Identification of a novel C 33 botryococcane and C 33 botryococcanone in the Maoming Basin, China

Preprint *in* Organic Geochemistry · October 2018

DOI: 10.1016/j.orggeochem.2018.07.005

7 authors, including:

Jing Liao
Chinese Academy of Sciences
7 PUBLICATIONS **21** CITATIONS
SEE PROFILE

Lu Hong
Chinese Academy of Sciences
57 PUBLICATIONS **649** CITATIONS
SEE PROFILE

Qiao Feng
Shandong University of Science and Technology
23 PUBLICATIONS **118** CITATIONS
SEE PROFILE

Youping Zhou
Shaanxi University of Science & Technology, Xi'an. CHINA
20 PUBLICATIONS **514** CITATIONS
SEE PROFILE

Organic Geochemistry 124 (2018) 103–111

Contents lists available at ScienceDirect

Organic Geochemistry

journal homepage: www.elsevier.com/locate/orggeochem

Identification of a novel C_{33} botryococcane and C_{33} botryococcanone in the Maoming Basin, China

Jing Liao [a,b], Hong Lu [a,*], Qiao Feng [b], Youping Zhou [c,*], Quan Shi [d], Ping'an Peng [a], Guoying Sheng [a]

[a] State Key Laboratory of Organic Geochemistry, Guangzhou Institute of Geochemistry and Institutions of Earth Science, Chinese Academy of Sciences, Guangzhou 510640, China
[b] Shandong University of Science and Technology, Shandong Provincial Key Laboratory of Depositional Mineralization & Sedimentary Minerals, Qingdao 266000, China
[c] Isotopomics in Chemical Biology and Shaanxi Key Laboratory of Chemical Additives for Industry, School of Chemistry & Chemical Engineering, Shaanxi University of Science & Technology, Xi'an 710021, China
[d] State Key Laboratory of Heavy Oil Processing, China University of Petroleum, Beijing 102249, China

ARTICLE INFO

Article history:
Received 17 October 2017
Received in revised form 14 June 2018
Accepted 11 July 2018

Keywords:
Botryococcanone
Botryococcane
Botryococcene biosynthesis
NMR
Cyclobutyl cation intermediate
Botryococcus braunii
Maoming oil shale

ABSTRACT

A novel C_{33} botryococcane in a sedimentary sample from the Maoming Basin in China was found to have a methyl group positioned β to the quaternary carbon C-10 in its structure based on IR, MS and NMR analyses and Lindeman-Adams modelling. This structure is inconsistent with the prediction from the currently accepted botryococcene biosynthetic pathway and different from the skeleton for the C_{33} botryococcanes suggested in the literature. A C_{33} botryococcan-24-one with the same carbon skeleton as the C_{33} botryococcane was also found to co-occur in the sediment sample. The location of the carbonyl group at C-24 is different from that of a previously reported botryococcenone from an Australian collection of race B of *Botryococcus braunii* where it is positioned at C-15. We propose a biogeochemical pathway to explain the occurrence of this unique skeleton in the Maoming Basin sediment. The biochemical component of the pathway involves electrophilic or nucleophilic attack between two farnesyl diphosphates (FPPs) leading to direct or indirect formation of a C_{30} botryococcene via a cyclobutane ring intermediate (1′-2-3-2′ cyclisation). A Retro-Prins reaction and subsequent methylation gives rise to C_{29} and C_{33} botryococcenes in sequence. The geochemical component involves first in-water-column (photo)-oxidation of the alkene to a C_{33} botryococcanone and subsequent reduction (hydrogenation) to stabilize the ketone as an alkane.

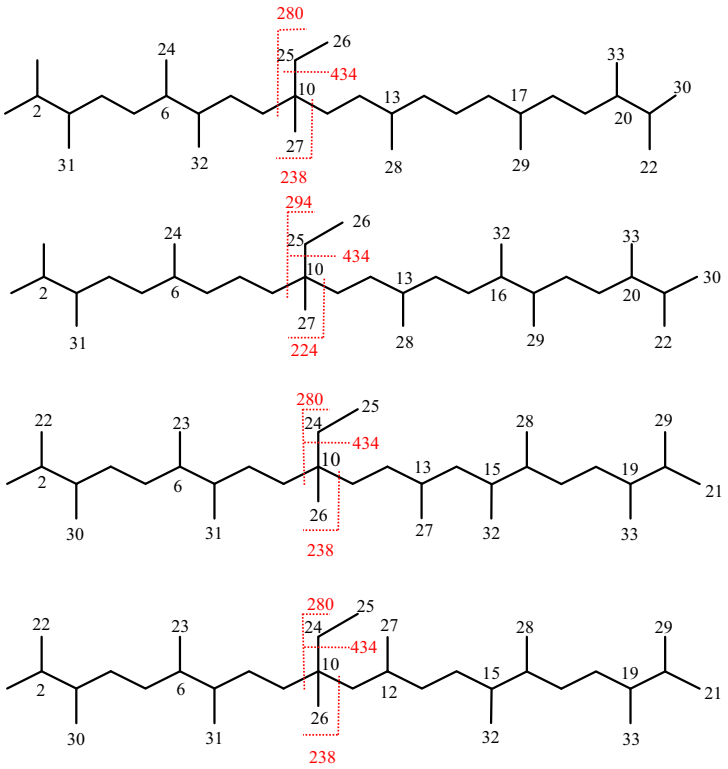
1. Introduction

Botryococcenes are geochemically important biomarkers for assessing depositional environment and geological age (Philp and Lewis, 1987; Guy-Ohlson, 1992; Volkman, 2014) as they are known to be synthesised only by race B of the freshwater unicellular green microalga *Botryococcus braunii* (Maxwell et al., 1968), which is known to contribute significantly to the botryococcanes (and other botryococcanoids) found in oil shales and oils. In field-grown or cultured *B. braunii*, botryococcenes are found to have a carbon range of C_{30}–C_{37} (Metzger et al., 1985a, 1985b; Okada et al., 1995; de Mesmay et al., 2008), and are often dominated by the C_{34} homologue(s) (Metzger and Largeau, 1999; Zhang et al., 2007; de Mesmay et al., 2008). This predominance of C_{34} botryococcenes in *B. braunii* is consistent with the C_{34} botryococcanes found in fossil samples. For example, C_{34} botryococcanes were first discovered in a Sumatran crude oil (Moldowan and Seifert, 1980) and subsequently in Australian coastal bitumens (McKirdy et al., 1986, 1994; Dowling et al., 1995) and the Kishenehn Formation in American Montana (Curiale, 1987). Grice et al. (1998) reported the occurrence of cyclobotryococcenes and structurally related organic sulfur compounds (OSC) in hypersaline sediments of Miocene/Pliocene age from the Sdom Formation in the Dead Sea. A novel (partially reduced) C_{34} botryococcene (1,6,17,21-octahydro botryococcene) from *B. braunii* was identified in a sediment from Sacred Lake, Mount Kenya (Huang and Murray, 1995). C_{32}–C_{34} botryococcenes were also detected in Florida Everglades freshwater wetland soil (Gao et al., 2007). C_{33}–C_{37} botryococcenes and partially reduced mono- and dicyclic unsaturated derivatives were reported in Holocene sediments from Lake Masoko in Tanzania (de Mesmay et al., 2008; Grossi et al., 2012).

* Corresponding authors at: State Key Laboratory of Organic Geochemistry, Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, 636 Biaoben Building, 511 Kehua Street, Tianhe District, Guangzhou 510640, China (H. Lu). Isotopomics in Chemical Biology, School of Chemistry & Chemical Engineering, Shaanxi University of Science & Technology, 418 Shixun Building, Xianqing Avenue, Weiyang District, Xi'an 710021, China (Y. Zhou).

E-mail addresses: luhong@gig.ac.cn (H. Lu), youping.zhou@sust.edu.cn (Y. Zhou).

https://doi.org/10.1016/j.orggeochem.2018.07.005
0146-6380/



16 (1C, 8CH, $12CH_2$, $12CH_3$)
(Brassell et al., 1986; Metzger et al., 1988; Summons and Capon, 1991)

17 (1C, 8CH, $12CH_2$, $12CH_3$)
(Summons and Capon, 1991; Metzger et al., 1988)

18 (1C, 9CH, $10CH_2$, $13CH_3$)

15 (1C, 9CH, $10CH_2$, $13CH_3$)

Fig. 1. The four possible carbon skeletons of C_{33} botryococcanes deducible from the mass spectrum obtained by Brassell et al. (1986).

Botryococcanes with an obvious odd carbon number predominance (C_{31} and C_{33}), although rare, were also reported in the Chinese Maoming Basin (Brassell et al., 1986). The identification of these botryococcanes was solely based on a comparison of their mass spectra with that of a C_{34} botryococcane standard (Moldowan and Seifert, 1980) and other botryococcane and botryococcene homologues (Metzger and Casadevall, 1983; Metzger et al., 1985a), and therefore was not conclusive. In the case of C_{33} botryococcane, two different structures (**16** and **17;** Fig. 1) were suggested by Brassell et al. (1986), Metzger et al. (1988) and Summons and Capon (1991). However, two equally possible structures (**15** and **18;** Fig. 1) are also consistent with the mass spectrum, and in particular the fragmentation pattern around the quaternary carbon (key ions at *m*/*z* 238, 280, and 434; Fig. 1). The four possible structures differ mainly in the location of the methyl group adjacent to the quaternary carbon C-10, i.e., C-28 (connected to C-13, γ to C-10) in **16** and **17**, C-27 in **18** (connected to C-13, γ to C-10) and **15** (connected to C-12, β to C-10).

To firmly establish the structure of the predominant C_{33} botryococcane in the Maoming Basin, we isolated the C_{33} botryococcane from the saturated fraction. We report here the structural reassessment of this alkane and a ketone, with the same carbon number and skeleton isolated from the polar fraction, by a combination of MS and NMR techniques and discuss their genetic relationship and possible geochemical and biosynthetic pathways leading to their formation in the Maoming Basin.

2. Material and methods

2.1. Sampling, separation and purification

The Eocene sedimentary sample used in this study was collected from an outcrop of the Maoming Basin located in the southwest of Guangdong Province in China. It was collected from the same formation at the same location from which botryococcanes were reported by Brassell et al. (1986) (Supplementary Fig. S1). The ground sample was Soxhlet-extracted with methanol/dichloromethane (1:9, v/v) for 72 h. The asphaltene fraction was centrifugally removed by precipitation in *n*-hexane. The maltene fraction was further separated into saturated, aromatic and polar fractions on a glass column (0.3 m × 1 cm i.d.) packed with silica (80–100 mesh) activated at 120 °C for 12 h and alumina activated at 450 °C for 4 h in tandem (4:1, v/v), using sequential elution with *n*-hexane (80 mL), *n*-hexane/dichloromethane (40 mL, 1:1, v/v) and methanol (40 mL). The polar fraction was further subfractionated on a column (0.3 m × 1 cm i.d.) packed with silica (100–230 mesh), activated at 120 °C for 12 h, by eluting with CH_2Cl_2/*n*-hexane (1:3 v/v, 40 mL, subfraction 1) and CH_2Cl_2/*n*-hexane (1:2 v/v, 40 mL, subfraction 2).

GC–MS analyses were then conducted on both subfraction 2 and the saturated hydrocarbon fraction. These fractions were then subjected to preparative gas chromatography (pGC) (Eglinton et al., 1996; Özek and Demirci, 2012; Zuo et al., 2013) with a DB-5 column (60 m × 0.53 mm i.d. × 1.5 μm film thickness) on an Agilent 7890 GC coupled to a Gerstel-preparative fraction collector (PFC) to isolate and purify the two target compounds. The preparative conditions used were as follows: He was used as carrier gas at a flow rate of 3.0 mL/min. The column was held at 80 °C for 2 min, then heated to 300 °C at 30 °C/min, and held for 40 min at 300 °C. The purified botryococcanone (approx. 2.0 mg, see Supplementary Figs. S2 and S4) and C_{33} botryococcane (approx. 3.5 mg, see Supplementary Figs. S1 and S3) were then subjected to FT-IR, HR-EIMS, and NMR analyses for further structural characterisation.

2.2. Instrumental analyses

FT-IR analysis of the purified botryococcanone was conducted on a FT-IR Tenor 27 spectrophotometer (Bruker Corporation, Billerica, USA) with the sample loaded as a KBr pellet. The spectrometer

was operated in absorption mode with absorptions in the range 475 cm^{-1} to 3675 cm^{-1} recorded at a resolution of 2 cm^{-1}.

HR-EIMS analyses of the botryococcane and botryococcanone were conducted on a Thermo Finnigan MAT95XP mass spectrometer (Germany) to determine the accurate mass. The HR-EIMS system was operated in the electron impact ionization mode (42 eV) at a mass resolution of R > 10,000 (10% valley).

^{1}H and ^{13}C NMR spectral analyses were conducted on a Bruker 600M AVANCE III spectrometer (operated at 600.19 MHz for ^{1}H and 150.92 MHz for ^{13}C). Broadband ^{13}C-decoupled spectra were recorded in $CDCl_3$, with TMS as internal standard. ^{1}H NMR chemical shifts were referenced to residual proton signal (7.26 ppm). ^{13}C NMR chemical shifts were referenced to the central line of ^{13}C multiplet (77.0 ppm). A combination of 1D and 2D experiments, ^{1}H–^{1}H correlation spectroscopy (COSY), heteronuclear single quantum correlation (HSQC), and heteronuclear multiple bond correlation (HMBC) were performed to assign the individual resonances. Distortionless enhanced polarization transfer (DEPT) spectra were used to determine the multiplicity of each ^{13}C nucleus.

GC–MS analyses of the saturated and polar fractions were performed on a Trace Ultra GC coupled to a Thermo DSQ-II mass spectrometer operating with an ionization energy of 70 eV and a *m/z* range of 50–600. An HP-5 column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) was used. The oven temperature was ramped from 80 °C (hold for 2 min) to 295 °C (hold for 20 min) at rate of 6 °C/min. He was used as the carrier gas with a constant flow of 1.2 mL/min.

3. Results and discussion

3.1. Structural elucidation of the novel C_{33} botryococcane and botryococcanone

Abundant C_{31} and C_{33} botryococcanes were found in the Maoming sediment extracts (Supplementary Fig. S1). The overall distribution of these hydrocarbons (including botryococcanes) in the saturated hydrocarbon fraction is very similar to that reported by Brassell et al. (1986) implying that we have essentially reproduced the profile of the saturated fraction obtained by these authors. A compound suspected to be the corresponding ketone of the C_{33} botryococcane was discovered and isolated from the polar fraction (see Supplementary Fig. S2 for the TICs of the polar fraction and subfraction 2). To confirm their skeletons, we first assigned the structure of the C_{33} botryococcane by NMR and then verified the skeleton of the C_{33} ketone by MS and NMR analyses.

C_{33} botryococcane. In order to ascertain its structure, in particular the substructure around the C-10 quaternary carbon (Fig. 1), the HMBC data were investigated in detail (Fig. 2, see also

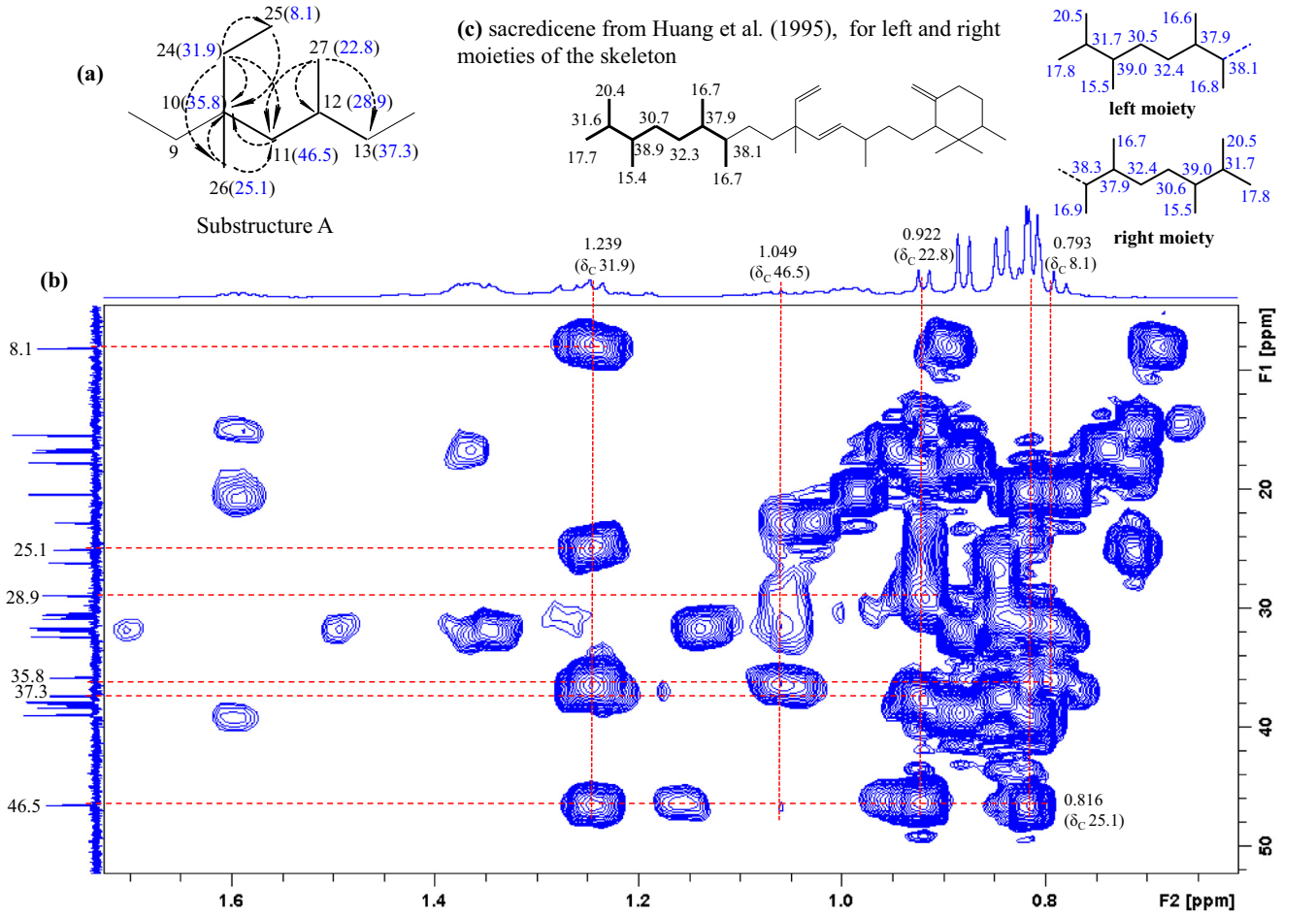


Fig. 2. The core substructure (a) around the quaternary carbon as deduced from the HMBC spectrum (b) for the C_{33} botryococcane (**15**) in this study. The position-specific chemical shifts in the left and right moieties in the C_{33} botryococcane identified here are very similar to that of the equivalent substructure of the sacredicene (c) identified by Huang et al. (1995).

Supplementary Table S1 and Supplementary Figs. S8–S10 for other NMR data). The correlations from H-24 (δ_H 1.239) to C-10 (δ_C 35.8), C-11 (δ_C 46.5), C-25 (δ_C 8.1) and C-26 (δ_C 25.1) indicated connections between C-10/C-11, C-10/C-24, C-10/C-26 and C-24/C-25 (Fig. 2a), which could also be further confirmed by the correlations from H-25 (δ_H 0.793) to C-10 (δ_C 35.8), H-26 (δ_H 0.816) to C-10 (δ_C 35.8), H-26 (δ_H 0.816) to C-11 (δ_C 46.5) and H-11 (δ_H 1.049) to C-10 (δ_C 35.8). Correlations from H-27 (δ_H 0.922) to C-11 (δ_C 46.5), C-12 (δ_C 28.9) and C-13 (δ_C 37.3) (Fig. 2b) established the connections C-11/C-12, C-12/C-13, and C-12/C-27. The values of the chemical shifts measured for the moieties left to C-8 and right to C-14 (Fig. 2c) are in good agreement with those reported by Huang et al. (1995) for a moiety of sacredicene, a botryococcane derivative sharing the same substructure **A** of the botryococcane we identified here. Thus, it is reasonable to assign the structure of C_{33} botryococcane as skeleton **15** (Fig. 1), which has a methyl group connected to a carbon β to C-10.

C_{33} botryococcanone. The mass spectrum (Fig. 3b) of the C_{33} ketone (see Supplementary Fig. S2 for the TIC of the polar fraction) displayed two distinct ions: *m/z* 254 and 296, both having an extra 16 Da added to the characteristic ions of *m/z* 238 and 280 for the C_{33} botryococcane as identified by Brassell et al. (1986) (Fig. 3a, see also Summons and Capon, 1991). Since cleavages around the sole quaternary carbon (C-10) in the botryococcane lead to the formation of the characteristic ions *m/z* 238/239 and 280/281 (Fig. 3a), which correspond to the loss of the two alkyl chains (C-1 – C-9) and (C-11 – C-21) (Fig. 3a), respectively, the characteristic ions *m/z* 254 and 296 could be explained by a McLafferty rearrangement around the quaternary carbon of a C_{33} botryococcanone with a carbonyl group positioned on the ethyl group (C-24) connected to quaternary carbon C-10 (Fig. 3c). Such a positioning of the carbonyl group is further supported by the very similar fragmentation pattern of C_{33} ketone to that of the C_{33} botryococcane (Fig. 3a): base peak *m/z* 71 (loss of the alkyl group $C_5H_{11}^-$ from C-3 and C-19, Fig. 3b), major peak *m/z* 127 (loss of $C_9H_{19}^-$ at C-6 and C-16, Fig. 3b) and the other ions *m/z* 57, 85, 99, 113, 141, 155, 169, 183, 197, 211, and 225).

HR-EIMS analysis (see Supplementary Fig. S6 for a partial mass spectrum) showed that the ketone has an accurate mass of 478.5108, in agreement with a calculated mass of 478.5114 for a formula of $C_{33}H_{66}O$, indicating one degree of unsaturation. FT-IR absorption (Supplementary Fig. S7) at 1704.8 cm^{-1} also indicated the presence of a carbonyl group (1705–1725 cm^{-1}) and excluded the possibility of an aldehyde group due to the absence of absorption characteristic bands for an aldehyde group (2820–2850 and 2720–2750 cm^{-1}). Therefore, the compound was initially identified as a C_{33} botryococcan-24-one (Fig. 3b).

In such a botryococcanone skeleton, one would expect strong correlations in the NMR chemical shifts (see Supplementary Table S2 and Supplementary Figs. S11–S13 for detailed 1D and 2D data) between the carbonyl carbon at C-24 and its neighbouring carbons and hydrogens at C-25, C-26, C-11 and possibly C-9 (Fig. 4). This is exactly what was observed with the HMBC correlation spectrum (Fig. 4b): carbon C-24 (δ_C 214.3) is correlated with H-9 (δ_H 1.500), H-11 (δ_H 1.673 and δ_H 1.358), H-26 (δ_H 1.102) and H-25 (δ_H 2.125). Note that the δ_C (214.3) of the C-24 (Fig. 4a and b) is close to that of the carbonyl carbon C-15 (δ_C 210.0) of the C_{33} botryococcenone identified by Summons and Capon (1991). The δ_C at 25.5 ppm for C-25 is consistent with the fact that C-25 is deshielded by the neighbouring carbonyl group (Breitmaier and Voelter, 1978; Cheng and Bennett, 1991; Cheng, 1994). Further, in the HMBC spectrum, it was observed that H-25 (δ_H 2.125) was correlated to the carbonyl carbon C-24 (δ_C 214.3), consistent with the expectation that a carbonyl at C-24 would give

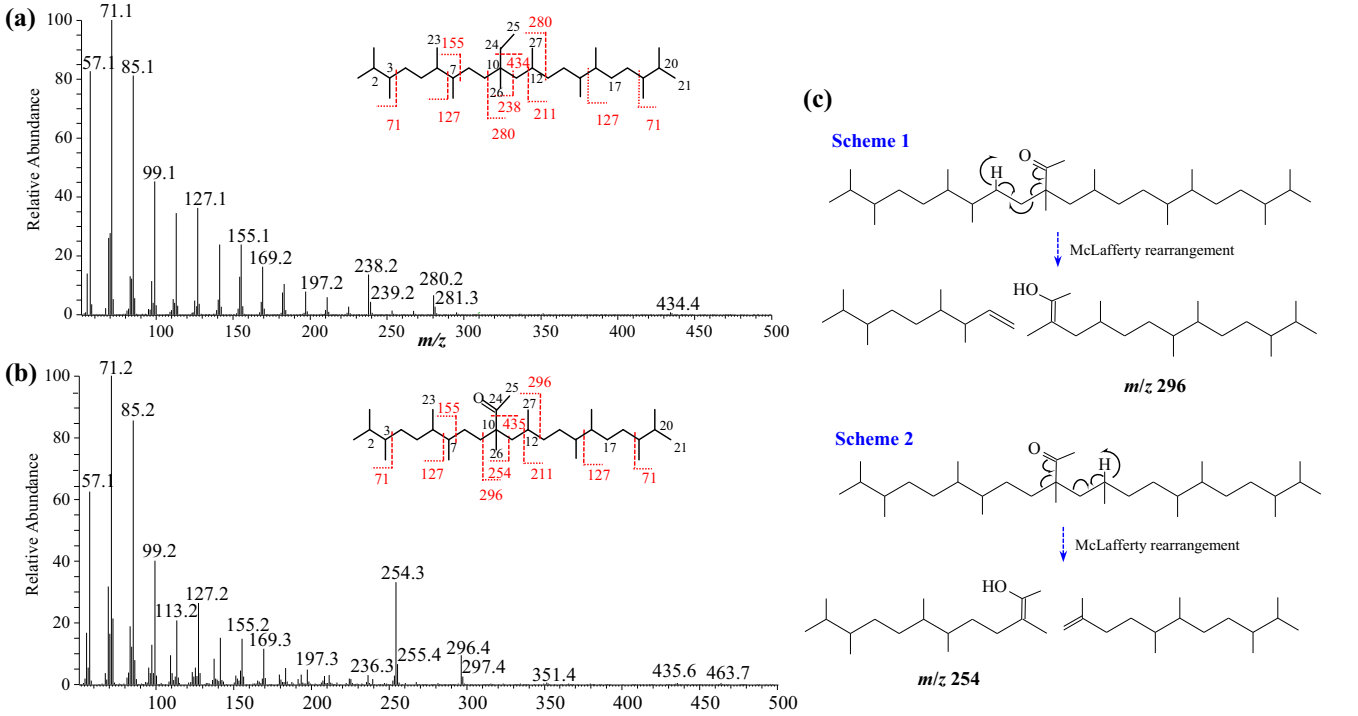


Fig. 3. The mass spectra for the novel C_{33} botryococcane (a) and C_{33} botryococcanone (b) identified in this study; (c) McLafferty rearrangement leading to the formation of fragment ions *m/z* 254 and 296 of C_{33} botryococcanone (b).

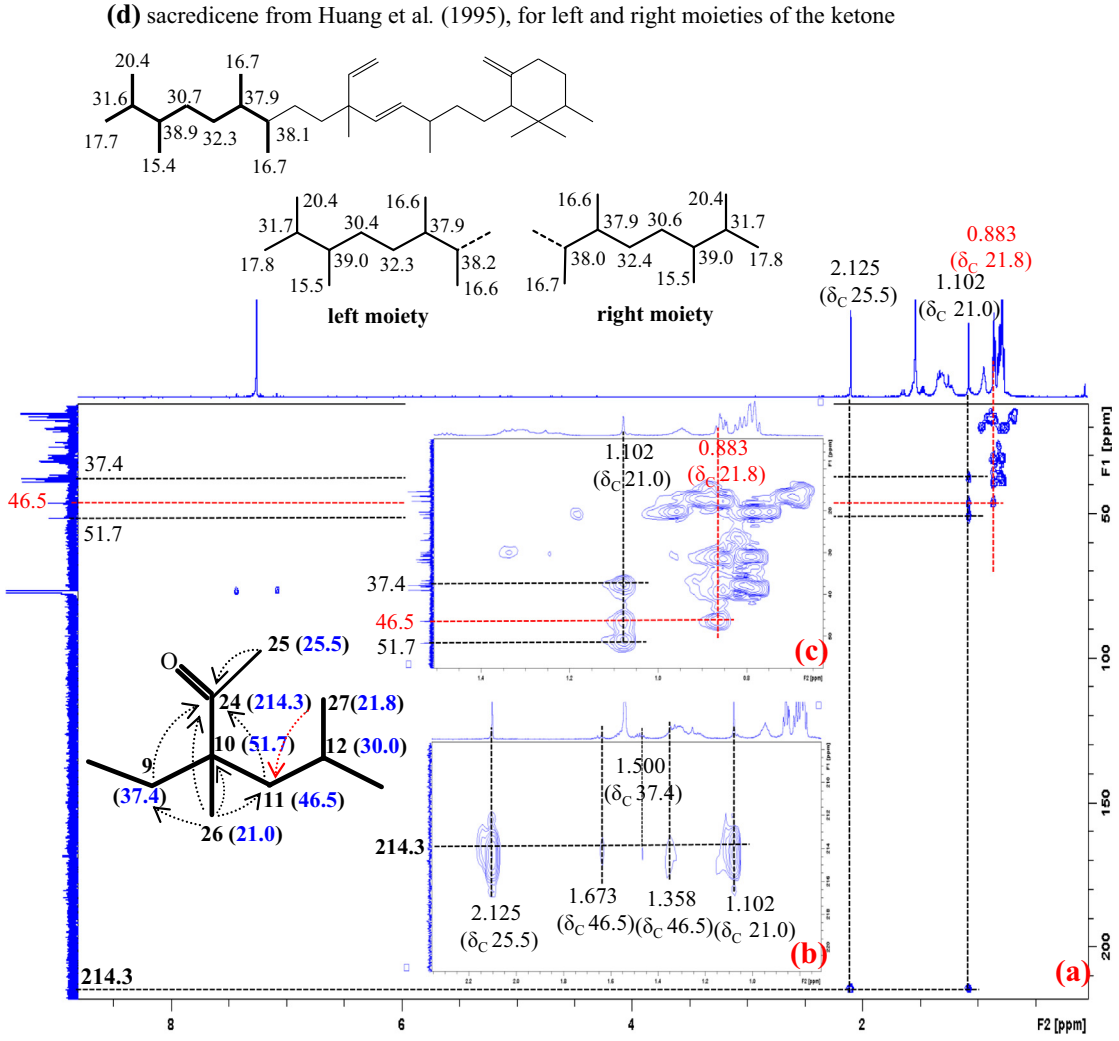


Fig. 4. Full (a) and zoomed-in partial (b and c) HMBC spectra of the identified C_{33} botryococcan-24-one showing the assignment of the carbonyl group to C-10 and a methyl group to C-12. The position-specific chemical shifts of the left and right moieties of the C_{33} botryococcan-24-one identified here (d) are also very close to that of the equivalent substructure of the sacredicene identified by Huang et al. (1995).

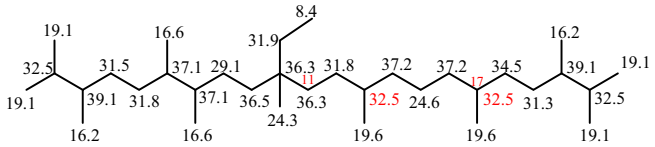
such a strong correlation (Fig. 4). The smaller values of δ_C at 21.0 ppm and δ_H at 1.102 ppm is consistent with the fact that C-26 is two bonds away from the carbonyl carbon. Therefore, the skeleton of the C_{33} botryococcanone can be established in which the carbonyl group is positioned on the ethyl group connected to the sole quaternary carbon (C-10), different from that of the botryococcenone identified by Summons and Capon (1991) where it is located on C-15.

Next, we explain why there should be a methyl group connected to a carbon β to C-10 (Fig. 3b), rather than γ to C-10. As C-11 (δ_C 46.5) is correlated with H-27 (δ_H 0.883), it is reasonable to position the C-27 methyl (δ_C 21.8) on C-12. Such a positioning successfully explains why C-9 and C-11 differ in their δ_H (up to 0.3 ppm) and δ_C (up to 9 ppm) and why H-9 is a singlet (δ_H 1.500) while H-11 is a doublet (δ_H 1.673 and 1.358). The δ_H and δ_C difference between C-9 and C-11 should not come from the C-24 carbonyl oxygen as, according to Lindeman and Adams (1971), Grant and Paul modelling (Breitmaier and Voelter, 1978), the δ_C difference between carbons β to a —$COCH_3$ group is 1 ppm. As the two substructures left to C-8 and right to C-14 are symmetrical and that their position-specific δ_C values are very close to a substructure of the sacredicene reported by Huang et al. (1995) (Fig. 4d), it is reasonable to assign the structure of C_{33} ketone to C_{33} botryococcan-24-one, with a methyl group β to C-10.

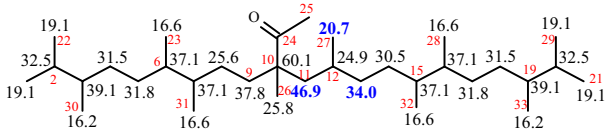
To further confirm the new skeletons of C_{33} botryococcanone and botryococcane in this study, we compared the Lindeman-Adams (Lindeman and Adams, 1971) modelled and measured carbon chemical shifts (Fig. 5) for the proposed new structures and that suggested by Brassell et al. (1986). The position-specific measured δ_C values (Fig. 5c and e) were very close to the calculated (empirical) values (Fig. 5b and d). By contrast, the measured δ_C values at C-11 (46.5 ppm) and C-16 (37.9 ppm) for the newly proposed skeleton **15** (Fig. 5e) differ significantly from the calculated δ_C values (C-11 (36.3 ppm) and C-17 (32.5 ppm)) for the suggested skeleton **15** (Fig. 5a) (Brassell et al., 1986).

3.2. Possible pathways for the formation of C_{33} botryococcane and botryococcanone

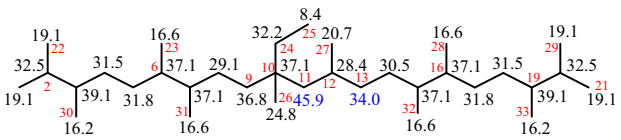
B. braunii is a phototrophic microalga usually found in surface waters. The commonly accepted pathway (**A** of Fig. 6) for the synthesis of (normal) botryococcenes (**6**) in *B. braunii* starts with the condensation of two farnesyl diphosphates (FPP, **1**) to form



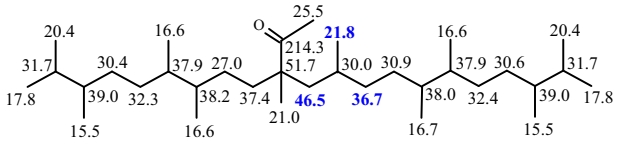
(a) calculated for the structure suggested by Brassell et al. (1986)



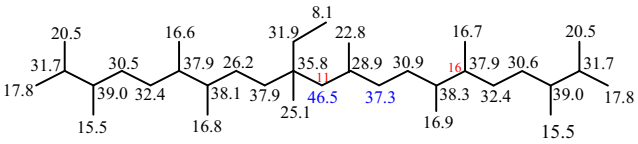
(b) calculated for the C_{33} botryococcanone identified in this study



(d) calculated for the C_{33} botryococcane identified in this study



(c) measured for the C_{33} botryococcanone identified in this study



(e) measured for the C_{33} botryococcane identified in this study

Fig. 5. The measured (c, e) and the calculated (b, d) δ_C values for the newly identified C_{33} botryococcane and C_{33} botryococcan-24-one. For comparison, the Lindeman-Adam modelling was also applied to the skeleton (a) proposed by Brassell et al. (1986). The position-specific chemical shift differences (Z_{iso}) brought by the substituent group —$COCH_3$ (Breitmaier and Voelter, 1978) for the modelled positions C-8, C-9, C-10, C-11, C-12 and C-26 of between the C_{33} botryococcane and C_{33} botryococcanone were −3.5, 1.0, 23.0, 1.0, −3.5 and 1.0 ppm, respectively.

presqualene diphosphate (PSPP, **2**). The condensation proceeds with the formation of a cyclopropylcarbinyl cation intermediate (**3**) by the attack of C1′ of one molecule of FPP (**1**) to the C2-3 double bond of another FPP (Rilling and Epstein, 1969; Rilling et al., 1971). Migration of C1′-3 and C1′-2 to C1′-1 and subsequent reduction by NADPH leads to the formation of squalene (not shown here; Van Tamelen and Schwartz, 1971; Coates and Robinson, 1972; Zhang and Poulter, 1995; Jarstfer et al., 1996; Blagg et al., 2002) while a reductive C1′-2 fission of the cyclopropane ring of **3** with NADPH leads to the formation of normal C_{30} botryococcene (**5**) with a C1′-3 linkage between the two FPPs (Huang and Poulter, 1989; Poulter, 1990). In this pathway, the formation of a botryococcenol with an —OH group located at various positions (hydroxybotryococcene, HBO; **4** as an example) as stable products or unstable intermediates is highly likely since in vitro cultivation experiments (Zhang and Poulter, 1995; Jarstfer et al., 1996, 2002; Pan et al., 2015) with recombinant squalene synthase (SQase) showed that HBO is a stable product although it is yet to be isolated from wild *B. braunii* populations. It is also highly likely that the often detected botryococcenes (**5** and **6**) from laboratory cultured or wild *B. braunii* are the in vivo dehydration product of HBO as the dehydration and subsequent reduction of alcohol by SQase has been confirmed by Pan et al. (2015), even though the experiment was conducted with bacteria. Successive methylation of **5** with S-adenosylmethionine-dependent methylase (SAM) can introduce up to 7 methyl groups (Metzger et al., 1985b, 1986) to give botryococcenes in the carbon range of C_{30}–C_{37} (**6**). The C1′-3 linkage means that no methyl group α or β to the quaternary carbon C-10 is possible as none of the α or β positions is methylated. Since the here identified C_{33} botryococcane (**15**) and C_{33} botryococcanone (**13**) both have a methyl group positioned β to the quaternary carbon C-10, it calls for a pathway different to the current one (**A** in Fig. 6) to explain the presence of these two new compounds in the Maoming Basin.

Here we propose a biosynthetic pathway (**B** in Fig. 6) leading to the synthesis of botryococcene with this unique skeleton followed by a geochemical pathway (**C** of Fig. 6) that converts them into the identified C_{33} botryococcane and botryococcanone. The pathway starts with the condensation of two FPPs (**1** in Fig. 6) by two nucleophilic attacks (one from the double bond between C-2 and C-3 to C1′ and the other from the double bond C-2′ and C-3′ to C-3) leading to the formation of a cyclobutylcarbinol cation (**7**, 1′-2-3-2′ cyclisation) with the positive charge located on the tertiary carbon (C**3**′). The cyclobutylcarbinol cation (**7**) can be also be indirectly formed from the cyclopropylcarbinyl cation intermediate (**3**) (Thulasiram et al., 2008, see Fig. 6). Subsequent C1′-2′ opening of the cyclobutane ring caused by electron migration shifts the positive charge to C1′ **(8)**. A Retro-Prins reaction (Al-Qallaf et al., 2000) could then give rise to the formation of C_{29} botryococcene (**10**) (Fig. 6) directly from **8** with the loss of a hydroxymethyl cation (**9**). Methylation of **10** leads to the formation of C_{33} botryococcene (**11**).

As the surface water is most likely to be oxic and can be penetrated by sunlight, once released to the surface water following algal death or active excretion, photo-mediated oxidation of **11** is likely. Indeed, Rontani and co-workers (e.g., Rontani et al., 1987, 2011; Rontani and Giusti, 1988; Rontani, 2001) provided evidence for photo-mediated oxidation of unsaturated lipids in the water column although the exact mechanism for such oxidation is still debatable. We suggest that the first product of (photo)oxidation is C_{33} botryococcen-24-one (**12**) as the ethenyl group of **11** is more easily oxidised (to a ketone) than those backbone-connected double bonds in the skeletons. Deeper in the water column or in the sediment, this oxidative process is expected to be gradually overtaken by reduction of the double bonds in **12** due to the decrease in oxygen and light levels, leading to the here identified C_{33} botryococcanone **13**. Further reduction of **13** converts it to a hypothetical C_{33} botryococcanol (**14**). Subsequent dehydration and

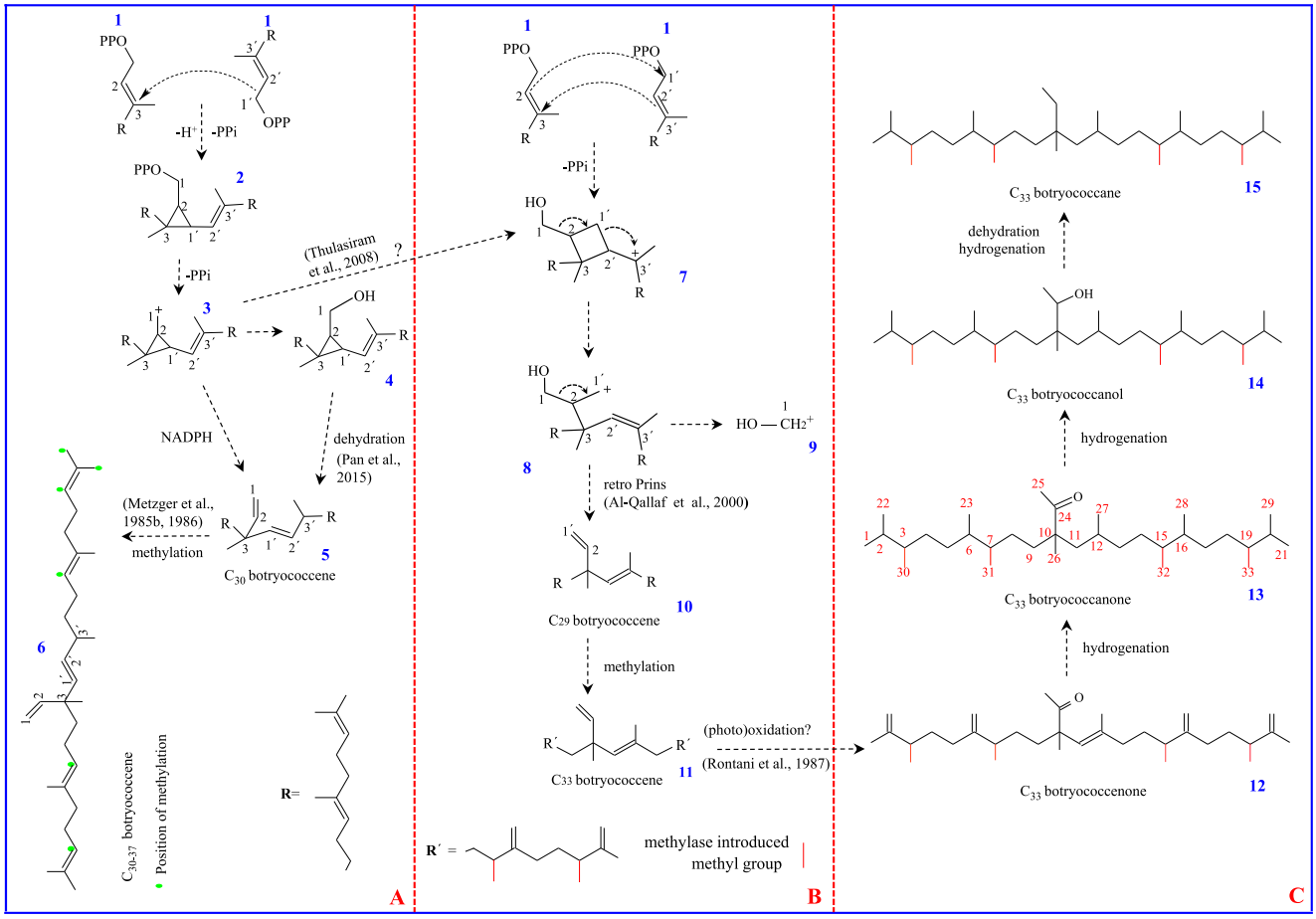


Fig. 6. Proposed biosynthetic and geochemical pathways for the formation of the new C_{33} botryococcane and C_{33} botryococcanone with unique carbonyl group location and methyl group positioned β to the quaternary carbon in the skeleton. **A** is the currently accepted biochemical pathway for biosynthesis of normal botryococcenes; **B** is the proposed biochemical pathway for the formation of the C_{33} botryococcene; **C** is the geochemical pathway for the formation of the C_{33} botryococcanone and botryococcane. The proposed pathway does not exclude other possibilities that may lead to the formation and preservations of these two new botryococcanoids in the Maoming Basin.

hydrogenation of **14** leads to the formation of C_{33} botryococcane (**15**).

4. Conclusions

Accepted (normal) botryococcene biosynthetic pathways involve the formation of a cyclopropylcarbinyl cation intermediate and predicts the absence of a β-positioned methyl group to the quaternary carbon in the skeleton. This prediction is, however, inconsistent with the firm structural establishment (1D and 2D NMR techniques, and Lindeman-Adams modelling) of a novel C_{33} botryococcane and a co-occurring C_{33} botryococcan-24-one with a uniquely positioned methyl group (β to the quaternary carbon) isolated by preparative gas chromatography.

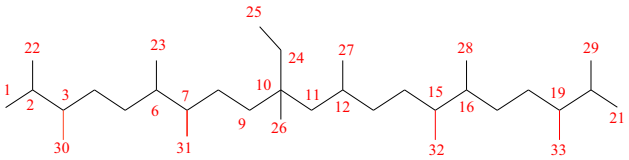
We propose a biosynthetic pathway where direct or indirect cyclobutanation (1′-2-3-2′ cyclisation) is a necessary step leading to the synthesis of C_{33} botryococcene with the unique skeleton of the identified alkane and ketone from two FPPs: electron migration induced C1′-2′ opening of the cyclobutane ring and subsequent Retro-Prins reaction with a loss of a hydroxymethyl cation gives rise to the formation of a C_{29} botryococcene. The C_{33} botryococcene formed by successive methylation in vivo could then be (photo)oxidised to a C_{33} botryococcenone in (oxic) shallow water, and reduced (hydrogenated) to a C_{33} botryococcane in (anoxic) deep-water or sediments.

Acknowledgements

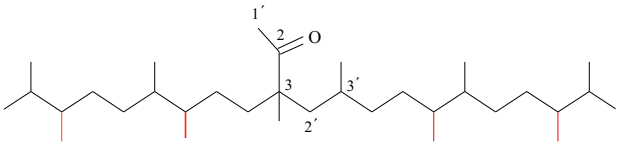
Drs. Yankuan Tian and Wenbin Zhang are thanked for their technical help with GC–MS and pGC instrumentation. This work was supported by The National Key R&D Program of China (2017YFC0603102), The Strategic Priority Research Program of the Chinese Academy of Sciences (XDA14010102), and NSFC grants (41673066, 41473045 and 41673045) to HL; a Shaanxi Provincial Talent 1000 Fellowship and a NSFC (41773032) to YZ and is presented in celebration of Prof. Tie-Guan Wang's 80th birthday. We thank the Associate Editor Philippe Schaeffer, Zhaohui Zhang and an anonymous reviewer for their very helpful comments which greatly improved the manuscript. This is contribution No. IS-2559 from GIGCAS and #5 from the Isotopomics in Chemical Biology group.

Appendix A

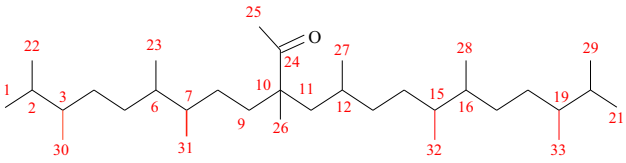
Carbon numbering used for interpretation of NMR data and mass spectra, and speculation about the biosynthetic pathway for the C_{33} botryococcane and C_{33} botryococcanone.



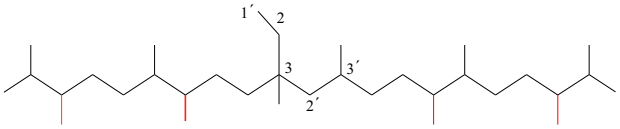
(a) The numbering of carbons in the C_{33} botryococcane as used for interpreting the NMR data and mass spectrum.



(b) The numbering of carbons in the C_{33} botryococcane as used in the tentative biosynthetic pathway (Fig. 6).



(c) The numbering of carbons in the C_{33} botryococcanone as used for naming and interpreting the NMR data and mass spectrum.



(d) The numbering of carbons in the C_{33} botryococcanone as used in the tentative biosynthetic pathway (Fig. 6).

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.orggeochem.2018.07.005.

Associate Editor—**Philippe Schaeffer**

References

Al-Qallaf, F.A., Hodson, L.F., Johnstone, R.A., Liu, J.Y., Lu, L., Whittaker, D., 2000. Heterogeneous liquid phase catalysis by metal (IV) phosphates of cyclic ether formation and a reverse Prins reaction. Journal of Molecular Catalysis A: Chemical 152, 187–200.

Blagg, B.S., Jarstfer, M.B., Rogers, D.H., Poulter, C.D., 2002. Recombinant squalene synthase. A mechanism for the rearrangement of presqualene diphosphate to squalene. Journal of the American Chemical Society 124, 8846–8853.

Brassell, S.C., Eglinton, G., Fu, J.M., 1986. Biological marker compounds as indicators of the deposition history of the Maoming oil shale. Organic Geochemistry 10, 927–941.

Breitmaier, E., Voelter, W., 1978. ^{13}C Nuclear Magnetic Resonance: Methods and Applications, Second Edition. Monographs in Modern Chemistry 5. Verlag Chemie.

Cheng, H.N., Bennett, M.A., 1991. Trends in shift rules in carbon-13 nuclear magnetic resonance spectroscopy and computer-aided shift prediction. Analytica Chimica Acta 242, 43–56.

Cheng, H.N., 1994. ^{13}C NMR spectral simulation and shift prediction. TrAC Trends in Analytical Chemistry 13, 95–104.

Coates, R.M., Robinson, W.H., 1972. Solvolysis of trans-2, 2-dimethyl-3-(2′-methylpropenyl) cyclobutyl tosylate. Model reactions relevant to squalene biosynthesis. Journal of the American Chemical Society 94, 5920–5921.

Curiale, J.A., 1987. Steroidal hydrocarbons of the Kishenehn Formation, northwest Montana. Organic Geochemistry 11, 233–244.

de Mesmay, R., Metzger, P., Grossi, V., Derenne, S., 2008. Mono- and dicyclic unsaturated triterpenoid hydrocarbons in sediments from Lake Masoko (Tanzania) widely extend the botryococcene family. Organic Geochemistry 39, 879–893.

Dowling, L.M., Boreham, C.J., Hope, J.M., Murray, A.P., Summons, R.E., 1995. Carbon isotopic composition of hydrocarbons in ocean-transported bitumens from the coastline of Australia. Organic Geochemistry 23, 729–737.

Eglinton, T.I., Aluwihare, L.I., Bauer, J.E., Druffel, E.R.M., McNichol, A.P., 1996. Gas chromatographic isolation of individual compounds from complex matrices for radiocarbon dating. Analytical Chemistry 68, 904–912.

Gao, M., Simoneit, B.R., Gantar, M., Jaffé, R., 2007. Occurrence and distribution of novel botryococcene hydrocarbons in freshwater wetlands of the Florida Everglades. Chemosphere 70, 224–236.

Grice, K., Schouten, S., Nissenbaum, A., Charrach, J., Sinninghe Damsté, J.S., 1998. A remarkable paradox: sulfurised freshwater algal (*Botryococcus braunii*) lipids in an ancient hypersaline euxinic ecosystem. Organic Geochemistry 28, 195–216.

Grossi, V., de Mesmay, R., Bardoux, G., Metzger, P., Williamson, D., Derenne, S., 2012. Contrasting variations in the structure and stable carbon isotopic composition of botryococcenes through the last glacial–interglacial transition in Lake Masoko (southern Tanzania). Organic Geochemistry 43, 150–155.

Guy-Ohlson, D., 1992. *Botryococcus* as an aid in the interpretation of palaeoenvironment and depositional processes. Review of Palaeobotany and Palynology 71, 1–15.

Huang, Y., Murray, M., 1995. Identification of 1,6,17,21-octahydrobotryococcene in a sediment. Journal of the Chemical Society, Chemical Communications, 335–336.

Huang, Y., Murray, M., Eglinton, G., Metzger, P., 1995. Sacredicene, a novel monocyclic C_{33} hydrocarbon from sediment of Sacred Lake, a tropical freshwater lake, Mount Kenya. Tetrahedron Letters 36, 5973–5976.

Huang, Z., Poulter, C.D., 1989. Stereochemical studies of botryococcene biosynthesis: analogies between 1′-1 and 1′-3 condensation in the isoprenoid pathway. Journal of the American Chemical Society 111, 2713–2715.

Jarstfer, M.B., Zhang, D.L., Poulter, C.D., 2002. Recombinant squalene synthase. Synthesis of non-head-to-tail isoprenoids in the absence of NADPH. Journal of the American Chemical Society 124, 8834–8845.

Jarstfer, M.B., Blagg, B.S., Rogers, D.H., Poulter, C.D., 1996. Biosynthesis of squalene. Evidence for a tertiary cyclopropylcarbinyl cationic intermediate in the rearrangement of presqualene diphosphate to squalene. Journal of the American Chemical Society 118, 13089–13090.

Lindeman, L.P., Adams, J.Q., 1971. Carbon-13 nuclear magnetic resonance spectrometry. Chemical shifts for the paraffins through C_9. Analytical Chemistry 43, 1245–1252.

Maxwell, J.R., Douglas, A.G., Eglinton, G., McCormick, A., 1968. The Botryococcenes–hydrocarbons of novel structure from the alga *Botryococcus braunii*, Kützing. Phytochemistry 7, 2157–2171.

McKirdy, D.M., Cox, R.E., Volkman, J.K., Howell, V.J., 1986. Botryococcane in a new class of Australian non-marine crude oils. Nature 320, 57–59.

McKirdy, D.M., Summons, R.E., Padley, D., Serafini, K.M., Boreham, C.J., Struckmeyer, H.I.M., 1994. Molecular fossils in coastal bitumens from southern Australia: signatures of precursor biota and source rock environments. Organic Geochemistry 21, 265–286.

Metzger, P., Casadevall, E., 1983. Structure de trois nouveaux "botryococcenes" synthetises par une souche de *Botrycoccus braunii* cultivee en laboratoire. Tetrahedron Letters 24, 4013–4016.

Metzger, P., Berkaloff, C., Casadevall, E., Coute, A., 1985a. Alkadiene- and botryococcene-producing races of wild strains of *Botryococcus braunii*. Phytochemistry 24, 2305–2312.

Metzger, P., Casadevall, E., Pouet, M.J., Pouet, Y., 1985b. Structures of some botryococcenes: branched hydrocarbons from the B-race of the green alga *Botryococcus braunii*. Phytochemistry 24, 2995–3002.

Metzger, P., David, M., Casadevall, E., 1986. Biosynthesis of triterpenoid hydrocarbons in the B-race of the green alga *Botryococcus braunii*. Sites of production and nature of the methylating agent. Phytochemistry 26, 129–134.

Metzger, P., Casadevall, E., Coute, A., 1988. Botryococcene distribution in strains of the green alga *Botryococcus braunii*. Phytochemistry 27, 1383–1388.

Metzger, P., Largeau, C., 1999. Chemicals of *Botryococcus braunii*. In: Cohen, Z. (Ed.), Chemicals from Microalgae. Taylor & Francis, London, pp. 210–211.
Moldowan, J.M., Seifert, W.K., 1980. First discovery of botryococcane in petroleum. Journal of the Chemical Society, Chemical Communications 19, 912–914.
Okada, S., Murakami, M., Yamaguchi, K., 1995. Hydrocarbon composition of newly isolated strains of the green microalga *Botryococcus braunii*. Journal of Applied Phycology 7, 555–559.
Özek, T., Demirci, F., 2012. Methods in molecular biology: Isolation of natural products by preparative gas chromatography. In: Satyajit, D.S., Lutfun, N. (Eds.), Natural Products Isolation. Humana Press, pp. 275–300.
Pan, J.J., Solbiati, J.O., Ramamoorthy, G., Hillerich, B.S., Seidel, R.D., Cronan, J.E., Almo, S.C., Poulter, C.D., 2015. Biosynthesis of squalene from farnesyl diphosphate in bacteria: three steps catalyzed by three enzymes. ACS Central Science 1, 77–82.
Philp, R.P., Lewis, C.A., 1987. Organic geochemistry of biomarkers. Annual Review of Earth and Planetary Sciences 15, 363–395.
Poulter, C.D., 1990. Biosynthesis of non-head-to-tail terpenes. Formation of 1′-1 and 1′-3 linkages. Accounts of Chemical Research 23, 70–77.
Rilling, H.C., Epstein, W.W., 1969. Mechanism of squalene biosynthesis. Presqualene, a pyrophosphorylated precursor to squalene. Journal of Biological Chemistry 91, 1041–1042.
Rilling, H.C., Poulter, C.D., Epstein, W.W., Larsen, B., 1971. Studies on the mechanism of squalene biosynthesis. Presqualene pyrophosphate, stereochemistry and a mechanism for its conversion to squalene. Journal of the American Chemical Society 93, 1783–1785.
Rontani, J.F., 2001. Visible light-dependent degradation of lipidic phytoplanktonic components during senescence: a review. Phytochemistry 58, 187–202.
Rontani, J.F., Giusti, G., 1988. Photosensitized oxidation of phytol in seawater. Journal of Photochemistry and Photobiology A: Chemistry 42, 347–355.
Rontani, J.F., Bonin, P., Giusti, G., 1987. Mechanistic study of interactions between photo-oxidation and biodegradation of *n*-nonylbenzene in seawater. Marine Chemistry 22, 1–12.
Rontani, J.F., Belt, S.T., Vaultier, F., Brown, T.A., 2011. Visible light induced photo-oxidation of highly branched isoprenoid (HBI) alkenes: significant dependence on the number and nature of double bonds. Organic Geochemistry 42, 812–822.
Summons, R.E., Capon, R.J., 1991. Botryococcenone, an oxygenated botryococcene from *Botryococcus braunii*. Australian Journal of Chemistry 44, 313–322.
Thulasiram, H.V., Erickson, H.K., Poulter, C.D., 2008. A common mechanism for branching, cyclopropanation, and cyclobutanation reactions in the isoprenoid biosynthetic pathway. Journal of the American Chemical Society 130, 1966–1971.
van Tamelen, E.E., Schwartz, M.A., 1971. Mechanism of presqualene pyrophosphate-squalene biosynthesis. Journal of the American Chemical Society 93, 1780–1782.
Volkman, J.K., 2014. Acyclic isoprenoid biomarkers and evolution of biosynthetic pathways in green microalgae of the genus *Botryococcus*. Organic Geochemistry 75, 36–47.
Zhang, D.L., Poulter, C.D., 1995. Biosynthesis of non-head-to-tail isoprenoids. Synthesis of 1′-1 and 1′-3 structures by recombinant yeast squalene synthase. Journal of the American Chemical Society 117, 1641–1642.
Zhang, Z., Metzger, P., Sachs, J.P., 2007. Biomarker evidence for the co-occurrence of three races (A, B and L) of *Botryococcus braunii* in El Junco Lake, Galápagos. Organic Geochemistry 38, 1459–1478.
Zuo, H.L., Yang, F.Q., Huang, W.H., Xia, Z.N., 2013. Preparative gas chromatography and its applications. Journal of Chromatographic Science 51, 704–715.