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STRUCTURAL ANALYSIS OF ACIDIC OLIGOSACCHARIDES DERIVED FROM THE METHYLATED, ACIDIC POLYSACCHARIDE ASSOCIATED WITH COCCOLITHS OF *Emiliania huxleyi* (LOHMANN) KAMPTNER

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ABSTRACT

A series of acidic oligosaccharides was obtained by graded, acid hydrolysis of the methylated, acidic polysaccharide associated with the coccoliths of the alga *Emiliania huxleyi* (Lohmann) Kamptner. After fractionation by ion-exchange chromatography, the structures of the oligosaccharides were determined by sugar analysis, g.l.c.–m.s. of the intact, permethylated oligosaccharide-alditols, and methylation analysis. The following oligosaccharides were characterised:

α-D-Gal*p*A-(1→6)-α-D-Man*p*-(1→3)-D-Man,
D-Gal*p*A-(1→4)-D-Gal*p*A-(1→6)-Man,
D-Gal*p*A-(1→4)-D-Gal*p*A-(1→2/6)-Man*p*-(1→3)-Man,
D-Gal*p*A-(1→4)-D-Gal*p*A-(1→3)-D-Xyl,
D-Gal*p*A-(1→2)-L-Man*p*6Me-(1→4)-D-Gal*p*A-(1→2)-L-Rha,
D-Gal*p*A-(1→2)-Man*p*-(1→4)-D-Gal*p*A-(1→2)-L-Rha,
D-Gal*p*A-(1→2)-L-Rha*p*-(1→4)-D-Gal*p*A-(1→2)-L-Rha,
D-Gal*p*A-(1→2)-L-Man*p*6Me-(1→4)-D-GalA,
D-Gal*p*A-(1→2)-Man*p*-(1→4)-D-GalA,
D-Gal*p*A-(1→2)-L-Man*p*6Me-(1→4)-D-Gal*p*A-(1→2)-L-Man6Me,
D-Gal*p*A-(1→2)-L-Man*p*6Me-(1→4)-D-Gal*p*A-(1→2)-Man,
D-Gal*p*A3Me-(1→2)-L-Man*p*6Me-(1→4)-D-Gal*p*A-(1→2)-L-Man6Me, and
D-Gal*p*A3Me-(1→2)-L-Man*p*6Me-(1→4)-D-GalA.

INTRODUCTION

The cell wall of the unicellular alga *Emiliania huxleyi* (Lohmann) Kamptner contains calcified plates, called coccoliths[1]. Sugar analysis of the water-soluble polysaccharide associated with these coccoliths revealed the presence of residues of L-galactose, D-glucose, D-mannose, L-mannose, L-rhamnose, L-arabinose, D-ribose, D-xylose, 6-*O*-methyl-D-mannose, 6-*O*-methyl-L-mannose, 2,3-di-*O*-methyl-L-rham-

nose, 3-*O*-methyl-D-xylose, 4-*O*-methyl-galactose, D-galacturonic acid, and 3-*O*-methyl-D-galacturonic acid[2]. In addition, the biopolymer contains ~3.8% of ester-bound sulphate[2]. The methylated, acidic polysaccharide is supposed to play a matrix role in the biocalcification process.

In order to obtain information on the primary structure of this macromolecule, it has been submitted to several degradation procedures, and we now report on the structural analysis of oligosaccharides obtained after graded hydrolysis with acid.

RESULTS AND DISCUSSION

The methylated, acidic polysaccharide of the alga was degraded by mild hydrolysis with 2M trifluoroacetic acid (3 h at 95°). The resulting products were fractionated by ion-exchange chromatography on Dowex-1 X2 ($formate^-$) resin. Washing with water yielded a neutral monosaccharide fraction, and mixtures (fractions I–III) of acidic oligosaccharides were obtained by stepwise elution with 0.25, 0.50, and 0.75M formic acid, respectively. Additional fractionations could not be performed because of the small amounts of material available (see Experimental).

For the identification of the various oligosaccharides, a number of methods were used. Sugar analysis was performed by g.l.c. of trimethylsilylated methyl glycosides[3]. Absolute configurations were determined by g.l.c. of trimethylsilylated (—)-2-butyl glycosides[4]. Permethylation of oligosaccharide-alditols was performed with sodium methylsulfinylmethanide in methyl sulfoxide and trideuteriomethyl-iodide[5]. For the methylation analysis, the carboxyl methyl esters of the permethylated, acidic oligosaccharide-alditols were reduced; after hydrolysis, the resulting methylated sugars were transformed into the corresponding alditol acetates[6]. All reduction steps were carried out with sodium borodeuteride. The permethylated oligosaccharide-alditols as well as the partially methylated alditol acetates were analysed by g.l.c. and g.l.c.–m.s.[6,7,8]. The symbols A and J introduced by Kochetkov and Chizhov[9], supplied with lower-case letters a, b, c, and d to indicate the monosaccharide units[10], are used in the discussion of the mass-spectral fragmentation patterns of the permethylated oligosaccharide-alditols present in the three formic acid fractions.

Fraction I (0.25M HCOOH). — Fraction I-A (R_{Man} 0.59) was isolated from I by preparative, paper chromatography, and was shown to consist of D-galacturonic acid and D-mannose in the molar proportions 0.3 : 1.0. After reduction, D-galacturonic acid, D-mannose, and mannitol were found in the molar ratios 0.3 : 0.6 : 0.4 (Table I). The ^{1}H-n.m.r. spectrum of reduced I-A showed two anomeric signals of equal intensity at δ 5.01 (1 H, $J_{1,2}$ ~1 Hz, α-D-mannose[11,12]) and 4.98 (1 H, $J_{1,2}$ 3.0 Hz, α-D-galacturonic acid), respectively, pointing to a trisaccharide-alditol. G.l.c. of the permethylated, reduced I-A gave one intense peak in the oligosaccharide region.

The mass spectrum obtained from the material in this g.l.c.-peak showed, *inter alia*, fragments at *m/e* 527 ($abcJ_1$), 464-429-394 (bcA_1-bcA_2-bcA_3), 314 (bcJ_1), 251-181 (cA_1-cA_3), and 245-210-175 (aA_1-aA_2-aA_3), suggesting the sequence GalA-Man-Man-ol-*1-d* (Scheme 1). The presence of the bcJ_1 fragment excludes a (1→3)

TABLE I

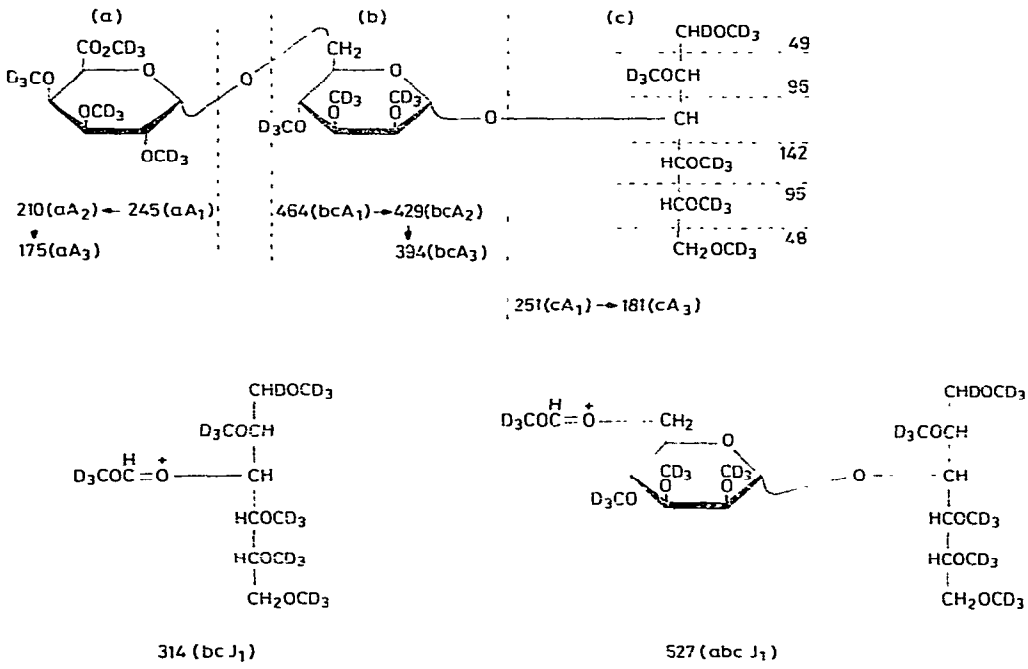
SUGAR ANALYSIS OF OLIGOSACCHARIDE FRACTIONS I–III

Component	T[a]	*Approximate molar ratio*[b]							
		I		*II*			*III*		
		P	*Q*	*P*	*Q*	*R*	*P*	*Q*	*R*
L-Rhamnose	0.38; 0.39	—	—	0.7	0.1	0.1	0.2	—	—
D-Xylose 3-*O*-Methyl-D-galacturonic acid[c]	0.47; 0.50	—	—	N.d.[d]	N.d.[d]	N.d.[d]	N.d.[d]	N.d.[d]	N.d.[d]
6-*O*-Methyl-L-mannose	0.55	—	—	1.9[e]	1.1[e]	1.1[e]	1.1[e]	0.9[e]	0.9[e]
Xylitol	0.60	—	—	—	0.3	0.3	—	0.4	0.4
D-Galacturonic acid	0.63; 0.69; 0.79; 0.81	0.3	0.3	5.4[f]	2.7	2.7	1.9	1.8	1.7
D-Mannose[g]	0.70; 0.75	1.0	0.6	0.7	0.5[h]	0.5[h]	0.7	0.3[h]	0.3[h]
L-Mannose[g]	0.70; 0.75	—	—	0.3	0.2[h]	0.2[h]	0.3	0.2[h]	0.2[h]
Rhamnitol	0.70	—	—	—	0.6[h]	0.6[h]	—	0.2[h]	0.2[h]
6-*O*-Methylmannitol	0.82	—	—	—	0.8[e]	0.7[e]	—	0.2[e]	0.2[e]
Galactonic acid	0.86[i]; 0.96	—	—	—	3.7	2.2	—	0.2	0.2
Mannitol	1.00; 1.09[j]	—	0.4	—	0.3	0.3	—	0.5	0.5
Galactitol-6,6-d_2	1.02	—	—	—	—	1.5	—	—	0.1

[a]Retention times of the trimethylsilylated methyl glycosides and trimethylsilylated alditols relative to pertrimethylsilylmannitol on an SE-30 glass-capillary column; in some cases, only *T* values for the main anomeric forms are given (temperature programme, 130→200° at 1°/min). [b]P, before reduction; Q, after one reduction-step; R, after two reduction-steps; values were calculated by using experimental, molar adjustment factors and are given relative to mannose + mannitol. [c]The identification of this constituent will be reported elsewhere. [d]Not determined; values for xylose and 3-*O*-methylgalacturonic acid could not be determined separately and the molar adjustment factor of the acid is unknown. [e]Calculated using the molar adjustment factor of mannose and mannitol, respectively. [f]Compared with columns Q and R, the value of total D-galacturonic acid is somewhat too low. An explanation cannot be given. [g]The ratio between the D and L form was determined from the butanolysis data. [h]After determination of the total amount of mannose + rhamnitol (both carbohydrates have the same molar adjustment factor), the ratio between mannose and rhamnitol (columns Q and R) was deduced from the values of mannose and rhamnose (column P) and of rhamnose or mannitol (columns Q and R). [i]Lactone form. [j]Small amount of 1(6)-mono-*O*-acetylmannitol, formed during the re-*N*-acetylation step in the methanolysis procedure[3].

linkage between GalA and Man[8]. The mass spectrum does not give information about the linkage between Man and Man-ol-*1-d*. Methylation analysis of the permethylated, reduced I-A demonstrated the presence of 1,2,4,5,6-penta-*O*-trideuteriomethylmannitol (8%), 2,3,4-tri-*O*-trideuteriomethylmannose (40%), and 2,3,4-tri-*O*-trideuteriomethylgalactose-6,6-d_2 (33%) (Table II). These results indicate the following structure for the main component in fraction I-A: α-D-Gal*p*A-(1→6)-α-D-Man*p*-(1→3)-D-Man.

Fraction II (0.50M HCOOH). — Fraction II is composed of L-rhamnose, D-xylose, 3-*O*-methyl-D-galacturonic acid, 6-*O*-methyl-L-mannose, D-galacturonic acid, D-mannose, and L-mannose, and was treated twice with borodeuteride. After



Scheme 1

the first reduction, the following alditols were detected: xylitol, rhamnitol, 6-*O*-methylmannitol, galactonic acid ("alditol", derived from galacturonic acid), and mannitol. The second reduction-step converted part of the galactonic acid into galactitol-*6,6-d*$_2$. This feature must be attributed to the presence of some methyl galactonate formed during the removal of boric acid with methanol after the first reduction. The molar carbohydrate compositions are presented in Table I. The formation of various alditols indicates that II is a mixture of several oligosaccharides. Reduced II was permethylated and part of it subjected to methylation analysis. As is evident from Table II, numerous partially methylated alditol acetates were found. The other part was studied directly by g.l.c.–m.s.; Fig. 1 shows a reconstructed ion-current chromatogram of permethylated, reduced II.

The mass spectrum of peak A showed, *inter alia*, fragments at *m/e* 526/523 (abcJ$_1$), 463-428 (bcA$_1$-bcA$_2$), 455/452-420/417 (baA$_1$-baA$_2$), 316 (bcJ$_1$), 253-183/182 (cA$_1$-cA$_3$), and 245/242-210-175 (aA$_1$-aA$_2$-aA$_3$), indicating the presence of two trisaccharide-alditols, namely, HexA-HexMe-Hex-ol-*1,6,6-d*$_3$ (II-A1) and HexA3Me-HexMe-Hex-ol-*1,6,6-d*$_3$ (II-A2). The two series of abcJ$_1$, baA$_1$-baA$_2$, and aA$_1$ fragments differing by 3 m.u., and present at equal abundances, point to trisaccharide-alditols that differ only in the presence of a naturally occurring methyl group in the terminal HexA. The observation of the abcJ$_1$ fragment at *m/e* 523 confirms directly the position of the naturally occurring methyl group in HexA3Me[8]. Using the sugar analysis (Table I), these oligosaccharide structures can be identified as GalA-Man6Me-

TABLE II

METHYLATION ANALYSIS OF REDUCED OLIGOSACCHARIDE FRACTIONS I–III

Trideuteriomethylated sugar[a]	T[b]	*Approximate mole %*		
		I	*II*	*III*
1,3,4,5-Rha[d]	0.21	—	4	1
1,2,4,5-Xyl[d]	0.22	—	2	8[e]
1,3,4,5,6-Man[d]	0.39	—	1	—
1,3,4,5-Man6Me[d]	0.39	—	8	4
1,2,4,5,6-Man[d]	0.41	8	—	4[f]
1,2,3,5,6-Gal-*6,6-d*$_2$[d]	0.44	—	4	1
1,2,3,4,5-Man[d]	0.46	—	—	6
1,2,3,4,5-Gal-*6,6-d*$_2$[d,g]	0.53	—	13	—
3,4-Rha	0.88	—	1	1
2,3,4,6-Man	1.00	8	2	3
2,3,4-Man6Me	1.00	—	2	1
1,2,3,5-Gal-*6,6-d*$_2$	1.05	—	—	3
3,4,6-Man	1.82	—	2	2
3,4-Man6Me	1.82	—	18	19
2,4,6-Man	1.88	6	—	—
2,3,4-Man	2.16	40	—	1
2,3,4-Gal-*6,6-d*$_2$	2.78	33	18	14
2,4-Gal3Me-*6,6-d*$_2$	2.78	—	5	4
2,4-Man	4.30	6	—	—
2,3-Gal-*6,6-d*$_2$	4.51	—	17	20
2,4-Gal-*6,6-d*$_2$	4.86	—	—	6

[a]1,3,4,5-Rha = 1,3,4,5-tetra-*O*-trideuteriomethylrhamnitol, 3,4-Rha = 3,4-di-*O*-trideuteriomethylrhamnose, *etc.* [b]Retention time of the corresponding alditol acetate relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol on 3% of OV-225 at 180°. [c]Ratios between the amounts of trideuteriomethylated alditol acetates and corresponding trideuteriomethylated/methylated alditol acetates were deduced from the mass spectra. [d]Part of this volatile compound and/or its acetate was probably lost during concentrations. [e]Contaminated with an unknown, non-carbohydrate compound. [f]The presence of a small proportion of 1,2,4,5-Man6Me could not be excluded. [g]Derived from free D-galacturonic acid.

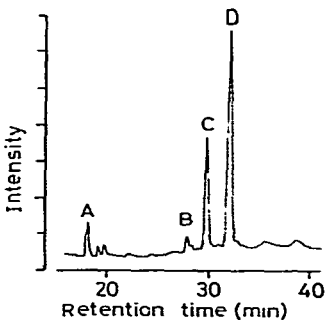


Fig. 1. Reconstructed ion-current chromatogram of fraction II after reduction and permethylation; A to D represent permethylated oligosaccharide-alditols, as discussed in the text.

Gal-ol-*1,6,6-d*$_3$ (II-A1) and GalA3Me-Man6Me-Gal-ol-*1,6,6-d*$_3$ (II-A2). With regard to the types of glycosidic linkage, the following information can be obtained from the mass spectrum. The detection of a bcJ_1 fragment excludes a (1→3) linkage between GalA(3Me) and Man6Me. The occurrence of alditol fragments at *m/e* 49, 50, 96, 97, and 143 are in accordance with a (1→4) linkage between Man6Me and Gal-ol-*1,6,6-d*$_3$. The latter alditol stems from galacturonic acid, originally present as the reducing unit in the parent oligosaccharides. These results, in combination with those obtained from the methylation analysis (Table II), lead to the following structures for the parent trisaccharides of II-A1 and II-A2, respectively: D-Gal*p*A-(1→2)-L-Man*p*6Me-(1→4)-D-GalA and D-Gal*p*A3Me-(1→2)-L-Man*p*6Me-(1→4)-D-GalA.

The mass spectrum of peak B showed, *inter alia*, signals at *m/e* 688 ($abcdJ_1$), 508 ($bcdJ_1$), 425-390-355 (baA_1-baA_2-baA_3), 281 (cdJ_1), 245-210-175 (aA_1-aA_2-aA_3), and 218 (dA_1), suggesting the sequence HexA-DeoxyHex-HexA-DeoxyHex-ol-*1-d* (II-B) for the oligosaccharide-alditol. The g.l.c. retention time is in accordance with that for tetrasaccharide-alditols (*cf.* peak D). The only information on the types of glycosidic linkage which can be derived from the mass spectrum is the exclusion of (1→3) linkages between the monosaccharide units by the presence of the $bcdJ_1$ and cdJ_1 fragments. Taking into account the sugar and methylation analysis data (Tables I and II), the following structure can be given for the parent tetrasaccharide of II-B: D-Gal*p*A-(1→2)-L-Rha*p*-(1→4)-D-Gal*p*A-(1→2)-L-Rha*.

The mass spectrum of peak C showed, *inter alia*, signals at *m/e* 721/718 ($abcdJ_1$), 508 ($bcdJ_1$), 458/455-423/420 (baA_1-baA_2), 245-210-175 (aA_1-aA_2-aA_3), and 218 (dA_1), indicating the presence of two oligosaccharide-alditols: HexA-HexMe-HexA-DeoxyHex-ol-*1-d* (II-C1) and HexA-Hex-HexA-DeoxyHex-ol-*1-d* (II-C2). The g.l.c. retention time is in accordance with that for tetrasaccharide-alditols (*cf.* peak D). The occurrence of HexMe in addition to Hex was deduced from the presence of two series of $abcdJ_1$, baA_1, and baA_2 fragments differing by 3 m.u. On the basis of the sugar analysis (Table I), the sequences for II-C1 and II-C2 are GalA-Man6Me-GalA-Rha-ol-*1-d* and GalA-Man-GalA-Rha-ol-*1-d*, respectively. Since the intensity patterns of the fragment ions of the two series in the mass spectrum are identical, the same types of glycosidic linkage occur in II-C1 and II-C2. From the relative abundances, it is concluded that the ratio between II-C1 and II-C2 is 7:3. Concerning the types of glycosidic linkage in the oligosaccharide-alditols, a (1→3) linkage between GalA and Man(6Me) can be excluded ($bcdJ_1$). These results, in combination with the methylation analysis (Table II), lead to the following structures for the parent tetrasaccharides of II-C1 and II-C2, respectively: D-Gal*p*A-(1→2)-L-Man*p*6Me-(1→4)-D-Gal*p*A-(1→2)-L-Rha and D-Gal*p*A-(1→2)-Man*p*-(1→4)-D-Gal*p*A-(1→2)-L-Rha. Comparison of the oligosaccharides makes it tempting to suggest that mannose has the same absolute configuration as 6-*O*-methyl-L-mannose.

The mass spectrum of peak D showed, *inter alia*, fragments at *m/e* 751/748/745

*The possibility of a (1→5)-linked D-Gal*f*A residue instead of a (1→4)-linked D-Gal*p*A cannot be excluded. The same holds for the other (1→4)-substituted D-Gal*p*A units reported herein.

(abcdJ_1), 541/538 (bcdJ_1), 455/452-420/417-385 (baA_1-baA_2-baA_3), 251/248 (dA_1), and 245/242-210-175 (aA_1-aA_2-aA_3). The observation of a few series of fragments differing by 3 m.u. points to the presence of a mixture of oligosaccharide-alditols consisting of compounds having various degrees of natural methylation. In view of the sugar analysis (Table I), the following six tetrasaccharide-alditol sequences have to be taken into account: GalA-Man6Me-GalA-Man6Me-ol-*l-d* (II-D1), GalA3Me-Man6Me-GalA-Man6Me-ol-*l-d* (II-D2), GalA-Man6Me-GalA-Man-ol-*l-d* (II-D3), GalA3Me-Man6Me-GalA-Man-ol-*l-d* (II-D4), GalA-Man6Me-GalA3Me-Man-ol-*l-d* (II-D5), and GalA3Me-Man6Me-GalA3Me-Man-ol-*l-d* (II-D6). The absence of non-terminal GalA3Me, as is clear from the methylation analysis (Table II), excludes II-D5 and II-D6. The occurrence of bcdJ_1 fragments in the mass spectrum excludes (1→3) linkages between GalA(3Me) and Man6Me in II-D1 to II-D4. The presence of signals at *m/e* 45, 48, 92, 95, 151, and 154, the latter two ions being derived from the primary fragments *m/e* 186 and 189, respectively, are in accordance with a (1→2) linkage between GalA and Man(6Me)-ol-*l-d*. In combination with the methylation analysis data the following parent oligosaccharides can be proposed for II-D1 to II-D4, respectively: D-Gal*p*A-(1→2)-L-Man*p*6Me-(1→4)-D-Gal*p*A-(1→2)-L-Man6Me, D-Gal*p*A3Me-(1→2)-L-Man*p*6Me-(1→4)-D-Gal*p*A-(1→2)-L-Man6Me, D-Gal*p*A-(1→2)-L-Man*p*6Me-(1→4)-D-Gal*p*A-(1→2)-Man, and D-Gal*p*A3Me-(1→2)-L-Man*p*-6Me-(1→4)-D-Gal*p*A-(1→2)-Man. From the peak-intensity ratios between *m/e* 751, 748, and 745 (11:55:34), 541 and 538 (10:90), 455 and 452 (62:38), 251 and 248 (11:89), and 245 and 242 (64:36) in the mass spectrum of peak D, the following composition can be estimated: 55%, II-D1; 34%, II-D2; 11%, II-D3; and ⩽1%, II-D4. With regard to the absolute configuration of the reducing mannose residue, the same suggestion can be made as for the parent oligosaccharide of II-C2.

Fraction III (0.75M HCOOH). — Fraction III consisted of L-rhamnose, D-xylose, 3-*O*-methyl-D-galacturonic acid, 6-*O*-methyl-L-mannose, D-galacturonic acid, D-mannose, and L-mannose. The following alditols were found after two successive reductions (*cf.* Fraction II): xylitol, rhamnitol, 6-*O*-methylmannitol, galactonic acid

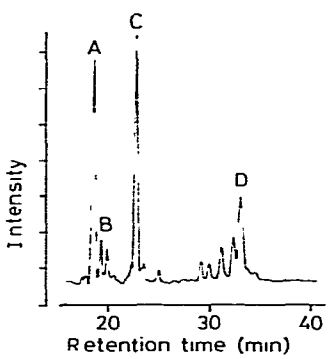


Fig. 2. Reconstructed ion-current chromatogram of fraction III after reduction and permethylation; A to D represent permethylated oligosaccharide-alditols, as discussed in the text.

("alditol", derived from galacturonic acid), mannitol, and galactitol-*6,6-d*$_2$ (see Table I). These alditols represent the various oligosaccharide-alditols in reduced III. After permethylation, part of reduced III was subjected to methylation analysis (see Table II). The other part was studied directly by g.l.c.–m.s.; Fig. 2 shows a reconstructed ion-current chromatogram of permethylated, reduced III.

The mass spectrum of peak A showed, *inter alia*, fragments at *m/e* 507 (M — $COOCD_3COCHOCD_3CH_2OCD_3$ or M — $COOCD_3CHO$ — $CHOCD_3CHOCD_3$), 494 (abcJ$_1$), 472-437-402 (baA$_1$-baA$_2$-baA$_3$), 396 (bcA$_2$), 267 (bcJ$_1$), 245-210-175 (aA$_1$-aA$_2$-aA$_3$), and 204-169-134 (cA$_1$-cA$_2$-cA$_3$), indicating the trisaccharide-alditol HexA-HexA-Pent-ol-*1-d* (III-A). The mass-spectral data exclude a (1→3) linkage between both HexA residues (bcJ$_1$). These results, in combination with those obtained from the sugar and methylation analysis (see Tables I and II), indicate the following structure for the parent trisaccharide of III-A: D-Gal*p*A-(1→4)-D-Gal*p*A-(1→3)-D-Xyl.

The mass spectrum of peak B showed, *inter alia*, the following signals: *m/e* 541/538/535 (abcJ$_1$), 443/440 (bcA$_2$), 423/420/417 (baA$_2$), 328 (bcJ$_1$), 265-195 (cA$_1$-cA$_3$), and 245/242-210-175 (aA$_1$-aA$_2$-aA$_3$). The occurrence of a few series of fragment ions differing by 3 m.u. shows that a mixture of various oligosaccharide-alditols is present, consisting of compounds having various degrees of natural methylation. Regarding the sugar analysis (Table I), the following four trisaccharide-alditols are possible: GalA-Man6Me-GalA-ol-*1-d* (III-B1), GalA3Me-Man6Me-GalA-ol-*1-d* (III-B2), GalA-Man-GalA-ol-*1-d* (III-B3), and GalA3Me-Man-GalA-ol-*1-d* (III-B4). In view of the same g.l.c. retention time for III-B1 to III-B4, it is probable that Man6Me and Man are involved in the same glycosidic linkages. The only information on the types of glycosidic linkage which can be derived from the mass spectrum is the exclusion of (1→3) linkages between GalA(3Me) and Man(6Me). The methylation analysis (Table II) gives evidence for the presence of terminal GalA(3Me), 1,2-linked Man6Me, 1,2- and 1,6-linked Man, and 1,4-linked GalA-ol-*1-d*. For reasons given above, 1,6-linked mannose can be excluded. From the peak-intensity ratios between *m/e* 541, 538, and 535 (14:59:27), 443 and 440 (14:86), 423, 420, and 417 (12:62:25), and 245 and 242 (73:27) in the mass spectrum of peak B, the following composition can be estimated: 59%, III-B1; 27%, III-B2; 14%, III-B3; and <1% III-B4. To summarise, peak B consists of the parent oligosaccharides D-Gal*p*A-(1→2)-L-Man*p*6Me-(1→4)-D-GalA (59%), D-Gal*p*A3Me-(1→2)-L-Man*p*6Me-(1→4)-D-GalA (27%), D-Gal*p*A-(1→2)-Man*p*-(1→4)-D-GalA (14%), and D-Gal*p*A3Me-(1→2)-Man*p*-(1→4)-D-GalA (<1%). Because of the similarity of these oligosaccharides, it is highly probable that mannose occurs in the L configuration.

The mass spectrum of peak C showed, *inter alia*, signals at *m/e* 596 (M — $CHDOCD_3CHOCD_3CHOCD_3$), 561 (596 — CD_3OH), 541 (abcJ$_1$), 478-443 (bcA$_1$-bcA$_2$), 472-437 (baA$_1$-baA$_2$), 314 (bcJ$_1$), 251-181 (cA$_1$-cA$_3$), and 245-210-175 (aA$_1$-aA$_2$-aA$_3$) which leads to the trisaccharide-alditol HexA-HexA-Hex-ol-*1-d* (III-C). The occurrence of the bcJ$_1$ fragment excludes a (1→3) linkage between the HexA residues, whereas a 1,5- or 1,6-linked Hex-ol-*1-d* is indicated by the presence

of *m/e* 49, 96, 143, and 190 (→154/155). These results, in combination with the sugar and methylation analysis data (Tables I and II), are in agreement with the parent trisaccharide structure D-Gal*p*A-(1→4)-D-Gal*p*A-(1→6)-Man.

The g.l.c. retention time of peak D indicates a tetrasaccharide-alditol (*cf.* Fig. 1, II-D). The mass spectrum of this peak showed, *inter alia*, fragments at *m/e* 754 ($abcdJ_1$), 656 ($bcdA_2$), 527 ($bcdJ_1$), 472-437 (baA_1-baA_2), 464-394 (cdA_1-cdA_3), 314 (cdJ_1), 251-181 (dA_1-dA_3), and 245-210-175 (aA_1-aA_2-aA_3). In combination with the carbohydrate composition (Table I), the following sequence can be derived: GalA-GalA-Man-Man-ol-*l*-*d* (III-D). The methylation analysis (Table II) demonstrates the occurrence of terminal GalA, 1,3- and 1,4-linked GalA, 1,2- and 1,6-linked Man, and 1,3- and 1,6-linked Man-ol-*l*-*d*. On the basis of the mass spectrum of the permethylated tetrasaccharide-alditol-*l*-*d*, the 1,3-linked GalA can be excluded because of the presence of the $bcdJ_1$ fragment. The 1,3-linked Man-ol-*l*-*d* is preferred, because of the absence of fragment ions typical for a 1,6-linked Man-ol-*l*-*d* (*cf.* the mass-spectral data for III-C). In addition, the presence of a cdJ_1 fragment excludes a (1→3) linkage between GalA and Man. In conclusion, the following structure can be proposed for the parent oligosaccharide of III-D: D-Gal*p*A-(1→4)-D-Gal*p*A-(1→2/6)-Man*p*-(1→3)-Man. Comparison with the structure determined for compound III-C makes it reasonable to consider structure III-D as an extension of III-C. This would lead to the suggestion that the internal Man is 1,6-linked.

Besides the oligosaccharides mentioned above, each of the fractions I, II, and III contains additional oligosaccharide material, as can be deduced from the sugar and methylation analysis data (Table I and II). These sugars probably occur as higher oligosaccharides, which are not sufficiently volatile to be analysed by g.l.c.–m.s..

The structures of the oligosaccharides demonstrate that the uronic acid residues are involved in several different monosaccharide sequences. This is a further illustration of the high complexity of the polysaccharide associated with the coccoliths of the alga *Emiliania huxleyi* (Lohmann) Kamptner.

EXPERIMENTAL

General methods. — Preparative, paper chromatography was performed on Whatman No. 3MM paper with ethyl acetate–acetic acid–pyridine–water (5:1:5:3). Detection of the oligosaccharides was effected with aniline oxalate[13].

G.l.c. of partially methylated alditol acetates was performed at 180° on a Pye 104 instrument equipped with a flame-ionisation detector and a glass column (2.00 m × 2.0 mm i.d.) packed with 3% of OV-225 on Chromosorb W HP (100–120 mesh); the nitrogen flow-rate was 20 ml/min.

G.l.c.–m.s. was performed with a combined Hewlett–Packard 5710A gas chromatograph/Jeol JMS-D300 mass spectrometer/Jeol JMA-2000 mass-data analysis system. Mass spectra (70 eV) were recorded with an ion-source temperature of 200°, an accelerating voltage of 3 kV, and an ionising current of 300 μA. For permethylated oligosaccharide-alditols, a glass column (2.00 m × 2.0 mm i.d.) packed with 3.8%

of SE-30 on Chromosorb W HP (100–120 mesh) was used; the column-oven temperature was held for 2 min at 200°, followed by an increase of 4°/min up to 300°. Partially methylated alditol acetates were analysed on a glass column (2.00 m × 2.0 mm i.d.) packed with 3% of OV-225 on Chromosorb W HP (100–120 mesh); the column-oven temperature was held for 2 min at 130°, followed by an increase of 2°/min up to 250°.

360-MHz ^{1}H-n.m.r. spectra were recorded with a Bruker HX-360 spectrometer, operating in the Fourier-Transform mode at a probe temperature of 25°. Before analysis, samples were exchanged three times in D_2O with intermediate lyophilisation. Chemical shifts (δ) are given relative to sodium 2,2-dimethyl-2-silapentane-5-sulfonate in D_2O as solvent (indirectly to acetone: δ 2.225).

Isolation of oligosaccharides obtained on graded hydrolysis with acid. — Native polysaccharide (74 mg) was hydrolysed with 2M trifluoroacetic acid (75 ml) for 3 h at 95°. After evaporation of the acid under diminished pressure, and lyophilisation, the residue was chromatographed on a column (0.9 × 11.5 cm) of Dowex-1 X2 (formate$^-$) resin (200–400 mesh). The resin was washed with water until the neutral sugars were removed (40 ml), the column was then eluted stepwise with 25-ml portions of 0.25M (fraction I), 0.50M (fraction II), and 0.75M (fraction III) formic acid, respectively. The yields of the oligosaccharide fractions I, II, and III were 3.0, 2.6, and 4.0 mg, respectively.

Sugar analysis. — Oligosaccharide fractions (20–100 μg) were analysed by g.l.c. after methanolysis[3] and butanolysis[4].

Reduction of oligosaccharides. — Oligosaccharide fractions (0.4–3.5 mg) were treated with sodium borodeuteride (25 mg) in water (4 ml) for 2 h at room temperature. After decomposition of the excess of borodeuteride with Dowex-50W X8 (H^+) resin, boric acid was removed by co-evaporation with methanol under reduced pressure.

Methylation of oligosaccharide-alditols. — Oligosaccharide-alditols were methylated with methylsulfinylmethanide–trideuteriomethyl iodide in methyl sulfoxide according to Hakomori[5]. The permethylated oligosaccharide-alditols, obtained *via* chloroform extraction, were directly analysed by g.l.c.–m.s.[7,8].

Methylation analysis. — Aliquots of the permethylated oligosaccharide-alditols were stirred overnight at room temperature with sodium borodeuteride (20 mg) in *p*-dioxane (2 ml) and absolute ethanol (0.75 ml). The excess of borodeuteride was decomposed with Dowex-50W X8 (H^+) resin and boric acid removed by co-evaporation with methanol. The resulting residues were hydrolysed (90% formic acid followed by 0.13M sulfuric acid), and the partially methylated monosaccharides were analysed as the corresponding alditol acetates by g.l.c. and g.l.c.–m.s.[6].

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