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Identification of 3-deoxy-*manno*-2-octulosonic acid, 3-deoxy-5-*O*-methyl-*manno*-2-octulosonic acid and 3-deoxy-*lyxo*-2-heptulosaric acid in the cell wall (theca) of the green alga *Tetraselmis striata* Butcher (Prasinophyceae)

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The main constituent of the cell wall complex carbohydrate of the scaly green alga *Tetraselmis striata* Butcher is shown to be 3-deoxy-*manno*-2-octulosonic acid (42%). In addition two other 2-keto-sugar acids are present, namely, 3-deoxy-5-*O*-methyl-*manno*-2-octulosonic acid (7%), the first methylated derivative of 3-deoxy-*manno*-2-octulosonic acid found in nature, and 3-deoxy-*lyxo*-2-heptulosaric acid (11%). The characterization of the three 2-keto-sugar acids has been carried out on the corresponding methyl ester methyl glycosides using GLC-MS and 500-MHz ^{1}H-NMR spectroscopy, and on the corresponding reduced alditol acetates using GLC-MS. Other monosaccharides occurring in the cell wall are D-galacturonic acid (14%), D-galactose (4%), D-gulose (2%), D-glucose (1%) and L-arabinose (1%).

The genera *Tetraselmis* and *Scherfelia* are unique among the green algae in their cell wall formation. The cells synthesize small non-mineralized scales in the Golgi apparatus, which are secreted and coalesce extracellularly to form the new cell wall or theca [1–4]. Little is known about the chemical composition of the theca in scaly green flagellates [5–7]. For the theca of *Tetraselmis tetrathele* galactose and galacturonic acid have been reported to be the main constituents (80–90%), with arabinose as a minor component (10–20%) [6]. During our studies on the biogenesis and chemical composition of the theca of *T. striata*, a strikingly different monosaccharide composition was found. Although galacturonic acid is present, here it will be reported that the acidic character of the theca of *T. striata* is mainly caused by the occurrence of three 2-keto-sugar acids.

MATERIALS AND METHODS

Isolation of the theca

Tetraselmis striata Butcher strain 443, kindly provided by Dr J. C. Green (Plymouth Culture Collection, Plymouth, England), was rendered axenic and cultured [8]. Cultures were concentrated using a Pellicon tangential-flow system (Millipore, 0.45 μm filter). Thecae, which are shed during cell division, were separated from cells by low-speed centrifugation (500 × *g*, 5 min), and then pelleted (4000 × *g*, 15 min). Several differential centrifugations with a Christ Biofuge B yielded a white pellet that contained no cells. To remove possible remnants of membranes the isolated thecae were extracted twice with methanol/chloroform/water (8:4:3, by vol.), washed with ethanol and water and then lyophilized [9]. The yield of isolated thecae varied over 1–2.5 mg dry mass/l culture, depending on the cell density of the culture. Isolated thecae are structurally intact as revealed by electron microscopy [3].

Monosaccharide analysis using (methyl ester) methyl glycosides

Theca samples (1 mg) were subjected to methanolysis using 1 M methanolic or trideuteromethanolic HCl (24 h, 85 °C) [10]. After neutralization with Ag_2CO_3, in each case the suspension was incubated with acetic anhydride (50 μl) for 18 h at room temperature. Then the mixture was centrifuged, the supernatant collected and the pellet washed twice with methanol. The combined supernatant was evaporated *in vacuo* at 35 °C, and the residue dried in a desiccator over P_2O_5. Prior to GLC analysis the sample was trimethylsilylated with pyridine/chlorotrimethylsilane/hexamethyldisilazane (5:1:1, by vol.) for 30 min at room temperature.

Purification of methyl ester methyl glycosides

Methanolysates were fractionated by HPLC on a Kratos liquid chromatograph consisting of two Spectroflow 400 solvent delivery systems, a Spectroflow 450 solvent programmer and a Rheodyne 7125 injection valve module. The separations were carried out on a CP-Spher C18 reversed-phase column (25 cm × 4.6 mm, Chrompack) using water/acetonitrile (96.5:3.5, by vol.) as eluent. The flow rate was 1 ml/min (8.6 MPa, room temperature). The eluate was monitored by a Spectroflow 783 programmable absorbance detector at 195 nm. In order to correlate HPLC peaks with GLC peaks, aliquots of the various lyophilized HPLC fractions were trimethylsilylated and subjected to GLC.

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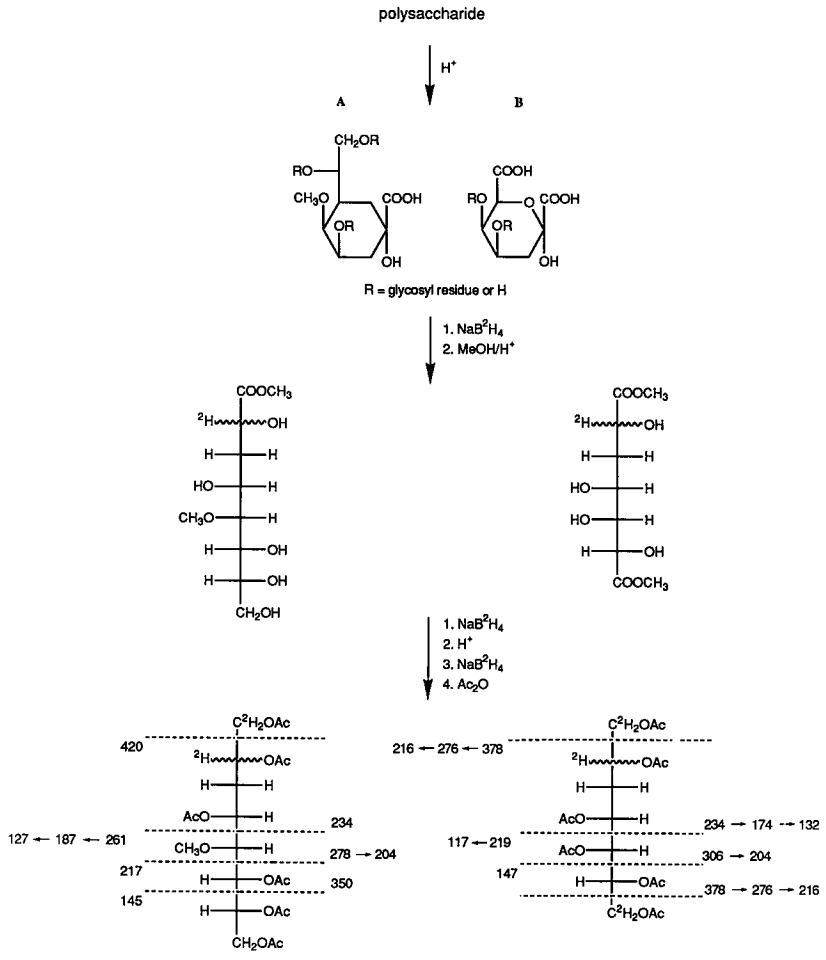
Abbreviations. dOclA, 3-deoxy-*manno*-2-octulosonic acid; 5-OMe-dOclA, 3-deoxy-5-*O*-methyl-*manno*-2-octulosonic acid; Me_3Si, trimethylsilyl; EI, electron impact; CI, chemical ionisation.



Scheme 1. *Formation and fragmentation pattern [GLC-(EI)MS] of peracetylated alditol derivatives of (A) 5-OMe-dOclA and (B) 3-deoxy-*lyxo*-2-heptulosaric acid*

Preparation of alditol acetates

Alditol acetates were prepared essentially as described [11]. Theca material (1 mg) was partially hydrolyzed with 1% acetic acid (2 h, 100°C) to cleave the ketosidic bonds (Scheme 1) and subsequently reduced with NaB^2H_4 (10 mg/0.15 ml 1 M ammonia; 2 h, room temperature). Boric acid was removed as trimethylborate by coevaporation with 1% methanolic acetic acid. The sample was methanolyzed with 1 M methanolic HCl (24 h, 85°C) and after evaporation of the solvent with a stream of dry N_2, the methyl ester groups were reduced with NaB^2H_4 in water (20 mg/0.2 ml; 2 h, room temperature). Boric acid was removed as described above. To hydrolyze methyl glycosides the residue obtained was treated with 2 M trifluoroacetic acid (2 h, 121°C). After evaporation of the acid with a stream of N_2, the residue was reduced with NaB^2H_4 and acetylated [12].

Gas-liquid chromatography/mass spectrometry

Capillary GLC was performed on a CP-Sil 5 WCOT fused silica column (25 m × 0.32 mm; Chrompack) using a Varian Aerograph 3700 gas chromatograph with an oven temperature program of 130–220°C at 4°C/min [10].

Combined GLC-(EI)MS was carried out on a Carlo Erba GC/Kratos MS 80/Kratos DS 55 system; electron energy, 70 eV; accelerating voltage, 2.7 kV; ionizing current, 100 μA; ion-source temperature, 225°C; BP-1 capillary column (25 m × 0.33 mm; S.G.E.; oven temperature program: 150°C for 2 min followed by 150–240°C at 2°C/min).

Combined GLC-(CI-NH_3)MS was performed on a Finnigan 400 GC-MS/INCOS DS system; ionizing pressure, 4 kPa; emission current, 310 mA; BP-1 capillary column (25 m × 0.22 mm; S.G.E.; oven temperature program: 70°C for 7 min followed by 70–130°C at 10°C/min and 130–200°C at 3°C/min; splitless injection).

500-MHz ^{1}H-NMR spectroscopy

Carbohydrates were repeatedly exchanged in ^2H_2O (99.96 atom % ^{2}H, Aldrich) with intermediate lyophilization. Resolution-enhanced ^{1}H-NMR spectra were recorded on a Bruker AM-500 spectrometer (Department of Chemistry, Utrecht University) operating at 500 MHz at a probe temperature of 27°C. Chemical shifts (δ) are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured indirectly to acetone in ^2H_2O (δ = 2.225 ppm).

Determination of the absolute configuration of monosaccharides

The absolute configurations of arabinose, gulose, glucose, galactose and galacturonic acid were determined gaschromatographically according to [13]. The trimethylsilylated (−)-2-butyl glycosides were analyzed on a capillary SE-30

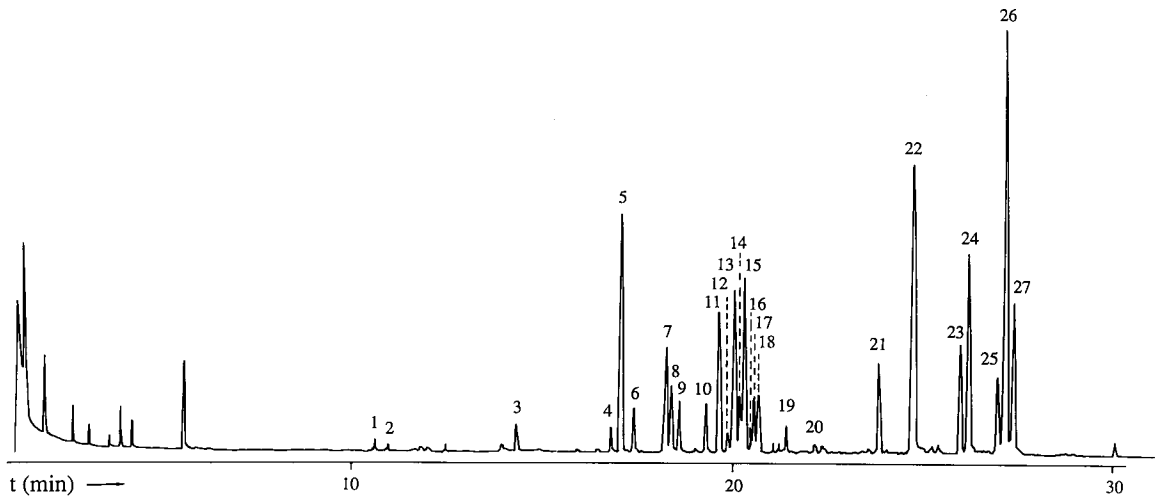


Fig. 1. *GLC-(EI)MS total-ion current chromatogram of trimethylsilylated (methyl ester) methyl glycosides derived from the theca of* Tetraselmis striata. The separation was carried out on a BP-1 WCOT fused silica capillary column (25 m × 0.33 mm) using an oven temperature program of 150 °C for 2 min followed by 150–240 °C at 2 °C/min and total ion current detection. Characterized monosaccharide: 1 and 2, arabinose; 3, 4 and 6, gulose; 5, 7, 15 and 17, galacturonic acid; 8, 10, 13 and 16, 3-deoxy-*lyxo*-2-heptulosaric acid; 9, 11, 12 and 18, galactose; 19 and 20, glucose; 21, mannitol (internal standard); 14, 22, 24, 25, 26 and 27, dOclA; 23, 5-OMe-dOclA

fused silica column (25 m × 0.32 mm, Chrompack) using an oven temperature program of 135–200 °C at 1 °C/min.

RESULTS

Isolation of the theca from axenic cultures is carried out by separating cells from shed thecae via a few centrifugation steps. Quantitative monosaccharide analysis shows the presence of D-galacturonic acid (14%), D-galactose (4%), D-gulose (2%), D-glucose (1%) and L-arabinose (1%). However, the major component is found to be 3-deoxy-*manno*-2-octulosonic acid (dOclA; 42%). Furthermore, two additional 2-keto-sugar acids are detected, namely, 3-deoxy-5-*O*-methyl-*manno*-2-octulosonic acid (5-OMe-dOclA; 7%) and 3-deoxy-*lyxo*-2-heptulosaric acid (11%). Owing to the lack of a standard for the heptulosaric acid, the molar adjustment factor of dOclA is used for quantification. The structures of the three 2-keto-sugar acids were determined by GLC-MS (EI and CI modes) and 500-MHz ^{1}H-NMR spectroscopy, as will be discussed in the following.

3-Deoxy-manno-*2-octulosonic acid*

In Fig. 1 the GLC-MS total-ion current chromatogram of the mixture of trimethylsilylated (methyl ester) methyl glycosides derived from the theca of *T. striata*, as obtained in the methanolysis procedure [10], is depicted. The relative intensities of the peaks 22, 24, 25, 26 and 27, and their relative retention times, are identical to those obtained from authentic dOclA (NH_4^+ salt, Sigma), derivatized in the same way. Furthermore, the EI mass spectra of the five peaks are the same as those recorded for the corresponding dOclA signals. The EI mass spectra of the pyranose (GLC peaks 25 and 26; Fig. 2A) and furanose (GLC peaks 24 and 27; Fig. 2B) ring forms show in the high-mass range several peaks confirming the molecular mass as 554 Da (*m/z* 539, M–Me; *m/z* 507, M–Me–MeOH; *m/z* 495, M–COOMe; *m/z* 464, M–Me_3SiOH; *m/z* 451, M–CH_2OMe_3Si). The pyranose ring form is indicated by the peak at *m/z* 320 (M–CH_2OMe_3Si-$CHOMe_3Si$-CHO). On the other hand, typical ions for the furanose ring form are *m/z* 349 (M–CH_2OMe_3Si-$CHOMe_3Si$), *m/z* 317 (M–CH_2OMe_3Si-$CHOMe_3Si$–MeOH), and *m/z* 307 (CH_2OMe_3Si-$CHOMe_3Si$-$CHOMe_3Si$). GLC peak 22 is assigned to 2,7-anhydro-dOclA [14], based on (CI-NH_3)MS (*m/z* 468, M+NH_4; *m/z* 450, M), (EI)MS (*m/z* 450, M; *m/z* 435, M–Me; *m/z* 420, M–CH_2O; *m/z* 391, M–COOMe; *m/z* 360, M–Me_3SiOH), and the finding that only one methyl group is introduced during methanolysis, as evidenced by trideuteromethanolysis. Two additional small peaks of dOclA (peaks 7 and 14) are not further identified; peak 7 coincides with one galacturonic acid peak.

Alditol acetate analysis (GLC-MS) shows the presence of 1,2,4,5,6,7,8-hepta-*O*-acetyl-3-deoxy-1,1,2-trideutero-octitol [11]. The relative retention time of the GLC peak is the same as for the GLC peak derived from authentic dOclA, subjected to the same derivatization procedure.

*3-Deoxy-5-*O*-methyl*-manno-*2-octulosonic acid*

GLC peak 23 in the total-ion current chromatogram (Fig. 1) was assigned to 5-OMe-dOclA. The CI(NH_3) mass spectrum indicates the molecular mass of the monosaccharide derivative to be 496 Da (*m/z* 514, M+NH_4; *m/z* 497, M+H). Monosaccharide analysis (GLC-MS) using trideutero-methanolysis, indicates the introduction of two methyl groups during the methanolysis step. The EI mass spectrum, depicted in Fig. 2C, shows in the high-mass range peaks at *m/z* 449 and *m/z* 437 corresponding with [M–Me–MeOH] and [M–COOMe], respectively. As compared to the dOclA spectra in Fig. 2A and 2B, several peaks have shifted 58 Da to lower *m/z* values, being the result of the replacement of an Me_3Si group by an Me group. The presence of the relatively intense peak at *m/z* 262 (M–CH_2OMe_3Si-$CHOMe_3Si$-CHO) is in accordance with a pyranose ring form, and indicates that the OMe group has to be attached at C-4 or C-5 (compare with *m/z* 320 in Fig. 2A). This is supported by the observation of peaks at *m/z* 146 (CHOMe-$CHOMe_3Si$) and *m/z* 159 (CHOMe-$COMe_3Si$-CH_2) (compare with *m/z* 204 and *m/z* 217 in Fig. 2A). In contrast to dOclA, no GLC peak for a

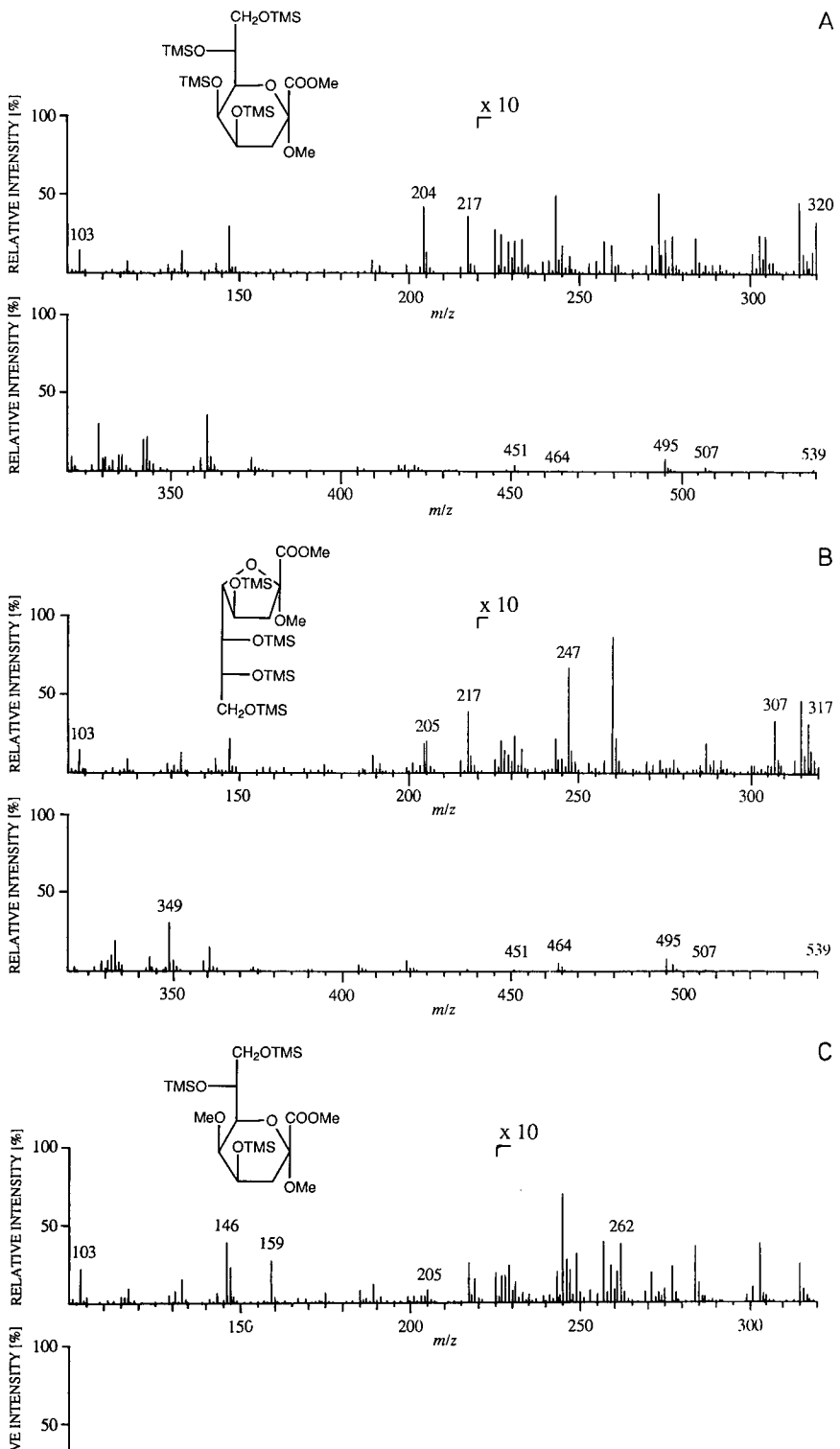


Fig. 2. *EI (70 eV) mass spectra of trimethylsilylated methyl ester methyl glycoside derivatives of (A) 3-deoxy*-manno-*2-octulopyranosonic acid; (B) 3-deoxy*-manno-*2-octulofuranosonic acid; and (C) 3-deoxy-5-*O-*methyl*-manno-*2-octulopyranosonic acid.* TMS ≡ Me_3Si

furanose ring form is observed, suggesting the methyl group to be located at C-5.

Alditol acetate analysis (GLC-MS) shows the occurrence of 1,2,4,6,7,8-hexa-*O*-acetyl-3-deoxy-5-*O*-methyl-1,1,2-trideutero-octitol. In the high-mass range the mass spectrum is dominated by cleavages of the C-4–C-5 [*m/z* 261 → *m/z* 187 (261 – AcOMe) → *m/z* 127 (187 – AcOH)] and the C-5–C-6 [*m/z* 278 → *m/z* 204 (278 – AcOMe)] bonds (see Scheme 1)

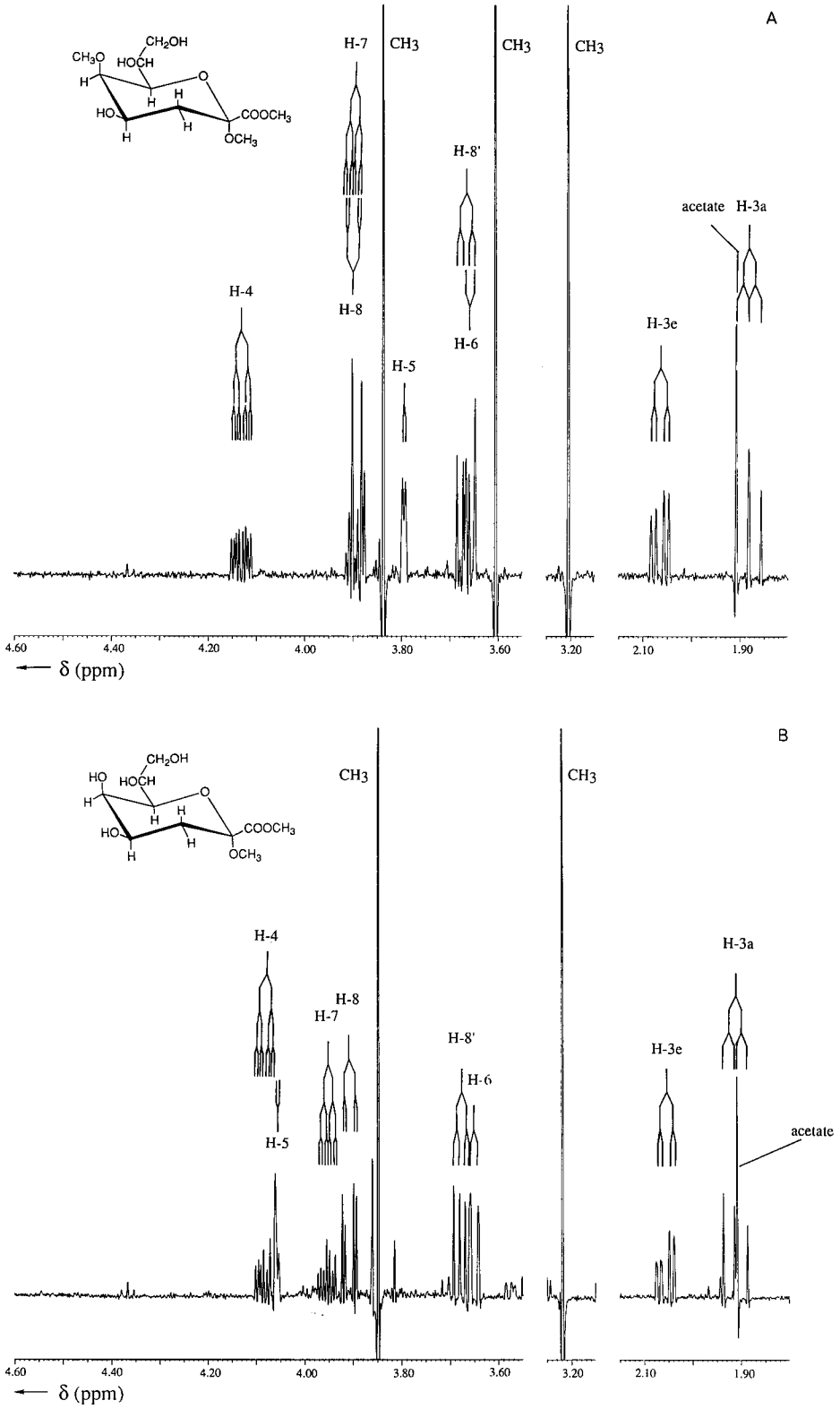


Fig. 3. *500-MHz ^{1}H-NMR spectrum of the methyl ester methyl glycoside of (A) 3-deoxy-5-*O*-methyl*-manno-*2-octulopyranosonic acid isolated from a theca methanolysate and (B) 3-deoxy-*α*-D-*manno-*2-octulopyranosonic acid recorded in ^2H_2O at 27°C*

[10]. Recently, EI-MS data for the non-deuterated substance have been reported [15].

The 500-MHz ^{1}H-NMR spectrum of the methyl ester methyl glycoside of 5-OMe-dOclA, isolated from a methanolysate of the theca, is depicted in Fig. 3A. In Table 1 the ^{1}H-NMR parameters of this compound are presented, together with those of the methyl ester methyl glycoside of α-D-dOclA*p* (axial OMe at C-2 in $^5C_2$ chair conformation [16–20]). Comparison of the H-3a and H-3e values of both methyl ester methyl glycosides indicates that the 5-*O*-methyl-

Table 1. *^{1}H-NMR data of the major methyl ester methyl glycosides of dOclA, 5-OMe-dOclA and 3-deoxy-*lyxo*-2-heptulosaric acid*
Chemical shifts (δ) are expressed downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate in ^2H_2O at 27°C and p^{2}H ≈ 7 acquired at 500 MHz, but were actually measured by reference to internal acetone (δ = 2.225 ppm)

Proton	Chemical shift of		
	5-OMe-dOclA	dOclA	3-deoxy-*lyxo*-2-heptulosaric acid
	ppm		
H-3a	1.882	1.911	1.939
H-3e	2.065	2.056	2.090
H-4	4.131	4.081	4.179
H-5	3.795	4.058	4.262
H-6	3.657	3.651	4.575
H-7	3.895	3.955	–
H-8	3.892	3.908	–
H-8′	3.666	3.675	–
OCH_3(C-2)	3.205	3.218	3.238
OCH_3(C-1)	3.837	3.848	3.843
OCH_3(C-5)	3.605	–	–
OCH_3(C-7)	–	–	3.881
Coupling constant			
	Hz		
$^2J_{3a,3e}$	−13.1	−13.3	−13.1
$^3J_{3a,4}$	11.9	11.5	12.0
$^3J_{3e,4}$	5.0	5.0	5.0
$^3J_{4,5}$	2.9	3.0	3.0
$^3J_{5,6}$	0.9	1.0	1.4
$^3J_{6,7}$	9.2	9.0	–
$^3J_{7,8}$	2.9	2.8	–
$^3J_{7,8'}$	6.4	6.0	–
$^2J_{8,8'}$	−12.4	−11.8	–
$^4J_{3e,5}$	< 1.0	1.2	–

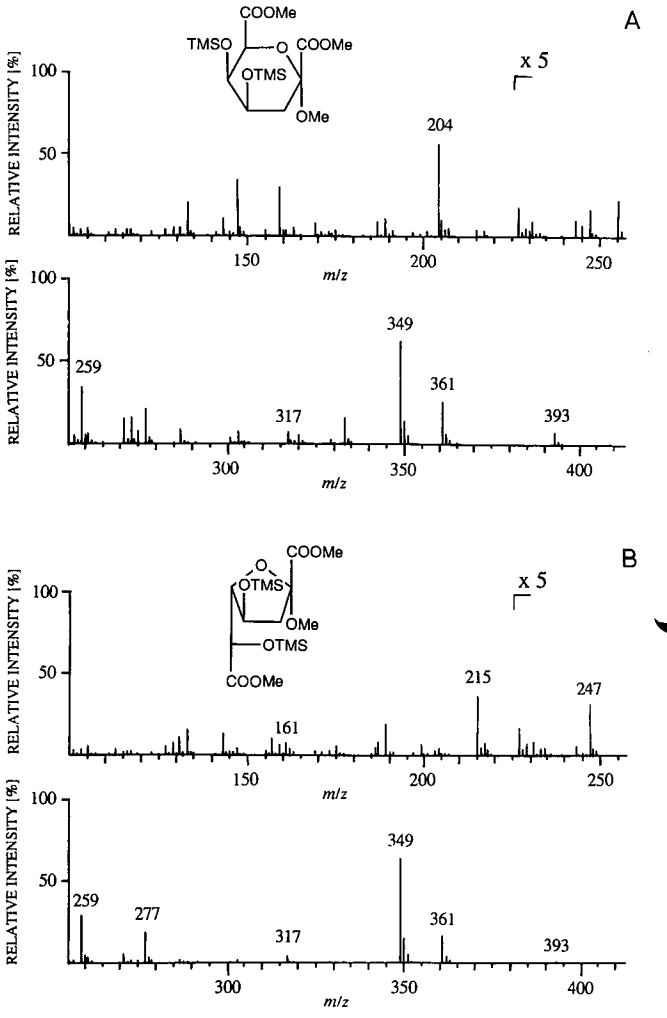


Fig. 4. *EI (70 eV) mass spectra of the trimethylsilylated methyl ester methyl glycoside derivatives of (A) 3-deoxy-*lyxo*-2-heptulopyranosaric acid, and (B) 3-deoxy-*lyxo*-2-heptulofuranosaric acid.* TMS ≡ Me_3Si

ated compound also has an axial OMe group at C-2 of a pyranose ring form (see also Fig. 3B). The various coupling constants in both methyl ester methyl glycosides are very similar, demonstrating the same stereochemical substitution pattern for the carbon skeleton in agreement with a *manno* configuration. As compared to the non-methylated product, the introduction of an Me group (δ = 3.605 ppm) at C-5 leads to a clear upfield shift for H-5 ($\Delta\delta$ = −0.264 ppm).

The results shown are identical to those for authentic 3-deoxy-5-*O*-methyl-D-*manno*-2-octulosonic acid, subjected to the same analytical identification procedures.

*3-Deoxy-*lyxo*-2-heptulosaric acid*

The four GLC peaks 8, 10, 13 and 16 in the total-ion current chromatogram (Fig. 1) belong to the third 2-keto-sugar acid, namely, 3-deoxy-*lyxo*-2-heptulosaric acid. CI(NH_3)-MS indicates the molecular mass of the trimethylsilylated methyl ester methyl glycosides to be 408 Da (*m/z* 426, M+NH_4; *m/z* 394, M+NH_4−MeOH). Monosaccharide analysis using trideuteromethanolysis and subsequent GLC-(EI)MS shows the introduction of three methyl groups during the methanolysis step, in accordance with the presence of two carboxyl functions besides the anomeric OH group. The EI mass spectra of the four GLC peaks show in the high-mass region fragment ions at *m/z* 393 (M−Me), *m/z* 361 (M−Me−MeOH), *m/z* 349 (M−COOMe), *m/z* 317 (M−COOMe−MeOH) and *m/z* 259 (349−Me_3SiOH). The occurrence of relatively intense peaks at *m/z* 247 (M−COOMe-$CHOMe_3Si$; shifted to *m/z* 253 in the deuterated analogue), *m/z* 215 (247−MeOH; shifted to *m/z* 218 in the deuterated analogue) and *m/z* 277 [M−Me−C(COOMe)-(OMe)CH_2; shifted to *m/z* 280 in the deuterated analogue] in the mass spectra of peaks 8 and 10 (Fig. 4B) is in accordance with the furanose ring form of a heptose 2-keto acid with carboxyl groups at C-1 and C-7 and a methylene group at C-3. The observation of the relatively intense peak at *m/z* 204 in the mass spectra of the GLC peaks 13 (Fig. 4A) and 16 is in agreement with the pyranose ring form of the same product.

Alditol acetate analysis (GLC-MS; Scheme 1B) shows the presence of 1,2,4,5,6,7-hexa-*O*-acetyl-3-deoxy-1,1,2,7,7-pentadeutero-heptitol [21], indicating the unknown product to be a 3-deoxy-2-heptulosaric acid.

The 500-MHz ^{1}H-NMR spectrum of the methyl ester methyl glycoside of the 3-deoxy-2-heptulosaric acid, isolated from a methanolysate of the theca, is shown in Fig. 5. The ^{1}H-NMR parameters are included in Table 1. The occurrence of two methyl ester groups is established by the OMe singlets

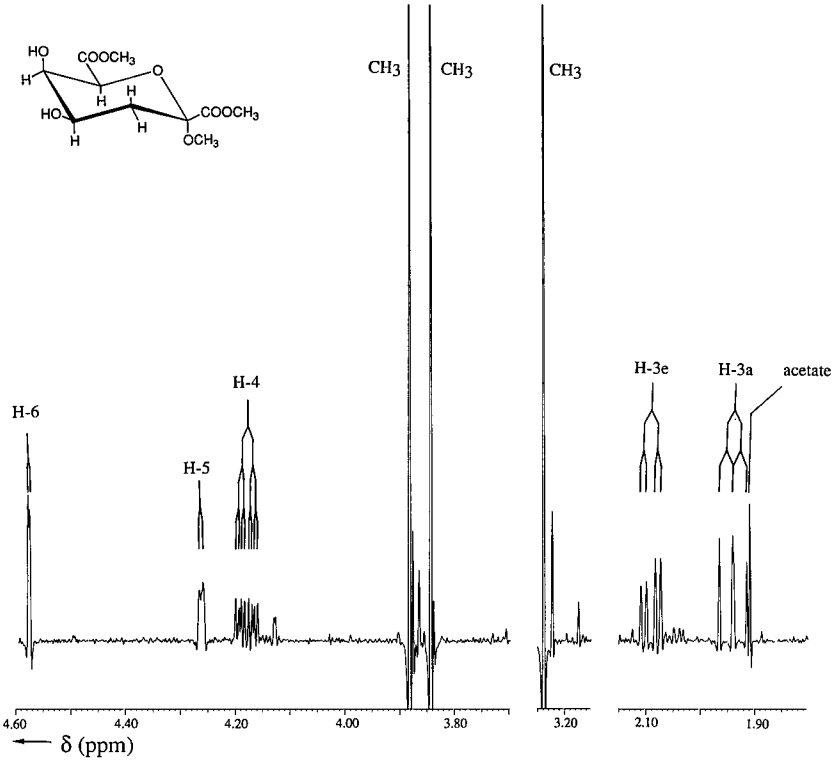


Fig. 5. *500-MHz ^{1}H-NMR spectrum of the methyl ester methyl glycoside of 3-deoxy-*lyxo*-2-heptulopyranosaric acid isolated from a theca methanolysate recorded in ^2H_2O at 27°C*

at δ = 3.843 ppm (assigned to C-1, see dOclA value) and δ = 3.881 ppm, whereas the OMe singlet of the methyl glycoside appears at δ = 3.238 ppm (see dOclA value). Based on a comparison of the chemical shift values of the methylene H-3 signals for dOclA, 5-O-Me-dOclA and 3-deoxy-2-heptulosaric acid (Table 1), it is tempting to assume that this derivative also has an axial OMe group at C-2 of a pyranose ring form. The coupling constants of the various protons of the skeleton pyranose ring form are in agreement with either a *lyxo* or a *ribo* configuration. Assuming a chair conformation with the carboxyl function in equatorial and H-6 in axial position [22], the 2-keto-sugar acid can be identified as 3-deoxy-*lyxo*-2-heptulosaric acid.

DISCUSSION

The various groups of algae exhibit a diverse spectrum of cell wall forms. Among the green algae the best characterized cell wall is the crystalline glycoprotein cell wall of chlorophycean algae [23], particularly *Chlamydomonas* (e.g. [24–28]). The cell wall of *Chlamydomonas* is entirely composed of hydroxyproline-rich glycoproteins, with carbohydrate chains consisting of galactose, arabinose, glucose and mannose [28]. Based mainly on electron microscopic data [2], it has been argued that the cell walls of *Tetraselmis* and *Chlamydomonas* are structurally similar and evolutionary related [29]. However, the data presented in this study show that the cell wall of *Tetraselmis* has quite a different sugar composition, consisting mainly of dOclA, 5-OMe-dOclA and 3-deoxy-*lyxo*-2-heptulosaric acid (60% of dry mass). The total carbohydrate content is about 80% of dry mass and the protein content is about 4% (unpublished results). The differences in cell wall sugar composition indicate that *Chlamydomonas* and *Tetraselmis* may not be so closely related as was previously thought. It has to be noted that monosaccharide analysis of the theca of *Tetraselmis tetrathele*, which was demonstrated earlier to contain galactose, galacturonic acid and arabinose [5, 6], give the same sugar composition as found for *Tetraselmis striata* (unpublished results). Perhaps the relatively harsh hydrolysis conditions used earlier prevented identification of the 2-keto-sugar acids.

dOclA is a constituent of lipopolysaccharides (inner core) and some capsular polysaccharides of Gram-negative bacteria [16]. Naturally occurring methylated dOclA has to our knowledge never been reported before, but was synthesized as a model compound for methylation analysis [15] and the periodic acid/thiobarbituric acid assay [30]. A 3-deoxy-2-heptulosaric acid was found in the lipopolysaccharide of *Acinetobacter calcoaceticus* [31], but no information is available about its configuration. Since only axenic cultures have been used for isolation of the thecae, contamination by bacteria as a possible source of dOclA can be excluded. Recently, dOclA [11, 21] and 3-deoxy-D-*lyxo*-2-heptulosaric acid [21] have been reported to occur as constituents of the rhamnogalacturonan II of different angiosperms and gymnosperms.

The findings described in this report give rise to two main questions: (a) Are dOclA-oligomer regions (repeating units?) present in the cell wall complex carbohydrate of *T. striata*. (b) How does the presence of dOclA relate to the fine structure of the theca or its unique mode of biogenesis. At present, we can conclude that the theca of *T. striata* is not only unique in its morphology and biogenesis, but also in its chemical composition and structure.

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