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HYDROCARBONS, ALDEHYDES AND TRIACYLGLYCEROLS IN SOME STRAINS OF THE A RACE OF THE GREEN ALGA *BOTRYOCOCCUS BRAUNII*

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Key Word Index—*Botryococcus braunii*; Chlorophyceae; alga; *n*-alkadienes and trienes; aldehydes; botryals; triacylglycerols.

Abstract—The composition of the hexane extracts of six cultured strains of the green alga *Botryococcus braunii* is reported. These strains originating from Bolivia, France and from culture collections, belonged to the A race of this alga, which is characterized by the production of odd *n*-C_{23}–C_{31} alkadienes and trienes. Besides hydrocarbons, botryals, even C_{52}–C_{64} α-branched, α-unsaturated aldehydes synthesized via an aldol condensation of even *n*-C_{26}–C_{32} mono-unsaturated aldehydes, and triacylglycerols occurred in all strains.

INTRODUCTION

In the green colonial alga *Botryococcus braunii*, the A race regroups strains which synthesize odd unbranched C_{23}–C_{31} dienes and trienes [1, 2]. From *in vivo* and *in vitro* analyses performed on algae originating from the culture collection at Cambridge, U.K., it was pointed out that the oil droplets surrounding the colonies, quickly extracted with hexane, consisted nearly exclusively of hydrocarbons [3]. However, more recent analyses carried out on other A strains, axenic [4] or otherwise [1] showed that hydrocarbons in some cases accounted for only 25% of the hexane extract. Moreover, we established recently that unusual even C_{52}–C_{64} aldehydes, termed botryals, comprised a larger proportion of the hexane extract than the hydrocarbons [5].

Continuing our screening on *B. braunii*, we have isolated new A strains from water samples collected in Bolivian and French lakes. Depending on the strain origin, it appeared that the hydrocarbon concentration fluctuated between 3 to 88% of the hexane extract; this extract furnishes the main part of the oil produced by the alga and is located in the external walls surrounding the cells. Herein we report the distribution of some of the main neutral lipids identified in the hexane extracts of laboratory grown cultures.

RESULTS AND DISCUSSION

Isolation and cultures

Alkadiene- and triene-producing algae have been isolated from water samples collected in five lakes; one was situated in France (Coat ar Herno in Brittany) and four in the Bolivian Andes (Challapata, Overjuyo, Pata Khota and Titicaca). Up to now, the A race has been recognized in two Australian lakes [6], in one pool of the Atlas region in Morocco [1], in four lakes of the Morvan region in France [1, 2] and in one lake in the U.K. (Maddingley Bricks Pits), from which the Cambridge Collection strain originated.

All the samples were subjected to purification after growing on petri dishes. Thereafter colonies were removed, inoculated separately in liquid medium and the daughter subcultures further analysed for their hydrocarbon content and composition. With the exception of the cultures originating from the Overjuyo lake, all the subcultures originating from a same sample exhibited a very close hydrocarbon fingerprint by GC analysis. Accordingly, the *B. braunii* population of each sampling could be considered as homogeneous. From the Overjuyo sample, besides one strain belonging to the A race, five strains of the B race were isolated, as previously reported [7]; they produced different mixtures of botryococcenes, triterpenoid hydrocarbons of general formula C_nH_{2n-10}, with $30 \leqslant n \leqslant 37$. These isolations confirm the cosmopolitan occurrence of the A race under different climatic environments, as has been reported for the B race [1, 6–9].

Hexane extractable lipids

Algae were harvested after three weeks of culture, i.e. when they were in the stationary phase which corresponds generally to the highest lipidic content observed during the growth of green algae [10–12]. The hexane extractable lipid contents and the data for specific compound classes are given in Table 1; the axenic strain from the culture collection at Austin (U.S.A.) has been investigated for comparison. The yield of hexane soluble material varied greatly with the origin of the strain, ranging from *ca* 18% of the dry weight for the Challapata strain, to 66% for that from Overjuyo.

Hydrocarbons

Elution of hexane extracts on silica gel CC with hexane gave pure hydrocarbon fractions. The variation noticed

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Table 1. Lipid content and composition of six strains of *B. braunii* A race

	Geographic origin					
	Bolivia				France	Collection strain Austin U.S.A.
	Challapata	Overjuyo (strain 6)†	Pata Khota	Titicaca	Coat ar Herno	
Hexane extractable lipids (a)	17.8	66.1	59.1	39.8	60.6	43.4
Hydrocarbons (a)	6.5	1.9	51.9	12.8	1.6	8.9
(b)	36.4	2.9	87.8	32.1	2.6	20.4
Aldehydes* (a)	1.3	3.9	n.d.	18.1	1.6	11.1
(b)	7.1	5.9	n.d.	45.4	2.6	25.5
Triacylglycerols (a)	6.4	1.7	n.d.	3.1	1.9	6.3
(b)	35.7	2.5	n.d.	7.8	3.1	14.6

n.d. Not determined.
*Botryals, as defined in text.
(a) % dry wt; (b) % hexane extract.
†Strains 1–5 from Overjuyo were as described in ref. [7].

for the hydrocarbon content was very marked: major lipid components of the Pata Khota strain (*ca* 88% of extract and 52% of dry wt), hydrocarbons were less abundant in the Challapata, Titicaca and Austin strains. They appeared as very minor components of the Coat ar Herno and Overjuyo strains (2.6 and 2.9% of extract and 1.6 and 1.9% of dry wt, respectively).

The composition of hydrocarbon fractions was examined by GC-MS and the location and stereochemistry of the double bonds deduced from co-injections with authentic materials [2] (Table 2). The Challapata, Pata Khota and Titicaca strains exhibited a hydrocarbon distribution, essentially the *E* and *Z* dienic series, very close to that observed for a strain originating from the Morvan region in France [2], with the 29:2 isomers as dominant constituents. The Coat ar Herno and Overjuyo strains showed a very similar hydrocarbon fingerprint with no detectable *trans* dienes, a predominance of the *n*-C_{27} family including three trienes, and the absence of *n*-C_{31} compounds.

Aldehydes.

The second class of compounds eluted on silica gel CC were botryals, a series of even carbon number C_{52}–C_{64} α-branched, α-unsaturated aldehydes [5]; they probably originate from the condensation of very long chain monounsaturated fatty aldehydes (even C_{26}–C_{32}), as suggested by the incorporation pattern of [1,2-^{13}C] acetate. Formation of aldols would lead via dehydration to the botryals **1** and **2**; these two families of isomers, easily separated by preparative silica gel TLC, exhibit, respectively, a *cis* and a *trans* formyl group relative to the alkyl chain.

Botryals (**1**, **2**) were the dominant components of the hexane extract of the Titicaca strain (45.4% of oil, 18.1% of dry wt), but minor products of the Coat ar Herno strain (2.6% of oil, 1.5% of dry wt). They were always found in a ratio in favour of **2** (Table 3), i.e. the isomers for which the formyl and the bulkier groups are *trans*, as generally observed for acid- or base-catalysed dehydration of aldols [13].

Because the mixture of botryals was not resolved by normal or reversed-phase HPLC and not eluted on GC columns, the chain length distribution was estimated from the relative intensities of the $[M]^+$ ions observed in the EI spectra of the mixtures **1** and **2** (Table 3). However, according to the condensation pattern of C_{26}–C_{32} aldehydes, and to the absence of symmetry in the structure of botryals, it must be considered that if the $[M]^+$ ions corresponded to one molecular species for C_{52} (C_{26}–C_{26}) and for C_{64} (C_{32}–C_{32}), they could arise from two molecular species for C_{54} (C_{26}–C_{28} and C_{28}–C_{26}) and for C_{62}, three for C_{56} and C_{60} and four for C_{58}. From this mass spectral procedure, a C_{58} predominance was found in the botryals isolated from the Austin and the Titicaca strains, while the Overjuyo strain, exhibited a less clear distribution.

The presence of these peculiar lipids in all A strains, especially in the axenic strain originating from the culture collection of Austin, ensure us that they were not produced by accompanying bacteria in the cultures. Moreover, the isolation of metabolites arising from a condensation pathway generally creates a problem of origin. Analogies existing between botryals and mycolic acids, incline us to consider that botryals are true metabolites of the algae. Indeed, mycolic acids, important lipid constituents of the cell wall of some bacteria are α-branched, β-hydroxy acids synthesized by a head to head condensation of long chain fatty acids [14] as demonstrated with a cell-free extract [15]. They occur as C_{32}–C_{36} compounds in *Corynebacterium* [16], as C_{34}–C_{66} in *Nocardia* [17] and as C_{78}–C_{85} in *Mycobacterium* [18].

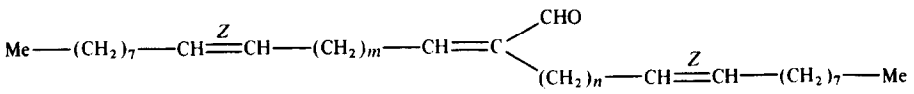
Finally, the localization of botryals in the outer walls of the alga, highly probable due to their quick extraction with a non polar solvent, suggest an important role for these compounds in *B. braunii* cell wall synthesis. Following epoxidation of the 'in chain' double bonds, they could furnish the basic carbon skeleton of the biopolymer forming the chemically resistant part of the cell wall [5].

Triacylglycerols

Triacylglycerols (TAGs) were detected in each strain by TLC analysis. With the exception of the Challapata strain, they did not constitute a dominant class of compounds in the hexane extractable lipids. Occurring widely in organisms in which they constitute storage lipids, their

Table 2. Composition of hydrocarbon mixtures from A strains of *B. braunnii* as determined by GC-MS

Hydrocarbons	Location and stereochemistry of the double bonds	Geographic origin				
		Bolivia				France
		Challapata	Overjuyo (strain 6)	Pata Khota	Titicaca	Coat ar Herno
23:2	1, 14 (*Z*)	—	2.1	0.8	—	1.0
23:2	1, 14 (*E*)	—	—	0.4	—	—
25:2	1, 16 (*Z*)	0.9	10.1	5.6	0.1	2.5
25:2	1, 16 (*E*)	0.5	—	3.1	0.7	—
25:3	—	—	8.7	—	—	2.4
27:2	1, 18 (*Z*)	4.9	30.9	8.2	3.1	31.0
27:2	1, 18 (*E*)	5.5	—	10.7	13.9	—
27:3	—	—	5.7	—	—	10.4
27:3	—	—	20.3	—	—	25.2
27:3	—	0.5	2.8	—	—	4.1
29:2	1, 20 (*Z*)	47.4	13.0	42.4	25.3	11.4
29:2	1, 20 (*E*)	16.8	—	19.7	32.6	—
29:3	1, 20 (*Z*), 22 (*Z*)	4.4	2.0	—	—	2.3
31:2	1, 22 (*Z*)	13.2	—	6.3	19.3	—
31:2	1, 22 (*E*)	—	—	—	3.8	—
31:3	—	1.7	—	—	—	—
others	—	4.2	4.4	2.8	1.2	9.7



m: odd from 15 to 21
n: even from 14 to 20

1 CHO *cis* relative to the alkyl chain
2 CHO *trans* relative to the alkyl chain

Table 3. Distribution of botryals in three A strains of *B. braunnii* according to their carbon number

Strains	Relative ratio 1/2	Botryals	Carbon number 52	54	56	58	60	62	64
Overjuyo 6	21/79	**1**	22	17	22	27	12	—	—
		2	14	20	38	19	9	—	—
Titicaca	12/88	**1**	4	12	23	30	16	9	6
		2	3	15	22	33	21	5	1
Austin	9/91	**1**	4	13	21	29	17	8	8
		2	3	14	22	33	19	6	3

localization in the outer walls of the alga, as suggested by their extraction with hexane, is nevertheless surprising.

TAGs were analysed by reversed-phase HPLC. By this technique they elute in ascending order of increasing PN, the partition number defined by PN=CN–2ND, where CN is the number of acyl carbon atoms and ND the number of double bonds [19–21]. The TAG distribution according to PN is given for two strains in Table 4; PN values from 44 to 58 were observed.

Acyl groups of TAGs were determined by transesterification with sodium methoxide–methanol and GC analysis. In the Challapata strain the following acids were identified: even monounsaturated predominated with 18:1 (67.1%), 20:1 (9%), 22:1 (1.1%), 24:1 (1.1%), 26:1

Table 4. Triacylglycerol distribution according to Partition Number (PN) in two strains of *B. braunnii*

PN	TAG*	Strain origin Challapata	Austin
44	—	1.7	traces
	—	0.9	—
46	LOO	6.0	0.5
	LPO	1.1	1.0
48	OOO	47.6	64.0
	POO	12.7	4.4
50	—	0.9	—
	—	6.2	2.4
	SOO	1.8	traces
52	—	1.4	1.2
54	—	0.4	0.8
	—	0.4	0.7
56	—	1.3	—
	—	—	1.3
	—	2.5	2.5
58	—	—	7.7
	—	10.3	12.1
others	—	4.8	2.0

*The order of acyl groups does not reflect their position on the glycerol. L = 18:2, P = 16:0, O = 18:1, S = 18:0.

(1.4%) and 28:1 (9.6%); 16:0 (6.8%), 18:0 (0.7%) and 18:2 (3.2%) were also present. Co-injections with some vegetable oils allowed to identify some compounds, from which triolein (PN = 48) appeared the dominant species. By taking into account the fatty acid analysis, it may be assumed that in the TAGs of the Challapata strain, the compound with PN 58 had 18:1–18:1–28:1 acyl groups. There are very few reports on the molecular composition of TAGs from microalgae: they concern three green algae [12], four freshwater chrysophytes [22] and three freshwater dinoflagellates [23]. These microorganisms did not exhibit molecular species containing very long chain acyl moieties, more recently found in TAGs of some higher plants, for example in crucifer seeds [24].

CONCLUSIONS

Lipid analysis of hexane extracts obtained from six strains of *B. braunii* belonging to the A race showed besides the presence of *n*-alkadienes, trienes, botryals and triacylglycerols for some strains. The hydrocarbon distributions observed confirm the existence of chemical variation in the A race of *B. braunii*, as noticed for the B race of the same alga [1, 7]. The similarities existing between botryals and mycolic acids suggest for these peculiar aldehydes of *B. braunii* an important role in the cell wall synthesis. Triolein dominate in TAGs; this could be related to the abundance in this alga of oleic acid [4], a direct precursor of the hydrocarbons [25].

EXPERIMENTAL

Origin of samples and cultures. Collections were made at the following sites. Bolivia: barrier lake of Challapata (Nov. 1985), pH 7.8, H_2O temp 16°; lake in the Overjuyo valley (Nov. 1985), pH 7.3, H_2O temp. 11°, lake of Pata Khota (Nov. 1985), pH 6.9, H_2O temp. 6°; lake of Titicaca (Nov. 1985), pH 8.4, H_2O temp. 15°; France, lake of Coat ar Herno, in Brittany (1982). The Austin strain was obtained from the Austin Culture Collection (University of Texas).

The isolation technique for the wild samples and the culture conditions (batch air-lift 1% CO_2), were as previously described [1]. After three weeks, the biomass was harvested and dried under vacuum at 50°.

Lipid extraction and analysis. Dried algae were extracted × 2 with hexane for 1 hr at room temp. The combined hexane extracts, concd under vacuum were purified by CC on silica gel (180 mg of products for 12 g adsorbent). Elution with 45 ml hexane afforded pure hydrocarbons. Further elution with 60 ml hexane–Et_2O (19:1) furnished a fraction containing aldehydes; TAG, were found in a third fraction eluted with 75 ml of hexane–Et_2O (23:2). Prep. TLC on silica gel of fr 2 (eluent hexane–Et_2O, 23:2) afforded botryals **1** (R_f 0.63) and botryals **2** (R_f 0.58). TAGs were purified from fraction 3 in the same way (eluent hexane–Et_2O, 17:3). The conditions used for GC-MS analyses of hydrocarbons were as reported in ref. [1]. MS of botryals were recorded at 70 eV in the EI mode. Conditions for HPLC of TAGs and for their transesterification are described in ref. [12].

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