Structure determination of the exopolysaccharide of Lactobacillus fermentum TDS030603—A revision

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The highly viscous neutral exopolysaccharide, produced in large amounts by Lactobacillus fermentum TDS030603 in MRS broth and in a chemically defined medium supplemented with glucose, was investigated by GLC(-EIMS) monosaccharide and methylation analysis, periodate oxidation, MALDI-TOF mass spectrometry, and 1D/2D NMR spectroscopy. The GLC(-EIMS) analyses showed that the EPS contained d-glucose and d-galactose in an averaged molar ratio of 2.6:1.0, consisting of terminal d-Glc p, 3-substituted d-Glc p, 2,3-disubstituted d-Glc p, and 6-substituted d-Gal p in an averaged molar ratio of 1.3:1.0:1.1:1.1, with a trace of terminal d-Gal p (0.1). Combined with the NMR data of the EPS (previously reported constituent analysis: terminal Glc p, 3-substituted Glc p, 2,3-disubstituted Glc p, and 6-substituted Gal p in an averaged molar ratio of 1.2:1.1:1.0:1.1) and oligosaccharides isolated after partial acid hydrolysis, a branched tetrasaccharide repeating unit, with some heterogeneity in the side chain, having the following structure is proposed:

\[ \rightarrow (\rightarrow 3)-\beta-D-Glc p-(1 \rightarrow 3)-\alpha-D-Glc p-(1 \rightarrow n) \]

\[ \uparrow \]

2

\[ \uparrow \]

1

\[ \alpha-D-Gal p \]

6

\[ \uparrow \]

1

\[ \alpha-D-Glc p \]

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1. Introduction

Exopolysaccharides (EPSs) produced by dairy lactic acid bacteria, carrying the GRAS (generally recognized as safe) status, are of increasing interest to the food industry, being often used to improve the texture and consistency of fermented dairy products. In addition to these features, some of the EPSs have been reported to have beneficial health properties such as anti-tumor, anti-ulcer, and immune-stimulating activities.

In earlier studies, it has been found that the neutral exopolysaccharide produced in large amounts by Lactobacillus fermentum strain TDS030603 yields a high degree of ropiness, being a serious candidate for use as an additive to increase the viscosity of food. The strain originated from a collection of lactic acid bacterial strains, isolated from traditional fermented milk products at the Obihiro University of Agriculture and Veterinary Medicine. Besides structural analysis studies of the EPS and studies on the effects of carbohydrate source on its physicochemical properties, also attention was paid to the characterization and expression analysis of the EPS gene cluster. The structural determination data, reported six years ago, were interpreted as belonging to one major and one minor EPS, both built up from a Glc-/Gal-containing tetrasaccharide repeating unit (two \( \alpha \) and two \( \beta \) anomeric residues). The major EPS should contain terminal Glc p, 3-substituted Glc p, 2,3-disubstituted Glc p, and 6-substituted Gal p. The minor EPS...
should contain terminal Galp, 3-substituted Glcp, 2,3-disubstituted Glcp, and 6-substituted Galp. For the major EPS, two possible structures for the backbone were proposed, built up from two Glc and one Gal residue with one Glc residue as side chain connected to one of the Glc backbone residues. For the minor EPS the same backbone possibilities were proposed, but now with one Gal residue as side chain connected to one of the Glc backbone residues.

Here, we report on a revised structure of the EPS of \textit{Lb. fermentum} strain TDS030603, showing that the earlier idea of one major and one minor EPS was not correct. In fact, a detailed study, making use of monosaccharide and methylation analysis, partial acid hydrolysis, periodate oxidation, MALDI-TOF mass spectrometry, and 1D/2D NMR spectroscopy, indicated one branched EPS structure (tetrasaccharide repeating unit with a disaccharide backbone), being slightly heterogeneous in its side chain.

2. Results and discussion

2.1. Preparation of EPS from \textit{Lb. fermentum} TDS030603

The EPSs \textit{EPS-MRS} and \textit{EPS-CDM}\textsubscript{Glc}, produced by \textit{Lb. fermentum} TDS030603 in MRS broth and in chemically defined medium supplemented with glucose, respectively, were isolated via absolute

Table 1

<table>
<thead>
<tr>
<th>Partially methylated alditol acetate</th>
<th>( R_t )</th>
<th>Structural feature</th>
<th>Molar ratio ( % )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,5-Di-O-acetyl-2,3,4,6-tetra-O-methyl-hexitol-1-d</td>
<td>1.00</td>
<td>Glcp(1→</td>
<td>29</td>
</tr>
<tr>
<td>1,5-Di-O-acetyl-2,3,4,6-tetra-O-methyl-hexitol-1-d</td>
<td>1.03</td>
<td>Galp(1→</td>
<td>2</td>
</tr>
<tr>
<td>1,3,5-Tri-O-acetyl-2,4,6-tri-O-methyl-hexitol-1-d</td>
<td>1.16</td>
<td>→3)Glc(1→</td>
<td>23</td>
</tr>
<tr>
<td>1,5,6-Tri-O-acetyl-2,3,4,tri-O-methyl-hexitol-1-d</td>
<td>1.24</td>
<td>→6)Galp(1→</td>
<td>22</td>
</tr>
<tr>
<td>1,2,3,5-Tetra-O-acetyl-4,6-di-O-methyl-hexitol-1-d</td>
<td>1.28</td>
<td>→2,3)Glc(1→</td>
<td>24</td>
</tr>
</tbody>
</table>

\( a \) \( R_t \), GLC retention times relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-glucitol-1-d (1.00) on EC-1.

\( b \) Calculated on the basis of FID responses (peak areas), taking into account similar molar adjustment factors.

Figure 1. 500-MHz 1D \( ^{1} \text{H} \) NMR spectra of native \textit{EPS-MRS} (A) and \textit{EPS-CDM}\textsubscript{Glc} (B) produced by \textit{Lb. fermentum} TDS030603 in MRS broth and in chemically defined medium supplemented with glucose, respectively, recorded in \( D_2O \) at 334 K. The HOD signal at 4.40 ppm was suppressed.
ethanol precipitation, followed by subsequent anion-exchange and size-exclusion chromatography.  

2.2. Composition of EPS-MRS and EPS-CDMGlc

Monosaccharide analysis, including absolute configuration determination, of both EPS-MRS and EPS-CDMGlc revealed the presence of D-glucose (Glc) and D-galactose (Gal) in a molar ratio of 2.6:1.0, in agreement with earlier data [Glc:Gal = 2.5:1 (whey-mediated broth, EPS-WMB), 2.6:1 (EPS-MRS), 2.6–2.8:1 (EPS-CDMGlc, EPS-CDMGal, EPS-CDMLac, and EPS-CDMSuc)].

As the native EPSs did not dissolve in dimethyl sulfoxide, the partially hydrolyzed (0.5 M TFA; 5 min, 100°C) EPSs EPS50-MRS and EPS50-CDMGlc were used for methylation analysis. Terminal Glc p, 3-substituted Glc p, 2,3-disubstituted Glc p, and 6-substituted Gal p residues were found in roughly similar molar ratios as those reported earlier for EPS-WMB (Table 1), suggesting highly similar/identical branched polysaccharide structures.

2.3. NMR spectroscopy of EPS-MRS and EPS-CDMGlc

The native EPSs EPS-MRS and EPS-CDMGlc showed high viscosity by dissolving them in D2O, thus only low-concentration samples could be used for NMR measurements. Due to a carbohydrate signal at δ 4.73, which is in the close neighborhood of the HOD signal at 300 K, all NMR spectra were recorded at 334 K (HOD signal at 4.40 ppm). As is evident from Figure 1, the 500-MHz 1H NMR spectra of both native EPS probes are identical, and highly similar to those published earlier for EPS-MRS, EPS-WMB, EPS-CDMGlc, EPS-CDMGal, EPS-CDMLac, and EPS-CDMSuc recorded at 343 K. In the anomic regions four H-1 signals are present (Fig. 1), denoted A at δ 5.674, B at δ 5.325, C at δ 4.985, and D at δ 4.731, respectively, according to decreasing chemical shift values. The δ values suggest α-configurations for A, B, and C, and β-configuration for D. The signal at δ 4.516 is not stemming from an anomeric proton (HSQC, δC 70.1; see below).

As the EPS50-MRS and EPS50-CDMGlc samples, used for methylation analysis, had a higher solubility and a lower viscosity in D2O, also 1D 1H NMR spectra of these probes were recorded at 334 K (Fig. 2). Both EPSs gave identical spectra, highly similar to the spectra of the native samples (Fig. 1). The four anomic signals A–D had the same chemical shifts as mentioned above. Both EPS50-MRS and EPS50-CDMGlc were further investigated by 2D NMR spectroscopy. TOCSY with different mixing times (20, 50, 75, 100, and 200 ms) and HSQC experiments were performed to obtain the complete assignment of the 1H and 13C chemical shifts of the constituent residues A–D (Table 2), whereas NOESY measurements were carried out to generate monosaccharide sequence data (Table 3). The combined 1H and 13C anomic δ values support the occurrence of α-configurations for A, B, and C, and β-configuration for D. In Figure 3, the 1D 1H NMR, the HSQC, and the TOCSY (200 ms) spectra, as well as the relevant part of the NOESY spectrum of EPS50-MRS are depicted.


Table 2

H and 13C NMR chemical shifts of monosaccharide residues A–D in EPS5-CDMGlc and EPS5-MRS, recorded in D2O at 334 K

<table>
<thead>
<tr>
<th>Residue</th>
<th>H-1</th>
<th>C-1</th>
<th>H-2</th>
<th>C-2</th>
<th>H-3</th>
<th>C-3</th>
<th>H-4</th>
<th>C-4</th>
<th>H-5</th>
<th>C-5</th>
<th>H-6a</th>
<th>C-6</th>
<th>H-6b</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5.674</td>
<td>5.94</td>
<td>4.19</td>
<td>4.02</td>
<td>3.63</td>
<td>4.13</td>
<td>3.86</td>
<td>3.76</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>1.96</td>
<td>73.3</td>
<td>75.8</td>
<td>75.9</td>
<td>69.8</td>
<td>72.7</td>
<td>61.8</td>
<td>3.71</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>C</td>
<td>5.32</td>
<td>5.89</td>
<td>4.02</td>
<td>4.02</td>
<td>3.96</td>
<td>4.51</td>
<td>3.91</td>
<td>3.68</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>4.985</td>
<td>3.57</td>
<td>3.72</td>
<td>3.44</td>
<td>3.74</td>
<td>3.85</td>
<td>3.77</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a In ppm relative to the signal of internal acetone at δ 2.225 for 1H and at δ 31.08 for 13C.
b Ring carbons involved in linkages are indicated in gray.

Comparison of the TOCSY spectra of EPS5-MRS with increasing mixing times (only the final spectrum of 200 ms is shown in Figure 3) allowed the assignment of the sequential order of the chemical shifts belonging to the same spin system.

The TOCSY A H-1 track (δ 5.674) showed the scalar coupling network H-2,3,4,5,6a. The H-6b value was found via the H-6a signal in the HSQC spectrum. Combined with the downfield shifts for A C-2 (δ 73.5; Me α-o-GlcP, δc 72.98) and A C-3 (δ 79.9; Me α-o-GlcP, δc 74.18), deduced from the HSQC spectrum, residue A corresponds with the (2,3)-α-o-GlcP-(1→ unit, observed in the methylation analysis. The relatively small downfield shift for A C-2 is due to the fact that also A C-3 is substituted, causing an additional small upfield shift for A C-2 (in agreement with a CASPER simulation).

The TOCSY B H-1 track (δ 5.325) revealed cross-peaks with B H-2,3,4,5,6a. The cross-peaks with B H-6a,6b were present on the B H-5 track at δ 4.516, which also contained the cross-peak with B H-4. The magnetization transfer on the H-1 track went only as far as B H-4, suggesting that B is the α-o-Galp residue. The relatively large 1H downfield shift of B H-5 (δ 4.516) is remarkable and could be due to the substitution at C-6 and/or a peculiar structure of the polysaccharide fragment containing this B residue. For residue B, the 1H and 13C (B C-6, δ 68.0; Me α-o-Galp, δc 62.28) NMR data are in agreement with a (→6)-α-o-Galp-(1→ unit.

The TOCSY C H-1 track (δ 4.985) revealed cross-peaks with C H-2,3,4,5,6a, yielding a set of chemical shifts, typical for a terminal α-o-GlcP-(1→6)-residue.

Finally, the set of cross-peaks with D H-2,3,4,5,6a,6b, as seen on the TOCSY D H-1 track (δ 4.731), matched that of a (→3)-β-o-GlcP-(1→ unit.

The 3-substitution is supported by the D C-3 signal at δ 83.1 (Me β-o-GlcP, δc 76.8).

To deduce information about the sequence of the monosaccharide residues in the EPS, a 2D NOE spectrum was recorded (Fig. 3), and the observed intra- and inter-residue cross-peaks are listed in Table 3. The intra-residue cross-peaks between A H-1 and A H-2, B H-1 and B H-2, and C H-1 and C H-2 confirmed the α-configurations for the two o-GlcP and d-Galp residues A and C, and the d-Galp residue B, whereas the β-configuration for the D o-GlcP residue was supported by the intra-residue cross-peaks between D H-1 and D H-3 and between D H-1 and D H-5. On the A H-1 track an inter-residue cross-peak with D H-3 indicated an A(1→3)D linkage [−3]-α-o-GlcP-(1→3)-β-o-Galp]. The observed cross-peak with D H-4 reflects the proximity in space of this proton to A H-1, due to the A(1→3)D linkage. On the D H-1 track an inter-residue cross-peak with A H-3 showed a D(1→3)A linkage [−3]-β-o-GlcP-(1→3)-α-o-Galp]. On the B H-1 track an inter-residue cross-peak with B H-2 supported a B(1→2)A linkage [−6]-α-o-Galp-(1→2)-β-o-Galp, and on the C H-1 track inter-residue cross-peaks with B H-6a and 6b corresponded with a C(1→6)B linkage [α-o-GlcP-(1→6)-α-o-Galp]. The inter-residue cross-peaks between A H-1 and B H-1 reflect the proximity in space of the 2,3-disubstituted α-o-GlcP A and 6-substituted α-o-Galp B residues. A structural interpretation of a part of the EPS is shown in Figure 4, including the repeating unit and the observed NOE connectivities.

Table 3

Intra- and inter-residue NOE connectivities observed for EPS5-MRS (see Figs. 3 and 4)

<table>
<thead>
<tr>
<th>NOE Type</th>
<th>Feature</th>
</tr>
</thead>
<tbody>
<tr>
<td>A H-1–A H-2</td>
<td>Intra α-o-GlcP unit</td>
</tr>
<tr>
<td>A H-1–B H-1</td>
<td>Inter Close in space</td>
</tr>
<tr>
<td>A H-1–D H-3</td>
<td>Inter −2,3)-α-o-GlcP-(1→3)-β-o-GlcP-(1→</td>
</tr>
<tr>
<td>A H-1–D H-4</td>
<td>Inter Close in space</td>
</tr>
<tr>
<td>A H-1–B H-2</td>
<td>Intra α-o-Galp unit</td>
</tr>
<tr>
<td>A H-1–A H-1</td>
<td>Inter Close in space</td>
</tr>
<tr>
<td>B H-1–A H-2</td>
<td>Inter −6)-α-o-Galp-(1→2,3)-α-o-GlcP-(1→</td>
</tr>
<tr>
<td>C H-1–B H-2</td>
<td>Intra α-o-GlcP unit</td>
</tr>
<tr>
<td>B H-1–B H-6a/6b</td>
<td>Intra α-o-Galp-(1→6)-α-o-Galp-(1→</td>
</tr>
<tr>
<td>D H-1–D H-3</td>
<td>Inter β-o-Galp unit</td>
</tr>
<tr>
<td>D H-1–D H-5</td>
<td>Inter α-o-Galp-(1→6)-α-o-Galp-(1→</td>
</tr>
<tr>
<td>D H-3–A H-3</td>
<td>Inter −3)-β-o-GlcP-(1→3,2)-α-o-o-GlcP-(1→</td>
</tr>
</tbody>
</table>

a Anomeric configurations and linkages in bold reflect the observed NOE cross-peaks.

Partial acid hydrolysis of native EPS-MRS and EPS-CDMGlc (0.5 M TFA; 20 min, 100 °C) yielded in each case a complex mixture of oligosaccharides, which was fractionated on Bio-Gel P-2. Twenty 10-min fractions were collected and checked by MALDI-TOF-MS, showing the same spectra (data not shown), being identical with the proposed side chain. Also here, it probably indicates a minor heterogeneity in the EPS, missing the terminal α-o-GlcP- unit in the side chain.
2. Periodate oxidation

Monosaccharide analysis of the polymeric material, obtained after periodate oxidation and subsequent NaBH₄ reduction of EPS₅-MRS, showed the only presence of glucose.

3. Conclusion

Taking together the monosaccharide analysis (on average D-Glc: D-Gal = 2.6:1.0) and the methylation analysis (on average Glcp(1→3)Glc(1→6)Galp(1→2,3)Galp(1→) = 1.2:1.0:1.1:1:0.2) data of this study and the earlier studies, combined with the detailed 1D/2D NMR analyses of the EPSs and the partial acid hydrolysate, a branched tetrascarhide repeating unit for the EPS from Lactobacillus fermentum TDS030603 is indicated, independent on the used culture media, as follows:

\[
\text{D} \rightarrow [\text{→3}]-\beta-D-Glc-p-(1\rightarrow3)-\alpha-D-Glc-p-(1\rightarrow)\text{A}
\]

\[
\text{B} \quad \downarrow
\]

\[
\text{C} \quad 2 \quad \alpha-D-Galp \quad 6 \quad \uparrow \quad 1
\]

The finding of small amounts of terminal Gal residues supports heterogeneity in the side chain. The reproducible finding of the Gal:Glc molar ratio, being lower than 3:1, indicated that for a few percentage not only the terminal Glc residue in the side chain is absent, but also the whole Glc-Gal side chain. A remarkable feature of the structure is that the non-branched D-Glc residues in the backbone have \(\beta\)-configuration and the branched ones \(\alpha\)-configuration. Looking back at the earlier reported structures, it can be ascertained that in the 1D NMR analysis the peaks as \(\delta\) 5.674 (here A H-1) and \(\delta\) 4.516 (here B H-5) were interpreted in the wrong way. The proposed revised EPS structure was simulated in CASPER (www.casper.organ.su.se/casper) for reference \(^1\)H and \(^13\)C chemical shift values, and the calculated data were in close agreement with the measured chemical shifts.

Recently, the composition of an EPS produced by Lb. fermentum F6 was reported, also containing glucose and galactose, however, in a molar ratio of 4:3.

4. Experimental

4.1. Production, isolation, and purification of exopolysaccharide from Lb. fermentum TDS030603

Lb. fermentum TDS030603 was pre-cultured aerobically in Man-Rogosa-Sharpe (MRS) medium for 24 h at 30°C. Then, the cells were harvested by centrifugation and washed thoroughly with sterilized phosphate buffered saline. The washed cell suspension was inoculated into 1 L of either MRS or a chemically defined medium, to which 1% (w/v) glucose was added as a sole carbon source (CDMGlc), yielding optical densities of 0.2 at 600 nm. After aerobic cultivation for 48 h at 30°C, in each case the cells were removed by centrifugation (17,000 \(g\), 1 h, 4°C). EPS-MRS or EPS-CDMGlc secreted into the supernatant, was precipitated by the addition of an equal volume of ice-cold ethanol, collected by centrifugation (17,000g, 1 h, 4°C). EPS-MRS or EPS-CDMGlc was dialyzed overnight against pure water at 4°C, and then lyophilized. The lyophilized EPSs were dissolved in 10 ml 50 mM Tris–HCl (pH 8.7) and purified by a batch method using a 20-ml slurry of DEAE A-50 equilibrated with the same buf-

Figure 3. 1D \(^1\)H NMR and 2D HSQC, TOCSY (mixing time, 200 ms), and NOESY (mixing time, 200 ms) spectra of EPS₅-MRS, recorded in D₂O at 334 K. In the 2D spectra, relevant cross-peaks are included (see text). HSQC: A₁ refers to a cross-peak between A H-1 and A C-1, etc. TOCSY: A₁ corresponds to the diagonal peak of residue A H-1; A₂ refers to a cross-peak between A H-1 and A H-2, etc. NOESY: A₁ corresponds to the diagonal peak of residue A H-1; A₁-D₃ (encircled) refers to an inter-residue cross-peak between A H-1 and D H-3; A₂ refers to an intra-residue cross-peak between A H-1 and A H-2, etc.
Figure 4. Schematic presentation of the observed $^1$H intra- (blue) and inter-residue (red) NOE connectivities (see Table 3) in a part of the *Lb. fermentum* TDS030603 EPS structure, including the repeating unit.

Figure 5. 500-MHz 1D $^1$H NMR spectrum of the EPS-derived disaccharide $\alpha$-D-Glc-(1→6)-$\alpha$-Galp, recorded in D$_2$O at 334 K. The HOD signal at 4.40 ppm was suppressed.

Table 4

<table>
<thead>
<tr>
<th>Residue</th>
<th>H-1</th>
<th>H-2</th>
<th>H-3</th>
<th>H-4</th>
<th>H-5</th>
<th>H-6a</th>
<th>H-6b</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-Glc-(1→6)-</td>
<td>4.943</td>
<td>1.57</td>
<td>3.72</td>
<td>3.42</td>
<td>3.74</td>
<td>3.86</td>
<td>3.76</td>
</tr>
<tr>
<td>$\alpha$-Glc-(1→6)-</td>
<td>99.1</td>
<td>72.2</td>
<td>73.9</td>
<td>70.4</td>
<td>72.6</td>
<td>61.4</td>
<td>61.1</td>
</tr>
<tr>
<td>$\beta$-Gal</td>
<td>5.254</td>
<td>3.81</td>
<td>3.84</td>
<td>4.03</td>
<td>4.259</td>
<td>3.89</td>
<td>3.72</td>
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<tr>
<td>$\beta$-Gal</td>
<td>93.3</td>
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<td>69.8</td>
<td>70.3</td>
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<tr>
<td>$\beta$-Gal</td>
<td>87.3</td>
<td>72.7</td>
<td>73.5</td>
<td>69.6</td>
<td>73.9</td>
<td>77.4</td>
<td></td>
</tr>
</tbody>
</table>

* In ppm relative to the signal of internal acetone at $\delta$ 2.225 for $^1$H and at $\delta$ 31.08 for $^{13}$C.

b Ring carbons involved in linkages are indicated in gray.
fer. The non-adsorbed fractions were collected, thoroughly dialyzed against pure water, and lyophilized. **EPS-MRS** was further purified on a column of Toyopearl HW-55F (2.6 x 100 cm, 15 mL/h, pure water), dialyzed against pure water, and lyophilized. Both EPSs were stored in a desiccator until use.

### 4.2. Chemical analyses

Monosaccharide analyses, absolute configuration determinations, methylation analyses of partially hydrolyzed (0.5 M TFA; 5 min, 100 °C) polysaccharide samples, and periodate oxidation of **EPS-MRS** were carried out as described previously.**

### 4.3. Mild partial acid hydrolysis of EPS-MRS

Polysaccharide samples (5 mg/mL) were treated with 0.5 M trifluoroacetic acid (TFA) for 5 min at 100 °C, and then the solutions were concentrated to dryness under a stream of nitrogen. The resulting products were dissolved in water and lyophilized.

### 4.4. Preparation of oligosaccharide fragments from EPS-MRS

A polysaccharide sample (~20 mg/2 mL) was treated with 0.5 M TFA for 20 min at 100 °C. After evaporation of the solution to dryness under a stream of nitrogen, the generated mixture of oligosaccharides was dissolved in water and lyophilized. The residue, dissolved in 2 mL water, was fractionated on a Bio-Gel P-2 column (94 cm x 1 cm), eluted with 10 mM ammonium bicarbonate at a flow rate of 13 mL/h. Fractions of 10 min were collected and checked for carbohydrate by an orcinol/H2SO4 spot test and for oligosaccharide mass distribution by matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

### 4.5. MALDI-TOF mass spectrometry

MALDI-TOF-MS, in positive-ion mode, of oligosaccharide materials was performed on an AXIMA™ mass spectrometer (Shimadzu Kratos Inc., Manchester, UK), equipped with a nitrogen laser (337 nm, 3 ns pulse width). Samples were prepared by mixing on the target 0.5 μL oligosaccharide solution with 0.5 μL 2.5-dihydroxybenzoic acid (10 mg/mL) in 50% aqueous acetonitrile as matrix solution.

### 4.6. NMR spectroscopy

Resolution-enhanced 1D/2D NMR spectra were recorded in D2O on a Bruker DRX-500 spectrometer (Bijvoet Center, NMR Spectroscopy, Utrecht University) at a probe temperature of 334 K. Before analysis, samples were exchanged twice in D2O (99.9 atom% D, Cambridge Isotope Laboratories, Inc., Andover, MA) with intermediate lyophilization, and then dissolved in 0.6 mL D2O. Suppression of the HOD signal was achieved by applying a WEFT pulse sequence for 1D experiments and by a pre-saturation of 1 s during the relaxation delay for 2D experiments. The 2D TOCSY spectra were recorded using an MLEV-17 mixing sequence with spin-lock times of 20–200 ms. The 2D NOESY spectra were recorded using standard Bruker XWINNMR software with mixing time of 200 ms. The carrier set was set at the downfield edge of the spectrum in order to minimize TOCSY transfer during spin-locking. Natural abundance 2D 13C–1H HSQC spectra (1H frequency 500.0821 MHz, 13C frequency 125.7552 MHz) were recorded without decoupling during acquisition of the 1H FID. Resolution enhancement of the spectra was performed by a Lorentzian-to-Gaussian transformation for 1D spectra or by multi-plication with a squared-ball function phase shifted by π/(2.3) for 2D spectra, and when necessary, a fifth order polynomial baseline correction was performed. Chemical shifts (δ) are expressed in ppm by reference to internal acetone (δ 2.225 for 1H and δ 31.08 for 13C).

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### References