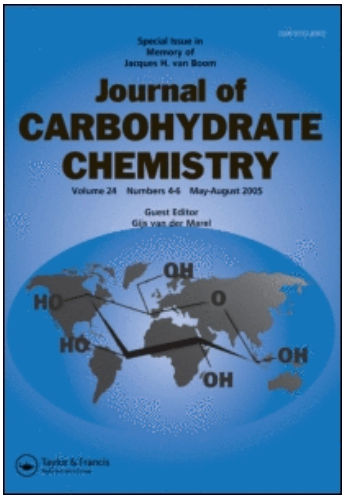

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ISOLATION AND STRUCTURAL ANALYSIS OF NEW FRUCTANS PRODUCED BY CHICORY

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ABSTRACT

This report describes a new series of oligosaccharides, which is formed in chicory roots under forcing conditions and during *in vitro* experiments using purified chicory 1-FFT (fructan:fructan 1-fructosyl transferase). It was demonstrated that the three smallest members of this new series (disaccharide, trisaccharide and tetrasaccharide) contain exclusively β-D-fructosyl residues after hydrolysis. The present data demonstrate that the smallest compound is levanbiose and that the other oligomers of this new series of fructans do not belong to the linear 2→6 linked levan-oligosaccharides nor to the linear 2→1 linked inulo-oligosaccharides. A combination of several chromatographic techniques yielded a fraction that contained only the compound with degree of polymerisation (DP) 2 (levanbiose, β-D-fructofuranosyl-(2→6)-D-fructofuranose), and a mixture of DP 3 of the new series and 1-kestose. Using homonuclear and heteronuclear 2D NMR experiments the complete ^{1}H and ^{13}C NMR assignments of levanbiose and DP 3 were obtained. From High Performance Anion Exchange Chromatography (HPAEC) and NMR experiments of DP 3 of the new series it was concluded that the molecule contains a β-D-fructofuranosyl residue 2→1 linked to the non-reducing moiety of levanbiose, and thus has to be named β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl-(2→6)-D-fructofuranose. The simple and regular pattern of the HPAEC retention times of the new oligosaccharides suggests that it is a homologous series of oligomers build by 2→1 elongation with β-D-fructofuranosyl residues at the non-reducing residue of levanbiose.

INTRODUCTION

During the last decade many researchers world-wide have sought for new food and non-food applications for fructans. Many crops and microorganisms produce fructans with structures (Figure 1) that are strongly species-specific. Knowledge of these structures will facilitate the development of applications. Fructans with predominately 2→1 linked or 2→6 linked β-D-fructofuranosyl residues are named in-

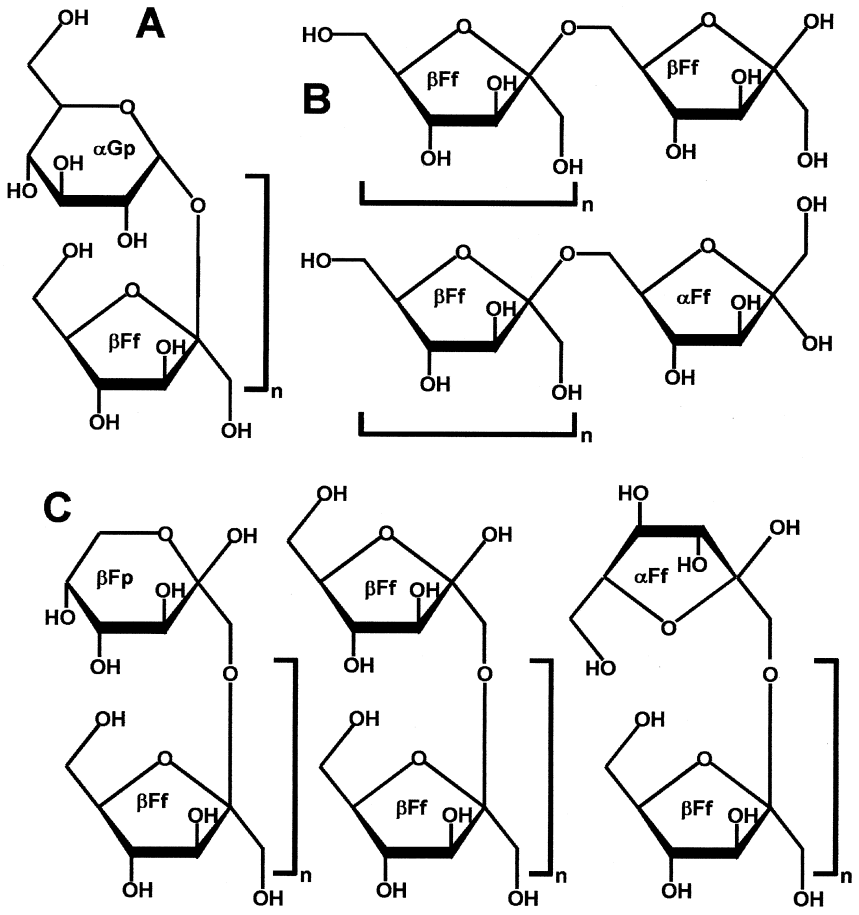

Figure 1. The structures of fructans: the non-reducing inulin oligosaccharides which consist of (2→1)-linked β-D-fructofuranosyl residues, terminated by an α-D-glucopyranosyl unit in a (1↔2) linkage are depicted in 1A and will be designated GF_n. Fructosyl-only levan oligosaccharides (1B) consist of 2→6 linked β-D-fructofuranosyl residues and are terminated by an α-D-fructofuranosyl or a β-D-fructofuranosyl residue and will be designated F^{l}_{n}. In 1C the Inulo-oligosaccharides, designated F^{i}_{n}, are depicted which consist of 2→1 linked β-D-fructofuranosyl residues, terminated by an α-D-fructofuranosyl residue, a β-D-fructofuranosyl residue or a β-D-fructopyranosyl residue. Fructosyl-only fructans with both 2→1 and 2→6 linkages, which belong to the graminans, will be designated F^{g}_{n}.

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ulin or levan, respectively. *Poaceae* and *Liliaceae* fructans contain both 2→1 and 2→6 linkages(1–3) and are named graminan.(3) Inulin is most often a mixture of linear oligo- or polysaccharides which consist of (2→1)-linked β-D-fructofuranosyl residues, terminated by an α-D-glucopyranosyl unit in a (1↔2) linkage (GF_n, Figure 1). Many reports are available which describe inulin oligosaccharides that consist of (2→1)-linked fructofuranosyl residues only(4–6) (F_n, Figure 1). These oligosaccharides are often named inulo-oligosaccharides or inulo-n-oses.

Inulin isolated from chicory contains mainly oligosaccharides of the homologous GF_n series. A relatively small part of chicory inulin consists of oligosacharides of the F_n series.(6) The ratio GF_n / F_n and the average degree of polymerisation ($\overline{DP}$) is dependent on harvest time and storage conditions of the chicory roots.(7) Recently, it was concluded from HPAEC-PAD experiments that inulin obtained from forced chicory roots also contains an additional third series of fructan.(8) At least the three smallest of these carbohydrates consist only of fructosyl residues. Hitherto, the structure of this new fructan type was unknown. Data presented in this work show that the disaccharide is levanbiose (β-D-fructofuranosyl-(2→6)-D-fructofuranose, Figure 2a) and the trisaccharide is a, until now, not described derivative thereof (β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl-(2→6)-D-fructofuranose, Figure 2b). This is the first report of the occurrence of levanbiose in chicory, remarkable because chicory produces normally only fructans with exclusively 2→1 linkages between the β-D-fructofuranosyl residues.

The structure of the new trisaccharide was determined using NMR spectroscopy. In order to facilitate the assignment of the NMR signals of the new trisaccharide, the complete ^{1}H and ^{13}C NMR assignments of the disaccharide were obtained. The assignments of the ^{13}C NMR signals of the inner residues in levan are available from the literature.(9) For the ^{13}C NMR signals of the α-anomer of levanbiose (Figure 2) and some of the ^{13}C NMR signals of the β-anomer of levanbiose, assignments were obtained(10) by comparison with ^{13}C NMR data of related compounds. The assignments of four of these signals had to be corrected. This is the first report of the assignment of the ^{1}H NMR signals of levanbiose and the ^{1}H and ^{13}C NMR signals of the new trisaccharide (β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl-(2→6)-D-fructofuranose). All ^{1}H and ^{13}C NMR signals of both anomeric forms of both compounds have now been assigned to specific atoms.

By simulation of the experimentally obtained ^{1}H NMR signals of levanbiose, accurate coupling constants were obtained which contain information on the three dimensional structure of levanbiose in aqueous solution. Finally, the dependency of the ^{1}H NMR signals of levanbiose on the barium chloride concentration was determined. These data may contain information on the three dimensional structure of this disaccharide.(11)

RESULTS AND DISCUSSION

In order to obtain pure levanbiose the isolation procedure started with an α-glucosidase digestion of a raw extract of carbohydrates isolated from forced

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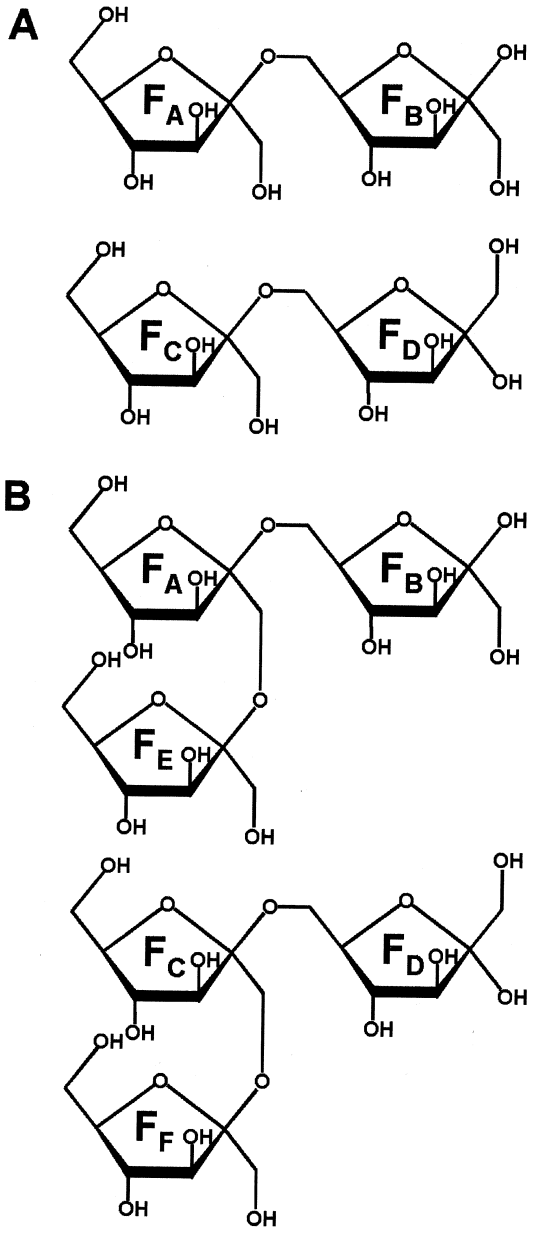

Figure 2. Structure of the two anomeric forms of levanbiose (A) and (β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl-(2→6)-D-fructofuranose (B).

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chicory roots. After this digestion the sucrose to levanbiose ratio was decreased by 88 % according to HPAEC-PAD analysis (Figure 3). Further fractionation on an ion-exchange column, in the Ca^{2+} form, resulted in a levanbiose and the new trisaccharide (β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl-(2→6)-D-fructofuranose) containing sample (Figure 3) with a reduction in the inulobiose to levan-

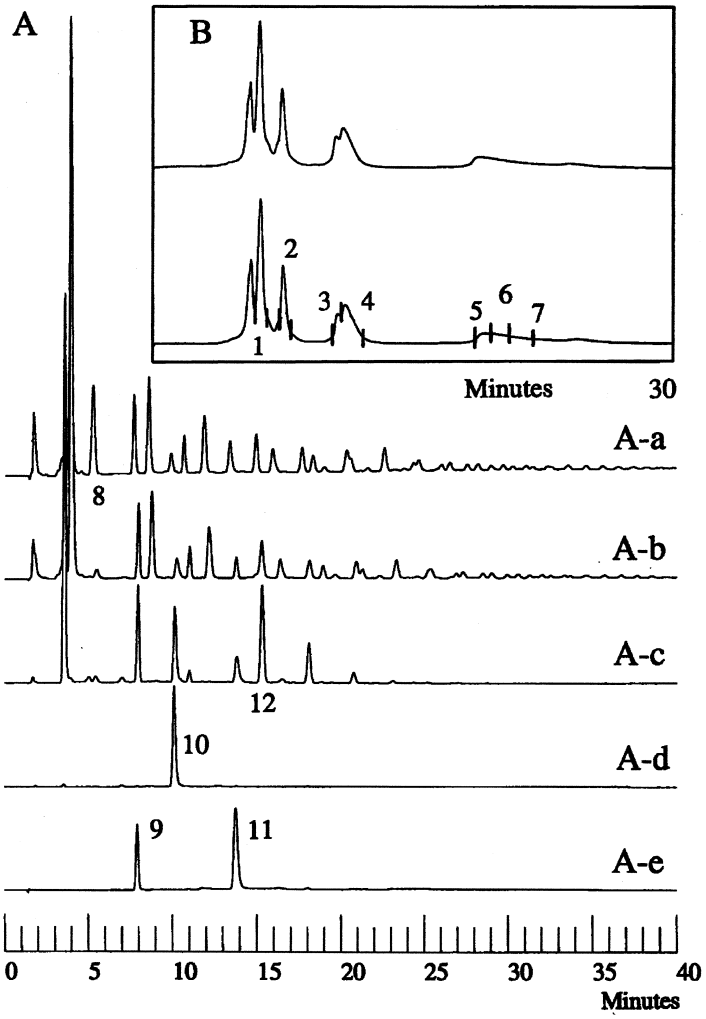

Figure 3. A: HPAEC-PAD chromatogram of the carbohydrate mixture produced by forced chicory roots (A-a), after the digestion with α-glucosidase (A-b), after fractionation on an ion-exchange column (A-c), and fractions obtained by RP-18 HPLC fractionation (A-d and A-e) of fraction A-c. B: RP-18 HPLC chromatogram obtained by the isolation of levanbiose and the new trisaccharide. Compounds: 1 = monosaccharides, 2,10 = levanbiose (β-D-fructofuranosyl-(2→6)-D-fructofuranose), 3,11 = the new trisaccharide (β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl-(2→6)-D-fructofuranose), 4,9 = 1-kestose (GF_2, Figure 1), 5-7,12 = F_4^i (Figure 1), 8 = sucrose (GF, Figure 1).

biose ratio by 99.6 %, due to the relative retention of inulobiose on the column. This sample was finally fractionated using RP-18 HPLC. An almost pure fraction of levanbiose was obtained together with a fraction that contained the new trisaccharide and 1-kestose in a molar ratio of approximately 2:1 according to HPAEC-PAD analysis. This was in good agreement with ^{13}C NMR analysis of the C2 signals.

In a prior attempt to isolate levanbiose, a comparable procedure has been used as described in the experimental part but without the digestion by α-glucosidase. As a result thereof a fraction of levanbiose was obtained which was severely contaminated by three fold of sucrose, and appeared to be not suitable for a complete NMR analysis. Besides the sucrose content, the composition of the fractions obtained after α-glucosidase digestion did not differ significantly from those obtained without the enzyme digestion, according to HPAEC-PAD analysis.

Retention times using HPAEC-PAD analysis of the new fructan oligomers with DP up to 8 were compared with those of non-reducing inulin oligosaccharides, inulo-oligosaccharides, and fructose-only levan oligosacharides. The retention time of the new chicory dimer was the same for levanbiose. Longer oligomers of the new fructans did not overlap with oligomers of the other series, with the exception of F_3^g (same retention time as GF_4) and F_4^g (same retention time as GF_5). The precise values of the retention times obtained in this work were slightly dependent on composition and concentration of the sample and on the precise composition of the solvent and the temperature, but in all cases the retention times of the carbohydrates (Figure 1) had the following sequence: $G < F < GF < GF_2 < F_2^i < F_2^l < GF_3 < F_3^i < GF_4$, $F_3^g < F_3^l < F_4^i < GF_5$, $F_4^g < F_4^l$, $F_5^i < GF_6 < F_5^g < F_6^i < GF_7$, $F_5^l < F_6^g < GF_8$, $F_7^i < F_6^l < F_7^g < GF_9 < F_8^i < F_8^g$. In the literature(8) another HPAEC-PAD gradient was used that showed that the trisaccharide and tetrasaccharide of the new fructan series does not belong to the non-reducing inulin oligosaccharides.

HPAEC-PAD analysis of hydrolysis products of the three smallest members of the new series (DP 2–4, in the literature(8) referred to as x, y, and z) showed that they only contain fructose residues.(8) From all these it can be concluded that the new trisaccharide is neither levantriose nor inulotriose and that the new tetrasaccharide is neither levantetraose nor inulotetraose, although these new oligosaccharides were shown to contain only fructose after hydrolysis.

The set of obtained ^{13}C NMR frequencies of the new chicory disaccharide is consistent with the set of ^{13}C NMR frequencies of levanbiose.(10) From the chemical shift values and the relative intensities of the ^{13}C NMR signals of the C2 atoms it can be concluded that the disaccharide has two anomeric forms in aqueous solution due to the existence of a reducing end, with a β / α ratio of 1:3.9, and that the disaccharide contains one non-reducing fructofuranosyl residue, for both anomeric forms. The absence of ^{13}C NMR signals in the region 74–66 ppm, being typical for C3, C4 and C5 atoms of fructopyranose residues,(1,4,12,13) shows that the reducing end has no free 6-OH group. This confirms that the reducing end is substituted at C6, and that the disaccharide is definitely levanbiose.

The ^{13}C NMR spectrum of the new trisaccharide is very close to that of levanbiose with six signals extra with frequencies typical for an additional non-reducing β-D-fructofuranosyl residue. Thus the trisaccharide contains a levanbiose moiety and an extra 2→1 linked β-D-fructofuranosyl moiety. From all these observations, however, it cannot be concluded whether the extra fructofuranosyl residue is 2→1 linked to the reducing or the non-reducing fructofuranosyl residue of the levanbiose moiety. This can be solved using a heteronuclear 2D NMR technique (HMBC), providing inter-residue correlations. For this purpose, first the complete sequential assignment of the ^{1}H and ^{13}C NMR signals of levanbiose was determined (Table 1 and 2).

For assignment of the ^{1}H and ^{13}C NMR signals of levanbiose, a strategy was used similar to the spectrum interpretation of GF_3 and GF_4 as described elsewhere,(14,15) using homonuclear and heteronuclear 2D NMR spectra. However, due to severe overlap the assignment of the individual spin systems and the sequential analysis could not be done separately, and was too complex to describe here in detail. Furthermore, due to the severe overlap, a third dimension had to be added to the ^{13}C and ^{1}H chemical shift values. The introduction of a third dimension was achieved by stepwise addition of barium chloride. This addition results in

Table 1. ^{1}H NMR Assignments and Coupling Constants for Levanbiose. See Figure 2a for Labelling of Fructosyl Residues

$[Ba^{2+}]$	0	0.29	0.62		0	0.29	0.62
$F_A-\delta$				$F_A-{}^{3}J_{H,H}$			
H1′	3.748	3.744	3.749	$J_{1',1}$	−12.2	−12.2	−12.4
H1	3.691	3.717	3.742	$J_{3,4}$	7.8	8.7	7.8
H3	4.171	4.181	4.202	$J_{4,5}$	8.3	7.7	8.1
H4	4.103	4.117	4.138	$J_{5,6}$	3.3	3.4	3.2
H5	3.867	3.876	3.890	$J_{5,6}$	6.7	7.1	6.8
H6′	3.805	3.805	3.804	$J_{6,6}$	−12.0	−12.3	−12.3
H6	3.677	3.666	3.656				
$F_B-\delta$				$F_B-{}^{3}J_{H,H}$			
H1′	3.586	3.598	3.621	$J_{1,1}$	−12.2	−12.1	−12.1
H1	3.543	3.571	3.607	$J_{3,4}$	7.7	8.2	8.1
H3	4.103	4.093	4.082	$J_{4,5}$	9.8	7.3	7.5
H4	4.122	4.128	4.133	$J_{5,6}$	3.2	2.7	2.7
H5	3.886	3.909	3.933	$J_{5,6}$	6.8	7.4	7.7
H6′	3.915	3.935	3.961	$J_{6,6}$	−10.8	−11.5	−10.4
H6	3.636	3.628	3.625				
$F_D-\delta$				$F_D-{}^{3}J_{H,H}$			
H1′	3.653	3.668	3.689	$J_{1,1}$	−12.3	−12.3	−12.3
H1	3.653	3.668	3.683	$J_{3,4}$	4.6	4.9	4.5
H3	4.084	4.099	4.118	$J_{4,5}$	6.2	6.7	6.6
H4	4.003	3.996	3.991	$J_{5,6}$	3.1	3.1	2.9
H5	4.148	4.170	4.194	$J_{5,6}$	6.5	7.0	7.6
H6′	3.930	3.955	3.980	$J_{6,6}$	−10.7	−10.5	−10.6
H6	3.647	3.638	3.632				

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Table 2. ^{13}C NMR Assignments for Levanbiose. See Figure 2a for Labelling of Fructosyl Residues

$[Ba^{2+}]$	0	0.10	0.20	0.29	0.39	0.50	0.62
$F_A-\delta$							
C1	61.38	61.38	61.39	61.40	61.38	61.38	61.42
C2	104.93	104.89	104.86	104.83	104.80	104.77	104.73
C3	78.18	78.25	78.32	78.39	78.44	78.51	78.60
C4	75.99	75.94	75.91	75.87	75.82	75.78	75.77
C5	82.49	82.46	82.43	82.41	82.38	82.36	82.33
C6	63.83	63.77	63.72	63.68	63.64	63.59	63.56
$F_B-\delta$							
C1	63.83	63.96	64.06	64.16	64.24	64.34	64.47
C2	103.10	103.09	103.09	103.09	103.09	103.09	103.09
C3	76.42	76.48	76.54	76.60	76.63	76.69	76.77
C4	76.09	76.05	76.02	76.00	75.95	75.92	75.91
C5	80.79	80.72	80.66	80.61	80.54	80.48	80.42
C6	63.83	63.81	63.81	63.82	63.81	63.81	63.81
$F_C-\delta$							
C1	61.38	61.38	61.39	61.40	61.38	61.38	61.42
C2	104.93	104.89	104.86	104.85	104.83	104.81	104.77
C3	78.12	78.23	78.32	78.39	78.44	78.51	78.60
C4	76.09	76.05	76.02	76.00	75.91	75.84	75.84
C5	82.49	82.46	82.46	82.46	82.43	82.40	82.39
C6	63.83	63.77	63.72	63.68	63.64	63.59	63.56
$F_D-\delta$							
C1	64.00	63.96	64.06	64.05	64.06	64.07	64.10
C2	106.28	106.29	106.31	106.32	106.35	106.35	106.33
C3	82.77	82.74	82.72	82.70	82.67	82.64	82.63
C4	78.07	78.07	78.07	78.07	78.06	78.06	78.05
C5	82.12	82.10	82.08	82.06	82.03	82.01	81.98
C6	62.80	62.79	62.78	62.77	62.76	62.74	62.73

changes of the chemical shift values that are different for the various ^1H and ^{13}C atoms. The change in chemical shift appeared to be linear with increasing barium chloride concentrations for both ^1H signals (Figure 4b) and ^{13}C signals (Figure 4a), similar to those described for inulin oligosaccharides.(11) This linearity made it easy to correlate a region with low overlap in a specific 2D NMR spectrum (obtained at a certain barium chloride concentration), to a region with low overlap in another 2D NMR spectrum (measured using a different barium chloride concentration).

For sequential assignment the anomeric region in the HMBC is crucial. In this region also cross peaks were found showing a correlation between the C2 signal of the non-reducing end with the H6 signals of the two anomeric forms of the reducing end. These cross peaks prove the existence of a 2→6 linkage.

In Figure 5, the anomeric region is depicted of spectra obtained for levanbiose, without barium chloride (sample BA0), and with barium chloride (samples BA3 and BA6, with Ba^{2+} concentrations of 0.29 mol / L and 0.62 mol / L, respectively). An easy starting point for the assignment is formed by the C2 signals. From

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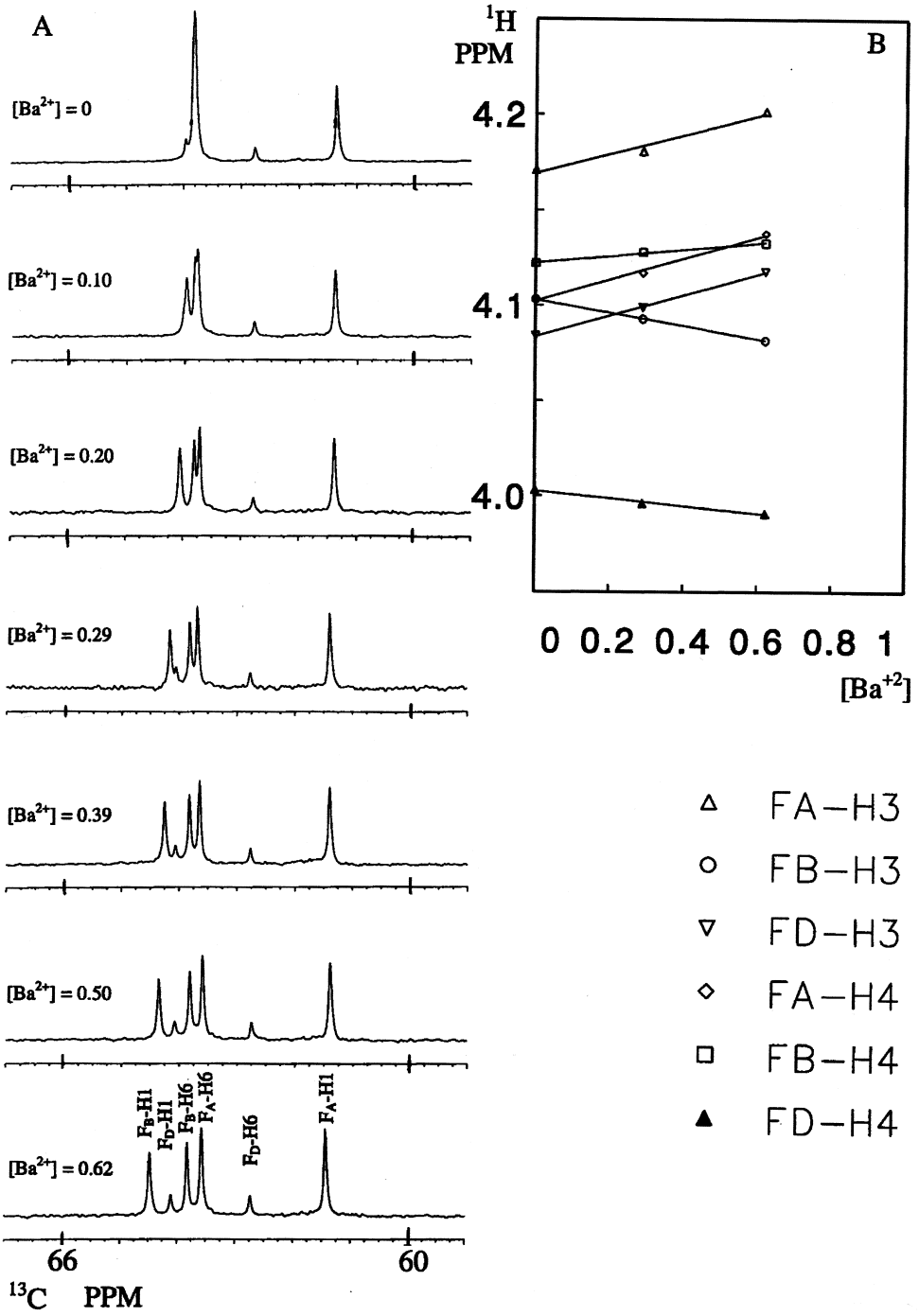

Figure 4. Dependence of ^{13}C chemical shift values (A) and ^{1}H chemical shift values (B) on the Ba^{2+} concentration.

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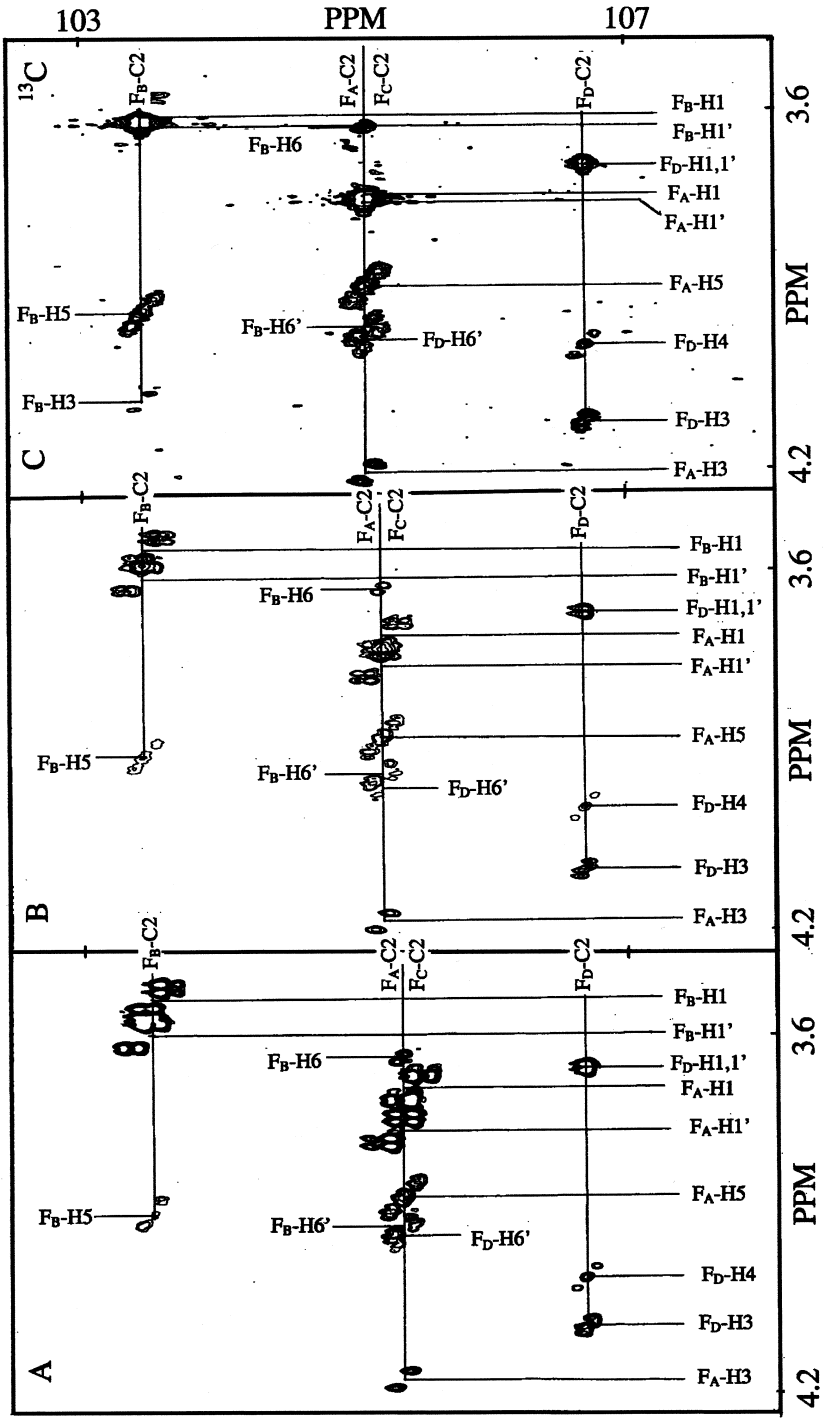

Figure 5. HMBC spectra of β-D-fructofuranosyl-(2→6)-D-fructofuranose obtained using three different $BaCl_2$ concentrations (A: $[Ba^{2+}] = 0$, B: $[Ba^{2+}] = 0.29$ and C: $[Ba^{2+}] = 0.62$ mol / L. Only the area that contains cross peaks with the C2 resonances are shown.

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the relative intensities, and also from the chemical shift values, it is clear that the signals with the lowest and the highest frequencies (in the anomeric region) belong to the two anomer forms of the reducing end. The remaining C2 signal belongs to the non-reducing fructofuranosyl residue for both anomer forms, which have apparently the same frequency. Thereby, the sequential assignment of the anomer signals is complete. For sequential analysis, the H1 signals are also important. They are easily recognised in a COSY spectrum because they only show cross peaks with H1 atoms that belong to the same CH_2-group. In the anomeric region of the HMBC spectra (Figure 5) each C2 atom showed cross peaks with just one couple of H1 atoms. This supports the absence of a 2→1 linkage and, therefore, the existence of a 2→6 linkage. Furthermore, these cross peaks enabled the sequential analysis of the H1 signals.

The H3 signals are recognised by their coupling patterns in relation to their chemical shift values. Because the H3 atoms can give only a cross peak with the C2 atoms of the same fructofuranosyl residue, they could be assigned sequentially. Because the H1 and H3 atoms give cross peaks with several other C atoms in the remaining part of the HMBC spectrum, those other C atoms could also be assigned sequentially. In order to obtain a complete assignment, it was also necessary to relate the ^{1}H signals to each other using the COSY- and relayed-COSY spectra. Because of severe overlap, it was often necessary to jump to comparable regions in spectra obtained at a different barium chloride concentration. Finally, the first complete assignment of the ^{1}H and ^{13}C NMR signals of the two anomeric forms of levanbiose was obtained (Table 1 and 2).

Comparison of the here described assignment of the ^{13}C NMR signals of levanbiose with a ^{13}C NMR spectrum interpretation described in the literature,(10) shows that the assignments(10) of the C1 atoms of the two anomeric forms have to be interchanged (F_D-C1 and F_B-C1, see Figure 2a), as well as the assignment(10) of the C4 atom of the non-reducing residue with the C4 atom of the β-form of the reducing residue (F_A-C4 and F_B-C4, see Figure 2a).

Because part of the spectra of the new trisaccharide (β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl-(2→6)-D-fructofuranose) closely resembles the spectra of the disaccharide (levanbiose), and because overlap was less severe for the new trisaccharide, a complete sequential assignment could be obtained without COSY spectra and without the use of barium chloride. The spectra of the new trisaccharide also contained signals of 1-kestose. In most cases they had a lower intensity and could be excluded from the analysis because their assignments are known.(14)

The anomeric region of the HMBC spectrum of the new trisaccharide is depicted in Figure 6. Just as was observed for levanbiose, two easily recognised C2 signals of a reducing end showed cross peaks with just one couple of H1 atoms that belong to the same fructofuranosyl residue. The two remaining C2 signals are typically for non-reducing fructofuranosyl residues. One of those C2 atoms show cross peaks with the H6 atoms of the reducing end, comparable to the observation for the reducing end of levanbiose. Therefore, it can be concluded that this C2 atom is located at the non-reducing fructofuranosyl residue which is 2→6

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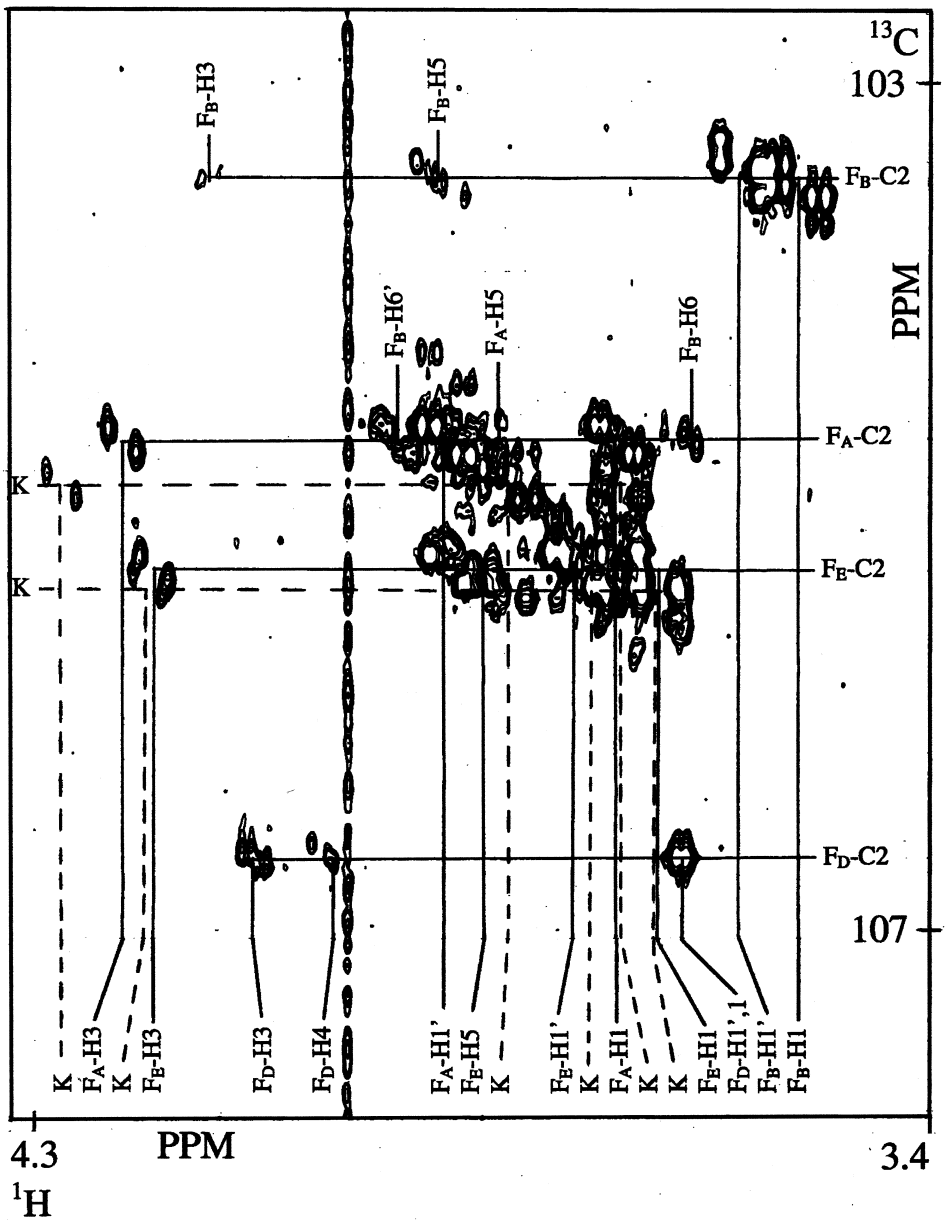

Figure 6. HMBC spectrum of β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl-(2→6)-D-fructofuranose. Only the area that contains cross peaks with the C2 resonances are shown. The signals with label K are from 1-kestose.

linked to a reducing fructofuranosyl residue. The C2 atom of this non-reducing fructofuranosyl residue shows a cross peak with just one couple of H1 atoms which, therefore, belong to the same residue. These H1 atoms show also cross peaks with the remaining non-reducing fructofuranosyl residue. This means that the second non-reducing fructofuranosyl residue is 2→1 linked to the first non-

Table 3. ^{1}H NMR Assignments of the Trisaccharide β-D-Fructofuranosyl-(2→1)-β-D-fructofuranosyl-(2→6)-D-fructo-furanose. See for Labelling of Fructosyl Residues Figure 2b

δ	F_A	F_B	F_D	F_E
H1′	3.884	3.594	3.652	3.755
H1	3.715	3.535	3.652	3.673
H3	4.211	4.102	4.078	4.180
H4	4.097	4.131	4.004	4.100
H5	3.850	3.893	4.147	3.854
H6′	3.798	3.935	3.946	3.765
H6	3.667	3.642	3.665	3.728

reducing fructofuranosyl residue. The C2 atom of this second non-reducing fructofuranosyl residue shows also cross peaks with a second couple of H1 atoms. They can, therefore, only be located in the second non-reducing fructofuranosyl residue. Hereby, a complete sequential assignment of the C2- and H1-signals is obtained. Comparable to the disaccharide, as in the case of the new trisaccharide, all C2 atoms gave cross peaks with the H3 atoms belonging to the same residue, resulting in a sequential assignment of all H3 signals. Using the sequential assignment of the H3 and H1 signals, the remaining part of the HMBC spectrum revealed the complete sequential assignment of the ^{1}H and ^{13}C NMR signals of the new trisaccharide (Table 3 and 4). This is the first structure determination of a fructofuranosyl-only trisaccharide which contains both a 2→1 and a 2→6 linkage. For simulation of the ^{1}H NMR spectra of levanbiose, all frequencies of the multiplets of all the protons were determined from the COSY spectra. This was done for the sample without barium chloride (BA0) and the samples with barium chloride added (BA3 and BA6, with Ba^{2+} concentrations of 0.29 mol / L and 0.62 mol / L, respectively). The simulated subspectra of the different spin systems were scaled and added, in order to obtain complete spectra, and compared with the experimentally obtained 1D ^{1}H NMR spectra (Figure 7). There is a good agreement between the simulated and experimentally obtained spectra given that 28 protons of levanbiose have signals in one very small spectral region of less than 0.7 ppm, and given that the frequencies were determined from 2D spectra, and given that it cannot be excluded that the small ^{1}H NMR sig-

Table 4. ^{13}C NMR Assignments of the Trisaccharide β-D-Fructofuranosyl-(2→1)-β-D-fructofuranosyl-(2→6)-D-fructo-furanose. See for Labelling of Fructosyl Residues Figure 2b

δ	F_A	F_B	F_C	F_D	F_E	F_F
C1	61.68	63.85	61.68	64.02	61.68	61.68
C2	104.33	103.11	104.33	106.31	104.94	104.94
C3	78.77	76.43	78.77	82.76	78.02	78.02
C4	75.93	76.12	76.00	78.08	75.61	75.61
C5	82.49	80.74	82.49	82.09	82.34	82.34
C6	63.75	63.96	63.75	62.97	63.48	63.48

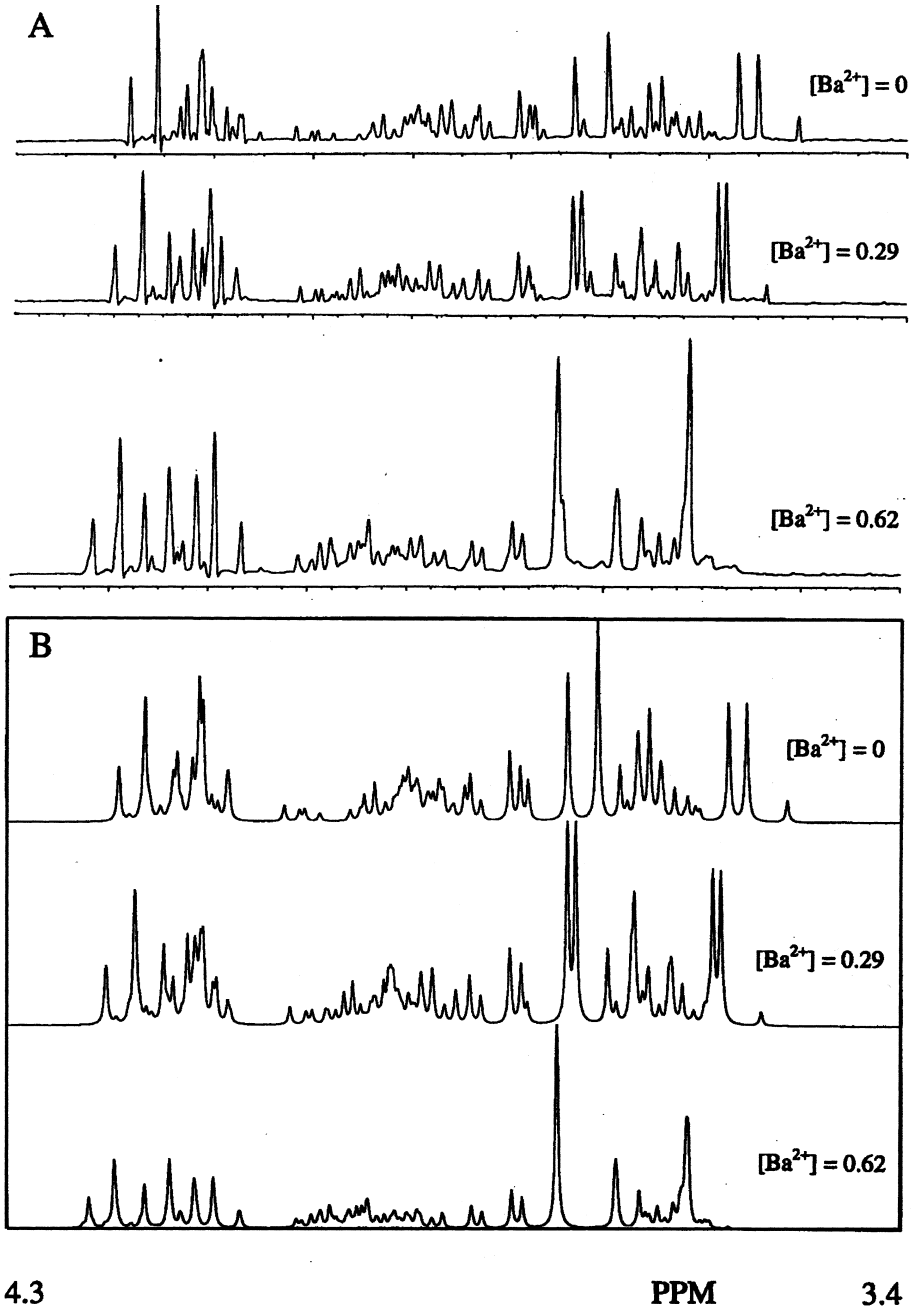

Figure 7. Experimental (A) and simulated (B) ^{1}H NMR spectra of levanbiose with Ba^{2+} concentrations of 0, 0.29 and 0.62 mol / L.

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nals of residue C (the non-reducing residue of the disaccharide with an α-anomeric reducing end, see Figure 2a) are slightly different from those of the more abundant residue A (the non-reducing residue of the disaccharide with a β-anomeric reducing end). This means that the determined coupling constants (Table 1) are useful for conformational analysis. For the new trisaccharide it was impossible to determine all frequencies of the multiplets of the different ^{1}H NMR signals. Therefore, for this compound no values for the coupling constants are given.

This report describes the first observation of 2→6 linkages in carbohydrates produced by chicory, and make it possible to speculate on the active site of the enzyme. It was shown(8) that these fructose-only oligosaccharides, with at that time unknown structure (designated x, y, z), were synthesised together with inulo-oligosaccharides both in forced chicory roots and in *in vitro* experiments. For the *in vitro* experiments chicory inulin was incubated using purified chicory 1-FFT together with high fructose concentrations. A large fructose / sucrose ratio is biologically relevant during sprouting and forcing conditions. Now it can be concluded that chicory 1-FFT uses fructose as acceptor to produce both inulobiose and levanbiose. This can be explained by the high degree of symmetry of fructose (Figure 8). The most common ring conformation of β-D-fructofuranose in aqueous solution is the $^{4}T_{3}$ structure.(15,16) After an imaginary rotation of 180 degrees of this molecule situated in the active site (for the acceptor residue) of chicory 1-FFT around an axis through the middle of C3–C4 and the ring oxygen O5, a comparable structure is obtained. Thereby the role of OH1 and OH6 is exchanged. Only the small OH2 group breaks this symmetry, resulting in a lower yield of levanbiose (and therefore the new fructan series) on comparison to inulobiose (and the inulo-oligosaccharides). If such a rotation is applied to the non-reducing residue of levanbiose, the symmetry is broken by the bulky reducing fructofuranosyl residue. Apparently, this results in severe steric hindrance because no levantriose formation was observed although it was demonstrated that inulobiose is a much better acceptor than fructose.(8)

The model described above for the substrate position in the 1-FFT active site might also explain the structure found for the new trisaccharide. Because the new trisaccharide is neither inulotriose nor levantriose it can theoretically only be produced by transferring a fructofuranosyl residue to OH1 present in both the reducing or the non-reducing fructofuranosyl residue of levanbiose. If the reducing end has to fit in the active site a bulky fructofuranosyl residue at the opposite site of the anomeric centre is present, which might prevent reaction. The non-reducing end has only a bulky group at the same site of the ring on O2, which is also the case for other good 1-FFT acceptors like inulo-oligosacharides, sucrose and inulin oligosaccharides containing a terminal glucose residue. Therefore, levanbiose will only be substituted by a third fructofuranosyl residue on the OH1 atom of the non-reducing end. It is reasonable to assume that only this third residue will act as acceptor to produce longer oligomers of this new fructan series. This assumption is supported by the simple and regular elution profile of these compounds during HPAEC-PAD analysis.

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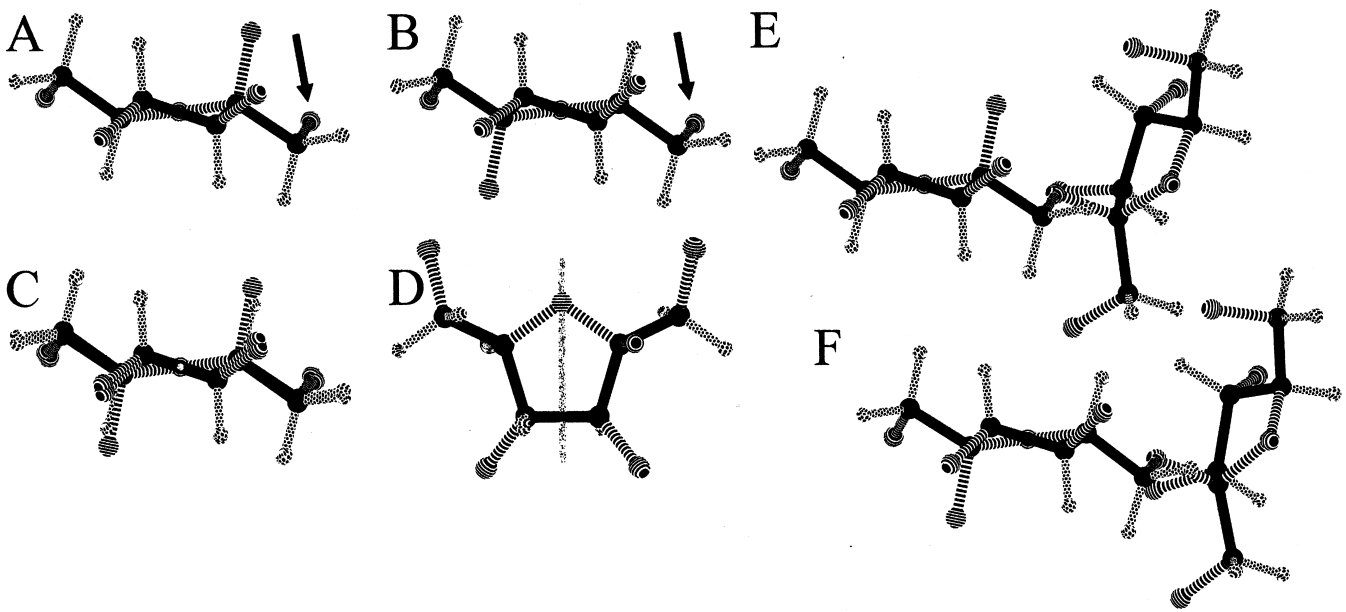

Figure 8. Visualisation of the high degree of symmetry of the fructose molecule in relation to the enzymic synthesis of both inulobiose and levanbiose. A: A fructose molecule with the conformation of the reducing end of inulobiose. B: A copy of the same molecule, rotated 180 degrees around an axis perpendicular to the plane of paper. C: A superposition of these two molecules. D: The same superposition, after 90 degrees vertical rotation of the axis of rotation as depicted in fig. C. E: Inulobiose, obtained from a Molecular Mechanics study.(17) F: Levanbiose, obtained from a Molecular Mechanics study.(18) Substitution of the acceptor molecule depicted in A at O1 (see arrow) will result in levanbiose (E). After 180 degrees rotation (B) the acceptor molecule will be substituted at O6 (see arrow), which results in levanbiose (F). To facilitate easy comparison, the hydroxymethyl groups on position six of the reducing ends are rotated -120 degrees towards another staggered conformation for all these structures. Molecular mechanics calculations show that these different staggered conformations are energetically accessible.([1]7,18) Although it will not prevent enzymic modification, it should be noted that, especially in the case of levanbiose, these are not the most abundant structures due to a higher energy content.

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EXPERIMENTAL

Digestion of Sucrose

An amount of 35 g carbohydrate syrup, obtained from forced chicory roots,(8) was dissolved by the addition of 80 mL aqueous buffer (0.1 M sodium acetate / acetic acid, 99:1, pH 6.6). After the addition of 300 U α-glucosidase (in 6 mL buffer, Boehringer-Mannheim GmbH) the solution was shaken for 12 h at 37°C. Subsequently, 100 U α-glucosidase (in 2 mL buffer) was added. After 3 h shaking at 37°C the solution was boiled for 5 min.

Fractionation of α-Glucosidase Digest

For fractionation of the oligosaccharides on a strongly acidic cation exchange (Dowex X2-400, 2% cross-linkage, 200–400 mesh, brought in Ca^{2+} form using 0.2 M $CaCl_2$ in water) column (30 × 5 cm) the obtained digest was diluted with water to a final volume of 125 mL. The flow of the eluent (water) was kept at 5 mL/min. For each of the 12 runs 10 mL was injected. Fractions were collected between 29 and 38 min, 38 and 46 min, 46 and 57 min, 57 and 68 min, 68 and 80 min, 80 and 91 min, 91 and 103 min, 103 and 114 min, and between 114 and 136 min. After each run of 160 min the column was regenerated by elution with 0.2 M $CaCl_2$ solution and water during 20 and 100 min, respectively. The pooled fraction collected between 80 and 91 min was lyophilised (yield 1.132 g) for further fractionation using reversed phase HPLC, and will be referred to as X2F6.

Isolation of Levanbiose and the New Trisaccharide (β-D-Fructofuranosyl-(2→1)-β-D-fructofuranosyl-(2→6)-D-fructofuranose)

The fraction (X2F6) of the α-glucosidase digest, obtained by cation exchange chromatography, with the highest concentration of levanbiose was dissolved in 9 mL water. After centrifugation at 12000 g during 15 min the supernatant was filtered (syringe filter, 0.45 μm) before fractionation on a reversed phase HPLC column (Alltech ECONOSIL C18, 250 × 22 mm, particle size 10 μm). For all separations the flow of the eluent (milli-Q quality water) was kept at 10 mL/min, and for each run (30 min at 21°C) 1 mL of the filtrate was injected. Fractions were obtained between 5.9 and 6.6 min, 7.3 and 7.9 min, 10.4 and 10.8 min, 10.8 and 12.1 min, 18.5 and 19.5 min, 19.5 and 20.5 min, and between 20.5 and 22.0 min. The eluent collected between 7.3 and 7.9 min of 8 runs was pooled. Lyophilisation yielded 78 mg levanbiose (according to HPAEC-PAD). The pooled fraction obtained between 10.4 and 10.8 min yielded after lyophilisation 38 mg of a mixture of the new trisaccharide (β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl-(2→6)-D-fructofuranose) and 1-kestose. According to the area of the peaks from the C2 signals in the ^{13}C NMR spectra this pooled fraction contains about two times more of the new trisaccharide than 1-kestose.

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Partial Hydrolysed Levan

A solution of 250 mg Levan (obtained from Aerobacter levanicum, Sigma) in 10 mL water was stirred and kept at 60°C. One minute after the addition of 100 μL HCl (37 %) the hydrolysis was stopped with 65 μL NaOH (50 %). The pH was brought to 7 with 0.1 M HCl and the reaction mixture was concentrated under reduced pressure.

Partial Hydrolysed Inulin

A solution of 100 g dahlia inulin (Sigma) in 600 mL water was heated to a temperature of 60°C. After the addition of 6 mL HCl (37 % in water) and stirring for 3 min at 60°C, 6 mL of an aqueous NaOH solution (12.5 g NaOH in 25 mL) was added. The pH was brought to 7 using a 1 M aqueous HCl solution and the reaction mixture was cooled down to room temperature. During the addition of 200 mL methanol the solution was stirred. The resulting suspension was stored at room temperature for 4 days without stirring. The precipitate was isolated by filtration, washed with acetone and dried under reduced pressure, and will be referred to as H3MP. The yield was 38 g white powder.

HPAEC-PAD Analysis

For High Performance Anion Exchange Chromatographic analysis 25 μL was injected of a solution with a typical carbohydrate concentration of ca 20 mg/L for a specific component or ca 200 mg / L for mixtures of oligosaccharides. The HPAEC column (DIONEX, Carbopac PA1, 4 × 250 mm) is equipped with a guard column (DIONEX, Carbopac PA1 4 × 50 mm). Detection is achieved via Pulsed Amperometric Detection (PAD). The flow was 1 mL / min and the temperature was 20°C. For PAD detection the applied potential of a pulse was kept at 0.1, 0.7, and −0.1 V during, respectively, 0.4, 0.2, and 0.4 s. The signal was integrated between 0.2 and 0.4 s after the beginning of the pulse. An eluent gradient of 60 min was used. The gradient started at 25 mmol sodium acetate until 400 mmol sodium acetate while the sodium hydroxide concentration was constant at 100 mmol during 45 minutes. After this period the sodium acetate concentration was kept at 400 mmol for 15 minutes.

HPAEC-PAD analysis was applied to all fractions obtained by Ca^{2+}-column anion exchange chromatography and RP-18 HPLC. Furthermore, for identification purposes also chicory inulin with and without the new fructans, inulo-oligisaccharides and levan oligosaccharides were injected. Finely, because relative retention times are more reliable than absolute retention times, mixtures of those fractions were also injected for determination of the elution sequence of the individual oligosaccharides.

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NMR Analysis, Preparation of Samples

Deuterium enriched barium chloride was prepared from $BaCl_2.2H_2O$ by exchange in D_2O. After dissolving the salt in D_2O it was concentrated under vacuum. This was repeated two times followed by recrystallisation from D_2O. A solution of 39 mg levanbiose in 1 mL D_2O was lyophilised. The residue was dissolved in 0.5 mL D_2O resulting in a volume of 0.528 mL. After performing NMR measurements on this solution, referred to as BA0, deuterium enriched barium chloride was added in 6 steps. The solutions obtained by the cumulative addition of 13.3 mg, 26.6 mg, 40.0 mg, 54.9 mg, 71.7 mg, and 91.3 mg barium chloride are referred to as BA1, BA2, BA3, BA4, BA5, and BA6, respectively. This resulted in a barium chloride concentration of 0.10, 0.20, 0.29, 0.39, 0.50, and 0.62 mol / L for those solutions, respectively. After each step NMR experiments were performed. The volume of solution BA6 was 0.589 mL.

NMR Analysis, Acquisition

^1H and ^{13}C NMR spectra were obtained with a Bruker DPX-300 spectrometer (ATO-DLO, Wageningen) operating at 300.131 MHz for ^1H and 75.477 MHz for ^{13}C. The probe temperature was kept at 300 K. Chemical shift values are expressed in ppm downfield from the signal for 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), but were actually measured relative to that of internal acetone (2.225 ppm for ^1H, and 31.55 ppm for ^{13}C). Processing of NMR data was performed using the Bruker software package UXNMR. All HMBC experiments were performed without suppression of the one-bond correlations.

For levanbiose, 1D ^1H and ^{13}C NMR spectra were obtained without barium chloride (sample BA0) and for all different barium chloride concentrations (samples BA1-BA6). The ^1H NMR spectra were obtained by acquisition of 32 scans of 16K data points and a spectral width of 4800 Hz. For the ^{13}C NMR spectra, 300 scans of 32K data points were accumulated using a spectral width of 19960 Hz.

The following 2D NMR spectra were recorded for levanbiose without barium chloride (sample BA0), and for two different barium chloride concentrations (samples BA3 and BA6, with Ba^{2+} concentrations of 0.29 mol / L and 0.62 mol / L, respectively). Gradient HMBC spectra were obtained with 2048 experiments of 2K data points by accumulation of 32 scans with a spectral width of 4800 Hz. For the acquisition of gradient COSY and gradient HOHAHA spectra with a spectral width of 850 Hz in both dimensions, 8 scans and 16 scans of 2K data points were accumulated, respectively.

Using the largest barium chloride concentration (sample BA6) for levanbiose, four additional 2D-NMR experiments were performed. A gradient HMQC spectrum was obtained by accumulation of 2048 experiments of 24 scans of 2K data points using a spectral width of 850 for ^1H and 700 Hz for ^{13}C. For a DQF-COSY spectrum, a relayed COSY spectrum and a double relayed COSY spectrum, a spectral width of 850 Hz was used in both dimensions. These spectra were ob-

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tained by the acquisition of 2048, 1536, and 512 experiments of 2K data points, respectively.

For the new trisaccharide (β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl-(2→6)-D-fructofuranose) an ^{1}H NMR spectrum was obtained by acquisition of 32 scans of 16K data points and a spectral width of 4800 Hz. For the ^{13}C NMR spectrum, 22344 scans of 32K data points were accumulated using a spectral width of 19960 Hz. A gradient HMBC spectrum of 2K data points in both dimensions was obtained, using 32 scans for each experiment and a spectral width of 1200 and 7000 Hz for ^{1}H and ^{13}C, respectively. For reasons of comparison a 1D ^{13}C NMR spectrum of 1-kestose was recorded by accumulation of 360 scans of 32K data points and a spectral width of 19960 Hz.

Simulation of ^{1}H NMR Spectra of Levanbiose

For scaling of the simulated subspectra of the different residues, a ratio of 3:1 was used for the β-anomeric form (Figure 2a) and α-anomeric form (Figure 2a) of the reducing end, respectively. Significant differences between the ^{1}H NMR signals of the non-reducing end of the two anomeric forms were not found. Therefore during calculations they were assumed to be the same. As each fructosyl residue contains two separated spin systems (H-3,4,5,6′,6 and H-1′,1) this means that for levanbiose six subspectra were calculated. Simulation of the experimentally obtained ^{1}H NMR spectra was performed using a local version of a LAOCOON type program. In order to obtain a complete spectrum the subspectra were scaled and added. This was done for levanbiose without (sample BA0) and with barium chloride added (samples BA3 and BA6).

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