Tetrahedron Letters,Vol.25,No.37,pp 4123-4126,1984 0040-4039/84 $3.00 + .00
Printed in Great Britain

BIOSYNTHESIS OF TRITERPENOID HYDROCARBONS IN THE ALGA BOTRYOCOCCUS BRAUNII

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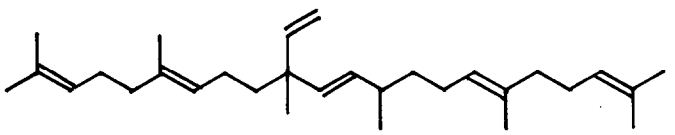
Abstract - ^{14}C-labelled tracers were incorporated into botryococcenes, triterpenoid hydrocarbons produced by a variety of the unicellular alga Botryococcus braunii. Data obtained with L-leucine might support for the biosynthesis the possibility of a non-mevalonoid pathway.

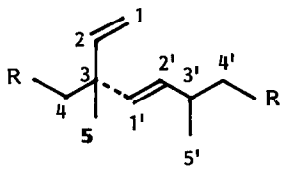
The unicellular alga, Botryococcus braunii, is well known for its high hydrocarbon content[1]. It was recently established that under name "B.braunii" there are in fact two varieties with very close morphologies[2] : one producing odd, long, unbranched alkadienes and trienes (from C_{25} to C_{31}), the other synthezising mainly acyclic and branched, highly-unsaturated hydrocarbons termed botryococcenes, of general formula C_nH_{2n-10} ($30 \leqslant n \leqslant 37$).

The structures of ten of these last compounds have been determinated[3-6] ; the data show they have in common the C_{30} carbon skeleton 1 under represented :



1

and suggest an isoprenoid origin. Moreover, a biosynthetic pathway which imply a tail-to-tail linkage of two C_{15} units, involving an 1'-3 condensation, was proposed[5] as for Artemisia ketone[7], or for isodigeranyl[8] : 2



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However, because growing in laboratory Botryococcus strains producing botryococcenes was until recently unsuccessful, this pathway remains hypothetical. Sometime ago, we succeeded in culturing wild strains of botryococcene-producing algae, and we have started a study about botryococcene biosynthesis. The recent publication of a paper related to the same topic[9] prompts us to report the first results we have obtained by supplying the alga with radioactive precursors.

Cultures of B.braunii were grown on a chemically defined medium[10], at 25°C, illuminated 24h/24h. The harvested cells were dried under vacuum and hydrocarbons extracted with solvents. As for the alkadienic strains, two pools of hydrocarbons were recovered according to the polarity of the solvent system used for their extraction and hence to their location in the cells.[11] The strain yielded essentially C_{30}, C_{31} and C_{32} (major) botryococcenes, as already described[3]. The main pool located in the outer wall of the alga (30 to 40 % of the dry biomass), was extracted with hexane (twice, one hour). Further extraction with $CHCl_3$-CH_3OH 1-1 (24 hours) gave a minor fraction of botryococcenes (1 to 2 % of the dry wt) present in the cells as cytoplasmic inclusions. Each hydrocarbon pool was then purified as already described for straight chain hydrocarbons[10].

In a preliminary experiment 10 µCi of sodium (2-^{14}C) D.L.-mevalonate (MVA) was fed to four batch cultures with the same amount of biomass, in the exponential growth phase* (100 mg/l. dry wt). Incorporation into botryococcenes was monitored after fixed feeding periods (Table I). In a second run of experiments (Table II) a higher amount of biomass was used (600 mg/l), the alga being also in the exponential phase. Feedings were performed separately with sodium (2-^{14}C) MVA, sodium (2-^{14}C) MVA + Tween 20 (so as to ensure a better penetration of the precursor into the biosynthetic sites), sodium (U-^{14}C) acetate and (U-^{14}C) L-leucine (10 µCi of each precursor was added). $AgNO_3$-SiO_2 TLC showed that the recovered radioactivity of the hydrocarbon fractions was located in the botryococcenes.

TABLE I - Incorporation of sodium (2-^{14}C) MVA* into botryococcenes.

Feeding time h	% incorporation** External pool	% incorporation** Internal pool	α***
5	0.045	0.014	3.2
24	0.149	0.018	8.1
48	0.237	0.027	8.6
144	0.358	0.026	13.6

*Specific activity of (2-^{14}C) D.L.-MVA : 39.5 mCi/mMole.

**Calculated as the only (3R)-enantiomer.

*** α = external pool/internal pool.

The results of the first experiments show that MVA, an usual precursor of isoprenoid compounds is incorporated into botryococcenes up to 0.35 % in six days. This level is higher than those observed in monoterpene biosynthesis studies both for some higher

*The highest hydrocarbon productivity was observed during this phase.

plants as Cinnamomum camphora[12] and for microorganisms such as the fungus Ceratocystis moniliformis[13], but lesser than those sometimes obtained for incorporation of MVA into higher terpenes[14].

It can be observed also that the radioactivity of the external pool is much higher than that of the internal one, whatever feeding time may be (Table I). The increase of α with time could mean that internal botryococcenes should migrate from the internal pool to the external one. This result, if it were confirmed, would be opposite to that observed with the "alkadienic strains", for which it was shown that two distinct sites of hydrocarbon biosynthesis are involved[11].

For the second run, (2-^{14}C) MVA incorporates at the same rate within 24 h (0.2 %) as in the first run (Table II). Attempts to increase incorporation of MVA by adding Tween was unsuccessful. Comparative incorporation experiments with (5-^{14}C) MVA and (2-^{14}C) MVA, at present under progress in our laboratory, could support a degradation of these tracers prior incorporation.

Sodium (U-^{14}C) is rapidly incorporated : near 12 % in 24 h. (By comparison, sodium (2-^{14}C) acetate is incorporated to 0.027 % in 24 h into geraniol by Pelargonium roseum[12] and 0.2 to 1 % into monoterpenoids by C. moniliformis[13]). The high level of incorporation observed with sodium acetate is certainly to be related to the strong orientation of the alga metabolism toward terpenoid production.

TABLE II - Incorporation of three presumed precursors* into the external botryococcenes in 24 hours.

Precursors	Sodium (2-^{14}C) MVA	Sodium (2-^{14}C) MVA +2% Tween 20	Sodium (U-^{14}C) acetate	(U-^{14}C) L-Leucine
Incorporation** %	0.20	0.066	11.76	5.23

*Specific activities : sodium (2-^{14}C) D.L.-MVA ; 39.5 mCi/mMole ; Sodium (U-^{14}C) acetate : 94 mCi/mMole ; (U-^{14}C) L-leucine : 250 mCi/mMole.

**Calculations take into account that to build up a C_5 moiety : from MVA, only the 3R-enantiomer is incorporated ; from acetate, three molecules participate, from which one carbon atom over six is lost ; from L-leucine, loss of one carbon atom.

Leucine, a discussed precursor of monoterpenoid biosynthesis by higher plants[15] was also tested. (U-^{14}C) L-leucine is incorporated to 5.23 % into botryococcenes. This level, very high in regard to previous reports[12,13], suggests the possibility of a non-mevalonoid pathway.

The structures of all botryococcenes suggest that alkylation should be subsequent to the linkage of the C_{15} units[4], the C_{30} botryococcene exhibiting the role of a precursor of all higher metabolites of this series. This point of view is supported by the radioactivity distributions evaluated for experiments with leucine at different incubation times. Indeed, when the incorporation level is maximum after 24 hours and remains stable up to 72 hours, the radioactivity distribution between the C_{30} and the higher botryococcenes shows an

important variation during this same period of time. Thus, C_{30} labelling declined from 46 % to 14 % (of the whole radioactivity) while C_{31} and C_{32} labelling increased to a same extent. As we were writing this paper, we were kindly informed by Dr. F.R. WOLF that his group at Lawrence Berkeley Laboratory, University of California, have reached similar results from $^{14}CO_2$ labelling experiments.

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(Received in France 25 June 1984)