PERGAMON

Phytochemistry 50 (1999) 541–548

PHYTOCHEMISTRY

Very-long-chain (C_{28}) highly unsaturated fatty acids in marine dinoflagellates

Maged P. Mansour *, John K. Volkman, Daniel G. Holdsworth, Anne E. Jackson, Susan I. Blackburn

CSIRO Division of Marine Research, G.P.O. Box 1538, Hobart, Tasmania, 7001, Australia

Received 27 July 1998; revised 27 July 1998

Abstract

Very-long-chain (C_{28}) highly unsaturated fatty acids (VLC-HUFA), octacosaheptaenoic acid (28:7(*n*-6)(4,7,10,13,16,19,22)) and octacosaoctaenoic acid (28:8(*n*-3)(4,7,10,13,16,19,22,25)) were identified in seven marine dinoflagellate species: *Prorocentrum mexicanum*, *P. micans*, *Scrippsiella* sp., *Symbiodinium microadriaticum*, *Gymnodinium* sp., *G. sanguineum* and *Fragilidium* sp. The proportion of these fatty acids accounted for less than 2.3% of the total fatty acids in these species. The main VLC-HUFA in *S. microadriaticum* was 28:7(*n*-6); in the other species it was 28:8(*n*-3), with *Scrippsiella* sp., *Gymnodinium* sp. and *Fragilidium* sp. containing only 28:8(*n*-3) and *Gymnodinium sanguineum* containing a small proportion of 28:7(*n*-6) in addition to 28:8(*n*-3). These findings show that some marine dinoflagellates have the ability to produce VLC-HUFA, but precursors of these VLC-HUFA with chain lengths between 22 and 28 carbon atoms were below the detection level. Both 28:7(*n*-6) and 28:7(*n*-3), as well as other VLC-HUFAs, have been detected previously in Baltic herring; our results suggest that they may have originated from microalgae.

Keywords: Marine dinoflagellates; Very-long-chain highly unsaturated fatty acids; Chain-elongation; Chemical ionization GC-MS; Desaturation; Dimethyl oxazoline derivatives; Hydrogenation; Fatty acids

1. Introduction

Very long-chain fatty acids with more than 22 carbon atoms (i.e. VLC-FA) appear to be widely distributed in higher plants and animal (Poulos, 1989; Rezanka, 1989; Tiffany, 1987): they have been found in seed oils, plant waxes, cutin, suberin, skin, hair, wax-like glands, retina, brain, liver, sperm and testis (Rezanka, 1989) and meibomian gland secretions (Tiffany, 1987). They have also been found in some autotrophic and heterotrophic lower organisms such as microalgae, fungi, sponges and bacteria (Rezanka, 1989), although there are few reports. Most of the identified very-long-chain fatty acids (VLC-FA) are either saturated or monounsaturated. However, VLC-HUFA (>3 double bonds) have been identified in higher animal tissue such as bovine retina, which contains fatty acids with carbon atoms and unsaturation as high as 36:6(*n*-3) (Rezanka, 1989). VLC-HUFA (up to 40:7) are found at elevated levels in brain tissue of human patients with Zellweger's syndrome (Poulos, 1989). The biosynthesis of VLC-HUFA is still speculative and their role is unknown.

Recently, VLC-HUFA were characterised in Baltic herring (*Clupea harengus*) (Linko & Karinkanta, 1970; Rezanka, 1990a,b); two of the fatty acids identified were 28:7(*n*-3) and 28:7(*n*-6). Here we report the presence of 28:7(*n*-6) and the fully unsaturated fatty acid 28:8(*n*-3) as the only detectable VLC-HUFA in seven species of marine dinoflagellates.

2. Results

2.1. Identification of VLC-HUFA

The two VLC-HUFA (28:7(*n*-6) [peak O, Fig. 1(a)] and 28:8(*n*-3) [peak P, Fig. 1(a)] were observed in GC

* Corresponding author. Fax 61-3-6232-5123; e-mail: peter.mansour@marine.csiro.au

0031-9422/98 $19.00
PII: S0031-9422(98)00564-0

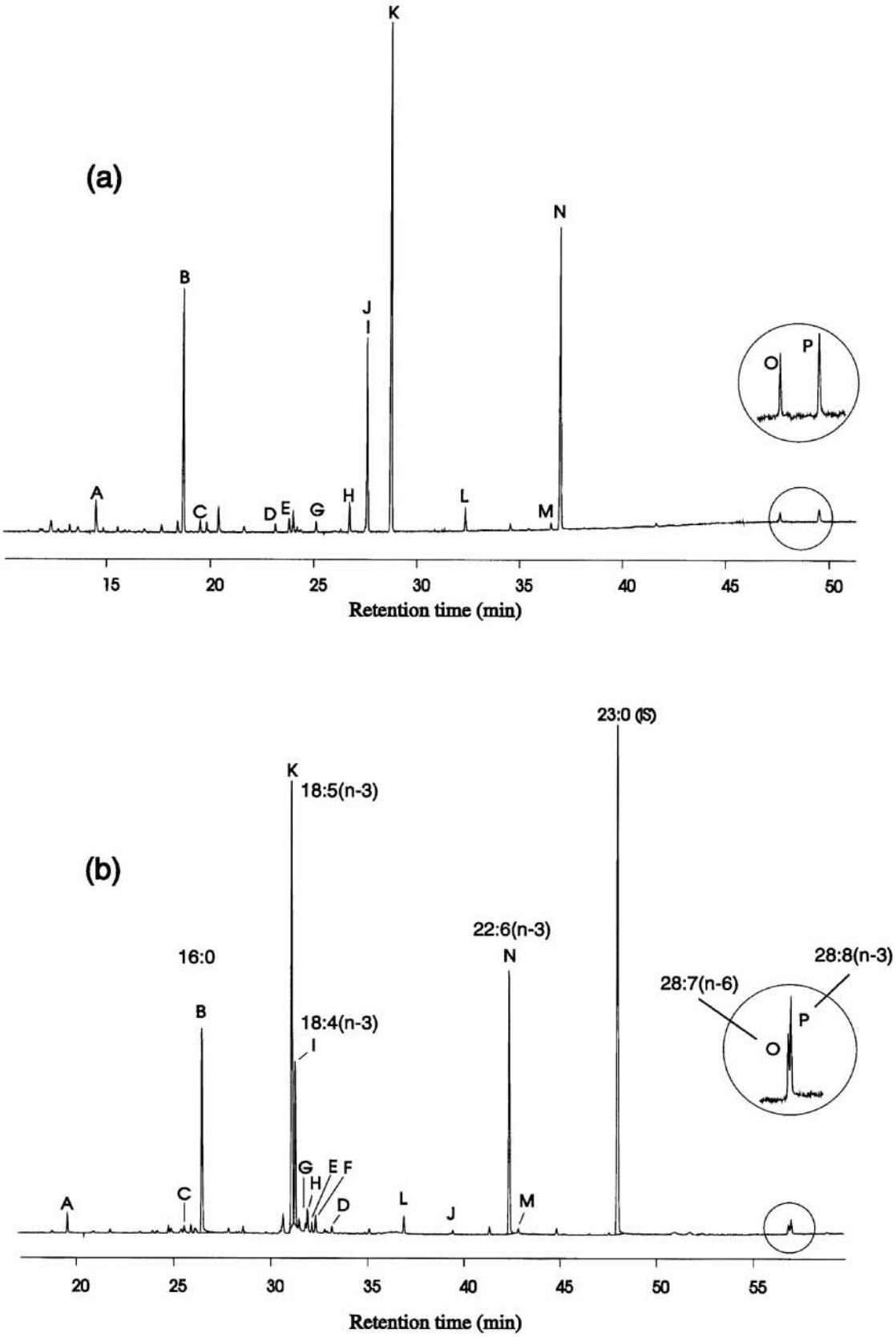

Fig. 1. Chromatograms of FAME from *Prorocentrum micans* on: (a) polar (BPX-70) and (b) non-polar (HP-1) capillary columns. For peak identifications of minor fatty acids refer to Table 1.

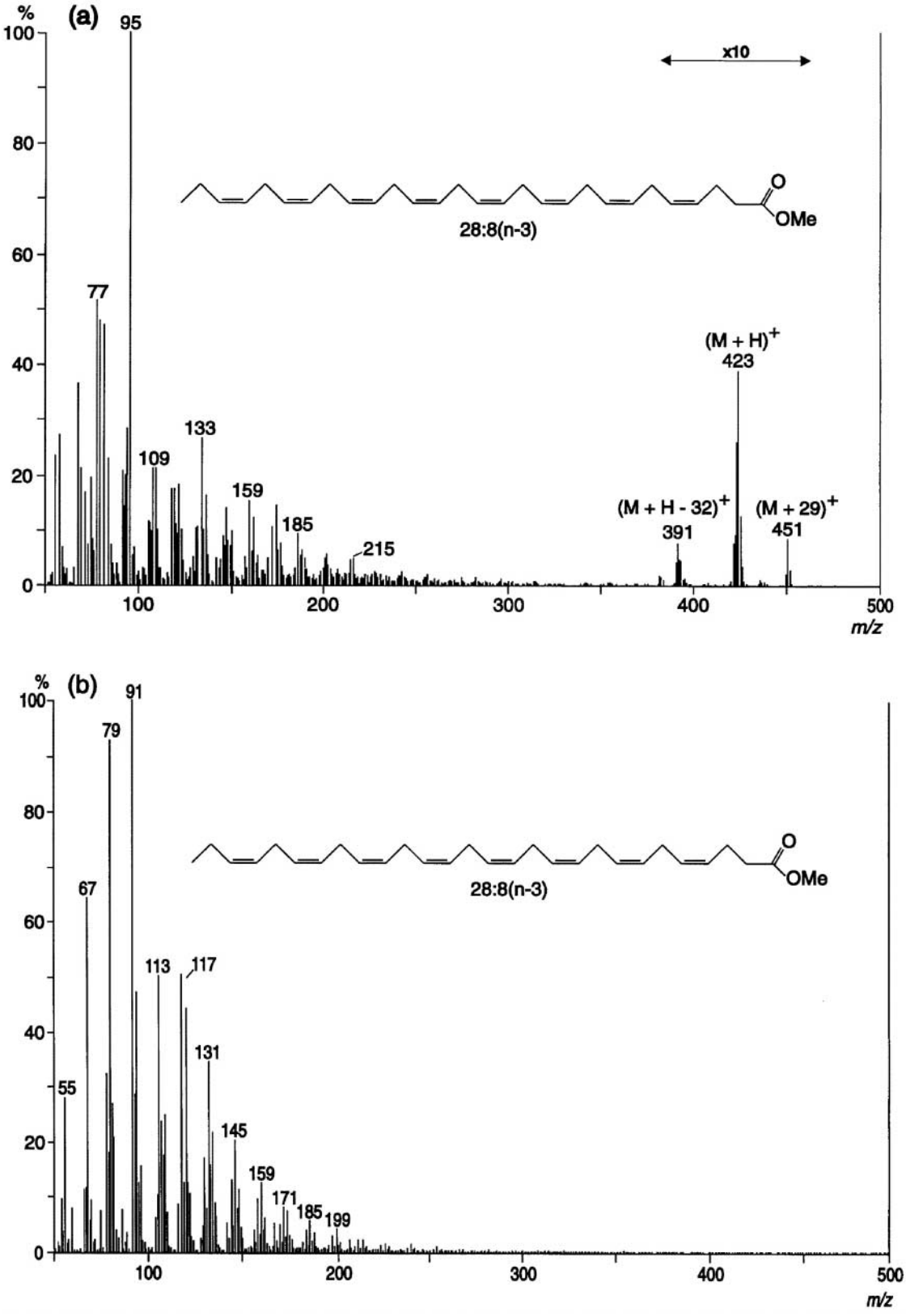

Fig. 2. (a) CI MS and (b) EI MS of 28:8(*n*-3) FAME from *Prorocentrum micans*.

traces from a polar BPX-70 column. These compounds, which emerged more than 10 min after docosahexaenoic acid (22:6(*n*-3)), accounted for less than 2.3% of the total fatty acids in a transmethylated total lipid extract. They were also resolved on a non-polar HP-1 column [Fig. 1(b)]. However, the polar BPX-70 column had a higher selectivity towards these VLC-HUFA.

The EI-mass spectra of these compounds gave the characteristic diagnostic ions for polyunsaturated FAME at *m*/*z* 79, 91 and a typical fragmentation pattern [Fig. 2(b)]. As expected the M^+ ion was not discernible. The presence of these compounds in a saponified fraction confirmed the presence of a carboxyl group and hence their identity as fatty acids.

2.2. Determination of chain length

After hydrogenation of the FAME, the two VLC-HUFA (peaks O and P) were not seen in the gas chromatogram, but a new peak appeared with an ECL value of 28.0, indicating a chain length of 28 carbon atoms without branching. The EI-mass spectrum of the hydrogenated VLC-FAME contained the fragment ions characteristic of saturated FAME: *m*/*z* 74, 87 and a weak but discernible M^+ ion at *m*/*z* 438, together with diagnostic ions at *m*/*z* 395 $[M-43]^+$ and 407 $[M-31]^+$ (results not shown).

2.3. Molecular weight, degree of unsaturation and fatty acid series

The methane CI-mass spectrum of peak P [Fig. 2(a)] had the characteristic ions: *m*/*z* 423 $[M+H]^+$; 391 $[M+H-32]^+$; 451 $[M+29]^+$ consistent with 28:8 FAME. Similarly the CI-mass spectrum for peak O contained the ions: *m*/*z* 425 $[M+H]^+$; 393 $[M+H-32]^+$; and 453 $[M+29]^+$, confirming the identity of this compound as 28:7 FAME (Rezanka, 1989). A methane CI-mass spectrum for 28:7(*n*-3) FAME found in Baltic herring (Rezanka, 1990b) is generally similar to that found here but with some differences, probably reflecting differences in mass spectrometer operating conditions and differences between instruments.

The proportion of the intensities of the ions *m*/*z* 109 and 151 could be used to distinguish between the FAME of the *n*-3 and *n*-6 series (Rezanka, 1990b; Fellenberg, Johnson, Poulos & Sharp, 1987) such that, if the ratio of the intensities of *m*/*z* 109/151 is >1, this indicates a *n*-3 FAME. We found the *m*/*z* 109/151 ratios for 22:6(*n*-3), 28:7 and 28:8 to be 2.5, 1.1 and 5, respectively, supporting their identification as 28:7(*n*-6) and 28:8(*n*-3). The ratio of *m*/*z* 109/151 for 28:7(*n*-3), previously identified in Baltic herring (Rezanka, 1990b), was 2.7, which gives further confidence in our assignments. Additional supporting evidence can be seen in Table 1 where the difference in equivalent carbon lengths (Δ ECL) for the two VLC-HUFA on

Table 1
Fatty acid composition of *Prorocentrum mexicanum* and *P. micans* with equivalent carbon lengths (ECL) derived on BPX-70 and HP-5 bonded-phase capillary GC columns

Fatty acid	Peak[a]	ECL		% composition	
		BPX-70	HP-5	*Prorocentrum mexicanum*	*Prorocentrum micans*
14:0	A	14.00	14.00	0.9	1.8
16:0	B	16.00	16.00	15.7	14.9
16:1(*n*-7)	C	16.32	15.80	0.5	0.8
18:0	D	18.00	18.00	0.9	0.5
18:1(*n*-9)	E	18.26	17.69	1.1	0.8
18:1(*n*-7)	F	18.35	17.44	2.1	1.3
18:2(*n*-6)	G	19.58	17.62	3.5	0.7
18:3(*n*-3)	H	18.85	17.69	0.3	1.7
18:4(*n*-3)	I	19.97	17.58	15.3	12.7
20:0	J	20.00	20.00	0.7	0.6
18:5(*n*-3)	K	20.51	17.55	36.4	37.6
20:5(*n*-3)	L	22.13	19.35	1.1	1.5
22:5(*n*-3)	M	24.00	21.43	0.0	0.4
22:6(*n*-3)	N	24.22	21.34	18.3	22.0
28:7(*n*-6)	O	29.02	26.85	0.1	0.8
28:8(*n*-3)	P	29.87	26.94	1.7	1.3
Others				1.4	0.7
Total				100.0	100.0

Table 2
The VLC-HUFA composition of five marine dinoflagellate species

Species	VLC-HUFA as % of total FA	
	28:7(*n*-6)	28:8(*n*-3)
Prorocentrum mexicanum	0.1	1.7
Prorocentrum micans	0.8	1.3
Scrippsiella sp.	0.0	1.7
Symbiodinium microadriaticum	0.7	0.2
Gymnodinium sp.	0.0	2.2
Gymnodinium sanguineum	0.1	1.4
Fragilidium sp.	0.0	2.0

For complete FA composition see Mansour et al., 1999 (submitted).

polar and non-polar columns can be compared with the Δ ECL of the only other *n*-6/*n*-3 FAME pair (18:2(*n*-6) and 18:3(*n*-3)). The Δ ECL for 28:7(*n*-6)/28:8(*n*-3) and 18:2(*n*-6)/18:3(*n*-3) on a polar column was 0.85 and 0.73, respectively, while on a non-polar column the values were 0.09 and 0.07.

2.4. Determination of position of double bonds

Double bonds were located by EI GC-MS of 4,4-dimethyloxazoline (DMOX) derivatives of FAME by identifying the difference of 12 dalton, which interrupts the regular 14 dalton pattern, produced by successive chain cleavages of methylene units (Fay & Richli, 1991). This rule, which was formulated by Anderson and co-workers (Andersson & Holman, 1974; Andersson, Christie & Holman, 1975), states that: if an interval of 12 dalton, instead of the regular 14 dalton, is observed between the most intense peaks of clusters of fragments containing *n* and *n*-1 carbon atoms in the acid moiety, a double bond occurs between carbons *n* and $n+1$ in the molecule. Peak O (Fig. 1) was identified as 28:7 (*n*-6) (4,7,10,13,16,19,22) (Fig. 3) and peak P (Fig. 1) as 28:8(*n*-3) (4,7,10,13,16,19,22,25) (Fig. 3). Interestingly we found that in the EI-mass spectrum of both VLC-HUFA and also for 22:6(*n*-3) that the $[M-1]^+$ ion was more abundant than the $[M]^+$ ion. It was also observed that fatty acids with fewer than four double bonds had an abundant $[M]^+$ ion, while fatty acids with four or five double bonds had $[M-1]^+$ and $[M]^+$ ions in similar abundances. These results suggest that, under these ionization conditions, hydrogen abstraction occurs more easily for fatty acids with more than five double bonds to form a stabilised $[M-1]^+$ ion, in preference to forming the $[M]^+$ ion.

2.5. Concentrations of 28:7(n-6) and 28:8(n-3)

The total of these two VLC-HUFA in the seven dinoflagellate species did not exceed 2.2% of the total fatty acids (Tables 1 and 2), and the individual proportions of the two varied greatly among the species. The full fatty acid analysis of *Prorocentrum mexicanum* and *P. micans* are given here; fatty acid data for the five other dinoflagellate species in an earlier report (Mansour, Volkman, Jackson & Blackburn, 1999). The major fatty acids in all species were 16:0, 18:4(*n*-3), 18:5(*n*-3) and 22:6(*n*-3), although the relative proportions varied between the species. *Prorocentrum micans* and *Symbiodinium microadriaticum* were the only species to contain high proportions of 28:7(*n*-6) and three species: *Scrippsiella* sp., *Gymnodinium* sp. and *Fragilidium* sp. had 28:8(*n*-3) as the sole VLC-HUFA (Table 2). In none of the species could we find detectable precursor fatty acids beyond 22:6(*n*-3) after GC analysis of transmethylated lipid extract. Analysis of $AgNO_3$-TLC-purified FAME of *Prorocentrum micans* also did not reveal any FAME precursors.

3. Discussion

3.1. Analysis of VLC-HUFA

Confirmation of the identities of the VLC-HUFA was difficult, due to the lack of pure standards and the small quantities present. However, a combination of $AgNO_3$-TLC, EI/CI GC-MS of FAME and EI GC-MS of DMOX derivatives proved to be useful for elucidation of the structure of these VLC-HUFA. Reports on VLC-HUFA are relatively recent (Rezanka, 1989; Rezanka, 1990a,b) and reflect recent advances in GC techniques and a greater awareness that fatty acids eluting after 22:6(*n*-3) might be present in gas chromatograms. Moreover, such compounds are minor components and require more elaborate spectrometric techniques to confirm their identity.

3.2. Biosynthesis and food-chain link

Our data show, for the first time, that VLC-HUFA occur in dinoflagellates, but the mechanism of their biosynthesis and their function are unknown (Rezanka, 1989). In a limited examination of representatives of marine microalgae other than dinoflagellates, we did not detect VLC-HUFA in a diatom (*Chaetoceros muelleri*), a haptophyte (*Pavlova pinguis*) or a thraustochytrid (heterotrophic "marine fungus") analysed under the same conditions (unpublished results), but further analyses of other species need to be carried out. It is not known why 28:7(*n*-6) and 28:8(*n*-3) are the only two VLC-HUFA in these dinoflagellates and why some species contain only 28:8(*n*-3).

The lack of any obvious precursors is intriguing and makes it difficult to elucidate how these compounds

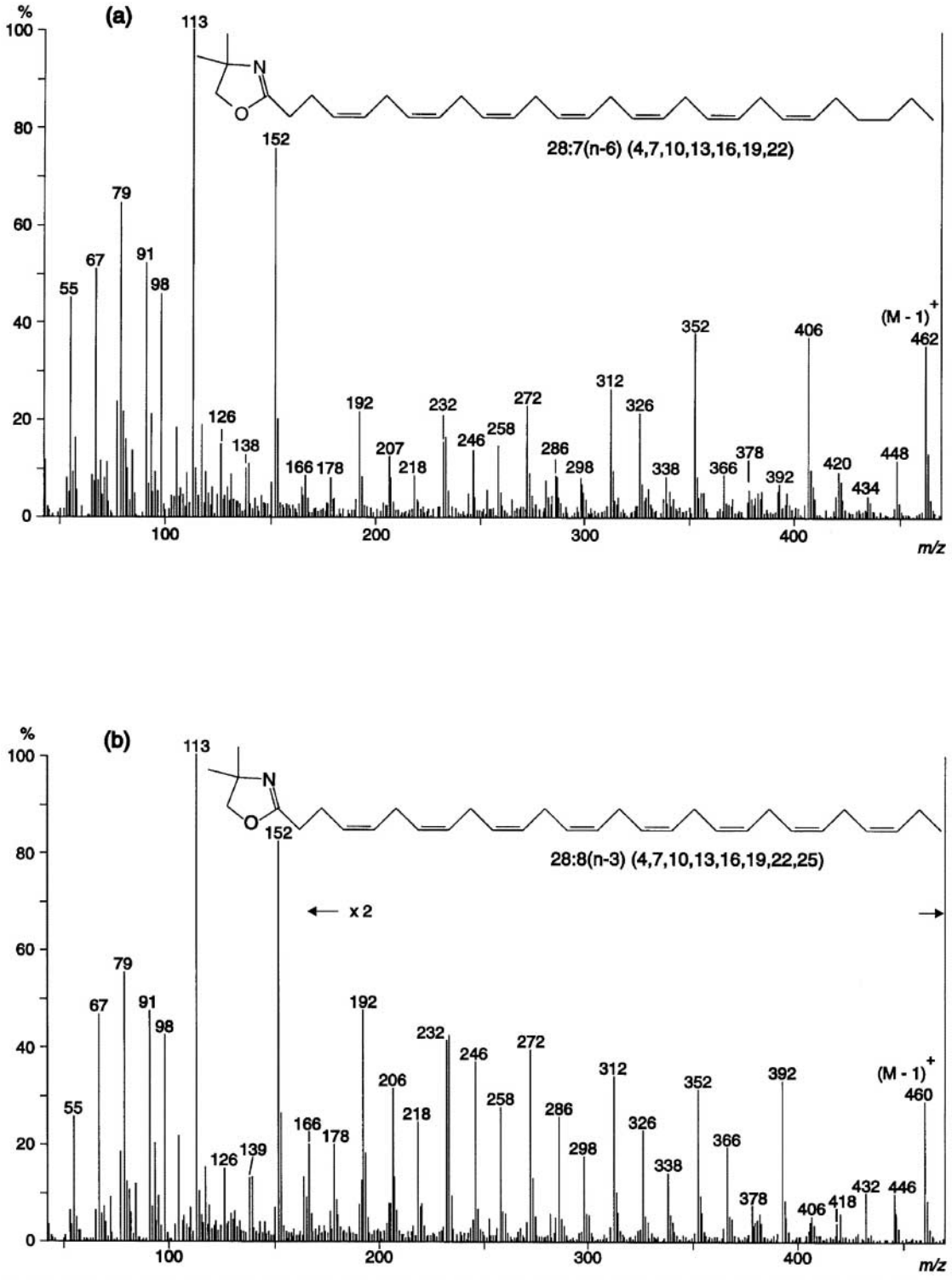

Fig. 3. (a) EI MS of 28:7(*n*-6) and (b) 28:8(*n*-3) as their DMOX derivatives from *Prorocentrum micans*.

might be synthesised. Conventional theory suggests successive chain elongation and desaturation of 22:6(*n*-3) as a possible mechanism for biosynthesis of these VLC-HUFA but no precursors were found. Either these VLC-HUFA are not formed by chain elongation or the precursor FAME are present only in very low proportions (< 0.1% of total fatty acids).

It is intriguing that the only other *n*-6 fatty acid in these dinoflagellates is 18:2(*n*-6), which is present in such small proportions (0.3–5.2%) that it seems unli-

kely that 28:7(*n*-6) was formed by successive chain elongation and desaturation steps of this fatty acid. It seems logical to assume that the biosynthesis of 28:7(*n*-6) and 28:8(*n*-3) are linked. One possibility is that 28:7(*n*-6) may be desaturated to 28:8(*n*-3), and because there is room for only one more methylene-interrupted double bond, by necessity it must be located in the *n*-3 position. Alternatively 28:8(*n*-3) could be formed first and then hydrogenated to 28:7(*n*-6). This second possibility, although unconventional, could explain why two species have 28:8(*n*-3), but no detectable 28:7(*n*-6).

Both 28:7(*n*-6) and 28:7(*n*-3) have been detected in Baltic herring (Rezanka, 1990b) which raises the question of whether these VLC-HUFA are metabolic products of the fish and/or are acquired through the diet. Interestingly, ten VLC-HUFA precursors with C_{24}, C_{26} and C_{28} chain-lengths were also identified in herring tissue lipids (Rezanka, 1990b) and some were present in much higher proportions than 28:7(*n*-6) (0.43%) and 28:7(*n*-3) (1.9%). These were 24:4(*n*-3) (5.65%), 24:5(*n*-3) (6.09%), 24:6(*n*-3) (3.91%), 26:5(*n*-3) (2.16%) and 26:6(*n*-3) (3.04%). A dietary source seems likely, since the VLC-HUFA composition in Baltic herring shows seasonal changes (Linko & Karinkanta, 1970).

Buzzi, Henderson and Sargent (1997) recently suggested that the formation of 22:6(*n*-3) in trout liver does not involve Δ^4-desaturation of 22:5(*n*-3), but rather proceeds via the Δ^6-desaturation of 24:5(*n*-3) to 24:6(*n*-3) and subsequent chain shortening. The high abundance of 18:5(*n*-3) found in several dinoflagellates (*Prorocentrum mexicanum*; *P. micans; Scrippsiella* sp., Mansour et al., 1999; *Gymnodinium* sp., Mansour et al., 1999) may similarly be formed by the chain-shortening of 20:5(*n*-3). Clearly, further research on the pathways of polyunsaturated fatty acid biosynthesis in marine organisms is still needed.

4. Experimental

4.1. Culturing and harvesting

Seven marine dinoflagellate strains were obtained from the CSIRO Collection of Living Microalgae: *Prorocentrum mexicanum* Tafall CS-292, isolated from a water sample, Wilson Inlet, Western Australia; *Prorocentrum micans* Ehrenberg CS-293, isolated from a water sample, La Jolla, California; *Scrippsiella* sp. CS-295/c, from ship's ballast water; *Symbiodinium microadriaticum* Freud CS-155 originally isolated from the sea anemone, *Condylactis* sp. from Jamaica; *Gymnodinium* sp. CS-380/1 isolated from a water sample, Devonport, Tasmania; *Gymnodinium sanguineum* Hirasaki CS-35, isolated from a water sample, La Jolla, California; and *Fragilidium* sp. CS-382 isolated from a water sample, Devonport, Tasmania. All strains were clonal and non-axenic. Duplicate 1 l. batch cultures of each strain were grown in 2 l. glass Erlenmeyer flasks capped with Steristoppers (Imbros Pty. Ltd.). The strains were grown at 18.5° under 80 μmol photons PAR $m^{-2} s^{-1}$ of cool white fluorescent light in GSe medium, a modification of the GP medium of Loeblich (1975), with 10^{-8} M selenium (as selenite) and salinity adjusted to 28 psu with Milli-Q water (Blackburn, unpublished data). In order to minimize cell lysis we did not aerate or shake the cultures since some dinoflagellates are quite fragile (Blackburn, unpublished results). The 2×1 l. cultures were combined and harvested by centrifugation at 3000–4500*g* for 10 min. at 7 days, estimated at being at late-logarithmic phase from a detailed examination of *Gymnodinium* sp. CS-380/1 (Mansour *et al.*, unpublished data).

4.2. Extraction and transmethylation

The algae were extracted with $CHCl_3$–MeOH–H_2O (1:2:0.8) by a modified version of Bligh and Dyer's method (Bligh & Dyer, 1959). FAME were formed by heating in MeOH–HCl (10:1) at 80° for 2 h and extracted into hexane–$CHCl_3$ (4:1,×3).

4.3. $AgNO_3$-TLC

FAME were separated according to the degree of unsaturation, using a double development in hexane–Et_2O–HOAc (94:4:2) on silica gel (7 g) plates loaded with 3% $AgNO_3$ (w/w). Bands were visualised under 366 nm UV light after spraying with 2′,7′-dichlorofluorescein, and extracted into hexane–$CHCl_3$ (4:1,×3). The FAME were washed with saturated NaCl and 2 M NH_3 to remove Ag^+ and 2′,7′-dichlorofluorescein, respectively.

4.4. Hydrogenation

FAME were reconstituted in iso-octane (3 ml) and bubbled with hydrogen for 3 hr in the presence of 30 mg PtO_2. After the addition of Milli-Q H_2O the saturated FAME were extracted with iso-octane (×4).

4.5. DMOX derivatization

FAME were heated with 2-amino-2-methyl-1-propanol at 210° for 2 h (Liebich, Schmieder, Wahl & Woll, 1994) and extracted into hexane–$CHCl_3$ (4:1,×3) after partitioning with Milli-Q H_2O.

4.6. Gas chromatography

GC was performed on an HP 5890 gas chromatograph fitted with an BPX-70 bonded phase capillary column (50 m; 0.32 mm i.d.; 0.25 μm film thickness), a FID and an on-column injector. Samples were injected at 45°; after 2 min the oven temperature was raised at 30° min^{-1} to 120° and then at 3° min^{-1} to 240°, where it was held for 20 min. FAME, hydrogenated FAME and DMOX derivatives were also analysed on an HP-1 non-polar column (50 m; 0.32 mm i.d.; 0.17 μm film thickness) with the same conditions as above except the final temperature was 310°.

4.7. Mass spectrometry

EI GC-MS was performed on a Fisons MD-800 with an on-column injector set at 45°. The sample was injected into a retention gap attached to an HP-5 Ultra 2 bonded phase column (50 m; 0.32 mm i.d.; 0.17 μm film thickness). The initial temperature of 45° was held for 1 min, followed by temperature programming at 30° min^{-1} to 140° then at 3° min^{-1} to 310°, where it was held for 12 min. Helium was used as the carrier gas. Mass spectrometer operating conditions were: electron impact energy 70 eV; transfer line 310°; source temperature 250°; scan rate 0.8 scans s^{-1} and mass range 40–650 dalton. CI GC-MS was achieved with methane and the following modifications to the operating conditions: electron impact energy 35 eV, source temperature 200°.

Acknowledgements

We thank Graeme Dunstan and Dr Malcom Brown for suggestions on the manuscript and Dr Peter Nichols for useful discussions about the DMOX reaction.

References

Andersson, B. A., Christie, W. W., & Holman, R. T. (1975). *Lipids, 10,* 215.
Andersson, B. A., & Holman, R. T. (1974). *Lipids, 9,* 185.
Bligh, E. G., & Dyer, W. J. (1959). *Canadian Journal of Biochemistry and Physiology, 37,* 912.
Buzzi, M., Henderson, R. J., & Sargent, J. R. (1997). *Comparative Biochemistry and Physiology, 116B,* 263.
Fay, L., & Richli, U. (1991). *Journal of Chromatography, 541,* 89.
Fellenberg, A. J., Johnson, D. W., Poulos, A., & Sharp, P. (1987). *Biomedical and Environmental Mass Spectrometry, 14,* 127.
Mansour, M. P., Volkman, J. K., Jackson, A. E., & Blackburn, S. I., *Journal of Phycology* (submitted).
Liebich, H. M., Schmieder, N., Wahl, H. G., & Woll, J. (1994). *Journal of High Resolution Chromatography, 17,* 519.
Linko, R. R., & Karinkanta, H. (1970). *Journal of the American Oil Chemists' Society, 47,* 42.
Loeblich, A. R., III (1975). *Journal of Phycology, 11,* 80.
Poulos, A. (1989). *Progress in Lipid Research, 28,* 35.
Rezanka, T. (1989). *Progress in Lipid Research, 28,* 147.
Rezanka, T. (1990a). *Journal of Chromatography, 513,* 344.
Rezanka, T. (1990b). *LC GC International-Magazine of Separation Science, 3,* 46.
Tiffany, J. M. (1987). *Advances in Lipid Research, 22,* 1.