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LONG-CHAIN ALKENES AND ALKENONES IN THE MARINE COCCOLITHOPHORID *EMILIANIA HUXLEYI*

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Abstract—Very long-chain *n*-alkenes and *n*-alkenones have been identified in the ubiquitous marine alga *Emiliania huxleyi*. The alkenes range from C_{31} to C_{38} and are almost exclusively odd-chain. Dienes, trienes and tetraenes were identified but no monoenes were found. The ketones ranged from C_{37} to C_{39} and consisted of both alken-2-ones and alken-3-ones, with trienes more abundant than dienes. Examination of three different forms of the alga, i.e. motile, sessile and coccolith, indicated that these ketones are formed throughout the growth cycle with only minor variations in the relative proportions of the individual compounds. Although these novel compounds have not been reported previously in organisms, they are widespread in marine sediments and may be useful biological markers for *E. huxleyi* input to sediments.

INTRODUCTION

Since its first appearance in the late Pleistocene [1] [*ca* 220000 BP] the microscopic alga *Emiliania huxleyi* (Lohm.) Hay et Mohler [2] has become ubiquitous in the oceans and often dominates the phytoplankton biomass in areas such as the surface waters off Bermuda [3, 4]. Large numbers of its coccolith (calcium carbonate) tests are to be found in many Pleistocene deposits, a notable example being in the Black Sea [5]. Its taxonomic position has changed several times and the alga has been variously named *Pontosphaera huxleyi*, *Coccolithus huxleyi* and most recently *E. huxleyi* [2, 6]. It is at present placed in the Haptophyta (Prymnesiophyta) order Isochrysidales, family Gephyrocapsaceae [2].

Although several studies [7–14] of lipid extracts of this species have been made, comprehensive analyses have been lacking. Chemotaxonomic features which distinguish this alga include the presence of an unusual carotenoid 19′-hexanoyloxyfucoxanthin [7–9] and a water soluble acidic polysaccharide containing a number of unusual sugars [14]. Other studies have examined the fatty acid composition [12] and demonstrated the presence of *n*-heneicosahexaene and pristane [10, 11]. In view of the widespread distribution of this alga in the marine environment, and the possibility that chemotaxonomic studies could assist in its classification, a more comprehensive lipid study was undertaken.

Our initial studies [13] showed that the major non-saponifiable lipids were very long-chain di- and tri-unsaturated alken-2-ones (**1**) and alken-3-ones (**2**). These compounds had not previously been reported in organisms but were known to be present in a number of marine sediments [13, 15]. In many of these sediments an origin from *E. huxleyi* seems probable [13], although several sediments pre-date the first arrival of *E. huxleyi* in the fossil record.

In this paper we report further compositional data for the ketone distributions at three different development stages of the alga and in addition the identification of a suite of novel straight-chain alkenes (**3**) extending in chain-length to C_{38}. These two groups of compounds, together with 24-methylcholesta-5,22(E)-dien-3β-ol [13] account for most of the non-saponifiable lipids of this alga.

RESULTS AND DISCUSSION

Hydrocarbons

A previous analysis of the hydrocarbons of *E. huxleyi* (as *Coccolithus huxleyi*) reported the presence of *n*-heneicosahexaene (*n*-C_{21}:6), pristane and trace quantities of *n*-alkanes (C_{14}–C_{25}) [10, 11]. The present work has confirmed the presence of *n*-C_{21}:6 but it was found to be only a minor component (*ca* 2%) of the hydrocarbon fraction. In addition to squalene (*ca* 1%) a number of very long-chain ($>C_{30}$) components were recognized in the capillary GLC analyses. These compounds would almost certainly have been missed by Blumer *et al.* [10, 11] since the packed GLC columns then in use were only capable of detecting up to C_{25}. With the high resolution capillary columns now available the range of study is extended to at least C_{45}, and perhaps higher [16], and in view of the novel compounds reported here, a re-examination of earlier work seems warranted.

Primary identification of these compounds has relied upon interpretation of mass-spectra, GLC co-injection and hydrogenation data since the amounts isolated from the laboratory cultures have been insufficient for more rigorous characterization. The position and geometry of the double bonds remain to be determined. Mass spectra were obtained by capillary GC–MS and these showed ion-clusters centred on $C_nH_{2n-2}^+$ and molecular ions consistent with alkenes (Table 1). A monotonic decrease with increasing *m/e* value was observed and no

Table 1. Percentage composition of long-chain alkenes in *E. huxleyi* at different growth stages

	Formula	MW	ECL†	Percentage composition		
				Motile	Sessile	Coccolith
Alkene-*						
31:2	$C_{31}H_{60}$	432	30.63	3.8	5.1	10.5
31:2	$C_{31}H_{60}$	432	30.72	3.8	2.9	4.4
31:2	$C_{31}H_{60}$	432	30.93	25.6	27.2	32.7
33:3	$C_{33}H_{62}$	458	32.59	6.1	3.3	5.0
33:3	$C_{33}H_{62}$	458	32.90	9.6	7.1	5.4
33:4	$C_{33}H_{60}$	456	32.35	1.1	0.2	1.5
33:4	$C_{33}H_{60}$	456	32.66	3.4	1.8	3.7
37:3	$C_{37}H_{70}$	514	36.41	42.2	47.4	32.4
38:3	$C_{38}H_{72}$	528	37.40	4.4	5.0	4.4
				100.0	100.0	100.0
Alken-2-one						
37:3	$C_{37}H_{68}O$	528	36.22	35.1	35.8	30.8
37:2	$C_{37}H_{70}O$	530	36.43	18.4	13.7	15.9
38:3	$C_{38}H_{70}O$	542	37.25	9.9	4.4	8.1
38:2	$C_{38}H_{72}O$	544	37.45	2.0	1.8	2.1
Alken-3-one						
38:3	$C_{38}H_{70}O$	542	ND	21.8	29.6	24.4
38:2	$C_{38}H_{72}O$	544	ND	9.9	12.1	16.5
39:3	$C_{39}H_{72}O$	556	ND	1.9	1.7	1.4
39:2	$C_{39}H_{74}O$	558	ND	1.0	0.9	0.8
				100.0	100.0	100.0

* The shorthand nomenclature used is no. of carbon atoms: no. of double bonds.
† Equivalent chain length (ECL) values refer to GLC analysis on a OV-1 glass WCOT capillary column.
ND. Not determined.

1a $n = 35$ and 36 $C_nH_{2n-3}-\overset{\overset{O}{\|}}{C}-CH_3$ **1b** $n = 35$ and 36 $C_nH_{2n-5}-\overset{\overset{O}{\|}}{C}-CH_3$

2a $n = 35$ and 36 $C_nH_{2n-3}-\overset{\overset{O}{\|}}{C}-CH_2CH_3$ **2b** $n = 35$ and 36 $C_nH_{2n-5}-\overset{\overset{O}{\|}}{C}-CH_2CH_3$

3a $n = 31$ C_nH_{2n-2} **3b** $n = 33, 37, 38$ C_nH_{2n-4}

3c $n = 33$ C_nH_{2n-6}

$$CH_3(CH_2)_{13}CH{=}CH(CH_2)_5CH{=}CH(CH_2)_{12}\overset{\overset{O}{\|}}{C}CH_3$$

4

$$CH_3(CH_2)_{13}CH{=}CH(CH_2)_5CH{=}CH(CH_2)_{12}\overset{\overset{O}{\|}}{C}CH_2CH_3$$

5

$$CH_3(CH_2)_{13}CH{=}CH(CH_2)_5CH{=}CH(CH_2)_5CH{=}CH(CH_2)_5\overset{\overset{O}{\|}}{C}CH_2CH_3$$

6

fragmentations suggestive of branched-chain structures were present. Three C_{31} alkenes (all dienes), four C_{33} alkenes (2 trienes and 2 tetraenes), one C_{37} triene and one C_{38} triene were characterized (**3** and Table 1). Catalytic hydrogenation produced only the expected C_{31}, C_{33}, C_{37} and C_{38} *n*-alkanes. The ratio of these alkanes was consistent with the compositional data presented in Table 1 indicating little or no loss of the polyunsaturated alkenes on the all-glass capillary GLC system.

Alkenes (or olefins) are seldom reported in studies of algal lipids and, apart from *n*-C_{21}:6, are generally monosaturated and fall within the chain-length range C_{15}–C_{21} [11, 17, 18]. Exceptions include the hydrocarbons of the golden-brown alga *Botryococcus braunii* which produces C_{27}, C_{29}, C_{31} and C_{33} dienes and a C_{29} triene [19]; the blue-green alga *Anacystis montana* which contains C_{19}–C_{29} monoenes with C_{27} preponderating [19]; and the green alga *Scenedesmus quadricauda* which contains a C_{27} monoene [17]. The isolation in the present work of C_{37} and C_{38} trienes is apparently without precedent.

The biosynthesis of alkenes by algae has not been extensively studied [10, 11, 20]. One reasonable mechanism is via decarboxylation of an unsaturated fatty acid containing one carbon atom more than the alkene. This process has been put forward to account for the presence of the *n*-C_{21}:6 alkene in many algae [10, 11, 20] despite the absence in some cases of the required 22:6 fatty acid precursor. This acid was not detected in the present study and no trace of the required long-chain unsaturated fatty acid precursors of the long-chain alkenes could be found. These results do not exclude fatty acid decarboxylation but it seems probable that additional mechanisms for alkene biosynthesis may be involved.

Ketones

In a previous paper [13] we reported the isolation from *E. huxleyi* of C_{37} and C_{38} alken-2-ones (**1**), and C_{38} and C_{39} alken-3 ones (**2**). In this paper we report additional data concerning the distribution of these compounds in morphologically distinct forms of the alga (Table 1), as well as comments on their biosynthesis. Data on double bond positions have not been determined, but recently de Leeuw *et al.* [15] determined these in three of the ketones isolated from an organic-rich Black Sea sediment known to contain many coccoliths of *E. huxleyi*. The structures presented were: heptaconta-15, 22-dien-2-one (**4**), octaconta-16,23-dien-3-one (**5**) and octaconta-9,16,23-trien-3-one (**6**).

Saturated alkan-2-ones are quite common in nature, occurring widely in soils and sediments [22], where they are thought to derive from microbial oxidation of *n*-alkanes. In higher plants, mid-chain ketones are often found and recent results [21] suggest that they may be synthesized by elongation of a suitable fatty acid precursor which is subsequently decarboxylated. Unsaturated ketones have not previously been isolated and this is thought to be due to the elongation system excluding unsaturated fatty acids [25].

The co-occurrence of C_{37} and C_{38} tri-unsaturated alkenes and alken-2-ones strongly suggests a biosynthetic relationship but it is noteworthy that the ratio of these chain-lengths is quite different in the two compound classes (Table 2). However, oxidation of the alkenes fails to account either for the presence of di-unsaturated ketones or C_{38} and C_{39} alken-3-ones. One possibility is that the alken-3-ones are formed from the alken-2-ones by addition of an extra carbon atom. This is consistent with the double bond data presented by de Leeuw *et al.* [15] (compare structures **4** and **5**). However, ratio analyses of selected components (Table 2) again show that the distributions, while similar, are not superimposable. Moreover, no C_{31} or C_{33} ketones were detected despite the abundance of the potential precursor alkenes (Table 1) and no long-chain secondary alcohols, which should be biosynthetic intermediates in the alkene to ketone conversion, could be detected. Further studies are required to elucidate the biosynthesis of these novel compounds.

Table 2. Selected ratios comparing alkene and ketone compositions at different growth stages of *E. huxleyi*

	Motile	Sessile	Coccolith
Alkene/ketone	0.10	0.22	0.06
Methyl ketones/ethyl ketones	1.9	1.3	1.3
Methyl ketones—TRI/DI*	2.2	2.6	2.2
Ethyl ketones—TRI/DI	2.2	2.4	1.5
Alkenes—37:3/38:3	9.6	9.5	7.4
Methyl ketones—37:3/38:3	3.6	8.1	3.8
Ethyl ketones—38:3/39:3	11.5	17.4	17.4

*TRI: Total tri-unsaturated components; DI: Total di-unsaturated components.

Changes in environmental conditions are well known to affect the lipid composition of microscopic algae. However, the possible effects of changes in cell morphology have not been examined. In this study we have produced three physiologically and morphologically distinct forms of *E. huxleyi* in a single culture and have harvested these at the same time, thus ensuring identical environmental conditions. An examination of the alkene and alkenone compositional data shown in Table 1 shows that both lipid classes are produced throughout the growth cycle and do not arise simply as a response by the organism to adverse environmental conditions. While there are variations in the proportions of individual components these are relatively minor and few systematic trends are apparent. The alkenones are markedly more abundant than the alkenes in each of the three samples (Table 2). The greater abundance of di-unsaturated ketones in the coccolith form may have geochemical significance since it is this form which is most likely to accumulate in marine sediments.

We have studied two other Prymnesiophyceae algae, *Hymenomonas carterae* (Braarud et Fagerl.) Braarud and *Crystallolithus hyalinus* Gaarder et Markali, using these capillary GLC and GC–MS techniques. Neither of these algae, however, was found to contain the alkenes or alkenones. These novel lipids thus offer a further chemotaxonomic discriminant between *E. huxleyi* and other Prymnesiophytes. A re-examination of other marine organisms is now required to test whether these unusual lipids might serve as a biological marker for an input of *E. huxleyi* to marine sediments.

EXPERIMENTAL

Algal cultures. An axenic culture of *Emiliania huxleyi* from the Plymouth Laboratory culture collection was grown in full-strength 'f/2' medium [23] at 15° under continuous aeration with filtered air. Cell counts, determined using a Coulter Counter, showed that the culture was actively growing, cell numbers increasing from 12.5×10^3 to 24.5×10^6 cells per ml over a period of thirteen days. At this stage the majority of cells were present as a sediment in the culture flask, the supernatant containing only 5.1×10^6 cells per ml. Examination of a sample of this supernatant under the microscope showed that the cells were actively motile and a total of 12.9×10^9 cells was removed by filtering 2.55 l. of the supernatant through a Whatman GF/C pad previously fired at 500° for 24 hr. The supernatant was then carefully decanted from the flask leaving the brownish white sediment. A known volume (1 l.) of sea water was then added to the flask and part of the sediment was suspended in this by gentle shaking. Examination of a sample of this material under a microscope showed that it contained sessile cells which had not yet formed coccoliths. Filtration of this suspension (1 l.) through a further Whatman GF/C pad gave a sample containing 9.83×10^9 of these sessile cells. The white sediment that remained in the culture flask was then treated with a further sample (340 ml) of sea water and eventually brought into suspension by prolonged, vigorous shaking. A sample of this material was examined under the microscope using a combination of bright field, polarised light and phase contrast and was found to contain cells that had formed coccoliths. A sample of 3.25×10^8 of these coccolithophorized cells was subsequently collected by filtering 340 ml of this white suspension through another Whatman GF/C pad.

Compound isolation and identification. Total lipids were extracted from the GF/C pads into CH_2Cl_2–MeOH (1:1, 200 ml) with sonication and saponified in aqueous KOH–MeOH (pH 12) under reflux [24]. Non-saponifiable lipids were extracted into hexane–Et_2O (9:1, 50 ml) and separated by TLC on Si gel into hydrocarbon and ketone fractions. These were analysed using splitless injection on a 20 m × 0.3 mm i.d. WCOT glass capillary column coated with OV-1. The oven temperature was programmed from 120° to 280° at 4°/min. using helium at 1.5 cm^{-3}/min as the carrier gas. FID and injector temperatures were 350°. Peak areas were measured by electronic integration. Alkenes were hydrogenated by dissolving an aliquot in EtOAc containing PtO_2 and passing H_2 through the suspension for three hours at 20°.

GC–MS used a quadrupole filter instrument operating at 350 μA, 40 eV electron energy and an ion-source temperature of 250°. The OV-1 WCOT column was interfaced directly to the ion source using glass-lined stainless steel tubing. Mass spectra were acquired at 1 sec intervals and processed using an on-line INCOS data system. Alkenes were identified from retention time, comparison with literature MS and hydrogenation data. Ketones were identified from MS, hydrogenation, and methoxime formation as described previously [13].

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