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STRUCTURAL STUDIES ON AN ANTITUMOR POLYSACCHARIDE FROM *Microellobosporia grisea*

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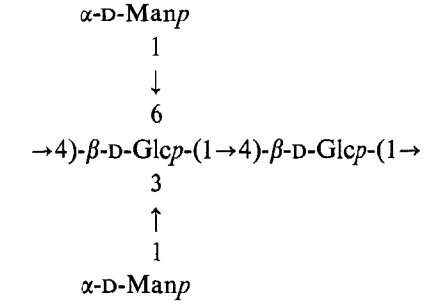
ABSTRACT

The structure of the antitumor polysaccharide from the actinomycete *Microellobosporia grisea* has been investigated. By methylation and periodate-oxidation studies, the polysaccharide was shown to consist of (nonreducing) D-mannosyl groups, (1→4)-linked D-glucosyl residues, and 3,6-branched, (1→4)-linked D-glucosyl residues in the approximate molar ratios of 2:1:1. Periodate oxidation of the polysaccharide, followed by borohydride reduction and mild hydrolysis with acid yielded glycerol, erythritol, 2-*O*-β-D-glucopyranosyl-D-erythritol, and 5-*O*-β-D-glucopyranosyl-2,4-bis(hydroxymethyl)-1,3-dioxane, which were isolated in the molar ratios of 2.0:0.14:0.74:0.35. Partial hydrolysis of the polysaccharide gave α-D-Man*p*-(1→6)-D-Glc*p*, β-D-Glc*p*-(1→4)-D-Glc*p*, α-D-Man*p*-(1→3)-D-Glc*p*, and β-D-Glc*p*-(1→4)-[α-D-Man*p*-(1→3)-]-D-Glc*p*. From these results, it is proposed that the polysaccharide is mainly composed of tetrasaccharide repeating-units having the following structure.



INTRODUCTION

Extracellular, antitumor polysaccharides have not been obtained from actinomycetes, except for a polysaccharide found by our laboratory; it was previously shown[1] that the actinomycete *Microellobosporia grisea* produces a water-soluble, extracellular polysaccharide exhibiting potent antitumor activity against both Ehrlich carcinoma and MM46 syngeneic adenocarcinoma, solid tumors in mice. This poly-

saccharide was found[1] to be a mannoglucan, composed of D-glucosyl and D-mannosyl residues in the molar ratio of 1.14:1.00, and having $[\alpha]_D$ +65° and a molecular weight of $\sim 1 \times 10^6$.

Polysaccharides having antitumor activity against certain allogeneic tumors, particularly Sarcoma 180 in mice, have been obtained from various sources[2], including bacteria, fungi, yeasts, lichens, and higher plants, but the relationship between their chemical structures and antitumor activities is not thoroughly clear. Thus, it was of interest to establish the structure of this polysaccharide, and to investigate the structure–antitumor activity relationship. In continuation of the previous study[1], we now report studies on the glycosidic linkages in this antitumor polysaccharide.

RESULTS

Methylation analysis. — The polysaccharide underwent complete methylation by the method of Hakomori[3], as indicated by the absence, from the i.r. spectrum of the product, of absorption in the region 3500–3400 cm^{-1}. Hydrolysis of the permethylated polysaccharide gave three methylated sugars, which were identified as 2,3,4,6-tetra-*O*-methyl-D-mannose, 2,3,6-tri-*O*-methyl-D-glucose, and 2-*O*-methyl-D-glucose as the alditol acetates by g.l.c.–m.s., and by demethylation analysis. By demethylation of these methylated sugars, the tetra-*O*-methylated sugar gave mannose plus a trace of glucose, and the tri- and mono-*O*-methylated sugars afforded only glucose. As shown in Table I, the molar ratios of these methylated sugars indicate that the polysaccharide is a highly branched mannoglucan composed of (nonreducing) D-mannosyl groups, (1→4)-linked D-glucosyl residues, and 3,6-doubly branched, (1→4)-linked D-glucosyl residues in the approximate molar ratios of 2:1:1.

Periodate oxidation, and complete Smith degradation. — On complete oxidation, the polysaccharide consumed 1.22 mol of periodate and released 0.46 mol of formic

TABLE I

METHYLATION ANALYSIS OF THE POLYSACCHARIDE

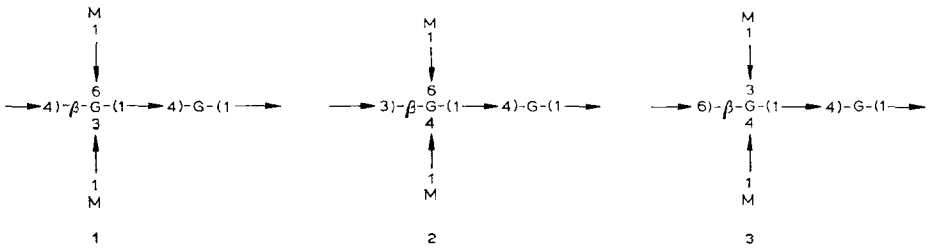
O-Methylated sugar (as alditol acetate)	T_R[a]	*Molar ratio*	*Mode of linkage*
2,3,4,6-Tetra-*O*-methyl-D-mannose	**1.00**	1.00	Man*p*-(1→
2,3,6-Tri-*O*-methyl-D-glucose	2.33	0.50	→4)-Glc*p*-(1→
			↓ 6
2-*O*-Methyl-D-glucose	6.74	0.47	→4)-Glc*p*-(1→ ↑ 3

[a]Retention time relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol

acid per sugar residue, in agreement with the respective values of 1.27 and 0.51 mol expected from the results obtained by the methylation analysis (see Table I).

The polyalcohol derived from the polysaccharide by periodate oxidation followed by reduction of the product with borohydride was completely hydrolyzed with acid. P.c. and t.l.c. of the hydrolyzate revealed the presence of glycerol, erythritol, and glucose, and the absence of mannose. G.l.c. confirmed their identities, and indicated the molar ratios of glycerol : erythritol : glucose to be 2.0 : 1.1 : 1.0, which is close to the ratios 2.0 : 1.0 : 1.1, determined colorimetrically after separation by p.c. On inspection of Table I, it is clear that the glycerol must arise from the (nonreducing) D-mannosyl groups, the erythritol from the (1→4)-linked D-glucosyl residues, and the glucose (that survived treatment with periodate) from the 3,6-di-*O*-glycosylated, (1→4)-linked D-glucosyl residues. Thus, the results obtained by complete Smith degradation were consistent with those deduced both from the methylation and the periodate-oxidation studies.

Controlled Smith degradation. — Some information on the linkages between the doubly branched D-glucosyl residues and their neighboring sugar residues was obtained by controlled Smith degradation[4], involving mild, acid hydrolysis of the polyalcohol derived from the polysaccharide by periodate oxidation, and borohydride reduction of the products. The polyalcohol derived from the polysaccharide was hydrolyzed with 0.25M sulfuric acid for 24 h at room temperature. T.l.c. revealed the presence of two unknown products, CSD-1 and CSD-2, in addition to glycerol and a small proportion of erythritol; CSD-1 and CSD-2 showed R_{Glc} 0.83 and 1.27, respectively. Colorimetric determination after separation by p.c. gave a 2.0 : 0.14 molar ratio of glycerol to erythritol; this suggests that most of the doubly branched D-glucosyl residues are flanked by (1→4)-linked D-glucosyl residues, as shown in



(G = D-glucopyranosyl residue, M = D-mannopyranosyl group)

Scheme 1 Possible tetrasaccharide repeating-units for the polysaccharide.

Scheme 1, as only in this case can the erythritol derived from the (1→4)-linked D-glucosyl residues not be released from the polyalcohol by mild hydrolysis with acid. In this instance, the polyalcohol must be extremely depolymerized by the mild hydrolysis, and give deoxyerythritolyl D-glucoside(s). In fact, the controlled Smith degradation of the polysaccharide resulted in a remarkable depolymerization, and

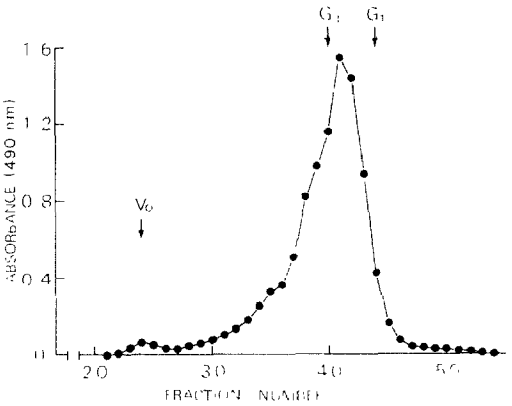


Fig. 1. Gel filtration of the mild-acid hydrolyzate of the polyalcohol on Sephadex G-25. [The polyalcohol (10 mg) was hydrolyzed with 0.25M sulfuric acid (2.0 mL) for 24 h at room temperature. The hydrolyzate, made neutral with Dowex-1 X-8 (CO_3^{2-}) resin, was evaporated to dryness, dissolved in 0.02M acetic acid (0.4 mL), and applied to a column (1.5 80 cm) of Sephadex G-25. The column was eluted with 0.02M acetic acid, and 2.4-mL fractions were analyzed by the phenol–sulfuric acid method. Arrows indicate the elution positions of G_1 (glucose) and G_3 (maltotriose).]

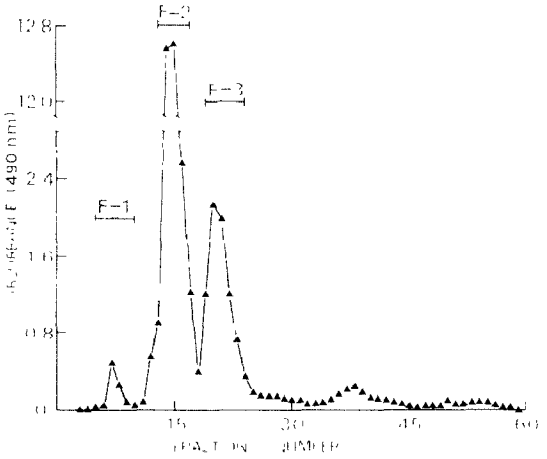


Fig. 2. Fractionation of the products of controlled, Smith degradation on Dowex-1 X-2 (OH^-) resin. [The polyalcohol (1.0 g) was subjected to hydrolysis with 0.25M H_2SO_4 for 24 h at room temperature. After crystallization of a large proportion of CSD-1, the rest was applied to a column (1.5 27 cm) of Dowex-1 X-2 (OH^-) resin. The column was eluted with water, and 9.8-mL fractions were analyzed by the phenol–sulfuric acid method.]

CSD-1 and CSD-2, both expected to be deoxyerythritolyl D-glucosides were eluted in the region of disaccharides by gel filtration on a column of Sephadex G-25 (see Fig. 1).

In order to isolate the products of controlled, Smith degradation, particularly CSD-1 and CSD-2, the polyalcohol (1.0 g) was subjected to the mild, acid hydrolysis already described. Of the resulting products, a large proportion of CSD-1 crystallized

TABLE II

ANALYSES OF CSD-1 AND CSD-2

Analysis	*Assignment*	*Compound*	
		CSD-1	*CSD-2*
^{1}H-n.m.r.[a]	Anomeric	4.57 (d, *J* 8.0 Hz)	4.54 (d, *J* 8.0 Hz)
	Acetal		4.42 (dd, *J* 4.0, 11.0 Hz)
^{13}C-n.m.r.[b]	Anomeric	102.5	103.4
	Acetal		100.0
	Glucosyloxylated	81.3	79.7
	Primary	60.6, 61.1, 62.2	60.5, 60.8, 62.0
	Other	69.5, 70.8, 73.2, 75.6, 75.8	69.2, 69.7, 70.3, 73.2, 75.7, 76.0
F.d.m.s. (*m/z*)[c]		569 ($2M^+ + 1$), 285 ($M^+ + 1$), 163	653 ($2M^+ + 1$), 327 ($M^+ + 1$), 295 ($M^+ - CH_2OH$), 163

[a]Chemical shifts (δ values) in p.p.m. from sodium 4,4-dimethyl-4-silapentane-1-sulfonate at 24° in D_2O. [b]Chemical shifts (δ values) in p.p.m. from Me_4Si at 24° (CSD-1) or 50° (CSD-2) in D_2O. [c]Selected ions.

from the ethanol solution of the products, and it was recrystallized from methanol (yield, 190 mg). The rest, including the uncrystallized portion of CSD-1, was fractionated into three fractions (F-1, F-2, and F-3) on a column of Dowex-1 X-2 (OH^-) resin, as shown in Fig. 2. Fraction F-1 contained glycerol and a small amount of erythritol, as indicated by t.l.c., and was separated into glycerol and erythritol by preparative p.c. Fractions F-2 and F-3 respectively consisted of pure CSD-2 and pure CSD-1. Thus, glycerol (270 mg), erythritol (20 mg), CSD-1 (242 mg), and CSD-2 (133 mg) were isolated from the mild, acid hydrolyzate of the polyalcohol (1.0 g) derived from the polysaccharide.

The D-glucoside CSD-1 had m.p. 189° and $[\alpha]_D$ −17° (*c* 1.0, water), and was characterized as 2-*O*-β-D-glucopyranosyl-D-erythritol[4]. The D-glucoside CSD-2 ($[\alpha]_D$ +11°; *c* 2.6, water) was crystallized as its hexaacetate (m.p. 155°), and was characterized as 5-*O*-β-D-glucopyranosyl-2,4-bis(hydroxymethyl)-1,3-dioxane[4]. Their identities were confirmed by n.m.r. spectroscopy, and f.d.-mass spectrometry (see Table II). The identities of these compounds clearly indicated that the doubly branched, (1→4)-linked D-glucosyl residues are present as single, 3,6-di-*O*-substituted units flanked by β-(1→4)-linked D-glucosyl residues, as shown in Scheme 1. These results indicated that the polysaccharide consists of tetrasaccharide repeating-units, such as sequences **1**, **2**, and **3** (see Scheme 1), and the presence of free erythritol in the mild-acid hydrolyzate showed that a small proportion (14%) of the (1→4)-linked D-glucosyl residues are located as contiguous (1→4)-linked units, or side chains, or both. The sequences **1**, **2**, and **3** would give the same analytical results, in so far as studies by methylation, periodate oxidation, and Smith degradation are concerned;

some information on the further structural features was obtained by partial acid-hydrolysis and by acetolysis of the polysaccharide.

Partial acid-hydrolysis, and acetolysis. — The polysaccharide was subjected to partial hydrolyses in order to elucidate its further structural features. The resulting oligosaccharides in the partial acid-hydrolyzate and acetolyzate were fractionated, on a column of activated carbon, into B-1 ($[\alpha]_D$ +65 ; *c* 1, water) and B-2 (+34°), and B-3 (+92°) and T-1 (+72°), respectively showing R_{Glc} 0.40, 0.54, 0.61, and 0.24 in t.l.c. (solvent *C*). The results obtained for these oligosaccharides by chemical analyses are summarized in Table III. Sugar, nonreducing terminus, and methylation analyses indicated that B-1, B-2, and B-3 are disaccharides consisting of D-Man*p*-(1→6)-D-Glc*p*, D-Glc*p*-(1→4)-D-Glc*p*, and D-Man*p*-(1→3)-D-Glc*p*, respectively, and that T-1 is a trisaccharide, either D-Glc*p*-(1→4)-[D-Man*p*-(1→3)-]-D-Glc*p* or D-Glc*p*-(1→3)-[D-Man*p*-(1→4)-]-D-Glc*p*. Trisaccharide T-1 was shown to have the former structure, based on the results of ^{1}H-n.m.r. analysis and α-D-mannosidase digestion, as described later. The identities of these oligosaccharides favor sequence **I** in Scheme 1 as the tetrasaccharide repeating-unit for the polysaccharide.

The glucobiose B-2 crystallized from 95% ethanol (m.p. 225°), and was characterized as cellobiose (lit.[5] m.p. 225°, $[\alpha]_D$ +35°). The linkage configurations of B-1, B-3, and T-1 were determined primarily by ^{1}H-n.m.r. spectroscopy. Signals common to these oligosaccharides were observed at δ 5.23–5.27 ($J_{1,2}$ low to 4.0 Hz) and 4.65–4.68 ($J_{1,2}$ 7.5–8.0 Hz), due respectively to the α- and β-anomeric protons of the D-glucose residues (at the reducing ends). The mannosylglucoses B-1 and B-3 showed one additional signal, ascribable to the anomeric proton of the α-linked D-mannosyl

TABLE III

CHEMICAL ANALYSES OF THE OLIGOSACCHARIDES

Oligo-saccharide	*Sugar (Glc/Man)*[a]	*Nonreducing terminus*	*Methylation analysis*		*Mode of linkage*
			O-Methylated sugar[b] *(as alditol acetate)*		
B-1	0.88	Mannose	2,3,4,6-Man[c]	(1.0)	Man*p*-(1→
			2,3,4-Glc	(0.9)	→6)-Glc*p*-(1→
B-2	Glucose only	Glucose	2,3,4,6-Glc	(1.0)	Glc*p*-(1→
			2,3,6-Glc	(0.9)	→4)-Glc*p*-(1→
B-3	0.97	Mannose	2,3,4,6-Man	(1.0)	Man*p*-(1→
			2,4,6-Glc	(0.9)	→3)-Glc*p*-(1→
T-1	2.04	Glucose, Mannose	2,3,4,6-Glc 2,3,4,6-Man	(1.0)	Glc*p*-(1→ Man*p*-(1→
			2,6-Glc	(0.5)	→4)-Glc*p*-(1→

[a] Molar ratio of glucose to mannose. [b] Values in parentheses indicate approximate molar ratios. [c] 2,3,4,6-Man = 2,3,4,6-tetra-*O*-methyl-D-mannose, etc.

TABLE IV

CHEMICAL SHIFTS[a] AND COUPLING CONSTANTS ($J_{1,2}$, Hz) OF THE ANOMERIC PROTONS[b]

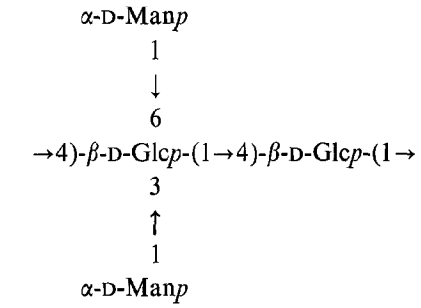
Oligo-saccharide	*Anomeric proton*		*Glycosidic proton*	
	H-1α (Glc)	*H-1β (Glc)*	*H-1′β (Glc)*	*H-1′α (Man)*
B-1	5.23 (4.0)	4.65 (8.0)		4.92 (~1)
B-3	5.27 (low)	4.68 (7.5)		5.27 (low)
T-1	5.24 (4.0)	4.68 (8.0)	4.50 (8.0)	5.36 (~1)

[a]In p.p.m. from sodium 4,4-dimethyl-4-silapentane-1-sulfonate. [b]In D_2O at 81.3°.

group, at δ 4.92 ($J_{1,2}$ ~1 Hz) and 5.27 ($J_{1,2}$ low), respectively. The branched mannosylglucobiose T-1 showed two additional signals, at δ 5.36 ($J_{1,2}$ ~1 Hz) and 4.50 ($J_{1,2}$ 8.0 Hz) ascribable to the anomeric protons of the α-linked D-mannosyl group and the β-(1→4)-linked D-glucosyl residues[6], respectively. These ^{1}H-n.m.r. data in the anomeric region are given in Table IV.

These linkage configurations were confirmed by digestion with the α-D-mannosidase from *Turbo cornutus*, which is an exoglycosidase, specific for α-D-linked D-mannose residues, that releases D-mannose from the nonreducing end[7]. This enzyme released mannose and glucose from both B-1 and B-3, and mannose and cellobiose from T-1, in agreement with the results obtained by the chemical analyses and ^{1}H-n.m.r. spectroscopy. Thus, B-1 was characterized as α-D-Man*p*-(1→6)-D-Glc*p* (lit.[23] $[\alpha]_D$ +70°), B-3 as α-D-Man*p*-(1→3)-D-Glc*p*, and T-1 as β-D-Glc*p*-(1→4)-[α-D-Man*p*-(1→3)-]-D-Glc*p*.

From all of the foregoing results (obtained by methylation, Smith-degradation, and partial-hydrolysis studies), it is deduced that the structure of the repeating unit of the polysaccharide is as shown.



DISCUSSION

The structure of an extracellular, antitumor polysaccharide from *Microellobosporia grisea* was analyzed by methylation, Smith-degradation, and partial-

hydrolysis techniques. The structural analyses showed that the polysaccharide has a unique structure, differing from those of other antitumor polysaccharides from various sources[2]. The 7-D-mannopyranosyl groups, the side chains in the polysaccharide, should play an important role in its water-solubility and antitumor activity, as its main chain is a water-insoluble (1→4)-β-D-glucan (cellulose), which has been reported to be inactive[5]. The results of methylation and Smith-degradation analyses led us to propose the three possible sequences **1**, **2**, and **3** (Scheme 1) as the structure of the repeating unit of the polysaccharide. Based on the identities of the oligosaccharides obtained by partial acid-hydrolysis and acetolysis, sequence **1** was preferred to the other two. This was supported by the observation that the D-mannosyl groups at O-3 of the doubly branched D-glucosyl residues were released, to yield single-branched, 6-*O*-substituted (1→4)-linked D-glucosyl residues by mild hydrolysis with, *e.g.*, 0.2M HCl during 7 h at 80° (unpublished result). The configurations of the linkages in the polysaccharide, which were confirmed by the results of controlled, Smith degradation and partial hydrolyses, were supported by i.r. spectroscopy: the polysaccharide gave absorption bands at 880 and 810 cm^{-1}, respectively ascribable to β-glucosidic and α-mannosidic linkages, as already described[1].

Of the controlled, Smith-degradation products, CSD-2 [5-*O*-β-D-glucopyranosyl-2,4-bis(hydroxymethyl)-1,3-dioxane], which is a glycolaldehyde acetal of CSD-1 (2-*O*-β-D-glucopyranosyl-D-erythritol), was first isolated[6] from the mild acid-hydrolyzate of oat-glucan polyalcohol, in addition to CSD-1. Thereafter, no-one has reported CSD-2 as a degradation product of polysaccharides, except for its relative, CSD-1. Interestingly, the controlled Smith-degradation of the polysaccharide, as herein described, yielded both CSD-1 and CSD-2, which were isolated in the molar ratio of ~1:0.5. The formation of a relatively large proportion of CSD-2 may be related to the unique structure of this polysaccharide, compared with that of the linear (1→3;1→4)-β-D-glucan from oats, giving[4] CSD-1 and CSD-2 in the molar ratio of 1.0:0.11.

EXPERIMENTAL

Materials. — The polysaccharide used in this work was isolated from the culture filtrate of *Microellobosporia grisea*, as described previously[1].

General methods. — Melting points were determined with a capillary melting-point apparatus and are uncorrected. Paper chromatography (p.c.) was performed on Toyo No. 51 filter paper by the ascending method with the solvent systems (v/v): (*A*) 5:2:7 ethyl acetate–pyridine–water and (*B*) 4:1.5:1 1-butanol–ethanol–water. Preparative p.c. was conducted on Whatman No. 3 MM paper. Sugars and alditols were detected with alkaline silver nitrate[10]. Thin-layer chromatography (t.l.c.) was performed on Kieselgel $60F_{254}$ (Merck) by the ascending method, using the solvent systems (v/v): (*C*) 5:3:1 1-butanol–2-propanol–water and (*D*) 1:1 benzene–acetone. Sugars were detected with *p*-anisaldehyde–sulfuric acid[11], and alditols with ammoniacal silver nitrate.

Gas–liquid chromatography (g.l.c.) was performed with a Hitachi model 163 gas chromatograph equipped with a flame-ionization detector. Separation was accomplished in a glass column (2 m × 3 mm, i.d.) packed with 3% of OV-225 on Gas Chrom Q (100–120 mesh), using nitrogen at 60 mL/min as the carrier gas. The column temperature was kept at 190° and 170° for alditol acetates and partially methylated alditol acetates, respectively, and programmed from 80 to 190° at the rate of 5°/min for Smith-degradation products. G.l.c.-mass spectrometry (g.l.c.–m.s.) was performed with the chromatographic column coupled to a JEOL JMS-D300 mass spectrometer at an ionization potential of 70 eV. Field-desorption mass-spectrometry (f.d.m.s.) was performed with a JEOL JMS-OISG2 mass spectrometer at an emitter current of 13–17.5 mA.

N.m.r. spectra for solutions in D_2O were recorded in the pulsed, Fourier-transform mode with a Varian XL-200 spectrometer. As references, external tetramethylsilane (Me_4Si) was used for proton-decoupled, ^{13}C-n.m.r. spectroscopy (50.2 MHz), and internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate for ^1H-n.m.r. spectroscopy (200 MHz). Chemical shifts are given in p.p.m. downfield from the reference standards.

Other analytical methods used in this work were as described previously[1].

Methylation analysis. — Prior to methylation, the sample was dried over P_2O_5 *in vacuo* for 5 h at 70°. The dry polysaccharide (200 mg) was methylated according to the procedure of Hakomori[3] as modified by Sandford and Conrad[12]. Complete methylation was confirmed by absence of OH absorption in the infrared (i.r.) spectra in carbon tetrachloride. The fully methylated polysaccharide thus obtained was precipitated from the chloroform solution by addition of five volumes of petroleum ether (yield, 176 mg), and hydrolyzed sequentially in 90% formic acid and then 0.25M sulfuric acid[13]. The resulting, methylated sugars were converted into the corresponding alditol acetates[14], and analyzed by g.l.c.[15] and g.l.c.–m.s.[16].

The methylated sugars were also fractionated by preparative t.l.c. (solvent *D*). The methylated sugars obtained were each demethylated with boron trichloride according to the method of Allen *et al.*[17], and then analyzed by t.l.c. (solvent *C*) and g.l.c. as the alditol acetates[18].

Periodate oxidation. — The polysaccharide (100 mg) was oxidized with 30mM sodium metaperiodate (100 mL) in the dark at 5°. The periodate consumption was determined at various time-intervals by a spectrophotometric method[19], and the formic acid production by titration with 10mM sodium hydroxide.

Preparation of the polyalcohol. — The polysaccharide (3.0 g) was oxidized with 60mM sodium metaperiodate (1.0 L) for 12 days at 5° in the dark. The reaction was then stopped with ethylene glycol, and the mixture was dialyzed against de-ionized water at 5°. The oxidized polymer in the non-dialyzable solution was reduced with sodium borohydride (4.5 g) for 1 day at room temperature. After decomposition of the excess of borohydride by addition of acetic acid (final pH, 5.5), the solution was again dialyzed as already described. The non-dialyzable solution was concentrated, and the concentrate centrifuged. The derived polyalcohol, in the supernatant liquor

(50 mL), was precipitated with ethanol (1.0 L), and dehydrated over P_2O_5 *in vacuo* (yield, 2.6 g).

Smith degradation. — The polyalcohol (20 mg) was completely hydrolyzed with 0.5M sulfuric acid for 5 h at 100°. The hydrolyzate was made neutral with Dowex-1 X-8 (CO_3^{2-}) resin, passed through a column of Dowex-50 X-8 (H^+) resin, and evaporated to dryness *in vacuo*. The complete Smith-degradation products thus obtained were examined by p.c. (solvents *A* and *B*) and t.l.c. (solvent *C*), extracted with water after separation by p.c. (solvent *A*), and determined colorimetrically, sugars were determined with phenol–sulfuric acid[20], and alditols with chromotropic acid[21]. For g.l.c. analysis, these products were converted into the corresponding alditol acetates[18].

In the controlled Smith-degradation, the polyalcohol (20 mg) was subjected to mild hydrolysis with 0.25M H_2SO_4 during 24 h at room temperature. The hydrolyzate was treated as already described, and the resulting degradation products were determined colorimetrically after p.c. separation (solvent *A*).

Isolation of the controlled, Smith-degradation products. — The polyalcohol (1.0 g) derived from the polysaccharide was hydrolyzed with 0.25M sulfuric acid (200 mL) for 24 h at room temperature. The hydrolyzate was made neutral with Dowex-1 X-8 (CO_3^{2-}) resin and evaporated to a syrup. Addition of ethanol (40 mL) to the syrup yielded a crystalline compound, which was recrystallized from methanol to give CSD-1 (190 mg).

Anal. Calc. for $C_{10}H_{20}O_9$: C, 42.25; H, 7.09. Found: C, 41.89; H, 6.85.

The ethanolic mother liquor was evaporated to a syrup, which was dissolved in water (2 mL), and fractionated, on a column (1.5 × 27 cm) of Dowex-1 X-2 (OH^-) resin (200–400 mesh), according to Austin *et al.*[22], to give a mixture (270 mg) of glycerol and erythritol, CSD-2 (133 mg), and CSD-1 (52 mg). The mixture was further fractionated by preparative p.c. (solvent *A*), to give glycerol (210 mg) and erythritol (20 mg). The syrupy compound CSD-2 (40 mg) was acetylated with 1:1 acetic anhydride–pyridine (0.8 mL), to give a crystalline compound, which was recrystallized from ethanol to yield the hexaacetate (55 mg). f.d.-m.s. data (selected ions): *m/z* 621 ($M^+ + CH_3CO$), 579 ($M^+ + 1$), 577 ($M^+ - 1$), and 518 ($M^+ - 1 - CH_3CO_2$).

Anal. Calc. for $C_{24}H_{34}O_{16}$: C, 49.83; H, 5.92. Found: C, 49.67; H, 5.95.

Partial hydrolysis with acid. — The polysaccharide (3.0 g) was hydrolyzed with 165mM sulfuric acid (500 mL) for 7 h at 100°, and the acid was neutralized with Dowex-1 X-8 (CO_3^{2-}) resin. The resulting, neutral solution was concentrated to ~50 mL, and the concentrate was applied to a column (2.0 × 40 cm) of activated carbon. The saccharides were eluted stepwise with water (2.0 L), 1:99 ethanol–water (3.0 L), and 1:39 ethanol–water (5.0 L). Fractions (100 mL) were collected, and analyzed by the phenol–sulfuric acid method[20]. The fraction eluted with water contained monosaccharides (glucose and mannose) and oligosaccharides B-1 (50 mg) and B-2 (180 mg), which were respectively eluted with 1:99, and 1:39

ethanol–water; each showed a single spot in t.l.c. (solvent *C*), and was isolated by freeze-drying.

Acetolysis. — The polysaccharide (5.0 g) in formamide (100 mL) was acetylated with acetic anhydride (180 mL) and pyridine (200 mL) for 2 days at 30°. The mixture was poured into 2% hydrochloric acid (5.0 L), with cooling, and the resulting precipitate was collected, washed successively with 0.5% hydrochloric acid, water, and methanol, and dried *in vacuo*. A solution of the solid in chloroform (200 mL) was passed through a column (3.0 × 40 cm) of silica gel (Merck), and the effluent and washing (chloroform) were combined, and evaporated to dryness *in vacuo*, to give acetylated material (6.5 g) which was dissolved in acetic anhydride (150 mL), and acetolyzed with sulfuric acid (15 mL) and acetic acid (150 mL) for 9 days at 30°. The mixture was poured onto ice cubes (~700 mL), made neutral with sodium carbonate, and extracted with chloroform (4 × 1.0 L). The extracts were combined, successively washed with 1% sodium carbonate and water, dried (anhydrous sodium sulfate), and evaporated to dryness. A suspension of the residue in methanol (30 mL) was centrifuged to remove insoluble materials, and the supernatant liquor was evaporated to dryness *in vacuo*, to give acetylated oligosaccharides (6.4 g).

The acetylated oligosaccharides (6.4 g) were dissolved in redistilled methanol (100 mL), and deacetylated with 5M sodium methoxide (1.0 mL) for 2 h at room temperature. The mixture was made neutral with acetic acid, evaporated to dryness, and the residue dissolved in water (30 mL), and applied to a column (2.0 × 55 cm) of activated carbon. The saccharides were eluted stepwise with water (3.0 L), 1:99 ethanol–water (4.5 L), and 1:39 ethanol–water (7.5 L). Fractions (300 mL) were collected, and analyzed by the phenol–sulfuric acid method[20]. Oligosaccharides B-3 (136 mg) and T-1 (293 mg), respectively eluted as a single peak with 1:99 ethanol–water and as the first peak with 1:39 ethanol–water; each showed a single spot in t.l.c. (solvent *C*), and was isolated by freeze-drying. A mixture (248 mg) of T-1 and B-2 was eluted as the second peak with 1:39 ethanol–water.

Chemical analyses of the oligosaccharides. — The oligosaccharides (~3 mg), each permethylated by the method of Hakomori[3], were hydrolyzed with 0.25M sulfuric acid, and the resulting methylated sugars were analyzed as the alditol acetates by g.l.c.[15] and g.l.c.–m.s.[16]. The sugar composition of the oligosaccharides was analyzed as the alditol acetates[18] by g.l.c. after hydrolysis with 0.5M H_2SO_4 for 5 h at 100°. Analyses of nonreducing termini were conducted by t.l.c. (solvent *C*) of hydrolyzates (0.5M H_2SO_4 for 5 h at 100°) of the oligosaccharides reduced with sodium borohydride.

α-D-*Mannosidase digestion.* — Each of the oligosaccharides (~1 mg) in 50mM citrate–phosphate buffer, pH 4.0 (1.0 mL), was incubated with α-D-mannosidase (30 mU) from the liver of *Turbo cornutus* (Seikagaku Kogyo, Ltd.) for 1 day at 37°. The mixture was desalted with Dowex-50 X-8 (H^+) and Dowex-1 X-8 (CO_3^{2-}) resins under cooling. The resulting, neutral solution was concentrated, and then subjected to t.l.c. (solvent *C*).

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