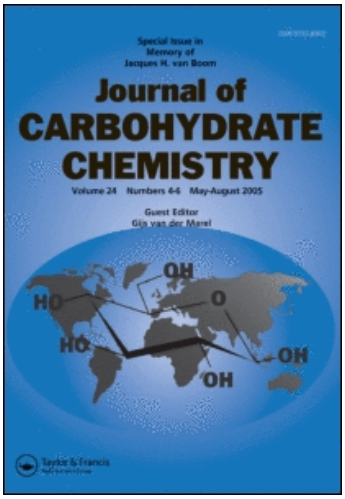

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MD CALCULATIONS ON NYSTOSE COMBINED WITH NMR SPECTROSCOPY ON INULIN RELATED OLIGOSACCHARIDES

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ABSTRACT

Molecular Dynamics (MD) calculations have been performed on nystose in water. According to these calculations the glycosidic linkages of the molecule are flexible. Structures obtained with MD calculations are compared with NMR data of several inulin related oligosaccharides and inulin, resulting in a model for the conformation of their fructofuranosyl residues. To extend the set of available NMR data of inulin related oligosaccharides, the complete assignment of the ^{1}H and ^{13}C NMR signals of ß-D-fructofuranosyl-(2->1)-ß-D-fructofuranosyl-(2->1)-ß-D-fructofuranosyl-(2->1)-ß-D-fructofuranosyl-(2->1)-α-D-glucopyranoside has been given here, using several 2D homo- and heteronuclear NMR experiments. Accurate coupling constants have been obtained by simulation of the 600 MHz 1D NMR spectra.

INTRODUCTION

Inulin, a storage carbohydrate found in many plant species,[1,2] consists of (2->1) linked ß-D-fructofuranosyl residues terminated by a α-D-glucopyranoside unit in a (2->1) linkage (Fig. 1). New industrial crops are available *e.g.* Chicory and Jerusalem Artichoke, which give inulin yields comparable to those of sucrose from sugar beet, and starch from potatoes.[3] However, despite this abundance, few commercial applications are known in comparison with for example starch. Understanding of the conformational behaviour of inulin, and its physico-chemical properties in relation to the structure could facilitate the development of applications.

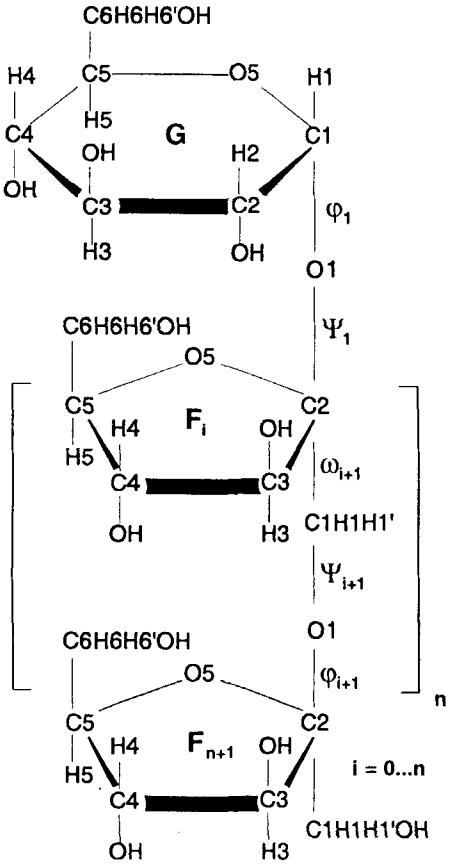

Fig. 1. Primary structures of inulin related oligosaccharides. For sucrose (GF), 1-kestose (GF_2), nystose (GF_3), and GF_4 n is 0, 1, 2, or 3, respectively. The fructofuranosyl residues (designated F) in inulin will be numbered, using the fructofuranosyl residue which is attached to the glucopyranosyl residue (designated G) as starting point. The ring protons of the terminal fructofuranosyl unit of GF_3 will accordingly be referred to as F3-H-3,4,5. In order to discriminate for example between the two protons which are bound to the C-6 of the first fructofuranosyl ring, the proton which has the largest NMR frequency will be designated as F1-H-6'. The dihedral angles φ_0, Ψ_0, and ω_1, are defined by G-O-5—G-C-1—G-O-1—F1-C-2, G-C-1—G-O-1—F1-C-2—F1-C-1, and G-O-1—F1-C-2—F1-C-1—F1-O-1 ,and the dihedral angles ω_i, Ψ_i, and φ_i are defined by F(i-1)-O-1—Fi-C-2—Fi-C-1—Fi-O-1, Fi-C-2—Fi-C-1—Fi-O-1—F(i+1)-C-2, and Fi-C-1—Fi-O-1—F(i+1)-C-2—F(i+1)-C-1, respectively.

Until now only low resolution X-ray data on mats of microcrystals and electron diffraction measurements of microcrystals of inulin have been reported.[4] In combination with Molecular Mechanics (MM) calculations on ß-D-fructofuranosyl-(2->1)-ß-D-fructofuranoside (inulobiose) a five fold helical structure has been suggested[4]. Computer modelling has shown[5]

that several types of helices are possible, without considerable steric hindrance. However, no preferred structure could be derived. With respect to small inulin related oligosaccharides the crystal structure of 1-kestose[6] and nystose[7] have been published. Moreover, MM calculations *in vacuo* have been performed for inulobiose,[8] 1-kestose,[9] and nystose.[7]

NMR spectroscopy is a useful tool for obtaining experimental data containing information about molecular conformations in solution. Assignment of the NMR-signals is necessary for developing spatial structural information of such molecules. In the context of this paper ^{1}H and ^{13}C assignments for inulin related oligosaccharides are available.[9-16] ^{13}C NMR signals assignments for GF_4 have been published.[10,15] In the case of 1-kestose, the NMR data concerning the fructofuranosyl-rings have been related to MM calculations.[9]

In the search for a model of inulin in aqueous solution, MD calculations have been performed on nystose, the smallest inulin related oligosaccharide with a fructofuranosyl-residue between two fructofuranosyl-units, by which all the characteristics of the primary structure of inulin are present. To determine the conformation of the fructofuranosyl-rings, NMR data of inulin and the inulin related oligosaccharides 1-kestose, nystose and ß-D-fructofuranosyl-(2->1)-ß-D-fructofuranosyl-(2->1)-ß-D-fructofuranosyl-(2->1)-ß-D-fructofuranosyl-(2->1)-α-D-glucopyranoside (GF_4) are compared with the results of the MD calculations. As an extension of the known NMR data from 1-kestose and nystose, the complete assignment of the ^{1}H and ^{13}C NMR signals of GF_4 is reported here, obtained by the combined use of several homo- and heteronuclear 2D NMR experiments. The ^{1}H-^{1}H coupling constants were obtained by simulation of 600 MHz 1D spectra.

RESULTS AND DISCUSSION

MD calculations[17,18] were performed with nystose in water. In Fig. 2 the dihedral angles are shown, which determine the conformation of the glycosidic bonds within the period of simulation (run A and B). The dihedral angles F1—C-2—F1—C-1—F1—O-1—F2—C-2 (ψ_1) and F2—C-2—F2—C-1—F2—O-1—F3—C-2 (ψ_2) correspond mainly with the *trans* conformation, and G—O-5—G—C-1—G—O-1—F1—C-2 (φ_0) fluctuates around 100°. The other dihedral angles of the glycosidic linkages occupy more than one staggered conformation, reflecting the flexible behaviour of the glycosidic bonds of the molecule.

The overall shape of the molecule during run A remains roughly linear, whereas during run B it varies from curved, almost circular, *via* linear to a bent conformation again. Several dihedral angles change during both runs. The torsion angles which determine the orientation of the primary alcohol groups show many transitions in both calculations.

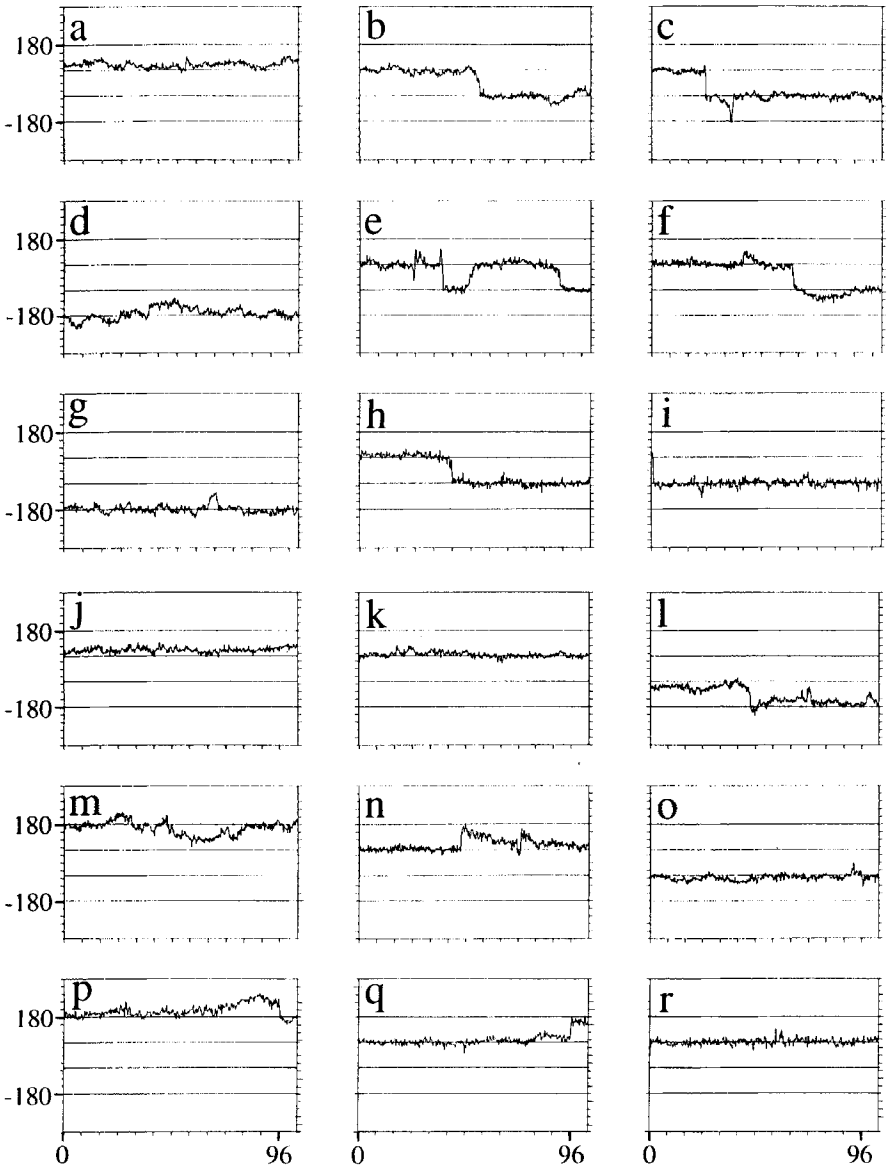

Fig. 2. The dihedral angles of nystose (Fig. 1) φ_0, Ψ_0, ω_1, Ψ_1, φ_1, ω_2, Ψ_2, φ_2, *and* ω_3, *which determine the conformation of the backbone, are depicted as a function of time for run B (a-i) and run A (j-r), respectively.*

The puckering of the fructofuranosyl-rings have been calculated on basis of the five dihedral angles.[19,20] Many transitions are observed from the north $^4T_3$-^4E pucker to conformations around $^0T_2$ and $^3T_2$ for runs A and B, respectively. As expected, the glucopyranosyl-ring adopts a rigid $^4C_1$ chair.

Table I. $^3J_{HH}$ *Coupling constants of the protons of nystose which determine the ring structures, measured by NMR and calculated from MD data.*

	G				F1		F2		F3	
	H1-H2	H2-H3	H3-H4	H4-H5	H3-H4	H4-H5	H3-H4	H4-H5	H3-H4	H4-H5
run A	2.6	10.0	9.2	9.3	8.8	8.1	5.9	6.1	7.9	7.1
run B	2.6	9.9	9.1	9.3	6.5	6.5	4.8	5.0	6.7	5.8
experimental	3.9	10.3	9.0	10.0	8.7	8.6	8.4	8.1	8.6	7.9

To compare the calculated ring-structures with experimentally obtained parameters, coupling constants were calculated from the dihedral angles of the theoretical ring conformations using an improved Karplus relation.[21] The $^3J_{HH}$ values from all time steps of the simulation were averaged with the exception of the equilibration period of the first 30 ps. These averaged coupling constants were compared with the $^3J_{HH}$ values measured by means of NMR spectroscopy[22] (Table I).

From the calculated data of the glucopyranosyl-ring only the $^3J_{1,2}$ value differs more than 1 Hz from the experimental one. The theoretical $^3J_{3,4}$ and $^3J_{4,5}$ values belonging to the F1 and F3 fructofuranosyl-rings are in close match with the experimental data. However, for run B a significant discrepancy has been observed. The largest difference is found for F2 in both runs. From these results it can be concluded that the GROMOS force field has to be optimized to make calculations of the fructofuranosyl-rings more consistent with NMR data.

To determine the conformation of the fructofuranosyl-rings of inulin and inulin related oligosaccharides, the coupling constants from which the ring-puckering can be derived, have been measured by NMR. Literature data have been used for 1-kestose and nystose. ^{13}C and ^1H NMR signals for GF_4, isolated from Jerusalem Artichoke and considered to be pure on the basis of HPLC (Dionex), have been firmly assigned by several 1D and 2D techniques. In Table II these data are summarized, together with the coupling constants, obtained by simulation of the ^1H NMR spectrum.

The spin systems in the ^1H NMR spectrum have been assigned using a 2D HOHAHA spectrum, as described earlier for nystose.[22] HMQC[23] and HMBC[24] techniques have been applied to distinguish between the various fructofuranosyl residues of GF_4.

In the HMBC spectrum the fructosyl C-2 signal at 104.50 ppm has a cross peak with G-H-1 and stems therefore from the fructofuranosyl residue (F1) attached to the glucopyranosyl ring. Cross peaks of the H-3 and H-1,1' protons with this F1-C-2 signal shows

Table II[a]. ^{1}H chemical shift values of GF_4

ppm	G	F1	F2	F3	F4
H-1	5.431	3.728	3.730	3.720	3.684
H-1'	-	3.836	3.879	3.883	3.752
H-2	3.535	-	-	-	-
H-3	3.761	4.274	4.217	4.230	4.187
H-4	3.468	4.043	4.074	4.098	4.101
H-5	3.84	3.88	3.86	3.86	3.86
H-6	3.81	3.79	3.76	3.74	3.75
H-6'	3.81	3.81	3.82	3.83	3.84

Table II[b]. ^{13}C chemical shift values of GF_4

ppm	G	F1	F2	F3	F4
C-1	93.78	62.36	62.23	62.00	61.80
C-2	72.48	104.50	104.32	104.32	104.96
C-3	73.90	78.10	78.85	78.69	78.10
C-4	70.55	75.19	75.63	75.63	75.76
C-5	73.74	82.55	82.39	82.39	82.39
C-6	61.46	63.48	63.48	63.48	63.48

Table II[c]. ^{1}H-^{1}H coupling constant values of GF_4.

Hz	G	F1	F2	F3	F4
H-1-H-1'	-	-10.1	-10.5	-10.3	-12.2
H-1-H-2	3.9	-	-	-	-
H-2-H-3	10.4	-	-	-	-
H-3-H-4	9.3	8.7	8.2	8.2	8.2
H-4-H-5	9.4	8.5	8.1	8.1	8.1
H-5-H-6'	3[b]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]
H-5-H-6	3[b]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]

a, Can not be determined because of overlap.
b, Averaged values due to degeneration of the H-6 resonances.

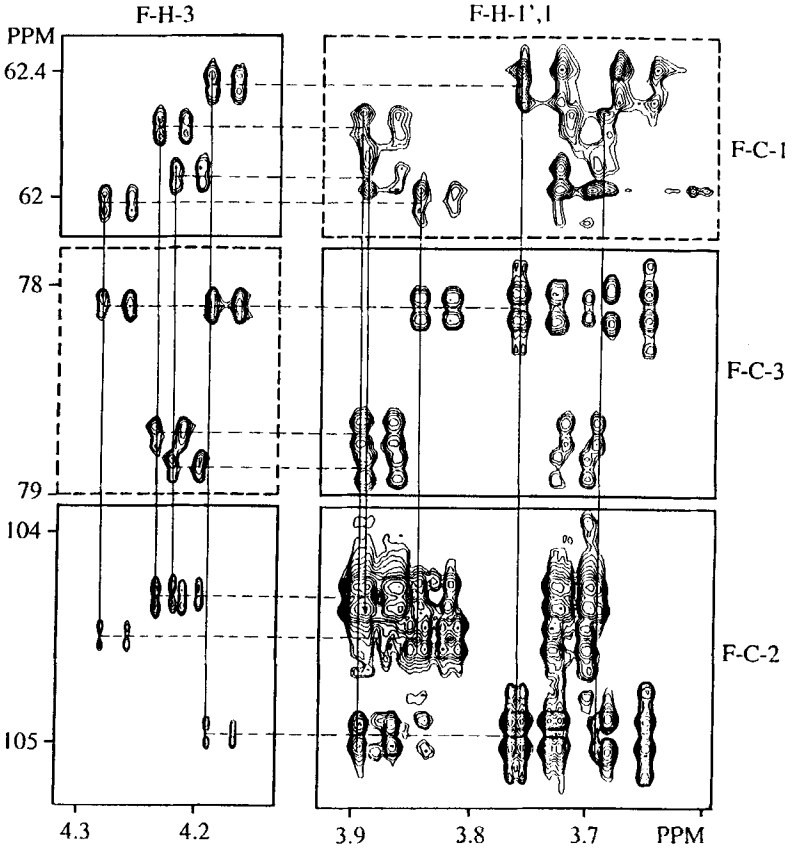

Fig. 3. 400 MHz HMBC spectrum of GF_4. The areas surrounded by dotted lines are taken from a 400 MHz HMQC spectrum of GF_4 and are shifted over $^1J_{CH}/2$. The left areas contain cross peaks with the F-H-3 protons and the areas on the right with F-H-1,1'. These protons have cross peaks with the F-C-1 signals (top), the F-C-3 signals (middle), and the F-C-2 signals (bottom).

these atoms belong to the same F1-residue. The F-H-1,1' protons at 3.752 ppm and 3.684 ppm have cross peaks only with one C-2 of a fructofuranosyl ring. This means that this carbon atom has to be of the terminal fructofuranose unit (F4). On the track of this F4-C-2 resonance cross peaks can also be found with an other H-1,1' spin system and a F-H-3 proton, thereby identifying the third fructofuranosyl residue (F3). The F3-H-1,1' resonances give cross peaks with F3-C-3 in the HMBC spectrum and F3-C-1 in the HMQC spectrum (Fig. 3). F3-H-3 can be assigned from a cross peak with F3-C-1 in the HMBC spectrum and from a cross peak with F3-C-3 in the HMQC spectrum. This enables discrimination between the H-3 signals of

the F2 and F3 residues, which is not possible from the HMBC spectrum alone, because the signals for F2-C-2 and F3-C-2 coincide. This completes the ^{1}H assignment of GF_4. The assumption that the sequence of signals in the HMBC and HMQC spectra are the same is confirmed by a HMBC spectrum without suppression of the one bond coherences.[25] Using the ^{1}H NMR data, the HMQC spectrum enables the complete ^{13}C assignment of GF_4. With respect to previous investigations[15] the assignments of the C-2 atoms of F1 and F4 have to be interchanged as well as those of C-1 of F1 and F2.

In order to obtain accurate coupling constant values, which are important for conformational analysis, the experimental 600 MHz ^{1}H spectrum of GF_4 has been simulated. Chemical shifts and coupling constants have been optimized by an iterative process.[26] Because of overlap in the 1D spectra of GF_4 the coupling constants with the fructofuranosyl H-6 protons could not be determined unambiguously. The agreement between the observed and the theoretical spectra is good in the region of interest.

From the experimental $J_{3,4}$ and $J_{4,5}$ values of 8 - 9 Hz, and the corresponding torsion angles, it can be concluded that all the fructofuranosyl-rings exist in a conformation close to $^{4}T_3$. This $^{4}T_3$ conformation has also been found for the fructofuranosyl residues of 1-kestose[9] in solution, and for fructose rings *in vacuo*.[27] The coupling constants of sucrose, 1-kestose, nystose[22] and inulin are in the same region. This points therefore to a general north conformation for the fructofuranosyl rings in inulin related oligosaccharides.

CONCLUSION

The ^{1}H and ^{13}C NMR signals of GF_4 have been assigned completely. The $^{4}T_3$ conformation seems to be general for the fructofuranosyl-rings in inulin and inulin related oligosaccharides in solution. From MD calculations it is concluded that the glycosidic linkages in nystose are flexible. The GROMOS force field used, has to be adapted to give better agreement with the conformation of the fructofuranosyl rings based on experimental NMR data. Further experimental studies on the conformational behaviour of the glycosidic linkages are needed to improve the model for hydrated inulin.

EXPERIMENTAL

General Methods. ^{1}H and ^{13}C NMR spectra were recorded on BRUKER AMX-400-WB (ATO-DLO/RIKILT-DLO, Wageningen), BRUKER AM-500 (Department of NMR spectroscopy, Utrecht University), and BRUKER AM-600 (SON high resolution NMR facility, Nijmegen) spectrometers. Prior to NMR spectroscopy 5 - 30 mg of the samples were

exchanged in 99.75%D D_2O with intermediate lyophilisation, finally using 99.96%D D_2O. The probe temperature was kept at 300 K. Chemical shifts (δ) are expressed in ppm downfield from 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) but were actually measured relative to internal acetone (2.225 ppm for ^1H spectra and 31.55 ppm for ^{13}C spectra). Processing of NMR-data was performed on a μVAX/VMS cluster with the Triton 2D-3D-NMR software package. Molecular Dynamics calculations were performed using the GROMOS software package[18] on a μVAX/VMS cluster.

For separation on a DIONEX unit, eluents were prepared using Milli-Q quality water, 50% carbonate free aqueous sodium hydroxide and anhydrous sodium acetate. A CarboPac PA1 (4 x 250 mm) column was used together with a CarboPac PA1 (3 x 25 mm) guard column. The system was equipped with a DIONEX Pulsed Electrochemical Detector.

Isolation of GF_4. Inulin from Jerusalem Artichoke was precipitated from a 85% aqueous ethanol solution and the precipitate removed by filtration. After concentration of the filtrate, GF_4 was isolated from the resulting mixture by HPLC using a semi-preparative RP 18 HPLC column, with an eluent flow (water) of 9 mL/min at room temperature.[28]

The isolated fractions were analyzed on a Carbopac PA1 column (4 x 250 mm) by means of anionic column chromatography, eluting with a gradient of 0.1 M aqueous sodium hydroxide, 0.5 M aqueous sodium acetate, and water running from 20/5/75 to 20/80/0 by volume in 45 minutes.

NMR measurements. A 100 MHz 1D ^{13}C- and a 600 MHz 1D ^1H spectrum of GF_4 and a 500 MHz 1D ^1H spectrum of inulin were recorded. At 500 MHz a 2D HOHAHA of GF_4 was recorded by acquisition of 512 experiments of 2K data points and a spectral width of 1500 Hz in both dimensions. At 400 MHz HMQC,[23] and HMBC[24] spectra of GF_4 were obtained. In an additional HMBC experiment the one bond correlations were not suppressed.[25] With a time domain of 2 K data points for each spectrum, 2048 experiments were used of 40, 64, and 60 scans, respectively. Magnitude calculation was performed for the HMBC experiments in the ω2 dimension. The delay for developing long range coherences was 100 ms.

Simulation of ^1H NMR. spectra. Subspectra arising from each spin system of GF_4 were simulated by a local version of a LAOCOON program[26] on a μVAX/VMs cluster. In order to obtain a complete ^1H spectrum, the simulated subspectra were scaled and superimposed.

Molecular dynamics calculations.[17] The GROMOS[18] program package was used for the MD calculations on nystose. For the anomeric C-2 atoms of the fructofuranosyl units, extensions to the standard force field were applied as described in the literature.[29] Two

simulations of 104 ps and 100 ps, referred to as run A and B, respectively, were performed using a different starting conformation. The structures used as starting points were built from a combination of a MM structure of inulobiose[8] and the crystal structure of 1-kestose.[6] For run A, dihedral angles of the fructofuranosyl glycosidic linkages were set according to the values of the MM structure of inulobiose. The values from the crystal structure of 1-kestose were used for run B. For the latter, the dihedral angles F2—C-2—F2—C-1—F2—O-1—F3—C-2 and F3—C-4—F3—C-5—F3—C-6—F3—O-6 were changed from 170 to 182 degrees and from 63 to 86 degrees, respectively. This was needed to increase the distance F3-C6—F1-O4 from 1.23, which is shorter than a covalent bond, to 2.01 Å in order to decrease repulsion. The molecules were placed in a periodic box (truncated octahedron). After filling the boxes with 387 and 356 water molecules for run A and B, respectively, the energy of the system was minimized. The simulation was started with velocities taken from a Maxwellian distribution at 300 K.

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