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Substituent distribution in highly branched dextrins from methylated starches

Yuri E.M. van der Burgt [a], Jack Bergsma [b], Ido P. Bleeker [b], Paul J.H.C. Mijland [b], Johannis P. Kamerling [a,*], Johannes F.G. Vliegenthart [a]

[a] *Department of Bio-Organic Chemistry, Bijvoet Center, Utrecht University, PO Box 80.075, NL-3508 TB Utrecht, The Netherlands*

[b] *AVEBE Research and Development, AVEBE-weg 1, NL-9607 PT Foxhol, The Netherlands*

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Abstract

Granular potato starch and amylopectin potato starch were methylated to molar substitutions (MS) up to 0.29. Extensive alpha-amylase digestion gave mixtures of partially methylated oligomers. Precipitation of larger fragments by methanol yielded mainly α-limit dextrins (84–99%). Methanol precipitates were extensively digested with beta-amylase yielding α,β-limit dextrins. The average substitution level of branched glucose residues in the dextrins thus obtained was determined after per deuteriomethylation by using FAB mass spectrometry, and compared with that of the linearly linked glucose residues. The present work demonstrates that methylation does not show any preference for substitution at either branched or linearly linked glucose residues, taking into account the inherently lower amount of substitution sites at branched residues. The results corroborate earlier studies wherein it was found that substituents in branched regions are distributed almost randomly. In addition, the data enable the determination of the average degree of branching of partially methylated dextrins.

Keywords: Methylated starches; Amylopectin potato starch; Branching; Amylolysis; Substituent distribution

1. Introduction

Starch, the main energy reserve of higher plants, consists of amylose and amylopectin. Amylose is considered as an essentially (1→4)-linked α-D-glucan, whereas amylopectin contains up to 5% of branched α-D-glucose residues. The short amylopectin side chains are (1→6)-linked to longer chains [1] and arranged in double helices, building up the organised crystalline framework of the starch granule [2,3]. Molecular mass distribution, amylose and amylopectin content, and the degree of branching of these molecules depend on the botanical source of the starch granules [1,4,5].

Chemical modifications of starches provide starch products that fulfil various demands [6]. Detailed information on the distribution of substituents can contribute to the understanding of relations between molecular structure and functional properties, thus opening ways to more-rational derivatisation processes. Previously, we reported that in methylated starches, crystalline linear amylopectin side chains, which play an important role in the retrogradation of gelatinised starches, contain fewer substituents than amorphous branched parts [7,8]. The aim of the present study is to

* Corresponding author. Tel.: +31-30-2533479/2184; fax: +31-30-2540980.

E-mail address: kame@boc.chem.uu.nl (J.P. Kamerling).

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examine the substitution pattern in branched regions of methylated starches in more detail to determine whether preferences exist for substitution sites at branched glucose residues.

2. Results and discussion

Preparation of methylated starches.—Methylated potato starches (**P**) and methylated amylopectin potato starches (**A**) were prepared by methylation of starch granules in an alkaline aqueous suspension using dimethyl sulfate [8]. The molar substitution (MS) values of the starch derivatives studied are listed in Table 1. The MS values and the monosaccharide compositions of the intact granules of **P10**, **P20**, **P30**, **A10**, **A20** and **A30** were determined by using GLC and have been reported previously [7].

Preparation of α,β-dextrins from methylated starches.—Gelatinised methylated starches **P10**–**P30** and **A10**–**A30** were extensively digested with alpha-amylase from *Bacillus subtilis* [9], yielding mixtures of (1→4)-α-D-glucans of different sizes with varying degrees of (1→6) branching (DB). The so-called α-limit dextrins, which are highly branched and have a degree of polymerisation (DP) > 8 (as determined by high-performance liquid chromatography (HPLC) [7]), were separated from the linear oligomers by precipitation with methanol. The MS values of the α-limit dextrins were determined after quantifying the amount of cross contamination during methanol precipitation (Table 1) [7]. The methanol precipitates thus obtained (further referred to as α-dextrin fractions) of **P10**–**P30** and **A10**–**A30**, mainly containing α-limit dextrins (84–99%) [7], were extensively digested with β-amylase from *Bacillus cereus*, yielding α,β-limit dextrins and small oligomers [9,10]. For each sample, the DP decreased during β-amylolysis, as could be demonstrated by ^{1}H NMR spectroscopy [11,12]. The α,β-limit dextrins obtained from **P10**–**P30** and **A16**–**A30** were separated from liberated maltose by using Bio-Gel P-2 chromatography. Small (1→4)-linked oligomers with or without single (1→6) branching, originating from cross contamination during the methanol precipitation [7] and with DP up to 4, as determined by MALDI-TOF mass spectrometry, co-eluted with the maltose fraction. Determination of the substitution level by using monosaccharide analysis shows that the MS values of the α,β-limit dextrins are higher than those of the corresponding α-limit dextrins (Table 1). This can be rationalised from the mode of action of beta-amylase, because the binding of the maltosyl groups that are subsequently cleaved will be sterically hindered by the presence of methyl substituents. Not only branching points stop the digestion, but also substituted glucose residues. Since predominantly non-substituted maltose is released

Table 1
MS values of α-limit dextrins and α,β-limit dextrins from **P10**–**P30** and **A10**–**A30**

Samples	Code	MS [a] (granule)	MS (α-limit dextrins)	MS (α,β-limit dextrins)	ΔMS [b] (%)	MS [c] ('α,β-limit dextrins')
Methylated potato starch	**P10**	0.103	0.142	0.179	26.1	0.20–0.24
Methylated potato starch	**P20**	0.211	0.287	0.314	9.4	0.30–0.31
Methylated potato starch	**P30**	0.296	0.380	0.415	9.2	0.35–0.38
Methylated amylopectin potato starch	**A10**	0.097	0.172	0.198	15.1	0.22–0.25
Methylated amylopectin potato starch	**A20**	0.191	0.287	0.327	13.9	0.33–0.34
Methylated amylopectin potato starch	**A30**	0.293	0.421	0.449	6.7	0.33–0.37

[a] Molar substitution (MS) is defined as mole of substituents/mole of glucose residues. MS varies from 0 (native starch) to 3 (permethylated linear starch). All MS values are determined by using GLC in triplo ($s < 0.01$).

[b] ΔMS = [MS (α,β-limit dextrins) − MS (α-limit dextrins)/MS (α-limit dextrins)] × 100%.

[c] Calculated MS of virtual α,β-limit dextrins using Eq. (1), with $0 < MS_{terminal} < 0.15$.

from the α-limit dextrins, the α,β-limit dextrins will have higher MS values. As can be seen from Table 1, the relative increases in MS values (ΔMS) are larger for the lower substituted α-limit dextrins. This observation is in agreement with the steric hindrance of beta-amylase already mentioned, which is expected to increase with the MS. The P-2 maltose fraction contained small amounts of methylated glucans, probably originating from the earlier mentioned cross contamination. However, from these data it cannot be excluded that beta-amylase also liberates maltose groups of low methylation level. In conclusion, β-amylolysis of α-limit dextrins from **P10**–**P30** and **A10**–**A30** results in a relative enrichment of partially methylated glucose residues in the generated α,β-limit dextrins as compared with the α-limit dextrins.

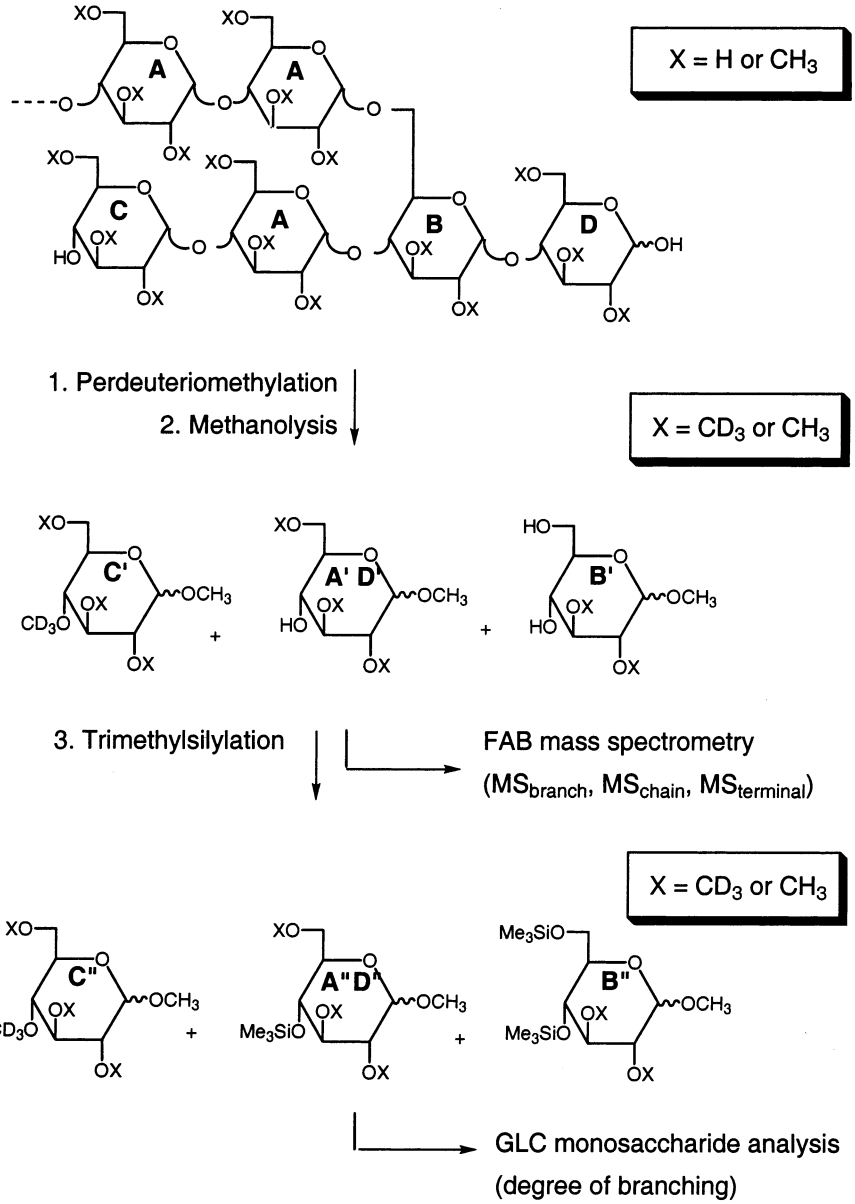
FAB mass spectrometric determination of average substitution levels of constituting residues in per(deuterio)methylated α,β-limit dextrins.—The α-dextrin fractions (mainly containing α-limit dextrins) and the α,β-limit dextrins obtained from **P10**–**P30** and **A10**–**A30** were perdeuteriomethylated with CD_3I [13], then methanolysed with methanolic HCl [14,15]. This derivatisation procedure yielded 12 mixtures all containing the three chemically different methyl glucosides (Scheme 1), namely per(deuterio)methylated methyl glucosides (**C**′), (deuterio)methylated methyl glucosides having HO-4 free (**A**′ and **D**′), and (deuterio)methylated methyl glucosides having HO-4 and HO-6 free (**B**′).

Each mixture of three chemically different methyl glucosides (Scheme 1) was analysed by FABMS. This resulted in three sets of sodium-cationised pseudomolecular ions in the FAB spectrum. The first set had the main peak at *m*/*z* 251 (**B**′, with $X = CD_3$), the second at *m*/*z* 268 (**A**′/**D**′ with $X = CD_3$), and the third at *m*/*z* 285 (**C**′, with $X = CD_3$). Each set contains three pseudomolecular ions having zero, one, or two CH_3 groups. Within each set, all three compounds show the same ionisation efficiency because they are chemically identical. Therefore, their FAB intensities represent the amount of each compound present in one set. Note that the intensities of the pseudomolecular ions of the three different sets of methyl glucosides (**B**′, **A**′/**D**′, and **C**′) cannot be compared with each other, because of their chemical inequivalency. FAB spectra of mixtures derived from **A10**–**A30** are shown in Fig. 1.

The sodium-cationised pseudomolecular ion at *m*/*z* 251 and its satellite ions at *m*/*z* 248 and 245 (trace amounts) originate from residues linked at O-1, O-4, and O-6 (**B**′ in Scheme 1, with $X = H$ or CH_3). Their peak intensities correspond to the amounts of non-, mono-, and disubstituted residues, respectively. From these data the average substitution level of glucose residues at branching points (MS_{branch}) can be calculated. The sodium-cationised pseudomolecular ion at *m*/*z* 268 and its satellite ions at *m*/*z* 265 and 262 originate from residues linked at O-1 and O-4, or the reducing end (**A**′ and **D**′ in Scheme 1, respectively, with $X = H$ or CH_3). Using the ratios of the peak intensities, the average substitution level of glucose residues in linear $(1\rightarrow4)$-linked chains (MS_{chain}) can be determined. The results are summarised in Table 2.

In branched glucose residues HO-6 is not available for substitution. Therefore, MS_{branch} is corrected (resulting in $MS_{branch,cor}$) by assuming that HO-6 of branched glucose residues is methylated in similar amounts as HO-6 of linearly linked glucose residues (taken from monosaccharide analysis of α,β-limit dextrins). Interestingly, the corrected substitution levels of branched glucose residues ($MS_{branch,cor}$) differ only slightly from those of linearly linked glucose residues (MS_{chain}) (see Table 2). Therefore, no significant preference for substitution at either branched or linearly linked glucose residues can be concluded. In an earlier report we have shown that methyl substituents are distributed almost randomly in branched regions of methylated starches (α-limit dextrins) [7]. The results shown here are in good agreement with a random distribution of methyl substituents over branched regions during methylation.

The sodium-cationised pseudomolecular ions at *m*/*z* 285 and the satellite ions at *m*/*z* 282 and 279 (trace amounts) originate from residues linked at O-1 only (**C**′ in Scheme 1, with $X = H$ or CH_3). Since the amounts of mono- and disubstituted residues are too low for quantification, in all α,β-limit dextrins the



Scheme 1. Methodology for the analysis of methylation at branching points in α-dextrin fractions and in α,β-limit dextrins obtained from **P10**–**P30** and **A10**–**A30**, and for the determination of the degree of branching (DB).

percentage of non-substituted glucose residues at the non-reducing end must be higher than 90%. Therefore, the average substitution level of terminal glucose residues is lower than 0.15 ($MS_{terminal} < 0.15$). This implies that alpha- and beta-amylase both have a diminished ability for hydrolysis of the glycosidic linkages of saccharides in which the terminal non-reducing glucosyl group is methylated.

Determination of degree of branching.—The degree of branching (DB) of hydrolysates of methylated starches cannot be determined by 1D ^{1}H NMR spectroscopy because of overlap of (partially) methylated →4)-Glc-(1→6)- residues with non-substituted →4)-Glc-(1→ residues, as is evident from Fig. 2. However, the DB may be determined by using GLC. For this purpose, each mixture of methyl glucosides obtained after perdeuteriomethylation and methanolysis of α,β-limit dextrins from **P10**–**P30** and **A10**–**A30** was trimethylsilylated and quantitatively analysed by GLC, using empirically determined molar response factors [16,17] (Fig. 3). In this way, the ratio for the residues **B**″, **C**″, and **A**″ + **D**″ (Scheme 1, with X = H or CH_3) was determined. From this the

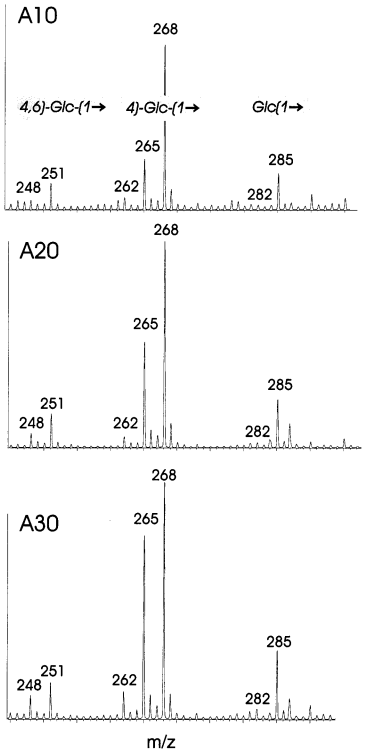


Fig. 1. FAB spectra of sodium-cationised methyl glucosides obtained from **A10**, **A20**, and **A30** via perdeuteriomethylation and methanolysis. The original linkage of the methyl glucosides is indicated for **A10**, the corresponding structures are given in Scheme 1.

DB could be derived (multiplied by 100% then gives the percentage of branched glucose residues present in the original polymer). The same was done for the α-dextrin fractions from **P10**–**P30** and **A10**–**A30**. The average DB values are given in Table 3.

From Table 3 it is clear that the DB values

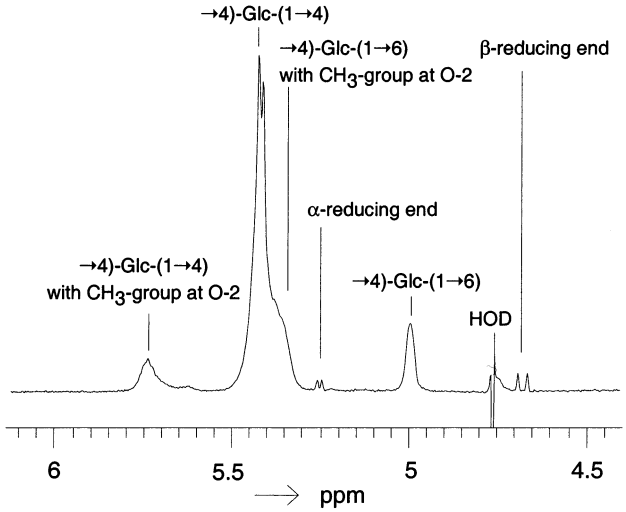


Fig. 2. ^{1}H NMR spectrum of the methanol precipitate of **A20** (only signals from anomeric protons are shown).

of all α-dextrin fractions increase after β-amylolysis. However, this increase shows no correlation with the MS. Using MS_{branch} and MS_{chain} from Table 2, and DB values from Table 3, the MS values of 'α,β-limit dextrins' (reconstructed virtual α,β-limit dextrins) were calculated according to Eq. (1). MS values thus obtained are summarised in Table 1.

$$\text{MS ('}\alpha,\beta\text{-limit dextrins')} = \text{DB} \times MS_{branch} + \text{DB} \times MS_{terminal} + [100 - (2 \times \text{DB})] \times MS_{chain} \quad (1)$$

The substitution level of terminal glucose residues ($MS_{terminal}$) is estimated to be between 0 and 0.15 (see above). As may be seen in

Table 2
Substitution levels of residues originally linked at O-1, O-4, and O-6, and of residues originally linked at O-1 and O-4

Sample	MS_{branch} values (residues linked at O-1, O-4, and O-6) [a]	MS contribution of 6-O-substituted residues [b]	$MS_{branch,cor}$ values (residues linked at O-1, O-4, and O-6) [c]	MS_{chain} values (residues linked at O-1 and O-4)	ΔMS [d] (%)
P10	0.239	0.022	0.261	0.287	10.0
P20	0.297	0.037	0.334	0.318	−4.8
P30	0.373	0.051	0.424	0.450	6.1
A10	0.265	0.029	0.294	0.277	−5.8
A20	0.298	0.057	0.355	0.367	3.4
A30	0.397	0.055	0.452	0.471	4.2

[a] A third decimal is included for clarity. However, the accuracy of the measurements justifies two decimals only.

[b] Positional molar substitution of 6-position (from monosaccharide analysis, counting one substituent of 6-*O*-methyl-, 2,6-*O*-dimethyl-, 3,6-*O*-dimethyl-, and 2,3,6-*O*-trimethyl methyl glucoside each).

[c] Calculated by adding the MS contribution of 6-*O*-substituted residues to MS_{branch}.

[d] $\Delta MS_{chain} = [MS_{chain} - MS_{branch,cor}/MS_{branch,cor}] \times 100\%$.

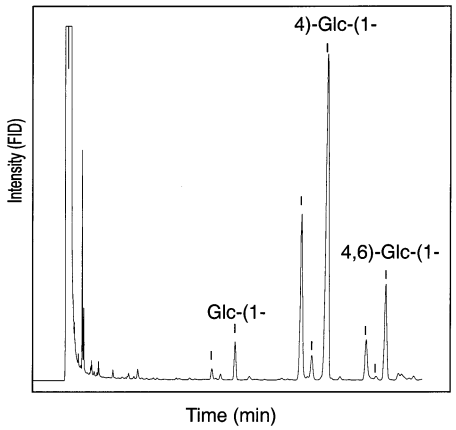


Fig. 3. Gas chromatogram of a mixture of trimethylsilylated methyl glucosides (from α,β-limit dextrins of **A10**). The labelled peaks are of interest. The first two peaks correspond to per(deuterio)methylated methyl glucosides [originating from residues linked at O-1 only (**C**″, α/β-pyranose)], the next three with monosilylated (deuterio)methylated methyl glucosides [originating from residues linked at O-1 and O-4 (**A**″, α/β-pyranose/furanose)] and the last three with disilylated (deuterio)methylated methyl glucosides [originating from residues linked at O-1, O-4, and O-6 (**B**″, α/β-pyranose/furanose)].

Table 1, the measured MS value of an α,β-limit dextrin differs significantly from the one of a reconstructed virtual α,β-limit dextrin. This is mainly due to the inaccurate value of $MS_{terminal}$. To obtain a more specific value of $MS_{terminal}$, detailed information is needed on which partially methylated maltose fragments are tolerated by beta-amylase.

3. Materials and methods

Preparation of α,β-limit dextrins from methylated starches.—Potato-starch granules and amylopectin potato-starch granules were methylated in an alkaline aqueous suspension by using Me_2SO_4 (see Table 1). The methylated starches obtained, **P10**–**P30** and **A10**–**A30** were gelatinised and extensively digested with alpha-amylase from *Bacillus subtilis* [9] (BAN 240L from Novo). Oligomers with DP > 8 precipitated by adding MeOH (5 vol equiv) [7]. The MeOH precipitates contained mainly α-limit dextrins and were extensively digested with beta-amylase from *Bacillus cereus* (Amano Pharmaceutical, Japan) in a shaking waterbath at 55 °C and pH 5.5, using 0.5 mass% of enzyme. α,β-Limit dextrins were isolated after separation from smaller oligomers (DP < 5) by using Bio-Gel P-2 column chromatography.

Analytical procedures.—Monosaccharide analysis was carried out by subjecting partially methylated oligomer or glucan samples to methanolysis (methanolic 1 M HCl, 18 h, 85 °C). The resulting mixtures of methyl glucoside derivatives were trimethylsilylated (1:1:5 hexamethyldisilazane–chlorotrimethylsilane–pyridine), identified by GLC–mass spectrometry, and quantified by GLC using empirical molar-response factors [7]. GLC analyses were performed on a WCOT CP-SIL 5CB fused-silica capillary column (25 m × 0.32 mm) by using a temperature program of 110–230 °C at 4°C/min and maintaining at 230 °C for 5 min. GLC–mass spectrometry of (*O*-methylated) glucose derivatives, measured as trimethylsilylated methyl glucosides, was carried out on an MD800/8060 system (Fisons instruments; electron energy, 70 eV), equipped with a DB-1 fused-silica capillary column (30

Table 3
Degrees of branching of α-dextrin fractions and α,β-limit dextrins from **P10**–**P30** and **A10**–**A30**

Sample	DB [a] (α-dextrin fractions)	DB [a] (α,β-limit dextrins)	ΔDB [b] (%)
P10	11.9	14.8	24.4
P20	11.7	12.5	6.8
P30	9.7	11.5	18.6
A10	11.8	14.2	20.3
A20	11.4	12.3	7.9
A30	8.7	10.9	25.3

[a] The degree of branching (DB) is defined as the percentage of glucose residues that are branched (calculated by dividing the amount of residues linked at O-1, O-4 and O-6 by the total amount of methyl glucosides in dextrin multiplied by 100%).

[b] ΔDB = [DB (α,β-limit dextrins) − DB (α-dextrin fractions)/DB (α-dextrin fractions)] × 100%.

m × 0.32 mm, J&W Scientific), using a temperature program of 110–230 °C at 4 °C/min and maintaining at 230 °C for 5 min.

Partially methylated oligomers (α-limit dextrins obtained after α-amylolysis of **P10**, **P20**, **P30**, **A10**, **A20**, and **A30** and α,β-limit dextrins obtained after β-amylolysis of α-dextrin fractions) were permethylated with CD_3I, as described previously (Me_2SO–NaOH) [13]. After methanolysis (methanolic 1 M HCl, 18 h, 85 °C) the methyl glucosides were analysed as such by FAB mass spectrometry; for gas-chromatographic purposes (determination of DB) the methyl glucosides were trimethylsilylated as already described before injection. FAB mass spectrometry was performed on a Jeol JMS SX/SX 102A four-sector mass spectrometer, operated at 10 kV accelerating voltage, equipped with a Jeol MS-FAB 10 D FAB gun operated at a 10 mA emission current, producing a beam of 6 keV xenon atoms. The per(deuterio)methylated oligosaccharide samples were measured over a mass range of m/z 10–1200 in a matrix of *m*-nitrobenzyl alcohol saturated with NaI, using the standard resolution of 1500. Prior to NMR analysis, samples were exchanged once in D_2O (99.9 atom% D, Isotec), then lyophilised and dissolved in 99.96 atom% D_2O (Isotec). 1D ^1H NMR spectra were recorded on a Bruker AC-300 spectrometer (Department of Organic Chemistry, Utrecht University), equipped with a 5 mm broad-band probe, at a probe temperature of 27 °C. Chemical shifts are expressed in ppm downfield from the signal for external Me_4Si, but were actually measured by reference to external acetone (δ 2.225). The data were collected in 16 K complex data sets and zero-filled to 32 K. After Fourier transformation, using a Gaussian multiplication, the spectra were integrated with Bruker software.

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