Pergamon

Phytochemistry, Vol. 49, No. 3, pp. 691–695, 1998

Printed in Great Britain
0031-9422/98/$—see front matter

PII: S0031-9422(98)00229-5

CELL WALL-SPECIFIC ω-HYDROXY FATTY ACIDS IN SOME FRESHWATER GREEN MICROALGAE

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(*Received* 16 *February* 1998; 16 *February* 1998)

Key Word Index—*Tetraedron minimum*; *Scenedesmus communis*; *Pediastrum boryanum*; freshwater algae; ω-hydroxy fatty acids; DMDS-derivatisation; cell wall.

Abstract—Ester-bound lipids from the cell walls of the green algae, *Tetraedron minimum*, *Scenedesmus communis* and *Pediastrum boryanum*, were analysed by gas chromatography-mass spectrometry. The double bond positions of the C_{30}, C_{32} and C_{34} mono- and C_{30} and C_{32} diunsaturated ω-hydroxy fatty acids obtained were determined by derivatisation with dimethyl disulphide (DMDS) prior to mass spectrometric identification. All unsaturated ω-hydroxy fatty acids have a double bond located at the ω9-position, whilst the C_{30} and C_{32} diunsaturated acids have a second double bond at the ω19 and ω18 positions, respectively. Unsaturated ω-hydroxy fatty acids are released upon saponification of the isolated cell walls in all three algae, but the distribution patterns of these compounds differ between species. ω-Hydroxy fatty acids are the main building blocks of the highly cross-linked constituent of the cell walls, herein termed algaenan, in which linear chains of esterified monomers are ether cross-linked at the position of the double bonds. Because none of these monomeric compounds were observed in the cytosolic fraction of the cells, it is believed that they are rapidly secreted and incorporated in the aliphatic cell walls of these algae.

INTRODUCTION

Non-hydrolysable, insoluble biopolymers termed algaenans have been encountered in cell walls of several green freshwater and marine microalgae, such as *Tetraedron minimum*, *Scenedesmus obliquus*, *S. communis*, *S. quadricauda*, *Pediastrum boryanum*, *Botryococcus braunii*, *Chlorella vulgaris* and several *Nannochloropsis* species [1–3]. Because of their high resistance to microbial attack these algaenans are selectively preserved upon settling in the water column and burial in the sediments. Upon subsequent cracking in the subsurface they may form a major source of petroleum [3, 4]. Since these green microalgae are widespread in nature, detailed studies of their lipid composition may help to discriminate between contributions of different types of algae to organic matter in sediments, thereby allowing the reconstruction of past environments and climates. Furthermore, analyses of lipids associated with the aliphatic cell walls of these algae may contribute to an understanding of the biochemical pathways involved in algaenan formation by obtaining information about the chemical structures of their monomeric precursors.

In the present study, ester-bound fatty acids of the aliphatic cell walls of *T. minimum*, *S. communis* and *P. boryanum* were identified by gas chromatography-mass spectrometry in combination with chemical derivatisation using dimethyl disulphide (DMDS) to determine the position of double bonds. Our results show that long-chain unsaturated ω-hydroxy fatty acids are intrinsic cell wall components and represent the main constituents of the ester-bound lipid fraction of the cell walls.

RESULTS AND DISCUSSION

To distinguish between cell wall-bound lipids and lipids of other cell components, cell walls were analysed separately from the cell's interior. To isolate the cell walls of the algae, the cytoplasm was removed by the use of a Potter homogenizer, after which the cell walls were collected by centrifugation. By subsequent treatment with α-amylase, according to a method used by Burczyk and Loos [5], the remaining starch gran-

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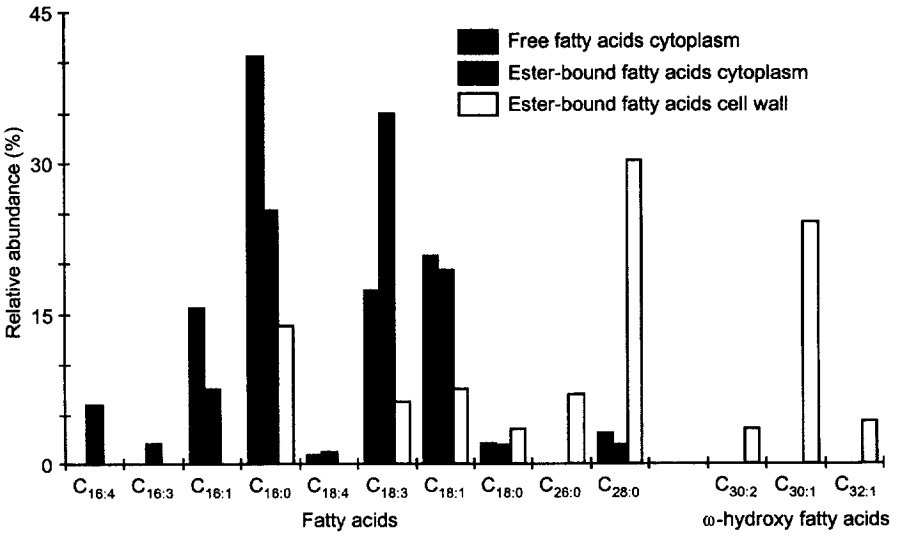

Fig. 1. Relative amounts of free and ester-bound fatty acids and unsaturated ω-hydroxy fatty acids from the cytoplasm and cell walls of *Scenedesmus communis*. Fatty acids in each fraction are plotted as the percentage of the sum of fatty acids in that fraction.

ules were removed enzymatically. A sulphuric acid treatment was subsequently used to remove the cell wall polysaccharides, after which a final saponification step released the ester-bound lipids from the polymer matrix. The compounds isolated after saponification were C_{16}-C_{28} long-chain fatty acids, which were also identified in the corresponding cytoplasm, and a number of long-chain mono- and diunsaturated ω-hydroxy fatty acids, only present in the cell wall ester-bound lipid fraction (Fig. 1). The monounsaturated ω-hydroxy fatty acids have chain lengths of C_{30}, C_{32} and C_{34}, whereas the diunsaturated ω-hydroxy fatty acids are only present as C_{30} and C_{32} compounds. All mono- and diunsaturated ω-hydroxy fatty acids show the same typical fragmentation pattern upon mass spectrometry [Fig. 2(a) and (c)]. Characteristic ions are $[M]^{\bullet+}$, $[M\text{-}15]^{+}$ (loss of a methyl radical), $[M\text{-}31]^{+}$ (loss of a methoxyl radical) and $[M\text{-}47]^{\bullet+}$ (loss of a methyl radical and methanol). The loss of a methyl radical is typical for trimethylsilyl (TMSi)-derivatised alcohols and the loss of a methoxyl radical is characteristic for methyl esters. The absence of characteristic secondary alcohol fragment ions reveals that the alcohol group is situated at the terminal position of the molecule and the M_r indicates the presence of one or two double-bonds. However, it is not possible to deduce the position of the double bonds from these mass spectra. Therefore, derivatisation of the double bonds with DMDS was performed yielding methyl thioethers, which give characteristic fragments revealing the position of the original double bond [6].[Fig. 2(b) and (d)]. All these compounds prove to have a ω9-double bond independent of chain length or degree of unsaturation. The second double bond of the diunsaturated ω-hydroxy fatty acids is positioned at ω19 and ω18 for the C_{30} and C_{32}, respectively.

Analysis of the lipids removed by acid hydrolysis showed that only traces of mono- and diunsaturated ω-hydroxy fatty acids were removed by this procedure. Therefore, the mono- and diunsaturated ω-hydroxy fatty acids removed by the subsequent base hydrolysis can be used to quantify these compounds. The amounts of mono- and diunsaturated ω-hydroxy fatty acids relative to the total biomass are reported in Table 1 and show that there is a significant difference in concentration of these compounds in the three algal species. Although the ratios between the indi-

Table 1. Amount of long-chain components isolated from *T. minimum*, *S. communis* and *P. boryanum* cell walls as in mg g^{-1} dry biomass

	T. minimum	*S. communis*	*P. boryanum*
$C_{30:1}$ ω9 ω-hydroxy fatty acid	0.10	0.03	2.8
$C_{30:2}$ ω9,19 ω-hydroxy fatty acid	0.08	trace	1.1
$C_{32:1}$ ω9 ω-hydroxy fatty acid	0.11	0.01	2.2
$C_{32:2}$ ω9,18 ω-hydroxy fatty acid	–	–	1.3
$C_{34:1}$ ω9 ω-hydroxy fatty acid	0.65	–	–

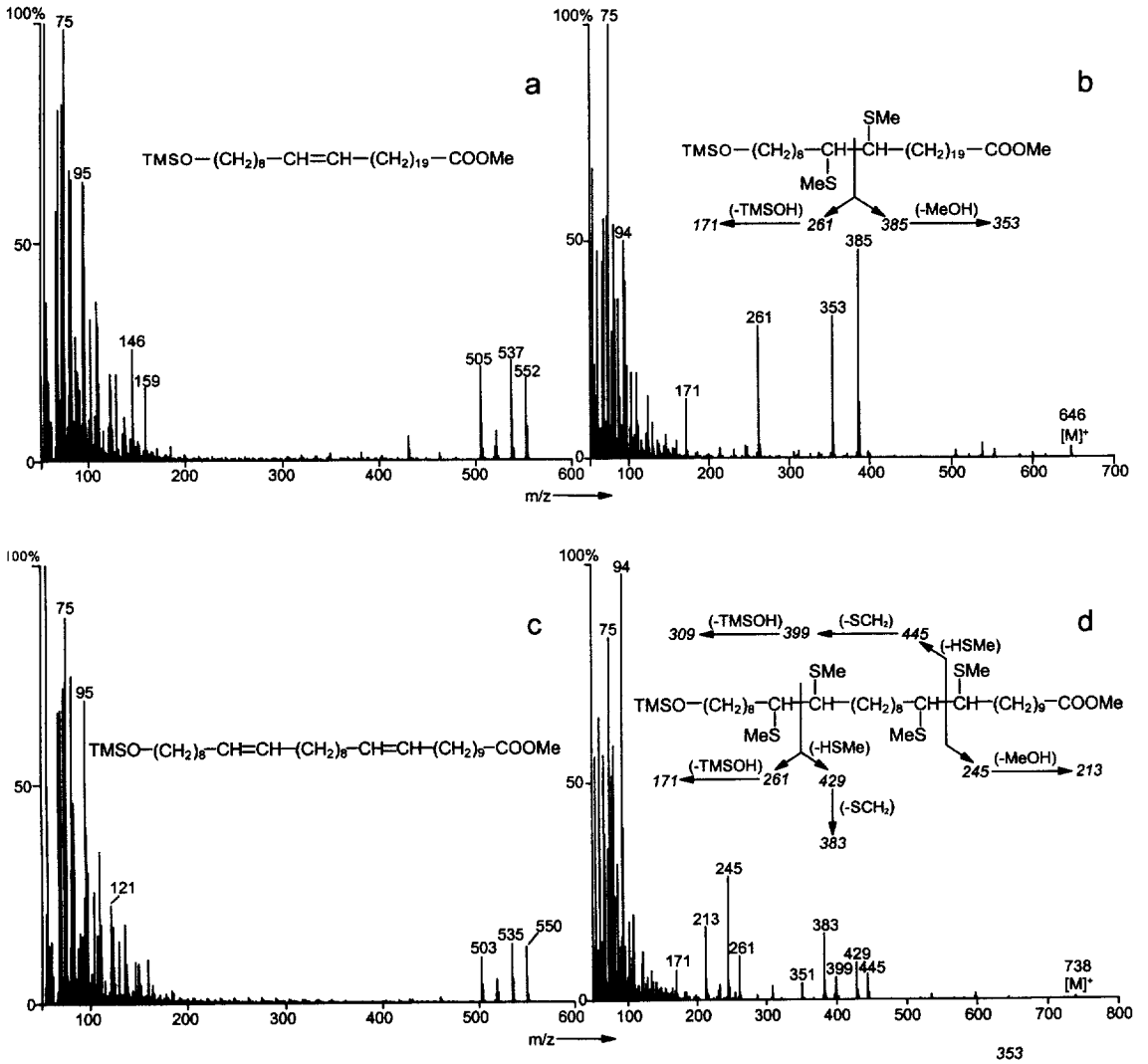

Fig. 2. Mass spectra of the C_{30} ω9 monounsaturated ω-hydroxy fatty acid methyl ester (a) before and (b) after DMDS derivatisation and the C_{30} ω9,19 diunsaturated ω-hydroxy fatty acid methyl ester (c) before and (d) after DMDS derivatisation.

vidual ω-hydroxy fatty acids seem to be species specific and can possibly be used to provide a qualitative estimate of the contribution of these algae to sediments, quantitative analysis can only be performed if the amount of these compounds produced by the algae is independent of the growth conditions. Although to our knowledge only higher plants produce ω-hydroxy fatty acids [7], the possibility that other organisms can also biosynthesise such compounds has to be taken into consideration when using ω-hydroxy fatty acids as biomarkers.

The long-chain unsaturated ω-hydroxy fatty acids identified in our study were specifically obtained from the cell walls of the investigated algae and were not observed in the cytoplasm (Fig. 1). This suggests that there is transport of these compounds to the cell wall or that they are even biosynthesised at this location. This indicates that they are probably involved in the formation of the aliphatic cell walls of these algae. Indeed, the chemical structure of the algaenans produced by these algae indicates that long-chain mono- and diunsaturated ω-hydroxy fatty acids are the main monomeric units of this biopolymer (unpublished results). The relation between long-chain hydroxy fatty acids and algaenans was already observed by Gelin *et al.* [8] who investigated the free and ester-bound lipids of *Nannochloropsis* species, a marine algae which also biosynthesises an aliphatic cell wall. Hydroxy fatty acids are also well known constituents of cutin, which is the main structural biopolymer in the plant cuticle, formed by a reticulated network of oxygenated fatty acids [7]. The mid-chain hydroxy long-chain fatty acids obtained from the *Nannochloropsis* species were suggested to be biosynthesised *via* a pathway similar to that of cutin monomers and proposed to be involved in the biosynthesis

of this algaenan. However, the main building blocks of cutin are C_{16} and C_{18} ω-hydroxy fatty acids, which are intermolecularly linked predominantly *via* ester-bonds to form an aliphatic biopolymer. The much longer C_{30}-C_{34} unsaturated ω-hydroxy fatty acids identified here are linked *via* a combination of ester- and ether-linkages (unpublished results), providing a macromolecular network that is more resistant but physically and chemically still similar to that of cutin. Therefore, it is proposed that at least some of the biological functions of cutins and algaenans are comparable, for example, in protecting the organisms against microbial attack. Because of these similarities it is also suggested that the algal mono- and diunsaturated ω-hydroxy fatty acids identified in this study are probably biosynthesised in a similar fashion, *via* a fatty acid synthetase, desaturase and ω-hydroxylase pathway. However, instead of retaining its relative position to the alcohol or acid group, the ω19 double bond of the C_{30} diunsaturated ω-hydroxy fatty acid shifts to the ω18 position in the C_{32} di-unsaturated ω-hydroxy fatty acid. This suggests that the C_{32} di-unsaturated ω-hydroxy fatty acid is not simply biosynthesised from the C_{30} diunsaturated ω-hydroxy fatty acid by C_2 chain elongation.

EXPERIMENTAL

Culture conditions and isolation process

Axenic strains of *T. minimum*, *S. communis* and *P. boryanum* were obtained from the Sammlung von Algenculturen at the University of Göttingen (SAG). All three algae were grown in 1 batch cultures at 19° using the Kates and Jones medium [9] aerated with 2% CO_2 enriched air at a light/dark regime of 16/8 hr. Algae were collected at the end of their log phase growth by centrifugation. Cytoplasm was removed by mechanically breaking the cell walls using a Potter homogenizer in combination with glass beads (diameter ~45 μm), which enhanced the grinding effect of this device. After this treatment collected cell walls were treated with α-amylase in NaOAc buffer (0.2 M, pH 5.4) to remove the remaining starch grains [5]. Residual cell walls were washed with NaOAc buffer, sat NaCl sol and demineralised H_2O (3 ×). Isolated cell wall material was extracted ultrasonically with 10 ml MeOH, 10 ml MeOH-CH_2Cl_2 (1:1, 2 ×), 10 ml CH_2Cl_2-hexane (1:1, 2 ×) and 10 ml hexane (1 ×). The residue obtained after centrifugation was stirred with 10 ml 12 M H_2SO_4 for 1 hr, subsequently washed with 10 ml 2× dist. H_2O (3 ×) and resuspended ultrasonically in 10 ml 2 M H_2SO_4. After refluxing for 2 hr under stirring, residual material was washed with 10 ml 2× dist. H_2O (3 ×), 10 ml MeOH (2 ×), 10 ml CH_2Cl_2-hexane (1 ×) and finally with 10 ml hexane (1 ×). The residue was resuspended in 7.5 ml KOH in MeOH (1M, 4% H_2O) and allowed to reflux for 1 hr. The residue was subsequently extracted with 5 ml MeOH (4 ×), 5 ml CH_2Cl_2-hexane (1:1, 2 ×) and 5 ml hexane (1 ×). The combined extracts were transferred to a separatory funnel, acidified with 30 ml 0.25 M HCl and the organic phase removed. The aq. layer was subsequently extracted with 10 ml CH_2Cl_2 (2 ×) and 10 ml hexane (2 ×). The combined extracts were dried (Na_2SO_4) and the solvents. An aliquot of this sample was treated with CH_2N_2 and N,O-*bis*(trimethylsilyl)-trifluoroacetamide (BSTFA)-pyridine to derivatise carboxylic acids and alcohol groups, respectively, prior to GC/MS analysis.

Extraction of the cytoplasm

An aq. suspension of cytoplasmatic material (10 ml) was extracted ultrasonically with 10 ml CH_2Cl_2 (2 ×) and 10 ml hexane (2 ×). The combined organic layers were dried (Na_2SO_4) and the solvents evapd. An aliquot was derivatised as described above prior to GC/MS analysis. The residue after extraction was isolated by centrifugation and refluxed in 5 ml KOH in MeOH (1 M, 4% H_2O) for 1 hr. The residue was removed by centrifugation and the supernatant acidified with 5 ml 1 M HCl in a separatory funnel. The residue was washed with 3 ml CH_2Cl_2 and this layer was transferred to the separatory funnel to extract the aq. phase. The aq. layer was once more extracted with 3 ml CH_2Cl_2 and next with 3 ml CH_2Cl_2-hexane (1:1, 2 ×). The combined extracts were dried over (Na_2SO_4) and an aliquot was derivatised as described above prior to GC/MS analysis.

DMDS derivatisation

Following the procedure described in ref.[6], *ca* 2 mg of compounds removed from the cell walls by saponification as described above was dissolved in hexane (100 μl) and reacted with dimethyl disulphide (100 μl) and an ethereal I_2 soln (60 mg ml^{-1}). The reaction mixt. was kept at 50° for 24 hr and then dild with hexane (0.5 ml). The reaction was quenched with an aq. $Na_2S_2O_3$ soln (2 ml, 5%) and the hexane layer was collected. The aq. layer was extracted twice with EtOAc (0.5 ml), the organic fras combined, dried Na_2SO_4 and the solvents were evapd. The compounds were further derivatised, prior to GC/MS analysis, using the methods described above.

Gas chromatography and gas chromatography-mass spectrometry

GC was performed on a FID instrument and an on-column injector. A 25 m fused silica capillary column coated with CP-Sil 5 (0.32 mm I.D.; film thickness 0.12 μm) was used with He as carrier gas. The FID temp. was 320° and the oven was programmed from 70° to 130° at 20° min^{-1}, then to 320° at 4° min^{-1} and held at this temp. for 15 min. For the analysis of the DMDS-derivatisation products, a 6 m fused silica capillary column coated with CP-Sil 5 (0.32 mm I.D.; film thickness 0.12 μm) was used with He as carrier

gas. The oven was programmed from 70° to 320° at 10° min^{-1} and held at this temp. for 30 min. For GC/MS analysis the column was coupled to the EI ion source of a mass spectrometer (mass range m/z 40–800 at a resolution of 1000; cycle time 1.8 sec; ionisation energy 70 eV).

Acknowledgement—This study was partly supported by The Netherlands Geosciences Foundation (GOA) with financial aid from the Netherlands Organisation for Scientific Research (NWO) and by a PIONIER grant to Jaap S. Sinninghe Damsté. We thank Marlen Dekker (NIOZ) for GC/MS analyses. This paper is NIOZ contribution 3224.

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