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Distribution of methyl substituents over branched and linear regions in methylated starches

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Abstract

Granular potato starch and amylopectin potato starch were methylated in aqueous suspension to molar substitutions (MS) up to 0.29. A method was developed to determine the MS of both branched and linear regions. After exhaustive digestion of the methylated starches with α-amylase, the highly branched fraction with a degree of polymerisation (DP) > 8 was separated from the linear oligomers by selective precipitation of the former in methanol. The substitution levels of branched and linear regions were determined. It was found that methylation takes place preferably at the branched regions of amylopectin and that amylose is higher substituted than linear regions of amylopectin. The distribution of methyl substituents in trimers and tetramers was determined by FABMS and compared to the outcome of a statistically random distribution. The results provided evidence for heterogeneous substituent distributions. Quantification of the degree of heterogeneity of the branched and linear regions showed a much larger deviation from random distribution in the linear regions.

Keywords: Methylated starches; Amylopectin potato starch

1. Introduction

Starch is widely used as a renewable, raw material in products, that are applied in the food, paper, textile, adhesives, plastic, pharmaceutical and petrochemical industries. It can be isolated from several plants and is composed of the α-D-glucopyranans amylose and amylopectin. Amylose is essentially linear with (1→4) linkages, whereas amylopectin is branched due to additional (1→6) linkages. For many applications, starch is modified

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to improve its functionality. This can be done enzymatically, physically or chemically. In the chemical derivatisation of starch the hydroxyl groups are partially substituted [1]. Although such processes have been carried out on an industrial scale for over 50 years, the information on topochemical aspects is scarce. Detailed information on the distribution of substituents can contribute to the understanding of relationships between molecular structure and functional properties, thus opening ways to 'tailored derivatives' by selecting derivatisation procedures. To acquire structural data about starch derivatives, different levels of organisation in terms of substituent distribution have to be studied, namely, branched and linear regions, crystalline and amorphous domains, amylose and amylopectin, and distribution in granules.

The aim of this study is the determination of substitution level and substituent distribution in branched and linear regions of methylated starches. This aspect is of interest since functional properties of starch derivatives such as pasting temperature, gelatinisation and cooking characteristics, solids–viscosity relationships and retrogradation tendencies are determined by the type of substituent, the level of substitution and topochemical aspects. For example, retrogradation of gelatinised starches is amongst other aspects determined by interactions between linear starch chains. In particular, the long-chain amylose molecules play a predominant role in this process. Chemical derivatisation is essential for many applications to avoid retrogradation. In this context the chemical derivatisation of linear starch regions of both amylopectin and amylose is of importance.

Here, we report a method for the separation of mixtures of branched and linear regions, obtained from methylated potato starch and methylated amylopectin potato starch after α-amylolysis [2]. Furthermore, the isolated branched and linear regions were studied with regard to substitution level and distribution of substituents.

2. Results and discussion

Monomer compositions of methylated starches.—Methylated potato starch and methylated amylopectin potato starch samples were prepared by methylation of starch granules in an aqueous suspension using dimethyl sulfate. The various starch derivatives **P4**–**P30** and **A4**–**A30** with varying degrees of methylation are listed in Table 1. The molar substitution (MS) values were determined by the Morgan–Zeisel method [3] and via a monosaccharide analysis [4]. The Morgan–Zeisel assay is based on ether-cleavage with hydrogen iodide resulting in the formation of methyl iodide, which is led by a CO_2-flow into a trap of a Ag(I)–methanol solution; afterwards the remaining Ag(I) is determined. For the monosaccharide analysis, the samples were subjected to methanolysis and trimethylsilylation yielding seven (partially methylated) methyl glucoside monomers (methyl 2,3,6-tri-*O*-methylglucoside was only found in trace amounts and therefore neglected). The monomers were characterised by GLC-mass spectrometry and quantified by GLC (Table 2). The quantitative data were used for the calculation of the MS values of all methylated starches. As is evident from Table 1 both methods provide similar results.

Using the monomer compositions of **P4**–**P30** and **A4**–**A30** (Table 2), statistical distributions of the methyl substituents over the eight theoretically possible monomers were calculated, assuming constant and independent reactivities of HO-2, HO-3 and HO-6 [5]. For all methylated starches more non- and disubstituted monomers and less monosubstituted monomers were observed than expected on the basis of a statistically random distribution (defined as homogeneous [5]). This can

Table 1
Methylated potato starch and methylated amylopectin potato starch

Samples	Code	MS[a] (Morgan–Zeisel)	MS[a] (GLC)
Methylated potato starch	**P4**	0.042	0.044
Methylated potato starch	**P6**	0.057	0.063
Methylated potato starch	**P8**	0.080	0.076
Methylated potato starch	**P10**	0.102	0.103
Methylated potato starch	**P20**	0.202	0.211
Methylated potato starch	**P30**	0.270	0.296
Methylated amylopectin potato starch	**A4**	0.038	0.048
Methylated amylopectin potato starch	**A6**	0.066	0.067
Methylated amylopectin potato starch	**A8**	0.078	0.089
Methylated amylopectin potato starch	**A10**	0.097	0.097
Methylated amylopectin potato starch	**A20**	0.191	0.191
Methylated amylopectin potato starch	**A30**	0.290	0.293

[a]The molar substitution (MS) is defined as mol of substituents/mol of Glc monomer. The MS varies from 0 (native starch) to 3 (permethylated linear starch).

Table 2
Monomer compositions of methylated potato starch (**P4**–**P30**) and methylated amylopectin potato starch (**A4**–**A30**)

Samples	Glc[a]	Glc2 Me	Glc3 Me	Glc6 Me	Glc2,3 Me_2	Glc2,6 Me_2	Glc3,6 Me_2
P4	95.7	2.5	0.9	0.8	0	0.1	0
P6	93.9	3.8	1.2	0.9	0.1	0.1	0
P8	92.6	4.7	1.4	1.1	0.1	0.1	0
P10	90.1	6.3	1.9	1.3	0.2	0.2	0
P20	80.7	12.2	3.3	2.0	0.9	0.7	0.2
P30	73.7	16.4	4.2	2.4	1.7	1.3	0.3
A4	95.4	2.7	0.9	0.8	0.1	0.1	0
A6	93.5	4.1	1.3	0.9	0.1	0.1	0
A8	91.5	5.3	1.7	1.1	0.2	0.2	0
A10	90.7	5.9	1.8	1.2	0.2	0.2	0
A20	82.5	11.0	3.1	1.8	0.8	0.6	0.2
A30	74.0	16.2	4.2	2.3	1.8	1.2	0.3

[a]All monomers are characterised as trimethylsilylated methyl glucopyranosides ($\alpha + \beta$) and are given in molar percentages.

be explained by assuming that some areas in a starch granule are better accessible during derivatisation than others, resulting in a heterogeneous substituent distribution along the polymer. However, other parameters could be responsible for this difference between observed and expected monomer compositions, such as vicinal substituent effects and variable reactivities of hydroxyl groups during methylation. Therefore, heterogeneity in the distribution of substituents should be determined in oligomer fragments.

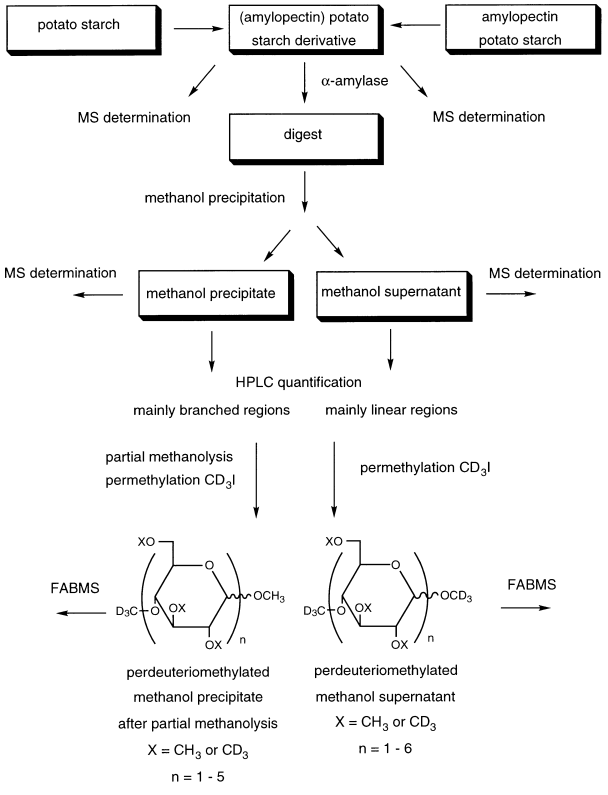
As is clear from Table 2, in all methylated starches the HO-2 group had by far the highest positional degree of substitution. This preference, with respect to the position in glucose, is independent of the MS. It means that topochemical effects could not be demonstrated at the level of monomers, as was already reported [6].

Methodology for the analysis of branched and linear regions.—All steps in the separation of branched and linear regions of methylated starches and the characterisations thereof are depicted in Scheme 1.

To determine the average degree of polymerisation (DP) and degree of branching (DB) of mixtures of α-D-glucopyranans ^{1}H NMR spectroscopy was used. The DP is calculated by dividing the integral of the anomeric proton signals by the sum of the integrals of the H-1α and H-1β signals of the glucose unit at the reducing end [7–9]. The DB is estimated by dividing the integral of the anomeric proton signals by the integral of the signal of (1→6)-linked Glc monomers. Note that thus obtained DB values are higher than the actual degree of branching, because 2-*O*-methylated (1→6)-linked Glc monomers can not be taken into account due to overlap of signals with those of nonsubstituted (1→4)-linked Glc monomers.

Characterisation of products obtained by α-amylolysis of methylated starches.—The methylated gelatinised starches **P4**–**P30** and **A4**–**A30** were extensively digested with α-amylase from *Bacillus subtilis* [2], yielding mixtures of (1→4)-α-D-glucopyranans of different sizes with varying degrees of branching. The dextrose equivalents (DE) of the α-amylase digests were determined by the method of Luff–Schoorl [10,11]. The underlying principle is based on oxidation of all reducing sugars with Cu(II). For each sample the average degree of polymerisation (DP) was calculated from the DE value [12]. With increasing MS of the starting materials the average DP values of the digests after α-amylolysis increase (Fig. 1). Apparently, methylation of hydroxyl groups decreases the ability for binding of α-amylase at subsites, thus limiting the number of (1→4) linkages that can be cleaved.

For the investigation of the possible occurrence of partially methylated reducing Glc units in the



Scheme 1. Methodology for the analysis of branched and linear regions in **P4**–**P30** and A4-A30.

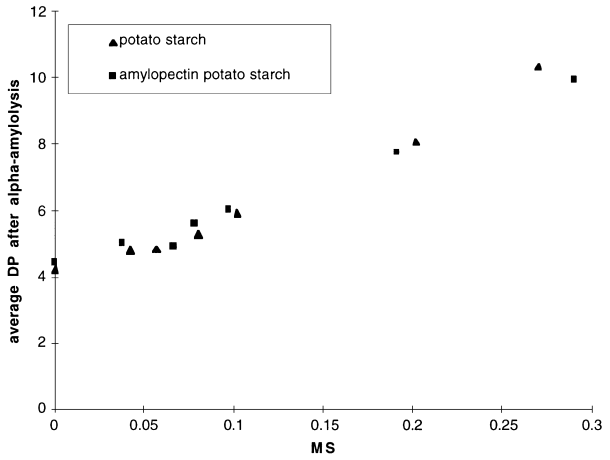


Fig. 1. Average DP values of digests of the starting methylated starches after α-amylolysis versus MS. The MS values were measured by Morgan–Zeisel [3] (Table 1) and the DP values via Luff–Schoorl [10,11]. Native potato starch and amylopectin potato starch (MS = 0) were used as references.

α-amylase digests of the methylated starches with the highest MS values (**P20**, **P30**, **A20** and **A30**), generated oligosaccharides were converted into their corresponding oligosaccharide alditols [13]. Then, the mixture of reduced oligomers obtained was hydrolysed, the liberated (partially methylated) glucose residues were converted into aldononitriles [4,14,15], and the mixtures of glucitols and gluconononitriles were analysed by GLC-mass spectrometry after acetylation. It was found that methyl substituents were present mainly in the gluconononitriles; only a trace of 6-*O*-methylglucitol was found. It is therefore concluded that α-amylolysis hardly occurs at methylated glucose sites; only 6-*O*-methylation is tolerated.

Separation of branched and linear starch regions.—In order to analyse the substitution level of branched and linear starch regions, the glucan mixtures obtained after α-amylolysis of methylated starches were treated with methanol, yielding in each case a precipitate and a supernatant (Scheme 1). ^{1}H NMR spectroscopy of the precipitate showed the presence of mainly large highly branched D-Glc oligomers, whereas the supernatant contained only smaller (1→4)-linked D-Glc oligomers, hardly containing (1→6) linkages. The presence of highly branched oligomers in the methanol precipitate was further supported by ^{1}H NMR studies after incubation with the debranching enzyme pullulanase [2].

It should be noted, however, that precipitation of the larger glucans in methanol is not fully selective, nor is it complete. As a result, the methanol precipitate is 'contaminated' with low amounts of small oligomers, whereas the supernatant is 'contaminated' with substantial amounts of large oligomers. To analyse this cross-contamination the methanol precipitates and the methanol supernatants of **P4**–**P30** and **A4**–**A30**, respectively, were subjected to HPLC. As a typical example in Fig. 2 the fractionation of **P10** is shown.

In pilot experiments it was established that after 11.5 min only (1→4)-linked linear oligomers elute (fraction 2; DP G8–G1). Up to 11.5 min (fraction 1; DP > G8) large and highly branched oligomers elute. After α-amylolysis of methylated amylopectin potato starch no long linear oligomers are expected, because of the absence of the highly polymeric amylose. Therefore, fraction 1 isolated from **A4**–**A30** corresponds only with branched regions. However, in fraction 1 isolated from methylated potato starch (**P4**–**P30**) some linear glucopyranans, originating from incomplete digestion of amylose, might occur. Differences found between substitution levels of fraction 1 and frac-

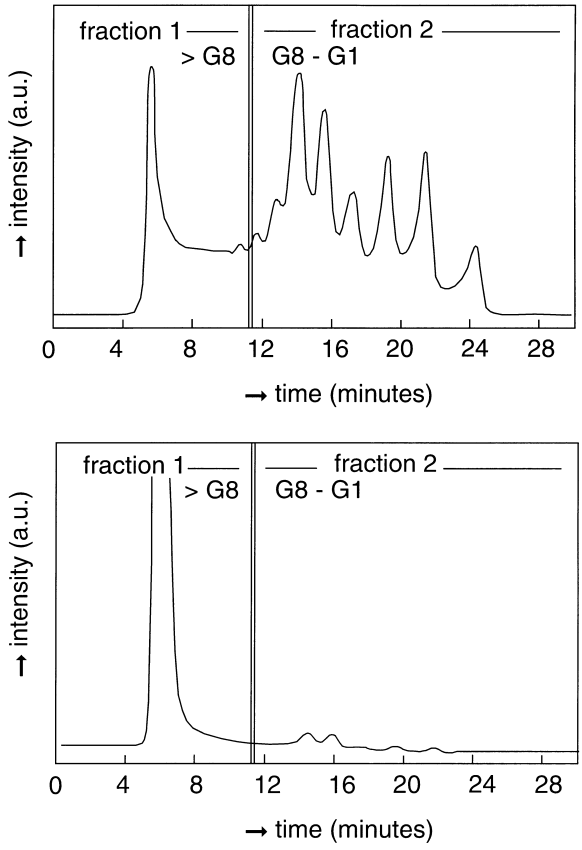


Fig. 2. HPLC elution profile of methanol supernatant (upper) and methanol precipitate (lower) isolated from **P10**. See Experimental for the analysis parameters.

tion 2 in potato starch and amylopectin potato starch, respectively, may therefore be attributed mainly to different substitution levels of amylose and amylopectin.

Substitution level of branched and linear regions from methylated starches.—The MS values of the methanol precipitates and the methanol supernatants of **P4**–**P30** and **A4**–**A30**, respectively, were measured by monosaccharide analysis [4] (Table 3). The molar ratios of the partially methylated monomers in the glucans of the methanol precipitates and the methanol supernatants of **P4**–**P30** and **A4**–**A30**, respectively, did not differ from the ones in the corresponding starting materials (Table 2).

The cross-contamination as mentioned above (Fig. 2) of branched and linear regions in the methanol precipitates and the methanol supernatants has been quantified by HPLC for each sample (Table 3), making use of the following equations:

$$\begin{aligned} MS_{mp} = {} & MS_{br} \cdot \text{fraction1 (in mp)} \\ & + MS_{lr} \cdot \text{fraction2 (in mp)} \end{aligned} \quad (1)$$

$$\begin{aligned} MS_{ms} = {} & MS_{br} \cdot \text{fraction1 (in ms)} \\ & + MS_{lr} \cdot \text{fraction2 (in ms)} \end{aligned} \quad (2)$$

where mp = methanol precipitate, ms = methanol supernatant, br = branched regions, lr = linear regions.

Combining eqs (1) and (2) the MS of the branched glucopyranans (MS_{br}) and the MS of the linear glucopyranans (MS_{lr}) can be calculated. In this way, the MS values of the branched and the linear regions of 12 different methylated starches were acquired and the results are summarised in Table 3. In methylated potato starch (**P4**–**P30**) the MS of the branched regions is 60–140% higher than the MS of the linear regions. This preference for the branched regions is probably related to a better accessibility or higher reactivity of these regions. An even larger difference in levels of substitution between branched and linear regions is observed in amylopectin potato starch (**A4**–**A30**; 140–260%). The latter finding may be attributed to a high reactivity of the amylose fraction in potato starch, which is predominantly present in the amorphous starch domains [16]. It implies that in amylopectin molecules of methylated starch the level of substitution in branched regions is higher than in linear regions. Previously, it was proposed that 2-hydroxypropyl and phosphate groups are located at branched regions of chemically modified starch [17]. However, no quantification could be made, whereas with the method described here (Scheme 1) the MS of branched and linear regions can be determined separately.

Table 3
Molar substitutions of branched and linear regions from methylated potato starch and amylopectin potato starch

Sample	% fraction 1 (in mp)	% fraction 1 (in ms)	MS (mp)	MS (ms)	MS (br)	MS (lr)
P4	85.7	15.4	0.059	0.043	0.062	0.039
P6	84.3	17.8	0.087	0.059	0.094	0.052
P8	86.0	20.0	0.125	0.073	0.136	0.057
P10	94.3	22.1	0.139	0.100	0.142	0.088
P20	96.8	45.0	0.282	0.200	0.287	0.129
P30	98.1	51.6	0.377	0.292	0.380	0.198
A4	91.8	20.9	0.077	0.044	0.081	0.034
A6	87.5	26.3	0.113	0.063	0.123	0.042
A8	89.0	27.6	0.133	0.075	0.143	0.049
A10	89.1	23.6	0.162	0.099	0.172	0.076
A20	98.4	40.7	0.284	0.171	0.287	0.091
A30	99.2	44.2	0.419	0.251	0.421	0.116

mp = methanol precipitate, ms = methanol supernatant.
br = branched regions, lr = linear regions.

Determination of distribution of substituents in methylated highly branched D-*glucopyranans and methylated linear glucopyranans with FABMS.*—The distribution of substituents in oligomer fragments represents the situation in the corresponding polymer backbone [5,18]. In order to prepare suitable derivatives for FABMS analysis, the oligomers in the methanol supernatants derived from **P20**, **P30**, **A20** and **A30** were permethylated with CD_3I [19], whereas those in the methanol precipitates derived from **P20**, **P30**, **A20** and **A30** were permethylated with CD_3I after partial methanolysis. Although oligomers of specific DP are chemically identical, mass spectrometry can distinguish between CH_3 groups present in the starch derivative and newly introduced CD_3 groups. It can be anticipated that all per(deuterio)methylated trimers show the same behaviour in terms of ionisation. Therefore, quantification of all FAB intensities in the trimer pseudo-molecular ion region ($[M + Na^+]$) is valid. The same holds for per(deuterio)methylated tetramers. It should be noted that quantification is more accurate in the trimer pseudo-molecular ion region than in the tetramer one, because of higher signal to noise ratios. Since resolution decreases using larger oligomers, FABMS analysis was restricted to trimers

and tetramers. The data obtained from FABMS analysis were compared to the expected distributions according to binomial statistics [5]. In the calculation methyl substitutents are distributed randomly over equal and independent glucose monomers. A typical analysis of trimers derived from **P20** is shown Fig. 3. For the methanol supernatant (mainly linear regions) the observed amount of nonsubstituted oligomers is lower than expected on the basis of a random distribution, whereas the amount of monosubstituted oligomers is higher. This forms a clear indication for the heterogeneous substitution during methylation of granular starch. For the methanol precipitate (mainly branched regions) the observed contribution of non- and highly substituted oligomers is slightly lower than calculated, whereas the amount of mono- and trisubstituted oligomers is slightly higher. The trends in branched regions are less pronounced than those in linear regions.

Previously, evidence was obtained for the blockwise substitution in granular methylated starches by GPC analysis after α-amylolysis followed by glucoamylolysis, however, without distinguishing branched from linear regions [16]. Our results from FABMS studies render quantification of the degree of heterogeneity in the substitution pattern of both branched and linear regions possible (Table 4). The degree of heterogeneity is expressed in the parameter H_n, where n corresponds with the DP of the per(deuterio)methylated oligomers [5]. If the observed and calculated mole fractions are identical (Fig. 3), the substituent distribution is homogeneous ($H_n = 0$). Increasing differences between observed and calculated mole fractions give rise to an increase of the value of H_n. All heterogeneity values in Table 4 are normalised by dividing by their corresponding MS values as calculated from FABMS data. In this way heterogeneities of samples

Table 4
Heterogeneity of substituent distribution in methylated potato and amylopectin potato starches

Sample	P20	P30	A20	A30
H_3/MS(trimers) ms	0.847	1.029	0.950	1.016
H_3/MS(trimers) mp	0.063	0.141	0.069	0.183
H_4/MS(tetramers) ms	0.926	0.956	0.749	0.941
H_4/MS(tetramers) mp	0.258	0.467	0.310	0.433

mp = methanol precipitate, ms = methanol supernatant.
Calculation of H_3 and H_4 is described in ref [5]. Homogeneous distribution results in $H_n = 0$; with increasing H_n heterogeneity of substituent distribution increases.

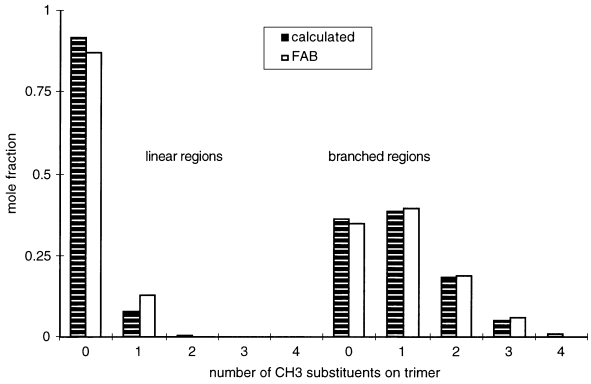


Fig. 3. Calculated (striped bars) and observed (white bars) substituent distribution in trimers from the methanol supernatant (mainly linear regions) and the methanol precipitate (mainly branched regions) of **P20**. Note that the branched regions contain more substituents than the linear regions.

with different degrees of substitution can be defined. It is evident from Table 4 that methyl substituents in branched regions are almost distributed randomly (i.e. according to binomial statistics), whereas in the linear regions a higher degree of heterogeneity is observed.

3. Conclusion

Methylation of granular starch in an aqueous suspension using dimethyl sulfate takes place preferably at the branched regions of amylopectin. However, the linear regions of amylopectin are substituted more heterogeneously (i.e. not randomly) than the branched regions. Amylose is higher substituted than linear regions of amylopectin as can be concluded from differences in substitution level between methylated potato starch and amylopectin potato starch.

4. Experimental

Digestion of methylated starches.—Methylated starch (14 g; prepared from potatoes or amylopectin potatoes by methylation of starch granules in an aqueous suspension using dimethyl sulfate, see Table 1) was suspended in Millipore water (300 mL), and $CaCl_2$ (30 mg) was added. The suspension was gelatinised by cooking for 15 min at 120 °C. After cooling down, the pH was set at 6.2. Then, the gelatinised starch was digested for 21 h with 0.1 mass% BAN 240L (Novo Nordisk A/S) in a shaking bath at 68.5 °C and constant pH (6.2). In

the first 4 h the pH was re-adjusted several times. α-Amylase-digested methylated starch was debranched with 2 mass% Optimax L300 (Genencor) at pH 4.5 and 57 °C for 24 h.

Methanol precipitation of highly branched D-*glucopyranans in α-amylolysis digests.*—MeOH(1.5 L) was slowly added to an aqueous solution of α-amylase-digested methylated starch (300 mL). The suspension was stirred at room temperature for 3 h. Then, the supernatant was separated from the sticky precipitate, and both fractions were concentrated.

Analytical procedures.—Monosaccharide analysis including GLC-mass spectrometry was carried out as described [4]; for the quantification by GLC empirical molar response factors were applied [20,21].

For the detection of (partially methylated) glucose units at the reducing end, first partially methylated oligoglucopyranan samples, obtained after α-amylolysis of the different methylated starches, were reduced with $NaBD_4$ in 0.5 M NH_4OH [13]. The reduced partially methylated oligoglucopyranan samples were hydrolysed with 2 M CF_3CO_2H (2 h, 100 °C), and the generated saccharide mixtures were converted into glucononitrile derivatives by treatment with NH_2OH in *N*-methylimidazole (30 min, 80 °C) [14,15]. The mixtures of (partially methylated) glucitols and glucononitriles were acetylated with Ac_2O (10 min, 20 °C), and analysed by GLC-mass spectrometry after working-up.

Partially methylated oligoglucopyranans from methanol precipitates (after partial methanolysis; 1.5 h, 60 °C, methanolic 0.5 M HCl) and methanol supernatants were permethylated with CD_3I as described previously (Me_2SO/NaOH) [19], and analysed by FABMS.

GLC analyses were performed on a WCOT CP-SIL 5CB fused-silica capillary column (25 m×0.32 mm) using a temperature program of 110–230 °C at 4 °C/min. HPLC analyses were performed on an Aminex HPX-42A column (300×7.8 mm) at 85 °C, eluted with water at a flow rate of 0.4 mL/min and RI detection. GLC–mass spectrometry of partially methylated glucitol acetates and glucononitrile acetates was carried out on an MD800/8060 system (Fisons instruments; electron energy, 70 eV), equipped with a DB-1 fused-silica capillary column (30 m×0.32 mm, J&W Scientific), using a temperature program of 110–230 °C at 4 °C/min. (*O*-Methylated) Glc derivatives were analysed as trimethylsilylated methyl glucosides with the same system, using the same temperature program. 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.

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).

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