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Note

Structure of the polysaccharide Zanflo elaborated by *Erwinia tahitica* ATCC 217 11

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The polysaccharide Zanflo from *Erwinia tahitica* ATCC 217 11 (S-10) [1] is one of many being prepared for industrial applications. It is valuable because of its fermentation efficiency and product properties. These include better viscosity than xanthan gum and larger compatibility with dyes. We have now elucidated the structure of the carbohydrate backbone of Zanflo, using NMR studies, methylation analysis, partial acid hydrolysis, and uronic acid degradation.

Previous analysis of Zanflo showed glucose, galactose, glucuronic acid, fucose, and *O*-acyl groups as components [1]. A hydrolysate using hydrolysis with 2 M CF_3CO_2H gave fucose, glucose, and galactose in the proportions 27:42:31 (Table 1, column A). GLC analysis of a sample that had been treated with methanolic hydrogen chloride also showed glucuronic acid. The absolute configurations of the sugars were determined according to Gerwig et al. [2] on a premethanolysed sample of S-10 and showed L-fucose, D-glucose, D-galactose, and D-glucuronic acid.

Methylation analysis showed the presence of terminal D-galactose, 4-substituted L-fucose, and 3,4-substituted D-glucose (Table 2, column A). When the native polysaccharide was methylated and then carboxyl-reduced, an additional 4-substituted D-glucuronic acid was demonstrated (Table 2, column B). From these data, a repeating unit of four sugar residues is indicated.

^{1}H NMR spectra of native Zanflo (Fig. 1) showed, *inter alia*, the presence of the methyl group of a 6-deoxyhexose and a complex anomeric region. The reason for this complexity

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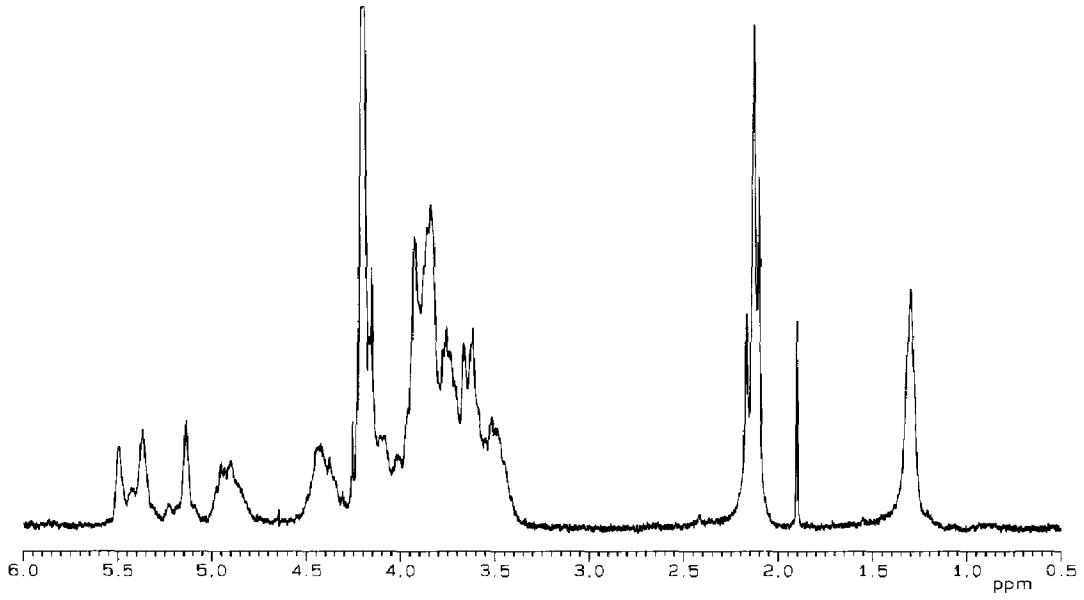

Fig. 1. ^{1}H NMR spectrum at 270 MHz of native S-10.

was indicated by the presence of at least three signals for *O*-acetyl groups at δ 2.10, 2.13, and 2.17, since protons on acetoxylated carbons often give signals in the anomeric region. Signals from *O*-acetyl groups appeared in the ^{13}C NMR spectrum at δ 21.0, 21.1, and 21.3. The integral of the *O*-acetyl signals corresponded to ca. 1.5 equiv. In the spectrum of the *O*-deacetylated polysaccharide (Fig. 2), signals for five protons were observed in the anomeric region. The signals at δ 4.41 and 4.49 were clearly from β-linked residues as the $J_{1,2}$ values were 7.5 and 7.8 Hz, respectively. The corresponding ^{13}C NMR values were δ 102.7 and 103.9. Two unresolved signals at δ 5.39 and 5.41 should, from the chemical shifts

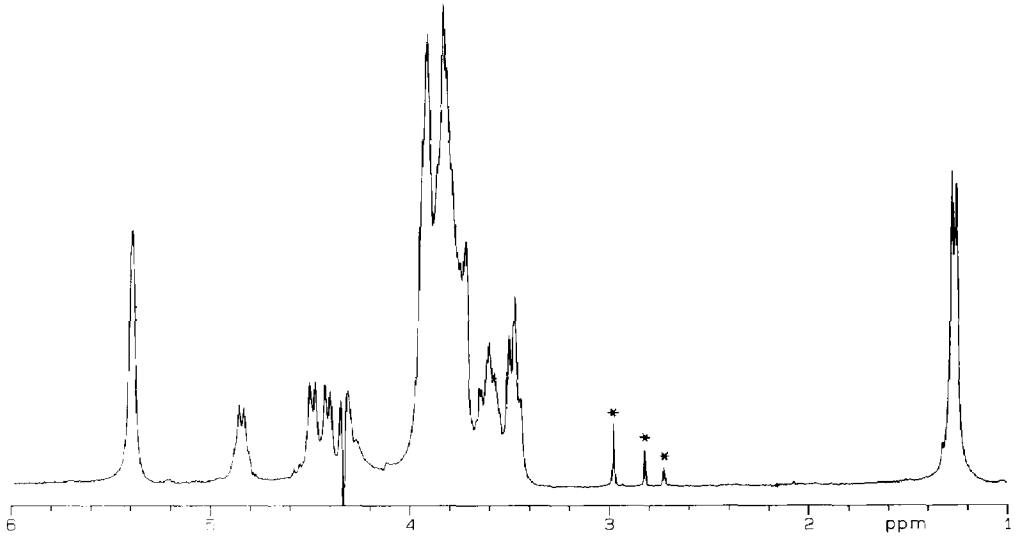

Fig. 2. ^{1}H NMR spectrum at 270 MHz of *O*-deacetylated S-10: * = unknown.

Table 1
Sugar analysis of S-10 and oligosaccharides thereof [a]

Sugar	Detector response		
	A	B	C
L-Fuc	27	33	18
D-Glc	42	65	42
D-Gal	31	2	40

[a] Key: A, native polysaccharide; B, trisaccharide from partial hydrolysis; C, tetrasaccharide from partial hydrolysis.

and the low $J_{1,2}$ values, be α-linked. In the ^{13}C NMR spectrum, the corresponding signals were found at δ 99.4 and 99.2, respectively. The residue with its H-1 signal at δ 5.39 was shown to be the 4-substituted fucose residue as the chemical shifts of its H-4/C-4 were δ 3.94/82.3, H-5/C-5 δ 4.84/67.4, and H-6/C-6 δ 1.26/16.0. No other signals in the low-field region for C-4 carbons were observed and consequently all the sugars are pyranoid.

Uronic acid degradation, that is, methylation, treatment with base under anhydrous conditions, followed by the addition of methyl iodide [3], gave a product that on hydrolysis showed terminal L-fucose, 4-substituted L-fucose, and terminal D-galactose as the main components (Table 2, column C). Some 3,4-substituted D-glucose was also observed. The presence of two L-fucose derivatives indicated that the uronic acid degradation was not complete and that the D-glucuronic acid had been linked to the 4-position of L-fucose. The decrease of 3,4-substituted D-glucose also indicated that the sugar had been liberated and degraded. The following structural element is thus demonstrated.

$$\rightarrow 3)\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-D-Glc}p\text{A-}(1\rightarrow 4)\text{-L-Fuc}p\text{-}(1\rightarrow$$
$$\qquad\qquad\quad 4$$
$$\qquad\qquad\quad \uparrow$$

To determine whether the terminal D-galactose residue was linked to the 3- or the 4-position of the branching D-glucose residue, oligosaccharides from partial acid hydrolysis were investigated. After hydrolysis using 0.5 M CF_3CO_2H for 3 h at 100°C, the resulting oligosaccharides were reduced with sodium borohydride and separated on a column of Bio-Gel P-2. This yielded, *inter alia*, a peak in the disaccharide region. The material was collected, permethylated, and analysed by GLC-EIMS. A GLC peak near to the retention time of permethylated lactitol indicated that it was a disaccharide-alditol. A peak in the mass spectrum at m/z 233 (aA_1) revealed that the non-reducing end was D-glucuronic acid and peaks at m/z 59 and 133 corroborated the 4-linkage of the L-fucitol residue. The ^1H NMR spectrum of the disaccharide showed, in addition to a signal for a 6-deoxy group at δ 1.31 (3 H), a signal for an anomeric proton at δ 4.61 ($J_{1,2}$ 7.7 Hz), demonstrating that the D-glucuronic acid was β-linked. The structural element β-D-Glc*p*A-(1→4)-L-Fuc is thus established.

Hydrolysis using 0.1 M CF_3CO_2H for 2 h at 100°C yielded, *inter alia*, larger oligosaccharides. The mixture of oligosaccharides was reduced and subjected to gel filtration on a column of Bio-Gel P-2. One of the oligosaccharides was a trisaccharide-alditol whose ^1H NMR spectrum showed signals at δ 5.44 ($J_{1,2}$ 4.0 Hz), 4.61 ($J_{1,2}$ 7.7 Hz), and 1.31 (3 H). A hydrolysate contained L-Fuc and D-Glc as major components (Table 1, column B) and

Table 2
Methylation analysis of S-10 and some degradation products [a]

Sugar [b]	Detector response				
	A	B	C	D	E
1,2,3,5-Fuc-ol				41	17
2,3,4-Fuc			23		
2,3-Fuc	18	21	19		
2,3,4,6-Glc				43	1
2,3,4,6-Gal	39	25	52	4	39
2,3,6-Glc				12	43
2,6-Glc	42	28	7		
2,3-Glc		25			

[a] Key: A, methylated polysaccharide; B, methylated and carboxyl-reduced polysaccharide; C, uronic acid-degraded polysaccharide; D, acidic trisaccharide; E, acidic tetraccharide.
[b] 2,3,4-Fuc = 2,3,4-tri-*O*-methyl-L-fucose, etc.

the methylation analysis (Table 2, column D) showed 4-substituted L-fucitol and terminal D-Glc as main components in addition to some terminal D-Gal and 4-substituted D-Glc, which can be explained by the presence of some tetrasaccharide-alditol in the sample (see below). From these data and those given above, it can be concluded that the structure of the trisaccharide-alditol is α-D-Glc*p*-(1→4)-β-D-Glc*p*A-(1→4)-L-Fucitol.

A tetrasaccharide-alditol was also isolated and its ^{1}H NMR spectrum showed signals at δ 5.42 ($J_{1,2}$ 4.0 Hz), 4.64 ($J_{1,2}$ 8.1 Hz), 4.44 ($J_{1,2}$ 7.7 Hz), and 1.30 (3 H). It contained, *inter alia*, L-Fuc, D-Gal, and D-Glc as shown by hydrolysis and GLC analysis (Table 1, column C). Methylation analysis of the tetrasaccharide-alditol showed 4-substituted L-fucitol, terminal D-Gal, and 4-substituted D-Glc (Table 2, column E). The structure of the tetrasaccharide-alditol must therefore be β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→4)-β-D-Glc*p*A-(1→4)-L-Fucitol. The linkage between the terminal D-Gal residue and the branch point D-Glc residue in the polysaccharide is consequently also 1→4, and the complete structure of the *O*-deacetylated polysaccharide is

→ 3)-α-D-Glc*p*-(1 → 4)-β-D-Glc*p*A-(1 → 4)-α-L-Fuc*p*-(1 →
4
↑
1
β-D-Gal*p*

No attempts were made to locate the ca. 1.5 equivalents of *O*-acetyl groups per repeating unit. The backbone of Zanflo is the same as that of *Klebsiella* type 16 [4].

1. Experimental

General methods.—Concentrations were performed under diminished pressure at < 40°C or under a stream of air or N_2. For GLC, a Hewlett-Packard 5890 instrument fitted with a

flame-ionisation detector was used. GLC-EIMS was performed on a Hewlett-Packard 5970 MSD instrument.

Alditol acetates and partially methylated alditol acetates were analysed on an HP-5 capillary column (25 m×0.20 mm), using the temperature program 180°C (1 min) →250°C at 3°C/min, and for the disaccharide 210°C (3 min) →250°C at 3°C/min. Analysis of the trimethylsilylated (+)-2-butyl glycosides was performed on the same column but the temperature program 130°C (1 min) →220°C at 3°C/min was used.

Gel permeation chromatography was performed on Bio-Gel P-2 or Sephadex G50 columns, using water buffered with 0.07 M pyridinium acetate of pH 5.4 as eluent, and monitored by a differential refractometer.

Preparation of O-*deacetylated polysaccharide.*—The polysaccharide was dissolved in 0.1 M NaOH and kept at room temperature for 16 h. After neutralisation, it was dialysed extensively against deionised water and freeze-dried.

NMR spectroscopy.—In order to decrease the molecular weight of the polysaccharide, it was kept in 0.1 M CF_3CO_2H at 100°C for 10 min. After evaporation of the acid and neutralisation, a product that came with the void volume on gel filtration on Sephadex G-50 was obtained. NMR spectra of polysaccharide solutions in D_2O were recorded at 70°C using a JEOL GSX-270 instrument. Chemical shifts are reported in ppm, using sodium 3-trimethylsilylpropanoate-d_4 (δ_H 0.00) or acetone (δ_C 31.00) as internal references. $^1H,^1H$- and $^{13}C,^1H$-COSY were performed using Jeol standard pulse-sequences.

Sugar and methylation analysis.—Hydrolysis of native and methylated Zanflo was performed by treatment with 0.5 M CF_3CO_2H at 100°C overnight. The sugars in the hydrolysates were converted into alditol acetates and partially methylated alditol acetates. Carboxyl-reduction of methylated polysaccharide (0.6 mg in 0.7 mL dry THF) was performed by treatment with $LiBH_4$ (3 mg) at 80°C for 2 h. The reaction was quenched with CH_3CO_2H and the methylated material recovered by partition between $CHCl_3$ and H_2O.

The absolute configuration of the sugars were determined according to Gerwig et al. [2].

Uronic acid degradation.—Carefully dried methylated polysaccharide (1 mg) was dissolved in Me_2SO (0.5 mL) and treated with a trace of *p*-toluenesulfonic acid and 2,2-dimethoxypropane (0.1 mL). Lithium methylsulfinylmethanide was generated in situ by the addition of butyl-lithium in hexane (0.2 mL; 2.5 M) to the solution, which was kept at room temperature for 1 h. After cooling, methyl iodide was added and the material was recovered and hydrolysed.

Partial acid hydrolysis.—The *O*-deacetylated polysaccharide (25 mg) was kept in 0.5 M CF_3CO_2H at 100°C for 3 h to give the disaccharide. For larger oligosaccharides, a sample (25 mg) was hydrolysed with 0.1 M CF_3CO_2H at 100°C for 2 h. After removal of the acid by evaporation and neutralisation, the product mixture was reduced with $NaBH_4$ and worked up as usual. Gel filtration on a column of Bio-Gel P-2 (2.5×70 cm) yielded in the first case, *inter alia*, a disaccharide-alditol (1 mg) and, in the second case, *inter alia*, a trisaccharide-alditol (1 mg) and a tetrasaccharide-alditol (2 mg). The methylated disaccharide-alditol had t_R 0.94 (lactitol = 1.0).

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