by acceptor reaction with the B-742 preparation.

Although they partially overlap, three resonances are distinguishable around 99 ppm (peaks 3, 4 and 5). They correspond to α-(1→6)-linked residues which may be slightly affected by different neighbouring effects.

At 96.7 and 92.8 ppm are encountered the resonances corresponding to the reducing end residue . They have the same chemical shifts as those observed in the spectrum of maltose and are assigned to the C-1β (peak 7) and C-1 α (peak 9) carbon of a glucose residue substituted at C-4.

The resolved resonances observed in this region present about equal intensities (the resonances due to the α and β configurations being added) and thus account for a d.p. 6 reducing oligosaccharide as expected from the retention time of $B_6$ (**Fig. 2a**).

*Region from 75 to 85 ppm*

The resonances associated with the bonded C-2, C-3, C-4 of the glucose residue as well as the resonances of the C-3, C-5, C-2 of the β form of the reducing glucose of the molecule arise in this region. By comparison of the $B_6$ spectrum with maltose and panose spectra, the resonances at 77.07 (peak 14), 75.4 (peak 15) and 74.9 ppm (peak 16) are attributed to the carbons 3, 5 and 2 (respectively) of the β form of the reducing glucose residue .

The resonances at 78.5 (peak 12) and 78.2 (peak 13) are related to a bonded C-4. The resonance due to the α anomer (peak 12) is slightly displaced from the resonance due to the β anomer (peak 13). That confirms that $B_6$ contains a maltose residue at the reducing end.

The signal at 81.4 ppm (peak 10), also displayed on the B-742 dextran (S fraction) spectrum,[22] is associated with a bonded C-3 and gives additional proof of the presence of an α-(1→3) glucosidic bond in the oligosaccharide.

*Region from 60 to 70 ppm*

On the basis of the analysis of the anomeric carbon region, the molecule should contain three bonded C-6 and three free C-6 atoms ( not involved in a linkage). Four resonances are encountered in the region corresponding to the free C-6 atoms. The signals observed at 61.7 and 61.6 ppm (peak 19) arise from the C-6 of the reducing end. Although poorly resolved, two additional resonances appear at 61.4 ppm (peak 20) and 61.3 ppm (peak 21). From this observation, it can be concluded that the molecule contains three free C-6 carbons. In the region corresponding to the bonded C-6, only two signals are displayed, but the intensity of the resonance at 66.25 ppm is higher than the intensity of the resonance at 66.9 ppm and probably accounts for two different C-6 carbons.The assignment of the different chemical shifts clearly allows one to conclude that $B_6$ is a d.p. 6 oligosaccharide composed of three α-(1→6), an α-(1→3) and an α-(1→4) glucosidic bonds (the latter being located at the reducing end). On the other hand, the glucanase-resistant oligosaccharides may have a branched structure. A linear oligosaccharide composed of α-(1→6), α-(1→3) and α-(1→4) linkages should not have presented any resistance to the glucoamylase action.[31-33] However, those results give no information about the position of the α-(1→3) and the α-(1→6) linkages in the molecule. To better characterize the oligosaccharide, we measured the spin-lattice relaxation times of the anomeric carbons.

*Spin-lattice relaxation time ($T_1$) measurement of the anomeric carbons*

By reflecting the average interaction of a carbon nucleus with its environment, $T_1$ gives very useful information about molecular motion and molecular flexibility. The carbon nuclei which occupy a position in the more mobile part of a molecule relax more slowly than the carbons located at the more rigid part. The magnitude of $T_1$ can thus be correlated to a given position of a carbon atom.[26,34-37] Seymour *et al.* [36,37] demonstrated that $T_1$ measurement could be used to assign the resonance arising from the di-*O*-substituted residues and the non-reducing end residue in the dextran polymers. An approach similar to the one described by Seymour *et al* . [36,37] was applied here. The data reported in Tables 3 and 4 were interpreted considering that a) a higher $T_1$ value reflects a higher mobility in the molecule, b) the residues at the extremities of the molecule present a higher freedom of motion than the residues which occupy the center of the molecule, c) the increase of substitution on a residue decreases its mobility.

The high $T_1$ values of the α-(1→3)-linked moiety (0.36 s) and one of the α-(1→6)-linked moieties (0.37 s) clearly indicate that these residues are located at two non-

TABLE 3.

Relaxation Time of the Anomeric Carbons of Oligosaccharide $B_6$

| Peaks | 1 | 2 | 3 | 4 | 5 | 7 | 9 |
|---|---|---|---|---|---|---|---|
| ppm | 100.6 | 100.2 | 98.9 | 98.7 | 98.6 | 96.7 | 92.8 |
| $T_1$, msec | 300 | 360 | 300 | 370 | 260 | 630 | 400 |
| Carbon | C-1→4 | C-1→3 | C-1→6 | C-1→6 | C-1→6 | C-1β | C-1α |

TABLE 4.

Relaxation Time of the Anomeric Carbons of Oligosaccharide $B_5$

| Peaks | 1 | 2 | 3 | 4 | 7 | 9 |
|---|---|---|---|---|---|---|
| p.p.m. | 100.7 | 100.16 | 98.9 | 98.6 | 96.71 | 92.8 |
| $T_1$, msec | 270 | 290 | 320 | 360 | 420 | 370 |
| Carbon | C-1→4 | C-1→3 | C-1→6 | C-1→6 | C-1β | C-1α |



reducing extremities. It further implies that the α-(1→3) linkage is in fact a branch linkage. The low $T_1$ value (0.26 s) observed for one of the α-(1→6) linkage suggests that this residue is trisubstituted and located between the α-(1→4)-linked residue and the third α-(1→6) linked residue which both have a $T_1$value of 0.3 s. The most probable structure obtained on the basis of those results is shown on **Figure 5a.**

**$^{13}C$ NMR of the oligosaccharide $B_5$**

As shown on **Figure 4**, the $^{13}C$ NMR spectra of the oligosaccharides $B_5$ and $B_6$ display a lot of common resonances which were generally assigned to the same residues. However, two additional resonances, 97.01 (peak 6) and 93.1 ppm (peak 8) in the spectrum of $B_5$, can be assigned to the C-1β and C-1α forms of another reducing residue.

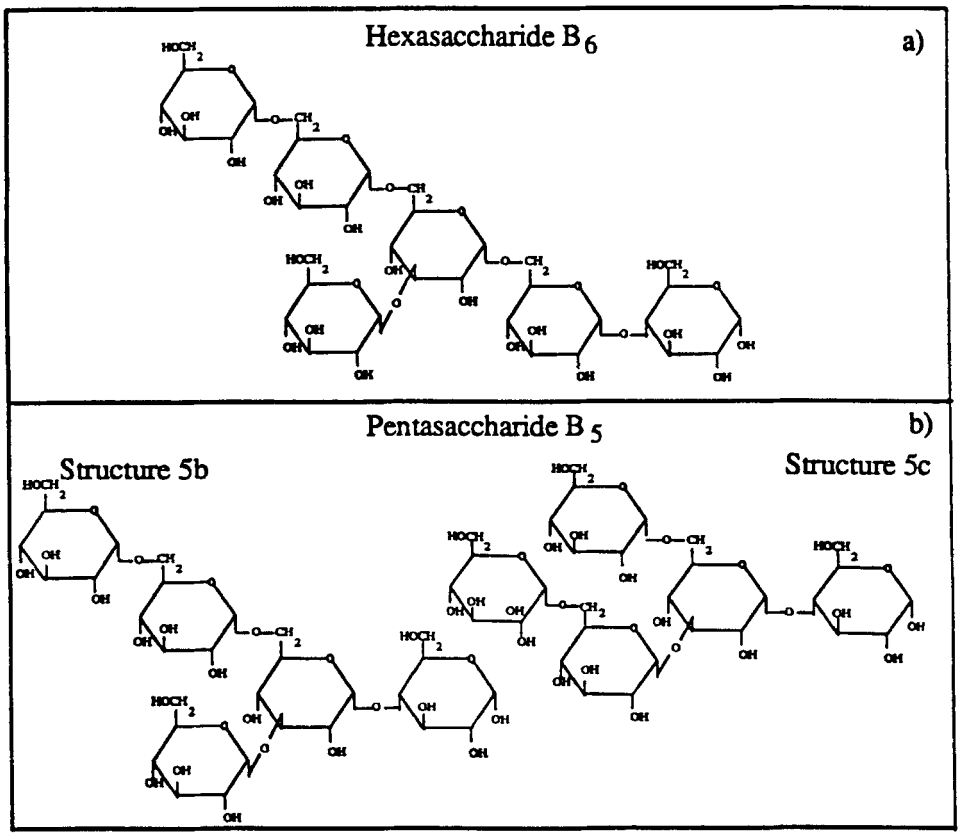


**FIG. 5. Structures proposed for oligosaccharides $B_6$ (a) and $B_5$ (b) in accordance with the results obtained from the enzymatic hydrolysis and the $^{13}C$ NMR.**

The preparation contains two different components. The percentages of each component were calculated from the intensities of the peaks 7 and 9 relative to the peaks 6 and 8, the composition of the preparation corresponds to 80-85 % for the major component (referred as $B_5$) and 15-20 % for the minor one. Those results are in agreement with the purity of $B_5$ determined from the HPLC chromatogram (**Fig. 3b**).

The oligosaccharide $B_5$ gives rise to the major resonances observed in the spectrum. In the anomeric region, six major resonances (1, 2, 3, 4, 7, 9) are identified with a d.p. 5 reducing oligosaccharide. One signal disappeared in the region of the α-(1→6)-linked residues. This indicates that $B_5$ contains two α-(1→6) linkages instead of three for $B_6$. Like $B_6$, $B_5$ presents an α-(1→4) linkage at the reducing end (peaks 1, 12 and 13) and an α-(1→3) linkage (peaks 2 and 11).

The major resonance at 80.8 ppm (peak 11) which arises from the bonded C-3 of $B_5$ is slightly displaced upfield compared to the resonance of the bonded C-3 of $B_6$. This fact suggests that the 3-*O*-substituted residues of the oligosaccharides $B_5$ and $B_6$ are affected by different neighbouring influences. To elucidate this point, we measured the $T_1$ of the anomeric carbons of $B_5$ (Table 4). The low value, displayed by the residue linked to the reducing end through an α-(1→4) bond, suggests that this residue is disubstituted at both O-3 and O-6 positions. The chemical shift variation observed for the bonded C-3 would be due to the fact that the di-*O*-substituted residue is an α-(1→4) linked residue in $B_5$ and not an α-(1→6) residue as in $B_6$. The $T_1$ values also indicate that an α-(1→6) is encountered at the non-reducing end of the oligosaccharide. Two possible structures are finally proposed for $B_5$ ( major component). The small difference observed between the $T_1$ values of the peaks 2 and 3 makes it difficult to know which residue constitutes the other non-reducing extremity. The low value of the α-(1→3)-linked residue can be explained either by the rigidity due to the close α-(1→4) linkage (Structure 5b) or by the fact that it is not an extremity of the molecule (Structure 5c). A complementary analytical method is required to provide additional information about the structure.
The approach described by Cheetham et al.,[38] which permits to distinguish the anomeric protons of glucosyl and isomaltosyl side-units in dextran using 500 MHz $^1$H NMR, may assist in choosing between structures 5b and 5c.

**$^{13}$C NMR of the fraction $B_4$**

As shown on the HPLC chromatogram (**Fig. 3b**), this fraction contained a mixture of oligosaccharides. The $^{13}$C spectrum confirms this result (**Fig. 4c**). As in the case of $B_5$, the four resonances displayed between 97 and 92 ppm indicate that two types of reducing oligosaccharides are present in the mixture. The oligosaccharide which contains a maltose residue at the reducing end is the minor component as shown by the weak intensities of the peaks 1, 7, 9, 12, 13, 19. The major component of the preparation has an α-(1→6) linkage at the reducing end. The high intensity of the resonances related to the α-(1→3) bond (peaks 2, 10, 11) further confirms that the both components contain an α-(1→3) linkage.

## DISCUSSION

In a previous work, Cote and Robyt[24] reported that the glucosyltransferases from the dialyzed and concentrated culture supernatant of *L. mesenteroides* NRRL B-742 ( B-742 CSDC) effected acceptor reaction in the presence of maltose and that the reaction led to the synthesis of the same series of oligosaccharides as the series obtained with the B-512F dextransucrase.[1, 9, 10]

The B-742 preparation which was used in the present work can be considered to be equivalent to the B-742 CSDC[24] because 98 % of the activity of the supernatant was recovered in the dextran phase obtained by phase partition with polyethyleneglycol (P.E.G.). When maltose was added to sucrose digest at an S/M ratio of 2, this preparation was found to catalyze the synthesis of oligosaccharides similar to those obtained with the B-742 CSDC. The study further showed that by varying the S/M ratio used in the acceptor reaction, it was possible to affect the size of the oligosaccharides. This feature which has previously been demonstrated for other glucosyltransferases[2,9,10,13,14,18] also characterizes the B-742 preparation.

By increasing the S/M ratio, we synthesized oligosaccharides of higher d.p. and clearly showed that beyond d.p. 5 both linear and α-(1→3)-branched oligosaccharides were produced by this preparation. The results reported by Cote and Robyt[24] were obtained using reaction conditions which did not allow the synthesis of oligosaccharides of d.p. higher than 5. In their study, the acceptor reaction was carried out with an S/M ratio of 0.42 and that further made impossible the observation of branched products.

The oligosaccharide ($B_6$) which did not belong to the linear series was proved by $^{13}C$ NMR to be a branched hexasaccharide. The structure of this product which is in agreement with the $^{13}C$ NMR spectra, the spin lattice relaxation time measurement and the enzymatic hydrolysis is depicted on **Figure 5a**. Such a structure has never been described before and is rather unusual due to the presence of the α-(1→4) linkage at the reducing end (coming from the maltose acceptor), the presence of α-(1→6) linkages and the α-(1→3) branched linkage. No other *L. mesenteroides* strain was shown to catalyze the synthesis of α-(1→3) branched linkage in the presence of small size sugar acceptors.

The $^{13}C$ NMR analysis of oligosaccharide $B_6$ revealed that the branching occurred on the residue antepenultimate to the reducing end. The oligosaccharide $B_5$, which appeared after the glucanase treatment, has an α-(1→3) branching on the residue penultimate to the reducing end (residue coming from the original maltose acceptor). Furthermore, some oligosaccharides produced after the glucanase treatment were found to have an α-(1→6) linkage at the reducing end and an α-(1→3) linkage as it is the case for the major component in the preparation $B_4$. This type of product clearly results from the endohydrolysis of an acceptor reaction product of higher d.p. which had an α-(1→3) linkage close to the non-reducing end. All those observations show that the glucose residue can be transferred onto several O-3 hydroxyl groups along the oligosaccharide chain.

Walker[39] studied the acceptor reaction catalyzed by the glucosyltransferase from *S.mutans* OMZ 176 in the presence of isomaltooligosaccharides. She reported that the synthesis of α-(1→3) branch linkages was catalyzed, in significant amounts, only in the

presence of acceptor having a d.p. higher than 7. The branching always occurred on the residue penultimate to the reducing end. More recently, the dextransucrase from *L. mesenteroides* NRRL B-512F was shown to catalyze the transfer of glucose residue either to the reducing end or to the non-reducing end of maltodextrin acceptors by synthesis of α-(1→6) linkage.[15] The results of our study differ significantly from those of Walker[39] and Fu.[15] They reflect very well the various specificities which can be found among the bacterial glucosyltransferases.

From the results obtained with the B-742 preparation, it is not possible to know precisely which glucosyltransferase is responsible for the synthesis of the α-(1→3) branched linkage. Two different dextrans are synthesized by the B-742 extracellular glucosyltransferases: the S fraction, which has 28 % of α-(1→3) branched linkages and 8 % of α-(1→4) linkages, and the L fraction which contains 14 % of α-(1→4) branched linkages.[23, 24] The glucosyltransferase which produces the highly α-(1→3) branched dextran, is probably the one which catalyzed the synthesis of the α-(1→3) branched oligosaccharides. No evidence of branched α-(1→4) linkages appears in our study. However, we cannot conclude that no α-(1→4) linkage has been synthesized by acceptor reaction because the glucoamylase, which was used to eliminate the linear oligosaccharides, may have also hydrolyzed the α-(1→4) linkages which could have been synthesized by acceptor reaction.

The study presented here shows that the glucosyltransferases from *L. mesenteroides* NRRL B-742 produced, by acceptor reaction, both linear and branched oligosaccharides. Unusual oligosaccharides are synthesized and can be isolated from the linear product using a glucanase treatment. The yield of the hydrolysis-resistant oligosaccharide was found to reach 19 %. The carbon 13 NMR study further confirmed that those products had α-(1→3) branch linkages. This technique was found to be very useful to determine the structures of the products and to demonstrate the complexity of the acceptor reaction catalyzed by the glucosyltransferase preparation from *L. mesenteroides* NRRL B-742.

## EXPERIMENTAL

### Glucosyltransferase Production

The glucosyltransferases were produced by culture of *L. mesenteroides* NRRL B-742 in a 2 liter fermentor. The medium used had the following composition: sucrose (40 g/L); yeast extract (20 g/L); $K_2HPO_4$ (20 g/L); $MgSO_4$,7 $H_2O$ (0.2 g/L); $MnSO_4$, $H_2O$ (0.01 g/L); $CaCl_2$ (0.02 g/L); Na Cl (0.01 g/L); $FeSO_4$,$H_2O$ (0.01 g/L). The culture

medium (1.5 L) was inoculated with 100 mL of a 15 h preculture. The temperature was maintained at 27 °C. The culture was stopped at the end of the growth when the pH reached the value of 4.5.

### Glucosyltranferase Purification

The pH of the culture medium was readjusted to 5.3 at the end of the culture, then the cells were removed by centrifugation. The final culture glucosyltransferase activity of the supernatant was 0.3 U/mL. The extracellular glucosyltransferases from *L. mesenteroides* NRRL B-742 were further purified by aqueous two phase partition between the dextran produced in the supernatant and polyethyleneglycol (P.E.G., Mw=1,500) using a process described by Paul et *al.*.[40] The P. E .G.(50 % w/v in $H_2O$) was added dropwise up to a final concentration of 20 %. The supernatant was then centrifuged at 16000 g for 15 min and 98 % of the total extracellular activity was recovered in the dextran rich phase. The activity of the preparation obtained was 0.025 U/mg of dextran and 2 U/mg of protein. The purification process did not permit the separation of the two glucosyltransferases produced by *L. mesenteroides* NRRL B-742 and the P. E .G. purified glucosyltransferases were referred as the B-742 preparation in this work. A glucosyltransferase unit (U) is defined as the amount of enzyme that catalyzes the formation of 1 μmole of fructose per min at 30 °C, pH 5.3 in the presence of sucrose (100 g/L). The proteins were assayed using the method of Lowry *et al.*[41] and the dextran was measured using the anthrone assay.

### Oligosaccharide Synthesis

The reactions were carried out at 30 °C in sodium acetate buffer 20 mM (pH 5.2), sucrose (100 g/L), B-742 preparation (1 U/mL), maltose (50 g/L or 14.3 g/L). The reactions were stopped after the total consumption of sucrose by heating at 75 °C for 15 min. Then, the glucosyltransferase was eliminated using ultrafiltration with an Amicon hollow fiber system (membrane cut-off=100,000), this ultrafiltration also permits to eliminate the endogenous or newly synthesized high molecular weight dextrans.

### Oligosaccharide Purification

Fructose was first removed by chromatography on a strongly acidic resin charged with calcium (Duolite C-204F). Then, the oligosaccharides were hydrolyzed using the conditions described below. The glucose formed was then digested by baker's yeast during 5 h at 30 °C. An amount of wet baker's yeast equal to the amount of glucose was used. The baker's yeast was then removed by centrifugation and the oligosaccharide preparation was ultrafiltrated using an Amicon hollow fiber system, the separation of the different oligosaccharides was then achieved by preparative HPLC.

### Oligosaccharide Hydrolysis

Two glucanases were simultaneously used to ensure the hydrolysis of the linear acceptor products synthesized: the endodextranase Sigma Grade I (E. C. 3.2.1.11) which

hydrolyzes only the α-(1→6)-D-glucosidic bonds [42,43] and the glucoamylase Novo (E. C. 3.2.1.3 ) from *A. Niger* which was shown to hydrolyze the α-(1→4), α-(1→3) and α-(1→6) linkages at respectively decreasing rate to produce glucose from the non-reducing end of linear glucooligosaccharides.[31,32] The acceptor-reaction mixture was first diluted to a total concentration of sugars of 40 g/L and was then incubated for 15 h at 37 °C with the glucoamylase (3 AGU /mL) and the dextranase (20 U/mL). The amount of glucanases used was previously shown to catalyze the hydrolysis of a 30 g/L oligodextran solution in 2 h. An endodextranase unit is defined as the amount of enzyme which catalyzes the hydrolysis of one μmole of isomaltose per min at 37 °C and pH 6. A glucoamylase unit is defined as the amount of enzyme which hydrolyzes one μmole of maltose per min at 25 °C.

**HPLC Analysis**

The oligosaccharides were analyzed using a Millipore-Waters system consisting of an M 6000 A pump, a U6K injector and a R-410 differential refractometer. A C18 column was used, and the elution was achieved either with ultrapure water or with a mixture of ultrapure water and methanol depending on the degree of polymerization of the oligosaccharides. The preparative chromatography was also carried out with a C18 column Partisil 10 ODS3 M40/50 (Whatman). The system consisted of a Gilson 303 pump, a Gilson 131 differential refractometer, a Rheodyne injection valve and a Pharmacia F300 fraction collector. Four mL of the oligosaccharides (150 g/L) were usually injected and eluted with ultrapure water at a flow rate of 15 mL/min.

**$^{13}C$ NMR**

The $^{13}C$ NMR spectra of the oligosaccharides ( 20 to 50 g/L in deuterated water) were recorded at 303 K with a Brucker AM 300 spectrometer. The spectral width was 15 KHz, the acquisition time was 0.54 s. The chemical shifts were expressed in ppm relative to the methyl signal of acetone in water which was used as standard at δ=31.07 ppm relative to the methyl signal of 4,4-dimethyl-4-silapentane-1-sulfonate. The inversion-recovery method was used for $T_1$ determination (two pulse sequences $(T...180...t...90)_n$) with at least 10 t values. The peak heights of the different signals were measured as a function of the delay time (t) and these data were then analyzed by computer to determine the relaxation rates.

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