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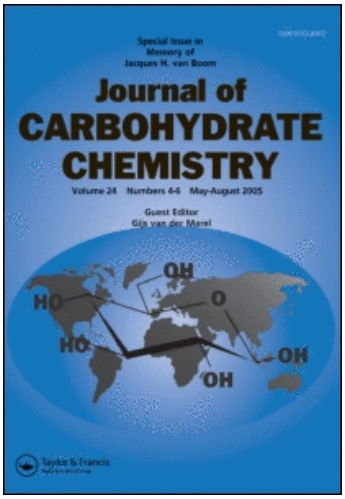

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The Preparation and the Assignment of the ^{1}H and ^{13}C NMR Spectra of Methylated Derivatives of Inulin Oligosaccharides

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THE PREPARATION AND THE ASSIGNMENT OF THE ^{1}H AND ^{13}C NMR SPECTRA OF METHYLATED DERIVATIVES OF INULIN OLIGOSACCHARIDES.

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ABSTRACT

The complete assignment of the ^{1}H and ^{13}C NMR spectra for permethylated sucrose, 1-kestose and nystose are given. Methylation of these inulin oligosaccharides did not change the conformation of the ring structures. A partially methylated derivative of nystose has been prepared using water as the solvent. The difference in reactivity of the various hydroxyl groups of nystose appeared to be very small.

INTRODUCTION

Commercially available inulin (from Chicory root) contains oligosaccharides consisting of (2->1)-linked ß-D-fructofuranosyl residues, mostly terminated at the reducing end by an α-D-glucopyranosyl unit in a (1<->2)-linkage (Fig. 1), and has typically an average degree of polymerisation (DP) of 7-8.[1] In the search for new

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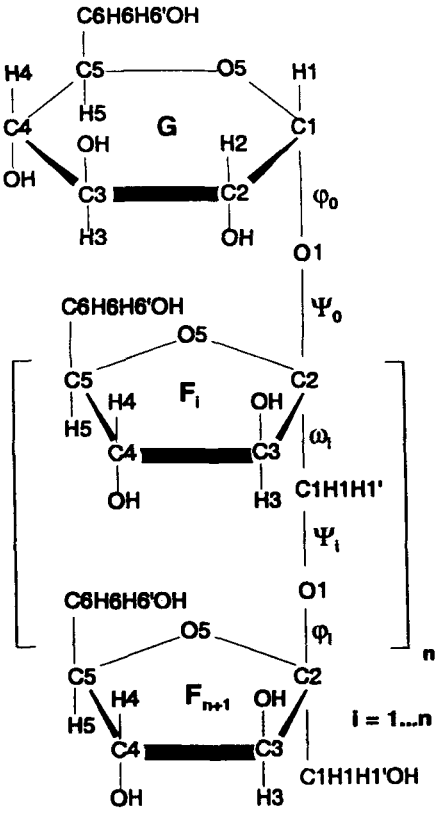

Fig. 1. Primary structure of oligosaccharides present in inulin. For sucrose (GF), 1-kestose (GF_2), and nystose (GF_3) n is 0, 1, or 2, respectively. The fructofuranosyl residues (designated F) in inulin will be numbered, using the fructofuranosyl residue which is attached to the glucopyranosyl residue (designated G) as a starting point.

applications of inulin, structure-function relationships are studied. The range of possible applications can be broadened through the preparation of various derivatives. Therefore the relation between the properties of inulin derivatives and the size, localization, and nature of the substituents and the DP of the oligosaccharides has to be studied.

To start this study the methylation of inulin oligosaccharides was examined. Until now, only the complete methylation of a mixture of inulin oligosaccharides, in organic solvents, had been published.[2-6] These product are not soluble in water which can limit their scope of applications. Therefore, in this report the preparation is

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described of partially methylated nystose in an aqueous system. Since nystose has an internal fructofuranosyl residue attached to two other fructofuranosyl moieties it can be regarded as a model for such residues in inulin. It can be expected that the partially methylated oligosaccharides have a better solubility in water than the fully methylated oligosaccharides. For NMR analysis the partially methylated compounds can be converted into fully methylated derivatives using ^{13}C enriched methyl iodide,[7,8] which enables also the determination of the regioselectivity for complicated mixtures of oligosaccharides with the same DP.

NMR spectroscopy is a useful tool for conformational analysis of molecules in solution. Vicinal (three bonds) coupling constants and NOE's provide information about dihedral angles and interproton distances, respectively. However, in order to obtain this structural information the assignment of the NMR signals to individual atoms in the molecule is a prerequisite. Although partial ^{1}H and ^{13}C assignments of permethylated sucrose have been published,[6] these are not available for permethylated inulin oligosaccharides. For NMR analysis, fully methylated sucrose, 1-kestose and nystose have been prepared. In this study the complete sequential ^{1}H and ^{13}C NMR assignments of permethylated sucrose, 1-kestose and nystose are described.

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RESULTS AND DISCUSSION

Preparation of derivatives. Methylation of sucrose, 1-kestose and nystose in DMF yielded fully methylated compounds which were not soluble in water. Derivatisation of nystose in water, however, resulted in a partially methylated product which was readily soluble in water and could not be extracted using dichloromethane. Further methylation of this compound with ^{13}C enriched methyl iodide (28.28 %) enabled analysis of the degree of methylation using ^{13}C NMR. In order to estimate the relative reactivities of the different OH groups in nystose, the localization of methyl groups in partially methylated nystose had to be determined. Therefore, the NMR signals of the methyl groups in permethyl nystose were assigned.

NMR assignments of permethylated sucrose. Using the anomeric G-H-1 and the F-H-3 doublets as starting points the COSY spectrum allowed the assignments of G-H-2,3,4,5 and F-H-4,5. The HMQC spectrum led to the identification of the corresponding ^{13}C signals.

In the HMBC spectrum cross peaks were found between G-H-1 and F-C-2 and between F-C-2 and F-H-1',1. The G-H-4 and F-H-5 resonances showed cross peaks with the secondary carbon resonances G-C-6 and F-C-6, respectively, which were recognized in the APT spectrum. Using the HMQC spectrum enabled the identification of F-C-1, F-H-6',6 and G-H-6',6. From these assignments (Table 1) it could be concluded that F-C-6 and F-C-1 have the same chemical shift. This was confirmed by the APT spectrum which showed for these atoms one peak with an intensity of about two times that of G-C-6.

The protons of the methyl groups were identified *via* cross peaks with the nearest carbohydrate signals in the HMBC spectrum. The HMQC spectrum revealed the corresponding ^{13}C assignments of the methyl groups. Because F-C-1 and F-C-6 have the same chemical shift, it was not possible to discriminate between F-Me-H-1 and F-Me-H-6 nor between F-Me-C-1 and F-Me-C-6.

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NMR assignments of permethylated 1-kestose. The DQF-COSY spectrum revealed the assignments of G-H-2,3,4,5 and F-H-4, using G-H-1 and F-H-3 as starting points. From the DQF-COSY alone it was not possible to discriminate between the two fructofuranosyl residues.

In the HMBC spectrum, G-H-1 showed a cross peak with a quaternary carbon which, therefore, was assigned to the anomeric carbon of the fructofuranosyl residue adjacent to the glucopyranosyl residue (F1-C-2).

The APT spectrum showed two secondary carbon signals which showed cross peaks with the F-H-3 resonances in the HMBC spectrum and were, therefore, assigned to the F-C-1 atoms. One of these C-1 resonances had a chemical shift 11 ppm larger than the other one and had a cross peak with a methyl group in the HMBC spectrum and was accordingly identified as F2-C-1. The assignment of F1-C-1,2 enabled the discrimination of the signals of the two fructofuranosyl residues.

Using the preceding assignments the HMBC spectrum allowed the identification of G-C-1,2,3,4,5, F1-H-3,4, F2-H-3,4, F1-H-1',1, F2-H-1',1 and F2-C-2. Two tertiary carbon signals, which were recognized in the APT spectrum, showed in the HMBC spectrum cross peaks with the F-H-3,4 resonances and were assigned to F1-C-5 and F2-C-5. The remaining secondary carbon signals had cross peaks in the HMBC spectrum with the F-H-4 or G-H-4 resonances and were assigned to F1-C-6, F2-C-6

Table 1a: ^{1}H NMR Assignments of permethylated sucrose, 1-kestose, and nystose:

Atom	Sucrose		1-Kestose			Nystose			
	G	F	G	F1	F2	G	F1	F2	F3
H-1'	-	3.597	-	3.712	3.524	-	3.719	3.750	3.487
H-1	5.530	3.373	5.541	3.712	3.468	5.541	3.719	3.666	3.432
H-2	3.116	-	3.142	-	-	3.145	-	-	-
H-3	3.426	4.014	3.463	4.108	3.938	3.376	4.119	4.028	3.946
H-4	3.165	3.811	3.221	3.788	3.716	3.217	3.699	3.608	3.662
H-5	3.929	3.915	3.942	3.899	3.899	3.947	3.908	3.879	3.871
H-6'	3.602	3.676	3.618	3.658	3.612	3.618	3.649	3.595	3.558
H-6	3.534	3.549	3.542	3.658	3.612	3.542	3.578	3.595	3.558
Me-H-1	-	3.409(3)	-	-	3.390	-	-	-	3.388
Me-H-2	3.439	-	3.440	-	-	3.442	-	-	-
Me-H-3	3.602	3.477	3.618	3.484	3.458	3.614	3.495	3.481	3.455
Me-H-4	3.537	3.450	3.547	3.464	3.429	3.545	3.462	3.436	3.435
Me-H-6	3.404	3.380(3)	3.403	3.371	3.375	3.401	3.371	3.367	3.376

Table 1b: ^{13}C NMR Assignments of permethylated sucrose, 1-kestose, and nystose:

Atom	Sucrose		1-Kestose			Nystose			
	G	F	G	F1	F2	G	F1	F2	F3
C-1	88.58	73.36	88.86	62.73	73.71	88.76	62.82	63.44	73.97
C-2	81.07	103.47	81.25	103.68	103.68	81.19	103.75	103.68	103.58
C-3	82.49	84.54	82.73	84.43	86.18	82.72	84.26	85.77	86.22
C-4	78.70	83.22	78.97	83.51	85.08	78.93	83.45	85.08	84.55
C-5	69.64	78.59	69.91	78.54	78.63	69.92	78.51	78.18	78.42
C-6	70.63	73.36	70.85	73.76	73.51	70.80	73.75	73.36	73.09
Me-C-1	-	58.60(4)	-	-	58.98	-	-	-	58.98
Me-C-2	57.43	-	57.68	-	-	57.64	-	-	-
Me-C-3	59.81	57.71	60.12	57.93	57.91	60.14	57.96	57.92	58.03
Me-C-4	59.50	57.59	59.85	57.96	57.69	59.87	57.94	57.66	57.74
Me-C-6	58.38	58.29(4)	58.73	58.60	58.55	58.72	58.60	58.52	58.61

Table 1c: ^{1}H-^{1}H NMR Coupling constants of permethylated sucrose, 1-kestose, and nystose:

Coupling constant	Sucrose		1-Kestose			Nystose			
	G	F	G	F1	F2	G	F1	F2	F3
J-1,1'	-	10.8	-	n.d.(1)	10.3	-	n.d.(1)	9.6	10.0
J-1,2	3.6	-	3.6	-	-	3.7	-	-	-
J-2,3	9.6	-	9.7	-	-	9.6	-	-	-
J-3,4	n.d.(2)	7.4	n.d.(2)	7.6	6.9	n.d.(2)	7.9	6.9	7.0
J-4,5	8.8(5)	7.4	8.8(6)	7.7	6.8	9.1(7)	7.9	7.0	6.9
J-5,6	10.1(5)	n.d.(2)	10.0(6)	n.d.(2)	n.d.(2)	10.1(7)	n.d.(2)	n.d.(2)	n.d.(2)
J-5,6'	n.d.(2)	6.5	n.d.(2)	n.d.(2)	n.d.(2)	n.d.(2)	n.d.(2)	n.d.(2)	n.d.(2)
J-6,6'	n.d.(2)	10.4	n.d.(2)	n.d.(2)	n.d.(1)	n.d.(2)	n.d.(2)	n.d.(1)	n.d.(1)

1:Could not be determined because of degeneration.
2:Not determined due to severe overlap.
3-7:May be reverse.

and G-C-6. The HMQC spectrum enabled the identification of F1-H-6, F2-H-6 and G-H-6.

Identification of the protons of the methyl groups took place *via* cross peaks with the nearest carbohydrate signals in the HMBC spectrum. The HMQC spectrum revealed the corresponding ^{13}C assignments of the methyl groups.

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NMR assignments of permethylated nystose. Using the easily identifiable G-H-1 signal as a starting point, the DQF-COSY spectrum of nystose enabled the identification of the G-H-2,3,4,5 signals. The characteristic doublets of the F-H-3 signals revealed the corresponding H-4 signals. The other resonances were hard to find in the DQF-COSY spectrum due to severe overlap. The corresponding ^{13}C signals G-C-1,2,3,4,5 and the F-C-3,4 signals were identified using the HMQC spectrum.

The other tertiary ^{13}C signals were recognised in the APT spectrum and assigned to the F-C-5 atoms. These ^{13}C resonances show cross peaks in the HMQC spectrum, enabling the identification of the F-H-5 resonances.

Discrimination between the secondary ^{13}C signals and the methyl groups was performed using the APT spectrum. Three of the secondary ^{13}C resonances showed in the HMBC spectrum cross peaks with the F-H-3,4 resonances, and one with the G-H-3 resonance, and were assigned to the F-C-6 atoms and G-C-6, respectively. The HMQC spectrum revealed the identification of the corresponding H-6 signals.

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The assignments of the remaining secondary ^{13}C resonances to the F-C-1 atoms were confirmed via the cross peaks in the HMBC spectrum with the F-H-3 resonances. Using the HMQC spectrum the corresponding F-H-1 resonances were identified. These H-1 resonances showed cross peaks in the HMQC spectrum with the easily identifiable quaternary F-C-2 resonances, thereby confirming the discrimination between the C-6 and C-1 signals. This means that the non-sequential assignment, except the methyl resonances, is complete.

The ^{1}H resonances of the methyl groups were assigned via the three-bond cross peaks in the HMBC spectrum with the nearest carbohydrate ^{13}C resonances (Fig. 2). The one bond correlations in the HMQC spectrum enabled the assignment of the corresponding ^{13}C resonances of the methyl groups.

The signals belonging to the three different fructofuranosyl residues could be discriminated as follows. One of the three F-C-1 atoms had a chemical shift about 10 ppm larger than the other two, very close to the F-C-6 atoms. This resonance had, therefore, been assigned to F3-C-1 which was the only C-1 atom attached to a methyl group and, thereby, resembling the C-6 atoms. In the HMBC spectrum the corresponding F3-H-1,1' resonances showed only cross peaks with the C-2 signal

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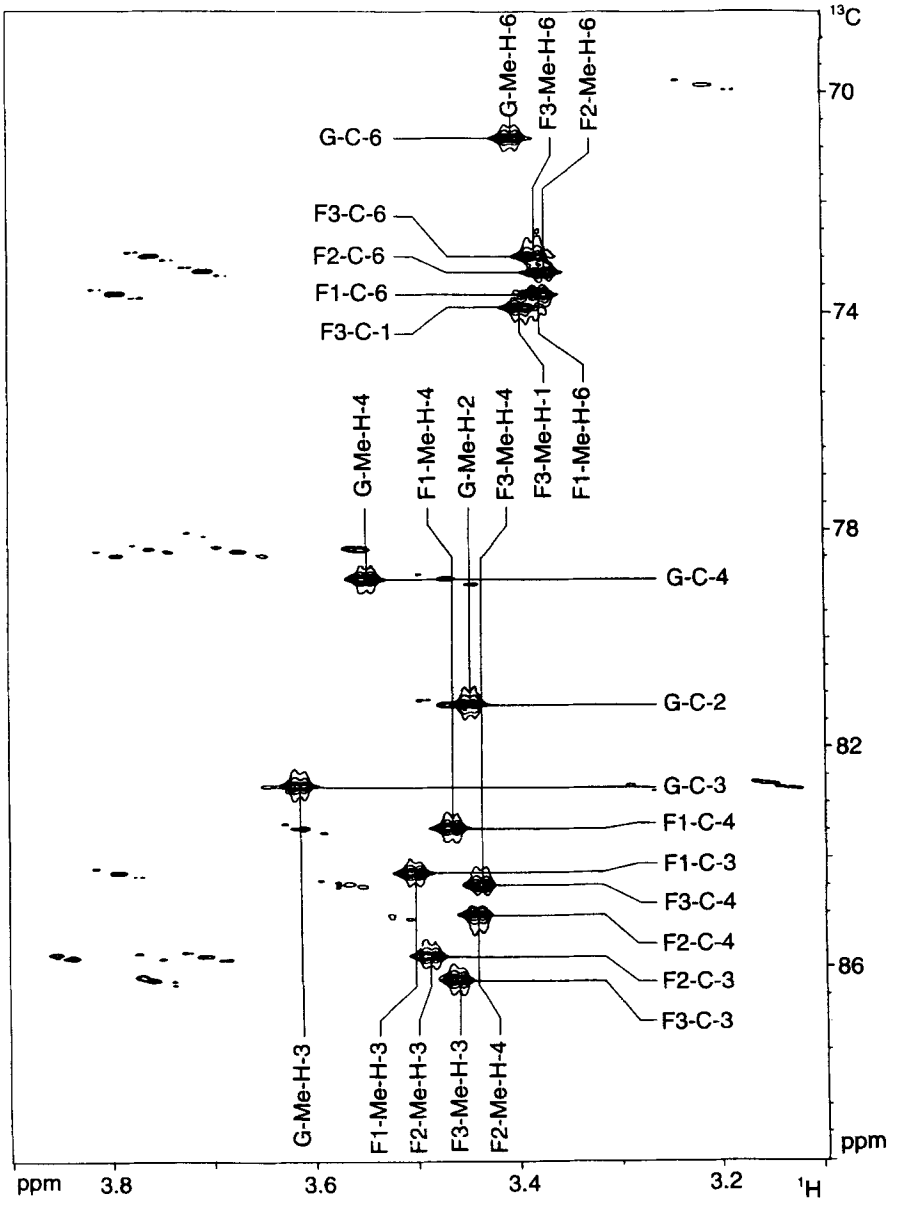

Fig. 2. HMBC spectrum of permethylated nystose. Only the area which contains cross peaks of the methyl resonances with the nearest carbohydrate ^{13}C resonances is shown.

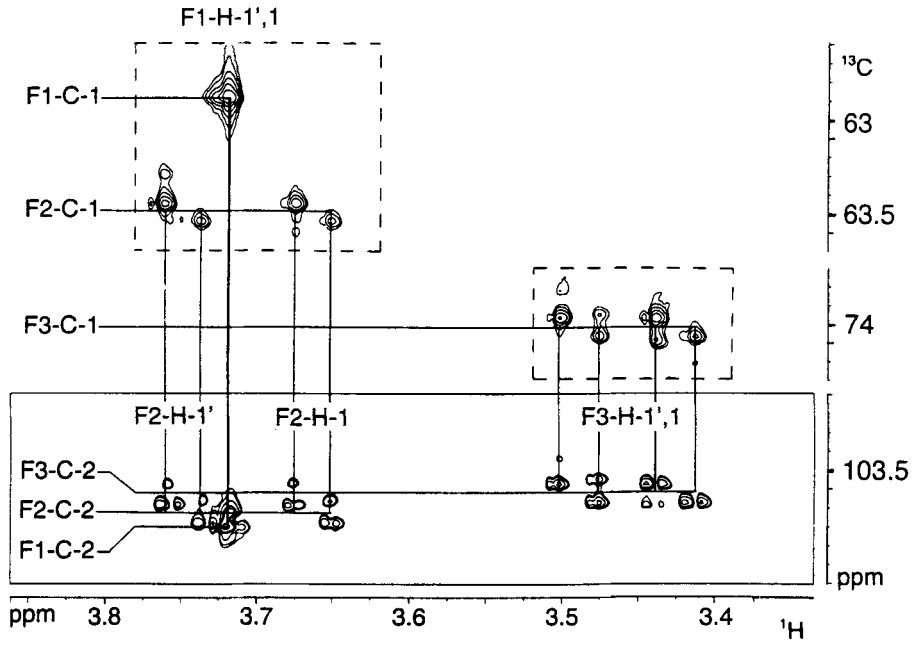

Fig. 3. HMBC spectrum of permethylated nystose. Only the areas which contain cross peaks with the fructofuranosyl H-1,1' protons are shown. The cross peaks in the areas surrounded by dotted lines are taken from the HMQC spectrum and are shifted over $^1J_{CH}/2$.

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having the lowest chemical shift (Fig. 3). The assignment of the F3-C-2 resonance was confirmed by a small cross peak with the protons of a methyl group via four bonds.

The F-C-2 atom with the largest chemical shift showed cross peaks in the HMBC spectrum with G-H-1, F1-H-5, and, via four bonds, with G-H-2. Consequently, this C-2 resonance was assigned to the fructofuranosyl residue (F1) adjacent to the glucopyranosyl residue and enabled discrimination from the remaining inner fructofuranosyl residue (F2). The data are summarized in Table 1.

Analysis of ring conformations. The ^{1}H-^{1}H vicinal coupling constants (J) were determined directly from the spectra, without simulation of the spin systems (Table 1c). This was assumed to be accurate for at least the glucopyranosyl $J_{1,2}$ and $J_{2,3}$ values and the fructofuranosyl $J_{3,4}$ values, because the large differences in chemical shift values allow a first order approximation.

All fructofuranosyl $J_{3,4}$ and $J_{4,5}$ coupling constants of permethyl sucrose, 1-kestose and nystose have been compared with the corresponding reported values[9] for the underivatized oligosaccharides. This resulted in a small average difference of 1.3, 1.2 and 1.1 Hz for sucrose, 1-kestose and nystose, respectively, which means that the $^{4}T_{3}$ pucker of the fructofuranosyl rings is preserved upon methylation. Also the $^{4}C_{1}$ chair conformation of the glucopyranosyl rings did not change as a result of methylation.

Analysis of methylated nystose. The material obtained by partial methylation of nystose in an aqueous system was too heterogeneous for analysis using NMR. Further methylation with ^{13}C enriched methyl iodide in organic solvent afforded fully methylated nystose which was homogeneous according to NMR. The use of ^{13}C enriched methyl iodide in the second step (in organic solvent) enabled calculation of the degree of methylation after the first step (in aqueous system).

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In the quantitative spectra of permethyl nystose and of ^{13}C enriched permethyl nystose, the ^{13}C NMR signals, except the methyl resonances, were integrated. The sum of these integrals has been used to scale the two spectra. The methyl signals were quantified via a fit of a sum of Lorentzian line shapes to the spectrum, because of spectral overlap of some methyl peaks.

Using the peak areas of the methyl groups of permethyl nystose and the scaling term, the peak areas of ^{13}C enriched permethyl nystose were calculated for a specific degree of methylation in the first step (in aqueous system). The calculated peak areas for a degree of methylation of 0 % and 100 % were compared with experimentally obtained values of the corresponding peak areas of ^{13}C enriched permethyl nystose. This resulted in the degree of methylation values (Fig. 4) for the different hydroxyl groups of the partially methylated nystose, obtained by the methylation in an aqueous system.

The average degree of methylation for all hydroxyl groups is 24 %. Differences in degree of methylation (Fig. 4) of the hydroxyl groups of partially methylated nystose are not very large, which means that the variation in reactivity of the different hydroxyl groups in water is small. This small regioselectivity can be a result of the large excess of base (6 equivalents OH^{-} for each R-OH group) which was needed to obtain a reasonable reaction time. The average degree of methylation for the

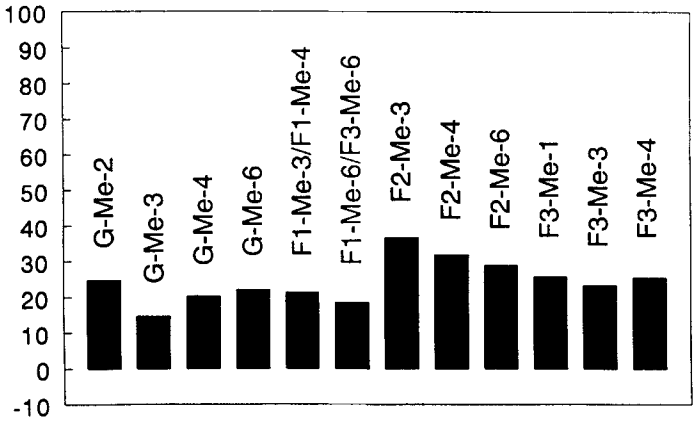

Fig. 4. Degree of methylation (%) of all hydroxyl groups in partial methylated nystose (from left to the right 25, 15, 20, 22, 21, 19, 37, 32, 29, 26, 23 and 25 %). Due to spectral overlap for F1-Me-6/F3-Me-6 and F1-Me-4/F1-Me-3 averaged values have been given.

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glucopyranosyl residue (20 %) is somewhat lower than the average degree of methylation for the fructofuranosyl residues (25 %). For the primary hydroxyl groups of the fructofuranosyl moieties (average degree of methylation 22 %) a slightly smaller reactivity has been observed than for the secondary hydroxyl groups (average degree of methylation 27 %). The highest values were found for F2-OH-3 and F2-OH-4, and the lowest value for G-OH-3.

CONCLUSION

Nystose, partially methylated with an average degree of methylation of 24 % is readily soluble in water, whereas fully methylated nystose is not. This suggests that the solubility of inulin can be easily modulated by a suitable degree of methylation.

The mixture of partially methylated nystoses was too heterogeneous for analysis with NMR. Further methylation with ^{13}C enriched methyl iodide resulted in homogeneous material, which enabled the determination of the degree of methylation for the partially methylated nystose.

EXPERIMENTAL

NMR spectroscopy. ^{1}H and ^{13}C NMR spectra were recorded on BRUKER AMX-400-WB (ATO-DLO/RIKILT-DLO, Wageningen), BRUKER DPX-300 (ATO-DLO, Wageningen) and BRUKER AC-200 (ATO-DLO, Wageningen) spectrometers at 295-300 K. Chemical shifts (δ) are expressed in ppm downfield from internal TMS or were actually measured relative to internal chloroform (76.9 ppm for ^{13}C spectra). For NMR measurements 100-200 mg compound was dissolved in 0.5 mL $CDCl_3$. Processing of NMR data was performed using the BRUKER software packages UXNMR, XWIN-NMR and WINNMR.

APT spectra for permethyl nystose and ^{13}C enriched permethyl nystose were measured at 50.323 MHz by accumulation of 16,000 scans with 65,536 data points and a spectral width of 15,152 Hz. Quantitative 1D ^{13}C NMR spectra for these compounds were obtained by the acquisition of 2944 scans of 65,536 data points using a repetition time of 20 s, gated decoupling, and a spectral width of 3012 Hz. APT spectra for permethyl sucrose and 1-kestose were recorded by 3000 scans of 16384 data points and a spectral width of 15152 Hz. 1D ^{1}H-NMR spectra were recorded for all compounds at 400.137 MHz.

Gradient DQF-COSY spectra[10] were obtained for permethylated 1-kestose and nystose at 400.137 MHz with 2048 experiments of 2 scans and 2048 data points and a spectral width of 2500 Hz in both dimensions. A gradient COSY spectrum of permethylated sucrose was measured at 300.131 MHz with 1024 experiments of 4 scans and 1024 data points and a spectral width of 4006 Hz for ω_1 and ω_2.

Gradient HMBC[11] spectra of permethylated sucrose, 1-kestose and nystose were recorded at 400.137 MHz by acquisition of 2048 experiments of 48, 48 or 16 scans, respectively, with 2048 data points. The spectral width was 6038 and 1202 Hz for ω_1 and ω_2, respectively. A gradient HMQC[12] spectrum of permethylated nystose was obtained with 64 scans for each experiment and the same spectral width and resolution as the HMBC spectrum. Gradient HMQC spectra of permethylated sucrose and 1-kestose were measured at 400.137 MHz by the accumulation of 1024 experiments of 56 scans with 2048 data points. The spectral width was 25156 for ^{13}C and 3546 Hz for ^{1}H.

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Isolation of inulin oligosaccharides. A mixture of inulin oligosaccharides with degree of polymerisation (DP) of 3, 4, and 5 ('NEOSUGAR' obtained from Meiji Seika Ltd.) was fractionated, at room temperature (ca. 20 °C), using a RP-18 HPLC column of 25 x 5 cm and water/methanol (99:1 v/v) as eluent. During the total run time of 36 min the flow was kept at 35 mL/min. For each run 2.6 g was injected (using a 20% m/v solution) and fractions of 63 mL, 105 mL, and 157.5 mL were collected for DP 3, DP 4, and DP 5, respectively. Lyophilisation yielded the isolated oligosaccharides which were 92, 86, and 69 % pure according to HPAEC-PAD[1] for DP 3, DP 4, and DP 5, respectively. The impurities of these fractions consisted mainly of inulin oligosaccharides with a higher or lower DP, which were removed by column chromatography after methylation.

Complete methylation of sucrose, 1-kestose and nystose. To a solution of 1.0 g (2.9 mmol) sucrose and 1.7 g (71 mmol) NaH in 100 mL DMF 11 g (78 mmol) methyl iodide was added and the mixture was stirred for 18 h at room temperature (ca 20 °C). For extraction, 0.5 L methanol was added, followed by 0.5 L dichloromethane and 1 L water. The aqueous phase was extracted three times with 150 mL dichloromethane. The combined organic phase was washed with 1 L water and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (35 x 3 cm) by eluting with dichloromethane-ethyl acetate. Using the same procedure as for sucrose, 1.5 g (3.0 mmol) 1-kestose and 0.60 g (0.90 mmol) nystose were methylated with 17 g (120 mmol) and 13 g (92 mmol) methyl iodide, respectively. The permethyl derivative of sucrose (1036 mg, 78 % yield), $[\alpha]_D$ +63.9° (*c* 1.01, $CHCl_3$), was pure according to TLC and ^1H NMR spectroscopy. Part (574 mg) of the total yield of the permethyl derivative of 1-kestose (1410 mg, 72 %) and part (256 mg) of the total yield of the permethyl derivative of nystose (620 mg, 80 %) contained small amounts of the permethyl derivatives of nystose (DP 4) and 1-kestose (DP 3), respectively. The rest of the permethyl derivatives of 1-kestose and nystose were pure according to TLC and ^1H NMR spectroscopy, $[\alpha]_D$ +20.7° (*c* 1.01, $CHCl_3$) and $[\alpha]_D$ +2.4° (*c* 1.00, $CHCl_3$), respectively.

Anal. DP 2 Calcd for $C_{20}H_{38}O_{11}$: C, 52.8; H, 8.43. Found: C,52.2; H, 8.50. Anal. DP 3 Calcd for $C_{29}H_{54}O_{16}$: C, 52.9; H, 8.26. Found: C,51.9; H, 8.27. Anal. DP 4 Calcd for $C_{38}H_{70}O_{21}$: C, 52.8; H, 8.18. Found: C,52.4; H, 8.24.

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Methylation of nystose in aqueous solution. To a solution of 1.2 g (1.8 mmol) nystose in 56 mL 10 % (m/v) NaOH (155 mmol) in water 3.0 g (21 mmol) methyl iodide was added and the mixture was stirred for 18 h at room temperature (ca 20 °C). The aqueous solution was extracted two times with 100 mL dichloromethane. After concentration under reduced pressure, the combined organic phase did not contain methylated carbohydrates. To the aqueous phase 200 mL strongly acidic cation exchanger was added. After filtration, the acidic solution was neutralized using 2 g 10% NaOH in water. Lyophilisation yielded 5 g product (including salts).

Further methylation of partially methylated nystose. To a solution of 2.5 g crude partially methylated nystose (containing 0.90 mmol) and 2.0 g (83 mmol) NaH in 60 mL DMF, a solution of 3.02 g (21.3 mmol) methyl iodide and 1.17 g (8.19 mmol) ^{13}C-methyl iodide (99.0 atom % ^{13}C, ISOTEC) in 10 mL DMF was added. After 7.5 h stirring at room temperature (ca 20 °C) 4.5 g (32 mmol) methyl iodide was added, and stirring at room temperature was continued for 18 h. After extraction and column chromatography part (23 mg) of the total yield (300 mg, 47 %) contained small amounts of the permethyl derivative of an inulin oligosaccharide with DP 5. The remainder was pure according to TLC and ^{1}H NMR spectroscopy.

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