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TETRAMETHYLSQUALENE, A TRITERPENE FROM *BOTRYOCOCCUS BRAUNII* VAR. *SHO WA*

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Key Word Index-Botryococcus braunii var. *showa*; Chlorophyceae; alga; irregular triterpenes; methylated squalene.

Abstract-A new triterpene, tetramethylsqualene, produced by Botryococcus ***braunii*** var. ***showa***, was isolated and characterized by NMR and mass spectroscopy. The compound is a squalene derivative with non-isoprenoid methyls at C-3, C-7, C-18, and C-22. Tetramethylsqualene was a minor component (ca 0.2%) of normal cultures; however, its concentration increased 20-fold in cultures supplemented with 2.1 mM L-methionine.

INTRODUCTION

Botryococcus braunii is a fresh water green alga with a cosmopolitan distribution. It has an extremely high hydrocarbon content (up to 75% of its biomass) [1, 2] and has been implicated in the formation of several oil-rich geological deposits [3, 4]. The B-race of ***B. braunii*** synthesizes a family of l'-3 linked triterpenes called botryococcenes [5]. The major component of the oil isolated from the alga is usually a C_{34} tetramethylated triterpene given the familial name botryococcene (1) [6]. The mechanism for biosynthesis of the botryococcene skeleton is thought to be closely related to the formation of squalene from two molecules of farnesyl diphosphate [7]. In ***B. braunii*** the parental triterpene is successively methylated to generate a family of acyclic and cyclic members [5, 8, 9].

In addition to the botryococcenes, we routinely found small quantities of squalene (0.2–0.7% of the total botryococcene fraction) in isolates from ***B. braunii*** var. ***showa*** [5,10]. Metzger and coworkers [11] recently reported the occurrence of a mono-methylated squalene on the basis of GC-MS studies of hydrocarbons from another botryococcene-producing strain. We now report the formation of a tetramethylsqualene in our cultures upon supplementation with L-methionine.

RESULTS AND DISCUSSION

During the course of feeding experiments with ***B. braunii*** var. ***showa***, we studied the effects of L-methionine on the distribution of hydrocarbons. In addition to a slight retardation of growth as the final concentration of L-methionine in the media was increased to 2.1 mM (see Fig. 1), there was a noticable shift in the population of botryococcenes to the higher members of the family [9], and the amounts of some compounds normally found in low concentrations increased noticeably.The largest percentage change was seen for an unknown compound (2), which increased from ca 0.2% in unsupplemented cultures to 4.4% in cultures containing 2.1 mM L-methionine (Fig. 1). This compound was selected for further characterization.

Repeated injection of the crude botryococcene fraction from methionine-enriched cultures on a 5μ ODS-Hypersil HPLC column gave 2.5 mg of pure 2. The compound eluted after braunicene [7], and when analysed by GC (DB-5, 245") eluted later than squalene and all of the botryococcenes. A high resolution mass spectrum of 2 had a molecular ion at ***m/z*** 466.4536, consistent with the formula $C_{34}H_{58}$. A ^{13}C NMR spectrum gave 17 sharp peaks, and a HET2D-J spectrum revealed 4 CH_3, 7 CH_2, 3 CH, and 3 C units. Of that group, 2 CH,, 1 CH, and 3 C were olefinic carbons. These results suggest that 2 is a tetramethylated triterpcne with a two-fold element of symmetry.

Inspection of the 500 MHz ^1H NMR spectrum of 2 (see Table 1) indicated that the compound was not a botryococcene because of the absence of characteristic resonances for the vinyl moiety and the quaternary methyl formed by the 1′–3 linkage. Two sets of methyls (6 1.004 and 1.026 *d*, *J* = 6.8 Hz) were attached to aliphatic methines, while the remaining methyls (6 1.585 and 1.663) were on double bonds. The methines appeared at 62.17 and 2.07 as cleanly resolved sextets, indicating that each was adjacent to an aliphatic methylene moiety. Two sets of multiplets centred at 64.72 and 4.69 had chemical shifts typical of the methylene protons of the unsymmetrically disubstituted double bonds in botryococcenes, while a broad signal at 65.137 had the appearance and chemical shift typically seen for olefinic hydrogens on trisubstituted double bonds in isoprenoids [12]. The remaining protons were located in methylene units centred at 62.00 and 1.90 and in overlapping multiplets between 1.37-1.58 (relative ratio I : 2: 2).

The connectivities of the individual protonated carbons were established from COSY spectra. Beginning at the end of the isoprenoid chain, the methyl at δ 1.663 (C-25, C-30) was coupled to the upfield peak of the olefinic methylene resonance at 4.69 (C-l, C-24), which, in turn, had a crosspeak to the sextet centred at 2.17 (C-3, C-22). The sextet was coupled to the diastereotopic methylene protons centred at 61.40 and 1.50 (C-4, C-21) and the methyl doublet at 1.026 (C-31, C-34). Crosspeaks were

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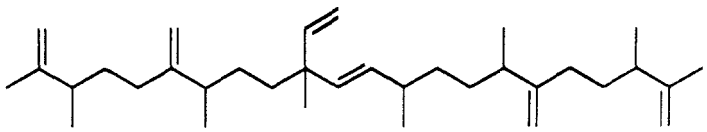

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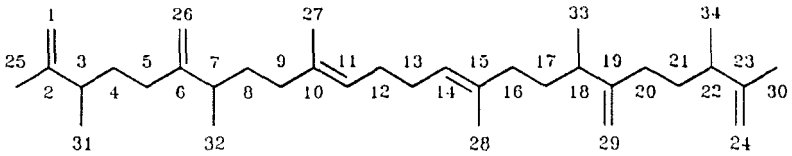

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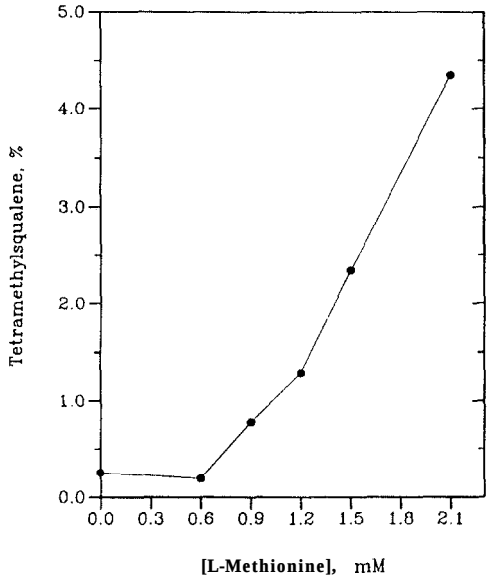

Fig. 1. Percentage of tetramethylsqualene (2) in the non-polar hydrocarbons from B. *braunii versus* the concentration of L-methionine in the media.

observed from the methylene resonance at δ 1.90 (C-S, C-20) to the signals at 61.40 and 1.50, to the oletinic methylenes at 64.72 (C-26, C-29), and to the sextet at 62.07 (C-7, C-18). This latter signal was also coupled to the methyl doublet at δ 1.004 (C-32, C-33) and diastereotopic methylene resonances at δ 1.35 and 1.50 (C-8, C-17), which were, in turn, strongly coupled to each other. The signal centred at 61.91 (C-9, C-16) had cross peaks back to the C-8 (C-17) methylenes, to the oletinic proton at 65.137 (C-l 1, C-13), to the oletinic methyls at 61.585 (C-27, C-28), and to the methylenes at 62.00 (C-12, C-13). Thus, COSY connectivities which established a squalene structure with additional methyls at C-3, C-7, C-18, and C-22 typical of the methylated centres found in botryococcenes were observed for all protonated carbons in the symmetrically methylated farnesyl units.

Additional connectivities and the stereochemistries of the trisubstituted double bonds were established from NOE and single-frequency decoupling experiments. An NOE was observed from the vinyl proton at C-l 1 (C-14) to the C-9 (C-16) methylene at δ 1.91 but not to the C-27 (C-28) methyl at 61.585. Thus, the C-10/C-11 (C-14/C-15) double bond had an E-configuration. When the signal at 65.137 was decoupled, the multiplet at δ 2.00 (C-12, C-13) sharpened to a singlet, while only minor changes were noticed at δ 1.91. These observations reinforce the structure deduced from COSY spectra.

The mass spectrum of 2 was also consistent with the proposed structure. A high mass peak formed by cleavage of the diallyic C-12/C-13 bond was seen at *m/z* 233. Peaks at *m/z* 149 and 151 are typical of the higher methylated acyclic botryococcenes and probably arise from cleavage between C-7 and C-8 (C-17 and C-l 8) followed by expulsion of hydrogen to generate a stable. highly conjugated cation.

Tetramethylsqualene (2) is an isomer of the common acyclic C_{34} botryococcene **1.** Metzger and coworkers reported that feeding experiments with L-methionine gave high levels of incorporation in the non-isoprenoid methyl groups in 1 [9], and it is commonly assumed that the methylation reactions in B. *braunii* are electrophilic alkylations involving S-adenosyl methionine [5, 7, 9]. Pulse-chase experiments further suggest that the methyls are added one at a time in a sequential manner [5, 9]. The richness of the methylation patterns observed among the botryococcenes could be accommodated by the action of a few isoprenoid transmethylases with rather low substrate specificities or by a large family of highly specific enzymes. Our data are more compatible with the former proposal. Under normal conditions, little squalene is found in the botryococcene fraction, and one would anticipate that much of the squalene made by B. *braunii* serves as a precursor for sterol biosynthesis. However, when the alga is incubated in the presence of high levels of methionine under conditions which should result in higher intracellular concentrations of S-adenosyl methionine, the relative concentration of 2 increased dramatically. It is reasonable to assume that the activities of the transmdthylases would increase in response to higher

Table 1. NMR spectra of tetramethylsqualene

Assignment	^{13}C (δ)*	^{1}H (δ, J (Hz)) †	COSY cross peaks†	HET2D-J*
C-l, C-24	109.270	4.69 (4H, m)	2.17, 1.663	CH_2
C-2, C-23	149.700			C
c-3, c-22	40.967	2.17 (2H, *sextet*, 6.8)	4.69, 1.50, 1.40, 1.026	CH
c-4, c-21	33.407	1.50 (2H, m)	2.17, 1.90, 1.40	
		1.40 (2H, m)	2.17, 1.90, 1.50	CH_2
c-5, c-20	31.638	1.90 (4H, m)	4.72, 1.50, 1.40	CH_2
C-6, C-19	154.601			C
C-7, C-18	39.546	2.07 (2H, *sextet*, 6.8)	4.72, 1.50, 1.35, 1.004	CH
C-8, C-17	37.488	1.50 (2H, m)	2.07, 1.91, 1.35	
		1.35 (2H, m)	2.07, 1.91, 1.50	CH_2
C-9, C-16	33.968	1.91 (4H, m)	5.137, 1.50, 1.35	CH_2
c-10, c-15	135.126			C
c-11, c-14	123.888	5.137 (2H, *br c*, 6.8)	2.00, 1.91, 1.585	CH
c-12, c-13	28.234	2.00 (4H, *m*, 6.8)	5.137, 1.91, 1.585	CH_2
C-25, C-30	18.886	1.663 (6H, *br s*)	4.69	CH_3
C-26, C-29	107.092	4.72 (4H, m)	2.07, 1.90	CH_2
C-27, C-28	15.990	1.585 (6H, *br s*)	5.137, 2.00, 1.91	CH_3
c-31, c-34	19.740	1.026 (6H, *d*, 6.8)	2.17	CH_3
C-32, C-33	20.136	1.004 (6H, *d*, 6.8)	2.07	CH_3

*Recorded at 100 MHz.
†Recorded at 500 MHz.

concentrations of substrate by a simple K_M effect. Since botryococcenes are terminal metabolites, increased methylation should shift in the distribution of products to higher methylated derivatives without altering the total production of botryococcenes. In contrast, enhanced methylation of squalene diverts material from sterol biosynthesis and increases the relative amount of methylated squalene in the hydrocarbon fraction of the organism. If substrate specificities for the botryococcene transmethylases are indeed low, one would anticipate that the hydrocarbon fraction may contain a variety of methylated squalene derivatives.

EXPERIMENTAL

General. NMR spectra were recorded in $CDCl_3$ at 26" and were referenced to int. TMS. COSY spectra were obtained according to the procedure of ref. [13] with a delay, D_3, of 50 msec between the second 90° pulse and the acquisition period to enhance weak long-range interactions. Heteronuclear two dimensional J-resolved spectra (HET2D-J) were recorded according to the procedure of ref. [14]. Steady state NOE difference spectra were recorded with 6 sec pre-irradiation and 6 sec acquisition period. A steady state of 4 and block size of 16 was used to ensure optimal cancellation between the on- and off-resonance spectra. Mass spectra were obtained at 17eV ionizing voltage in the EI mode. A DB-5 fused silica capillary column (30 m x 0.25 mm) was used for all GC work. A 30 x 0.78 cm 5μ ODS-Hypersil column (70000 HETP/m) and a Waters chromatograph with detection at 214nm was used for all HPLC work. UV traces were digitized every 2sec on a Radiomatic Flo-One detector through the analog input channel and integrated using Flo-One software.

Feeding and isolation. Cultures of *B. braunii* var. *showa* were grown in media aerated with 1–2% CO_2 enriched air as previously reported [7]. L-Methionine was added to each of 800 ml cultures daily for 6 days starting on day 6, and the incubation was continued for 7 days following the final addition. Final concns of added L-methionine varied from 0 to 2.1 mM. Cells were harvested by filtration through 5μ Nitex cloth, washed with deionized water, lyophilized, and extracted by sonication in hexane–Me_2CO (1: 1) as previously described [7]. The extracts were passed through a short column of silica gel and eluted with four column vols of hexane. Individual samples were analysed by GC and HPLC (elution with MeCN).

A portion of the material isolated from cells grown on 2.1 mM methionine was fractionated by repeated injections (5 mg/injection) on ODS-Hypersil using MeCN as the mobile phase. Fractions containing the last peak to elute were combined and rechromatographed to give 2.5 mg of a colourless oil; $[\alpha]_D^{25}+6°$ ($CHCl_3$, c 0.21); mass spectrum (EI, 17 eV, *m/z*, rel. int.): 466 $[M]^+$ (1.4), 314 (1.7), 233 (2.4), 232 (1.2), 231 (1.7), 217 (2.0), 163 (10.7), 151 (11.4), 149 (42.6), 137 (13.5), 123 (47.3), 121 (44.2), 109 (62.2), 95 (100), 81 (57.7), and 69 (15.5); HRMS (EI, 17 eV), m/z 466.4536 ($C_{34}H_{58}$, 466.4539).

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