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TWO CYCLOBOTRYOCOCCENES FROM THE B RACE OF THE GREEN ALGA *BOTRYOCOCCUS BRAUNII*

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Key Word Index—*Botryococcus braunii*; Chlorophyceae; algae; botryococcenes; triterpenoid hydrocarbons.

Abstract–Two new botryococcenes, triterpenoid hydrocarbons of general formula C_nH_{2n-10} with $n = 30 - 37$, have been isolated from the oil produced by two strains of the B race of the green alga *Botryococcus braunii*. The structures of these C_{32} and C_{34} compounds were elucidated by spectroscopic methods, especially ^1H and ^{13}C NMR; spectral assignments have been further corroborated by means of 2D ^1H–^1H and ^1H–^{13}C chemical shift correlation experiments. These highly branched hydrocarbons exhibit a six carbon ring in their carbon skeleton.

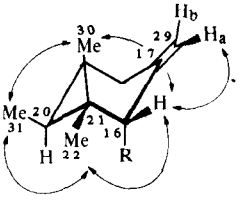
INTRODUCTION

The strains of the green colonial alga, *Botryococcus braunii*, are classified into races according to the nature of the hydrocarbons they synthesize. The recently identified L race yields a single hydrocarbon, a $C_{40}H_{78}$ tetraterpene, named lycopadiene [1, 2]. The A race synthesizes series of odd C_{23}–C_{31} *n*-alkadienes and trienes [3, 4] whereas the B race is characterized by the production of C_{30}–C_{37} triterpenoid hydrocarbons, the botryococcenes, of general formula C_nH_{2n-10} [3]. Up to now, *ca* 50 botryococcenes have been identified in the hexane extracts of wild or laboratory grown strains [2, 3, 5], and 10 structures have been determined among them [6–8]. Biosynthetic studies have shown that C_{31}–C_{37} botryococcenes are formed by methylation from their lower homologues [9]; the C_{30} compound **1**, probably synthesized by a 1′–3 condensation of two C_{15} units, acts as the precursor for all the higher metabolites.

In a preceding paper, we described the isolation from wild samples of new B strains; some of them were characterized by the production of some cyclobotryococcenes [2] as previously found in an Australian sample [8]. Here, we report the structure of two of these compounds, based on mass spectra analysis and ^1H and ^{13}C NMR investigations.

RESULTS AND DISCUSSION

GC/MS analyses of the hydrocarbon mixtures extracted from two *B. braunii* strains originating from Bolivia and the Ivory Coast, allowed us to characterize eight botryococcenes ranging from C_{32} to C_{34} [2]. From these mixtures, two new compounds, $C_{32}H_{54}$ **2** and $C_{34}H_{58}$ **3**, were further obtained as pure liquids after separation on a reversed phase C_{18} column. The IR spectra of both compounds exhibit bands for terminal methylene at 3070, 1645, 995, 910 and 890 cm^{-1} and a *trans* double bond at 975 cm^{-1}.



Scheme 1. Nuclear Overhauser effects in **2**.

Catalytic reduction of both compounds leads to the formation of two botryococcanes, spectroscopically similar in pairs by mass analysis, thus suggesting stereochemical isomerism induced by hydrogenation. On EI mass spectrometry, botryococcanes exhibit a series of three doublets in the mass region $m/z > 200$ [7, 8]; they are related to the losses of one ethyl group and two alkyl chains bound to the quaternary carbon 10. Accordingly the peaks at *m/z* 419, 418 for **2** and 447, 446 for **3** indicate that they contain a ring moiety. Furthermore, comparison of the other doublets of high mass, with that registered for their non-cyclized C_{32} and C_{34} analogues [8], shows that the ring is located in the long chain (regarding carbon 10) for **2** (*m/z*: 279, 278) and in the short chain for **3** (*m/z*: 239, 238).

According to their ^1H NMR spectra, compounds **2** and **3** show signals corresponding to the following groups: ABX system –C–CH=CH_2 (5.99 or 5.97 1H *dd*, 5.13 or 5.15 1H *dd*, 5.17 or 5.25 1H *dd*), –C–CH =CH–CH(Me)– (5.61 or 5.57 1H *dd*, 5.45 or 5.40 1H *dd*, 2.22 or 2.19 1H *m*, 1.14 or 1.12 3H *d*) with an *E* geometry for the disubstituted double bond ($J_{11,12} = 16$ Hz), –C–Me (1.26 or 1.23 3H *s*) (Tables 1 and 2). These signals are characteristic of partial structure **A** corroborated by the corresponding ^{13}C chemical shifts ; found in all botryococcenes [8] **A** arises from the 1′–3 condensation of two C_{15} units. Moreover, both ^1H and ^{13}C NMR

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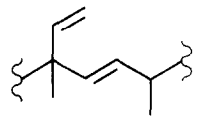
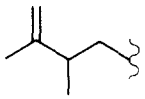


A



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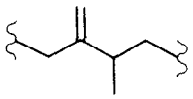

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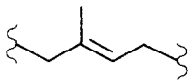


D

spectra exhibit terminal methylene and allylic methyl signals of type **B** and **C**.

In **3**, the long chain (regarding C_{10}) which does not contain the ring, comprises the **B** and **C** units as its C_{34} analogues [8]. The 2D homonuclear (COSY 45) and long range proton–carbon correlation experiments (HETCOSY), confirm partial structures **B** and **C** and allows us to combine them, together with **A**, as in **3** (Table 2). COSY and one bond HETCOSY experiments, show that protons 19 are non equivalent (δ at 1.52 and 1.65) ; moreover from long range HETCOSY, only *trans* correlations (relatively to the double bonds) are observed between protons 29 and carbon 16 or 18 and between

Table 1. NMR spectral data for C_{32} **2** (in C_6D_6, TMS int. ref.)

Assignment	^{1}H (400.13 Hz) shift	Associated protons by COSY	^{13}C (100.6 Hz) shift
1	4.90, 1H *m* 4.92, 1H *m*	1.72 (H-23); 2.24 (H-3)	110.6
2	–	–	150.3*
3	2.24, 1H *m*	1.11 (H-32); 1.52, 1.64 (H-4); 4.90, 4.92 (H-1)	41.7
4	1.52, 1H *m* 1.64, 1H *m*	2.10 (H – 5) ; 2.24 (H – 3)	34.2
5	2.10, 2H *dd* *J* = 8, 8 Hz	1.52, 1.64 (H – 4); 1.73 (H – 24); 5.38 (H – 7)	38.4
6	–	–	135.5
7	5.38, 1H *tq* *J* = 7, 1.5 Hz	1.73 (H – 24); 2.10 (H – 5); 2.21 (H – 8)	125.6
8	2.21, 2H *br m*	1.65 (H – 9) ; 1.73 (H – 24); 5.38 (H – 7)	24.2
9	1.65, 2H *br m*	2.21 (H – 8)	42.4
10	–	–	42.8
11	5.61, 1H *d* *J* = 16 Hz	2.22 (H – 13); 5.45 (H – 12)	136.6
12	5.49, 1H *dd* *J* = 16, 8 Hz	2.22 (H – 13); 5.61 (H – 11)	135.1
13	2.22, 1H *m*	1.14 (H – 28) ; 1.22, 1.46 (H – 14); 5.45 (H – 12) ; 5.61 (H – 11)	38.4
14	1.22, 1H *m* 1.46, 1H *m*	1.44, 1.62 (H – 15); 1.77 (H – 16); 2.22 (H – 13)	36.7
15	1.44, 1H *m* 1.62, 1H *m*	1.22, 1.46 (H – 14); 1.77 (H – 16)	25.0
16	1.77, 1H *dd* *J* = 11, 4Hz	1.22, 1.46 (H – 14); 1.44, 1.62 (H – 15); 4.90 (H – 29a)	57.4
17	–	–	150.1*
18	2.15, 2H *m*	1.34, 1.48 (H – 19); 4.75 (H – 29b)	31.8
19	axial:1.34, 1H *dddd* *J* = 12, 12, 12, 6 Hz equatorial 1.48, 1H *m*	1.48 (H – 19*eq*); 1.64 (H – 20); 2.15 (H – 18) 1.34 (H – 19ax); 1.64 (H – 20); 2.15 (H – 18)	32.9
20	1.64, 1H *m*	0.87 (H – 31); 1.34, 1.48 (H – 19)	35.5
21	–	–	37.5
22	1.01, 3H *s*	0.93 (H – 30)	27.6
23	1.72, 3H *br s*	4.90, 4.92 (H – 1)	19.4
24	1.73, 3H *d* *J* = 1.5 Hz	2.10 (H – 5); 2.21 (H – 8); 5.38 (H – 7)	16.5
25	1.26, 3H s		24.4

Table 1. *Continued*

Assignment	^{1}H (400.13 Hz) shift	Associated protons by COSY	^{13}C (100.6 Hz) shift
26	5.99, 1H *dd* J = 17.5, 10.5 Hz	5.13, 5.17 (H−27)	147.4
27	5.13, 1H *dd* J = 10.5, 1.5 Hz	5.99 (H−26)	112.2
	5.17, 1H *dd* J = 17.5, 1.5 Hz	5.99 (H−26)	
28	1.14, 3H *d* J = 6.5 Hz	2.22 (H−13)	21.8
29	a:4.90, 1H *m*	1.77 (H−16); 4.75 (H−29b).	110.2
	b:4.75, 1H *dd* J = 3, 1 Hz	2.15 (H−18); 4.90 (H−29a).	
30	0.93, 3H *s*	1.01 (H−22)	22.3
31	0.87, 3H *d* J = 6.5 Hz	1.64 (H−20)	16.5
32	1.11, 3H *d* J = 6.5 Hz	2.24 (H−3)	20.4

* Interchangeable

Signals indicated as *m* were unresolved or overlapped multiplets.

Table 2. NMR spectral data for C_{34} **3** (in C_6D_6, TMS int. ref.)

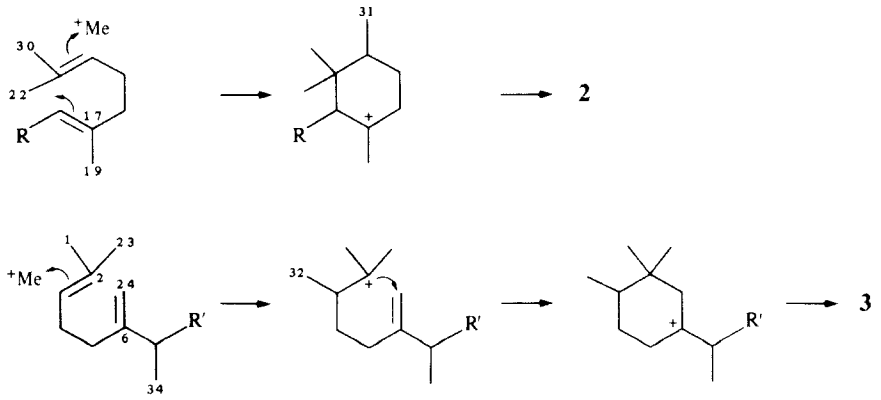
Assignment	^{1}H (400.13) shift	Associated protons by COSY	^{13}C (100.6 Hz) shift	Associated protons by long range HETCOSY
1	0.96, 3H *s*	1.11 (H−23)	24.0	1.11 (H−23); 1.54 (H−3)
2	–	–	35.2	0.96 (H−1); 1.11 (H−23); 5.28 (H−24)
3	1.54, 1H *m*	0.98 (H−32)	39.3	0.96 (H−1); 1.11 (H−23); 5.28 (H−24)
4	1.43–1.65, 2H *m*	1.89–2.02 (H−5)	28.8	0.98 (H−32)
5	1.89–2.02, 2H *m*	1.43–1.65 (H−4)	25.7	5.28 (H−24)
6	–	–	139.1	1.14 (H−34); 1.43–1.65 (H−4); 1.89–2.02 (H−5); 2.09 (H−7)
7	2.09, 1H *m*	1.14 (H−34); 1.43 (H−8)	42.3	1.14 (H−34); 5.28 (H−24)
8	1.43, 2H *m*	2.09 (H−7)	30.5	1.14 (H−34)
9	1.51, 2H *m*		40.0	1.23 (H−25); 5.57 (H−11)
10	–	–	42.6	1.23 (H−25); 5.15, 5.25 (H−27); 5.57 (H−11)
11	5.57, 1H *d* J = 16 Hz	2.19 (H−13); 5.40 (H−12)	137.1	1.23 (H−25); 5.40 (H−12)
12	5.40, 1H *dd* J = 16, 8 Hz	2.19 (H−13); 5.57 (H−11)	134.5	5.57 (H−11)
13	2.19, 1H *m*	1.12 (H−28); 1.60 (H−14); 5.40 (H−12); 5.57 (H−11).	38.3	1.12 (H−28); 5.57 (H−11)
14	1.40, 2H *m*	2.19 (H−13)	35.9	1.12 (H−28); 1.52 (H−15)
15	1.52, 2H *m*	2.20 (H−16)	34.3	1.17 (H−33)
16	2.20, 1H *m*	1.17 (H−33); 1.52 (H−15); 4.98 (H−29a)	41.0	1.17 (H−33); 4.96 (H−29b)
17	–	–	155.2	1.17 (H−33); 2.20 (H−16)
18	2.09, 2H *m*	1.52, 1.65 (H−19); 4.96 (H−29b)	32.5	4.98 (H−29a)
19	1.52, 1H *m*; 1.65, 1H *m*	2.09 (H−18); 2.27 (H−20)	34.3	1.10 (H−31); 2.09 (H−18)
20	2.27, 1H *m*	1.10 (H−31); 1.52, 1.65 (H−19); 4.91 (H−22a)	41.9	1.71 (H−30); 4.89 (H−22b)
21	–	–	150.2	1.10 (H−31); 1.71 (H−30)
22	a: 4.91, 1H *br s* b: 4.89, 1H *q* J = 1 Hz	2.27 (H−20). 1.71 (H−30).	110.6	1.71 (H−30)

Table 2. *Continued*

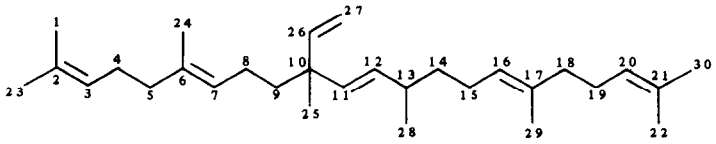
Assignment	^{1}H (400.13) shift	Associated protons by COSY	^{13}C (100.6 Hz) shift	Associated protons by long range HETCOSY
23	1.11, 3H *s*	–	30.2	0.96 (H – 1); 5.28 (H – 24)
24	5.28, 1H *br s*	1.89–2.02 (H – 5)	133.6	0.96 (H – 1); 1.11 (H – 23)
25	1.23, 3H *s*		24.5	5.57 (H – 11)
26	5.97, 1H *dd* $J = 17.5, 10.5$ Hz	5.15, 5.25 (H – 27)	147.6	1.23 (H – 25)
27	5.15, 1H *dd* $J = 10.5, 1.5$ Hz 5.25, 1H *dd* $J = 17.5, 1.5$ Hz	5.97 (H – 26)	111.9	
28	1.12, 3H *d* $J = 6.5$ Hz	2.19 (H – 13)	22.0	
29	a: 4.98, 1H *br s* b: 4.96, 1H *br s*	2.09 (H – 18); 2.20 (H – 16)	108.4	
30	1.71, 3H *br s*	4.9 (H – 22)	19.4	4.91 (H – 22a)
31	1.10, 3H *d* $J = 6.5$ Hz	2.27 (H – 20)	20.5	
32	0.98, 3H *d* $J = 6$ Hz	1.54 (H – 3)	16.8	
33	1.17, 3H *d* $J = 6.5$ Hz	2.20 (H – 16)	21.0	
34	1.14, 3H *d* $J = 6.5$ Hz	2.09 (H – 7)	20.6	

protons 22 and carbons 20 or 30. Compound **3** also retains a trisubstituted double bond (δ ^{13}C at 139.1 and 133.6 ; δ ^{1}H at 5.28 *br s*). COSY and HETCOSY experiments require its placement in a six-membered ring, as shown in **3**. Analysis of the COSY data show the following connectivities in the ring moiety : 3–32, 4–5–24 and 8–7–34. The overlapping of signals does not allow us to clearly observe correlations between H_3 and H_4 and between H_8 and H_9. For this same reason, NOEs observed between H_1–H_3, H_{24}–H_1, H_{23}–H_{24} and H_{24}–H_7, are insufficient to determine ring geometry.

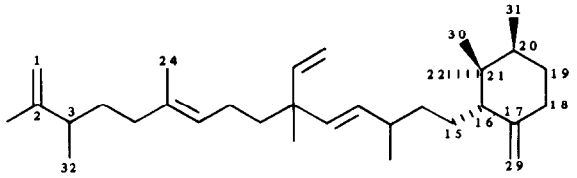
^{1}H and ^{13}C NMR spectra of **2** indicate the presence of partial structures **B** and **D**, joined together to form the short chain, regarding C_{10}, as established by COSY (Table 1). This experiment allows an unambiguous assignment of the ^{1}H signals of the ring moiety. Exocyclic methylene protons 29a and 29b are correlated to protons 16 and 18, respectively. These are correlated to the non-equivalent protons 19, which in turn are coupled to the methine proton 20, finally correlated to the protons of methyl 31. The stereochemistry of the ring was deduced by NOE difference spectroscopy (Scheme 1). Clear enhancements are obtained between H_{29a} (4.75 *dd*, $J_{29a,29b} = 1$ Hz; $J_{29a,18ax} = 3$ Hz and H_{16} (1.77 *dd*, $J_{16,15} = 11.4$ Hz), and also between this proton and those of the *gem* methyls 22 and 30, thus leading to an equatorial orientation for 16. Moreover, the absence of allylic coupling between protons 29 and 16, when it is observed between H-29 and H-18ax ($J = 3$ Hz, dihedral angle near 90°), is in agreement with the equatorial orientation of H-16 (dihedral angle near 0°). Positive NOEs are also observed between the protons of methyl 31 and those of the *gem* methyl 30 and 22, thus establishing an equatorial orientation for methyl 31, which is then confirmed by the



Scheme 2. Proposed mechanisms for the biosynthesis of **2** and **3**.



1



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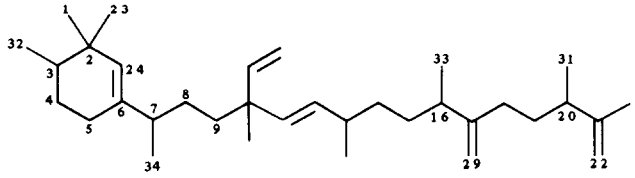

3

axial orientation of its *gem* proton 20 ($J_{20ax.19ax}$ = 12 Hz). From a Drieding model, it appears that the unexpected axial orientation of the large substituent at C-16 abolishes strong interactions existing for an equatorial orientation : H-15, H-14/H-29a and Me on C-21.

The structures of these two partially cyclic botryococcenes, for which cyclization probably occurs during the alkylation process (Scheme 2), in addition to the 10 previously determined, illustrate the chemical variability observed in the B. race of *B. braunii*.

EXPERIMENTAL

Botryococcenes **2** and **3** were produced by a Bolivian strain originating from Overjuyo lake (strain 5) and an Ivorian one (Ayame lake), cultivated in the laboratory [2]. They were separated from the botryococcene mixts by HPLC, using Resolve 5μ spherical C_{18} Waters (two 150 × 3.9 mm) [8]. Botryococcene samples were injected as solns in Me_2CO (20 μl, 10% in solvent). Mobile phase Me_2CO–MeCN (3:7), flow rate 60 ml/hr. Successive inj of samples were carried out and the compounds collected. R_ts (min): **2** 17.5; **3** 19.5.

Hydrocarbons were characterized using CI(NH_3) and EIMS. The apparatus was equipped with a fused silica column ; 25 m WCOT SE 52. The following column temps were used. Botryococcenes : prog. 220–260° at 2°/min (RR_ts/squalene : **2** : 0.90; **3**: 0.89); botryococcanes: 260°. EI/MS (probe 70 eV) *m/z* (relative intensity), botryococcanes derived from **2**: 448 $[M]^+$ (1), 419 $[M\text{-}C_2H_5]^+$ (4), 418 (2), 279 $[M-C_{12}H_{25}]^+$ (2), 278 (5), 225 $[M-C_{16}H_{31}]^+$ (3), 224 (6), 183 (5), 169 (3), 153 (12), 139 (19), 125 (12), 113 (24), 111 (22), 99 (24), 97 (50), 95 (21), 83 (45), 71 (29), 69 (100), 57 (59), 55 (45); botryococcanes derived from **3**: 476 $[M]^+$ (0), 447 $[M-C_2H_5]^+$ (1), 446 (1), 295 $[M-C_{13}H_{25}]^+$ (1), 294 (5), 237 $[M-C_{17}H_{35}]^+$ (2), 236 (6), 167 (7), 155 (5), 154 (4), 153 (4), 152 (4), 141 (7), 139 (7), 127 (11), 125 (18), 113 (11), 111 (25), 99 (16), 97 (16), 85 (40), 83 (36), 71 (75), 69 (59), 57 (100), 55 (41).

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