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OLIGOSACCHARIDES ISOLATED FROM *Agave vera cruz*

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ABSTRACT

The structures of naturally occurring and enzymically synthesized oligosaccharides, consisting of fructose and glucose residues and having d p 3–8 in the stem of *Agave vera cruz* have been investigated by using methylation analysis mass spectrometry, and p m r spectroscopy. The naturally occurring trisaccharides were identified as 1-kestose and neokestose, and the tetrasaccharides as nystose and at least one other related to neokestose The higher fractions consist of mixtures of (branched) oligosaccharides related to 1-kestose, neokestose, or 6-kestose as basic structures The enzymically synthesized trisaccharide was identified as 1-kestose and the tetrasaccharide as nystose The higher fractions consist of mixtures of linear oligosaccharides related to 1-kestose and neokestose

INTRODUCTION

In relation to its food value, the constituents of the stem of *Agave vera cruz* have been investigated in detail This stem is a rich source of polyfructans[1] Furthermore, a series of oligosaccharides comprising glucose (1 mol) and fructose (1 or more mol) was detected[2] Recently, the biosynthesis of these oligosaccharides has been studied[3,4] The enzyme preparation used contains at least two transfructosylases namely, a "sucrose–sucrose 1-fructosyltransferase' (SST), acting on sucrose to form a trisaccharide[3], and a "β-(2→1)-fructan-β-(2→1)-fructan 1-fructosyltransferase" (FFT), acting on 1-kestose to form a series of oligosaccharides with increasing fructose content[4]

We now report on the characterization of the naturally occurring and the enzymically synthesized oligosaccharides having d p 3–8

RESULTS

Determination of the d.p of the oligosaccharides

The naturally occurring oligosaccharide fractions* F_2G–F_7G and the enzymi-

*F_xG (x = 2–7) indicates the ratio of fructose and glucose residues ES denotes enzymically synthesized"

cally synthesized ESF_2G–ESF_6G gave no reaction with Somogyi's copper reagent, indicating that they are non-reducing. Hydrolysis with 0.1M HCl (15 min at 70°) or with dialysed yeast-invertase (EC 3.2.1.26) (B.D.H., 1:5 dilution, 5 h at 37°) yielded only glucose and fructose. In view of the specificity of invertase (β-D-fructofuranosidase), all fructose residues in the various oligosaccharides must have the β-D-furanose structure. Previously, it had been demonstrated that F_2G and ESF_2G were trisaccharides[3].

The d.p. of the higher, naturally occurring oligosaccharide fractions F_3G–F_7G was determined after hydrolysis with invertase (24 h at 37°, toluene as preservative). The total amount of reducing sugars was determined by the phenol–sulphuric acid method[5], and the amount of D-glucose with D-glucose oxidase[6]. The results are summarized in Table I. The homologous nature of the naturally occurring oligosaccharide fractions was further demonstrated by the paper-chromatographic technique of French and Wild[7]. In Fig. 1, the $\log [R_S/(1-R_S)]$ values are plotted against the determined d.p. for solvent *A*, showing a linear relationship. The same relationship was found for the enzymically synthesized oligosaccharide fractions ESF_2G–ESF_6G. Taking into account that ESF_2G is a trisaccharide and ESF_3G a tetrasaccharide (*vide infra*), the d.p. of the other fractions ESF_4G–ESF_6G is evident from Fig. 1.

Structural analysis of intact oligosaccharides by g.l.c., m.s., and p.m.r. spectroscopy

The trimethylsilyl (Me_3Si) derivative of F_2G gave one peak in g.l.c. on 3% of OV-17 and 3% of OV-25. However, t.l.c. (solvent *E*) of free F_2G showed two spots F_2G^I (R_F 0.36) and F_2G^{II} (R_F 0.41).

After isolation of both compounds and trimethylsilylation, the mass spectra of Me_3Si-F_2G^I and Me_3Si-F_2G^{II} proved to be identical with those reported for Me_3Si-1-kestose [*O*-α-D-Glc*p*-(1↔2)-*O*-β-D-Fru*f*-(1←2)-β-D-Fru*f*] and Me_3Si-neokestose [*O*-β-D-Fru*f*-(2→6)-*O*-α-D-Glc*p*-(1↔2)-β-D-Fru*f*], respectively[8]. Me_3Si-F_2G^{II} was also obtained by partial crystallization of Me_3Si-F_2G from acetone-d_6 at 4°. The presence of 1-kestose and neokestose in F_2G was further proved by p.m.r. spectroscopy[9] (Table II).

TABLE I

DETERMINATION OF THE d.p. OF THE NATURALLY OCCURRING OLIGOSACCHARIDE FRACTIONS

	Total reducing sugars (μg)	*Glucose (μg)*	*Fructose + Glucose / Glucose*[a]
F_3G	158.0	37.5	4.2 (4.0)
F_4G	252.5	49.6	5.1 (5.0)
F_5G	155.0	28.2	5.5 (6.0)
F_6G	144.0	20.6	6.9 (7.0)
F_7G	138.4	18.4	7.5 (8.0)

[a]The theoretical ratios are given in parentheses.

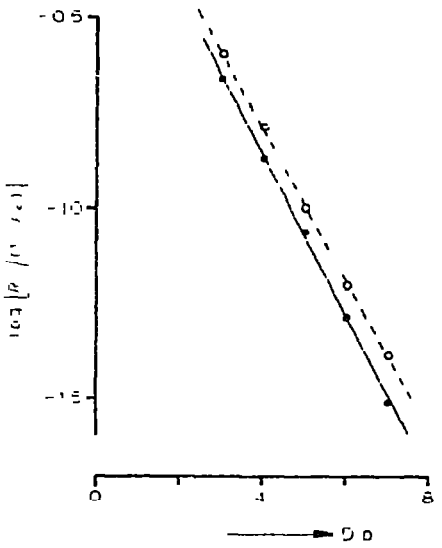

Fig 1 Paper-chromatographic mobilities of the naturally occurring and enzymically synthesized oligosaccharide fractions from *Agave vera cruz* in solvent *A* The log $[R_S/(1-R_S)]$ values are plotted against the determined d p R_S was calculated with the formula[7] $(1-R_S)^8 = 1-R_S^8$ in which R_S is the single ascent value and R_S^8 is the apparent value obtained after 8 multiple ascending runs $R^8_{S(sucrose)}$ values F_2G 0 79, F_3G, 0 63, F_4G 0 48 F_5G, 0 36, F_6G, 0 22 ESF_2G, 0 84, ESF_3G, 0 70, ESF_4G, 0 54, ESF_5G, 0 40 ESF_6G 0 30 ● = F_xG series, ○ = ESF_xG series

TABLE II

MOST IMPORTANT δ-VALUES OF THE Me_3Si DERIVATIVES OF F_3G^I, F_3G^{II}, ESF_2G, AND ESF_3G

	$H_G^{a,a}$	$H_{3F(1)}{}^b$	$H_{3F(2)}{}^b$	$H_{3F(3)}{}^b$
F_2G^I	5 36 (3 1 Hz)	4 45 (7 8 Hz)	4 27 (6 8 Hz)	
ESF_2G	5 36 (~3 Hz)	4 44 (~8 Hz)	4 27 (~7 Hz)	
1-Kestose[c]	5 35 (3 2 Hz)	4 43 (8 0 Hz)	4 26 (~7 Hz)	
F_2G^{II}	5 25 (3 3 Hz)	4 48 (7 8 Hz)	4 35 (7 8 Hz)	
Neokestose[c]	5 25 (2 9 Hz)	[d]	[d]	
ESF_3G	5 46 (3 3 Hz)	4 46 (7 7 Hz)	4 40 (7 4 Hz)	4 27 (7 0 Hz)
Nystose	5 46 (3 5 Hz)	4 46 (7 7 Hz)	4 40 (7 5 Hz)	4 26 (~7 Hz)

[a]Equatorial H-1 of the glucose residue, the coupling constants are given in parentheses [b]H-3 of the fructofuranose residues [c]Lit [9] data [d]Not determined

The Me_3Si derivative of F_3G gave two peaks in g l c on 3% of OV-17, 3% of OV-25, and 3 8% of SE-30 Separations by t l c. could not be achieved Because of the high molecular weight of the compounds, g l c –m s could not be applied Co-chromatography on 3.8% of SE-30 showed that one of the components had the same retention time as Me_3Si-nystose [*O*-α-D-Glc*p*-(1↔2)-*O*-β-D-Fru*f*-(1←2)-*O*-β-D-Fru*f*-(1←2)-β-D-Fru*f*]

The Me_3Si derivative of ESF_2G gave one peak in g l c on 3% of OV-17 and 3% of OV-25 T l c (solvent *E*) showed one spot with R_F 0 36. The mass spectrum[8]

and the p.m.r. spectrum[9] of Me_3Si-ESF_2G were identical to those of Me_3Si-1-kestose (Table II).

The Me_3Si derivative of ESF_3G gave one peak in g.l.c. on 3% of OV-17 and 3.8% of SE-30, having the same retention times as Me_3Si-nystose. The mass spectrum[8] and the p.m.r. spectrum[9,10] of Me_3Si-ESF_3G were identical to those of Me_3Si-nystose (Table II).

Structure analysis of oligosaccharides by the alditol acetate method (combined g.l.c.–m.s.)

The oligosaccharide fractions of the F_xG and ESF_xG series were investigated by the alditol acetate method of Lindberg and his co-workers[11,12].

F_2G was analyzed after fractionation by t.l.c. into F_2G^I and F_2G^{II}. After permethylation of the various oligosaccharide fractions, the products were hydrolysed and converted into their partially methylated alditol acetates by reduction and acetylation. Reduction of the partially methylated D-fructose gives rise to a mixture of D-glucitol and D-mannitol derivatives. On 3% of OV-225, the pairs of D-glucitol and D-mannitol derivatives obtained from 1,3,4,6-tetra-*O*-methyl-, 1,3,4-tri-*O*-methyl-, 3,4,6-tri-*O*-methyl-, or 3,4-di-*O*-methyl-D-fructose cannot be separated[13]. Moreover, the D-glucitol and D-mannitol derivatives from 1,3,4-tri-*O*-methyl- and 3,4,6-tri-*O*-methyl-D-fructose have the same retention times on 3% of OV-225 and cannot be distinguished by mass spectrometry, unless labelled with deuterium introduced by reduction with $NaBD_4$.

The results are summarized in Table III. The volatility of the alditols of 1,3,4,6-tetra-*O*-methyl-D-fructose is high, which may give rise to losses in the various evaporation procedures. Furthermore, it has been found in model experiments that degradation of fructose residues can occur during the hydrolysis of permethylated oligosaccharides. The primary fragmentations of the partially methylated alditol acetates obtained by using $NaBD_4$ as reducing agent are shown in Fig. 2. The position of the labelling is directly evident in the spectra of 1,3,4,6-Fru* (C-2; absence of *m/e* 118 and presence of *m/e* 162), 2,3,4,6-Glc (C-1, presence of *m/e* 118); 2,3,4-Glc (C-1, presence of *m/e* 118) and 1,3,4-Fru (C-2, absence of *m/e* 118 and presence of *m/e* 162). In the case of 3,4,6-Fru and 3,4-Fru, it is also necessary to consider the secondary fragmentations, *i.e.*, the elimination of acetic acid from *m/e* 190. Here the position of the label at C-2 was proved by comparison with the mass spectra of 3,4,6-Glc and 3,6-Glc labelled at C-1.

F_2G consists of a mixture of 1-kestose (F_2G^I) and neokestose (F_2G^{II}). F_3G consists of a mixture of nystose and at least one tetrasaccharide related to neokestose (a third fructose residue linked at C-1 of one of the other fructose residues of neokestose). The detection of 1,3,4,6-Fru, 2,3,4,6-Glc, 3,4,6-Fru, 1,3,4-Fru, 2,3,4-Glc and 3,4-Fru in the analysis of the fractions F_4G, F_5G, F_6G, and F_7G makes clear that each of these fractions consists of a mixture of (branched) oligosaccharides.

*1,3,4,6-Fru = 2,5-di-*O*-acetyl-1,3,4,6-tetra-*O*-methyl-D-glucitol + 2,5-di-*O*-acetyl-1,3,4,6-tetra-*O*-methyl-D-mannitol, 2,3,4,6-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, *etc.*

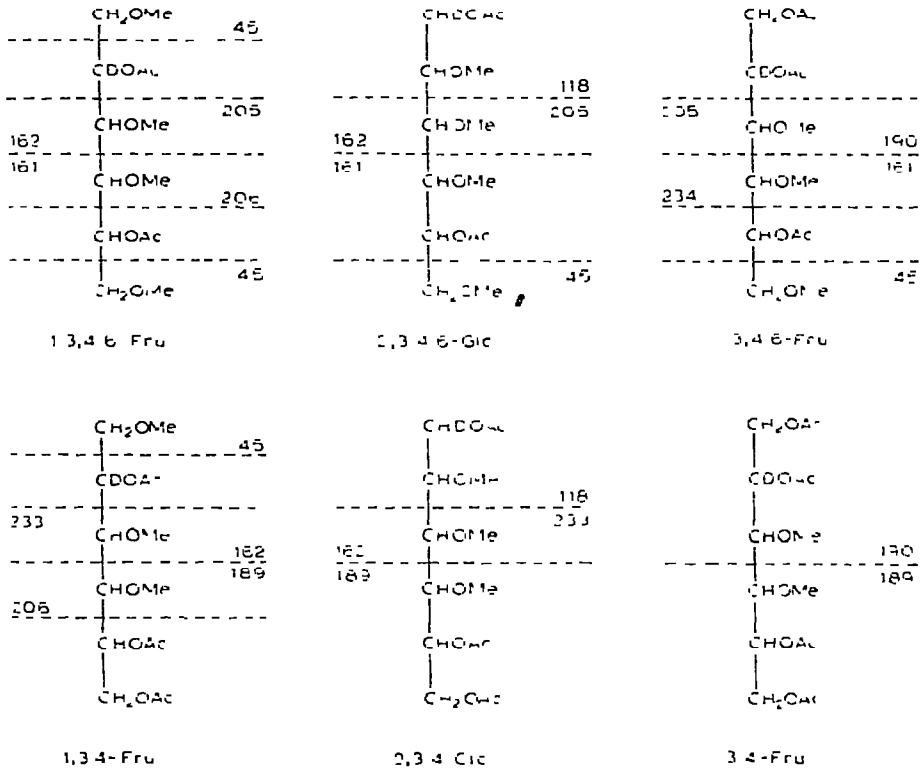

Fig. 2. Primary fragmentations of the partially methylated alditol acetates ($NaBD_4$ reduction). For an explanation of the abbreviations 1,3,4,6-Fru *etc.*, see the footnote on p. 278.

TABLE III

METHYLATION ANALYSIS OF THE NATURALLY OCCURRING AND ENZYMICALLY SYNTHESIZED OLIGOSACCHARIDE FRACTIONS

Oligosaccharide fraction	*Methylated sugar* (%)				
	1,3,4,6-Fru (T 0.75)	*2,3,4,6-Glc* (T 1.00)	*3,4,6-Fru* (T 1.87)	*2,3,4-Glc* (T 2.26)	*3,4-Fru* (T 4.63)
F_2G^I	28	34	38	—	—
F_3G^{II}	63	—	—	37	—
F_3G	28	7	39	26	—
F_4G	29	5	39[b]	19	7
F_5G	26	4	43[b]	16	10
F_6G	20	4	51[b]	15	10
F_7G	21	3	50[b]	14	12
ESF_2G	30	39	31	—	—
ESF_4G	24	14	54	8	—
ESF_5G	22	4	58	16	—
ESF_6G	13	1	64	22	trace

[a]For the abbreviations, see the text. [b]Besides 3,4,6-Fru, small proportions of 1,3,4-Fru (see text).

built up from 1-kestose, neokestose, or 6-kestose as the basic structure. The presence of small proportions of 1,3,4-Fru in addition to 3,4,6-Fru was deduced from the mass spectra of the deuterium-labelled alditols.

ESF_2G is identical with 1-kestose. Because of the presence of 1,3,4,6-Fru, 2,3,4,6-Glc, 3,4,6-Fru, and 2,3,4-Glc, the fractions ESF_4G, ESF_5G, and ESF_6G consist of mixtures of linear oligosaccharides, built up from 1-kestose and neokestose as basic structures. Only in the case of ESF_6G was a trace of 3,4-Fru observed, indicating the presence of branching points.

DISCUSSION

The presence of 1-kestose, 6-kestose, and neokestose, first encountered as products of invertase action on sucrose[14,15], has been demonstrated in many fructan-bearing plants[16]. Among these, 1-kestose is reported to play a significant role in inulin synthesis[17]. It is the major oligosaccharide formed during levan synthesis from sucrose by *Corynebacterium* species[18].

A number of fructo-oligosaccharides known to occur in several species of *Compositae*[19], *Gramineae*[20], *Amaryllidaceae*[21], and *Liliaceae*[16,22] are mixtures of isomeric compounds. For the fructan-bearing plants Jerusalem artichoke[17] and *Allium cepa*[16,23], the structures of some natural, but only a very few of the enzymically synthesized, oligosaccharides have been reported. The neokestose series of oligosaccharides was found only in *Asparagus cochinchinensis*[24]. The oligosaccharides present in rye haulms have also been investigated[25]. The presence of 1-kestose, 6-kestose, and *O*-α-D-Glc*p*-(1→2)-[*O*-β-D-Fru*f*-(2→6)]-*O*-β-D-Fru*f*-(1←2)-β-D-Fru*f* was demonstrated. The higher oligosaccharides are of the branched type with a non-reducing terminal D-glucose residue, and (2→1)- and (2→6)-linked β-D-Fru*f* residues. Higher oligosaccharides and even fructans with neokestose in the middle of the molecule have been reported in *Polygonatum odoratum*[26] and the Hawaiian ti plant *Cordyline terminalis*[27]. Moreover, in the latter species, the fructan is branched. Their pathways of synthesis are unknown.

The clear juice of the stem of *Agave vera cruz* contains two trisaccharides, namely 1-kestose and neokestose. In addition to the tetrasaccharide nystose, one or two other isomers, related to neokestose, namely *O*-β-D-Fru*f*-(2→1)-*O*-β-D-Fru*f*-(2→6)-*O*-α-D-Glc*p*-(1←2)-β-D-Fru*f* and/or *O*-β-D-Fru*f*-(2→6)-*O*-α-D-Glc*p*-(1←2)-*O*-β-D-Fru*f*-(1←2)-β-D-Fru*f*, are also present. Each of the oligosaccharide fractions with d.p. 5–8 contains, in addition to the higher homologues of 1-kestose and neokestose, isomers related to 6-kestose; branched structures are also present.

The investigation of the enzymically synthesized oligosaccharides indicates the formation of only one trisaccharide (1-kestose) and one tetrasaccharide (nystose) from sucrose and 1-kestose, respectively. Each of the oligosaccharide fractions with d.p. 5–7 contains linear, higher homologues of 1-kestose and neokestose. It seems possible that, beyond the tetrasaccharide nystose, the transfer specificity of the enzyme FFT is not confined to the terminal fructose residue. The fructose moieties can also

be successively transferred to C-6 of the glucose residue in nystose and its higher homologues, leading to two series.

There is no evidence for the biosynthesis of 6-kestose and neokestose by the enzyme preparation. It is not clear whether a separate branching-enzyme synthesizes the linkage *O*-β-D-Fru*f*-(2→6)-β-D-Fru*f*, although, in the methylated material of fraction ESF_6G, a trace of 3,4-Fru has been observed. The naturally occurring neokestose and its next higher homologues may arise from successive disproportionation of the homologous penta- and tetra-saccharides ($F_3GF + F_3GF \rightarrow F_4GF + F_2GF$, $F_2GF + F_2GF \rightarrow F_3GF + FGF$) or by stepwise hydrolysis ($F_nGF \rightarrow F_{n-1}GF + F$; $F_{n-1}GF \rightarrow F_{n-2}GF + F$; *etc*.). As already described, neokestose can arise also from the action of invertase on sucrose. Aspinall *et al*.[28] have shown that the major fructan (d.p. 32) in *Agave vera cruz* is a highly branched polysaccharide having a terminal glucose residue and both (2→1) and (2→6) linkages between the fructose residues. No evidence for a neokestose type of structure has been obtained.

Recently, it was found that each of the oligosaccharides 1-kestose and its higher homologues (d.p. 4–6) from *Agave vera cruz* could serve as a substrate for fructan synthesis.

EXPERIMENTAL

General methods. — Paper chromatography was carried out on Whatman No. 3MM paper with (*A*) 5:3:2 1-butanol–ethanol–water[29], (*B*) 6:1:3 1-propanol–ethyl acetate–water[30], and (*C*) 9:5:7 1-butanol–pyridine–water[31].

T.l.c. was performed on plates of a mixture of silicagel HR and Kieselguhr (Merck) [3:1, impregnated with a 0.07M sodium phosphate buffer[32] (pH 7)] with (*D*) 4:1:1 1-butanol–acetic acid–water and (*E*) 100:60 1-butanol saturated with water–methanol[21], or on coated plastic sheets (FR 1500 LS 254 silicagel, Carl Schleicher Schull) with solvent (*E*). Detection was effected with benzidine–acetic acid[33], benzidine–trichloroacetic acid[19], and urea–HCl[34].

G.l.c. of partially methylated alditol acetates was carried out at an oven temperature of 160° on a Pye 104 instrument equipped with a flame-ionization detector and a glass column (1.60 m × 4 mm) containing 3% of OV-225 on Chromosorb W-AW-DMCS (80–100 mesh). The flow rate for nitrogen was 40 ml/min. The retention times (*T*) are given relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

G.l.c.–m.s. of the partially methylated alditol acetates was carried out with a Jeol JGC-1100/JMS-07 combination (oven temperature, 150° for 10 min, followed by an increase of 2°/min up to 200°, column material, 3% of OV-225 on Chromosorb W-AW-DMCS, 80–100 mesh, column dimensions, 2.00 m × 3 mm, ion source temperature, 250°, electron voltage, 75 eV, ionization current, 300 μA; accelerating voltage, 3 kV).

Mass spectrometry of Me_3Si derivatives of tri- and tetra-saccharides was performed on AEI MS-9 and MS-902 mass spectrometers (electron voltage, 70 eV,

ion chamber temperature, 130–150°, ionization current, 500 μA, accelerating voltage 8 kV). The Me_3Si derivatives were prepared as described earlier[8]. The purity was tested by g.l.c. using 3% of OV-17, 3% of OV-25, or 3.8% of SE-30 on Chromosorb W-AW-DMCS (80–100 mesh).

P.m.r. spectroscopy of Me_3Si derivatives of tri- and tetra-saccharides (5–10 mg) was carried out with a Varian HA-100 spectrometer locked on tetramethylsilane, or a Varian XL-100 spectrometer (FT-technique) locked on deuterium of the solvent acetone-d_6. The chemical shifts are given relative to tetramethylsilane on the δ-scale (indirect to acetone-d_0, $\delta = 2.05$) at room temperature[9].

Isolation procedure — (*a*) *Naturally occurring oligosaccharides*. The clear juice of the stem of *Agave tequilana cruz* was prepared as described previously[1]. The precipitates obtained by treatment with 60, 80, and 88% ethanol, successively, consisted predominantly of inorganic salts, polyfructans, and higher oligosaccharides. The final supernatant was freed from ethanol, passed through columns of Dowex-50 X8 (sodium form) and Dowex-1 X8 (chloride form), and then adsorbed on a 1:1 charcoal–Celite column. The latter column was washed with 5% ethanol to remove monosaccharides and sucrose, and then eluted with 40% ethanol. The eluate containing oligosaccharides with d.p. 3–15 was concentrated and then subjected to preparative paper chromatography on Whatman No. 3MM paper in solvent *A* (7–8 multiple ascending runs) or solvent *B* (2–4 multiple descending runs).

Solutions of the isolated fractions F_2G–F_7G in methanol were poured into acetone, and the resulting, white, flocculent precipitates were dried in a vacuum desiccator over P_2O_5. The oligosaccharide fractions F_2G–F_7G, obtained in amounts of 1800, 530, 400, 220, 60, and 40 mg, respectively, were homogeneous by paper chromatography in solvents *A*, *B*, and *C*, and by t.l.c. in solvents *D* and *E*, except for F_2G which showed two spots in solvent *E*.

(*b*) *Enzymically synthesized oligosaccharides*. A solution (0.5 ml) of an enzyme preparation (5.8 mg) from the *Agave* stem-juice, free from hydrolytic activity[4], was incubated with a solution of 1 g of sucrose in 0.5 ml of 0.2M sodium acetate buffer (pH 5.6) at 37° for 7.5 h. After inactivation and removal of the proteins, the sample was transferred to a column (14.5 × 3.3 cm) of 1:1 charcoal–Celite. The column was eluted first with 5% ethanol to remove glucose, fructose, and sucrose, and subsequently with 25% ethanol to elute the trisaccharide ESF_2G. The trisaccharide was purified further by preparative paper chromatography, yielding ESF_2G (220 mg) with $[\alpha]_D$ +32.3° (*c* 3.34, water)[3].

The higher oligosaccharides were prepared by incubation of 200 mg of the trisaccharide in 0.3 ml of 0.2M sodium acetate buffer (pH 5.6) with 0.4 ml of the enzyme preparation (4.6 mg) at 37° for 24 h. After inactivation and removal of the proteins, the sample was subjected to repeated preparative paper chromatography (solvent *B*) until single spots were obtained in each case. Solutions of the isolated fractions ESF_3G–ESF_6G in methanol were poured into acetone, resulting in white, flocculent precipitates. After drying over P_2O_5, the amounts were 29, 20,

8, and 5 mg, respectively The oligosaccharide fractions were homogeneous by paper chromatography in solvents *A*, *B*, and *C*, and by t l c in solvents *D* and *E*.

Methylation analysis of oligosaccharides — Samples (2–10 mg) of the various oligosaccharides were methylated by the Hakomori procedure with methyl iodide–sodium methylsulphinylmethanide in methyl sulphoxide[11,35]. Each methylated oligosaccharide was recovered by chloroform extraction After concentration of the chloroform layers to dryness, the residues were treated with 1 ml of 90% formic acid for 15 min at 70°. Subsequently, the solutions were diluted with 9 ml of water and heated for 1 h at 70°. After concentration under reduced pressure at 40°, the residual formic acid was removed by co-distillation with water The partially methylated alditol acetates, prepared as described by Bjorndal *et al*[11], were investigated by g l c. and g l c –m s For the reduction step, $NaBH_4$ was used as well as $NaBD_4$.

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