



0016-7037(95)00281-9

C₃₂–C₃₆ polymethyl alkenes in Black Sea sedimentsJAAP S. SINNINGHE DAMSTÉ,^{1,2} ANNE-MARIE W. E. P. ERKES,³ W. IRENE C. RUPSTRA,¹
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(Received May 5, 1994; accepted in revised form September 7, 1994)

Abstract—A series of novel C₃₂–C₃₆ polymethylhentriacontenes has been identified in near-surface sediments of the Black Sea. Hydrogenation of these components indicated that they possess the 5,8,14,24,27-pentamethyl-, 8,14,24,27-tetramethyl-, 8,14,24-trimethyl-, 8,14-dimethyl-, and 14- and 15-methylhentriacontane C skeletons, which was confirmed by synthesis of an authentic standard of the C₃₄ member. The dominant C₃₆ member of this series contains eight double bonds. The ¹³C content of these polymethylhentriacontenes indicated that they are probably biosynthesized by photoautotrophs and that blooming or bicarbonate pumping affected the ¹³C content of the fixed C. Their structures suggest that they were biosynthesized by methylation of an unsaturated *n*-C₃₁ precursor at specific positions in a well-defined sequence. This biosynthetic pathway represents an alternative for the biosynthesis of isoprenoid-like components.

INTRODUCTION

Specific acyclic branched and isoprenoid hydrocarbons, in addition to hydrocarbons derived from steroids and triterpenoids, in sediments and crude oils have been widely used as indicators for the presence of specific organisms in palaeo-depositional environments. For example, the monomethylalkanes 7- and 8-methylheptadecane are markers for cyanobacteria (Han and Calvin, 1970), while specific long-chain dimethylalkanes (e.g., 3,9-, 9,13-, and 11,15-dimethylalkanes) may indicate contributions of insect waxes to sediments (Kenig et al., 1994).

The ubiquitous regular isoprenoids pristane and phytane are less specific markers since they can derive from a number of sources (e.g., Volkman and Maxwell, 1986; Goossens et al., 1984; Ten Haven et al., 1987). However, other isoprenoid hydrocarbons are highly characteristic of their sources. The C₂₅ regular isoprenoid, 2,6,10,14,18-pentamethylcosane (I), has been ascribed to halophilic bacteria (Waples et al., 1974), whereas the C₂₅ irregular isoprenoid, 2,6,10,15,19-pentamethylcosane (II), is thought to be derived from methanogenic bacteria (Brassell et al., 1981) although an algal source was recently suggested (Kohnen et al., 1992; Freeman et al., 1994). The C₄₀ regular isoprenoid, lycopane (III), is also probably derived from algae (McCaffrey et al., 1991; Kohnen et al., 1992; Wakeham et al., 1993; Freeman et al., 1994), whereas the C₄₀ irregular, head-to-head isoprenoid, ω,ω-bi-phytane (IV), is thought to originate from ether lipids of archaeobacteria (Moldowan and Seifert, 1979). C₂₀, C₂₅, and C₃₀ highly branched isoprenoid (HBI) alkanes (V–VII) and alkenes are widely distributed components (Rowland and Robson, 1990) of which the C₂₅ and C₃₀ members seem to be derived from diatoms (Nichols et al., 1988; Volkman et al., 1994; S. G. Wakeham, unpubl. results). The C₃₄ irregular isoprenoid, botryococcane (VIII), which in fact is not a real isoprenoid but is formed by four additional methylation reactions of a C₃₀ isoprenoid (Metzger et al., 1991), is specific

for contributions of the alga *Botryococcus braunii* race B (Moldowan and Seifert, 1980; Metzger et al., 1991).

Here we report the identification of a novel class of branched hydrocarbons, polymethylhentriacontenes, in surface sediments of the Black Sea and suggest their biomarker potential.

EXPERIMENTAL**Samples**

Sediments were collected in the central basin of the Black Sea during the 1988 expedition of the R/V Knorr. Unit I sediments were obtained from 30-cm box cores (BC 1, 43.5°N, 29°E; 170 m water depth; BC 10, 43°N, 34°E; 2200 m water depth). Unit IIa sediment was obtained from a 60-cm box core (BC 21, 43°N, 32°E; 2100 m water depth). Unit IIb sediments were taken from a giant gravity core (GGC 20; 43°N, 32°E; 2100 m depth).

Extraction and Fractionation

Samples were extracted and fractionated as described elsewhere (Wakeham et al., 1995). Briefly, samples were ultrasonically extracted with mixtures of dichloromethane (DCM) and methanol. An apolar fraction was isolated from the extract by column chromatography and was hydrogenated in ethyl acetate using as Adam's catalyst (PtO₂). The apolar fraction of sample BCI 5–10 cm was further fractionated by argentation thin layer chromatography on silica gel plates using hexane as the eluent. The bands were scraped off the TLC plate, extracted ultrasonically with ethyl acetate, and an aliquot of the polyene fraction (*R_f* = 0.18–0.58) was hydrogenated with PtO₂.

Gas Chromatography and Gas Chromatography-Mass Spectrometry

Gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) were performed as described elsewhere (Wakeham et al., 1995). Chemical ionization (CI) mass spectrometry was performed with isobutane as reagent gas.

Isotope Ratio Monitoring Gas Chromatography-Mass Spectrometry (irmGC-MS)

Compound-specific isotope analysis was performed using the irmGC-MS facility of Prof. J. M. Hayes (University of Indiana). Details have been described elsewhere (Hayes et al., 1990).

Synthesis

8,14,24-trimethylhentriacontane was synthesized using the sequence of reactions depicted in Fig. 1. 2-Nonanol was brominated with Br_2 /triphenylphosphine in refluxing THF for 3 h. 2-Bromononane (99% pure by GC) was isolated from the reaction mixture by column chromatography with 30% yield. 2-Nonyl magnesium bromide was prepared from 2-bromononane and Mg in refluxing THF (4 h) and coupled with 1,5-dibromopentane (THF, 50°C, 1 h) with LiCuBr_2 as a catalyst (Andringa et al., 1990). The resulting 1-bromo-6-methyltridecane was purified (99% by GC; overall yield 35%). The Grignard reagent of this bromide was prepared in refluxing diethylether (3 h) and was reacted with solid CO_2 to prepare 7-methyltetradecanoic acid. 1-Bromo-6-methyltridecane was also coupled with 2-lithiumthiophene (prepared by reaction of thiophene with *n*-BuLi in THF at 0°C) in THF (65°C, 20 h) using TMEDA as a catalyst. Both crude reaction mixtures and a small amount of dimethylformamide were dissolved in dichloromethane and, after cooling to -10°C, SnCl_4 was added. 2-(6-Methyltridecyl)-5-(1-oxo-7-methyltetradecyl)-thiophene was isolated from the reaction mixture by column chromatography (yield 72%). This component was methylated with MeLi in diethyl ether at 20°C for 2 h. The crude reaction mixture was dehydrated with triethylsilane in trifluoroacetic acid at 0°C using BF_3 etherate as a catalyst (Parnes et al., 1977) and pure 2-(2-(8-methylpentadecyl)-5-(6-methyltridecyl)-thiophene was isolated by column chromatography (yield 27%). This thiophene was desulphurized using Raney Ni (ethanol, reflux, 3.5 h) and the reaction mixture was hydrogenated with PtO_2/H_2 in ethyl acetate for 3.5 h. 8,14,24-Trimethylhentriacontane (90% pure by GC) was ob-

tained in good yield (86%). $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ (ppm relative to TMS): 0.84 (*d*, $J = 6.4$ Hz, 9 H, CH_3 at C-8, C-14 and C-24), 0.88 (*t*, $J = 6.9$ Hz, 6 H, H' 's at C-1 and C-31), 1.26 (broad, 55 H, H' 's at C-2-C-30). $^{13}\text{C-NMR}$ (100 MHz, CDCl_3) δ (ppm relative to TMS): 14.13 (C-1 and C-31), 19.74 (CH_3 at C-8, C-14 and C-24), 22.71 (C-2, C-30), 27.11 (C-6, C-10, C-12, C-16, C-22, C-26), 29.41 (C-4, C-28), 29.75, 30.02, 30.05 (C-5, C-17, C-18, C-19, C-20, C-21, C-27), 30.38 (C-11), 31.95 (C-3, C-29), 32.77 (C-8, C-14, C-24), 37.11 (C-7, C-9, C-13, C-15, C-23, C-25). The $^{13}\text{C-NMR}$ assignments were made using an attached proton test and by comparison with spectra of dimethylalkanes (Pomonis et al., 1989).

RESULTS AND DISCUSSION

Identification of polymethylhentriacontenes

A TLC fraction ($R_f = 0.18\text{--}0.58$) of the apolar fraction of the extract of a near-surface sediment of the Black Sea from the western part of the basin (BC1 5–10 cm) is dominated by one component (Fig. 2a). Its mass spectrum (Fig. 3a) indicates that it is a highly unsaturated hydrocarbon. No molecular ion was observed. GC-Cl-MS indicated a molecular weight of 490 daltons, although components with one unsaturation more and less are probably also present in smaller quantities. Hydrogenation (PtO_2, H_2) of this fraction afforded the hydrogenated counterpart of this component (IX, Fig. 2b). Its mass spectrum (Fig. 3b) reveals clearly that this component is a branched alkane. GC-MS with chemical ionization proved that its molecular weight is 506 daltons ($\text{C}_{36}\text{H}_{74}$) as suggested by the m/z 491 (M-15) in the EI mass spectrum. The enhanced intensities of m/z 85, 141, 239, 295, 393, and 449 relative to those in mass spectra of long-chain *n*-alkanes

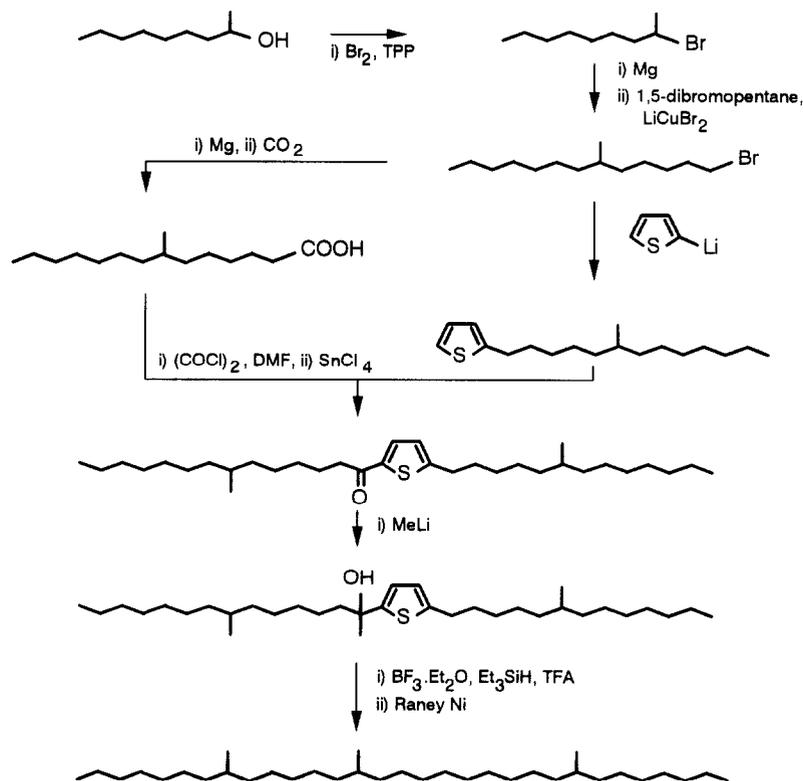


FIG. 1. Scheme for the synthesis of 8,14,24-trimethylhentriacontane.

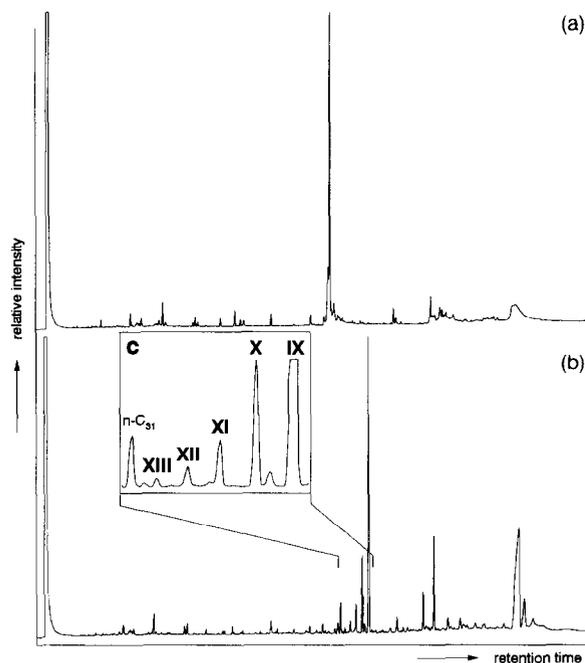


FIG. 2. Gas chromatograms of (a) the polyene fraction and (b) the hydrogenated polyene fraction of the apolar fraction of a surface sediment from the Black Sea (BC1 5–10 cm). Inset (c) shows enlargement of the region of interest. Roman numerals refer to structures listed in the Appendix.

suggested 5,8,14,24,27-pentamethyl-hentriacontane (IX) as the correct structure, although 5,8,14,17,23,26-hexamethyltriacontane could not be completely excluded. The smaller relative enhancement of m/z 239 and 295 argues, however, for the first possibility. Comparison of the measured pseudo Kovats retention index with the calculated value (Kissin et al., 1986) further supports this tentative identification (Table 1).

The hydrogenated fraction contains, in addition to the component described above, four related components (Fig. 2b; X–XIII). A similar fraction of composited apolar fractions of Black Sea surface sediments contained these four components in higher abundance relative to the C₃₆ component (Fig. 7 in Wakeham et al., 1995). Their EI (e.g., Figs. 3c–d) and CI mass spectra indicate that they are pseudo homologous with one to four fewer methyl groups. They were identified as 8,14,24,27-tetramethyl- (X), 8,14,24-trimethyl- (XI), 8,14-dimethyl- (XII), and a mixture of 14- (XIII) and 15-methylhentriacontane, respectively. Their measured pseudo Kovats indices are in good agreement with the calculated values (Table 1).

These tentative identifications were confirmed by synthesis of an authentic standard of the C₃₄ member (XI) of this pseudo homologous series (Fig. 1). The standard has a mass spectrum identical to that of the geological component and coelutes with the geological component on two different stationary phases (CP Sil-5 and DB-1701).

The acyclic C skeleton of the major component of the TLC fraction discussed above (Fig. 2a) is thus established. Since the molecular weight of this component is 490, the component must possess eight double bonds. Location of the double bond positions is not possible by mass spectrometry alone. However, the base peak at m/z 107 (C₈H₁₁; Fig. 3a) suggests that six of the eight double bonds are located at the terminal positions of the C skeleton. The indicated positions (see structure in Fig. 3a) would be consistent with formation of the major fragment ion at m/z 136 (C₁₀H₁₆) by a six-membered ring hydrogen transfer. The positions of the remaining two other double bonds are as yet unclear.

Analysis of hydrogenated apolar fractions of Black Sea sediments indicated the presence of polymethylhentriacontenes in sediments from Unit I and IIa but not in Unit IIb (Table 2).

Origin of Polymethylhentriacontenes

To the best of our knowledge the polymethylhentriacontenes have not been reported in either biota or sediments. Determination of the ¹³C content of the novel C skeletons indicated $\delta^{13}\text{C}$ values of -23.6 ± 0.3 , -20.9 ± 0.6 , and $-20.4 \pm 3.2\%$ PDB for 5,8,14,24,27-pentamethyl- (IX), 8,14,24,27-tetramethyl- (X), and 8,14,24-trimethyl-hentriacontane (XI), respectively. The indicated uncertainties are one standard deviation from the mean of duplicate measurements. The δ value for 8,14,24-trimethylhentriacontane can only be considered an estimate, in view of the large error caused by its relatively low concentration. The isotope values fall in a narrow range, consistent with an origin from one source organism as suggested by the strong structural similarities of these components. Comparison with other carbon isotopic values of lipids from surface sediments near the center of the basin (Freeman and Wakeham, 1992; Sinninghe Damsté et al., 1993; Freeman et al., 1994; Fig. 4) indicates that the polymethylhentriacontenes are, apart from isorenieratene (XIV) and C₂₅ HBI alkenes, the most ¹³C-enriched components.

Isorenieratene is isotopically heavy because it is a carotenoid pigment of photosynthetic green sulphur bacteria, which use the reversed tricarboxylic acid cycle to fix CO₂, leading to biomass anomalously enriched in ¹³C (Sirevag et al., 1977; Quandt et al., 1977; Summons and Powell, 1987; Sinninghe Damsté et al., 1993). The C₂₅ HBI alkenes are thought to be derived from diatoms (Nichols et al., 1988; Volkman et al., 1994; S. G. Wakeham, unpubl. results). They are enriched in ¹³C because of (1) localized reduction in [CO₂(aq)] during blooms, (2) bicarbonate pumping by the diatoms, or (3) both processes (Deuser, 1970; Fry and Wainright, 1991; Kohnen et al., 1992; Summons et al., 1993; Freeman et al., 1994; Schouten et al., 1994). Since the δ values of the polymethylhentriacontenes are in the range between those of the diatom-derived HBI alkenes and those of other lipids derived from phytoplankton (Fig. 4), an origin from phytoplankton seems likely. Since lipids are typically 3.5–4.0‰ depleted in ¹³C relative to total biomass (Hayes, 1993) and the weighted average carbon isotopic composition of the polymethylhentriacontenes is -23.1% , the ¹³C content of the biomass of their source organism can be estimated to be -19.5% . If this figure

* To emphasize structural relationships, the numbering of the positions of the methyl groups is not in accord with IUPAC rules.

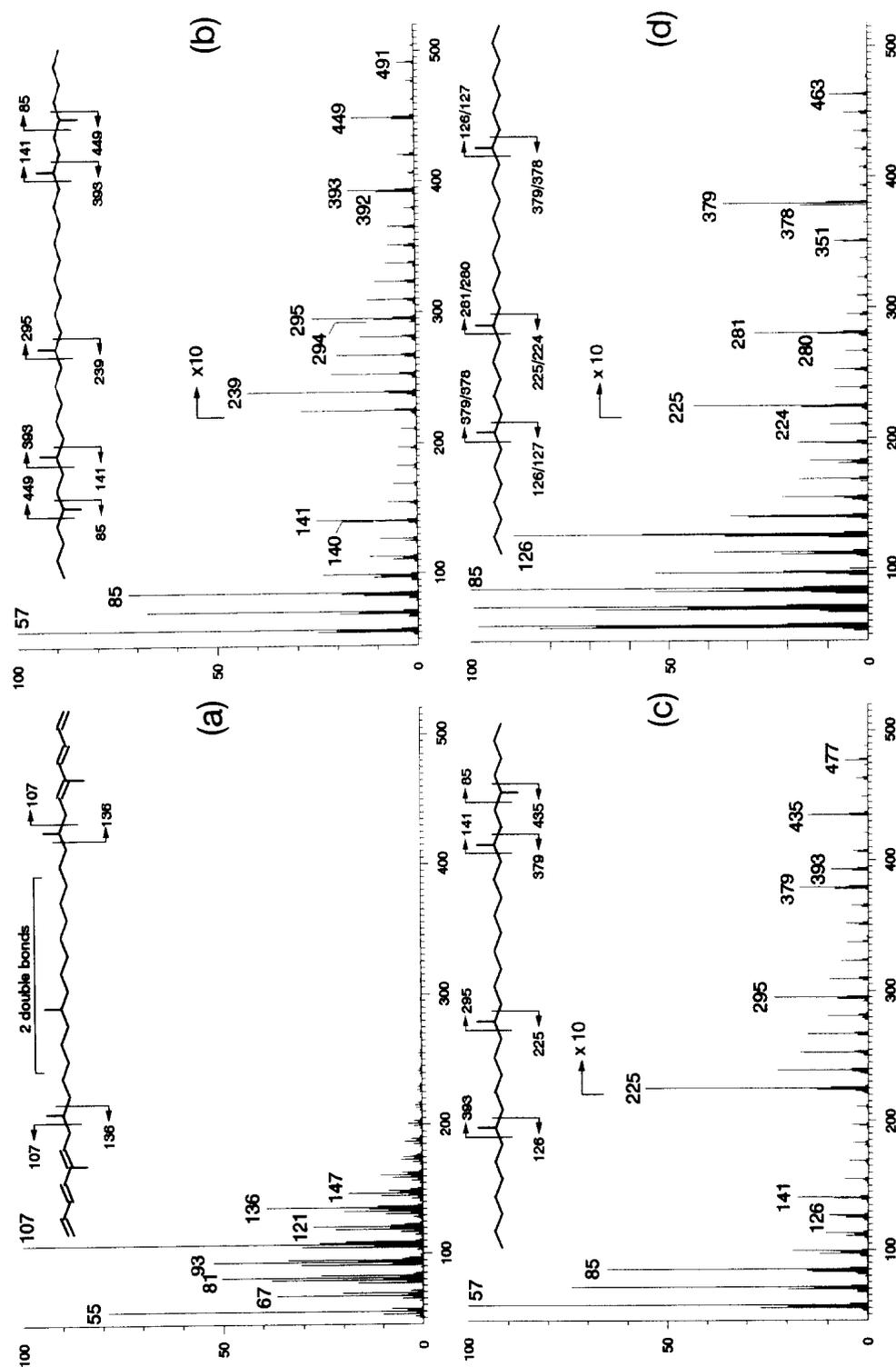


FIG. 3. Mass spectra (corrected for background) of (a) 5,8,14,24,27-pentamethylhentriacontane, (b) 5,8,14,24,27-pentamethylhentriacontane, (c) 5,8,14,24-trimethylhentriacontane, and (d) authentic 8,14,24-trimethylhentriacontane.

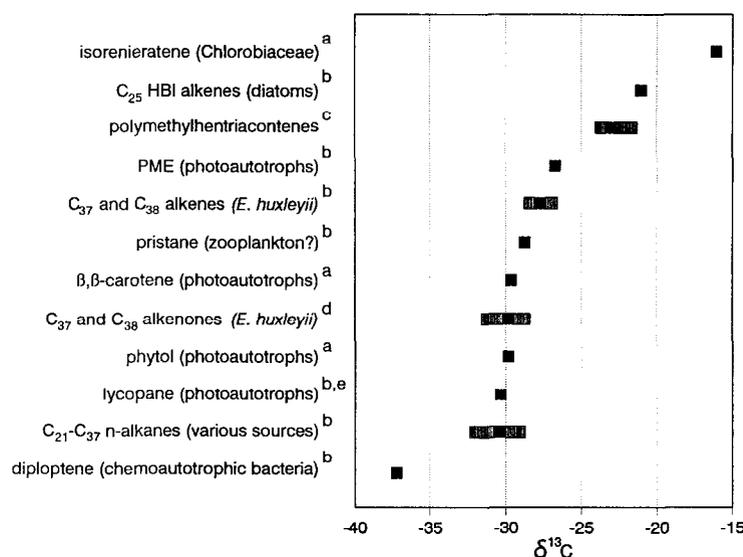


FIG. 4. Carbon isotopic compositions ($\delta^{13}\text{C}$ PDB) of selected lipids from surface sediments of the Black Sea. Data from: ^aSinninghe Damsté et al., 1993, ^bFreeman et al., 1994, ^cthis work, ^dFreeman and Wakeham, 1992, ^eWakeham et al., 1993. Sources of lipids are indicated in brackets. Values for isorenieratene, phytol and β,β -carotene were obtained from measurements of octahydroisorenieratene, phytane and β,β -carotane formed by desulphurization of polar fractions (Sinninghe Damsté et al., 1993).

is compared with calculated $\delta^{13}\text{C}$ values of autotrophs using the C_3 pathway of C fixation residing at different depths in the Black Sea water column (ca. -24 , -26.5 , -28 , and -37‰ at 5, 25, 35, and 50 m, respectively; Freeman et al., 1994), it follows that the biomass of the source organisms of the polymethylhentriacontenes falls outside this range. However, it should be emphasized that Freeman et al. (1994) based their biomass ^{13}C values on temperature, $[\text{CO}_2(\text{aq})]$ and DOC $\delta^{13}\text{C}$ data obtained at one station during Leg 3 of the RV Knorr expedition in 1988, data which represent only one moment during the annual cycle. These parameters may also change on an annual basis. However, the large difference (4.5‰) between the estimated ^{13}C content of the biomass and the calculated ^{13}C content of the biomass of autotrophs living at 5 m depth in the water column suggests that, as in case of diatoms, either blooming or bicarbonate pumping affected the isotopic composition of fixed C of the photoautotrophs bio-

synthesizing the polymethylhentriacontenes. Alternatively, given the number of reactive sites (i.e., the number of double bonds), the enriched ^{13}C content of the polymethylhentriacontenes might indicate that the analyzed material represents a fractionated residue due to an isotope effect associated with reactions occurring at double bonds (e.g., sulphurization, oxidation). Such an effect has been noted for free diaryl isoprenoids (Hartgers et al., 1994a,b). However, no reaction products of polymethylhentriacontenes have been identified in a detailed investigation of both low- and high-molecular-weight fractions of the organic matter in Black Sea sediments (Van Kaam-Peters, unpubl. results). Therefore, this latter explanation is deemed less likely.

Biosynthesis and Possible Biological Role of Polymethylhentriacontenes

The structures of the polymethylhentriacontenes suggest that they were biosynthesized by methylation of an unsaturated $n\text{-C}_{31}$ precursor at specific positions in a well-defined sequence. We may speculate that methylation started at position 14 (or 14') and proceeded via positions 8, 8', 5', and, ultimately, 5 (Fig. 5) indicating that the methylation is en-

Table 1. Calculated and experimental pseudo Kovats indices of several polymethylhentriacontenes.

component	calculated I ^a	experimental I ^b
5,8,14,24,27-pentamethylhentriacontane	3298 ^c	3293
8,14,24,27-tetramethylhentriacontane	3251 ^c	3249
8,14,24-trimethylhentriacontane	3205	3205
8,14-dimethylhentriacontane	3167	3168
14- and 15-methylhentriacontane	3129	3131

^a determined from the data of KISSIN et al. (1986)

^b CP Sil-5, 130° to 320°C at 4°C.min⁻¹

^c a correction factor of -3 was used to account for the interaction of the methyl groups at C-5' and C-8' and C-5 and C-8. This factor has been determined for 2,5-dimethyldecane (KISSIN et al., 1986) but should probably be -5 for the polymethylhentriacontenes.

Table 2. Occurrence of polymethylhentriacontenes in Black Sea sediments.

Sample	Approximate Age Range (y)	Occurrence
BC4 0-5 cm Unit I	0-300	✓
BC1 5-10 cm Unit I	200-500	✓
BC21 44-52 Unit IIa	3300-4400	✓
GGC20 17-22 Unit IIb	5200-6200	-

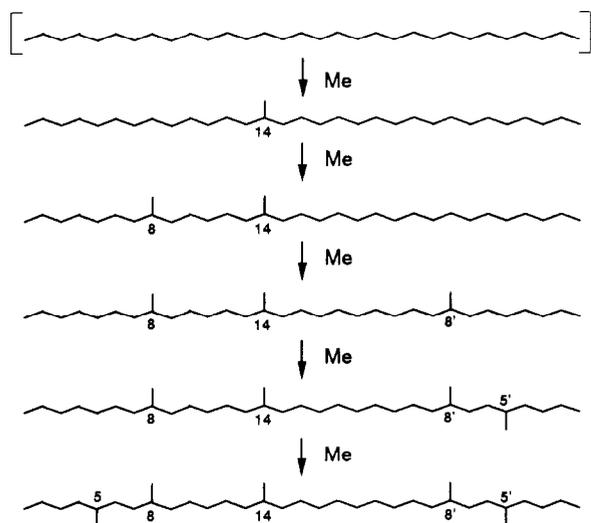


FIG. 5. Possible biosynthetic relationships between the carbon skeletons of the polymethylhentriacontenes. Note that double bonds are not indicated.

zymatically controlled. Notably, the proposed unsaturated n - C_{31} precursor is known to be a product of the abundant Black Sea alga *Emiliania huleyii* (Volkman et al., 1980) and is also present in the Black Sea water column and surface sediments (Wakeham et al., 1991), although the number of double bonds is less (≤ 3) than in 5,8,14,24,27-pentamethylhentriaconta-octaene (Wakeham et al., 1995). Furthermore, the isotopic compositions of C_{37} alkenes in the water column and surface sediments of the Black Sea differ from those of the polymethylhentriacontenes (Fig. 4; Freeman et al., 1994). Upon hydrogenation of the polyene fraction, some hentriacontane was formed, indicating that a polyene with a n - C_{31} skeleton is present in the sediments, but its $\delta^{13}C$ value (-32.2‰) indicates that it is probably not related to the polymethylhentriacontenes. Biosynthesis of branched hydrocarbons by methylation reactions is not unusual in photoautotrophs. For example, the cyanobacterium *Anabaena variabilis* appears to use methylation by S-adenosyl methionine in the synthesis of 7- and 8-methylheptadecanes (Fehler and Light, 1970). In the freshwater alga *Botryococcus braunii* race B, biosynthesis of C_{31} - C_{34} botryococcenes proceeds via methylations of a C_{30} precursor by S-adenosyl methionine (Metzger et al., 1987).

The structure of the end product of this series of methylation reactions, 5,8,14,24,27-pentamethylhentriaconta-octaene, is to some extent similar to that of isoprenoids. It possesses five methyl groups on an n - C_{31} chain. For comparison, squalene has six methyl groups on an n - C_{24} chain. Therefore, this sequence of methylation reactions may represent an alternative biosynthetic pathway for isoprenoid-like structures.

The biological role of the polymethylhentriacontenes is at present not clear but their molecular shape suggests that they may act as membrane rigidifiers. The degree of methylation (which varies according to our sedimentary data) may be a response to environmental conditions.

Acknowledgments—We thank the crew of R/V Knorr for assistance during sampling. Dr. B. Hay provided the unit 1 transition sapropel

and unit 2 sediments and helped in assigning sediment ages. Dr. I. King and Prof. J. M. Hayes are gratefully acknowledged for the determination of $\delta^{13}C$ values of the polymethylhentriacontenes and providing access to the irm-GC-MS instrument, respectively. Drs. J. M. Hayes and M. A. McCaffrey provided useful reviews. Mr. A. Sinninghe and Dr. J. A. Peters are acknowledged for NMR analysis. This work was partially supported by the Netherlands Organization for Scientific Research (NWO) through a PIONIER grant to JSSD and by the U.S. National Science Foundation (OCE-9022238 and OCE-9123420) and Office of Naval Research (N00014-90-J-1785) to SGW.

Editorial handling: J. T. Senftle

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APPENDIX

