Possible biosynthetic pathways for all *cis*-3,6,9,12,15,19,22,25,28-hentriacontanoaene in bacteria

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Abstract A very long chain polyunsaturated hydrocarbon, hentriacontanonaene (C31:9), was detected in an eicosapentaenoic acid (EPA)-producing marine bacterium, which was isolated from the mid-latitude seashore of Hokkaido, Japan, and was tentatively identified as mesophilic *Shewanella* sp. strain osh08 from 16S rRNA gene sequencing. The geometry and position of the double bonds in this compound were determined physicochemically to be all *cis* at positions 3, 6, 9, 12, 15, 19, 22, 25, and 28. Although C31:9 was detected in all of the seven EPA- or/and docosahexaenoic acid-producing bacteria tested, an EPA-deficient mutant (strain IK-1Δ8) of one of these bacteria had no C31:9. Strain

IK-1Δ8 had defects in the *pfaD* gene, one of the five *pfa* genes responsible for the biosynthesis of EPA. Although *Escherichia coli* DH5α does not produce EPA or DHA inherently, cells transformed with the *pfa* genes responsible for the biosynthesis of EPA and DHA produced EPA and DHA, respectively, but not C31:9. These results suggest that the Pfa protein complex is involved in the biosynthesis of C31:9 and that *pfa* genes must not be the only genes responsible for the formation of C31:9. In this report, we determined for the first time the molecular structure of the C31:9 and discuss the possible biosynthetic pathways of this compound.

Key words Decarboxylation · Eicosapentaenoic acid · Head-to-head condensation · Hentriacontanonaene · *pfa* genes · Polyunsaturated fatty acid · Polyunsaturated hydrocarbon · *Shewanella* sp.

Abbreviations

CI	Chemical ionization
CI-GC/MS	Chemical ionization-mass spectrometry
DHA	Docosahexaenoic acid
EI	Electron impact ionization
EI-GC/MS	Electron impact ionization-mass spectrometry

EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl ester
GC/MS	Gas chromatography-mass spectrometry
GLC	Gas-liquid chromatography
HPLC	High-performance liquid chromatography
PUHC	Polyunsaturated hydrocarbon
PUFA	Polyunsaturated fatty acid.

Introduction

A unique very long straight-chain polyunsaturated hydrocarbon (PUHC) with an odd number of carbon atoms, hentriacontanonaene (C31:9), was first discovered in marine Antarctic bacteria [1]. The same compound was reported in a deep-sea bacterium [2]. These C31:9-producing bacteria are psychrophilic and produce eicosapentaenoic acid (EPA) and/or docosahexaenoic acid (DHA) [1]. However, the molecular structure of C31:9 has not been determined, and the relationship between these *n*-3 polyunsaturated fatty acids (PUFAs) and C31:9 biosynthesis is not clear, although bacterial EPA and DHA are synthesized by the Pfa protein complex encoded by the *pfaA, pfaB, pfaC, pfaD*, and *pfaE* genes [3].

Another straight-chain PUHC with an odd number of carbons, all *cis*-3, 6, 9, 12, 15, 18-heneicosahexaene (C21:6), is distributed in marine planktonic algae and animals [4, 5]. The biosynthesis of C21:6 may occur through the decarboxylation of DHA [4] because the marine organisms possessing C21:6 contain DHA [4, 5] as well as the hydrocarbon derived from DHA (Δ4, 7, 10, 13, 16, 19) by decarboxylation. C21:6 from DHA should have double bonds at 3, 6, 9, 12, 15, and 18. Eukaryotes have a decarboxylation pathway to synthesize odd-carbon-number alkanes from even-carbon-number counterpart fatty acids via fatty aldehyde formation [6]. However, no

biochemical or molecular evidence on the biosynthesis of C21:6 is available, and information about C31:9, which is recognized as a biomarker of Antarctic bacteria [1], is limited.

We isolated an EPA-producing bacterium from the surface of seaweed collected at the mid-latitude seashore of Hokkaido, Japan. This isolate (designated strain osh08) included, in addition to low levels of EPA, a hydrocarbon suspected to be C31:9. In this study, we describe the physicochemical determination of the molecular structure of this compound including the position and geometry of the double bonds. We investigated the distribution of C31:9 using various types of marine and nonmarine microorganisms, including EPA- or DHA-producing bacteria and eukaryotes, a genetically engineered EPA-deficient mutant of *Shewanella* species, and *Escherichia coli* that had been transformed with the *pfa* genes responsible for the biosynthesis of EPA or DHA. We discuss the possible biosynthetic pathways of C31:9 with regard to the distribution of EPA, DHA, C31:9, and *pfa* genes in bacteria.

Experimental Procedures

Strains and Culture Conditions of Bacteria and Eukaryotes

Bacterial strains isolated from seaweed samples that had been collected at the mid-latitude seashore of Oshoro, Hokkaido, Japan (43.19 N, 141.00 E) were screened for their ability to produce long-chain

PUFAs such as EPA and DHA in moderate-temperature environments. The seaweed fragments (approximately 2 × 3 cm) soaked in seawater were vortexed. Portions (200 μL) of the seaweed sample were spread on the plates containing ZoBell agar medium (1 g/L peptone, 1 g/L yeast extract, 0.1 g/L $Fe_3(PO_4)_2$, and 0.15 g/L agar in 50% (by vol) seawater; [7]). Sea water used in this study was natural seawater that had been filtrated using filter paper (type No.02, Advantec, Tokyo, Japan). Plates were then incubated at 20 °C for several days. Bacterial colonies were subjected to methanolysis using 10% (by vol) acetyl chloride in methanol, as described below. One strain (osh08) capable of producing EPA was isolated, purified by repeated streaking of cells on the agar plates, and utilized for further investigations.

Strain osh08 was cultivated in the ZoBell liquid medium with shaking at 180 rpm at 25 °C for 1 day, unless otherwise stated. To investigate effects of temperature on growth and production of C31:9, strain osh08 was cultivated at 0, 4, 15, 20, 25, 30, 37, 40, and 43 °C.

The following strains were used: *Colwellia maris* ABE-1^T [8], *Moritella marina* MP-1^T (ATCC15381^T), *Shewanella marinintestina* IK-1^T [9], *Shewanella pneumatophori* SCRC-2738^T [10], *Shewanella benthica* (ATCC43992), *Pseudomonas alcaliphila* AL15-21^T [11] as marine bacteria; *Pseudomonas psychrophila* E-3^T [12], *Vibrio rumoiensis* S-1^T [13], *Shewanella oneidensis* MR-1^T (ATCC 700550^T), *Pseudomonas aeruginosa* WatG [14], *Rhodococcus erythropolis* (laboratory strain isolated from soil), *E. coli* DH5α (Takara Bio, Tokyo, Japan), and

Stenotrophomonas maltophilia (laboratory strain from soil) as nonmarine bacteria; a thraustochytrid-like microorganism strain 12B [15] and *Schizochitrium limacinum* SR21 [16] as marine eukaryotes; and a terrestrial eukaryote, *Saccharomyces cerevisiae* (baker's yeast). Nonmarine and marine bacteria were grown in Luria-Bertani (LB) medium and LB medium containing 30 g/L NaCl, respectively. Strain 12B, *S. limacinum* SR21, and yeast were grown in F medium, as described previously [15].

Extraction and Analysis of Lipids

Hydrocarbons of osh08 were first extracted with hexane in the fatty acid methyl ester (FAME) fraction after methanolysis of dry cells (approximately 15 mg) using 1 mL of 10% (by vol) acetyl chloride in methanol at 100 °C for 1 h. A mixture of 10 μg each of heneicosanoic acid (21:0, Sigma-Aldrich, Tokyo) and *n*-tetracosane (C24:0, Sigma-Aldrich) was used as an internal standard. To avoid coextraction of FAMEs, hydrocarbons were directly extracted from dry cells (approximately 10 mg) with 20 mL of a mixture of methanol/hexane (1:1, by vol) after sonic oscillation at 70 watts for 10 sec in an ice bath using a sonic disruptor (model, W-185; Branson, Danbury, CT). This procedure was repeated three times. The combined hexane extracts were used as crude hydrocarbon fraction.

The crude hydrocarbon fraction was applied to thin-layer chromatography (TLC) on silica gel plates (type 5721, Merck, Darmstadt, Germany) using a mixture of hexane/ethyl ether/acetic acid (90:10:1, by vol) as solvent. Squalene (Kanto Chemical, Tokyo), and squalane (Tokyo Kasei Kogyo, Tokyo), were used as standards.

Purification of C31:9 was performed by reversed-phase high-performance liquid chromatography (HPLC). Crude hydrocarbon extracts (1.5 mg aliquots) were dissolved in 1 mL of acetonitrile. The solution was filtered through a Millex-HV filter (pore size 0.45 μm, Millipore, Billerica, MA). A 300 μL aliquot of the filtrate was subjected to HPLC on a liquid chromatography system (model CCMP, Tosoh, Tokyo) equipped with an ODS column (type TSKgel ODS-80Ts, 15 cm long, 2.0 mm I.D., Tosoh) and an ultraviolet detector (model UV-8010, Tosoh), which had been equilibrated with acetonitrile. C31:9 was eluted with the same solvent at a flow rate of 1.0 mL/min, and absorbance at 210 nm of the eluate was monitored. The retention time of C31:9 was around 16.2 min. C31:9 with a purity of more than 99%, which was evaluated by gas-liquid chromatography (GLC; see below), was subjected to further analysis.

The *cis* and *trans* configurations of double bonds of C31:9 were determined using HPLC-purified hydrocarbon fraction (approximately 500 μg) by infrared spectrometry (IR; model FT/IR 660 Plus, Jasco, Tokyo) by the potassium bromide method. Assignment of individual peaks of C31:9 in IR was referred to [17–19].

The purified hydrocarbon fraction dissolved in hexane at 3.5 μg/mL was fully hydrogenated using H_2 gas at room temperature for 40 min. Platinum(IV) oxide at 5 mg/mL was used as a catalyst. Hydrogenated hydrocarbon was extracted with hexane. Partial hydrogenation was performed using hydrazine [20]. The hydrocarbon fraction (approximately 20 μg) was dissolved in 1 mL methanol and then mixed with 0.1 mL hydrazine and 0.1 mL 30% H_2O_2 (by vol). The reaction mixture was stirred for 90 min at 50 °C. To stop the reaction, 1 mL 5 M HCl was added, and the mixture was extracted with hexane. The extract was applied to silver nitrate TLC on silica gel (no 5721, Merck), as described previously [21] with a solvent system composed of hexane/benzene (1:1, by vol). Silica gels corresponding to spots whose mobility was close to that of authentic 1-dodecene (C12:1, Sigma-Aldrich) were scraped off the plate and extracted with a mixture of methanol/water/hexane (1:1:2, by vol). A hexane fraction containing hydrocarbons with one double bond was recovered. The location of double bonds in each monounsaturated hydrocarbon was determined by chemical ionization (CI) ion-trap mass spectrometry using acetonitorile as a regent gas (see below and [22–24]).

The FAME fraction including hydrocarbon, crude and purified hydrocarbons, and hydrogenated hydrocarbons were analyzed by GLC as described [25] on a gas chromatograph (model GC-353B, GL Sciences, Tokyo) equipped with a polar capillary column (type BPX70, 25 m long, 0.22 mm I.D., 0.25 μm film thickness, SGE Japan, Yokohama, Japan) unless otherwise stated, and flame ionization

detection with nitrogen as the carrier gas. The GLC oven temperature was 80 °C initially and programmed up to 240 °C at a rate of 4.0 °C/min. The oven temperature was held at the maximum for 60 min. The injector and detector temperatures were set at 221 °C. The data were analyzed using a D-2500 Chromato-Integrator (Hitachi, Tokyo). In order to detect PUFAs and PUHCs with very long retention times, GLC analysis was also performed using a nonpolar capillary column (type TC-1, 30 m long, 0.25 mm I.D., 0.1 μm film thickness) obtained from GL Science. The injector and detector temperatures were set at 250 °C and 340 °C, respectively. The oven temperature was first set at 50 °C, and then raised to 330 °C at the rate of 10 ºC/min. The maximum oven temperature was held for 10 min. Triacontanoic acid methyl ester (30:0) and tetratriacontane (C34:0) from Sigma-Aldrich were used as internal standards.

Relative fatty acid and hydrocarbon composition was expressed as weight percentage of the total combined weight of these compounds. The hydrocarbons and FAMEs were also analyzed by ion-trap mode gas chromatography-mass spectrometry (GC/MS) on a Varian system (model CP-3800 gas chromatograph and Saturn 2200 ion trap mass spectrometer, Varian Technologies Japan, Tokyo) under the same conditions as described previously [25]. Data were analyzed using a Saturn™ Software Workstation Version 5.52. In GC/MS analysis, two modes of electron impact ionization (EI) and CI were utilized and abbreviated as EI-GC/MS and CI-GC/MS, respectively. In CI-GC/MS, acetonitrile was used as an ionization reagent.

FAMEs were identified by comparing their retention times with those of authentic standards in GLC and by their EI-GC/MS analysis. To determine the position of double bonds of monounsaturated fatty acids, the FAME fraction was subjected to the I_2-catalyzed reaction for the formation of adducts with dimethyl sulfide according to the procedure of Shibahara et al. [26] The resultant dimethyl disulfide adducts were analyzed by EI-GC/MS, as described above. The position of double bonds of EPA was determined by EI-GC/MS analysis of its pyrrolidide derivative prepared, as previously [27].

DNA Procedures

For PCR amplification of 16S rRNA genes, osh08 cells were cultivated as described above. Genomic DNA was isolated by the method of Marmur [28]. PCR was performed using a Mastercycler® ep gradient Thermal Cycler (Eppendorf AG, Hamburg, Germany) in a total volume of 50 μL using *Ex Taq* DNA polymerase (Takara Bio, Tokyo, Japan) in the supplied buffer. For the amplification of the full length of 16S rRNA genes, primers 9F (5′–GAGTTTGATCCTGGCTCAG–3′) and 1541R (5′–AAGGAGGTGATCCAGCC–3′) were used. PCR was carried out according to the following program, an initial denaturation at 95 °C for 5 min followed by 30 cycles of 1 min at 95 °C, 1 min at 57 °C, and 2 min at 72 °C, concluding with a 5 min extension at 72 °C for the amplification of the 16S

rRNA gene. PCR products were analyzed by electrophoresis on 10 g/L agarose gels and were visualized by a UV transilluminator after staining with ethidium bromide (Nippon Gene, Tokyo).

PCR products that had been ligated to the pCR2.1-TOPO® vector using the TOPO TA cloning® Kit (Invitrogen) were subjected to cycle sequencing according to the manufacturer's protocol. Recombinant vectors were used to transform *E. coli* DH5α and transformants were selected by blue/white colony screening. Individual white colonies were grown at 37 °C overnight with rotary shaking in LB medium. Plasmid pCR2.1-TOPO carrying 16S rRNA gene from osh08 was isolated with the mini-preparation method [29]. Cycle sequencing was performed as described previously [30]. The sequence comparative searches were performed using the NCBI (http://www.ncbi.nlm.nih.gov/) databases. The osh08 16S rRNA gene sequence has been deposited in DDBJ/GenBank/EMBL with the accession number of AB447987.

Results

Extraction and Cellular Contents of Polyunsaturated Hydrocarbons and Fatty Acids

The compound suspected to be a very long chain hydrocarbon with multiple double bonds was extracted with hexane in the FAME fraction after methanolysis of osh08 cells grown at 25 °C. As

described below, this compound was identified as a PUHC with 31 carbons and 9 double bonds (hentriacontanonaene; C31:9). Δ9- Hexadecenoic acid (28.8% ± 0.7%) and hexadecanoic acid (14.5% ± 1.0%) were the major fatty acids and pentadecenoic, hexadecenoic, heptadecenoic and octadecenoic acids included two isomers with a double bond at different positions (Table 1). C31:9 was the sole hydrocarbon detected in the fraction. The contents of C31:9 and EPA (20:5) were 3.0% ± 0.6% and 0.5% ± 0.2%, respectively.

C31:9 was extracted directly from dry cells in the hexane fraction with a mixture of hexane and methanol (1:1, by vol). The content of this compound was 0.63 ± 0.02 μg/mg of dry cells for cells grown at 20 °C for 18 h (3.15 μg/mL of culture). The content of C31:9 after methanolysis was calculated to be 0.49 ± 0.01 μg/mg of dry cells (2.45 μg/mL of culture) for cells grown under the same conditions.

Effects of Temperature on the Growth of Strain osh08 and Content of EPA and C31:9

The osh08 cell grew in the temperature range of 4 °C to 40 °C, and the optimum growth occurred at 30 °C. No growth was observed at 0 °C or 43 °C. According to Wiegel's definition [31], this type of strain should be regarded as mesophilic bacteria that are tolerant to a temperature around 5 °C rather than as psychrotrophs, which can grow at 0 °C.

Table 2 shows the contents of C31:9 and EPA in the total FAME fraction including hydrocarbons after methanolysis of osh08 cells grown at various temperatures. The maximum production of C31:9 and EPA was at 25 °C and 4 °C, respectively. It is interesting that the content of C31:9 and EPA changed in an antiparallel mode with regard to their biosynthetic pathways (see below). To our knowledge, the highest temperature at which EPA is produced in bacteria is 28 °C for *Shewanella japonica* [32] and *S. pacifica* [33]. Although EPA comprises around 0.2% of total fatty acids, osh08 produced EPA at 37 °C, and the maximum temperature at which C31:9 was produced was also 37 ºC (Table 2).

Physicochemical Analysis of C31:9

In EI-gas chromatographic analysis of the FAME fraction including hydrocarbons, C31:9 occurred at a retention time of about 34 min (Fig. 1a), and no peak was detected after C31:9. The mass spectrum of C31:9 in EI-GC/MS was similar to that of the methyl esters of PUFAs such as arachidonic acid, EPA, and DHA, where fragment ions at an m/z of 79 and 91 were dominant (Fig. 2a), suggesting that the hydrocarbon contained multiple methylene-interrupted double bonds in the straight aliphatic chain. In CI/MS analysis of the C31:9 peak, an ion of $[M+54+H]^+$ at m/z 473 was prominent (Fig. 2b). The molecular weight of H_2-hydrogenated C31:9 (C31:0), whose retention time was around 30 min in an EI-gas chromatogram (Fig. 1b), was determined to be 436 by the occurrence of an $[M+40+H]^+$ ion

at m/z 477 in CI/MS (Fig. 2c). These results indicate that the hydrocarbon comprises 31 carbon atoms with 9 double bonds (C31:9, molecular weight, 418).

In GLC analysis of the total FAME fraction using a nonpolar capillary column, EPA and C31:9 appeared around 22 min and 28.5 min, respectively, and the retention times of 30:0 and C34:0 used as internal standards were about 26 min and 34 min, respectively. However, no peaks suspected to be 32:9 fatty acid were detected (data not shown).

The EI-gas chromatogram of partially hydrogenated hydrocarbons gave five peaks (peaks 1, 2, 3, 4, and 5 in Fig. 3) with the same molecular weight of 434, suggesting that the fraction was a mixture of hydrocarbons with one double bond at different positions.

The CI-mass spectrum of one isome (peak 1 in Fig. 3) was shown in Fig. 4a, where fragment ions at m/z 264 (a) and 278 (b) were evident. This result indicates the location of a double bond at 15 (or 16 when counted from the opposite end). Peak 2 was that of an isomer having a double bond at 12 or 19, because fragment ions at m/z 222 (a) and 320 (b) were detected (Fig. 4b). Peaks 3, 4, and 5 were determined to be isomers having double bonds at 9 or 22, 6 or 25, and 3 or 28, respectively (Figs. 4c to 4e). The results are summarized together with the relative intensity of each diagnostic ion in Table 3. The double bonds at 3, 6, 9, and 12 are equivalent to the locations at 28, 25, 22, and 19, respectively.

The infrared spectrum of HPLC-purified C31:9 is shown in Fig. 5. There were intense peaks at 2,963 and 2,855 cm^{-1} corresponding to C-H stretching of CH_3 groups, intense peaks at 2,921 and 2,360 cm^{-1} corresponding to C-H stretching of CH_2 groups, and peaks at 1,457 and 1,337 cm^{-1} corresponding to C-CH_2 and C-CH_3 groups, respectively [18]. A peak at 1,391 cm^{-1} corresponded to CH bending of the HC=CH *cis* double bond [19]. However, there was no peak at 970 cm^{-1} corresponding to CH bending of the HC=CH *trans* double bond. The intense peaks around 3,015 cm^{-1} and 1,670 cm^{-1} are regarded as those corresponding to CH stretching and C=C stretching, respectively, of the HC=CH *cis* double bond [17, 19]. The relatively intense peak at 1,731 cm^{-1} is considered to correspond to C=O and C-O stretching of carboxylate ester of the contaminating fatty acid methyl esters [17, 19]. Considering all these data together, we identified the compound as all *cis*-3, 6, 9, 12, 15, 19, 22, 25, 28-hentriacontanonaene (C31:9)(Fig. 6). The position and geometry of double bonds of C31:9 were determined for the first time in this study.

Distribution of C31:9 in Various Microorganisms and its Possible Biosynthetic Pathways

Table 4 shows the distribution of EPA, DHA, C31:9, and *pfa* genes in bacteria and some eukaryotic microorganisms. C31:9 was found in all tested marine bacteria that produce EPA or DHA, as also found by Nichols *et al*. [1]. The *pfa* genes responsible for the biosynthesis of EPA and DHA have

been cloned from *S. pneumatophori* SCRC-2738, *S. marinintestina* IK-1, and *M. marina* MP-1 [3]. *Shewanella oneidensis* MR-1^T grown at 20 °C for 18 h contained both EPA and C31:9 at 0.2 ± 0.1% and 2.0 ± 0.7%, respectively, of the total FAME and hydrocarbon fraction, although this strain, which was isolated from sediments of a freshwater lake, does not require NaCl for growth [34] and is recognized as mesophilic [35]. The chromosome of this bacterium (AE014299; see [36]) included *pfa* genes (Table 4). However, the marine bacterium *P. alcaliphila* AL15-21^T and nonmarine yeast produced neither PUFA nor C31:9. Two marine eukaryotes, which are known to produce very high levels of DHA and other PUFAs [15, 16], produced no C31:9. These eukaryotic microorganisms are expected to have *pfa*-like genes that are responsible for the biosynthesis of PUFAs because such genes have been cloned from *Schizochytrium* sp. ([37] and see accession numbers AF378327, AF378328, and AF378329). Although *E. coli* DH5α does not inherently produce EPA or DHA, the cells transformed with the *pfa* genes responsible for the biosynthesis of EPA and DHA produced EPA and DHA, respectively, but not C31:9 (Table 4 and see [3]). In contrast, the EPA-deficient mutant (strain IK-1Δ8) of *S. marinintestina* IK-1, in which one of the five *pfa* genes, *pfaD* gene, responsible for biosynthesis of EPA is defective [38], produced neither EPA nor C31:9. These results suggest that five *pfa* genes are involved in the biosynthesis of C31:9. However, *pfa* genes must not be the only genes responsible for the formation of C31:9 because *E. coli* recombinants carrying *pfa* genes had no C31:9 (Table 4).

Tentative Identification of Strain osh08

The 1534 bp nucleic acid sequence of the 16S rRNA gene of osh08 was determined and was 99.8% and 98.4% similar to that of *Shewanella basaltis*^{T} (EU143361) and *S. hafniensis*^{T} (AB205566), respectively. The strain was identified tentatively as *Shewanella* sp. strain osh08. Detailed characterization of this strain is in progress.

Discussion

In this study, an EPA-producing mesophilic strain (*Shewanella* sp. strain osh08) was isolated from the mid-latitude seashore of Hokkaido, Japan. This strain included a long-chain polyunsaturated hydrocarbon, hentriacontanonaene (C31:9), which has never been reported in bacteria other than psychrophiles [1, 2]. The geometry and position of the double bonds in this compound were determined by IR analysis to be all *cis* (Fig. 5) at positions 3, 6, 9, 12, 15, 19, 22, 25, and 28 (Table 3 and see Fig. 6). The same compound (C31:9) was detected in marine and nonmarine, and psychrophilc and mesophilic bacteria, which contained EPA and/or DHA (Table 4). Based on our present results, previous studies on the bacterial distribution of PUFAs and *pfa* genes [3], and data

on the predicted biosynthetic pathway of PUFAs in the polyketide synthesis mode [37, 39, 40], we will discuss the biosynthetic routes of C31:9.

When hydrocarbons are produced from fatty acids, decarboxylation of the precursor is necessary. Aliphatic hydrocarbons can be produced in eukaryotes by decarboxylation of corresponding fatty acids [6]. Some marine and freshwater algae produce a C21:6 hydrocarbon with double bonds at 3, 6, 9, 12, 15, and 18, and this PUHC is thought to be formed by decarboxylation of DHA [5], although no biochemical or molecular evidence is available. This speculation is acceptable because 1) the positions of the double bonds of C21:6 are consistent with the view of the decarboxylation of DHA with double bonds at Δ4, 7, 10, 13, 16, and 19; and 2) most organisms that have C21:6 also contain DHA [5].

Two biosynthetic routes can be proposed for the formation of C31:9 (see Fig. 7). One pathway is that C31:9 is synthesized by a head-to-head condensation mechanism, in which two molecules of Δ4, 7, 10, 13-hexadecatetraenoic acid (16:4) are condensed in a head-to-head mode. An additional double bond can be formed by the condensation of two fatty acid molecules, which releases CO_2 (Fig. 7a). The head-to-head formation of long chain nonisoprenoid hydrocarbons from fatty acids has been reported in both vivo [41] and in vitro [42] systems of *Sarcina lutea*, where a monounsaturated long chain hydrocarbon can be formed from two saturated fatty acid molecules by the following equation [41–43].

$$RCH_2COOH + R'CH_2COOH \rightarrow RCH{=}CHCH_2R' + CO_2$$

In the in vitro system, the head-to-head condensation requires coenzyme A, Mg^{2+}, ATP, NADPH, and either pyridoxal phosphate or pyridoxamine phosphate as cofactors [43]. Although we have no direct biochemical evidence on the role of this mechanism in the formation of C31:9, the number and positions of double bonds in C31:9, the antiparallel occurrence of EPA and C31:9 in osh08 cells, and the detection of 16:4 in EPA-producing recombinant *E. coli* [44] support this hypothetical pathway.

The other proposed biosynthetic pathway of C31:9 may occur by decarboxylation of a hypothetical very long chain PUFA, Δ4, 7, 10, 13, 16, 20, 23, 26, 29-dotriacontanonaenoic acid (32:9), which may be synthesized by the Pfa protein complex using 16:4 as a precursor (Fig. 7b). At the final step in this hypothetical biosynthetic process of C31:9, decarboxylation of the 32:9 fatty acid is necessary. No intermediate fatty acids other than 16:4 would be overlapped after 16:4 in the biosynthetic pathways of 32:9 and EPA (and DHA) because of the antiparallel occurrence of EPA and C31:9 in osh08 cells grown at various temperatures (below 25 °C, Table 2). However, no fatty acid corresponding 32:9 has been detected in the FAME fraction of osh08 under different GLC conditions using polar and non polar columns (see above). In both proposed pathways, the C31:9 molecule could have an ethylene-interrupted double bond between C15 and C19 (or C12 and C16) (see Figs. 6 and 7). With regard to the decarboxylation activity to synthesize C31:9, such activity would be present only in bacteria carrying *pfa* genes, because there was no C31:9 detected in two

PUFA-producing marine eukaryotes, which are expected to have eukaryote type *pfa* genes ([37] and Table 4).

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Figure legends

Fig. 1 EI-gas chromatograms of native hentriacontanonaene (C31:9; a) and its fully hydrogenated derivative, hentriacontane (C31:0; b).

Fig. 2. EI-mass spectrum of native C31:9 (a), and CI-mass spectra of C31:9 (b) and C31:0 (c). Acetonitrile was used as the ionization reagent in CI/MS.

Fig. 3 EI-gas chromatogram of a C31:1 mixture prepared from partially hydrogenated C31:9. Five peaks of the C31:1 isomers were numbered.

Fig. 4. CI-mass spectra of the C31:1 isomers. Panels a, b, c, d, and e are a spectrum of peaks 1, 2, 3, 4, and 5, respectively, in Fig. 3.

Fig. 5. IR spectrum of C31:9. There was no characteristic peak at 970 cm^{-1} corresponding to CH bending of the HC=CH *trans* double bond (a region indicated by arrow).

Fig. 6. Proposed structure of C31:9. The position of the double bonds of C31:9 (product) was counted from both methyl ends.

Fig. 7. Possible biosynthetic pathways of C31:9.

(a) C31:9 formation by head-to-head condensation of two Δ4, 7, 10, 13-hexadecatetraenoic acid (16:4) molecules. (b) C31:9 formation by decarboxylation of a hypothetical very long chain polyunsaturated fatty acid, Δ4, 7, 10, 13, 16, 20, 23, 26, 29-dotriacontanonaenoic acid (32:9), which may be synthesized by the Pfa protein complex using 16:4 as a precursor. Fatty acids in this figure are abbreviated, as in 16:4(4,7,10,13), where the number before the colon shows the number of carbon atoms and after the number of double bonds in the fatty acid. The distances of the double bonds from the carboxylic end of the fatty acid are indicated in parentheses. Fatty acid intermediates before 16:4 (4,7,10,13) are not shown. Compounds other than 16:4(4,7,10,13), 20:5(4,8,11,14,17) (EPA), 22:6(4,7,10,13,16,19) (DHA), and hentriacontanonaene **(**C31:9) are hypothetical. Boxed fatty acids are regarded as intermediates in the formation of C31:9.

Table 1 Fatty acid and hydrocarbon composition in osh08 grown at 25 ºC

Fatty acid and hydrocarbon[a]	Content (w/w, % total[b])
12:0	3.4 ± 0.3
iso13:0	2.8 ± 0.1
13:0	1.5 ± 0.3
14:0	3.3 ± 0.2
iso15:0	8.8 ± 0.5
15:0	8.0 ± 1.1
15:1(7)	1.2 ± 0.1
15:1(9)	1.0 ± 0.2
16:0	14.5 ± 1.0
16:1(7)	0.7 ± 0.1
16:1(9)	28.8 ± 0.7
17:0	3.5 ± 0.5
17:1(9)	9.3 ± 0.7
17:1(11)	0.7 ± 0.2
18:0	1.4 ± 0.1
18:1(9)	0.9 ± 0.1
18:1(11)	4.0 ± 1.0
20:5(5,8,11,14,17)	0.5 ± 0.2
C31:9	3.0 ± 0.6
Others	2.7 ± 0.5
Total	100

[a] Fatty acids are abbreviated, as in 16:1(9), where the number before the colon shows the number of carbon atoms and after the number of double bonds in the fatty acid. The distance of the double bond from the carboxylic end of the fatty acid is indicated in parenthesis. iso13:0 and iso15:0 are iso-branched fatty acids. C31:9 is hentriacontanonaene.

[b] Fatty acids and hydrocarbons were separately quantified using 21:0 and C24:0, respectively, as internal standards, and the sum of these compounds was regarded as 100%. The data indicated are means ± standard errors for three independent experiments.

Table 2 Eicosapentaenoic acid and hentriacontanonaene contents in osh08 cells grown at various temperatures

Growth temp., °C	EPA (w/w, % total[a])	C31:9（w/w, % total[a]）
4	1.9 ± 0.2	1.1 ± 0.1
15	0.9 ± 0.4	2.0 ± 0.2
20	0.6 ± 0.1	2.4 ± 0.2
25	0.5 ± 0.2	3.0 ± 0.6
30	0.3 ± 0.1	1.7 ± 0.1
37	0.2 ± 0.1	1.4 ± 0.2
40	ND[b]	ND

[a] Fatty acids and hydrocarbons were separately quantified using 21:0 and C24:0, respectively, as internal standards. The data indicated are means ± standard errors for three independent experiments.

[b] Not detected.

Table 3 Summary of diagnostic ions in the acetonitrile CI mass spectra of hentriacontamonoene isomers

C31:1 isomer	Peak no. in Fig. 3	m/z of fragment (relative intensity)	
		a[a]	b[a]
15(16)-C31:1	1	264 (7.3)	278 (6.9)
12(19)-C31:1	2	222 (8.9)	320 (6.2)
9(22)-C31:1	3	180 (5.9)	362 (4.2)
6(25)-C31:1	4	138 (7.7)	404 (2.8)
3(28)-C31:1	5	96 (8.3)	446 (3.0)

[a] A fragment shown in Fig. 4.

Table 4 Distribution of eicosapentaenoic and docosahexaenoic acids, hentriacontanonaene, and *pfa* genes in various microorganisms[a]

Organisms	Temp. (°C)	EPA	DHA	C31:9	*pfa* genes [Ref]
Marine bacteria					
Shewanella sp. osh08	20	+[d]	–[e]	+	ND[f]
Colwellia maris ABE-1^T	15	+	+	+	ND
Moritella marina MP-1	15	+	+	+	+ [3]
Shewanella benthica	15	+	–	+	ND
Shewanella pneumatophori SCRC2738^T	20	+	–	+	+ [3]
Shewanella marinintestina IK-1^T	20	+	–	+	+ [3]
Shewanella marinintestina IK-1Δ8	20	–	–	–	±[g] [38]
Pseudomonas alcaliphila AL15-21^T	20	–	–	–	ND
Nonmarine bacteria					
Pseudomonas psychrophila E-3^T	20	–	–	–	ND
Pseudomonas aeruginosa WatG	20	–	–	–	ND
Rhodococcus erythropolis	20	–	–	–	ND
Stenotrophomonas maltophilia	20	–	–	–	ND
Shewanella oneidensis MR-1^T	20	+	–	+	+ [36]
Escherichia coli DH5α	20	–	–	–	–
E. coli DH5α/pEPAΔ1[b]	20	+	–	–	+ [3, 44]
E. coli DH5α/pDHA3 plus pET21a::*pfaE*[c]	15	–	+	–	+ [3, 44]
Marine eukaryotes					
Thraustochytrid–like microorganism strain 12B	30	+	+	–	ND
Schizochitrium limacinum SR21	30	+	+	–	ND

Nonmarine eukaryotes					
Saccharomyces cerevisiae	30	–	–	–	–

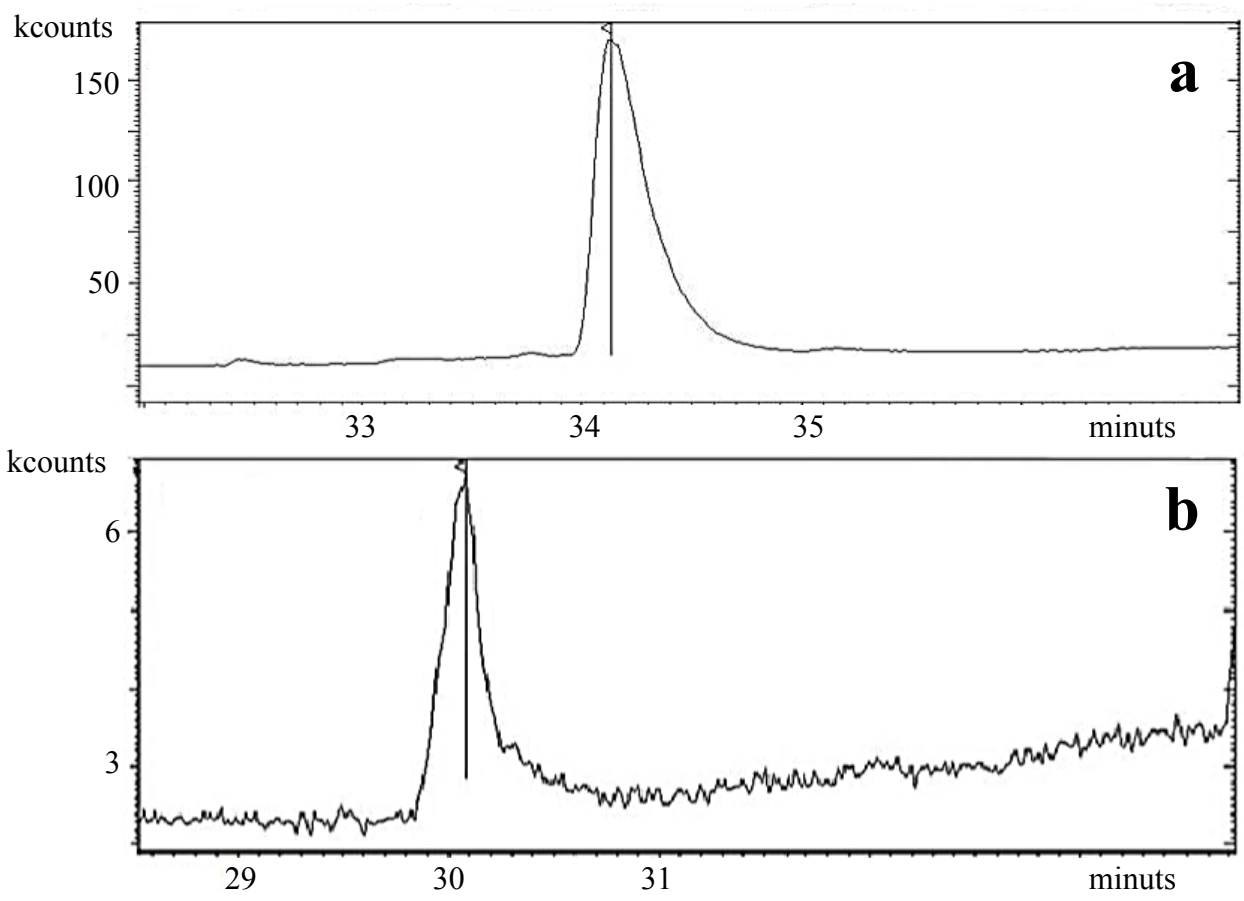
[a] For microorganisms other than osh08, the fatty acid and hydrocarbon fraction was prepared by methanolysis of wet or dry cells in the presence of only 21:0 as internal standard, as described in Materials and Methods

[b, and c] *E. coli* DH5α carrying pEPAΔ1 that contains *pfaA–E* genes responsible for the biosynthesis of EPA and *E. coli* DH5α carrying pDHA3 that contains *pfaA–pfaD* genes and *pfaE* gene responsible for the biosynthesis of DHA, respectively

[d, and e] Present and absent, respectively

ND[f] Not determined.

±[g] Present but only the *pfaD* gene is deficient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100
50
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34
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minuts
kcounts
6
3
b
29
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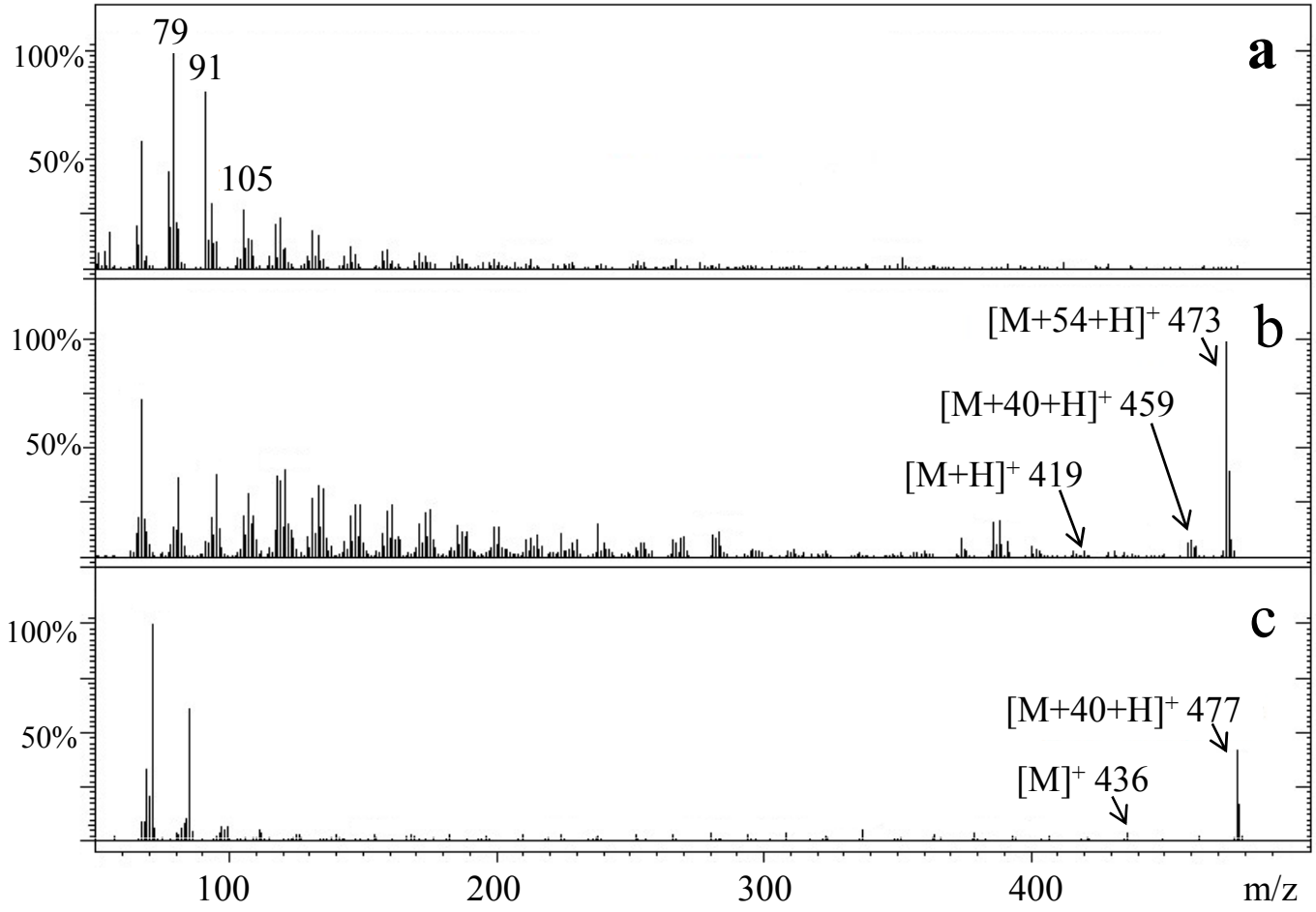
31
minuts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79
91
105
100%
50%
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50%
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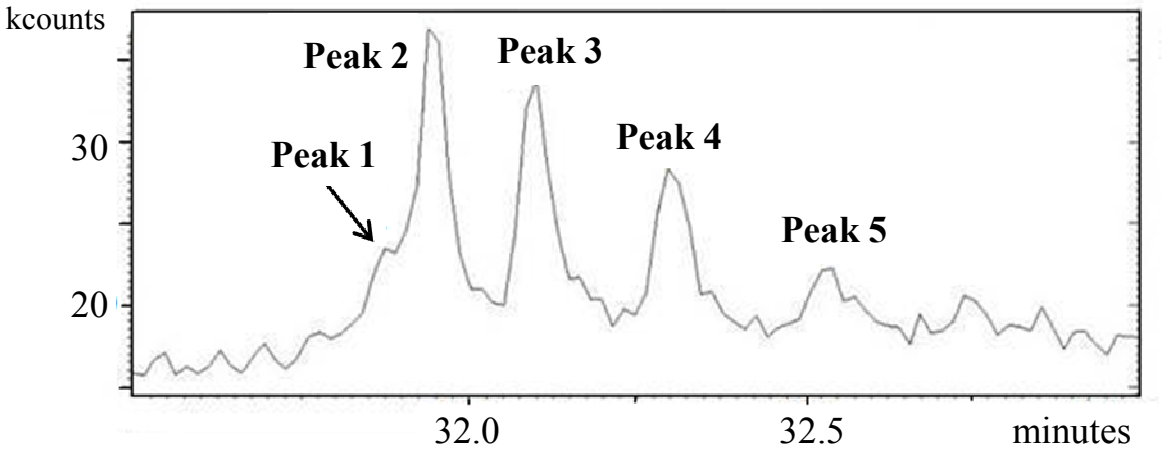
[M]+ 436
100%
50%
100
200
300
400
m/z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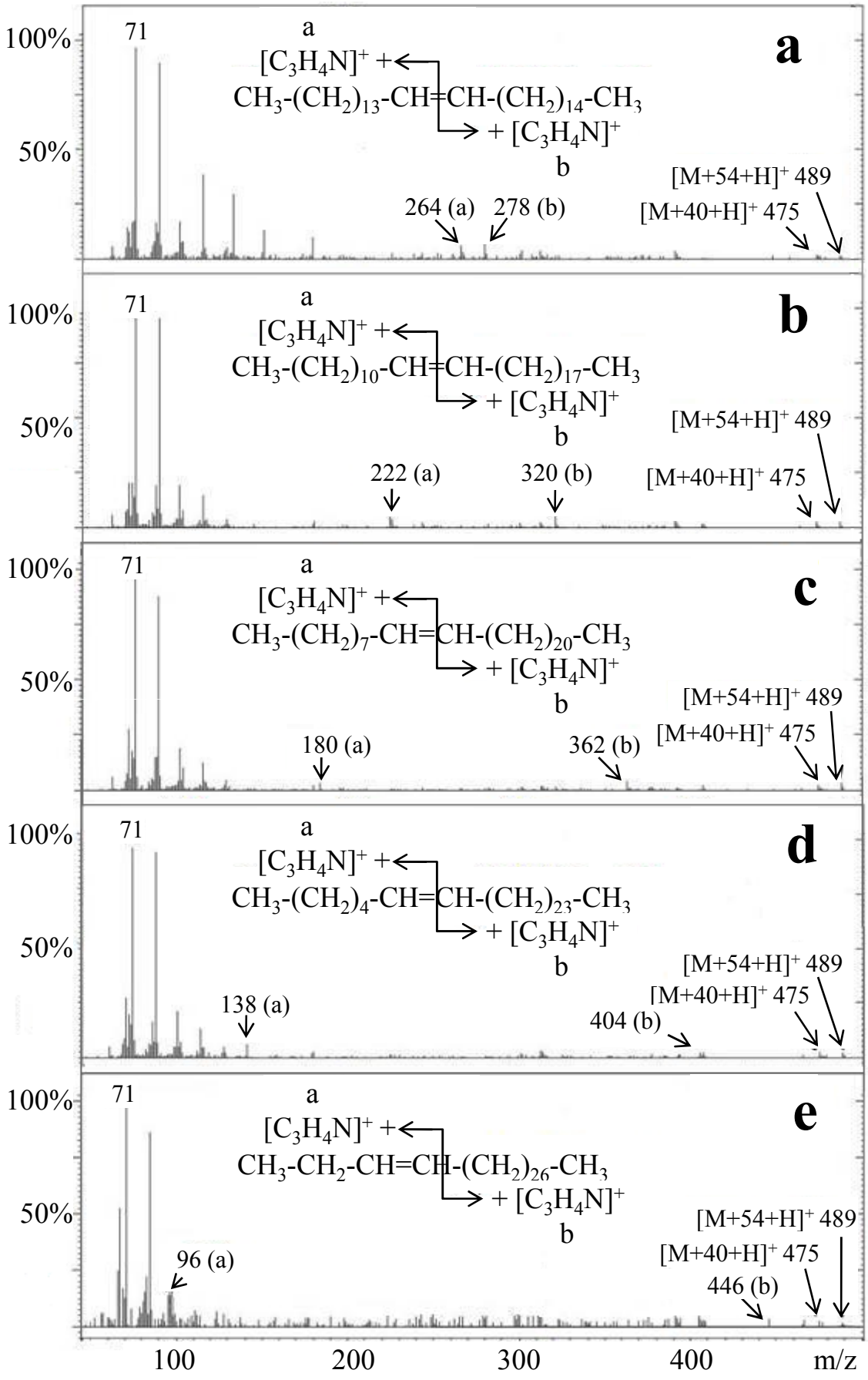
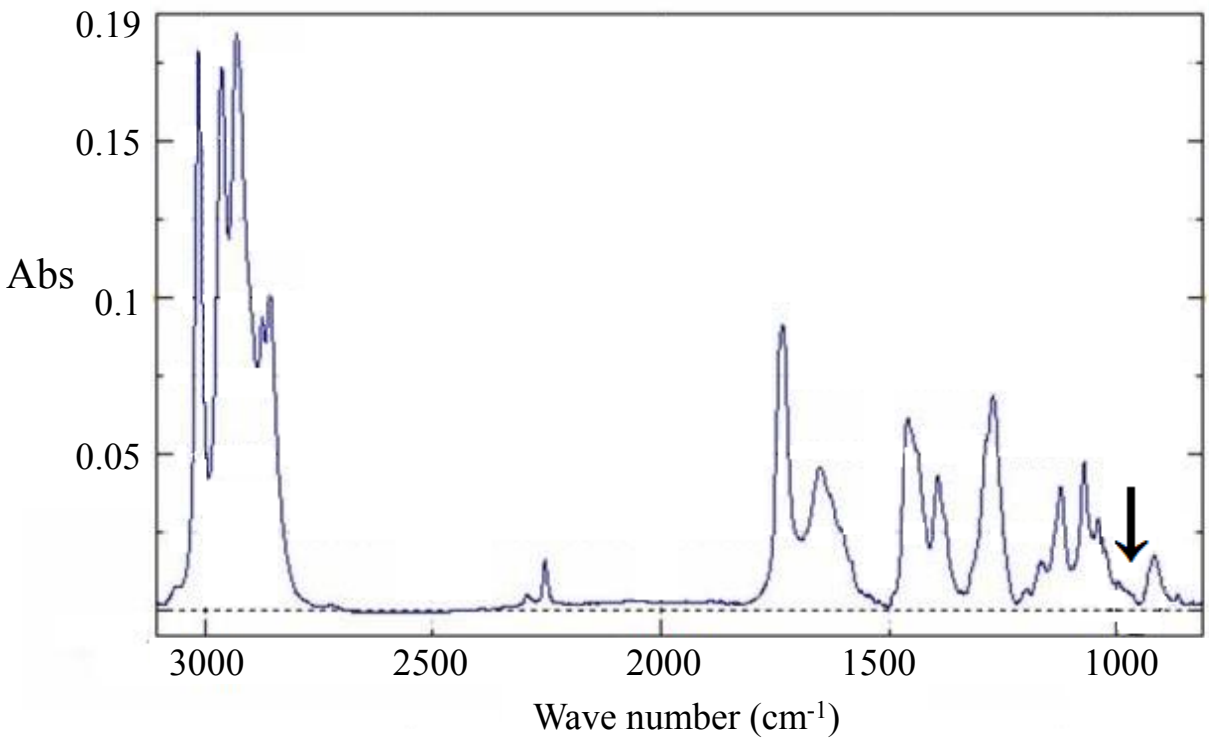
Peak 3
Peak 4
Peak 5
32.0
32.5
minutes


a
b
c
d
e
100%
50%
71
a
[C3H4N]+ +
CH3-(CH2)13-CH=CH-(CH2)14-CH3
+ [C3H4N]+
b
264 (a)
278 (b)
[M+54+H]+ 489
[M+40+H]+ 475
CH3-(CH2)10-CH=CH-(CH2)17-CH3
222 (a)
320 (b)
CH3-(CH2)7-CH=CH-(CH2)20-CH3
180 (a)
362 (b)
CH3-(CH2)4-CH=CH-(CH2)23-CH3
138 (a)
404 (b)
CH3-CH2-CH=CH-(CH2)26-CH3
96 (a)
446 (b)
100
200
300
400
m/z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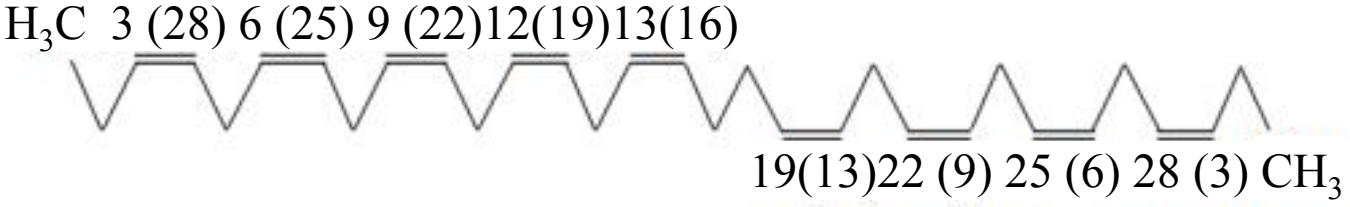
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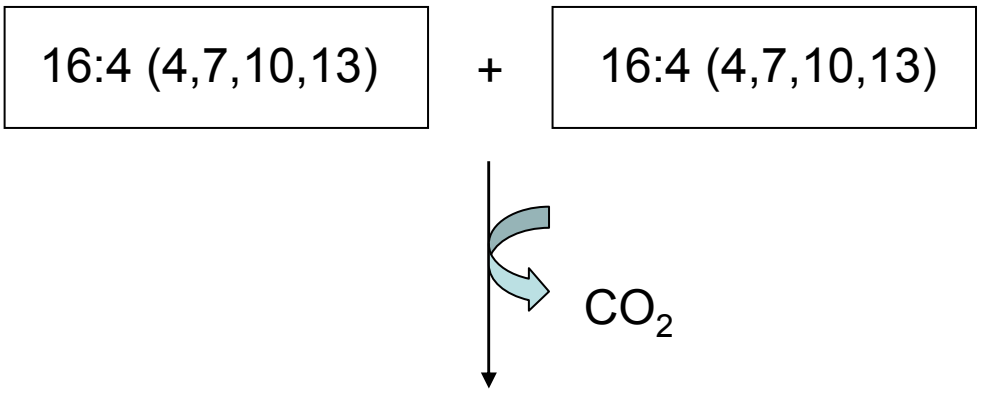
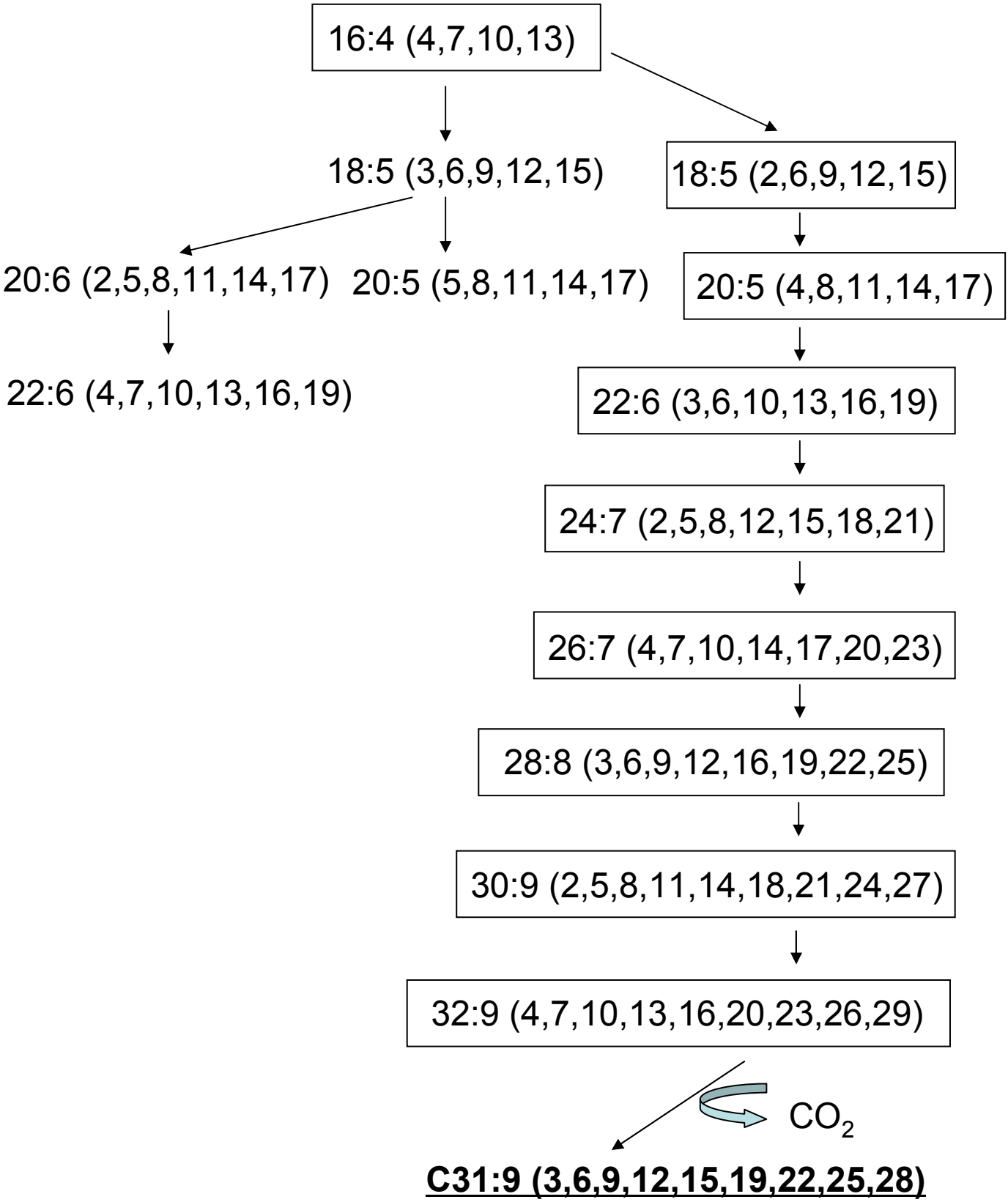
19(13)22 (9) 25 (6) 28 (3) CH3

a



C31:9 (3,6,9,12,15,19,22,25,28)

b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18:5 (3,6,9,12,15)
18:5 (2,6,9,12,15)
20:6 (2,5,8,11,14,17)
20:5 (5,8,11,14,17)
20:5 (4,8,11,14,17)
22:6 (4,7,10,13,16,19)
22:6 (3,6,10,13,16,19)
24:7 (2,5,8,12,15,18,21)
26:7 (4,7,10,14,17,20,23)
28:8 (3,6,9,12,16,19,22,25)
30:9 (2,5,8,11,14,18,21,24,27)
32:9 (4,7,10,13,16,20,23,26,29)
CO_2
C31:9 (3,6,9,12,15,19,22,25,28)