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Novel polyunsaturated *n*-alkenes in the marine diatom *Rhizosolenia setigera*

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Four previously unknown *n*-C_{25} and *n*-C_{27} heptaenes of the marine diatom *Rhizosolenia setigera* were isolated and identified using NMR spectroscopy. They possess six methylene interrupted (*Z*)-double bonds starting at C-3 and an additional terminal or *n*-2 (*Z*)-double bond. Structural and stable carbon isotopic evidence suggests that these polyenes are biosynthesized by chain elongation of the C22:6*n*-3 fatty acid, followed by decarboxylation and introduction of double bonds at specific positions.

Keywords: biosynthesis; NMR spectroscopy; polyenes; *Rhizosolenia setigera*; structural identification.

Microalgae are known to contain polyunsaturated alkenes [1]. The most common is *n*-$C_{21:6}$ [all-(*Z*) heneicosa-3,6,9,12,15,18-hexaene, **1** (Fig. 1)] [1–4], formed by decarboxylation of the 22:6*n*-3 polyunsaturated fatty acid [2]. Some diatoms biosynthesize *n*-$C_{21:5}$ (heneicosa-3,6,9,12,15–pentaene) [4]. A variety of longer chain polyenes has been reported [5–10] but the structures of only a few of these components have been elucidated fully. For example, the A race of the freshwater microalga *Botryococcus braunii* produces large amounts of odd-numbered carbon C_{23}-C_{31} alk-1-enes and related alkadienes with the second double bond in the *n*-9 position [9]. The biochemical function of these polyenes remains, however, enigmatic. Recently, we tentatively identified C_{25} and C_{27} polyenes in the marine diatom *Rhizosolenia setigera* [10]. Here we report their full structural identification and discuss their biosynthesis.

MATERIALS AND METHODS

Isolation of polyenes

Large cell masses of *R. setigera* (strain CCMP 1330 from the Provasoli-Guillard National Centre for Culture of Marine Phytoplankton, USA; isolated from Vineyard Sound, MA, USA) were grown at 12 °C in batch cultures under the conditions described previously [11]. Cells were harvested by filtration on precombusted glass fibre filters and extracted ultrasonically with hexane after freeze-drying. An apolar fraction (5.5 mg) was obtained from the extract (51 mg) by column chromatography (Al_2O_3, hexane/dichloromethane, 9 : 1 eluent). Subsequently, alkenes were isolated from this fraction by HPLC using a semipreparative reversed-phase column (Waters Symmetry Prep C_{18}; 7.8 × 150 mm, 7 μm) and a mobile phase of methanol/water (95 : 5; v/v) delivered at 2 mL·min^{-1}. Separation was monitored by UV detection at 210 nm. This resulted in a C_{25} and a C_{27} *n*-polyene fraction (1.9 and 0.3 mg, respectively). The two major components in the C_{25} *n*-polyene fraction were further purified by HPLC on a semipreparative normal phase column (Econosphere Silica; 10 × 250 mm, 10 μm, Alltech Associates Inc.) with a mobile phase of hexane delivered at 2 mL·min^{-1}. This resulted in fractions (≈ 85% pure by GC) of ≈ 0.3 mg of **2** and **3**.

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Abbreviations: APCI-MS, atmospheric pressure positive ion chemical ionization mass spectrometry; irm-GC/MS, isotope-ratio-monitoring gas chromatography/mass spectrometry.

Note: a web page is available at http://www.nioz nl/en/deps/mbt/info.html

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GC, GC/MS and isotope-ratio monitoring GC/MS

GC, GC/MS and isotope-ratio monitoring (irm)-GC/MS were performed using conditions described previously [10,12].

HPLC/atmospheric pressure positive-ion chemical ionization MS

The molecular mass of the polyenes was determined by HPLC/MS. Analyses were performed using a HP (Hewlett Packard, Palo Alto, CA, USA) 1100 series HPLC/MS equipped with an auto-injector and Chemstation chromatography software. Separation was achieved by a HP Eclipse XDB-C_{18} column (2.1 × 150 mm, 5 μm), maintained at 40 °C. The injection volume was 10 μL from solutions with a concentration of 1 mg·mL^{-1}. The *n*-polyenes were eluted with a linear gradient from MeOH/H_2O (4 : 1) to MeOH in 10 min (total run time 20 min); the flow rate was 0.6 mL·min^{-1}. Detection was achieved using atmospheric pressure chemical ionization (APCI)-MS of the eluent. Conditions for APCI-MS were as follows: nebulizer pressure, 45 p.s.i.; vaporizer temperature, 400 °C; drying gas (N_2) flow at 8 L·min^{-1} and temperature 350 °C; capillary voltage, −4 kV; corona, 6 μA. Positive ion spectra were generated by scanning *m/z* 200–500 in 0.7 s.

NMR spectroscopy

NMR spectroscopy was performed on a Bruker ARX400, a Varian Unity Inova 500 and a Bruker DRX600 spectrometer

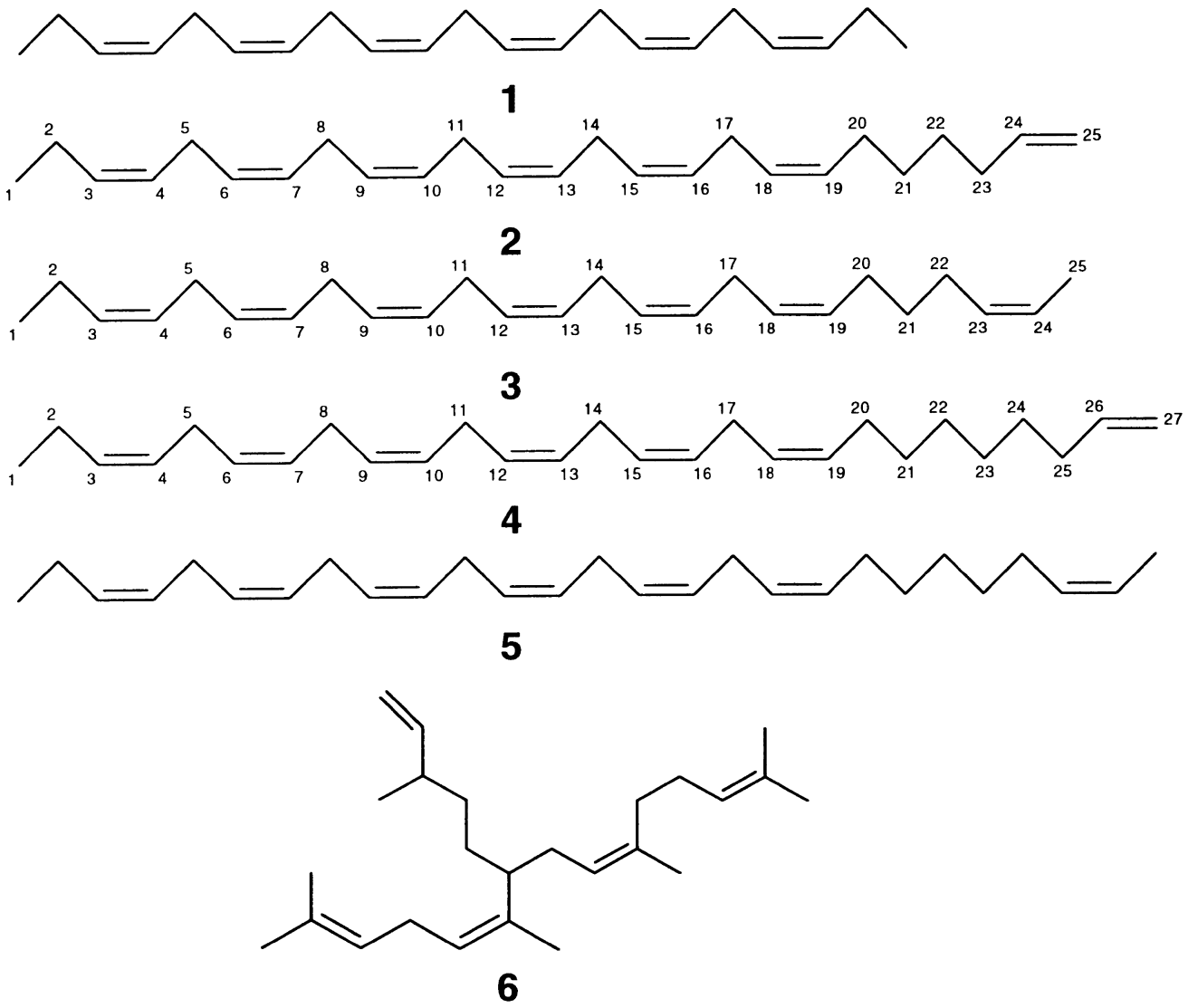

Fig. 1. Structures cited in the text.

equipped with a dual ^{1}H-^{13}C probe, an SWBB probe and an inverse TBI-Z probe with a pulsed field gradient accessory, respectively. All experiments were recorded at 300 K in $CDCl_3$. Proton and carbon chemical shifts were referenced to internal $CDCl_3$ (7.24/77.0 p.p.m.). In the two-dimensional ^{1}H-^{13}C COSY the number of complex points and sweep widths were 2000 points per 6 p.p.m. for ^{1}H and 512 points per 150 p.p.m. for ^{13}C. In the two-dimensional ^{1}H-^{1}H COSY the number of complex points and sweep widths were 2000 points per 5.5 p.p.m. Quadrature detection in the indirect dimension was achieved with the time-proportional-phase-incrementation method. The data were processed with the NMRSUITE software package. After apodization with a 90 shifted sinebell, zero filling to 512 real points was applied for the indirect dimensions. For the direct dimensions zero filling to 4000 real points, Lorentz transformations were used.

RESULTS AND DISCUSSION

Figure 2 shows a typical chromatogram of the apolar fraction of the extract of *R. setigera* cells grown at 12 °C. This fraction contained small amounts of heptadecane and significant amounts of heneicosa-3,6,9,12,15,18-hexaene (*n*-$C_{21:6}$; **1**), the C_{25} highly branched isoprenoid pentaene **6** reported earlier [11], and two clusters of peaks comprising C_{25} and C_{27} linear polyenes **2**–**5**. Hydrogenation of these polyenes resulted in formation of C_{25} and C_{27} *n*-alkanes, establishing their carbon skeletons. The number of double bonds of the *n*-polyenes, assessed by determination of the molecular mass with HPLC/APCI-MS, was found to be seven in all cases. *n*-C_{25} heptaenes **2** and **3** and the *n*-C_{27} heptaene **4** were isolated by HPLC and their structures were determined by high-field NMR.

Structural assignment of the C_{25} *n*-heptaene 3

Analysis by high field ^{1}H and ^{13}C NMR of **3** led to complete assignment of proton and carbon chemical shifts (Table 1). The ^{1}H NMR spectrum revealed the presence of 14 olefinic H, 10 di-allylic H, six allylic H, one olefinic CH_3, one aliphatic CH_3 and one aliphatic CH_2 group. Carbon multiplicities were established by an APT spectrum. This revealed that polyene **3** contains 25 carbon atoms with 14 olefinic CH, nine aliphatic

Table 1. ^{1}H (600 MHz) and ^{13}C (125 MHz) NMR data of all-(*Z*) pentacosa-3,6,9,12,15,18,23-heptaene 3 and all-(*Z*) pentacosa-3,6,9,12,15,18,24-heptaene 2 in $CDCl_3$.

	H-shift		C-shift					
			Heptaene **3**			Heptaene **2**		
C-number	Heptaene **3**	Heptaene **2**	CH_3	CH_2	CH	CH_3	CH_2	CH
1	1.0 (t, 3H, J = 7.5 Hz)	0.98 (t, 3H, J = 7.5 Hz)	14.29			14.26		
2	2.08 (m, 2H)	2.07 (m, 2H)		20.56			20.56	
3	5.39 (m, 1H)	5.38 (m, 1H)			132.05			132.04
4	5.39 (m, 1H)	5.38 (m, 1H)			127.91			127.91
5	2.82 (m, 2H)	2.81 (m, 2H)		25.64[b]			25.64[b]	
6	5.39 (m, 1H)	5.38 (m, 1H)			128.57[a]			128.57[a]
7	5.39 (m, 1H)	5.38 (m, 1H)			128.53[a]			128.52[a]
8	2.86 (m, 2H)	2.84 (m, 2H)		25.64[b]			25.64[b]	
9	5.39 (m, 1H)	5.38 (m, 1H)			128.24[a]			128.25[a]
10	5.39 (m, 1H)	5.38 (m, 1H)			128.24[a]			128.24[a]
11	2.86 (m, 2H)	2.84 (m, 2H)		25.62[b]			25.64[b]	
12	5.39 (m, 1H)	5.38 (m, 1H)			128.13[a]			128.14[a]
13	5.39 (m, 1H)	5.38 (m, 1H)			128.11[a]			128.11[a]
14	2.86 (m, 2H)	2.84 (m, 2H)		25.62[b]			25.64[b]	
15	5.39 (m, 1H)	5.38 (m, 1H)			127.88[a]			127.88[a]
16	5.39 (m, 1H)	5.38 (m, 1H)			127.88[a]			127.73[a]
17	2.82 (m, 2H)	2.81 (m, 2H)		25.54[b]			25.54[b]	
18	5.37 (m, 1H)	5.33 (m, 1H)			127.01			127.01
19	5.39 (m, 1H)	5.38 (m, 1H)			130.14			130.22
20	2.09 (m, 2H)	2.07 (m, 2H)		26.84			27.08	
21	1.4 (q, 2H, J = 7.5 Hz)	1.39 (m, 2H)		29.50			28.53	
22	2.06 (m, 2H)	1.39 (m, 2H)		26.45			29.09	
23	5.39 (m, 1H)	2.05 (m, 2H)			130.40		33.67	
24	5.41 (m, 1H)	5.81 (m, 1H)			124.06			138.97
25	1.60 (dd, 3H, J = 6.1, 0.9 Hz)	4.99 (dd, 1H, J = 17.2 & 1.9 Hz) 4.93 (d, 1H, J = 10.2 Hz)	12.80				114.29	

[a,b] Assignments may be interchanged.

CH_2 and two CH_3 units. Homonuclear (COSY) and inverse heteronuclear (HMQC, HMBC) two-dimensional NMR spectra were used to assign chemical shifts. These data indicate that six of the seven double bonds are methylene interrupted and that the position of the remaining double bond is more isolated. The position of this double bond was established by the inverse HMQC experiment which indicated connectivities of the characteristic quintet at 1.4 p.p.m. of the aliphatic CH_2 (H-21) and the olefinic CH_3 group (H-25) with C-23. The allylic long-range coupling observed in the ^{1}H-^{1}H COSY spectrum between H-25 and H-22 confirmed this assignment. The assignments also prove that the double bonds are at positions 3, 6, 9, 12, 15, 18 and 23. The stereochemistry of the double bonds in **3** was determined to be all-*Z* because the (di)allylic CH_2 units have chemical shifts at $\approx$ 26 p.p.m. (except C-2), which is 4 p.p.m. downfield from the chemical shift observed for typical allylic CH_2 units in a (*E*)-geometry ($\approx$ 30 p.p.m.) [13]. The C-2 allylic CH_2 shift, observed at 20.6 p.p.m., is characteristic for a 3*Z*-alkene [13]. These assignments compare favourably with an APT spectrum of the all-(*Z*) 22:6*n*-3 polyunsaturated fatty acid methyl ester. These stereochemical assignments were confirmed by one-dimensional NOE experiments, which indicated NOE interaction of allylic and diallylic protons, and the protons of C-25 and C-22, establishing the (*Z*)-stereochemistry of the double bonds at C-3, C-18 and C-23. Thus, **3** was identified as all-(*Z*) pentacosa-3,6,9,12,15,18,23-heptaene.

Structural assignment of the C_{25} *n*-heptaene 2

The ^{1}H NMR spectrum of the *n*-C_{25} heptaene **2** was in many respects similar to that of **3**. However, some essential differences were noted: the double doublet at 1.60 p.p.m. was absent, there was one more aliphatic CH_2 group, and there were clear signals from a terminal olefinic group. This is consistent with the change of the position of the double bond at C-23 in **3** to C-24 in **2**. This assignment is in full accordance with the obtained APT ^{13}C NMR spectrum and homonuclear (COSY) and inverse heteronuclear (HMQC) two-dimensional NMR

Table 2. Stable carbon isotopic composition of various lipids of *R. setigera* grown at 12 °C.

Compound	$\delta^{13}C$ (per mL)
Heneicosa-3,6,9,12,15,18-hexaene 1	−23.8 ± 0.3
C_{25} heptaenes **2** and **3**	−23.1 ± 0.1
C_{27} heptaenes **4** and **5**	−23.7 ± 0.3
Highly branched isoprenoid pentaene **6**	−24.9 ± 0.1
Phytol	−21.6 ± 0.2
$C_{16:0}$ fatty acid	−25.2 ± 0.7
$C_{20:5}$ fatty acid	−23.2 ± 0.2
$C_{22:6}$ fatty acid	−22.5 ± 0.2

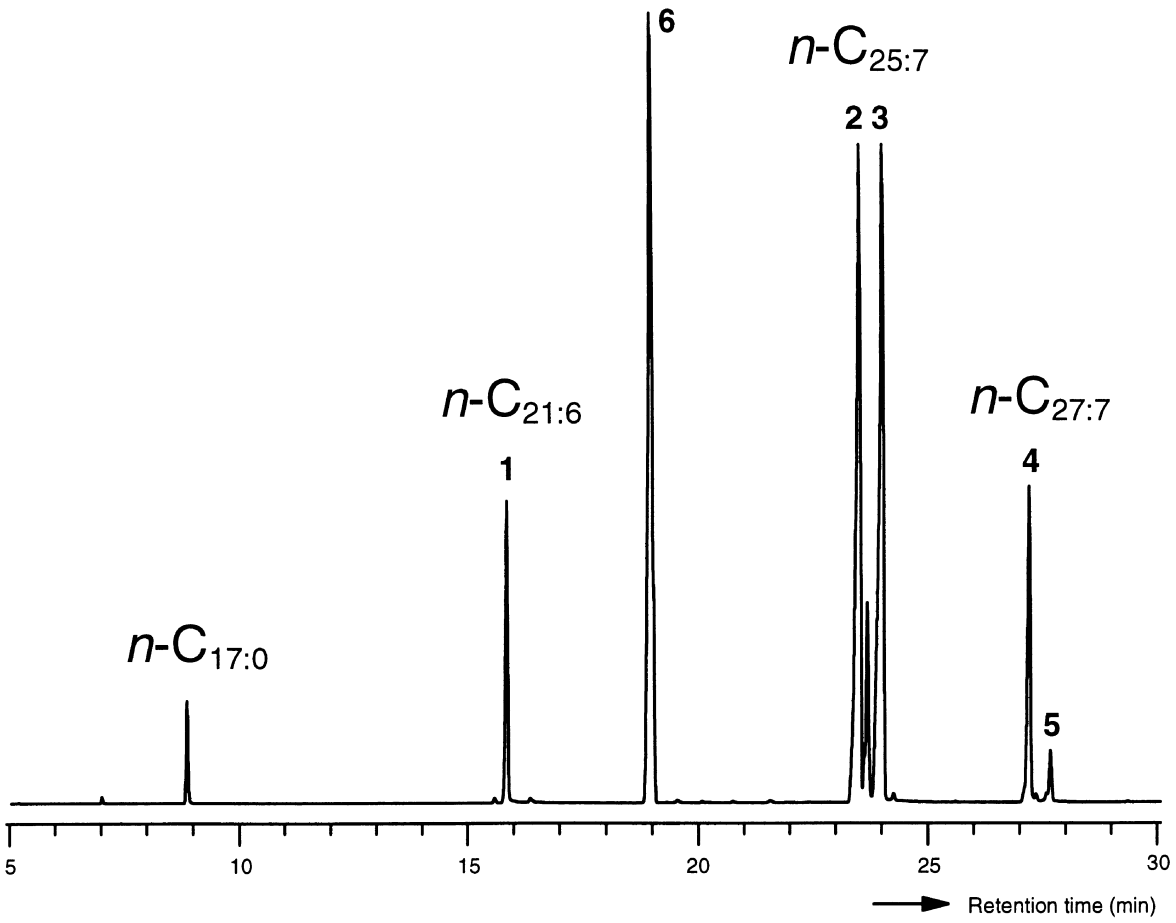

Fig. 2. Partial gas chromatogram of the apolar fraction of the extract of *R. setigera* cell masses grown at 12 °C.

spectra. Consequently, **2** was identified as all-(*Z*) pentacosa-3,6,9,12,15,18,24-heptaene, an identification that is in agreement with its GC-elution behaviour, eluting ≈ 25 Kovats units earlier than heptaene **3** [10]; (*Z*) alk-2-enes are known to be the least volatile alkene isomers and elute significantly later than alk-1-enes [14].

Structural assignments of C_{27} *n*-heptaenes 4 and 5

To investigate the effect of chain elongation on the structures of the C_{27} polyenes a fraction containing 88% of **4** and 11% of **5** was also analysed by NMR. The ^{1}H NMR spectrum was very similar to that of *n*-C_{25} heptaene **2** and showed the characteristic signals of a terminal double bond. The main difference was the presence of three aliphatic CH_2 units. This establishes the major C_{27} isomer to be all-(*Z*) heptacosa-3,6,9,12,15,18,26-heptaene (**4**). A small doublet in the ^{1}H NMR spectrum at 1.60 p.p.m. was also observed, indicating that the minor isomer **5** possesses a *n*-2 double bond. It is, thus, a homologue of **3**.

Biosynthetic relationships

The structural similarities (i.e. double bond positions and stereochemistry) between heptaenes **2**–**5** and *n*-$C_{21:6}$ (**1**) suggest that they are formed through the same biosynthetic intermediate,

Table 3. Relative abundance of hydrocarbons in *R. setigera* grown at different temperatures.

Growth temp (°)	Relative abundance (% of total)				
	n-$C_{17:0}$	*n*-$C_{21:6}$ (**1**)	HBI-$C_{25:5}$ (**6**)	*n*-$C_{25:7}$ (**2** + **3**)[a]	*n*-$C_{27:7}$(**4** + **5**)[b]
4[c]	2	15	37	6 (1.6 + 4)[d]	40 (19 + 20)[d]
12[c]	4	3	23	37 (10 + 26)	31 (7 + 23)
12	1	5	9	44 (27 + 9)	38 (14 + 21)
12	2	6	29	54 (25 + 24)	9 (1 + 8)
20[c]	4	8	17	59 (28 + 23)	12 (2 + 9)

[a] Values in parentheses give percentages for polyenes **2** and **3**. [b] Values in parentheses give percentages for polyenes **4** and **5**. [c] Reported previously [10]. [d] At 4 °C polyenes **2**–**5** actually contain one double bond fewer.

i.e. the 22:6*n*-3 polyunsaturated fatty acid [4]. For biosynthesis of the heptaenes this fatty acid should be elongated by two or three C_2 units and subsequently decarboxylated. Long-chain polyunsaturated fatty acids [i.e. 28:8(*n*-3) and 28:7(*n*-6)] have indeed been reported in marine dinoflagellates [15]. However, presumed intermediate polyunsaturated fatty acids for heptaenes **2**–**5** were not detected in *R. setigera* (i.e. concentrations are $< 1\%$ of the most abundant 22:6*n*-3 polyunsaturated fatty acid). A specific enzymatic reaction seems to be required to form the terminal or (*Z*) *n*-2 double bond after decarboxylation of the elongated polyunsaturated fatty acids. This may perhaps proceed via either a specific dehydrogenation reaction or via β-oxidation of the elongated polyunsaturated fatty acid, decarboxylation and dehydration to form either the terminal or *n*-2 (*Z*)-double bond. It is interesting to note that in the biosynthesis of 1,22-hentriacontadienes and 2(*Z*),22-hentriacontadienes in haptophyte microalgae similar enzymatic reactions are required; their biosynthesis is presumed to proceed via chain elongation and decarboxylation of 9-octadecenoic acid (oleic acid) [7]. This pathway has been convincingly demonstrated to be operative in the biosynthesis of C_{23}-C_{31} diunsaturated 1,*n*-9(*Z*)-alkadienes by *B. braunii*. These components showed strong label incorporation after feeding with ^{14}C-labelled oleic acid [9]. However, this microalga does not produce alkenes with (*Z*) *n*-2 double bonds, as do the haptophytes and the diatom *R. setigera*.

Supporting evidence for the biosynthetic relationship between hexaene **1** and heptaenes **2**–**5** was obtained by compound-specific stable carbon isotope measurements (Table 2) of the hydrocarbons **1**–**6**, some fatty acids and phytol. These data show that the carbon isotope values for hexaene **1** and heptaenes **2**–**5** are, within experimental error, identical and close to those of their alleged biosynthetic precursors, the polyunsaturated fatty acids. The values are different to those of the isoprenoid lipids **6** and phytol and the saturated C_{16} fatty acid.

Variability in lipid composition

Previously, we reported on the variability of the hydrocarbon composition of *R. setigera* grown at different temperatures [10]. An important observation was that the C_{25} and C_{27} linear polyenes produced at 4 °C contain one double bond fewer than heptaenes **2**–**5** biosynthesized at 12 and 20 °C. Because the GC patterns of the isomers produced at 4 °C are rather similar to those of the heptaenes (Fig. 1), it is unlikely that these hexaenes have lost the terminal or *n*-1 double in **2**–**5**. It seems more likely that these polyenes have been synthesized by an identical biosynthetic route as proposed for **2**–**5** but starting from the 22:5*n*-6 polyunsaturated fatty acid. These polyenes would consequently miss the C-18 double bond compared with **2**–**5**. This argument is consistent with the widespread occurrence of *n*-$C_{21:5}$ (heneicosa-3,6,9,12,15–pentaene) in other diatoms [4]. This isomer was, however, not observed in our experiments with *R. setigera*.

In our previous communication [10] we also reported a marked increase in the ratio of C_{25} over C_{27} polyenes with increasing growth temperature (Table 3). However, later experiments did not confirm this: two batch cultures grown at 12 °C resulted in quite different ratios. Moreover, there seems no consistent trend with temperature in the ratio of polyene isomers **2**–**5** (Table 3). Therefore, temperature cannot be the only factor determining the hydrocarbon composition of *R. setigera*. Until the physiological role of these hydrocarbons is determined, it remains difficult to understand what controls their distribution. Some oceanic *Rhizosolenia* species have been found to migrate between 80 and 100 m (nutrient-rich waters) pools (below) and nutrient-depleted surface waters [16–18]. Maybe, the polyenes play a role in the regulation of the buoyancy reported to allow the vertical migration [18]. This would explain our ambiguous results regarding ratio variations with temperature. Buoyancy regulation is difficult to follow in batch cultures but we intend to design experiments to this end.

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