Phenyl- and Cyclopentylimino Derivatization for Double Bond Location in Unsaturated C₃₇-C₄₀ Alkenones by GC-MS

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A method for the identification of double bond locations in polyunsaturated long chain alkenones adapted to nanogram amounts as currently analyzed by gas chromatography coupled to mass spectrometry (GC-MS) has been developed. The method is based on interpretation of the electron impact mass spectra of the imino derivatives of the carbonyl groups using either cyclopentyl or phenyl substituents. Other complementary derivatization methods such as elaidization and hydrogenation have also been used for structural characterization of these compounds. This application has led to the identification of a novel homologous series of di-, tri-, and tetraunsaturated ketones with carbon number chain lengths between 37 and 40 in coastal hypersaline sediments. The novel series identified shows a distribution in which the double bond position between different homologs is established by reference to the distance from the carbonyl group whereas the previously known alkenones were constituted by unsaturated homologs with double bonds located at defined distances of the terminal methyl. This difference points out to a dissimilar, but still unknown, biogenic precursor of these novel alkenones. (J Am Soc Mass Spectrom 2004, 15, 1161–1172) © 2004 American Society for Mass Spectrometry

Linear methyl and ethyl C₃₂-C₄₀ ketones with 2, 3, or 4 double bonds and *trans* configuration, also known as alkenones, have been found in both recent and ancient sediments throughout the world oceans. These compounds are synthesized by few species of microalgae belonging to the class of Haptophyceae [1–4]. Cultures of these species showed that the proportion of di- and triunsaturated alkenones changed linearly with water temperature [5, 6]. This finding and the ubiquity of C₃₇-C₃₉ alkenones in recent and past marine sediments has led to the widespread use of the C₃₇ homologs for the estimation of sea surface temperatures in climate change studies [7–10]. This method has become, at present, a reference standard for the measurement of paleotemperatures in paleo-oceanography. Its success also depends on the unprecedented resistance of these compounds to diagenetic alteration which, despite changes in concentration, does not involve modification of the relative proportion of di- and triunsaturated homologs [11].

Another important aspect for the usefulness of this alkenone ratio is the limited number of alkenone biosynthesizer species within the Haptophyceae group which simplifies the biogeochemical transcription of

paleotemperatures into sedimentary alkenone composition. However, there are cases of discrepant alkenone distributions in which the sedimentary composition provides clearly unreliable paleotemperature estimates, e.g., lacustrine sediments [12]. In these cases, contributions from species different than those currently found in open marine environments are at the likely origin of the deviations.

Detailed structural characterization of the alkenones found both in marine and freshwater environments are needed for adequate assessment of differences in chemical composition that could reflect distinct algal precursors. In this respect, isomers encompassing dissimilarities in double bond location cannot be differentiated by the current gas chromatography-mass spectrometry (GC-MS) methods since they are not resolved chromatographically and cannot be differentiated by electron impact (EI) or chemical ionization mass spectra. Derivatization methods must therefore be implemented. Surprisingly, despite the widespread use of these compounds for paleoclimatic purposes very few studies have been devoted to chemical characterization. However, the new methods must be adapted to the limited amounts available in paleoceanographic studies.

The first research on the structural characterization of the alkenones addressed the location of double bonds by mass spectral interpretation of the trimethylsilyl ethers (TMSiE) after hydroxylation with osmium tetrox-

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Figure 1. (a) Total ion current trace showing the distribution of C_{37} – C_{40} alkenones found in the recent sediments of a hypersaline pond. (b) The same distribution after elaidization of the double bonds with *p*-toluenesulfonic acid. Asterisks denote peaks of all-trans isomers.

ide (OsO_4) [13]. The structures of 15,22-heptatriacontadien-2-one, 16,23-octatriacontadien-2-one, and 9,16,23-octatriacontatrien-2-one were determined with this method. The position of the carbonyl group was recognized by McLafferty type rearrangements m/z (M-58 and M-72). The EI mass spectra of the O-methyl and O-ethyl oximes and the TMSiE of the alkenols obtained by $NaBH_4$ alkenone reduction allowed further differentiation between the ethyl and methyl ketones [13, 14]. This derivatization approach was also used for double bond location in a novel series of monounsaturated ketones (C_{35} – C_{38}) [15].

The all-*trans* configuration of the naturally occurring alkenones was confirmed by the total synthesis of (E,E,E)-8,15,22-heptatriacontatrien-2-one and (E,E)-

15,22-heptatriacontadien-2-one [16]. Recent studies on sediments of the Black Sea (5000–8000 y BP) involving the combined use of GC coupled to infrared spectroscopy (GC-IR), nuclear magnetic resonance (NMR), direct insertion electron impact mass spectrometry (Probe-EIMS), and dimethyldisulphide (DMDS) derivatization allowed double bond location of (16E,21E)-hexatriacontadien-3-one [17]. This compound was the major constituent of an homologous series of long chain diunsaturated ketones (C_{35} – C_{39}), analogous to another series with chain lengths from C_{37} to C_{42} found in ancient sediments off the North Atlantic west coast (95 and 105 Ma, Cretaceous) [18].

Unfortunately, the above reported methods have low yields and require high sample amounts. In addition, the adducts generated involve major molecular weight increases with respect to the underivatized compounds. These two problems evidence the need for alternative procedures. The present study undertakes a new ap-

Figure 2. Mechanisms of fragmentation in N-substituted imines containing double bonds induced by a positive charge located in the nitrogen atom suggested for derivatives of long chain alcohols (nicotines [30]) and fatty acids (4,4-dimethyloxazolines [29] and picolinyl esters [26]). R_2 = cyclopentyl (Cp) or phenyl (Ph). R_1 = methyl (Me) or ethyl (Et). R represents the rest of the aliphatic chain towards the methyl end.

R_2 = Phenyl, Cyclopentyl

R_1 = Me, Et

R = Alkenyl (C_{34} – C_{36})

Figure 3. Scheme of reaction for the formation of phenyl and cyclopentyl imino derivatives of alkenones.

Figure 4. Mass spectra of the N-phenyl (17 ng) and N-cyclopentyl imines (5 ng) of 8E,15E,22E-nonatriacontadien-2-one.

proach involving imino derivatization of carbonyl groups, namely phenyl- and cyclopentylimino derivatives, and location of the unsaturated functionalities by interpretation of the resulting electron impact mass spectra. This approach allows the determination of the position of unsaturated functionalities with alkenone amounts at the nanogram level. The method has been applied successfully to the structural determination of naturally occurring alkenones in hypersaline environments. To the best of our knowledge this is the first time that the occurrence of such compounds is reported in these coastal sedimentary environments.

Experimental

Materials and Reagents

The following analysis grade solvents, dichloromethane (DCM), methanol (MeOH), hexane (Hex), isooctane (i-O), ethyl acetate (AcOEt), and toluene (Tol), as well as molecular sieves (4 Å), potassium hydroxide (KOH), silicagel 40 (70–230 mesh), aluminium oxide 90 (neutral, 70–230 mesh), and anhydrous sodium sulphate were purchased from Merck (Darmstadt, Germany). Anhydrous tetrahydrofuran (THF), aniline (99.5%), cyclopentylamine (99%), sodium p-toluenesulfonate hy-

Figure 5. McLafferty rearrangements leading to the formation of the N-cyclopentyl- ($R_2 = \text{Cp}$) and N-phenylimino ($R_2 = \text{Ph}$) derivatives of the methyl ($R_1 = \text{Me}$) and ethyl ($R_1 = \text{Et}$) alkenones.

drate, and 1,4-dioxane (99+%, ACS Reagent) were from Aldrich (Milwaukee, MI). The synthetic standards (E,E,E)-8,15,22-heptatriacontatrien-2-one and (E,E)-15,22-heptatriacontadien-2-one were generously provided by Professor J. R. Maxwell, University of Bristol, UK [15].

Four Å molecular sieves were heated overnight at 500 °C, and reactivated by heating at 120 °C for 1 h prior to use. Aliquots of THF, aniline, and cyclopentylamine were stored overnight in activated molecular sieves before use. Two mL vials screw-capped with a Teflon lined silicone septum were used for the derivatization reactions.

Sampling

Sediment samples were collected from a shallow carbonate precipitation pond with water salinity in the order of 70 g/L, at the La Trinitat salt works, Ebro Delta, NE Iberian Peninsula [19].

Extraction and Fractionation

Freeze dried sediments were Soxhlet extracted with DCM/MeOH (2:1) for 8 h. The extracts were hydro-

lyzed overnight at room temperature in a 10% wt/vol solution of KOH in MeOH. Extraction with Hex yielded a fraction enriched in neutral compounds which was subsequently fractionated on a hybrid column (330 × 9 mm i.d.) packed with 8 g of aluminium oxide on top and 8 g of silica gel at the bottom. Both adsorbents were dried overnight at 280 and 120 °C and partially deactivated with 5% water. Low polarity compounds (hydrocarbons) were first eluted with Hex (20 mL) and, subsequently, with Hex/DCM (90:10, 20 mL) and Hex/DCM (80:20, 40 mL). Then, the ketone-enriched fraction was eluted with Hex/DCM (25:75, 20 mL). This fraction was dried under nitrogen, dissolved in isooctane and stored at -20 °C.

Alkenone Derivatization

Imino derivatives were prepared from the previously dried standards (6–20 mg) or samples (100 mg) by addition of 50 mL of aniline or cyclopentylamine and 250 mL of THF. The solution was dried by addition of either 10–15 beads of molecular sieves, a 4 mm layer of anhydrous sodium sulphate or anhydrous calcium chloride. Air was purged with nitrogen or argon before vial capping and, after wrapping with thick aluminium foil, the vials were kept in an oven at 90 °C overnight. Then, they were cooled to room temperature and stored at -20 °C. Before GC-MS analysis, aliquots of the crude reaction mixtures were diluted in toluene.

Epoxidation of the unsaturated ketones was performed by addition of an excess of freshly prepared solution of 3,3-dimethyldioxirane (DMDO) in acetone (75 mM) to the alkenone fractions, stirring for 15 min and drying [20]. Elaidization (cis/trans isomerization) was performed by refluxing the alkenone fractions in dioxane with p-toluenesulfinic acid [21]. Unsaturated ketones were hydrogenated on platinum oxide in AcOEt.

Instrumental Analysis

GC-MS was performed with a Fisons MD800 GCMS (THERMO Instruments, Manchester, UK). A 30 m capillary column coated with 5% phenyl- 95% methylpolysiloxane (0.25 mm i.d. and 0.25 mm film thickness; HP-5, Hewlett Packard, CA) was used. The carrier gas was He at a flow of 2.1 mL/min. Injection port and transfer line temperatures were 300 °C. Samples were injected in toluene. Two oven temperature programs were used. One started at 90 °C (1 min), raised at 15 °C/min to 150 °C and then at 4 °C/min to 310 °C with a final holding time of 30 min. The other started at 90 °C (1 min) increased at a rate of 20 °C/min to 320 °C and the final holding time was 60 min. The quadrupole mass spectrometer was operated in EI mode (70 eV), scanning between *m/z* 50–700/s. Ion source temperature was 200 °C.

Figure 6. Series of even-electron ions observed in the mass spectra of the N-cyclopentyl- ($R_2 = \text{Cp}$) and N-phenylimino ($R_2 = \text{Ph}$) derivatives of the methyl ($R_1 = \text{Me}$) and ethyl ($R_1 = \text{Et}$) alkenones.

Figure 7. Suggested mechanisms in the vicinity of the double bonds remotely induced by the positive charge located on the N from the N-cyclopentyl and N-phenyl imino methyl and ethyl alkenone derivatives. (a–c): formation of intense even-electron ions. (d, e): formation of the fragments labelled as x , x' , and x'' , ... in Figures 4 and 9. The positions originally occupied by the double bonds are indicated (see text for details). The positive charge located on the N may induce a H-rearrangement leading to the formation of a conjugated double bond system. D.b.m. refer to double bond migration. R = alkyl chain towards methyl end. As an example, the N-phenylimine of 15E,22E-heptatriacontan-2-one (Me 37:2, R_1 = Me, R_2 = Phe, x = 15, x' = 22, y = 11) gives rise to the following mass fragments (m/z): (a): $[M]^{\bullet+}$ = 605, $[M-1]^+$ = 604, $[x+2]$ = 326; (b) $[x+3]$ = m/z 340; (c) $[x-2]$ = 272; (d) and (e) $[x]$ = 298.

Results and Discussion

Analysis of the alkenone fraction from La Trinitat salt works by GC-MS showed a distribution of methyl and ethyl ketones with chain lengths between C_{37} and C_{40} (Figure 1a). This distribution was characterized by the predominance of the C_{37} homolog group maximized at the triunsaturated alkenone and by having the tetraunsaturated alkenone as second C_{37} compound. Similar distributions have been found in a hypersaline lake from the Tibet plateau [22], an alkaline lake in Turkey [23], and some eutrophic lakes in England [12].

In the ketone extracts considered for the present study, the carbonyl position depends on chain length parity. That is, odd and even carbon number alkenones were methyl and ethyl ketones, respectively. No traces of even-chain methyl ketones or odd-chain ethyl ketones were observed. This composition is different from that currently found in marine environments which is

characterized by containing both methyl and ethyl C_{38} homologs.

Elaidization and Hydrogenation

The process of elaidization [21] induces the acid-catalyzed *cis/trans* equilibration of all the double bonds from a molecule, generating distributions similar to those arising from a thermodynamic process, with predominance of *trans*- over *cis*-configurations. This technique, as applied here, is useful in the two extreme cases generally found in natural products. In the most common example, the originally fatty acids (all-*cis*) distribution is transformed into another dominated by the non-natural all-*trans* isomers. Conversely, when applied to olefins with all-*trans* configuration, the original compounds dominate over the newly generated (all-*cis*).

Table 1. Mass fragments of the C₃₇–C₄₀ alkenones found in the sediments from La Trinitat hypersaline ponds

Ketone	Structure	Ketones			Phenylimines							Cyclopentylimines						
		M-R1 ^a ECL ^b			M-R1 ^a Mc ^c X x' x'' x''' ^d							M-R1 ^a Mc ^c x x' x'' x''' ^d						
		M	m/z	DB5	M	m/z	m/z	m/z	m/z	m/z	m/z	M	m/z	m/z	m/z	m/z	m/z	m/z
Me37:4	8E,15E,22E,29E	526	511	3866	601	586	133	200	296	392	488	593	578	125	192	288	384	480
Me37:3	8E,15E,22E	528	513	3883	603	588	133	200	296	392		595	580	125	192	288	384	
Me37:2	15E,22E	530	515	3892	605	590	133	298	394			597	582	125	290	386		
Et38:4	9E,16E,23E,30E	540	511	3958	615	586	147	214	310	406	502	607	578	139	206	302	398	494
Et38:3	9E,16E,23E	542	513	3973	617	588	147	214	310	406		609	580	139	206	302	398	
Et38:2	16E,23E	544	515	3986	619	590	147	312	408			611	582	139	304	400		
Me39:3	8E,15E,22E	556	541	4073	631	616	133	200	296	392		623	608	125	192	288	384	
Me39:2	15E,22E	558	543	4087	633	618	133	298	394			625	610	125	290	386		
Et40:3	9E,16E,23E	570	541	4165	645	616	147	242	338	434		637	608	139	234	330	426	
Et40:2	16E,23E	572	543	4179	647	618	147	312	408			639	610	139	304	400		

^aM-R1 fragment due to the α cleavage to the carbonyl group, R1 = Me or Et, see Figure 2.

^bECL = Effective chain length relative to the homologous series of n-alkanes = $100 \cdot n + (RT_k - RT_{(n)}) / (RT_{(n+1)} - RT_{(n)})$. RT_(n) and RT_(n+1) are the retention times of the linear alkanes preceding and succeeding the ketone (RT_k).

^cMc McLafferty type rearrangement.

^dx, x', x'', x''' indicate the relative position of the unsaturations.

Previous work based on total synthesis indicated that the naturally occurring alkenones have all-*trans* configuration [16]. Keeping in mind these previous results, comparison of the chromatographic profiles of the original and elaidized alkenone distributions of the present study (Figure 1) confirm the all-*trans* configuration. However, full elucidation of the *cis-trans* composition would require access to large amounts of these alkenones for infrared spectroscopy and nuclear magnetic resonance methods.

Catalytic hydrogenation of this alkenone fraction generated four chromatographically resolved peaks with retention indices corresponding to a straight chain. Their mass spectra were characterized by molecular ions of *m/z* 534, 548, 562, and 576, indicating straight chain C₃₇, C₃₈, C₃₉ and C₄₀ aliphatic ketones, respectively. The C₃₇ and C₃₉ homologs exhibited base fragments of *m/z* 59 (McLafferty) and less intense M⁺ – 15 ions, evidencing methyl ketone structures. On the other hand, the base fragments of *m/z* 73 (McLafferty) and the less intense M⁺ – 29 ions of the C₃₈ and C₄₀ homologs indicated ethyl ketones.

The combination of these two derivatization techniques shows, therefore, that the C₃₇–C₄₀ alkenone fraction of these hypersaline sediments under study is composed of straight chain C₃₇ and C₃₉ methyl ketones as well as C₃₈ and C₄₀ ethyl ketones with two to four unsaturations and all-*trans* configuration.

Double Bond Derivatization

First attempts of double bond location of these C₃₇–C₄₀ alkenones were assayed with direct epoxidation by reaction with dimethyldioxirane [20] or formation of oximes [13, 14] but none of these methods provided useful structural information, e.g., oxime derivatives provide information on the position of the carbonyl group but not on the location of the double bonds.

Other derivatization methods such as formation of methylthioethers involved major analytical difficulties [24] due to the high retention time increases of the derivatives which hindered feasible GC-MS analysis.

Imino Derivatization of C₃₇–C₄₀ Di-, Tri-, and Tetraunsaturated Alkenones

In view of the above mentioned difficulties, a new method for double bond location was developed by analogy to those described for fatty acids upon GC-MS analysis of 4,4-dimethyloxazolidines and picolinyl esters [25–28]. Double-bond derivatization methods encompassing N-containing adducts involve EI-MS in which the positive charge is predominantly located on the N atom due to the lower ionization potential of non-bonding electron pairs. The location of the charge in these atoms induces remote fragmentations with H rearrangement or cyclization and minimizes double bond scrambling. Schemes for these types of fragmentation are shown in Figure 2a and b. Hence, the MS obtained from the diverse N-derivatives of unsaturated fatty acids [29] and alcohols [30] are characterized by homologous series formed by fragments differing in 14 da (CH₂) corresponding to the saturated sections of the aliphatic chains. The positions of the unsaturations are indicated by increments of 26 da, and in some cases of 12 da, within the series which is continued with addition of further 14 da until the end of the chain or location of another unsaturation.

As an analogue derivative for the ketone groups, an imine functionality was devised, and both aromatic and aliphatic functionalities, such as phenylimines and cyclopentylimines, respectively, were chosen for the study. The reversibility of the reaction scheme (Figure 3) required complete water removal. Thus, several desiccants were tested, such as activated 4 Å molecular

Figure 8. Background subtracted mass spectra of the N-phenylimines of 15E,22E-heptatriacontadien-2-one prepared from a synthetic standard and the ketone fraction of a sediment extract from a hypersaline pond.

sieves, anhydrous sodium sulphate, and anhydrous calcium chloride. All three provided quantitative yields, however, calcium chloride was discarded because of the disaggregation particles generated. Among the other two, anhydrous sodium sulphate is recommended over the molecular sieves since the latter may eventually catalyze undesired chemical reactions. However, this phenomenon was not detected during the present study.

The small scale of the samples (μg) did not require large laboratory apparatus. Thus, the reactions were

performed in the same 2 mL vials as were used for initial GC-MS analysis. Derivatization of alkenone standards provided quantitative yields in the case of both phenyl- and cyclopentylimino derivatives when kept under a dry, inert atmosphere. Absence of quantitative loss was assumed in the absence of free alkenones in the derivatized mixtures which were analyzed without further fractionation. The method is highly reproducible as long as dry solvents and reagents are stored in activated molecular sieves and the derivatized fractions are analyzed within one day after transfer to vials.

Figure 9. Mass fragmentograms corresponding to the base peaks (McLafferty rearrangements) in the spectra of the original methyl (Me) and ethyl (Et) alkenone mixtures (a), (b) and their respective N-cyclopentylimino (MeCp and EtCp; (c), (d) and N-phenylimino (MePh and EtPh; (e), (f) derivatives found in one sediment extract from a hypersaline pond.

These derivatives involved longer retention times than the initial products but GC-MS analysis was still feasible (Figure 4).

Mass Spectra of the Imino Derivatives

The validity of the method was tested on synthetic standards of 8E,15E-heptatriacontadien-2-one and 8E,15E,22E-heptatriacontatrien-2-one. The MS of the imines obtained from the long chain alkenones and other unsaturated carbonyl-containing lipid molecules, e.g., fatty acids, share a characteristic feature of the dominance of the ions containing the N-functionality over the ions without the heteroatom (Figure 4). The base peaks at m/z 133 and m/z 147, for methyl- and ethylphenylimines, respectively, and m/z 125 and 139 for methyl- and ethylcyclopentylimines, respectively, represent fragments formed by β -cleavage after a

McLafferty type γ -H rearrangement, equivalent to the m/z 58 and 72 ions observed in underivatized methyl and ethyl ketones [31] (Figure 5).

In addition to these odd ions, the spectra exhibit homologous series of fragments starting at m/z 118 and 132 for the methyl- and ethylphenylimines, respectively, and m/z 110 and 124 for the ethyl- and methylcyclopentylimines, respectively (Figure 6). The consecutive fragments, whether open chain or cyclic, are separated by 14 da in the saturated sections of the aliphatic chains and are continued by increments of 26 da whenever an unsaturation is found. Increments of 12 da are also observed but maybe it is difficult to picture a direct cleavage of a double bond. In these MS some peaks of the homologous series, labelled as $x + 2$, $x' + 2$, ..., $x + 3$, $x' + 3$, ..., and $x - 2$, show a higher intensity induced by the vicinity of the double bond. Their higher intensity is thought to derive from the conjugated 1,3-dienes in the first two (Figure 7a and b) and to the formation of an allylic radical in the third (Figure 7c). The presence of some ions with higher intensity (labeled as x , x' , x'' , ...) enhanced by the vicinity of the double bond, suggest that a similar mechanism may account for fragments arising from cleavage of the chain in the positions originally occupied by the double bond after migration to closer positions ($x - 2$, $x - 3$) towards the proximal end of the molecule (Figure 7d and e). Double bond migration in this type of N-bearing compounds and EI conditions is minimized but still takes place. An example of this process can be found in the spectra of 2-alkenylbenzoxazole derivatives of long-chain fatty acids [32], where the unsaturation location involved reproducible migration of the double bonds towards proximal allylic positions.

These diagnostic ions can be successfully used to locate unsaturations even in cases of low sample amount. For example, interpretable mass spectra were obtained by injection of 1 ng of alkenone derivatives in the scan mode. The low ionization potential of the N non-bonding electrons may account for the relatively high intensity of the molecular ion $[M]^{++}$ (Figure 4), although an even higher intensity was detected for the deprotonated even-electron molecular ion $[M - H]^+$. A plausible mechanism for the formation of this ion could be devised as an alternative pathway to the one leading to the formation of the mentioned $x + 2$ (Figure 7a, pathway 1). On the other hand, the product of α -cleavage in the ethylimines $[M - C_2H_5]^+$ is of a much higher relative intensity, comparable to the molecular ion, than the equivalent fragment observed in the methyl ketones $[M - CH_3]^+$, likely due to the loss of a larger fragment.

The most relevant mass spectral differences between cyclopentyl- and phenyl-imines encompass the loss of the cyclopentyl group ($M - C_5H_9$), whereas an analogous fragment is not observed in the phenylimines attributable to the aromatic character of the N-substi-

Table 2. Summary of the C₃₇–C₄₀ alkenones identified in the present study together with those previously described in the literature

Ketone	Structure	Method ^a	Site ^b	Method ^a	Site ^b	Method ^a	Site ^b	Method ^a	Site ^b	Method ^a	Site ^b
Me35:2						GCMS	Go	GCMS	Black Sea		
Me35:1	18E					TMSiE	Go				
Me35:1	19E					TMSiE	Go				
Me35:1	20E					TMSiE	Go				
Me35:1	21E					TMSiE	Go				
Et36:2						GCMS	Go				
Et36:2	16E,21E							COM	Black Sea		
Et36:1	19E					TMSiE	Go				
Et36:1	21E					TMSiE	Go				
Et36:1	22E					TMSiE	Go				
Me37:4	8E,15E,22E,29E			S	Sed/Eh		Go	GCMS	Black Sea	Im	ST
Me37:3	8E,15E,22E			S	Sed/Eh	TMSiE	Go	GCMS	Black Sea	Im	ST
Me37:2	15E,22E	TMSiE	WR	S	Sed/Eh	TMSiE	Go	GCMS	Black Sea	Im	ST
Me37:1	20E					TMSiE	Eh, Go, Ig				
Et38:4	9E,16E,23E,30E									Im	ST
Me38:4	9E,16E,23E,30E							GCMS	Black Sea		
Et38:3	9E,16E,23E	TMSiE	WR			TMSiE	Eh, Go, Ig	GCMS	Black Sea	Im	ST
Me38:3	16E,23E					TMSiE	Eh, Go, Ig	GCMS	Black Sea		
Et38:2	16E,23E	TMSiE	WR			TMSiE	Eh, Go, Ig	GCMS	Black Sea	Im	ST
Et38:2								GCMS	Black Sea		
Me38:2	16E,23E					TMSiE	Eh, Go, Ig	GCMS	Black Sea		
Et38:1	21E					TMSiE	Eh, Go				
39:2								GCMS	Black Sea		
Et39:3	10E,17E,24E					TMSiE	Eh, Go	GCMS	Black Sea		
Me39:3	8E,15E,22E					TMSiE	Go, Ig			Im	ST
Et39:2	17E,24E					TMSiE	Eh, Go, Ig	GCMS	Black Sea		
Me39:2	15E,22E					TMSiE	Go, Ig			Im	ST
Me39:2								GCMS	Black Sea		
Et40:3	9E,16E,23E									Im	ST
Et40:2	16E,23E									Im	ST
Reference		de Leeuw et al. (13)	Rechka et al. (15)	Rontani et al. (16)		Xu et al. (17)				This Work	

^aMethods: S, total synthesis; GC-MS, Identification of carbonyl position and number of unsaturations; COM, combined NMR, GC-IR and DMDS derivatisation; Im, imino derivatization method of the present study.

^bSites: WR, Walvis Ridge; Sed/Eh, Atlantic sediments and *Emiliania huxleyi* cultures; Eh, *E. huxleyi*; Go, *Gephyrocapsa oceanica*; Ig, *Isochrysis galbana*; Black Sea sediments; ST, Hypersaline system considered in the present study.

tuent (Figure 4b). In addition, there is a relatively higher intensity of the fragments formed in the vicinity of the phenylimino group (m/z 118, 132, 146, and 160 in Figure 4) when compared with those of the cyclopentylimino derivatives (m/z 110, 124, 138, and 152 Figure 4). The spectra of ethylphenylimines of tri- and tetra-unsaturated ketones, exhibit two intense even peaks and one odd ion, m/z 200//214//215 (Table 1, Figure 4a). Similarly, the spectra of the methyl alkenone homologs exhibit the series m/z 186//200//201 at high intensity. The formation of these mass fragments may be induced by an interaction of the aromatic ring with the double bond at position 8 by analogy with the occurrence of fragment pairs at m/z 138//139 and 152//153 in 4,4-dimethyl oxazolines prepared from polyunsaturated fatty acids [25, 29]. A relatively higher intensity of these fragments was observed mainly in the derivatives with double bonds on carbons 4 and 5. This proximity to the N atom may have produced stable small-sized fragments similar to those in Figure 2a and b with $y = 3$ for Δ^4 and $y = 4$ for Δ^5 . At larger distance to the imino group (Δ^8 , $y = 6$, Figure 6), the aromatic nitrogen substituent may stabilize the above men-

tioned fragments, as observed in the case of phenylimines but not among cyclopentylimines.

Sediment Samples

The phenylimino derivatives of 15E,22E-heptatriacontadien-2-one from one standard and one sediment extract from the hypersaline system are compared in Figure 8. Shown in Figure 9 are the stacked GC mass chromatograms obtained by selection of the base peaks of the methyl and ethyl ketones in free form (m/z 58 and 72, Figures 9a and b), derivatized as cyclopentylimines (m/z 125 and 139, Figures 9c and d) and phenylimines (m/z 133 and 147, Figures 9e and f). In all cases, the high sensitivity of these base peaks indicate that the mixture of alkenones is constituted of C₃₇ and C₃₉ methyl ketones (odd-chain length) and C₃₈ and C₄₀ ethyl ketones (even chain length).

Joint evaluation of the MS of the initial C₃₇–C₄₀ alkenone mixtures and both cyclopentyl and phenylimino derivatives has allowed the description of the structural composition of all the homologs found in the hypersaline sediments of La Trinitat. Their struc-

Figure 10. Mass spectra of the N-phenyl and N-cyclopentyl imines of 6,10,14-trimethylpentadecan-2-one.

tural assignments, diagnostic ions, and the effective chain length values (ECL) relative to a homologous series of n-alkanes in a 5% phenyl-95% methyl polysiloxane (DB-5) capillary column are summarized in Table 1.

Both original mixture and derivatives provide similar chromatographic resolution which increase progressively from cyclopentylimines to phenylimines, 0.42 to 0.47 ratio units, respectively (Figure 9). The reaction does not involve significant yield differences according to number of unsaturations. Thus, the $U^{K'}_{37}$, $C_{37:2}/(C_{37:2} + C_{37:3})$ index, remains unchanged when comparing the underivatized ketones (0.27), the phenylimino (0.26), and cyclopentylimino (0.27) derivatives.

Previously Known Long Chain Alkenones

A joint description of the alkenones found in the present study and those previously described in the literature (sediments and algae) is shown in Table 2. Among all these compounds, only three have been synthesized [16] and only one has been isolated in sufficient amounts to perform NMR studies [17] or GC-IR. Three of the C_{37} alkenones identified in the present study show the same structure as those obtained by total synthesis [16]. The longer C_{38} and C_{39} homologs exhibit unsaturations at positions different to those reported in the literature (Table 2). All the alkenones identified in this hypersaline sediment have a

common feature in terms of double bond position (8,15,22,29 and 9,16,23,30 for the methyl and ethyl ketones respectively) since they all exhibit the same distance relative to the carbonyl group. Conversely, in previously reported open sea sediment samples, the location of the double bonds occurs at a constant distance from the terminal methyl (Table 2). This difference suggests a dissimilar organism as the possible source of these C₃₇–C₄₀ alkenones in the hypersaline environments studied. This unusual distribution is reported in the present study for the first time.

Imino Derivatization of Regular Isoprenoid Alkenones

The imino derivatives are also sensitive to methyl substituent positions. This is illustrated in Figure 10 for the cyclopentyl- and phenylimino derivatives of 6,10,14-trimethylpentadecan-2-one. This isoprenoid alkenone has also been found in the hypersaline sediments considered in the present study and originates from phytol degradation in recent environments [33–35]. The spectra show the homologous series of fragments at m/z 110 + 14 y and 118 + 14 y mentioned above, interrupted by increments of 28 da due to the presence of methyl substituents.

Conclusions

A novel and simple method for the location of double bond positions in C₃₇–C₄₀ alkenones in sample amounts as those currently handled in EI GC-MS, e.g., 1 ng, has been developed. The method is based on the interpretation of the EI MS after derivatization of the carbonyl group to phenyl- or cyclopentylimines. Application of this method to coastal hypersaline sediments has allowed the identification for the first time of novel long chain alkenones such as 9,16,23,30-octatriacontatetraen-3-one, 16,23-tetracontadien-3-one, and 9,16,23-tetratcontatrien-3-one. The use of double bond elaidization has shown that the configurations of the unsaturations were all-*trans*. The structures of these alkenones suggest a distinct biological origin from that previously described in marine algal cultures or open sea recent or ancient sediments.

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