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CHEMOTAXONOMIC EVIDENCE FOR THE SIMILARITY BETWEEN *BOTRYOCOCCUS BRAUNII* L RACE AND *BOTRYOCOCCUS NEGLECTUS*

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Key Word Index—*Botryococcus braunii*; A and L chemical races; *Botryococcus neglectus*; Chlorophyceae; alga; hydrocarbons; *n*-alkadienes and trienes; lycopadiene; tetraterpene; chemosystematics; chemical variability.

Abstract—The hydrocarbon distribution patterns of nine strains of the green alga *Botryococcus* originating from four lakes and one pond are reported from GC-mass spectrometric analyses; they complement previously published investigations on *Botryococcus braunii*. Seven strains from four different origins (Australia, Bolivia, England and Portugal) contain very-long-chain hydrocarbons, mainly dienes and trienes, odd-carbon-numbered, ranging on the whole from C_{23} to C_{33}; these compounds are characteristic of the A chemical race of *B. braunii*. Depending on the strain, a C_{27}, C_{29} or C_{31} prevalence is observed. In the Australian strains, the presence of *n*-C_{29} and *n*-C_{31} trienes exhibiting two conjugated double bonds in the midchain in a *cis,trans* configuration is reported for the first time in this alga. Two strains of *Botryococcus neglectus* isolated from a water sample collected in an Indian lake produce *trans,trans*-lycopadiene as the main hydrocarbon; this acyclic tetraterpenoid is typical of the L chemical race of *B. braunii*. This result strongly suggests that *B. neglectus* and *B. braunii* L race constitute the same taxa. The hydrocarbon contents vary from about 28% of the dry weight in the English strain to less than 0.1% in the Indian ones. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

The hydrocarbons from *Botryococcus braunii* have been studied extensively since the 1970s and three types have been identified (for a review up to 1990, see ref. [1]): (i) odd very-long-chain hydrocarbons (mono- to tetraunsaturated), from C_{23} to C_{31}; (ii) branched triterpenoids from C_{30} to C_{37}, called botryococcenes; and (iii) an acyclic tetraterpenoid, lycopadiene.

Morphologically, the strains producing the three types of hydrocarbon do not differ markedly from one another, except for the presence of a dense matrix embedding the cells in algae yielding botryococcenes or lycopadiene [2–4], a somewhat less conspicuous pyrenoid and the absence of oily droplets surrounding the colonies in the lycopadiene-producing algae [4], which also exhibit smaller cells [5]. On the basis of these results and observations, we have classified the strains into three chemical races: A, B and L, with reference to alkadienes, botryococcenes and lycopadiene, respectively [1]. Some phycologists consider, however, that many algae of the genus *Botryococcus* are wrongly referred to the morphotype *B. braunii*. Thus, on the basis of some morphological variations, Komáreck and Marvan [6] recognized more than 10 species in the genus.

Recently, we have isolated from water samples collected in Australian and Bolivian lakes new strains of *B. braunii*. We report herein the analyses of their hydrocarbons. Two collection strains, not previously investigated to the best of our knowledge, were also analysed. Moreover, we have also obtained two strains of *B. neglectus*, originating from an Indian lake [7]; the results of our analyses could provide information on *Botryococcus* taxonomy.

RESULTS AND DISCUSSION

Hydrocarbons were analysed in seven cultured strains of *B. braunii*: two were obtained from culture collections (formerly originating from Grasmere lake in England and from a pond in Amieiro in Portugal), five were isolated from water samples collected in two lakes (Jillamatong in Australia and Ichu Khota in Bolivia). Two strains of *B. neglectus*, originating from Kulavai lake in India, were also investigated. Algae

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Table 1. Lipid and hydrocarbon contents of nine strains of *Botryococcus*

	B. braunii							*B. neglectus*	
	Australia Jillamatong				Bolivia	England	Portugal	India Kulavai	
	1	2	3	4	Ichu Khota	Grasmere	Amieiro	1	2
Heptane extractable lipids (% dry wt)	53.0	20.4	30.8	34.2	21.0	44.0	2.4	18.1	19.8
Hydrocarbons:									
% dry wt	15.3	5.6	9.5	8.3	0.9	27.6	0.09	0.08	0.07
% lipids	28.9	27.4	30.8	24.3	4.3	62.7	3.7	0.4	0.3

were harvested after 3 weeks of culture, that is, when they were in the stationary phase of growth. The heptane-extractable lipid contents and the hydrocarbon yields are given in Table 1.

The yields of oil extractable with heptane, a solvent allowing the recovery of the main part of the alga lipids stored in the thick outer walls [8], were 2.4–53% of the dry weight. With the notable exception of the Portuguese strain (2.4% of oil by dry weight), the obtained values were comparable with those previously reported for numerous strains of *B. braunii* [1, 9–12]. Light microscope observation revealed that the Amieiro strain was not pure but highly contaminated with a unicellular alga of undetermined species, affecting subsequent comparisons.

Much larger variations in hydrocarbon contents were noted. Thus, the content observed for the *B. braunii* strain from Grasmere is about 400 times larger than that in strain 2 of *B. neglectus* from Kulavai. Together with previous analyses, these data extend the range of hydrocarbon contents in cultured *Botryococcus* from less than 0.1% to up to 61% of the dry weight [3]. Moreover, hydrocarbons were major constituents of the heptane extracted lipids only in the Grasmere strain (*ca* 63%). Although especially known as a hydrocarbon-rich microorganism, it is now well established that *Botryococcus* also produces large amounts of other lipids. Indeed, some strains accumulate appreciable amounts of triacylglycerols [10] and some others exhibit a very high content of ether lipids of unusual structure (alkoxy and phenoxy ether lipids in the A race [11, 12], ditetraterpenoid ether lipids, the structural determination of which is in progress for the L race). Both types of compound were detected by TLC of the extracts of the present strains.

The seven strains of *B. braunii* (the four strains from Jillamatong and those from Ichu Khota, Grasmere and Amieiro) contained unbranched very-long-chain hydrocarbons (Table 2). On the whole, C_{17} and odd-carbon-numbered C_{23} to C_{33} compounds were detected by GC-mass spectrometric analyses. The heptadecane and heptadecene identified in the Amieiro strain very likely originated from contaminants since numerous microalgae and cyanobacteria are known to synthesize such compounds in low amounts [13]. The C_{33} diene and triene present in the Australian isolates are reported for the first time in cultures of algae of the A race. In experiments done to identify some trienes of the Jillamatong strains, the hydrocarbons from strain 4 were purified by reverse-phase HPLC. Two fractions enriched in trienes, I (C_{29} trienes + C_{27} diene, 43:7) and II (C_{31} trienes + C_{29} diene, 9:1), were examined by NMR.

The ^{1}H NMR spectrum of fraction I showed five dominant signals in the olefinic proton region. Among them, three were ascribed to protons of a non-conjugated terminal double bond: δ 4.92 (1H), 4.99 (1H) and 5.81 (1H). The two other signals, δ 6.25 (2H, *dd*, J = 7.3, 1.7 Hz) and 5.44 (2H, *ddt*, J = 7.3, 1.7, 7.1 Hz), were characteristic of protons of two conjugated double bonds in a (*Z*),(*Z*) stereochemistry. Moreover, ozonolysis of this fraction and GC-mass spectrometric analysis of the derived aldehydes established that the midchain unsaturations were in positions 20 and 22 and, therefore, that the main compound (**1**) of fraction I was nonacosa-1,20(*Z*),22(*Z*)-triene, previously identified in a collection strain [14].

The ^{1}H NMR spectrum of fraction II exhibited seven dominant resonances in the low-field region for the main hydrocarbon **2**, three for terminal unsaturation, as in **1**, and four others for two conjugated double bonds in the midchain, $-CH_2-CH_A=CH_B-CH_C=CH_D-CH_2-$, with δ at 5.30 (H_A, *ddt*), 5.65 (H_D, *dt*), 5.94 (H_B, *dd*) and 6.30 (H_C, *dddt*). The *cis,trans* configuration was evident from the coupling constant values, $^3J_{A,B}$ = 10.8 Hz and $^3J_{C,D}$ = 15.1 Hz. Moreover, identification by GC-mass spectrometry in the ozonolysis products of α,ω-dialdehyde C_{21}, established that the midchain double bonds of the C_{31} trienes were in positions 22 and 24. However, the precise relative stereochemistry, 22(*E*),24(*Z*) or 22(*Z*),24(*E*) remains unknown; it could be only determined via stereospecific or stereoselective synthesis of standards [15]. This is the first report for the occurrence of a triene with two midchain conjugated double bonds in a *cis,trans* configuration in *B. braunii* A race.

In addition, the ^{1}H NMR spectrum of fraction I showed minor signals corresponding to the major ones

Table 2. Composition of hydrocarbons of strains of A race of *B. braunii*

Hydrocarbon	Retention time/heptane (sec)	Location and stereochemistry of double bonds	Relative %				
			Australia Jillamatong*		Bolivia	England	Portugal
			Strain 1	Strain 4	Ichu Khota	Grasmere	Amieiro
$C_{17}H_{34}$	125	–	–	–	–	–	1.2
$C_{17}H_{36}$	130	–	–	–	–	–	2.2
$C_{23}H_{44}$	370	1,14 (*Z*)	Trace	Trace	Trace	2.1	Trace
$C_{25}H_{48}$	600	1,16 (*Z*)	0.1	Trace	0.2	3.7	0.6
$C_{25}H_{48}$	610	1,16 (*E*)	–	–	0.4	1.4	Trace
$C_{25}H_{50}$	670	1	–	–	0.7	–	–
$C_{27}H_{50}$	890	–	–	–	14.3	–	–
$C_{27}H_{52}$	900	1,18 (*Z*)	4.1	3.6	47.0	17.8	4.1
$C_{27}H_{52}$	910	1,18 (*E*)	–	–	29.8	5.9	1.1
$C_{27}H_{54}$	950	1	–	–	3.4	–	0.6
$C_{29}H_{56}$	1250	1,20 (*Z*)	52.8	16.3	2.6	62.7	35.9
$C_{29}H_{56}$	1260	1,20 (*E*)	–	–	–	–	5.8
$C_{29}H_{58}$	1310	1	Trace	Trace	–	–	0.9
$C_{29}H_{54}$	1320	–	2.4	1.7	–	–	6.3
$C_{29}H_{54}$ (**3**)	1330	1,20,22†	Trace	1.6	–	–	6.3
$C_{29}H_{54}$ (**1**)	1380	1,20(*Z*),22,(*Z*)	5.1	12.9	–	–	3.0
$C_{29}H_{54}$	1400	–	–	0.3	–	–	8.6
$C_{31}H_{60}$	1710	1,22(*Z*)	28.1	26.7	–	3.8	18.1
$C_{31}H_{60}$	1730	1,22(*E*)	–	–	–	–	1.7
$C_{31}H_{58}$ (**2**)	1820	1,22,24‡	6.0	28.6	–	–	–
$C_{31}H_{58}$ (**4**)	1840	1,22(*Z*),24(*Z*)	Trace	2.6	–	–	–
$C_{33}H_{64}$	2090	–	Trace	0.6	–	–	–
$C_{33}H_{62}$	2170	–	Trace	2.1	–	–	–
Other	–	–	1.4	3.0	1.6	2.6	3.6

* Strains 2 and 3 exhibit hydrocarbon profiles very similar to that of strain 1.
† 20(*Z*),22(*E*) or 20(*E*),22(*Z*).
‡ 22(*Z*),24(*E*) or 22(*E*),24(*Z*).

in fraction II, indicating, in turn, that the minor C_{29} triene **3** in fraction I would be a 20(*E*),22(*Z*) or 20(*Z*),22(*E*) isomer and the minor C_{31} triene **4** in fraction II would exhibit a 22(*Z*),24(*Z*) conjugated system.

Strains 1–3 from Jillamatong showed a very similar distribution, with a C_{29} prevalence (60% of total) and the predominance of dienes (C_{27} to C_{31}) with a *cis* midchain unsaturation (no *trans* dienes were detected); C_{29} and C_{31} trienes accounted for 13.5% of the total hydrocarbons. In sharp contrast, the hydrocarbons from strain 4 were dominated by C_{31} compounds (60% of total) and C_{29} and C_{31} trienes comprised nearly half of the hydrocarbons. Moreover, the analyses of the hydrocarbons from natural algae from Jillamatoong (R. Summons, unpublished results), also showed a C_{31} prevalence; however, the presence in these samples of tetra- and pentaenes, not detected in the four cultured strains, indicates that some other strains of the A race are also present in this lake.

The Ichu Khota strain exhibited a very high C_{27} prevalence (*ca* 95% of total hydrocarbons), which accounted for only 4.3% of the lipids; C_{27} (*E*) and (*Z*) dienes dominated.

In the Amieiro and Grasmere strains, C_{29} hydrocarbons predominated and (*E*) isomers were observed for C_{25} to C_{31} dienes in algae from Amieiro, while such isomerism was only found with C_{25} and C_{27} dienes in algae from Grasmere. Moreover, while this English strain did not synthesize trienes, four were detected in the Portuguese strain, two of which were also identified in the Jillamatong isolates.

The results of this survey confirm that strains of *B. braunii* of the A race may differ significantly in hydrocarbon production (1–61% of dry weight) and composition. Indeed, a C_{27}, C_{29} or C_{31} prevalence may occur and, if dienes, especially those with a $\omega 9(Z)$ configuration, generally dominate, some strains synthesize trienes preferentially or (and) can produce mono- and tetraenes [16]. Also, conjugated unsaturations can occur in the terminal position (trienes and tetraenes) [16] or midchain (trienes).

trans,trans-Lycopadiene (2,6(*R*),10(*R*),14,19,23(*R*),27(*R*),31-octamethyldotriaconta-14(*E*),18(*E*)-diene) was the main hydrocarbon in the two strains of Kulavai. In addition, a minor $C_{40}H_{78}$ compound was also identified; it accounted for 20% of the hydrocarbon fraction in strain 1 and 8% in strain 2. Due to its very low abundance in these algae, no structural determination was undertaken.

It is likely to be a *cis,trans* or a *cis,cis* isomer of *trans,trans*-lycopadiene. The occurrence of lycopadiene in these two strains of *B. neglectus* indicates a very close relationship with the L chemical race of *B. braunii*, the sole microalga known to synthesize this tetraterpenoid [4, 5]. Moreover, *B. neglectus* and *B. braunii* L races exhibit a quite similar morphology. The only possible difference would be the absence of pyrenoid in the former; however, in the so-called L chemical race, it could be observed exclusively by transmission electron microscopy [4], while *B. neglectus* was described only from light microscope observations [6, 7].

EXPERIMENTAL

General. EIMS: 70 eV. ^{1}H (250 MHz) and ^{13}C (62.5 MHz) NMR: $CDCl_3$, TMS as int. standard. CC: silica gel (70–230 mesh).

Strain origins, cultures and hydrocarbon extraction. The two collection strains originated from the CCAP of Ambleside, U.K. (No. 807/2; collected in Grasmere Lake, Cumbria, U.K.) and from the Department of Botany of the University of Coimbra, Portugal (No. 58; collected in a small pool near Amieiro, Portugal). The sampling of the other algae were made in the ephemeral (brackish water) lake of Jillamatong (New South Wales) in Australia, in the freshwater lake of Ichu Khota in Bolivia, and in the freshwater lake of Kulavai in Chingelput (Tamil Nadu) in India (strain 1, MCRC Bocc 4/1994; strain 2, MCRC Bocc 3/1994 [7]). The isolation techniques of the new strains, the culture conditions (batch air-lift, 1% CO_2), extraction with heptane (2 × 1 hr) and hydrocarbon purification by silica gel CC were as previously described [3].

Analyses and fractionation of hydrocarbons. GC-MS analyses were made with a quadrupole instrument equipped with a 25 m CPSil 5CB fused silica column, progr. from 230° to 290° at 2° min^{-1} for the *n*-dienes and trienes and from 260 to 300° at 4° min^{-1} for lycopadienes. HPLC was carried out using two Resolve 4μ spherical C_{18} Waters columns (150 × 3.9 mm), mobile phase Me_2CO–MeCN–THF (5:12:1), flow rate 2 ml min^{-1}, detection by RI. Repeated injection of hydrocarbons obtained from strain 4 of Jillamatong, allowed the recovery of frs I (15.5 min) and II (22 min).

Nonacosa-1,20(Z),22(Z)-*triene* (**1**). ^{1}H NMR: δ 6.25 (2H, *dd*, J = 7.3, 1.7 Hz), 5.81 (1H, *ddt*, J = 17.3, 10.2, 6.7 Hz), 5.44 (2H, *ddt*, J = 7.3, 1.7, 7.1 Hz), 4.99 (1H, *ddt*, J = 17.3, 2.1, 1.5 Hz), 4.92 (1H, *ddt*, J = 10.2, 2.1, 1.5 Hz), 2.16 (4H, *dt*, J = 7.0, 6.7 Hz), 2.03 (2H, *m*), 1.20–1.45 (overlapping CH_2), 0.88 (3H, *t*, J = 7.0 Hz). ^{13}C NMR: δ 139.2, 132.1, 123.6, 114.1, 33.9, 31.9, 31.8, 29.7, 29.6, 29.4, 29.3, 29.2, 29.0, 27.5, 22.7, 14.1.

Hentriaconta-1,22,24-*triene* (**2**). ^{1}H NMR: δ 6.30 (1H, *dddt*, J = 15.1, 10.5, 1.2, 1.3 Hz), 5.94 (1H, *dd*, J = 10.8, 10.5 Hz), 5.81 (1H, *ddt*, J = 17.3, 10.2, 6.7 Hz), 5.65 (1H, *ddt*, J = 15.1, 1.3, 7.0 Hz), 5.30 (1H, *ddt*, J = 10.8, 1.2, 7.5 Hz), 4.99 (1H, *ddt*, J = 17.3, 2.1, 1.5 Hz), 4.92 (1H, *ddt*, J = 10.2, 2.1, 1.5 Hz), 2.14 (2H, *m*), 2.08 (2H, *m*), 2.03 (2H, *m*), 1.20–1.45 (overlapping CH_2), 0.88 (3H, *t*, J = 7.1 Hz). ^{13}C NMR: δ 139.2, 134.6, 130.1, 128.7, 125.7, 114.1, 33.9, 33.0, 31.8, 29.8, 29.6, 29.5, 29.3, 29.2, 29.1, 29.0, 27.7, 22.7, 14.1.

Ozonolysis of frs I and II. CS_2 solns (2 ml) of I and II (2 mg) were treated with O_3 at −78° until the characteristic blue colour of O_3 persisted. Then, excess O_3 was removed under a N_2 stream and the reaction mixts treated with solid triphenylphosphine (5 mg). The resulting solns were allowed to warm to room temp. and, after concn under red. pres., analysed directly by GC-MS (CPSil 5CB, progr. from 70° to 250° at 2° min^{-1}. Fr. I: mainly heptanal and α,ω-nonadecanedial *m/z* (rel. int.): 296 $[M]^+$ (1), 278 $[M-H_2O]^+$ (2), 123 (17), 109 (35), 97 (45), 96 (57), 95 (75), 83 (57), 82 (83), 81 (77), 69 (62), 67 (68), 57 (75), 55 (100), 43 (63), 41 (75). Fr. II: mainly heptanal and α,ω eneicosanedial *m/z* (rel. int.): 324 $[M]^+$ (1), 306 $[M-H_2O]^+$ (2), 123 (18), 109 (36), 97 (47), 96 (61), 95 (70), 83 (61), 82 (90), 81 (75), 69 (64), 67 (65), 57 (79), 55 (100), 43 (67), 41 (74).

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