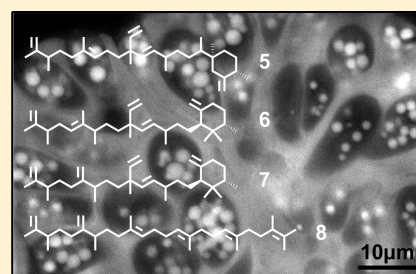


Isolation and Characterization of Cyclic C₃₃ Botryococcenes and a Trimethylsqualene Isomer from *Botryococcus braunii* Race BMehmet Tatli,[†] Mandar T. Naik,^{†,‡,§} Shigeru Okada,^{⊥,||} Lawrence J. Dangott,^{†,#} and Timothy P. Devarenne^{*,†,§}[†]Department of Biochemistry & Biophysics, [‡]Biomolecular NMR Laboratory, Department of Biochemistry & Biophysics, and [#]Protein Chemistry Lab, Department of Biochemistry & Biophysics, Texas A&M University, College Station, Texas 77843, United States[⊥]Laboratory of Aquatic Natural Products Chemistry, Graduate School of Agricultural and Life Sciences, University of Tokyo, Yayoi, Bunkyo, Tokyo 113-8657, Japan^{||}Japan Science and Technology Agency-Core Research for Evolutional Science and Technology (CREST), Gobancho, Chiyoda, Tokyo 102-0076, Japan

S Supporting Information

ABSTRACT: Three cyclic C₃₃ botryococcenes and one new trimethylsqualene isomer were isolated from the B race, Showa (Berkeley) strain of *Botryococcus braunii*, which is known to produce large amounts of isoprenoid hydrocarbons ranging in carbon number from 30 to 34. Their purity was determined by GC-MS, and structures were characterized by 1D and 2D NMR. One of these molecules, cyclic C₃₃-1 botryococcene (5), has an unusual connection of a methylenecyclohexane ring to the molecule backbone not seen before in botryococcenes. This report further adds to our knowledge of the wide range of isoprenoid hydrocarbon structures produced by *B. braunii*.



Botryococcus braunii is a green colonial microalga that has received great attention from scientists for several reasons. First, it produces large amounts of liquid hydrocarbons, which can be transformed into high-quality combustion engine fuels via hydrocracking and distillation with a fuel distribution of 67% gasoline, 15% aviation turbine fuel, and 3% residual oil.¹ Second, this species has been reported to be a major contributor to the formation of existing oil shale and coal deposits.^{2,3}

B. braunii race B, the focus of this study, produces the triterpene isoprenoids known as botryococcenes, and production is typically in the range of 30–50% of algal dry weight.^{4,5} The B race of *B. braunii* has been shown to make many isomers of botryococcenes originating from the C₃₀ botryococcene (1) molecule. For example, once produced 1 is successively methylated at positions C-3, C-7, C-16, and C-20, to generate C₃₁, C₃₂, C₃₃, and C₃₄ botryococcene (2) homologues in conventional strains, but up to C₃₆ and C₃₇ in some unique strains.^{4,6} Approximately 20 different isomers based on the C₃₁ to C₃₄ (2) botryococcene structures exist. These isomers occur in acyclic and cyclic forms, with the cyclic forms containing methylenecyclohexane rings.^{4,7} In the B race, C₃₀ squalene (3) is also methylated at positions C-3, C-7, C-18, and C-22 to generate C₃₁ monomethylsqualene, C₃₂ dimethylsqualene, C₃₃ trimethylsqualene, and C₃₄ tetramethylsqualene (4) homologues.^{8,9}

RESULTS AND DISCUSSION

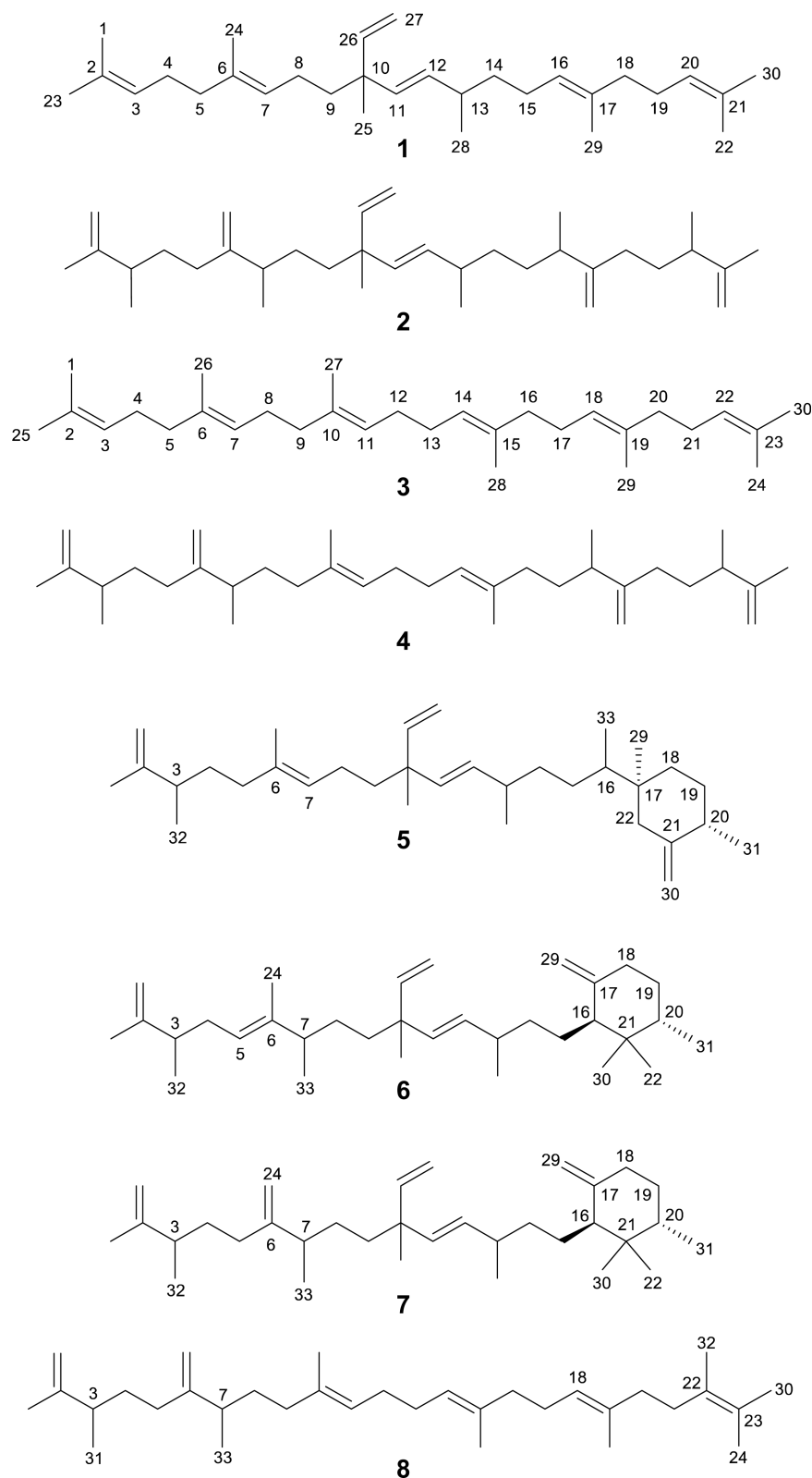
In the current study, we undertook an effort to identify new botryococcene and methylsqualene isomers from the Showa (a.k.a. Berkeley) strain of the B race of *B. braunii*. We would expect the presence of new B race hydrocarbons for several possible reasons: different growth conditions between laboratories may lead to the biosynthesis of alternative molecules, different strains of the B race may biosynthesize discrete hydrocarbons, or improvements in analytical techniques over the years may reveal new molecules not identified previously. From these studies three cyclic C₃₃ botryococcenes, cyclic C₃₃-1 botryococcene (5), cyclic C₃₃-2 botryococcene (6), and cyclic C₃₃-3 botryococcene (7), were purified and structurally characterized. A previous study used mass spectrometry to assign five botryococcene isomers to a single proposed structure similar to 6 and 7.¹⁰ Here we use 2D NMR to resolve two of these structures as 6 and 7. Furthermore, the structure of one new trimethylsqualene isomer, C₃₃-2 trimethylsqualene (8), is also described.

B. braunii hydrocarbons were purified using a combination of reversed- and normal-phase HPLC. An initial separation of a total hydrocarbon extract was achieved using reversed-phase HPLC, which generated eight peaks, labeled as A–H in Figure S1, Supporting Information. Analysis by GC-MS revealed all peaks, except peaks A and H, contained hydrocarbon mixtures

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Chart 1



(Figure S2). Hydrocarbon molecules were not detected by GC-MS for peak G (not shown), and it was not further studied. Next, individual molecules from peaks D–F were purified to homogeneity using a normal-phase HPLC silica column. The mixtures of molecules found in peaks B and C were not separable by silica column HPLC. Thus, a silver-nitrate-coated

silica-based HPLC column was used to purify these hydrocarbons to homogeneity as previously done to separate very similar botryococcene isomers.¹¹ Final purity of all isolated molecules to at least $\geq 95\%$ was confirmed by GC-MS.

Subsequent analysis of the purified hydrocarbons by GC-MS and 2D NMR spectroscopy identified the following previously

identified compounds (Table S1 and Figure S3): from peak A, C₃₀ botryococcene (1) at $m/z = 410.4$ (NMR data not recorded); from peak B, C₃₂ botryococcene at $m/z = 438.5$, braunicene at $m/z = 438.5$, and showacene at $m/z = 424.4$; from peak C, wolfcene at $m/z = 424.5$ and monomethylsqualene at $m/z = 424.5$; from peak D, dimethylsqualene at $m/z = 438.5$ and a C₃₄ botryococcene isomer at $m/z = 466.7$; from peak E, trimethylsqualene at $m/z = 452.5$; and from peak F, tetramethylsqualene at $m/z = 466.6$.

Additionally, compounds 5–8 were identified during this process. Peak H contained the cyclic C₃₃ botryococcene 5 (Figure S4A). Peak C contained the two additional cyclic C₃₃ botryococcene isomers 6 and 7 (Figure S4B and C). Peak F contained the trimethylsqualene isomer 8 (Figure S4D). Analysis by GC-MS in EI and CI mode confirmed parent ions for 5–8 at $m/z = 452.5$, 452.6, 452.7, and 452.5, respectively, and the presence of several signature fragments for isoprenoids at $m/z = 69$, 81, 95, and 121 (Figures S5 and S6). The molecular formula of compounds 5–8 was determined as C₃₃H₅₆ from HRMS in CI mode with parent ions at $m/z = 452.4372$, 452.4373, 452.4384, and 452.4372 (calculated as 452.4382) for 5–8, respectively (Figure S7).

Each hydrocarbon was subjected to ¹H and ¹³C NMR analysis. Initially, COSY and TOCSY data were acquired to resolve severe signal overlap characteristics of the hydrocarbon molecules. We have relied on HSQC to deduce ¹H and ¹³C chemical shifts as reported in Tables 1 and 2 and ¹³C chemical shifts in Tables S1–S4. The molecules shown in Figure S3 were previously reported in the literature,^{8,11–15} and the ¹³C chemical shifts we observed matched those reported (C₃₃ botryococcene in Table S2, all others in Table S1). Additionally, TOCSY-HSQC, H2BC, and HMBC were performed to elucidate the structures of molecules 5–8. The HSQC spectra of these molecules (Figure S8) were obtained by reducing the spectral width in the indirect dimension to produce intentional ¹³C resonance aliasing. The corresponding true ¹³C chemical shifts (Tables 1 and 2 and Tables S1–S4) were calculated by adding the spectral width to the observed shifts.

Here we have identified the differently cyclized molecule 5, which describes a new scaffold not reported in the literature for botryococcenes. Molecule 5 has a unique arrangement whereby the six-membered ring is attached to the molecule backbone at position C-17. In previous reports, botryococcenes containing cyclic rings on the same side of the molecule as in 5 the C-16 position is usually seen to bear the cyclic ring^{13,14,16} as seen in braunicene^{16,17} (Figure S3). A botryococcene with a similar methylenecyclohexane ring structure to 5 has been reported.^{16,18} However, this molecule is a C₃₄ botryococcene with the methylenecyclohexane ring attached to the molecule backbone at C-6. For molecule 5, the ring is formed from the connection of the methyl carbon in the terminal isoprene (C-22) unit and the third carbon (C-17) in the adjoining isoprene unit.

Due to this unique ring arrangement in 5, chemical shifts originating from the ring positions differ significantly from other samples studied in this and other reports. The structure of 5 is elucidated by exclusive use of NMR experiments (Figures 1 and S8). By comparison, the ¹³C chemical shifts for C₃₃ botryococcene^{11,19} and 5 are similar up to position C-13 (Tables 1 and S2). Then the chemical shifts for C-14, C-15, and C-16 show a slight deviation for 5, inferring that these two molecules are structurally similar until position C-16. Thereafter the NMR data are entirely different between the two

Table 1. ¹³C and ¹H NMR Spectroscopic Data (¹H 500 MHz, ¹³C 125 MHz, CDCl₃) for 5

position	δ_C , type	δ_H (J in Hz)
1	109.6, CH ₂	4.62
		4.71
2	150.2, C	
3	41.0, CH	2.10
4	33.6, CH ₂	1.34
		1.45
5	37.7, CH ₂	1.87
6	135.1, C	
7	124.8, CH	5.07
8	23.3, CH ₂	1.90
9	41.6, CH ₂	1.37
10	42.0, C	
11	136.0, CH	5.35
12	134.1, CH	5.17
13	37.5, CH	2.07
14	36.4, CH ₂	1.16
		1.34
15	28.7, CH ₂	0.86
		1.48
16	43.9, CH	1.11
17	38.4, C	
18a	35.4, CH ₂	[eq] 1.44, m
18b		[ax] 1.30, ddd (13.2, 12.6, 3.8)
19a	32.3, CH ₂	[eq] 1.62, m
19b		[ax] 1.14, m
20	37.6, CH	1.93, m
21	152.2, C	
22	46.4, CH ₂	[eq] 2.04, m
		[ax] 1.86, m
23	19.2, CH ₃	1.65
24	16.2, CH ₃	1.56
25	23.8, CH ₃	1.07
26	147.0, CH	5.80
27	111.3, CH ₂	4.93
28	21.8, CH ₃	0.97
29	18.4, CH ₃	0.66, s
30	106.0, CH ₂	4.61, d (4.8)
31	18.5, CH ₃	1.04, d (7.2)
32	19.9, CH ₃	1.00
33	13.8, CH ₃	0.80

molecules for carbons 17, 18, 19, 20, 21, and 22 of the ring structure (Table 1). Analysis by HMBC (Figure 1A), COSY (Figure 1B), HSQC-TOCSY (Figure S20), and H2BC (Figure S21) provide evidence that the six-membered ring is formed by C-17, C-18, C-19, C-20, C-21, and C-22. For example, long-range HMBC correlations between the H-29 methyl protons and C-16, C-18, and C-22 suggest such a ring formation. Moreover, COSY correlations indicate a connection between C-18 and C-19, and HMBC correlations from the H-30 exomethylene protons to C-20 and C-22 confirm this assignment. Those experiments also show the tertiary carbon C-20 methine is connected to the C-31 methyl group. Overall, our 2D NMR data demonstrate a novel botryococcene scaffold for 5.

Additionally, the relative configuration of the methylenecyclohexane ring in 5 was determined using selective 1D NOESY experiments and complemented with *J*-couplings (Table S3). The observed NOEs between the axial positions H-18b, H-20,

Table 2. ^{13}C and ^1H NMR Spectroscopic Data (^1H 500 MHz, ^{13}C 125 MHz, CDCl_3) for 6, 7,^a and 8

6			7 ^a			8	
position	δ_{C} , type	δ_{H} (J in Hz)	position	δ_{C} , type	δ_{H} (J in Hz)	δ_{C} , type	δ_{H} (J in Hz)
1	109.3, CH_2	4.53 4.70	1	109.7, CH_2	4.64, 4.73	109.7, CH_2	4.64, 4.73
2	149.6, C		2	150.1, C		150.0, C	
3	41.8, CH	2.13	3	41.2, CH	2.15	41.2, CH	2.16
4	33.0, CH_2	1.98 2.08	4	33.6, CH_2	1.50	33.6, CH_2	1.41, 1.51
5	124.1, CH	5.10	5	31.8, CH_2	1.87	31.9, CH_2	1.90
6	139.0, C		6	155.2, C		154.7, C	
7	34.7, CH	2.51	7	40.8, CH	2.00	39.8, CH	2.06
8	29.5, CH_2	1.23	8	33.5, CH_2	1.40	34.2, CH_2	1.35, 1.51
9	39.7, CH_2	1.23	9	39.2, CH_2	1.28	37.7, CH_2	1.89
10	41.3, C		10	41.8, C		135.4, C	
11	135.9, CH	5.29	11	135.9, CH	5.29	124.5, CH	5.16
12	134.4, CH	5.15	12	134.3, CH	5.15	28.5, CH_2	2.01
13	37.5, CH	2.01	13	37.5, CH	2.01	28.5, CH_2	2.01
14	36.0, CH_2	0.97 1.16	14	36.0, CH_2	0.97, 1.16	124.5, CH	5.16
15	32.3, CH_2	1.21 1.46	15	32.3, CH_2	1.21, 1.46	135.4, C	
16	56.8, CH	1.62, m	16	56.8, CH	1.62	40.0, CH_2	2.06
17	148.9, C		17	148.9, C		26.9, CH_2	2.12
18	31.2, CH_2	2.05, m	18	31.2, CH_2	2.05	124.5, CH	5.08
19a	24.1, CH_2	[eq] 1.42, m	19	24.2, CH_2	1.30, 1.42	135.4, C	
19b		[ax] 1.30, m	20	35.0, CH	1.61	37.7, CH_2	1.90
20	35.0, CH	1.61, m	21	36.4, C		33.6, CH_2	1.41, 1.51
21	36.4, C		22	27.1, CH_3	0.87	154.7, C	
22b	27.1, CH_3	[ax] 0.87, m	23	19.1, CH_3	1.66	131.6, C	
23	19.9, CH_3	1.68	24	107.6, CH_2	4.66, 4.73	17.9, CH_3	1.62
24	18.2, CH_3	1.53	25	23.8, CH_3	1.03	19.2, CH_3	1.65
25	23.8, CH_3	1.03	26	147.2, CH	5.76	107.5, CH_2	4.68, 4.75
26	147.2, CH	5.76	27	111.2, CH_2	4.90, 4.93	16.2, CH_3	1.60
27	111.2, CH_2	4.90 4.93	28	21.2, CH_3	0.93	16.2, CH_3	1.60
28	21.2, CH_3	0.93	29	109.3, CH_2	4.46, 4.55	16.2, CH_3	1.60
29a	109.3, CH_2	4.63, d (3.5)	30	21.9, CH_3	0.73	25.9, CH_3	1.69
29b		4.46, d (3.5)	31	16.0, CH_3	0.77	19.9, CH_3	1.00
30	21.9, CH_3	0.73, d (4.8)	32	20.0, CH_3	1.02	19.9, CH_3	1.00
31	16.0, CH_3	0.77, d (7.2)	33	20.6, CH_3	0.98	20.4, CH_3	1.00
32	19.6, CH_3	1.00					
33	19.8, CH_3	0.93					

^aNOE measurements to determine relative configuration of the cyclohexane ring were not performed since the ^1H and ^{13}C chemical shifts for the ring positions are identical to those of 6.

and H-22b, as well as between H-19b and the H₃-29 protons, in conjunction with the large vicinal *J*-coupling of 12.6 Hz seen for H-18b and H-19b suggest the cyclohexane ring exists in a stable chair conformation (Figure 1C). The *J*-coupling values for the acyclic portions of the molecule were not determined due to extreme overlap that has also been seen in previous studies.^{11,20}

On the basis of these studies, the ring formation for 5 can be speculated to occur as proposed in Figure 2. This mechanism involves C-16 methylation to generate a C-17 carbocation, ring closure between C-22 and C-17 creating a quaternary carbon at C-17 with production of a cyclohexyl cation at C-21, and finally hydrogen elimination at C-30.

The remaining two cyclic C₃₃ botryococcenes, 6 and 7, are very similar except for the location of a double bond. This double bond between C-5 and C-6 in 6 is seen to be relocated to between positions C-6 and C-24 in 7. This can be readily

seen in the multiplicity-edited HSQC spectra acquired on these samples (Figure S8B and C).

The selective 1D NOESY experiments carried out on 6 (Table S4) and 7 (data not shown) indicate relative ring configurations (Figure 1D) similar to that of braunicene as previously reported.^{14,17} This conclusion is also supported by the fact that all HSQC peaks corresponding to the cyclohexane ring (C-16 to C-21) of 6, 7, and braunicene superimpose on each other (Table 2 and Table S1).

The planar structure of 8 identified in our study closely resembles trimethylsqualene⁸ (Table S2 and Figure S3) with the following differences. The double bond between C-23 and C-24 of trimethylsqualene is shifted to between C-22 and C-23 in 8, generating the corresponding differences in chemical shifts seen for C-22 to 24 and C-30 (Table 2). The structural confirmation for molecules 5–8 reported here provides proof

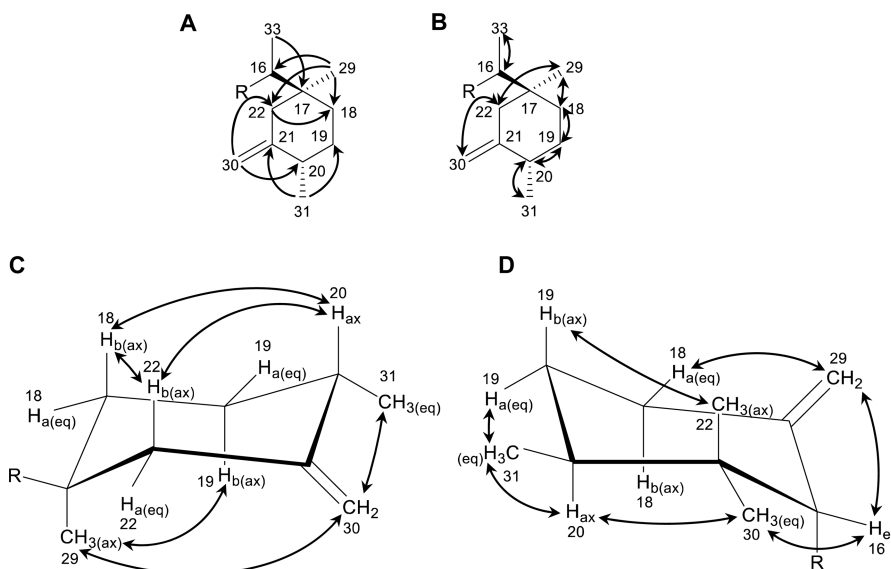


Figure 1. Selected correlations observed in 2D NMR experiments for **5**. Only significant correlations are shown. (A) HMBC correlations; (B) COSY correlations. (C and D) Relative configuration of the methylenecyclohexane ring of **5** and **6**, respectively. Arrows indicate strong NOE connectivities within each ring.

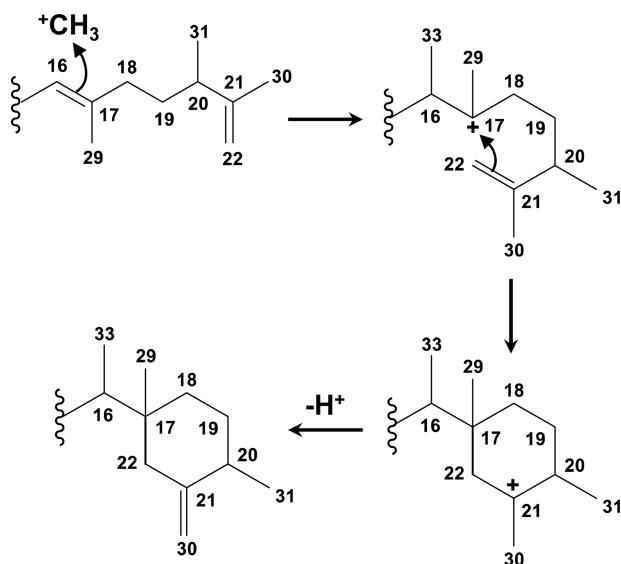


Figure 2. Proposed mechanism for ring formation in **5**.

of the impressive capacity of *B. braunii* race B to produce a variety of botryococcene and squalene isomers.

EXPERIMENTAL SECTION

General Experimental Procedures. A Chirascan CD spectrometer (Applied Photophysics) was used to collect ECD data. NMR spectra were obtained using 500 and 800 MHz Bruker Avance III HD spectrometers equipped with a 5 mm inverse detection TXI probe. GC-MS analysis was performed using a Bruker 436-GC-SCION SQ Premium system, and HRMS spectra were collected on a Waters Micromass AutoSpec Ultima MS in the CI mode. HPLC was conducted using a Cosmosil SC₁₈-AR-II column (Nacalai Tesque; 20 × 250 mm, 8 mL/min), a Develosil 60 silica column (Nomura Chemical, Japan; 20 × 250 mm, 8 mL/min), and a KANTO silver nitrate (AgNO₃) silica column (Kanto Chemical; 4.6 × 250 mm, 1 mL/min).

Culturing and Harvesting *Botryococcus braunii* Race B. Race B of *B. braunii*, Showa (Berkeley) strain,²¹ was grown in modified Chu13 medium²² at 22 °C under using 13 W compact fluorescent 65

K lighting with an intensity of 280 μmol photons/m²/s. Cultures were continuously aerated with filter-sterilized 2.5% CO₂ enriched air and grown under a 12 h light:12 h dark cycle. New cultures were inoculated with 100 mL of 6-week-old cultures into 750 mL of fresh medium. After 5 weeks, the cells were harvested by vacuum filtration using 10 μm nylon-mesh, frozen in liquid nitrogen, and stored at −80 °C.

Extraction and Purification of Race B Hydrocarbons. Five-week-old algal cells stored at −80 °C were freeze-dried, and hydrocarbons extracted first using *n*-hexane three times to remove extracellular hydrocarbons, followed by two extractions with CHCl₃/MeOH (2:1) to recover intracellular hydrocarbons. Solvent from both extracts was evaporated using a rotary evaporator, resuspended in a small volume of *n*-hexane, combined, and applied to a gravity-fed silica gel column. *n*-Hexane was used as the eluent for the gravity-fed silica gel column, and hydrocarbons were collected as the eluent from the column prior to the pigment front. The collected eluent was evaporated to dryness using a rotary evaporator, and the sample resuspended in acetone equal to 1 volume of the hydrocarbons without solvent. Hydrocarbons were separated using reversed-phase HPLC by injecting ~100 μL of sample onto a Cosmosil SC₁₈-AR-II column (Nacalai Tesque; 20 × 250 mm) with 100% MeOH as the mobile phase at a flow rate of 8 mL/min and detection at 210 nm. Individual peaks containing multiple molecules were collected, and these samples were further separated using normal-phase HPLC by injecting samples onto a Develosil 60 silica column (Nomura Chemical; 20 × 250 mm) with 100% *n*-hexane as the mobile phase at a flow rate of 8 mL/min and detection at 210 nm. Each peak was collected individually, and the purity and molecular weight of each sample determined by GC-MS analysis. Any samples that still contained multiple molecules were further separated by normal-phase HPLC using a KANTO silver nitrate (AgNO₃) silica column (Kanto Chemical; 4.6 × 250 mm) with 99.85%/0.15% *n*-hexane/MeCN as the mobile phase at a flow rate of 1 mL/min and detection at 210 nm. Retention times of the HPLC peaks were observed as follows: C₁₈ column HPLC retention times: peak A, ~50 min; peak B, ~52 min; peak C, ~58 min; peak D, ~64 min; peak E, ~68 min; peak F, ~71 min; peak G, ~79 min; and peak H (molecule **5**), ~86 min. Silica column HPLC retention times: C₃₂ botryococcene, ~7 min; wolficene, ~7 min; braunicene, ~8 min; trimethylsqualene, ~9 min; tetramethylsqualene, ~10 min; showacene, ~11 min; **8**, ~11 min; dimethylsqualene, ~13 min; and monomethylsqualene, ~14 min. Silver nitrate silica column HPLC retention times: C₃₄ botryococcene

isomer, ~4 min; 6, ~5 min; and 7, ~8 min. The approximate yields of the new isolated compounds based on total hydrocarbon amount were determined as 1.2 mg, 0.30% for 5; 2.4 mg, 0.60% for 6; 0.8 mg, 0.20% for 7; and 0.6 mg, 0.15% for 8.

Cyclic C₃₃-1 botryococcene (5): colorless liquid; ECD (0.000221 M, *n*-hexane) λ_{max} ($\Delta\epsilon$) 164 (−0.3) nm; ¹H NMR (500 and 800 MHz, CDCl₃) and ¹³C NMR (125 and 200 MHz, CDCl₃) data, Table 1, Table S3; HRCIMS *m/z* 452.4372 [M]⁺ (calcd for C₃₃H₅₆, 452.4382).

Cyclic C₃₃-2 botryococcene (6): colorless liquid; ECD (0.000221 M, *n*-hexane) λ_{max} ($\Delta\epsilon$) 164 (−1.9) nm; ¹H NMR (500 and 800 MHz, CDCl₃) and ¹³C NMR (125 and 200 MHz, CDCl₃) data, Table 2; HRCIMS *m/z* 452.4373 [M]⁺ (calcd for C₃₃H₅₆, 452.4382).

Cyclic C₃₃-3 botryococcene (7): colorless liquid; ECD (0.000221 M, *n*-hexane) λ_{max} ($\Delta\epsilon$) 164 (−1.9) nm; ¹H NMR (500 and 800 MHz, CDCl₃) and ¹³C NMR (125 and 200 MHz, CDCl₃) data, Table 2; HRCIMS *m/z* 452.4384 [M]⁺ (calcd for C₃₃H₅₆, 452.4382).

C₃₃-2 Tetramethylsqualene (8): colorless liquid; ECD (0.000221 M, *n*-hexane) λ_{max} ($\Delta\epsilon$) 164 (2.0) nm; ¹H NMR (500 and 800 MHz, CDCl₃) and ¹³C NMR (125 and 200 MHz, CDCl₃) data, Table 2; HRCIMS *m/z* 452.4372 [M]⁺ (calcd for C₃₃H₅₆, 452.4382).

GC-MS Analysis. GC-MS analysis was performed using a Bruker 436-GC-SCION SQ Premium system (Bruker Daltonics) with a 5% phenyl BR-5 ms capillary column (30 m × 0.25 mm, film thickness: 0.25 μ m) in EI (70 eV) or CI (methane gas at 20 psi) mode. The GC temperature conditions were as follows: started at 220 °C and held for 1 min, then raised to 280 °C with a rate of 5 °C/min, and finally increased to 300 °C at a rate of 2 °C/min and held for 10 min. The GC injection port was held at 280 °C. The carrier gas was helium at a flow rate of 2.58 mL/min. For the MS, 250 and 200 °C were the temperatures of the interface and ion source, respectively.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.6b00934.

Compound purification via HPLC methods and structural information using ¹H, ¹³C, HSQC, HMBC, ECD, HRMS, and GC-MS with EI and CI modes for all molecules reported in this study (PDF)

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Notes

The authors declare no competing financial interest.

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- (19) The structure of molecule 3–2 was incorrectly presented,¹¹ and the correct structure is shown in Figure S23 as determined by the corresponding author S. Okada, who is also a coauthor on the current study.
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