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MID-CHAIN HYDROXY LONG-CHAIN FATTY ACIDS IN MICROALGAE FROM THE GENUS *NANNOCHLOROPSIS**

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Key Word Index—*Nannochloropsis*; Eustigmatophyceae; microalgae; monohydroxy fatty acids; dihydroxy fatty acids; alkyl diols; bound lipids; hydroxylation, chain-elongation.

Abstract—Extractable and bound lipids of four species of microalgae from the genus *Nannochloropsis* have been examined by capillary GC-mass spectrometry. In addition to previously described unusual C_{28}–C_{34} alkyl diols and *n*-alcohols, small quantities of compounds identified as C_{28}–C_{34} monohydroxy fatty acids were detected both in free and bound form. For each carbon chain-length member of the series, a single positional isomer predominated, which was identified as the ω18-isomer from characteristic mass spectral fragment ions. The position of the hydroxyl group at ω18 rather than at a constant position relative to the carboxylic acid group indicates that the series results from chain-elongation (or perhaps chain-shortening) of a particular hydroxy fatty acid, rather than hydroxylation of a range of fatty acids. Furthermore, two dihydroxy fatty acids, identified as 15,16-dihydroxydotriacontanoic and 16,17-dihydroxytritriacontanoic acids, were also found in the products from acid hydrolysis of the cell residue. Possible biosynthetic correlations between these hydroxy and dihydroxy fatty acids and the long-chains diols also produced by these algae are discussed. Our data suggest that hydroxy acids may be precursors of the diols via reduction of the acid group. However, another pathway must be invoked in order to explain the formation of some diol isomers.

INTRODUCTION

In previous studies, we showed that species of microalgae from the class Eustigmatophyceae contain unusual long-chain alcohols and alkyl diols [1–3]. These compounds are present at both free and mainly ester-bound components; C_{30}–C_{32} homologues were identified in each series with even-chain constituents predominating. The main alkyl diol was the 1,15-C_{32} isomer which occurred with smaller amounts of a monounsaturated analogue. The long-chain alcohols exhibited a similar carbon number distribution, though with one more double bond, than the corresponding alkyl diol, e.g. the major monounsaturated C_{32} alcohol was dotriacont-15-en-1-ol. The presence of several other long-chain compounds was noted in our earlier studies but not identified at the time. We now report the identification of some of these compounds as long-chain saturated mono- and di-hydroxy fatty acids.

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RESULTS

Identification of monohydroxy fatty acids

The monohydroxy fatty acids (as TMSi-ether methyl esters) were first noted as peaks in a chromatogram of the total extractable lipids which had been transesterified with methanolic-HCl and reacted with BSTFA to form TMSi-ethers. These peaks were not present in a chromatogram of the neutral constituents formed after saponification of the total extract; however, they were present in the total fatty acids after saponification, confirming the presence of carboxylic acid and hydroxyl groups. They were estimated to account for less than 5% of the total extractable fatty acids.

The hydroxy acids were identified from mass spectra of their TMSi-ether methyl ester derivatives. Trimethylsilylation is a particularly useful method for identifying long-chain hydroxy acids and the fragmentation patterns are well established [4]. Mass spectra of mid-chain hydroxy acids (as TMSi-ethers, methyl esters) show ions at $[M-15]^+$ (loss of methyl), $[M-31]^+$ (loss of OMe) and $[M-47]^+$ (loss of methanol from $[M-15]^+$) [4]. The major ions in the mass

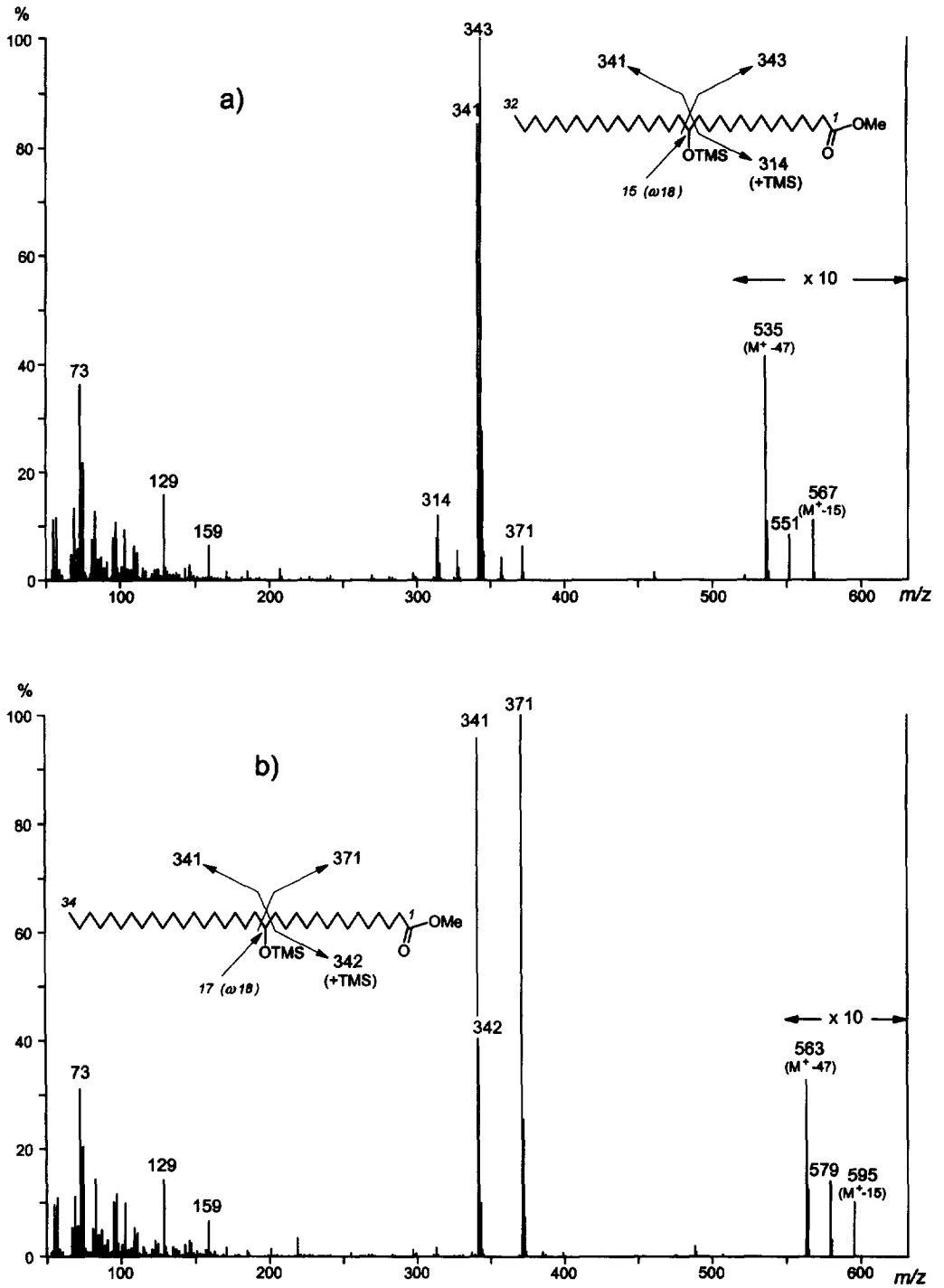

Fig. 1. Mass spectra of (a) C_{32} and (b) C_{34} monohydroxy fatty acids (as methyl esters, TMS-ethers).

spectrum originate from cleavage α to the mid-chain OTMSi group, with the ion containing the methyl ester function more abundant than the corresponding ion containing the terminal alkyl chain (Fig. 1). Mass spectra with similar characteristics have been reported for shorter-chain mid-chain hydroxy acids, such as the methyl ester of the TMSi-ether of 9-hydroxypentadecanoic acid from cutin of the plant, *Araucaria imbricata* [5]. Within the mid-chain hydroxy acid series the most abundant isomer at each carbon number was identified as the isomer with an hydroxyl group at the ω18 position, from the major fragment at *m/z* 341 in each spectrum (Fig. 1). This ion was accompanied by characteristic cleavage ions at *m/z* 287 (C_{28}), *m/z* 301 (C_{29}), *m/z* 315 (C_{30}), *m/z* 329 (C_{31}), *m/z* 343 (C_{32}), *m/z* 357 (C_{33}) and *m/z* 371 (C_{34}). The previously described [6] rearrangements leading to the even ions *m/z* 314 (C_{32}) and *m/z* 342 (C_{34}) are also very

characteristic ions for mid-chain hydroxy acids (Fig. 1). Other isomers were detected in trace amounts as observed for the C_{32} compound with the minor fragments *m/z* 371/313 and *m/z* 357/327, corresponding with the ω16 and ω17 isomers, respectively (Fig. 1). However, in many cases, these isomers were not detectable even using highly sensitive selected-ion monitoring GC-mass spectrometry (Table 1).

Identification of dihydroxy fatty acids

Dihydroxy fatty acids were only observed in both base and acid hydrolysates of the algal cell residues after non-bound lipids had been extracted. The chain-length distribution was more restricted than that of the monohydroxy fatty acids and their relative abundances were minor compared with those of the latter components. We could only identify C_{32} and C_{33} homologues with certainty, due to co-elution with other constituents. The C_{32} cluster comprised mainly 15, 16-dihydroxydotriacontanoic acid and the C_{33} cluster was essentially composed of 16,17-dihydroxytritriacontanoic acid. The former had characteristic ions at *m/z* 327 and 343 due to cleavage between the vicinal hydroxy groups, while the latter had ions at *m/z* 327 and 357 (Fig. 2). Other major ions in both spectra were $[M-15]^+$ (loss of Me), $[M-31]^+$ (loss of OMe), *m/z* 129 $[(CH_3)_3Si^+OCH_2CH{=}CH_2)]$, the rearrangement ion, *m/z* 147, which is common in di-OTMSi derivatives [4] and the rearrangement even ions, *m/z* 416 and *m/z* 430, involving the ω17-positioned hydroxyl groups in the C_{32} and C_{33} analogues, respectively. In both compounds, the hydroxyl groups were located mainly at the ω17 and ω18 positions. It should be noted that minor fragments due to isomers with mid-chain hydroxyl groups located at the ω18/ω19 and ω19/ω20 were also detected in the C_{32} cluster [Fig. 2(a)].

Compositional variations between extractable and bound lipids

There was little variation in the distribution of bound hydroxy acids, i.e. those released after KOH and HCl hydrolyses (Table 1, Fig. 3). These ranged from C_{28} to C_{34} with a maximum at C_{32} but without a strong dominance, whereas the extractable compounds were mainly the C_{30} and C_{32} hydroxy acids and for *N. oculata* almost exclusively C_{30}. However, the distribution of these extractable hydroxy acids was similar to those of the extractable and bound ω18 diols (Fig. 3).

Table 1. Relative concentrations of mid-chain (ω18) hydroxy fatty acids in *Nannochloropsis* species

	Nannochloropsis salina			*Nannochloropsis* sp.			*Nannochloropsis granulata*			*Nannochloropsis oculata*		
	Extract	Residue-base*	Residue-acid†	Extract	Residue-base*	Residue-acid†	Extract	Residue-base*	Residue-acid†	Extract	Residue-base*	Residue-acid†
C_{28} (Δ^{11})	—	tr	1	—	1	3	—	1	6	1	tr	—
C_{29} (Δ^{12})	—	2	3	—	8	7	—	8	11	—	—	—
C_{30} (Δ^{13})	43	9	9	73	19	20	63	32	20	91	62	82
C_{31} (Δ^{14})	tr	16	15	15	16	14	—	18	20	—	20	8
C_{32} (Δ^{15})	50	27	28	12	21	21	37	24	19	8	18	10
C_{33} (Δ^{16})	tr	25	24	—	9	14	—	8	12	—	—	—
C_{34} (Δ^{17})	7	21	20	—	26	20	—	9	9	—	—	—

* Extract obtained after treatment of extracted biomass with KOH.
† Extract obtained after treatment of KOH-treated, extracted biomass with HCl.
Data normalized to all measured isomers = 100%.

DISCUSSION

Biosynthesis of monohydroxy acids

Monohydroxy fatty acids are well known constituents of the polyester cutins and suberins of higher plants, but have not previously been found in micro-

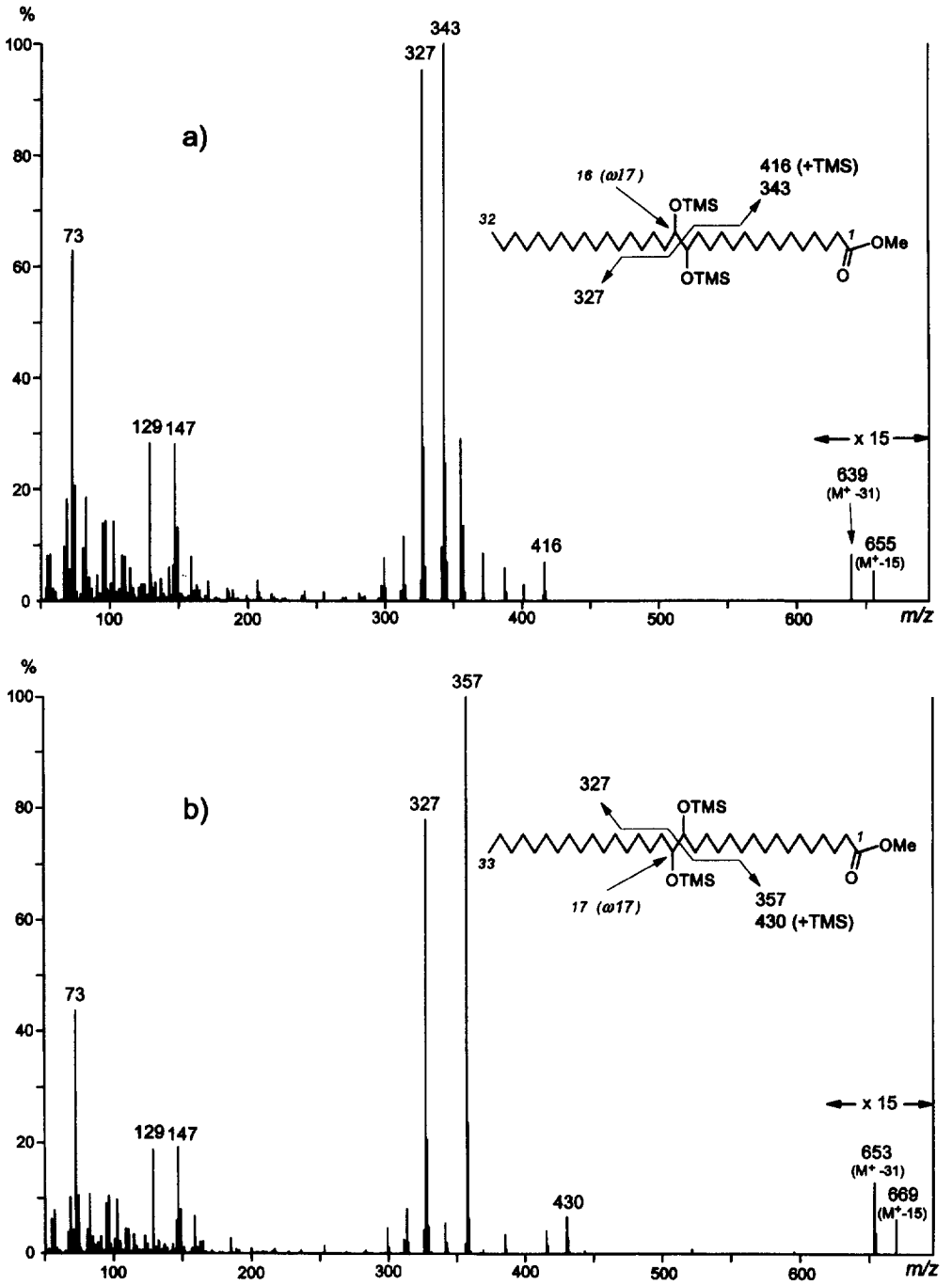

Fig. 2. Mass spectra of (a) C_{32} and (b) C_{33} dihydroxy fatty acids (as methyl esters, TMS-ethers).

algae. The most common constituents have an hydroxyl group located at the end of the chain (ω-hydroxy acids), but substantial amounts of acids having a mid-chain hydroxyl group also occur [7]. In all cases, the carbon numbers are lower than those found in *Nannochloropsis* and typically occur in the range C_{16}–C_{24}.

The major dihydroxy fatty acid with a mid-chain hydroxyl group in higher plant cutins is 10,16-dihydroxyhexadecanoic acid. This co-occurs with the 9,10-isomer and their relative abundance appears to be species-dependent. The presence of both isomers and lack of unsaturated C_{16} fatty acids in cutin has been interpreted as evidence for direct hydroxylation for the introduction of the mid-chain hydroxyl group [7]. In contrast, in the C_{18} series of fatty acids it has been suggested that oleic (18:1) and linoleic (18:2) fatty acids undergo hydroxylation at C-18, followed by epoxidation at Δ^9 and subsequent hydration of the epoxide to yield the mid-chain hydroxy fatty acid isomers [7].

The distribution of long-chain hydroxy acids in *Nannochloropsis* is quite different from that reported

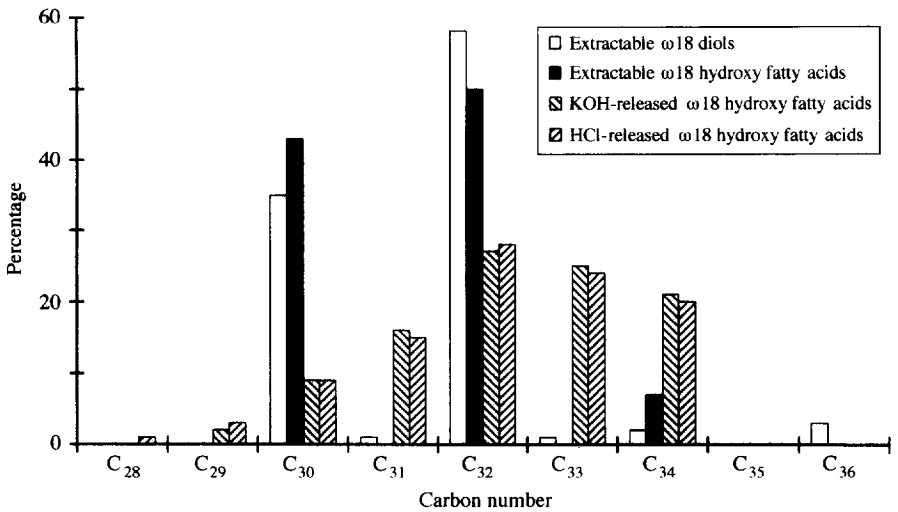

Fig. 3. Relative distributions of extractable ω18 hydroxy fatty acids released by KOH and HCl treatments of extracted residues together with extractable alkane diol distribution in *Nannochloropsis salina*.

in any other organism. In particular, the occurrence of long chain-lengths is without precedence. The position of the hydroxyl group at ω18 rather than at a constant position relative to the carboxylic acid group indicates that the series results from chain-elongation (or chain-shortening) of a particular hydroxy fatty acid. The predominance of the C_{30} homologue in the extractable lipids for three of the four *Nannochloropsis* species studied, raises the possibility that this compound is both chain-elongated and chain-shortened to produce the other members in the series. In contrast, the bound hydroxy acids have a different distribution, although to a lesser extent for *N. oculata*, and probably were linked to the insoluble matrix at different stage of biosynthesis. It should be noted that the culture of *N. oculata* was grown under different conditions from the three others; this could have been responsible for differences observed between the lipid distributions of the these species. Furthermore, the presence of relatively abundant bound odd carbon-numbered fatty acids is intriguing; their biosynthesis is difficult to explain on the basis of the classical chain elongation process.

Biosynthesis of dihydroxy fatty acids

A monomeric constituent of plant cutins 9,10-dihydroxyoctadecanoic acid [7]. The hydroxyl groups in this fatty acid occur at the same position as the double bond in oleic acid which is abundant in the lipids of many plants. By analogy, the 15,16-dihydroxy C_{32} fatty acid in *Nannochloropsis* might derive from a corresponding C_{32} monounsaturated fatty acid. However, no trace of this fatty acid could be detected. It is now recognised that at least some of the 9,10-dihydroxyoctadecanoic acid found in cutin hydrolysates is an artefact and that the actual fatty acid present in the original cutin is 9,10-epoxyoctadecanoic, which ring opens under strongly basic conditions to form the corresponding dihydroxy fatty acid [8]. Since we only observed the dihydroxy fatty acids after hydrolysis of the residues, this raises the possibility that the 16,17-dihydroxydotriacontanoic acid is formed from the corresponding 16,17-epoxydotriacontanoic acid. This compound could also serve as a precursor for the corresponding monohydroxy fatty acid. Long-chain epoxides are thought to be precursors of plant polyesters [8], and it is intriguing that it has now been demonstrated that all of the *Nannochloropsis* species studied here produce a highly resistant aliphatic biopolymer, which is thought to be mainly composed of long-chain alkyl units linked through two or three ether bonds at the ω17 and/or ω18 and the terminal positions [3, unpublished data]. However, we were unable to obtain any direct evidence for long-chain epoxides and, therefore, this hypothesis requires further testing.

Relationship with long-chain alkyl diols

In contrast to the distributions of hydroxy acids, the long-chain alkyl diols found in *Nannochloropsis* show a greater variety of positional isomerism. In the C_{30} diol, the hydroxyl group occurs at positions C-13 and C-15 (relative to the terminal hydroxyl group, i.e. ω18 and ω16, respectively, relative to the methyl group), whereas in the C_{32} diol the 1,15 isomer (i.e. ω18) comprises *ca* 95% of the isomers present. The similarity between the carbon number distribution between long-chain diols and hydroxy acids suggests that biosynthesis of both classes of compound may be linked, although the similarities are only strong for the extractable lipids from *N. salina* (Fig. 3). Concerning the three other species, and especially *N. oculata*, the C_{30} hydroxy acid is always predominant, which is not reflected in the diol distributions of these species. C_{30}–C_{32} long-chain diols have not been identified in other microalgae, but shorter-chain C_{22} 1,14-

diols have been identified in freshwater microalgae from the genus *Ochromonas* [9]. Labelling studies demonstrated that the 1,14-diols were formed by C-14 hydroxylation of the C_{22} saturated fatty acid, followed by reduction of the carboxylic acid group to the alcohol.

However, in *Nannochloropsis* it cannot be a simple conversion of the hydroxy acid to alkyl diols, because of the additional positional isomers in the diol series. For instance, the presence of C_{30} 1,13 and C_{32} 1,15 diols is readily explained by reduction of the fatty acid group in the hydroxy acids to the alcohol, whereas the presence of the C_{30} 1,15 diol cannot be explained by such a mechanism. It is also possible that, in some cases, the hydroxy acids were all reduced to their diol counterparts and, thus, not present in extracts. This phenomenon can be explained by both culture conditions and the nature of the species; further studies should thus be performed to answer these questions.

EXPERIMENTAL

Algal cultures. Cultures of *N. oculata* (strain CS-216) were obtained from the CSIRO Algal Culture Collection [10]. *N. salina* and *Nannochloropsis* sp. were obtained from the Culture Collection of the Natural Environment Research Council (U.K.) and *N. granulata* from the Culture Collection of Bigelow (U.S.A.). The three latter species and *N. oculata* were cultured according to previously described methods [3 and 10, respectively].

Lipid extraction and hydrolysis of residues. Successive ultrasonic extractions of freeze-dried algal biomass were carried out with MeOH ($\times 2$), MeOH–Cu_2Cl_2 (1:1, $\times 5$) and hexane ($\times 2$). The extract was then treated with MeOH–HCl–$CHCl_3$ (10:1:1) for 2 hr at 80° and the lipids, including the methyl-esterified acids, were extracted with hexane–$CHCl_3$ (1:1). Hydroxyl groups were then derivatized with pyridine–BSTFA to form the corresponding TMSi-ethers. GC-MS was used for the identification of components. Extracted residues were saponified with 1 N KOH in MeOH (reflux for 1 hr) and then hydrolysed with 1 N HCl (reflux for 2 hr). Both extracts obtained from these hydrolyses were derivatized. Acids were esterified with CH_2N_2 and hydroxyl groups silylated as described above. Derivatized extracts were analysed by GC-MS.

Chromatography. GC was performed on an instrument equipped with an FID and an on-column injector. A 25 m fused silica capillary column coated with CP-Sil 5 (0.32 mm i.d.; film thickness 0.45 μm) was used. He was used as carrier gas and the FID temp. was 320°. The oven was prog. from 70 to 130° at 20° min^{-1}, then to 320° at 4° min^{-1} and held at this temp. for 10 min. The column was coupled to the EI source of the mass spectrometer (mass range m/z 40–800 at a resolution of 1000); cycle-time 1.8 sec; ionisation energy 70 eV). Relative abundances of hydroxy esters were calculated from summed mass chromatograms of the principal fragment ions, rather than by direct measurement of GC-FID peak areas, in order to remove errors introduced by co-elutions. Quantitation of hydroxy acids in the base-treated residue was further complicated by co-elution with other components which also gave strong m/z 341 ions (C_{31} and C_{32}). Ion ratios in this case were adjusted by reference to clean MS obtained from other analyses. Also, the C_{30} diol in extractable lipid extracts co-eluted with the 32:1 alcohol but quantified using m/z 313 and 341 ions relative to the C_{32} diol.

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REFERENCES

1. Volkman, J. K., Barrett, S. M., Dunstan, G. A. and Jeffrey, S. W., *Organic Geochemistry*, 1992, **18**, 131.
2. Volkman, J. K., Brown, M. R., Dunstan, G. A. and Jeffrey, S. W., *Journal of Phycology*, 1993, **29**, 69.
3. Gelin, F., Boogers, I., Noordeloos, A. M. M., Sinninghe Damsté, J. S., Hatcher, P. G. and de Leeuw, J. W., *Geochimica et Cosmochimica Acta*, 1996, **60**, 1275.
4. Eglinton, G., Hunneman, D. H. and McCormick, A., *Organic Mass Spectrometry*, 1968, **1**, 593.
5. Hunneman, D. H. and Eglinton, G., *Phytochemistry*, 1972, **11**, 1989.
6. Capella, P., *Advances in Mass Spectrometry*, Vol. 4. Elsevier, London, 1968, p. 196.
7. Walton, T. J. and Kilattukudy, P. E., *Biochemistry*, 1972, **11**, 1885.
8. Kolattukudy, P. E., Walton, T. J. and Hushuaha, R. P. S., *Biochemical and Biophysical Research Communications*, 1971, **42**, 739.
9. Elovsen, J., *Biochemistry*, 1974, **13**, 3483.
10. Jeffrey, S. W., 1980, in *CSIRO Division of Fisheries and Oceanography Annual Report* (1977–1979). 1980, p. 22.