

## BOTRYOCOCCENE DISTRIBUTION IN STRAINS OF THE GREEN ALGA *BOTRYOCOCCUS BRAUNII*

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

**Abstract**—The B race of the green colonial alga, *Botryococcus braunii*, is characterized by the production of botryococcenes, i.e. triterpenoid hydrocarbons of general formula  $C_nH_{2n-10}$ ,  $n=30-37$ . Samples of this alga, collected in Bolivia, France, Ivory Coast, Philippines and Thailand, were grown in the laboratory and examined for their hydrocarbon composition. About 20 new compounds belonging to the botryococcene family have been identified by GC-MS. Thus, the very large variability related to the diversity of the alkylation pattern, leading from the  $C_{30}$  to the botryococcene series, was confirmed for the B race. Some  $C_{33}$  compounds characterized in the present study afford the same carbon skeleton as that of a fossil  $C_{33}$  botryococcane, recently discovered in a Chinese oil shale, thus emphasizing the geochemical interest of *B. braunii*.

### INTRODUCTION

The B race of the green colonial alga *Botryococcus braunii* produces large amounts of polyunsaturated, branched,  $C_{30}$ – $C_{37}$  triterpenoid hydrocarbons, termed botryococcenes, of the general formula  $C_nH_{2n-10}$  [1]. Large variations in the botryococcene composition have been noticed, according to the geographical origin [1–4] and the culture conditions [1]. The structures of some of these compounds [5–7] and a biosynthetic study performed on algae fed with C-labelled acetate and L-methionine [8], have shown that this variability can be related to the diversity of the methylation patterns which lead, from the parent  $C_{30}$  hydrocarbon, to the botryococcenes.

In continuation of our screening of this cosmopolitan alga, water samplings were collected in some freshwater lakes of Bolivia, France, Ivory Coast, Philippines and Thailand, from which new strains were isolated and grown. This paper deals with the GC-MS analyses of the botryococcene content of these new strains.

### RESULTS AND DISCUSSION

#### *Isolation and cultures*

Botryococcene-producing algae have been found in eight lakes situated in different geographical areas: two in the temperate climate zone (France: Vioreau and Pareloup lakes), one in the Bolivian Andes (Overjuyo valley) and five in tropical environments (Ivory Coast: Ayame, Taabo and Yamoussoukro, the Philippines: Katugday, and Thailand: Songkla Nakarin). Enough biomass was collected from the two last lakes to perform a

direct hydrocarbon analysis (data in Table 1). All the samples, with the exception of that from the Philippines, were subjected to purification after growing on Petri dishes. Thereafter, about 10 colonies were removed, inoculated separately in liquid medium, and the daughter subcultures further analysed for their hydrocarbon content.

Surprisingly, the isolates obtained from the sample originating from Thailand did not produce botryococcenes after purification, nor alkadienes and trienes, the two types of hydrocarbons respectively produced by the two known races, 'B' and 'A' of *B. braunii* [1]. Indeed, by the analyses of wild *Botryococcus* samples and of their derived subcultures, we demonstrated that the nature of the hydrocarbons remained unchanged at any stage of growth. The Thailand algae cultivated in the laboratory, yielded principally one hydrocarbon (98% of the whole), a  $C_{40}H_{78}$  tetraterpene, termed lycopadiene by reference to its basic carbon skeleton and to the presence of a double unsaturation [9], which indicates a new race of *Botryococcus*. From the analyses of the Thailand wild sample and of its derived subcultures, it appeared that this new race coexists with the B race in the Songkla Nakarin reservoir. Such lycopadiene-producing *Botryococcus* were also isolated from the Yamoussoukro lake. They exhibit a smaller cellular size (8–9 by 5  $\mu$ m) than that of alkadiene- or botryococcene-producing algae (13 by 7–9  $\mu$ m). In the case of the algae originating from Thailand, the explanation of the only isolation of lycopadiene strains lies perhaps in the conditions of the long transport, unfavourable to *Botryococcus*, with selection of the lycopadiene strains, initially present in low concentration.

The coexistence of two races of *Botryococcus* in the same lake was also observed with the Overjuyo sample. In this case, alkadiene-producing strains (A race), were

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Table 1 GC-MS analysis

$C_nH_{2n-10}$	Compound	Relative retention/ squalene	Bolivia* Overjuyo				
			1	2	3	4	5
32	1	0.678					
32	2	0.703					
33	3	0.727					
33	4	0.744					
33	5	0.756					
30	6‡	0.760		10.4			
31	7‡	0.770		5.6			
33	8	0.773					
33	9	0.788					
33	10	0.805					
33	11	0.810		12.1			
34	12‡	0.814			9.2	3.3	6.6
33	13	0.832					
33	14	0.833					
34	15‡	0.847	7.6		79.3	32.8	54.9
32	16‡	0.870	82.0	27.8	0.9	47.9	10.4
33	17‡	0.887	6.6	16.4	2.2	6.6	3.9
34	18	0.899					
32	19	0.900		17.8	2.2	6.6	18.7
35	20‡	1.011					
36	21	1.112					
36	22	1.168					
36	23‡	1.175					
n.i.			3.8	9.9	6.6	2.8	3.8

\*Cultivated strains

†Wild samples

isolated besides botryococcene-producing strains (B race). The hydrocarbon analyses of the A race will be submitted in a next paper.

#### Hydrocarbon analyses

The botryococcene fractions separated from the lipidic extracts were subjected to GC-MS analyses. In the algae examined, 23 botryococcenes were identified; they range from  $C_{30}$  to  $C_{36}$ . Apart from previously identified botryococcenes [1, 7, 8], 15 new compounds were detected (Table 1).

The Thailand and Philippines wild samples showed very similar hydrocarbon distributions. The predominant compound was the  $C_{34}$  botryococcene 15 (Scheme 1), found in algae of the Oakmere lake (England) [2, 5] and also in *Botryococcus* samples collected in some Australian [3, 7] and West-Indian [6, 7] freshwater lakes.

For the algae originating from the other countries, the analyses were performed after culture under standard conditions promoting a slow growth as in nature. Indeed, air lift cultures, with air supplied with  $CO_2$  propitious for a fast growth, lead to somewhat different botryococcene compositions, where the synthesis of hydrocarbons of low mass are favoured [8], and which does not correspond to the real fingerprint of the B race strains. While the subcultures of algae from France and Ivory Coast exhibited, for the same origin, fairly close hydrocarbon distributions, it appeared that the Bolivian isolates could

be classified into five strains related to their botryococcene patterns (Table 1).

In connection with this observation, it may be assumed that different botryococcene-producing populations exist in the Overjuyo lake, as previously observed in a Martinique one [1].

The Overjuyo strain 1 produced a simple mixture of botryococcenes  $C_{32}$  16,  $C_{33}$  17 and  $C_{34}$  15, while strains 3, 4 and 5 yielded in addition, the botryococcene  $C_{34}$  12 and a  $C_{32}$  compound 19, these three strains are distinguished from one another only by different relative percentages of their metabolites. In the strain 2, the botryococcene components were restricted to  $C_{30}$ - $C_{33}$  compounds.

The Parelopou strain\* exhibited the largest mass range, from  $C_{30}$  to  $C_{36}$ . Up to now the compound  $C_{35}$  was detected only in algae from the Darwin lake in Australia [1, 3], when compound 23 of the  $C_{36}$  group, was observed in this latter and also in three lakes of the French West Indies [1]. The Vireau strain, which synthesized six  $C_{33}$  besides two  $C_{32}$ , illustrated well the chemical variability of the *B. braunii* B race.

The Ayame and Yamoussoukro strains displayed close

\*In a previous study [1], we described the failure encountered with the Parelopou sample, because of fungal contaminations. Here, some new, always contaminated isolates could be examined, although they exhibited a very slow growth.

of botryococcenes

Geographic origin						
France*		Ivory Coast*		Philippines†		Thailand†
Pareloup	Vioreau	Ayame	Yamoussoukro	Katugday	Songkla Nakarin	
		3.1				
		1.9				
		6.2				
		3.4				
		2.5				
5.8						
8.6						
27.7		21.3		14.4		
48.3						
7.2		1.8		3.6		7.0
		7.4		2.9		
6.9						
27.0		8.9		27.3		82.4
11.2						90.3
12.5						
		53.2		46.0		
5.9						
12.8						
2.4						
4.2						
2.4	—	7.4		5.8	10.2	2.7

† Botryococcenes previously identified.

n 1 Compounds not identified due to poor resolution of GC peaks

$C_{33}$ – $C_{34}$  botryococcene patterns, while the other Ivorian strain, from Taabo lake (data not shown in Table 1), yielded a large variety of  $C_{32}$  and  $C_{33}$  botryococcenes showing a poorly resolved chromatogram on capillary columns.

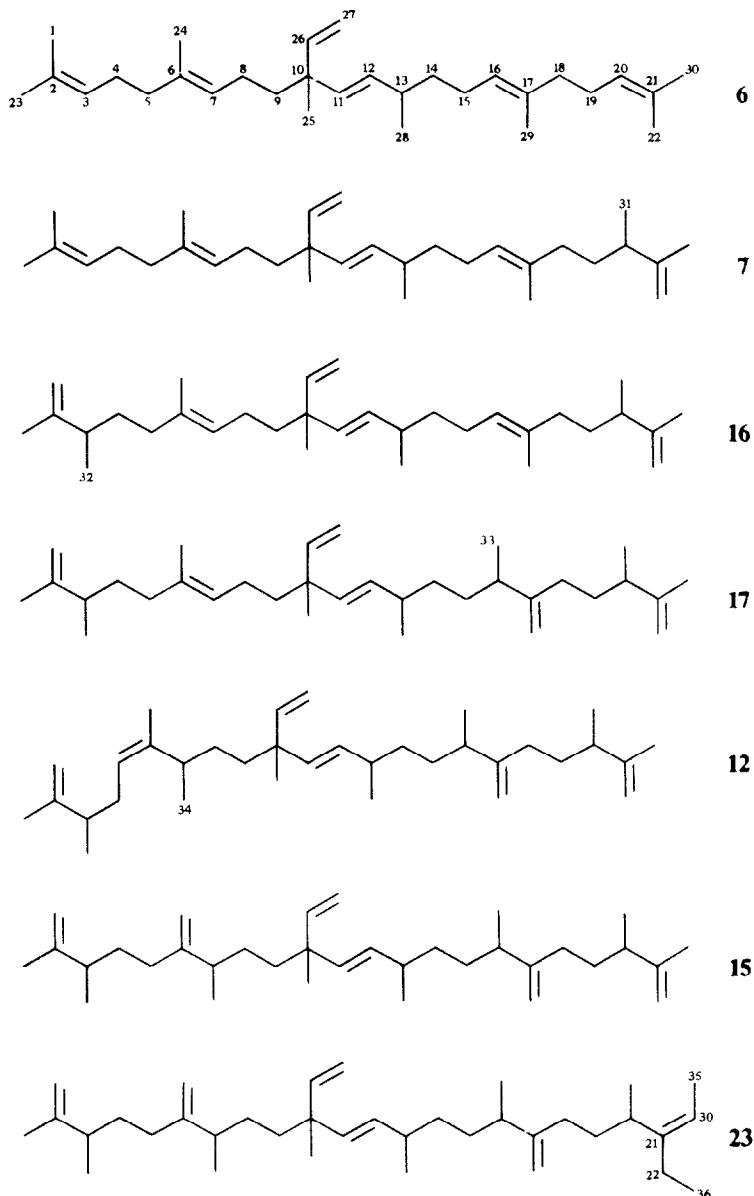
All these results, as well as those originating from other strains [1] and from wild algae [1, 3], emphasize the wide extent of the polymethylated triterpenes typical of the B race of *B. braunii*.

From a biosynthetic point of view, botryococcenes  $\geq C_{31}$  can be easily related to their common precursor the  $C_{30}$  compound **6**. Indeed, monomethylations can take place on positions 3, 7, 16 and (or) 20 of the  $C_{30}$  backbone **6**, the alkylation can give rise to double bond isomerism, as observed for methylation on C-7 leading to the botryococcenes **12** and **15**; successive methylations may exist on the same site as in the  $C_{36}$  botryococcene **23**, and finally, the methylation may be the starter of a concomitant partial cyclization [7].

GC-MS of the fully hydrogenated botryococcanes allows the demonstration of some alkylation patterns and the existence of some cyclized metabolites (Table 2) from ion doublets arising from fragmentation around the quaternary carbon  $R^1C(Me)(C_2H_5)R^2$  (Scheme 2). The catalytic reduction of the purified extracts leads, on the whole, to 16 botryococcanes from which 6 have been previously identified: **24**, **25**, **28**, **30**, **33** and **39** [7]. The  $C_{34}$  botryococcane **33**, has been formerly identified in some Australian and Sumatran crude oils [10, 11]. The

botryococcane **26** and **27** from the Vioreau strain exhibits ion doublets at  $m/z$  281, 280 and 225, 224, suggesting that methylation takes place on branching  $R^1$  and  $R^2$  (Scheme 2) as for their isomer **28**, but on other positions. The  $C_{33}$  triterpane **29**, derived from the Vioreau, Ayame and Yamoussoukro strains, displays an identical mass spectrum to that of a fossil  $C_{33}$ , the major botryococcane of a Chinese oil shale [12]. The structure **37** is ascribed to the botryococcane **C<sub>35</sub>** on the basis of its mass spectrum and of the role played by a given botryococcene as a precursor for its next highest homologues, here the  $C_{36}$  compounds which have identical carbon skeletons (Table 2). Finally, six botryococcanes exhibiting a ring moiety have been characterized:  $C_{32}$  **31** and **32**,  $C_{33}$  **34** and **35** and  $C_{34}$  **36** and **38**. They derive in pairs from the alkenes **19**, **11** (Overjuyo strain) and **18** (Ayame and Yamoussoukro strains), suggesting a stereochemical isomerism induced by the hydrogenation of an intra or an exocyclic double bond. Moreover, the comparison of their ion doublets with that registered for the non-cyclized analogues, shows that the ring is located on  $R^2$  for **31** and **32** (with methylation on  $R^1$  and on  $R^2$ ), on  $R^1$  for **34** and **35** (one methyl on  $R^1$  and two on  $R^2$ ) and for **36** and **38** (two methyls on  $R^1$  and on  $R^2$ ).

Up to now, only some  $C_{31}$ ,  $C_{33}$  and  $C_{34}$  botryococcanes were clearly recognized in some oil shales [10–13]; owing to the wide extent of the botryococcene series, it may be assumed that such other biological markers will be identified in lacustrine crude oils.



Scheme 1. Structures of some botryococcenes

## EXPERIMENTAL

**Origin of the samples.** Collections were made at the following sites: Bolivia lake in the Overjuyo valley (29 Nov 1985), pH 7.3, H<sub>2</sub>O temp 11°; France barrier lake of Pareloup in the Rodez region (31 Aug 1982), pH 6.8, H<sub>2</sub>O temp 24°; barrier lake of Viorceau in Brittany (1984); Ivory Coast barrier lake of Ayame (24 Feb 1984), pH 5.7, H<sub>2</sub>O temp 30.8°; barrier lake to Taabo (28 Feb 1984), pH 5.5, H<sub>2</sub>O temp 32.7°; lake of Yamoussoukro (29 Feb 1984), pH 5.5, H<sub>2</sub>O temp 27.7°; Philippines lake of Katugday (31 Mar 1985); Thailand reservoir of the Songkla Nakarin University (20 Jan 1985), pH 6.8, H<sub>2</sub>O temp 27°.

**Isolation and culture conditions** The isolation technique and the standard conditions for the cultures (un aerated at 22° and

under a light-dark cycle, 14 hr illumination per day) were as previously described [1].

**Hydrocarbon analyses** The conditions for the hydrocarbon extraction with hexane, the purification of botryococcene on Al<sub>2</sub>O<sub>3</sub> column, the hydrogenation to botryococcane and the conditions used for GC-MS were as reported in refs [1, 7].

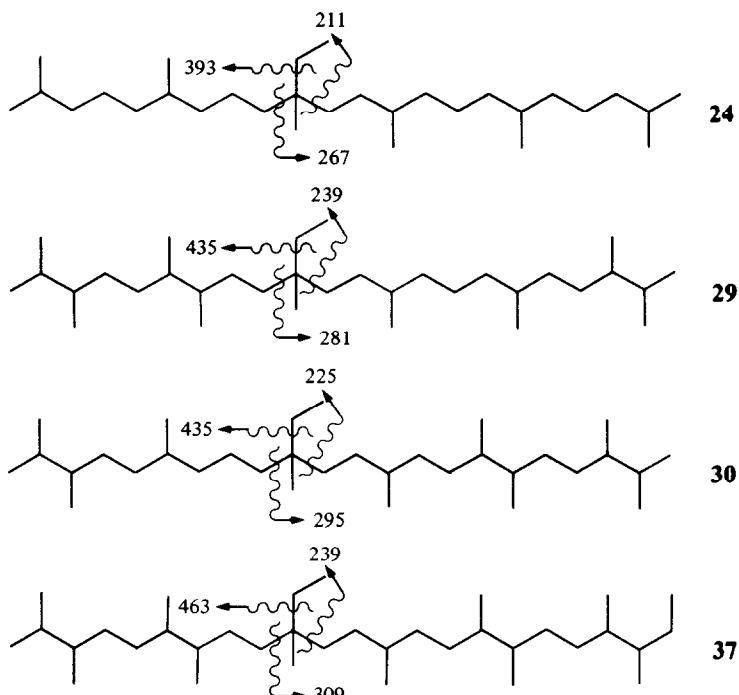
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Table 2. GC-MS of botryococcenes

Botryococcenes		RR <sub>f</sub> /squalane	Geographic Origin	Parent botryococcene	(M <sup>+</sup> , if present) and ion doublets
C <sub>30</sub> H <sub>62</sub>	24*	0.827	Pareloup, Overjuyo 2	6	393, 392 [M-C <sub>2</sub> H <sub>5</sub> ] <sup>+</sup> , 267, 266 [M-C <sub>11</sub> H <sub>23</sub> ] <sup>+</sup> , 211, 210 [M-C <sub>15</sub> H <sub>31</sub> ] <sup>+</sup>
C <sub>31</sub> H <sub>64</sub>	25*	1.055	Pareloup; Overjuyo 2	7	407, 406 [M-C <sub>2</sub> H <sub>5</sub> ] <sup>+</sup> , 281, 280 [M-C <sub>11</sub> H <sub>23</sub> ] <sup>+</sup> ; 211, 210 [M-C <sub>16</sub> H <sub>33</sub> ] <sup>+</sup>
C <sub>32</sub> H <sub>66</sub>	26	1.101	Vioreau	1 or 2	421, 420 [M-C <sub>2</sub> H <sub>5</sub> ] <sup>+</sup> ; 281, 280 [M-C <sub>12</sub> H <sub>25</sub> ] <sup>+</sup> ; 225, 224 [M-C <sub>16</sub> H <sub>31</sub> ] <sup>+</sup>
C <sub>32</sub> H <sub>66</sub>	27	1.219	Vioreau,	1 or 2	as for 26
C <sub>32</sub> H <sub>66</sub>	28*	1.315	Pareloup; Overjuyo (all strains), Taabo	16	as for 26
C <sub>33</sub> H <sub>68</sub>	29*†	1.375	Vioreau, Pareloup, Yamoussoukro	4, 5, 8, 10, 14, 9	435, 434 [M-C <sub>2</sub> H <sub>5</sub> ] <sup>+</sup> ; 281, 280 [M-C <sub>13</sub> H <sub>27</sub> ] <sup>+</sup> ; 239, 238 [M-C <sub>16</sub> H <sub>33</sub> ] <sup>+</sup>
C <sub>33</sub> H <sub>68</sub>	30*	1.507	Pareloup, Overjuyo (all strains), Taabo	17	435, 434 [M-C <sub>2</sub> H <sub>5</sub> ] <sup>+</sup> , 295, 294 [M-C <sub>12</sub> H <sub>25</sub> ] <sup>+</sup> ; 225, 224 [M-C <sub>17</sub> H <sub>35</sub> ] <sup>+</sup>
C <sub>32</sub> H <sub>64</sub>	31	1.616	Overjuyo 2, 3, 4	19	(448), 419, 418 [M-C <sub>2</sub> H <sub>5</sub> ] <sup>+</sup> , 279, 278 [M-C <sub>12</sub> H <sub>25</sub> ] <sup>+</sup> ; 225, 224 [M-C <sub>16</sub> H <sub>31</sub> ] <sup>+</sup>
C <sub>32</sub> H <sub>64</sub>	32	1.671	Overjuyo 2, 3, 4	19	as for 31
C <sub>34</sub> H <sub>70</sub>	33*†	1.726	Pareloup, Ayame; Yamoussoukro, Over- juyo 1, 3, 4, 5, Katugday; Songkla Nakarin	12, 15	449, 448 [M-C <sub>2</sub> H <sub>5</sub> ] <sup>+</sup> ; 295, 294 [M-C <sub>13</sub> H <sub>27</sub> ] <sup>+</sup> , 239, 238 [M-C <sub>17</sub> H <sub>35</sub> ] <sup>+</sup>
C <sub>33</sub> H <sub>66</sub>	34	1.849	Overjuyo 3	11	(462), 433, 432 [M-C <sub>2</sub> H <sub>5</sub> ] <sup>+</sup> , 295, 294 [M-C <sub>13</sub> H <sub>23</sub> ] <sup>+</sup> , 223, 222 [M-C <sub>17</sub> H <sub>35</sub> ] <sup>+</sup>
C <sub>33</sub> H <sub>66</sub>	35	1.945	Overjuyo 3	11	as for 34
C <sub>34</sub> H <sub>68</sub>	36	2.137	Ayame, Yamoussoukro	18	447, 446 [M-C <sub>2</sub> H <sub>5</sub> ] <sup>+</sup> ; 295, 294 [M- C <sub>13</sub> H <sub>25</sub> ] <sup>+</sup> , 237, 236 [M-C <sub>17</sub> H <sub>35</sub> ] <sup>+</sup>
C <sub>35</sub> H <sub>72</sub>	37	2.151	Pareloup	20	463, 462 [M-C <sub>2</sub> H <sub>5</sub> ] <sup>+</sup> ; 309, 308 [M-C <sub>13</sub> H <sub>27</sub> ] <sup>+</sup> , 239, 238 [M-C <sub>18</sub> H <sub>37</sub> ] <sup>+</sup>
C <sub>34</sub> H <sub>68</sub>	38	2.205	Ayame; Yamoussoukro	18	as for 36
C <sub>36</sub> H <sub>74</sub>	39*	2.603	Pareloup	21, 22, 23	477, 476 [M-C <sub>2</sub> H <sub>5</sub> ] <sup>+</sup> ; 323, 322 [M-C <sub>13</sub> H <sub>27</sub> ] <sup>+</sup> ; 239, 238 [M-C <sub>19</sub> H <sub>39</sub> ] <sup>+</sup>

\*Botryococcenes previously described [6, 7, 10, 11]

†Compounds recognized in crude oils [10,11]



Scheme 2 Structures of some botryococcenes and main fragmentations.

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