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ISOSHOWACENE, A C_{31} HYDROCARBON FROM *BOTRYOCOCCUS BRAUNII* VAR. *SHOWA*

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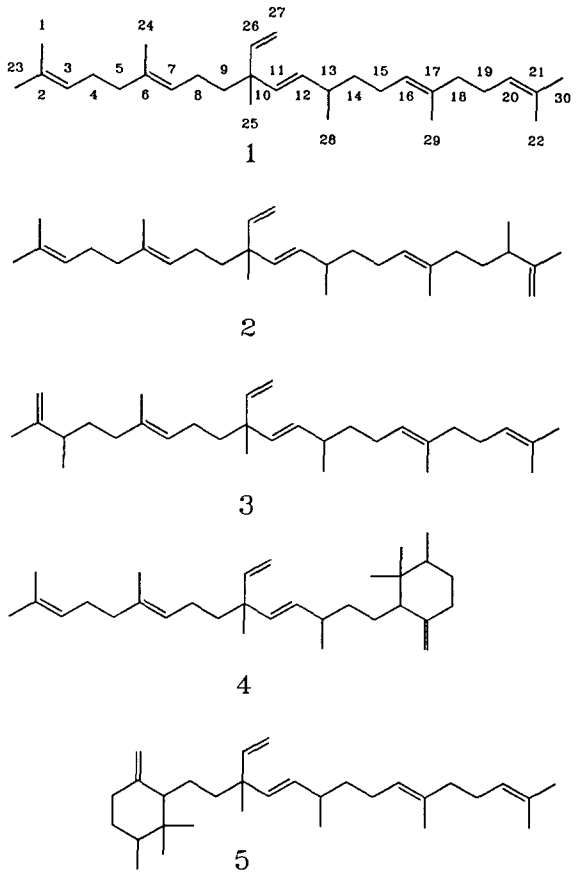
Key Word Index—*Botryococcus braunii* var. *showa*; Chlorophyceae; alga; botryococcenes; irregular triterpenes; structure.

Abstract—Two C_{31} acyclic hydrocarbons, showacene and isoshowacene, were obtained from a 16-day-old air-lift culture of *Botryococcus braunii* var. *showa*. The compounds were isolated as a 17:10 mixture by reversed-phase HPLC. Upon analysis by GC-MS and by ^1H and ^{13}C NMR, the compounds were found to be 1′-3 fused triterpenes of the botryococcene type bearing a single additional methyl at opposite ends of the isoprenoid chains.

INTRODUCTION

Botryococcus braunii is a colonial fresh water green alga found worldwide that produces and accumulates massive quantities of hydrocarbons [1, 2]. There are two forms of *B. braunii* which have similar morphologies but differ in the hydrocarbons contained in the oil sack that surrounds the algal cell. The L-form produces a series of linear odd-numbered C_{23}–C_{31} alkyl dienes and trienes [3], while the B-form synthesizes a family of C_{30}–C_{37} isoprenoids collectively called botryococcenes [3–5]. Each member contains an unusual 1′-3 fusion of farnesyl residues and all but the C_{30} parent are further modified by methylation with *S*-adenosylmethionine.

Methylations of double bonds in the parental hydrocarbon (**1**) generate a variety of olefinic and cyclic products. In most of the structures reported to date [6, 7], the farnesyl residue in the 1′ branch of the 1′-3 linkage is modified in preference to the 3′ residue, and hypermodification (more than three additional methyls) is only reported in the 1′ residue. There are three exceptions to this pattern. Recently, we reported the structure of isowolficene (**5**) [8], an unusual C_{31} botryococcene containing a cyclohexyl moiety presumably formed by an *S*-adenosyl methionine initiated cyclization at the terminal double bond in the 3′ arm. Brassell *et al.* [9] found several botryococcanes in the Eocene Maoming oil shale from Guangdong province in China, including a C_{31} botryococcane with a non-isoprenoid methyl at C-3 and a C_{33} botryococcane with non-isoprenoid methyls at C-3, C-7, and C-20. We now report the isolation of showacene† (**2**) and isoshowacene (**3**) from *B. braunii* var. *showa* [10], acyclic C_{31} botryococcenes with an additional methyl in the 1′ and 3′ farnesyl residues, respectively.



Scheme 1. C_{30} and C_{31} botryococcenes.

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†The C_{31} botryococcene **2** was first reported by Metzger *et al.* [7]. We suggest the name showacene for **2** and isoshowacene for **3**.

RESULTS AND DISCUSSION

The structure of showacene (**2**) was first reported by Metzger and coworkers [11]. It was initially assigned to

isoshowacene (**3**) and later corrected on the basis of GC-MS cleavage patterns at the quaternary centre in the perhydrogenated derivative [7]. Other C_{31} isomers were not reported in the strain of *B. braunii* they studied. In conjunction with biosynthetic studies in *B. braunii* var. *showa* (formerly referred to as the Berkeley isolate [3, 6, 8]), we had occasion to isolate a fraction by HPLC which gave a single peak on a 30 m × 0.25 mm fused silica DB-5 capillary column (245°). The material had a $[M]^+$ at *m/z* 424, and we initially assumed that it was the C_{31} botryococcene reported previously by Metzger *et al.* [7]. However, analysis of the 500 MHz ^{1}H NMR spectrum of the oil clearly indicated that it was a mixture of compounds. After considerable experimentation, GC conditions were discovered (DB-5; 50° for 2 min, 50° to 245° at 20°/min, hold at 245°) that gave two partially resolved peaks in a ratio of 17:10, both of which had $[M]^+$ at *m/z* 424.

In $CDCl_3$, a 500 MHz ^{1}H NMR spectrum of the mixture had two clearly resolved three-proton doublets at δ1.015 (J = 6.8 Hz) and 1.013 (J = 6.8 Hz) in a ratio of 17:10, respectively (see Table 1). These signals were typical of methyls derived from *S*-adenosylmethionine and attached to methine carbons adjacent to unsymmetrically disubstituted double bonds in other acyclic botryococcenes [7, 8]. Comparisons between NMR spectra of the mixture and **1** or polymethylated botryococcenes and the GC-MS results clearly indicated that the 'extra' carbon in the two components was a methyl group. When the ^{1}H NMR spectrum of the mixture was recorded in cyclohexane-d_{12}/hexafluorobenzene (1:9), substantial shifts were observed for several resonances. Not only were the C-31 methyls further separated at δ1.065 and 1.061, two peaks were observed for the C-25 quaternary methyl at δ1.143 and 1.147 in a relative ratio of 17:10. The signals for the vinyl proton at C-26 were also split

Table 1. ^{1}H NMR spectra of botryococcenes **1** and **2***

	^{1}H (ppm)		J_{H-H}(Hz)	COSY cross peaks	
Assignment	**2**	**3**		**2**	**3**
1	1.610 (3H, *br s*)	4.69 (2H, *br s*)		5.10, 2.07	2.12, 1.664
3	5.11 (1H, *m*)	2.12 (1H, *m*)		2.07, 1.688, 1.610	4.69, 1.37 1.35, 1.013
4	2.07 (2H, *m*)	1.37 (2H, *m*)		5.10, 1.98	2.12, 1.89
5	1.98 (2H, *m*)	1.89 (2H, *m*)		5.13, 2.07, 1.58	5.13, 1.37, 1.35
7		5.13 (1H, *m*)		1.98, 1.91 1.58	1.91, 1.89, 1.58
8		1.91 (2H, *m*)		5.13, 1.58, 1.39	5.13, 1.58, 1.39
9		1.39 (2H, *m*)			1.91
11		5.354 (1H, *dd*)	0.8, 15.5		5.203, 2.11
12		5.203 (1H, *dd*)	8, 15.5		5.354, 2.11
13		2.11 (1H, *ddm*)	8, 7		5.354, 5.203, 0.978, 1.30
14		1.30 (2H, *m*)			2.11, 1.96
15		1.96 (2H, *m*)			5.13, 1.58, 1.30
16		5.13 (1H, *m*)			1.98, 1.96, 1.58
18	1.90 (2H, *m*)	1.98 (2H, *m*)		5.13, 1.37	5.13, 2.07
19	1.37 (2H, *m*)	2.07 (2H, *m*)		2.12, 1.89	5.11, 1.98
20	2.12 (1H, *m*)	5.11 (1H, *m*)		4.69, 1.37 1.015	2.07, 1.688 1.610
22	4.69 (2H, *m*)	1.610 (3H, *br s*)		2.12, 1.664	5.11, 2.07, 1.688
23	1.688 (3H, *br s*)	1.664 (3H, *br s*)		5.10, 2.07, 1.610	4.69
24		1.58 (3H, *br s*)		5.13, 1.98, 1.91	5.13, 1.91, 1.89
25		1.085 (3H, *s*)			5.816†, 5.354†, 5.203†
26		5.816 (1H, *dd*)	10.7, 17.6		4.966, 4.947
27		4.966 (1H, *dd*)	1.0, 10.7		5.816, 4.947
		4.947 (1H, *dd*)	1.0, 17.6		5.816, 4.966
28		0.978 (3H, *d*)	7.0		2.11
29		1.58 (3H, *br s*)		5.13, 1.96, 1.89	5.13, 1.98, 1.96
30	1.664 (3H, *br s*)	1.688 (3H, *br s*)		4.69	5.11, 1.610
31	1.015 (3H, *d*)	1.013 (3H, *d*)	6.8		2.12

*Taken in $CDCl_3$, 26° at 500 MHz. Assignments are based upon the assumption that **2** is the more abundant isomer.

†Very weak.

into two sets of doublets of doublets at δ5.843 and 5.838 (J = 10.7, 17.6 Hz).

The equivalent or nearly equivalent ^1H and ^{13}C chemical shifts in 17:10 ratios for both components in the mixture listed in Tables 1 and 2 suggested that they had the same double bond stereochemistries. The *E* stereochemistry of the C-11–C-12 disubstituted double bond was assigned from the magnitude of the vicinal coupling constant (J = 15.5 Hz). Comparisons of ^1H and ^{13}C chemical shifts with those of **1** indicated *E* stereochemistries for the trisubstituted double bonds as well.

There are four acyclic monomethylated C_{31} regioisomers (methylation at C-3, C-7, C-16, or C-20) which would be expected to give spectra similar to the those summarized in Tables 1 and 2. Integration of the vinyl methyl resonances indicated three protons for each of the peaks at δ1.610, 1.664, and 1.688 and a six-proton peak centred at 1.58 ppm. The chemical shift of the signal at δ1.58 was identical to those for the methyls on internal *E*-trisubstituted double bonds in **1** [7] and the cyclic C_{31} isomers wolficene (**4**) and isowolficene (**5**) [8]. As the peak at δ1.58 in the mixture contained six protons, neither of the internal trisubstituted double bonds were methylated, and by a process of elimination, the additional methyls are located at C-3 or C-20.

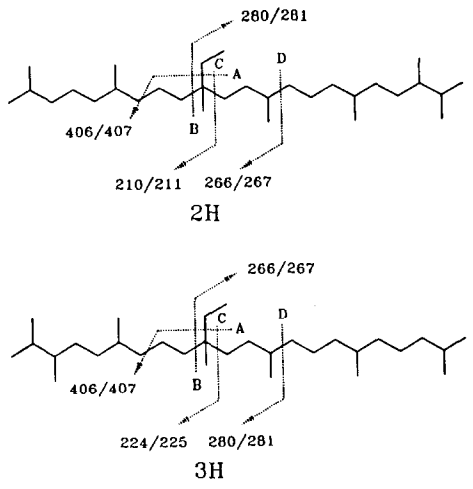
We reached similar conclusions upon examination of the ^{13}C spectra of **1** and the mixture of **2** and **3** (see Table 2). Chemical shift assignments for **1** from ^1H and ^{13}C HETCOR spectra [12] and substituent effects for the additional methyls [13, 14] were in agreement with those reported by Metzger and coworkers [7]. The ^{13}C spectrum of the mixture gave 53 of the possible 62 peaks expected for **2** and **3**. Resonances assigned to C-8, C-10, and C-24–C-29, and C-31 were not resolved. The remaining carbons appeared in pairs with relative intensities of *ca.* 17:10. Thus, it was possible to obtain chemical shifts for all carbons in the major and minor isomers. The assignments are based on the assumption that the more abundant isomer is **2**. The effects of methylation on β-ε carbons in the isoprenoid chains are illustrated by the differences in chemical shifts between **2** and **3**. Downfield shifts were seen at the β (C-19 in **2** and C-4 in **3**) and δ carbons (C-17 in **2** and C-6 in **3**), while upfield shifts were found at the γ (C-18 in **2** and C-5 in **3**) and ε positions (C-16 in **2** and C-7 in **3**). This alternating pattern of up- and downfield shifts at β, γ, δ, and ε carbons is similar to that seen by Couperus and coworkers [13] in model substituted alkenes. Small downfield shifts were also found for C-16–C-19 in **2** relative to **3**; however, the magnitudes of the effects are too small to be used to distinguish between regioisomers.

Structures of regioisomeric botryococcenes can usually be assigned from mass spectra of the related botryococcanes where cleavages at the quaternary centre produce doublet fragments characteristic of the 1′ and 3 isoprenoid chains (see Scheme 2) [5, 8, 9]. Unfortunately when the mixture of **2** and **3** was hydrogenated, the saturated isomers **2H** and **3H** could not be separated, and it was not possible to determine which regioisomer predominated by GC-MS. The major higher mass fragments from **2H** and **3H** arise by rupture of bonds to the quaternary centre. As illustrated in Scheme 2, cleavages **A**–**C** give ion doublets at *m/z* 406/407, 280/281, and 210/211 for **2H** and at *m/z* 406/407, 266/267, and 224/225 for **3H**. In theory, fragments from **B** and **C** should be diagnostic for the site of methylation. However, the

Table 2. ^{13}C NMR spectra of botryococcenes **1**–**3***

C	Chemical shifts (ppm) 1	2	3	δ_2–δ_3 (ppm)
1	17.791	17.765	109.285	−91.520
2	131.198	131.149	150.043	−18.894
3	124.293	124.341	40.806	83.585
4	26.811	26.844	33.441	−6.597 (β)
5	39.790	39.779	37.574	2.205 (γ)
6	134.630	134.595	134.894	−0.299 (δ)
7	124.724	124.793	124.663	0.130 (ε)
8	23.191	23.212	23.212	0.000
9	41.361	41.408	41.444	−0.036
10	42.078	42.086	42.086	0.000
11	135.704	135.762	135.736	0.026
12	133.632	133.662	133.687	−0.025
13	36.753	36.808	36.740	0.068
14	37.429	37.499	37.454	0.045
15	25.895	25.926	25.899	0.027
16	124.618	124.441	124.571	−0.130 (ε)
17	134.595	134.940	134.642	0.298 (δ)
18	39.793	37.574	39.806	−2.232 (γ)
19	26.813	33.441	26.825	6.616 (β)
20	124.291	40.806	124.367	−83.561
21	131.170	150.070	131.171	18.899
22	17.791	109.285	17.765	91.520
23	25.793	25.762	19.061	6.701
24	16.008	16.001	16.001	0.000
25	23.603	23.675	23.675	0.000
26	146.634	146.660	146.660	0.000
27	111.038	111.052	111.052	0.000
28	21.252	21.210	21.210	0.000
29	16.069	16.076	16.076	0.000
30	25.793	19.079	25.899	−6.820
31	—	19.756	19.756	0.000

*Taken in $CDCl_3$ at 125 MHz. Assignments are based upon the assumption that **2** is the more abundant regioisomer.



Scheme 2. Cleavages at the quaternary centres in **2H** and **3H** leading to major 1′ and 3′ fragments.

Table 3. Comparisons of fragment ion intensities in mass spectra* of the mixture of **2H** and **3H**

Cleavages			Relative intensity† GC-MS		
2H	**3H**	*m/z*	Probe	Early	Late
C	—	210/211	3.0	3.6	8.5
—	C	224/225	1.6	3.6	2.2
D	B	266/267	1.1	1.6	0.8
B	D	280/281	1.4	1.0	1.4
A	A	406/407	1.0	1.0	1.0

*EI spectra at 17 eV.
†Normalized to the $[M-C_2H_5]^+$ doublet at *m/z* 406/407.

intensity of the 266/267 and 280/281 pairs may contain a contribution from tertiary cleavage **D**, thereby compromising an analysis. The ion doublets at *m/z* 210/211 and 224/225 (process **C**) do not have an obvious interference by simple cleavage of **2H** or **3H**, and we chose to compare the intensities of these fragments.

As can be seen in Table 3, the relative intensity of the *m/z* 210/211 and 224/225 fragment ions was 19:10 in a probe sample, in good agreement with the relative intensities of NMR resonances and indicating that **2H** is the major regioisomer. In addition, mass spectral scans taken early and late during elution of **2H** and **3H** (at *ca* 25% of maximal intensity) during GC-MS runs indicated that partial fractionation had occurred. The intensities of *m/z* 210/211 and 224/225 doublets were comparable in the early spectrum, while the relative ratio increased to 3.9 in the late spectrum. The intensity patterns we saw for **2H** and **3H** in our sample were reversed in the mass spectrum of **3H** reported by Brassell *et al.* [9]. If the *m/z* 210/211 and 224/225 doublets arise exclusively from **2H** and **3H**, respectively, it is quite possible that the sample from Maoming oil shale also contains both compounds, except **3H** is the predominant regioisomer. From these results we conclude that **2** is the major C_{31} regioisomer in *B. braunii* var. *showa*.

Based on the structures of the four C_{31} botryococcenes from *B. braunii* var. *showa*, it is evident that methylations which lead to showacene (**2**), isoshowacene (**3**), wolficene (**4**), and isowolficene (**5**) occur readily in both farnesyl residues. The results open the possibility that a limited number of transmethylases with low specificities are involved in the *S*-adenosylmethionine-mediated methylations. Thus, the biosynthesis of botryococcenes beyond the C_{30} stage may involve a complex network of methylations that interconnect several species.

EXPERIMENTAL

NMR spectra were recorded at 500 MHz referenced to int. TMS. COSY spectra were obtained according to the procedure of ref. [15] with a delay, D3 of 50 msec, between the second pulse and the acquisition period to enhance weak long-range interactions, and heteronuclear chemical shift correlated spectra (HETCOR) by the procedure of ref. [12]. A DB-5 fused silica capillary column (30 m × 0.25 mm) was used for all GC and GC-MS work.

Air-lift cultures were grown and harvested as previously described [6]. Lyophilized cells (3.6 g) were stirred with 100 ml of 1:1 hexane–Me_2CO for 1 hr under N_2. The suspension was filtered and the residue re-extracted. A final extraction was performed with 100 ml of $CHCl_3$–MeOH (1:1). The extracts were combined and filtered to yield a deeply coloured oil. The residue was loaded onto a 2.5 × 16 cm silica gel column (Baker, 60–200 mesh) and eluted with four column volumes of dry hexane. Solvent was removed under vacuum to yield 1.08 g of a colourless oil. Further purification of 150 mg of the oil by repeated injection of 5 mg quantities on HPLC on a 5 μm ODS-Hypersil semi-prep. column (30 × 0.78 cm) gave five major peaks upon elution with MeCN. The second peak was collected, and solvent was removed under red. pres. to yield 11 mg of a colourless oil.

The mixture of hydrocarbons was reduced by combining 0.5 mg of sample, 100 mg of 10% Pt on C, and 2 ml of EtOAc in a 1.5 × 12 cm test tube. The tube was placed in a hydrogenation bottle, purged with H_2, pressurized to 50 psi and gently shaken. GC analysis indicated complete reduction within 20 min. The suspension was passed through a plug of silica gel and the gel washed with 5 ml of EtOAc. Solvent was removed under red. pres. to yield 0.4 mg of a colourless oil. Analysis by ^1H NMR indicated all of the double bonds had been reduced.

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