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Novel Unsaturated Triterpenoid Hydrocarbons from Sediments of Sacred Lake, Mt.Kenya, Kenya

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Abstract: A novel family of three isoprenoidal hydrocarbons (**1-3**), a $C_{34}H_{64}$ and two $C_{33}H_{60}$, have been isolated from lacustrine sediment deposited 5000-18,000 yrs BP (^{14}C dating) and their structures and stereochemistry determined from the interpretation of 1D and 2D ^{1}H and ^{13}C NMR and mass spectral data as well as degradation by ozonolysis. These compounds are attributed to algae, based on their close structural relationship with botryococcenes and other isoprenoidal hydrocarbons produced by the green microalga *Botryococcus braunii*. Copyright © 1996 Elsevier Science Ltd

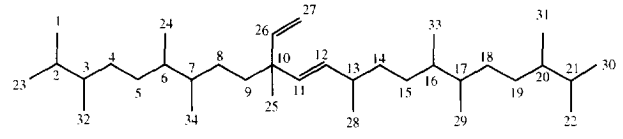
INTRODUCTION

Sacred Lake is a small (0.51km^2), shallow (5m) crater lake situated at 2350m a.s.l. on the slopes of Mt. Kenya, Kenya, in humid montane rain forest. Sediment cores from the lake (and several East African high mountain lakes) provide a high resolution record of changes in the Quaternary terrestrial and aquatic environment and ecology, and have been the subject of intensive interdisciplinary research.[1-4] Stratigraphic studies of the pollen and bulk carbon isotope record for Sacred Lake indicate a dramatic change in the surrounding vegetation from grassland (mainly C_4) in the last glacial to forest (C_3) at present.[3] Exceptionally high $\delta^{13}C$ values are encountered in this and other lake sediments (e.g. -4.44‰ vs PDB in Lake Bosumtwi, Ghana[4]). Such values are normally inaccessible to terrestrial plants, though they may be produced by algae under special conditions.[3-4]

Examination of lipid constituents and their individual carbon isotopic compositions in 15 horizons of Sacred lake sediments (^{14}C age between 20 to 30,000 yrs BP) has revealed the first direct evidence of substantial algal contribution to the sediment bulk carbon isotope record.[5-6] Two novel isoprenoid hydrocarbons, namely 1,6,17,21-octahydrobotryococcene **1** and a cyclic compound **2**, were identified in the sediment extracts. We reported the isolation and the structures of **1** and **2** in two preliminary communications[5-6] and proposed the name sacredicene for **2**. In this paper, we report the identification of an analogue of sacredicene, **3**, isolated from a sediment horizon deposited ca. 5,000 yrs BP and also the full NMR and MS characterisation for **1** and **2**. Since **2** and **3** have the same molecular formula and are structurally similar, we propose the names sacredicene A and B

for **2** and **3**, respectively. Ozonolysis of these compounds, followed by GC-MS analyses, confirmed the double bond positions in **1-3**.

Shifts in $\delta^{13}C$ values of compounds **1** and **2** measured by gas chromatography-isotope ratio mass spectrometry (GCIRMS) are positively correlated with those for the bulk carbon isotope record. $\delta^{13}C$ values in the range of -5.1 to -14.3‰ (vs. PDB) were found for 1,6,17,21-octahydrobotryococcene in the glacial horizons of Sacred Lake core SL1.[5-6] Compounds **1** and **2** exhibit a very close structural relationship with some botryococcenes which constitute a wide family of triterpenoid hydrocarbons of general formula C_nH_{2n-10} (n=30-37), produced specifically by the B race of the contemporary freshwater microalga, *Botryococcus braunii*.[7-14] Biosynthetic experiments indicated that a parent triterpene (botryococcene C_{30}), **4**, formed by condensation of two farnesyl units, is successively methylated by S-adenosylmethionine at C-3, C-7, C-16 and C-20, to yield all higher members of the botryococcene family.[15,16] In some cases the electrophilic methylation can give rise to the formation of cyclohexyl compounds.[9,10-12,14] Botryococcenes have not been previously reported in recent sediments, although *B. braunii* (B race) is widely distributed in freshwater lakes and renowned for producing high amounts of these hydrocarbons.[7] The low resistance of such polyunsaturated compounds to diagenesis could be related to their ability to polymerize or to their sensitivity towards microbial attack. However, the abundance of sacredicenes is so high in Sacred Lake sediments that, in a horizon ^{14}C dated 2200 yrs BP, almost 15mg of sacredicenes were extracted from a single gram of sediment.



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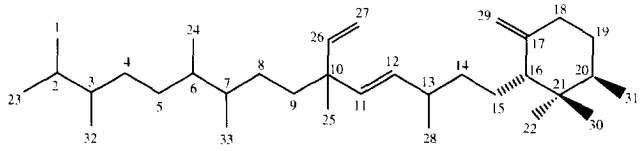

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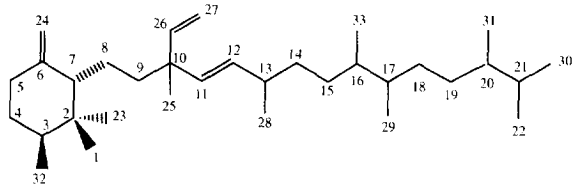

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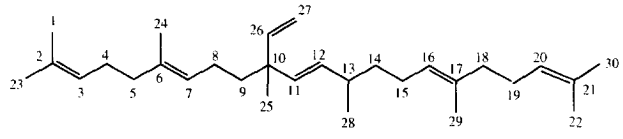

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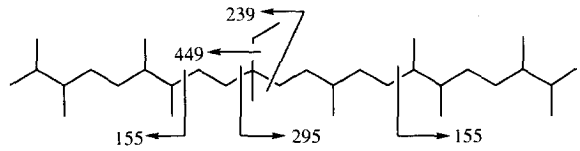
RESULTS AND DISCUSSION

The aliphatic hydrocarbon fractions were separated from the total extracts of the sediment samples by liquid chromatography, as indicated in the experimental section. Capillary gas chromatography and coupled gas chromatography-mass spectrometry (GC-MS) analysis of these fractions showed the presence of mainly *n*-alkanes (C_{21} to C_{33}), hopanes and hopene derivatives (C_{27}, C_{29}-C_{32}), an unresolved complex mixture occurring around the retention time for C_{21} *n*-alkane, and a series of highly branched compounds with molecular weights ranging from 442 to 474. Three major branched hydrocarbons **1-3** were isolated from sediment horizons containing relatively high abundances of the target compounds (^{14}C ages for these horizons: 14,500; 10,280; 5,000 yrs BP), using the procedures outlined in the experimental section. The highly branched nature of these C_{33} and C_{34} compounds was apparent from their pseudo Kováts retention indexes (see experimental section for definition) on a nonpolar HP Ultra-1 capillary column: **1** (2789.2); **2** (2759.7); **3** (2738.6), all eluting between the C_{27} and C_{28} *n*-alkanes.

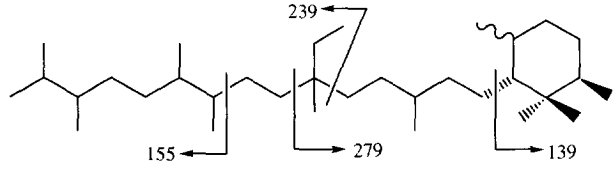
The chromatographic behaviour, mass spectra and NMR data indicated the following molecular formulae for the three compounds: **1** $C_{34}H_{66}$ with two degrees of unsaturation; **2** $C_{33}H_{60}$ and **3** $C_{33}H_{60}$ both with four degrees of unsaturation. The mass spectral fragmentation patterns of compounds **1** and **2** were informative, as they revealed cleavages allylic to the double bonds and adjacent to the quaternary centre C-10.[5-6] However, the mass spectrum of **3**[17] is more complicated than that of **2**: m/z 291, derived from the long chain side fragment which cleaved adjacent to the quaternary carbon centre C-10, weakened considerably to 3% (the corresponding m/z 273 ion in **2** is 21%), while the m/z 177 of a complicated origin becomes dominant. Comparison of mass spectra for sacredicenes **2-3** indicates that the intensity of m/z 177 may be strongly influenced by the distance between the cyclohexyl moiety and the quaternary carbon centre and possibly formed by re-arrangements involving the quaternary C-10.

Catalytic hydrogenation of **1** resulted in a single diastereomer **1H** which co-eluted with an authentic botryococcane on capillary GC,[5] while hydrogenation of **2** and **3** produced two diastereomers **2H** and two diastereomers **3H** in each case (diastereomer pairs have identical mass spectra). The diastereomers, readily separated by capillary GC and GC-MS, were very likely the result of the reduction of the methylene cyclohexyl double bond, giving rise to an axial or an equatorial methyl, depending on the stereochemistry of the hydrogenation. RuO_4 treatments[18] of **1H-3H** confirmed the lack of double bonds in **1H-3H**, which resulted in no further changes of the saturated compounds. The EI mass spectra of **1H-3H** showed major fragmentations around the quaternary centre C-10 and were particularly informative about the locations of the ring for **2** and **3**. For instance, diagnostic ions at m/z 223 and 295 in **3H**, in contrast to m/z 239 and 279 in **2H**, indicate the ring located in the short (**3**) and long chain (**2**) side, respectively. In addition, the major fragments at m/z 294 and 238 in **1H** are indicative of the presence of a long and a short acyclic chain, respectively, as previously observed from the mass spectra of the synthetic or fossil C_{34} botryococcane.[7,19]

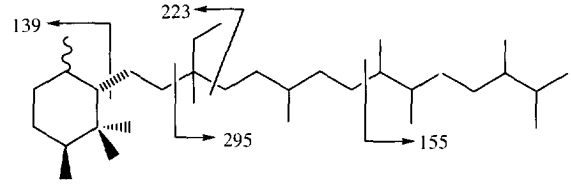
Both 1D and 2D NMR experiments (^1H, ^{13}C, DEPT, ^1H-^1H COSY, ^1H-^{13}C COSY, long range ^1H-^{13}C COSY, NOEDF, NOESY, HOHAHA difference) were performed in order to fully assign ^1H and ^{13}C chemical shifts for compounds **1-3** (Table 1) and determine the ring geometries. Full assignments for compound **3** benefited from comparisons of the data for **2** and **3**, since the cyclizations at one side of the chains in **2-3** helped clarify some previously interchangeable chemical shifts occurring at the long or short chain sides of **1**.



(1H)



(2H)



(3H)

The ^{1}H and ^{13}C NMR spectra of **1-3** show signals for two fragments, -C-CH=CH_2 and -C-CH=CH-CH(Me)- with an E geometry due to the large coupling constant of the olefinic protons (^{3}J=15.6), as illustrated in partial structure **5**. Such an arrangement is present in the middle of the chain of each botryococcene type hydrocarbon as a result of the 1'-3 condensation of two C_{15} isoprenoid units[20]. However, both compounds **2-3** show the presence of a terminal double bond >C=CH_2. In addition to mass spectral evidence, the presence of a ring in **2-3** was also suggested by ^{1}H and ^{13}C NMR data. For example, in **2**, presence of ^{13}C signals at δ56.7 (CH by DEPT; C-16) and δ36.8 (C by DEPT; C-21), and comparisons of ^{13}C NMR data with **1** are consistent with a cyclisation via these two carbon atoms (C-16 and C-21). Changes of ^{1}H chemical shifts and multiplicities for C_{22}, C_{30} and C_{31} in **2**, in comparison to **1**, further confirmed the presence of the ring. Compounds **2** and **3** have similar ^{13}C signals at δ57.0 (CH by DEPT; C-7) and δ36.9 (C by DEPT; C-2) and similar ^{1}H NMR in the methyl and olefinic regions, also indicating the presence of a ring.

^{1}H-^{1}H COSY gives connectivities appropriate for partial structures **5** and **6**. Since NMR spectra are analogous for compounds **1-3**, the discussion below will use compound **3** as an example (Table. 1). In compound **3**, the vinyl proton at C-26 was linked to the H-27 resonance at δ4.90 (J=17.4 Hz) and δ4.93 (J=10.7 Hz). A connectivity pattern began at H-11 with crosspeaks to the C-12 proton (J=15.6 Hz) and the C-13 methine (J=1.0 Hz). The signal for the C-13 methine had crosspeaks to the H-12 resonance (J=7.9 Hz), the C-28 methyl (J=6.7 Hz), and the non-equivalent C-14 methylene protons. The C-7 methine resonance coupled strongly to the C-8 proton signal at δ1.39 (J=11.3 Hz), weakly to C-9 methylene peak at δ1.23 (J=3.4 Hz), and trans across the exocyclic double bond to the C-24 signal (Hb) at δ4.509 (J=3.05 Hz), which was also coupled to the other C-24 resonance at δ4.676 (J=1.5 Hz). Both protons at C-24 gave crosspeaks to the two C-5 proton signals at δ2.04. COSY peaks also joined the non-equivalent methylenes at C-5 and C-4 and the C-3 methine to signals for

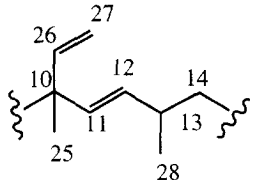
Table 1. NMR spectral data for compounds **1-3** (in $CDCl_3$, TMS int. ref.) isolated from sediments of Sacred Lake, Mt. Kenya.

Carbon No.	1		2		3	
	^1H (500)	^{13}C (125.1)	^1H (500)	^{13}C (125.1)	^1H (500)	^{13}C (125.1)
1	0.856,3H,d,6.8	20.43	0.857,3H,d,6.7	20.44	0.735,3H,s	21.68
2	1.57,1H,m	31.66	1.56,1H,m	31.64		36.92
3	1.22,1H,m	38.94	1.23,1H,m	38.92	1.59,1H,m	34.79
4	1.33,2H,m	30.71	1.34,2H,m	30.66	a: 1.16-1.24,1H.m e: 1.4-1.46,1H.m	32.06
5	1.34,2H,m	32.35	1.33,2H,m	32.26	2.04,2H,m	30.97
6	1.27,1H,m	37.92	1.28,1H,m	37.85		149.48
7	1.27,1H,m	38.16	1.28,1H,m	38.13	1.582,1H,dd,11.3,3.4	57.03
8	0.96-1.25,2H,brm	27.19	0.96-1.25,2H,brm	27.17	1.39,2H,m	20.70
9	1.23,2H,m	39.49	1.25,2H,m	39.52	1.23,2H,m	39.80
10		42.00		42.00		41.89
11	5.305,1H,dd,15.8,1.0	135.94	5.299,1H,dd,15.6,0.9	135.85	5.293,1H,dd,15.6,1.0	135.76
12	5.145,1H,dd,15.8,7.9	133.77	5.15,1H,dd,15.6,7.9	134.00	5.143,1H,dd,15.6.7.93	133.86
13	2.01,1H,m	37.33	2.02,1H,m	37.31	2.01,1H,m	37.34
14	1.16-1.25,2H,m	35.31	0.99,1.17,2H,m	35.80	1.12-1.2,1.23-1.28,2H,m	35.37
15	1.34,2H.m	32.29	1.42,2H,m	23.95	1.34,2H,m	32.34
16	1.29,1H,m	38.10	1.628,1H,dd,11.3,3.7	56.63	1.32,1H,m	38.05
17	1.30,1H,m	37.55		149.70	1.31,1H,m	37.59
18	1.34,2H,m	30.85	2.05,2H,m	30.94	1.39,2H,m	30.86
19	1.26,2H,m	30.44	a: 1.17-1.2,1H,m e: 1.45-1.48,1H,m	32.07	1.28,2H,m	30.46
20	1.26,1H,m	38.95	1.6,1H,m	34.73	1.23,1H,m	38.97
21	1.57,1H,m	31.63		36.80	1.57,1H,m	31.64
22	0.856,3H,d,6.8	20.42	0.871,3H,s	26.91	0.859,3H,d,6.71	20.45
23	0.786,3H,d,6.9	17.70	0.79,3H,d,7.0	17.73	0.872,3H,s	26.92
24	0.802,3H,d,6.4	16.69	0.806,3H,d,6.4	16.72	4.676,1H,m 4.509,1H,dd,3.05,1.0	109.33
25	1.03,3H,s	23.68	1.042,3H,s	23.67	1.031,3H,s	23.91
26	5.789,1H,dd,17.5,10.8	147.06	5.781,1H,dd,17.4, 10.6	147.04	5.763,1H,dd,17.4,10.7	147.16
27	4.944,1H,dd,10.8,1.5; 4.922,1H,dd,17.4,1.5	110.94	4.937,1H,dd,10.7,1.7; 4.916,1H, dd,17.3,1.8	110.97	4.925,1H,dd,10.7,1.52 4.903,1H,dd,17.4,1.53	110.88
28	0.963,3H,d,6.6	21.35	0.936,3H,d,6.7	20.97	0.959,3H,d,6.71	21.37
29	0.801,3H,6.5	16.58	4.671,1H,m; 4.51,1H,dd,2.8,1.2	109.10	0.804,3H,d,6.71	16.61
30	0.786,3H,d,6.9	17.74	0.735,3H,s	21.65	0.79,3H,d,6.75	17.73
31	0.781,3H,d,6.6	15.44	0.775,3H,d,5.8	15.82	0.79,3H,d,6.75	15.46
32	0.781,3H,d,6.6	15.44	0.783,3H,d,5.5	15.44	0.771,3H,d,7.02	15.79
33	0.81,3H,d,6.8	16.65	0.806,3H,d,6.4	16.72	0.803,3H,d,6.80	16.67
34	0.804,3H,d,6.4	16.73				

the C-4 methylene and C-32 methyl. In the rest of **3**, COSY crosspeaks connected C-30 methyl to the C-21 methine (J=6.75 Hz); H-22 to H-21 (J=6.75 Hz); H-31 to H-20 (J=6.75 Hz); H-29 to H-17 (J=6.71 Hz) and H-33 to H-16 (J=6.8 Hz).

NOE difference and 2D-NOESY experiments gave additional spatial connections and stereochemical properties of the ring moiety in **2-3**. For instance, in compound **3**, positive NOEs (≥ 2%) were observed between the protons of methyl 32 and those of the *gem* methyl 1 and 23, hence establishing an equatorial orientation for methyl 32. Similarly, the equatorial orientation for the proton at C-7 is confirmed by NOE enhancement after irradiation of the protons at 1 (0.735 ppm) and 23 (0.872 ppm). NOEs for protons at C-3 (1.59 ppm), C-7 (1.58 ppm), C-8 (1.39 ppm), C-1 (0.735 ppm) and C-32 (0.771 ppm) were observed upon irradiation of the C-23 protons. Likewise, irradiation of the C-1 protons resulted in NOEs for protons at C-7 (1.58 ppm), C-4 axial (1.2 ppm), C-24 Hb (4.51 ppm) and C-23 (0,872 ppm) (but not for C-3). Finally, irradiation of the C-32 protons gave NOEs for protons at C-3 (1.59 ppm), C4 (1.2 and 1.44 ppm), C-1 and C23. In the NOESY spectrum, significant negative (relative to the positive diagonal signals) crosspeaks were shown between olefinic Ha (4.51 ppm) and allylic C-7 proton (1.58 ppm) and between Ha and the C-1 protons; also between Hb (4.68 ppm) and the C-5 protons (2.04 ppm), and between Hb and C-4 equatorial (1.43 ppm). In addition, NOESY crosspeaks were observed between olefinic C-11 and C-12 protons and C-13 (2.01 ppm), C-28 (0.96 ppm) and C-14 (1.1 and 1.24 ppm) protons; between C-26 and C-25 protons (1.03 ppm); and between C-27 and C-25 (1.03 ppm), C-27 and C-9 protons (1.23 ppm).

A HOHDF (Homonuclear Hartmann-Hahn difference) experiment was performed by excitation of C-32 protons (0.771 ppm). It resulted in clear enhancement for protons at C-3, C-4 and C-5 and was useful in refining the chemical shift assignments, particular for the equatorial and axial protons at C-4. The coupling constant between H-4 axial (1.2 ppm) and H-3 proton (1.59 ppm) is significantly larger than that between H-4 equatorial (1..42 ppm) and H-3, as expected.



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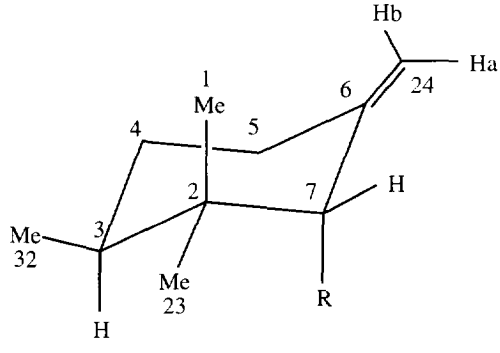

(6)

A 3.35 ppm upper-field shift of the ^{13}C resonance for C-8 (20.70 ppm) in sacredicene **3**, in comparison with C-15 (23.95 ppm) in sacredicene **2**, can be readily explained by a γ-effect of the additional vinyl group (C26=C27) on the quaternary C-10 in sacredicene **3**.

Ozonolysis The hydrocarbons **1-3** were subjected to ozonolysis in order to confirm the double bond positions, as illustrated in Fig. 1. The aldehydes and ketoaldehydes derived from reductive decomposition (using

triphenylphosphine) of the resulting polyozonides were directly analysed by GC-MS. The dialdehydes **7** and **9** were stable under GC-MS operating conditions. Table 2 gives the major MS fragments of cleavage products **7-10** (origins of some fragments are also shown in Fig. 1).

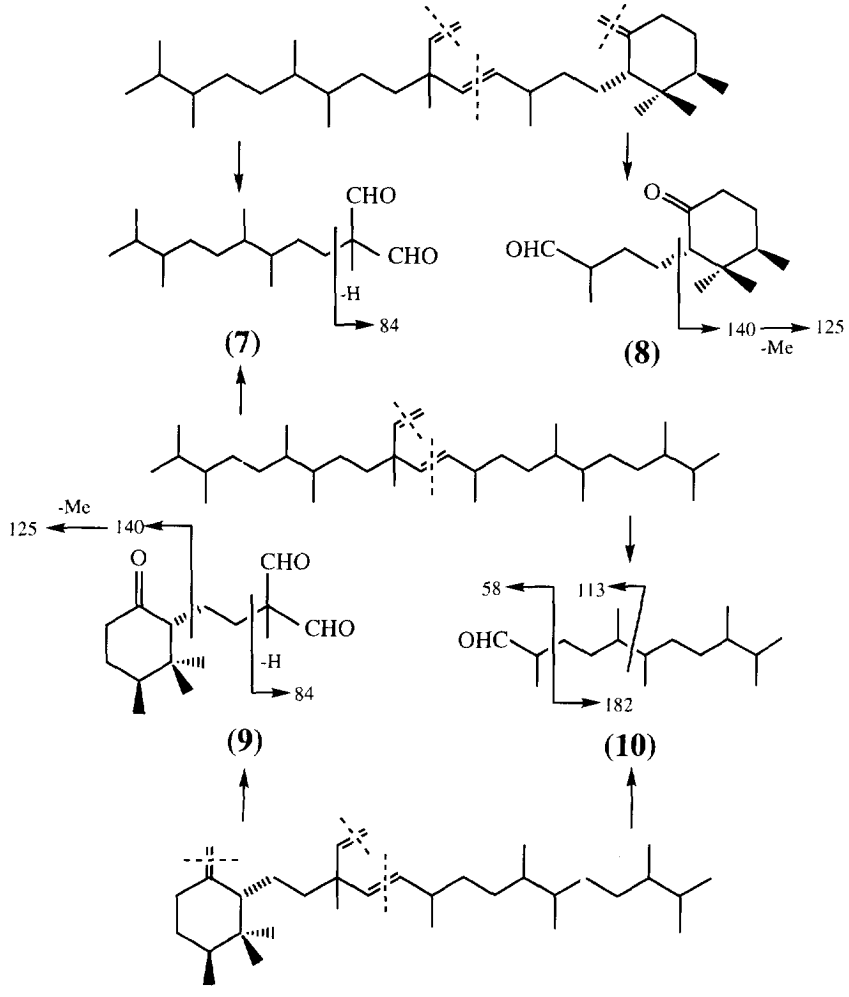

Fig. 1. Ozonolysis of unsaturated hydrocarbons **1-3.** Reductive decomposition of the polyozonides and the major MS fragments of the cleavage products

Biosynthesis: the 1,6,17,21-octahydrobotryococcene and sacredicenes identified in Sacred Lake sediments have not previously been identified in living *Botryococcus*. Their coexistence in some sediment horizons with unchanged botryococcenes[21] incline us to believe that they originate, directly or indirectly, from this microalga. **1-3** have new stereocentres (e.g. at C-6 and C-17 in **1**) which are not present in the parent C_{34} botryococcene, yet only exist as single diastereomers in the sediments as demonstrated by capillary GC and NMR

Table 2. Mass Spectral Data of Cleavage Products (relative intensities of major ions in parentheses; cf. Fig.1).

7	268 $[M]^+$ (1); 240 $[M\text{-}CO]^+$ (9); 184 (4); 154 (8); 142 (8); 123 (23); 113 (15); 95 (30); 84 $[OHC\text{-}C(CHO)=CH_2]^+$ (34); 71 (100); 57 (64); 43 (95)
8	224 $[M]^+$ (2); 209 $[M\text{-}Me]^+$ (6); 181 $[M\text{-}CO\text{-}Me]^+$ (26); 153 (8); 140 (38); 125 (100); 98 (20); 83 (35); 69 (19); 55 (44); 41 (30)
9	252 $[M]^+$ (0); 234 $[M\text{-}H_2O]^+$ (6); 206 (2); 153 (21); 140 (31); 125 (100); 98 (21); 84 $[OHC\text{-}C(CHO)=CH_2]^+$ (36); 83 (43); 55 (45); 41 (34)
10	240 $[M]^+$ (3); 182 (7); 154 (7); 123 (23); 113 (36); 95 (42); 85 (42); 71 (85); 58 (41); 57 (83); 43 (100)

data and, moreover, the C26=C27 vinyl—the most easily reducible double bond in botryococcenes,[5,7,8] is left unchanged. Both factors lead us to propose that their origin via an abiotic reduction of parent botryococcenes is rather unlikely. However, at the present time, there is no evidence for the production of such compounds by a variant of the B race of *B. braunii*. If that were the case the alkylation pathway leading from a parent C_{30} compound to **1-3** would be probably close to the one operating in the synthesis of 24β-methyl sterols with a saturated side chain reported for green algae.[22] Another possibility would be a microbial reduction of the disubstituted methylene double bonds located in the acyclic moieties of the botryococcenes.

EXPERIMENTAL

Sediment material: Core SL1 was collected from the deepest part of Sacred lake (altitude 2350m a.s.l., maximum water depth 5m, pH 5.0-6.1, vegetation: humid montane rain forest, mean annual rainfall ca. 1780 mm; length of the core 16.34m). Samples were sectioned at 1cm intervals and stored at 4°C before analyses. The ages of the sediment samples were determined by ^{14}C dating by conventional or accelerator mass spectrometer (AMS) as well as U/Th dates of tephras and peat for the bottom of the core. The oldest sample (bottom) was over 100,000 yrs BP (U/Th)[3].

Extraction and Isolation: Up to 13g (usually 5g or less) of the sediment samples were freeze-dried overnight and then extracted ultrasonically using a solvent system of sequentially decreasing polarity (MeOH×2, MeOH:CH_2Cl_2 (1:1)×2, CH_2Cl_2×2), in order to obtain total lipids. Carboxylic acids were then isolated from the extract by using solid phase extraction (Aminopropyl Bond Elute®), which quantitatively retains acids when total extracts are flashed through with CH_2Cl_2:isopropanol 2:1 (acids were subsequently recovered with 2% acetic acid in ether). The neutral fractions were chromatographed on silica gel flash column (hexane, CH_2Cl_2 and CH_2Cl_2:MeOH 1:1) or TLC (ethyacetate:hexane 7:1) to obtain 'aliphatic hydrocarbons'. The aliphatic hydrocarbons were urea-adducted to remove *n*-alkanes/alkenes and subsequently separated into several fractions by developing repeatedly over TLC plates impregnated with 15% $AgNO_3$, using different solvents (hexane; hexane:CH_2Cl_2 1:1; CH_2Cl_2). Fractions containing the highest abundance of target compounds were combined and further purified by reversed phase HPLC (C18, 20 cm× 4 mm, Waters; solvent: MeOH-MeCN (4:1), 1.5 ml min^{-1}; Refractive index detector). Compounds **1** (2mg), **2** (2mg) and **3** (9mg) were isolated and then checked by GC-FID, being generally over 90%.

Hydrogenation: 1,6,17,21-octahydrobotryococcene **1** and the sacredicenes **2-3** were catalytically reduced at 1 atm H_2 (catalyst: PtO_2), using 10% hexane in ethyl acetate as solvent. After stirring overnight, the catalyst was removed by filtration through a short silica gel column and solvent eliminated by rotary evaporation.

Ozonolysis: Hydrocarbons **1-3** (ca. 1-3mg), dissolved in CS_2, were ozonised at -78°C until the blue color of O_3 persisted. Excess O_3 was eliminated by bubbling N_2 through the cold solution. The ozonides were subsequently reduced by addition of triphenylphosphine and the reaction mixture then allowed to warm to room temperature. Solvent was evaporated under reduced presure and the compounds then analysed directly by GC-MS (with injector temperature 250°C).

Gas chromatography (GC): Analyses were carried out on a Varian 3400 GC fitted with split/splitless injector and FID. An HP Ultra-1 fused silica capillary column (50m×0.32mm; 0.17μm film thickness) was used. H_2 was used as carrier gas with a flow of ca. 2ml/min. Typical temperature programme was: 40 °C isothermal 1 min, 10 °C/min to 180 °C, then 4 °C/min to 300 °C, isothermal 20 min. The pseudo Kováts retention indexes were calculated according to the formula: $Index = 100n + 100 * \left[\frac{R_x - R_n}{R_{n+1} - R_n}\right]$, where x is the compound of interest; n is the carbon number for the nearest *n*-alkane eluting in front of x on GC; R denotes GC retention time.

Gas Chromatography-Mass Spectrometry (GC-MS): 70 eV EI analyses were performed on a Carlo Erba Mega gas chromatograph (on-column injection) interfaced directly with a Finnigan 4500 mass spectrometer. Typical conditions were: column (CPSil-5CB, 50m×0.32mm, film thickness 0.12mm, fused silica capillary; CHROMPACK), helium as carrier gas.

Gas Chromatography-Isotope Ratio-Mass Spectrometry (GC-IRMS): Compound specific carbon isotopic analyses were performed using a Varian 3400 GC attached to a Finnigan MAT Delta-S isotope ratio mass spectrometer via a combustion interface consisting of an alumina reactor (0.5 mm ID) containing copper and platinum wires (0.1 mm OD).

Nuclear Magnetic Resonance: 1D and 2D ^1H and ^{13}C NMR spectra were measured on a JEOL ALPHA-500 spectrometer. ^1H-^{13}C correlation spectra were measured with an inverse probe and field gradient accessory. The chemical shifts (δ) are reported in ppm downfield from TMS and are internally referenced to the $CDCl_3$ solvent. Coupling constants are given in Herz.

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17. Major MS fragments of (**3**) (GC-MS, EI, 70eV): m/z 57 (93), 71 (63), 81 (100), 95 (79), 109 (67), 123 (48), 137 (23), 149 (12), 153 (13), 177 (70), 189 (10), 217 (12), 291 (3), 374 (4), 441 (6), 456 (3). and (**3**) after catalytic hydrogenation: 57 (100), 71 (89), 83 (59), 97 (32), 113 (22), 127 (28), 139 (20), 153 (26), 169 (10), 222 (12), 294 (19), 433 (5), 462 (2).
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