Phytochemistry, Vol. 26, No. 1, pp 129–134, 1987.
Printed in Great Britain.

0031–9422/87 $3.00 + 0.00

BIOSYNTHESIS OF TRITERPENOID HYDROCARBONS IN THE B-RACE OF THE GREEN ALGA *BOTRYOCOCCUS BRAUNII*. SITES OF PRODUCTION AND NATURE OF THE METHYLATING AGENT

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(*Revised received* 23 *May* 1986)

Key Word Index—*Botryococcus braunii*; Chlorophyceae; alga; botryococcenes; triterpenoid hydrocarbons; sites of hydrocarbon biosynthesis; L-methionine; methylation.

Abstract– The B race of the green alga *Botryococcus braunii* is characterized by the production of large amounts of botryococcenes, i.e. triterpenoid hydrocarbons of general formula C_nH_{2n-10}, $n = 30$–37. The axenic strain used in this work produces botryococcenes ranging from C_{30} to C_{34} when fast growth is promoted by air-lift. Sequential extraction of hydrocarbons with solvents showed that botryococcenes accumulate in two distinct sites: externally in the successive outer walls forming a dense matrix and internally, probably in cyctoplasmic inclusions. Moreover, chase experiments after feeding the algae with sodium [1,2-^{14}C]acetate, and feeding experiments with L-[Me-^{14}C]methionine established the existence of an excretory process from the cells towards the matrix. The results of the radio GC analyses of the botryococcenes synthesized during the feeding experiments provided good evidence to show that the C_{30} botryococcene is the precursor of all the higher hydrocarbons, and that each intermediate botryococcene C_{31}–C_{33} is the precursor of its next highest homologue. L-Methionine acts as the methyl donor in the methylation process, leading from the C_{30} precursor to the botryococcene family. The ^{13}C NMR spectra of the botryococcenes produced when the algae were fed with L-[Me-^{13}C]methionine indicate that the methylation takes place on the C_{30} backbone in positions 3, 7, 16 and 20.

INTRODUCTION

It is now well established that the hydrocarbon-rich alga, *Botryococcus braunii*, is divisible into two hydrocarbon-distinct races [1]. Algae of the A race produce odd-numbered *n*-alkadienes and trienes from C_{23} to C_{31}, while those of the B race yield branched polyunsaturated hydrocarbons of general formula C_nH_{2n-10}, $n = 30$–37, termed botryococcenes. The studies reported up to now show some analogies between the two races. Their ultrastructures are rather similar and attempts to correlate organization with hydrocarbon type have failed [2]. In both races, hydrocarbons are synthesized during active growth [1, 3]. The major part of the hydrocarbons (90–95%) is extracted quickly with non-polar solvents [1, 3, 4]; additional amounts are obtained from extraction with more polar solvents. This sequential extraction suggested the existence of two hydrocarbon pools which was confirmed for alkadiene producing-algae using a Raman microprobe [5]. A pulse chase experiment with radiolabelled precursors established that there was a distinct biosynthetic site for each hydrocarbon pool and ruled out the possibility of migrations from one pool to the other [6].

By contrast, preliminary experiments on botryococcene biosynthesis provided some support for the idea of an excretory phenomenon in algae of the B race [7]. It was also suggested that botryococcenes C_{31}–C_{37} are formed by methylation from lower homologues of similar structure [7, 8]. In this respect the C_{30} botryococcene, probably synthesized by a 1′ 3 condensation of two C_{15} units [9], should act as the precursor of all the higher metabolites.

The present study was undertaken with an axenic strain of the B race isolated from algae sampled in Martinique [1], in an attempt to corroborate these assumptions. To this end, the relationship between external and internal pools was examined by means of chase experiments after feeding with sodium [1,2-^{14}C]acetate. In addition L-[Me-^{14}C] and L-[Me-^{13}C]methionines were fed to the cultures and the hydrocarbons analysed by radio GC and ^{13}C NMR.

RESULTS AND DISCUSSION

Localization and analysis of botryococcenes from an axenic B strain

An axenic strain of the B race was isolated from the previously described MLM2 strain originating from Martinique [1]. It was established from a single colony, and the daughter subcultures were grown for the first time under standard conditions (shaken and unaerated, see Experimental). No antibiotic or chemical treatment was applied, in order to avoid possible irreversible perturbation of hydrocarbon biosynthesis.

After a culture period of 2 months, the algae were harvested, dried and submitted to successive extractions

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with solvents of increasing polarity. The hexane extract contained most of the botryococcenes (32% dry wt), while an additional amount (2% dry wt) was obtained from the chloroform–methanol extract. The acetone extract of intermediate polarity contained only traces of hydrocarbons. By contrast with race A, we could not identify oily cytoplasmic inclusions as hydrocarbons by means of a Raman microprobe due to the presence of carotenoids, the bands of which would mask the low intensity ones of any botryococcenes present in these inclusions [5]. Nevertheless, the results of the sequential extraction point to the existence of an external pool extractable with hexane and of an internal pool only accessible with chloroform–methanol, as recently proposed by Wolf *et al.* [10] for another strain of the B race. The almost complete absence of botryococcenes in the acetone extract corroborates this hypothesis.

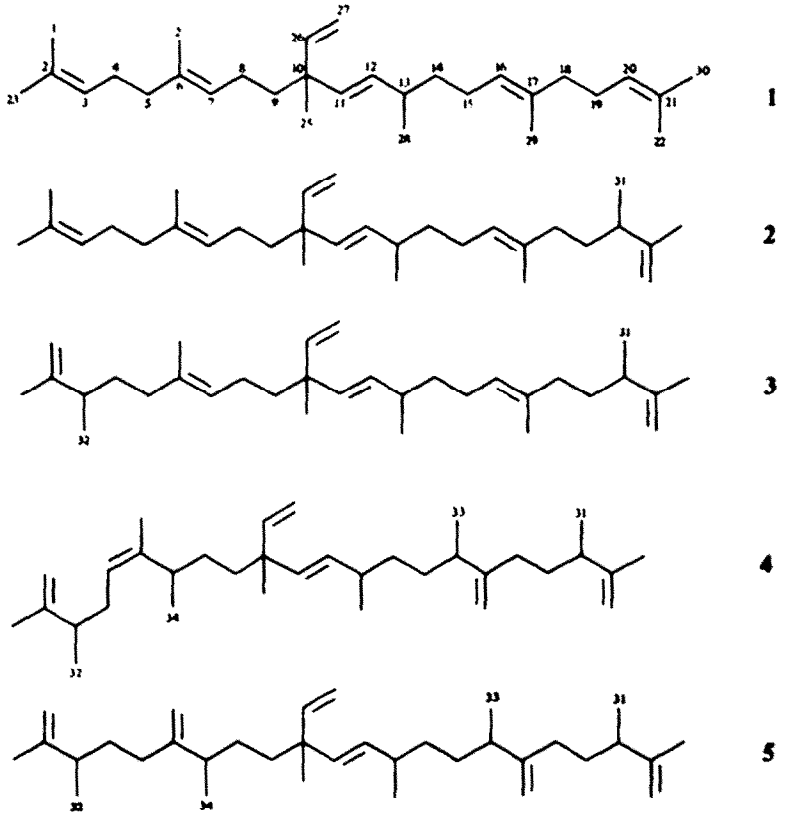
GC/MS analyses were performed on the purified hydrocarbons from the axenic strain grown under two sets of conditions: standard and batch air-lift, this latter technique bringing about an increase in growth during the exponential phase [1]. From these different cultures nine botryococcenes, C_{30}-C_{34}, and squalene (less than 1% of the hydrocarbon fraction) were identified (Table 1 and Scheme 1).

For the standard cultures, a similar distribution of botryococcenes was observed in the external pool and in the internal one. In both pools the C_{34} botryococcene, **4**, predominated. In these conditions only very small variations in the relative abundance of these hydrocarbons occured with culture duration.

By comparison with standard culture conditions, air-lift culture conditions affected the composition of the botryococcene mixtures both of the external pool (Table 1), as previously noticed for other B strains [1], and of the internal one, i.e. C_{34} botryococcenes decreased in favour of lower hydrocarbons especially those in the internal pool; two hydrocarbons, C_{32} (*RR*$_t$ 0.859) and C_{34} (*RR*$_t$ 0.768), not produced. Moreover, a somewhat different distribution was then observed between the pools: **4** was still preponderant in the external pool while **1** and **2** predominated in the internal one. In older cultures, the internal and external botryococcene mixtures tended to the same composition, which was rather similar to that observed under the standard conditions. The accumulation of the C_{30} hydrocarbon **1** in the cells was attributed to a greater efficiency of its biosynthesis relative to the alkylation process in the CO_2 enriched culture.

Feeding experiments with radiolabelled precursors

Chase experiments were performed in order to investigate the possible relationship between the two hydrocarbon pools and to examine the role of the C_{30} hydrocarbon **1** as a precursor of the higher homologues. After feeding with sodium [1,2-^{14}C]acetate over a 2 day period the algae were transfered into fresh 'cold' medium and growth allowed to continue for different periods of time. The radioactivities of the botryococcene pools at the end of the experiments were corrected for the presence of ^{14}C-labelled squalene the radioactivity content of which was evaluated from the areas of the radio GC peaks (Table 2).



Scheme 1. Structures of botryococcenes **1**–**5**.

Table 1. GC-MS analyses of botryococcenes from the B axenic strain cultivated under different conditions

C_nH_{2n-10} (*n*)	*RR*, (squalene = 1)	Compounds*	Standard culture† External pool	Standard culture† Internal pool	Air lift culture‡ External pool	Air lift culture‡ Internal pool
30	0.760	1			18.1	38.7
31	0.770	2			10.4	24.6
32	0.859		3.3	3.5		
32	0.870	3			7.9	7.7
32	0.882				4.2	3.1
33	0.873		4.9	4.4	10.8	6.6
34	0.768		4.3	4.4		
34	0.814	4	66.5	66.4	35.8	14.7
34	0.847	5	16.7	16.8	8.8	1.5
n.i.			4.3	4.5	4.0	3.1
% Dry wt			32	2	37	3

*Compounds 1-5 have previously been characterized [13].
†Two-month-old culture.
‡Seven-day-old culture.
n.i.: Compounds not identified due to poor resolution of GC peaks.

Table 2. Variation in botryococcene labelling during chase experiments*

Botryococcenes	Radioactivity (10^{-3} dpm) Time in 'cold' medium (hr) 0	2	7	30	100
Internal pool					
Total	185	86	38	16	1.5
C_{30}	37	10	4	n.d.†	n.d.
C_{31}	33	12	6		
C_{32}	43	17	7		
C_{33}	46	17	8		
C_{34}	26	30	13		
External pool					
Total	408	482	568	599	623
C_{30}	40	39	34	24	
C_{31}	29	29	28	18	
C_{32}	20	34	62	36	
C_{33}	25	44	46	42	
C_{34}	294	336	398	479	‡
α (External/internal)	2.2	5.6	14.9	37.4	415

*The distribution of the label inside each pool was determined through botryococcanes.
†Not determined, because of lack of sensitivity of the radio GC technique.
‡More than 90% of the total radioactivity was located in the C_{34}.

At time 0, the radioactivity content of the total external botryococcenes was about twice that of the total internal content. During the chase, the labelling ratio α (external/internal) increased, and after 100 hr, almost all the radioactivity was recovered in the hexane extract (external pool). These data were indicative of hydrocarbon excretion from the internal pool to the external a process, which does not occur for the alkadienes produced by the A race [6]. Moreover, the total hydrocarbon radioactivity (external + internal) exhibited a small increase during the chase: 593×10^3 dpm at time 0, against 624×10^3 dpm after 100 hr. This increase may have been due to botryococcene biosynthesis from degradative products of cellular components such as reserve lipid materials, and from close radioactive precursors such as C_{15} units biosynthesized during the last hours preceding the chase.

The distribution of the label between the hydrocarbons of different chain lengths was measured at each time point

(Table 2). However, due to the poor resolution of the botryococcenes on the packed columns used for radio GC, this analysis was performed on the fully hydrogenated botryococcanes which were better resolved. In the internal pool, there was a continuous decrease in the radioactivity in the C_{30}–C_{33} hydrocarbons, although the C_{34} hydrocarbon exhibited a very small increase during the first 2 hr. A somewhat different trend was observed for the external hydrocarbons. Indeed, with regard to the net increase of the label during the chase, three categories of external hydrocarbons could be recognized: the C_{30}–C_{31}, the C_{32}–C_{33} and the C_{34}. The first group lost radioactivity, while the activity reached a maximum after 7 hr and then declined in the record group, and continuously increased in the C_{34}. After 100 hr, the latter compound contained more than 90% of the label. In the light of this study, we can conclude that the C_{30} botryococcene is the precursor of all the higher hydrocarbons of the series. Wolf *et al.* came to the same conclusion from the results of a $^{14}CO_2$ pulse-chase experiment [8]. Furthermore, it also appears that each intermediate botryococcene, C_{31}–C_{33}, acts as a precursor of the next homologue. In this axenic strain, the C_{34} hydrocarbons are the end products of the biosynthetic pathway: **1** → **2** → **3** → → **4**, **5**. The variations in the external C_{30}–C_{33} activities probably result from the conjunction of two processes of different nature and of opposite effects. Thus the transformation of the lowest hydrocarbons into the highest hydrocarbons through methylation tends to decrease the radioactivity of the former, when the hydrocarbon transfer from the internal site to the external one counterbalances this decline, at least at the beginning of the chase. In other words, the C_{30} C_{33} activities at a given time depend on the relative rates of the excretory and methylation processes.

The distribution of the radioactivity in the internal and external hydrocarbon pools was also analysed after feeding L-[Me-^{14}C]methionine. The radioactivity of the external botryococcenes was higher than that of the internal ones (Tables 3). Nevertheless, the labelling ratio β (external/internal) showed important variations with time: a fast decrease after 4 hr followed by a regular and slow increase. Radio GC showed that no radioactivity was associated with C_{30}, whatever the feeding time or the pool considered. Initially, the C_{31} compound exhibited the highest labelling. In the hexane extract, the activities associated with the C_{31}–C_{33} hydrocarbons declined after reaching a maximum, when the C_{34} label increased. In the chloroform–methanol extract, after a slower rate of incorporation into the C_{31}–C_{34} hydrocarbons during the first 2 hr than that observed for the same external compounds, a rapid increase was noticed, followed by a slow decrease for all botryococcanes.

The incorporation of ^{14}C methyl groups from labelled methionine in the external pool as well as in the internal one (high β value and presence of botryococcenes > C_{30} in the two pools) and the irreversibility of the excretory process established in the chase experiment and confirmed by the increase of β after 4 hr, prove that methylation takes place in both pools.

Feeding experiments with L[Me-^{13}C]methionine

Supplying culture media with methionine is known to have a negative influence on some photosynthetic organisms, e.g. *Euglena*, growth restricted [11]; carrot cells, protein synthesis inhibited [12]. A similar effect was observed with the *B. braunii* B race: 0.5 mM methionine strongly inhibited the growth*. Moreover, the botryococcene composition was found to depend on the initial biomass concentration, the amount of added methionine and the incubation time as was shown in the two following experiments. In Experiment 1, the culture was initiated

* The very small amount of ^{14}C-labelled methionine used in the preceding experiment, about 0.1 μM, did not affect algal growth.

Table 3. Variation in botryococcene labelling after admistration of L-[Me-^{14}C]methionine*

	Radioactivity (10^{-4} dpm)				
	Time after feeding (hr)				
Botryococcenes	2	4	7	23	68
Internal pool					
Total	55	451	321	281	158
C_{31}	20	172	119	70	5
C_{32}	15	117	64	48	27
C_{33}	12	81	64	53	29
C_{34}	8	74	81	110	97
External pool†					
Total	792	902	1144	1386	1604
C_{31}	277	289	320	152	32
C_{32}	206	207	206	180	32
C_{33}	158	171	206	194	64
C_{34}	151	235	412	860	1476
β (External/internal)	14.4	2.0	3.6	4.9	10.1

* See the first footnote in Table 2.

† After 5 days more than 95% of the radioactivity was concentrated in the external C_{34}.

Table 4. Compositions of the botryococcene mixtures (external pool) in relation to the method of addition of L-[Me-^{13}C]methionine (% of the whole)

Compounds	Experiment 1 Continuous addition of methionine	Experiment 2 Single, large addition of methionine
C_{30} 1	5	8
C_{31} 2	8	6.7
C_{32} 3	87	32.4
C_{33} (*RR*, 0.873)	Traces	11.4
C_{34} 4	Traces	30.5
C_{34} 5	Traces	8.0
Others	Traces	3.0

from a small inoculum and small amounts of the labelled precursor were added over an 18 day growth period, thereafter a large amount of methionine was added and the culture continued for 5 more days. In Experiment 2, a single dose of labelled methionine was added to a well-developed culture. The effects of these treatments on the external botryococcenes are shown in Table 4. From the botryococcene mixture synthesized during Experiment 1, the hydrocarbon 3 was separated by reversed-phase HPLC. Its ^{13}C NMR spectra exhibited 11-fold enhancement of the signal at δ19.68, previously ascribed to C-31 and C-32 relative to that observed for the unlabelled compound [13].

Thus, the incorporation of ^{13}C methyl groups shows that methylation takes place on positions 3 and 20 of the C_{30} backbone, to give botryococcene 3; the protons lost during the alkylation originate from the vinylic methyl groups Me-1 and Me-22.

HPLC of the botryococcene mixture recovered from Experiment 2 did not allow a separation of isomers 4 and 5, as already observed when compounds differ from one another only in the positions of the double bonds. Nevertheless, in the ^{13}C NMR spectra of the mixture 4 + 5 (3:1) four signals predominated at δ19.68 (C-31 of 4), 19.54 (C-32 of 4) 19.78 (C-31, 32 of 5 and C-34 of 4*), and 20.22 (C-33 of 4 and 5).

All these observations demonstrate that the methylation takes place on positions 3, 7, 16 and 20 of the C_{30} backbone.

CONCLUSION

In *B. braunii* race B, feeding and chase experiments have provided evidence of the existence of an excretory process for botryococcenes from the cell towards the matrix; this phenomenon and the difference in rates, which probably exists, between C_{30} biosynthesis and the methylation step could explain the relative localization of the lowest and highest hydrocarbons during active growth, under conditions promoting fast-growing cultures. Feeding experiments with radiolabelled acetate and methionine demonstrated that each botryococcene > C_{30} originates from the methylation of a lower homologue, the methylation reactions occur both in the cell and in the matrix, the C_{30} hydrocarbon being the precursor of all compounds of this family.

The accumulation of different botryococcenes in nature or in culture is indicative of the specificity and diversity of the methylation systems in race B. From this point of view, our recent isolation from a wild sample of a new strain able to synthesize and to accumulate a partially cyclized botryococcene may be of some interest. As earlier hypothesized for the C_{34} cyclized botryococcene characterized in an Australian sample [13], the cyclization could be a direct consequence of the methylation process.

EXPERIMENTAL

Isolation and culture conditions of the axenic B strain. The axenic strain was derived from the previously described MLM2 strain originating from Martinique [1]. It was produced using dilution and rinsing techniques with a sterile modified CHU 13 medium [1]. After centrifugation, the process was repeated *ca* 10 times on the floating colonies collected with a sterile pipette. Then, the colonies were inoculated on agar (12 g/l.) plates: modified CHU 13 medium with an admixture of nutrients for bacterial growth (glucose 0.1 g/l. yeast extract 0.1 g/l., peptone from casein 0.2 g/l.). After 2 weeks of growth, an axenic *B. braunii* colony was removed and inoculated into liquid medium. Daughter subcultures were systematically examined using suitable media for bacteria growth as previously described [14]. The conditions for standard and batch air-lift (1% CO_2) cultures were as already reported [1].

Localization and GC-MS analyses of botryococcenes. The dry biomass was subjected to successive extractions with hexane (2 × 1 hr) Me_2CO (12 hr), $CHCl_3$–MeOH (1:1, 24 hr). The crude extracts were chromatographed on neutral Al_2O_3, activity II, with hexane as eluent to obtain botryococcenes. GC/MS analyses were performed as previously described [1, 13]. The *in vivo* Raman microprobe study was carried out as described in [5].

Feeding experiments. The algae were fed with sodium [1,2-^{14}C]acetate (94 mCi/mM), L-[Me-^{14}C]methionine (51 mCi/mM) and L-[Me-^{13}C]methionine (isotopic purity 90%), dissolved in H_2O and autoclaved.

The radio-labelled experiments were conducted as follows: cylindrical tubes containing 180 ml of culture medium were inoculated with identical amounts of algal biomass and the cultures performed under batch air-lift conditions (1% CO_2), at 25° with continuous illumination (470 μE/m^2 · sec). After one week, acetate (5 μCi) and methionine (10 μCi) were separately injected into each tube. Cultures with [^{14}C]methionine were taken for analysis after various times of growth. The algae were dried under vacuum at 60°, subjected to successive extractions with solvents and the botryococcene fractions purified as above. In the chase expt, algae grown in the presence of ^{14}C-acetate were harvested after 3 days under sterile conditions, washed with modified CHU 13 medium and resuspended in fresh cold medium and growth allowed to continue. The cultures were analysed after, different periods of time by the methods just described. Radioactivity was determined by liquid scintillation counting using 0.4% butyl-PBD in toluene. Radio GC was carried out on botryococcanes [13] using a SE 30 10% column at 280°.

Incorporation of L-[Me-^{13}C]methionine was achieved by two different methods. Experiment 1: to an air-lift culture of low

*When we studied 4 by ^{13}C NMR, a peak at δ17.96 was ascribed to carbon 34 [13]. The non-observation of any increase in intensity for this signal in the spectrum of the hydrocarbon mixture produced during Experiment 2 leads us to consider that the previously published assignments for C-24 and C-34 of 4 must be interchanged as follows: C-24 17.96 and C-34 19.78.

initial biomass (*ca* 50 mg/l.) 0.5 mg of methionine was added on days 1, 5 and 10, 1 mg on day 15, 2 mg on days 16, 17 and 18 and 100 mg on day 25. The culture was taken for analysis on day 30. Experiment 2: to a dense batch air-lift culture in linear growth phase (dry biomass of *ca* 2 g/l.), 100 mg of methionine were added. The culture was taken for analysis five days later. Hydrocarbons were extracted with hexane, purified as usual and separated by reversed-phase HPLC as described in ref. [13]. ^{13}C NMR (50 MHz) spectra were obtained from $CDCl_3$ solns using TMS as int. ref.

Acknowledgements—We wish to thank Dr. Brenckmann for the isolation of the axenic strains, Dr. Sellier and Miss Mauroy, Laboratoire de Spectrométrie de Masse, E.N.S.C., Paris, for GC/MS analyses, Dr. Dhamelincourt, Laboratoire de Spectrochimie Infrarouge et Raman, U.S.T., Lille, for Raman spectra and Dr. Vuilhorgue, Société Rhone-Poulenc, for assistance in recording ^{13}C NMR spectra. This work was supported by the CNRS, as a part of the 'Programme Interdisciplinaire de Recherche sur les Sciences pour l'Energie et les Matières Premières'.

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