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Botryolins A and B, two tetramethylsqualene triethers from the green microalga *Botryococcus braunii*

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Abstract

Two new triterpenoid polyethers with a tetramethylsqualene carbon skeleton, botryolins A and B, have been isolated from the green microalga *Botryococcus braunii*. Their structures were determined by means of spectral analyses including 2D NMR.

Keywords: *Botryococcus braunii*; Chlorophyceae; Alga; Botryolins; Triterpene ethers; Squalene

1. Introduction

The B race of the green microalga *Botryococcus braunii* is known to produce in abundance unusual triterpenoid hydrocarbons, named botryococcenes, a family of 1′-3 linked C_{30}–C_{37} compounds (Metzger et al., 1985) and also minor amounts of squalene and C_{31}–C_{34} methylated squalenes (Metzger and Casadevall, 1983; Huang and Poulter, 1989; Okada et al., 1995). The end product in the biosynthesis of methylated squalenes is a tetramethyl derivative with non-isoprenoid methyl groups at C-3, C-7, C-18 and C-22, as shown in **1**. Several derivatives of methylated squalenes have been isolated from two strains of the B race: a series of C_{32}–C_{34} monoepoxides, a diepoxide of tetramethylsqualene, both exhibiting epoxide functions in the central part of the molecules (Delahais and Metzger, 1997; Metzger, 1999), as well as some novel carotenoids, comprising a tetramethylsqualene moiety (Okada et al., 1996, 1997, 1998), probably derived from the mono- and di-epoxides.

In addition to the production of hydrocarbons, the biosynthesis of ether lipids is another important characteristic of *B. braunii* (Metzger and Largeau, 1999; Rager and Metzger, 2000): these compounds arise biosynthetically from the condensation of epoxides derived from hydrocarbons, unsaturated aldehydes and (or) alkenyl phenols. The present paper describes the isolation and the structure determination of two novel triterpenoid triethers named botryolins A (**2**) and B (**3**), both closely related to tetramethylsqualene **1**, from a strain of *B. braunii* originating from Ivory Coast (Metzger et al., 1990) and grown in the laboratory.

2. Results and discussion

The lipids stored in the outerwalls of the alga were extracted with heptane (Metzger and Largeau, 1999). After removal from this crude extract of a rubbery polymer (Berthéas et al., 1997; Metzger and Largeau, 1999), the remaining lipids were fractionated by column chromatography over alumina. The toluene eluate was purified by preparative silica gel TLC to give botryolins A (**2**) and B (**3**) in a yield of 0.16 and 0.20% of algal dry weight, respectively.

Botryolin A (**2**) had the molecular formula $C_{34}H_{60}O_3$ as deduced by HREIMS and ^{13}C NMR analyses. Its IR spectrum showed olefinic (1640 cm^{-1}), terminal methylene (3070, 890 cm^{-1}) and C–O (1120, 1080 cm^{-1}) absorption bands. The ^{13}C NMR spectrum contained

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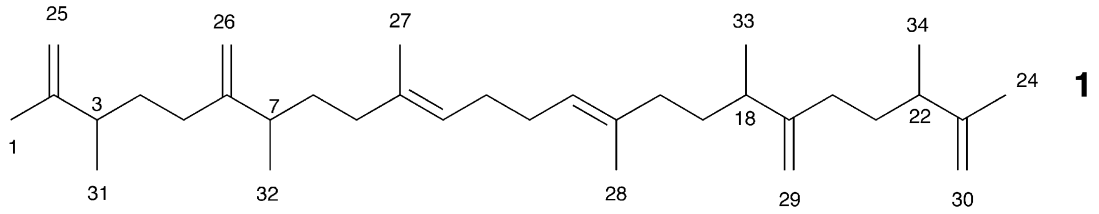

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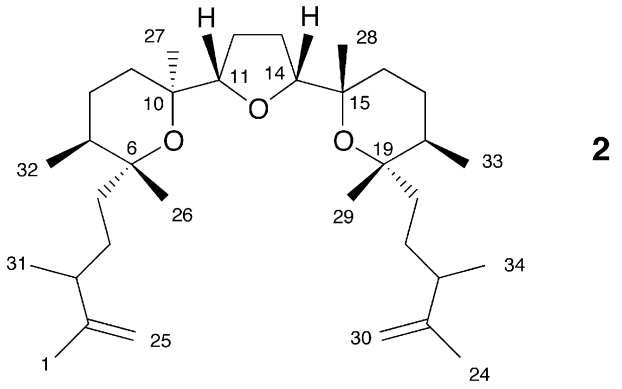

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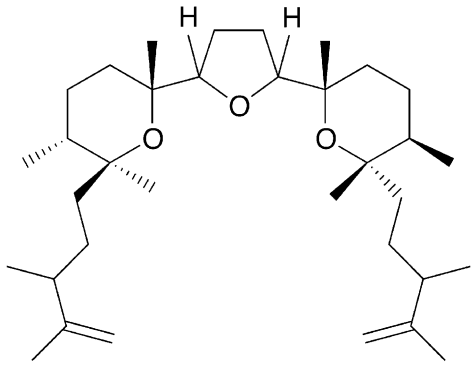

3

34 resonances, for $10\times CH_3$, $12\times CH_2$, $6\times CH$ and $6\times C$. The existence of two $>C=CH_2$ groupings was revealed by the presence of four olefinic carbon resonances: $2\times CH_2$ (δ_C at 109.3 and 109.4) and $2\times C$ (δ_C at 150.6). Furthermore, $2\times CH$ (δ_C at 81.4 and 88.0) and $4\times C$ (δ_C at 73.9, 74.4, 76.1 and 76. 8) signals were seen in the C–O region of the ^{13}C NMR spectrum (Table 1). On the basis of these latter data, and taking into account the absence of OH absorptions in the IR spectrum, together with the existence of five degrees of unsaturation, including two carbon–carbon double bonds, it was deduced that **2** must be tricyclic, probably a triether. The protons and carbons of **2** were assigned unambiguously from the results of COSY, TOCSY, HMQC and HMBC experiments. The ^1H NMR spectrum contained resonances associated with four methyl singlets (δ_H 1.00, 1.03, 1.05, 1.12), four methyl doublets (δ_H 0.77, 0.79, 0.99, 1.00), and two vinylic methyl groups (δ_H 1.63, 1.64). The ^1H NMR spectrum also contained signals for two oxymethines [δ 4.00 (H-11, *dd*, $J=6.7$, 7.0 Hz) and δ 3.48 (H-14, *dd*, $J=6.7$, 7.4 Hz)]. Interpretation of the COSY and TOCSY spectra showed these groups to be part of a tetrahydrofuran (THF) ring, a deduction further confirmed by HMBC correlations (Table 1). Furthermore, starting from H-11 and CH_3-32 (δ_H 0.79, δ_C 17.4), two and three bond HMBC correlations provided conclusive evidence for the existence of a tetrahydropyran (THP) substructure bearing three methyl groups at C-6, C-7 and C-10, and a side chain at C-6. In a same way, starting from H-14 and CH_3-33 (δ_H 0.77, δ_C 17.5), the presence of a second THP ring exhibiting similar substitutions, could be deduced. Finally, TOCSY and HMBC experiments showed that each side chain had a non-isoprenoid methyl group, at C-3 and C-22, respectively.

The coupling patterns of H-11 and H-14 of both THF botryolins A and B were almost identical, and so precluded their use for the determination of THF configuration (see for example: Alali et al., 1998). A ROESY spectrum recorded with **2**, however, revealed that H-11 and H-14 were on the same side of the molecule, and allowed a *cis* configuration to be assigned to the THF ring. Moreover, in the ROESY spectrum, the signal of H-11 exhibited ROE correlations with both the signals of CH_3-26 and CH_3-32. This indicated that these proton and methyl groups were on the same side of the molecule and that CH_3-26 and the C-10–C-11 bond linking the *cis*-THF to the THP ring were axially oriented, and, consequently, CH_3-32 in an equatorial orientation and the THP ring in chair conformation. In contrast, no ROE correlation was observed between H-14 and CH_3-29, but the strong correlations observed between CH_3-28 and CH_3-29, and H-17a (δ_H 1.50) with both CH_3-28 and CH_3-29, showed that H-17a, CH_3-28 and CH_3-29 were on the same side of the molecule. In the same way, the ROE observed between CH_3-29 and CH_3-33 established that this latter methyl group was also on the same side of the molecule as CH_3-29. Taking together, these results and the study of Drieding model indicated that the second THP ring was in chair conformation, with CH_3-28 and CH_3-29 axially oriented and CH_3-33 in equatorial oientation, leading, therefore, to the structure proposed for **2**.

Botryolin B (**3**) had the same molecular formula, $C_{34}H_{60}O_3$, as **2**, and showed a similar IR spectrum. The ^{13}C NMR spectrum exhibited 34 peaks and a DEPT

experiment showed that **3** also comprised the same numbers of CH_3, CH_2, CH and C as **2**. The NMR data (Table 1) of compounds **2** and **3** showed the two molecules to have a same planar structure, comprising a THF ring C–C linked to two pentasubstituted THPs. The EIMS spectra supported these deductions; among others they exhibited ions at *m/z* 223 and 293 which resulted in both cases from the cleavage of the C-10–C-11 and C-14–C-15 bonds. Furthermore, botryolin B exhibited in its ^1H and ^{13}C NMR spectra signals consistent with the THP in the right hand half of **2** (Table 1). The ^1H NMR spectrum displayed resonances for four methyl singlets (δ_H 1.05, 1.13, 1.15 and 1.17), four methyl doublets (δ_H 0.78, 0.90, 0.99 and 1.01), and two vinylic methyl groups (δ_H 1.63 and 1.64). The ^1H and ^{13}C NMR spectra showed signals for two $>C=CH_2$, with four protons at δ_H 4.66, 2×C at δ_C 150.4 and 150.6, and 2×CH_2 at δ_C 109.2 and 109.3. As for compound **2**, the ^1H NMR spectrum also showed two signals for two oximethines [δ 3.70 (H-11, *dd*, $J=6.6$, 7.0 Hz) and δ 3.52 (H-14, *dd*, $J=6.6$, 7.0 Hz)], which were part of a THF ring, according to COSY, TOCSY and HMBC data. The stereochemistry of these two THF protons, H-11 and H-14, could not be determined given the absence of coupling in the ROESY spectrum and, as above mentioned, the similarity of the ^1H–^1H coupling constant values. The attachement of two THP rings to C-11 and C-14 of THF was deduced from the HMBC spectrum of **3** (see Table 1). The comparison of the NMR data of botryolin B with those of **2**

Table 1
^{13}C (100 MHz) and ^1H (400 MHz) NMR data of botryolins A (**2**) and B (**3**) ($CDCl_3$)

	Botryolin A (**2**)			Botryolin B (**3**)		
Position	δ ^{13}C	δ ^1H (mult., *J* Hz)	HMBC[c]	δ ^{13}C	δ ^1H (mult., *J* Hz)	HMBC[c]
1	18.8[a], *q*	1.63 (*s*)	3, 25	18.8[a], *q*	1.64 (*s*)	3, 25
2	150.6[d], *s*		1, 3, 4, 25, 31	150.4, *s*		1, 3, 4, 25, 31
3	41.7[e], *d*	2.05 (*m*)	1, 4, 25, 31	42.1, *d*	2.03 (*m*)	1, 4, 25, 31
4	28.1, *t*	1.40 (*m*), 1.30 (*m*)	3, 5, 31	28.6, *t*	1.35 (*m*)	3, 5, 31
5	40.2, *t*	1.35 (*m*), 1.20 (*m*)	4, 26	36.6, *t*	1.40 H a (*m*), 1.15 H b (*m*)	4, 26
6	76. 8, *s*		5, 7, 26, 32	75.8, *s*		5, 7, 26, 32
7	35.9, *d*	1.54 (*m*)	8, 9, 26, 32	36.4, *d*	1.52 (*m*)	8, 9, 26, 32
8	24.4, *t*	1.51 (*m*), 1.41 (*m*)	7, 9, 32	23.9, *t*	1.78 (*m*), 1.44 (*m*)	7, 9, 32
9	33.0, *t*	1.98 H eq (*m*), 1.27 H ax (*m*)	8, 11, 27	29.0, *t*	1.86 H eq (*m*), 1.26 H ax (*m*)	8, 11, 27
10	74.4, *s*		8, 9, 11, 12, 27	74. 6, *s*		9, 11, 12, 27
11	81.4, *d*	4.00 (*dd*, 6.7, 7.0)	9, 12, 14, 27	85. 7, *d*	3.70 (*dd*, 6.6, 7.0)	9, 12, 14, 27
12	26.3, *t*	1.75 (*m*)	11, 13, 27	26.0, *t*	1.73 (*m*)	11, 13, 14
13	26.1, *t*	1.71 (*m*)	12, 14	26. 5, *t*	1.73 (*m*)	11, 12, 14
14	88.0, *d*	3.48 (*dd*, 6.7, 7.4)	11, 13, 28	87.9, *d*	3.52 (*dd*, 6.6, 7.0)	11, 13, 28
15	73.9, *s*		13, 14, 16, 28	74.0, *s*		13, 14, 16, 28
16	33.8, *t*	1.51 (*m*), 1.41 (*m*)	14, 17, 28	34.1, *t*	1.52 (*m*), 1.42 (*m*)	14, 17, 28
17	24.6, *t*	1.50 H ax (*m*), 1.42 H eq (*m*)	16, 18, 33	24.6, *t*	1.52 (*m*), 1.42 (*m*)	16, 18, 33
18	37.0, *d*	1.51 (*m*)	17, 20, 29, 33	37.1, *d*	1.52 (*m*)	17, 29, 33
19	76.1, *s*		18, 20, 29, 33	76.1, *s*		18, 20, 29, 33
20	40.5, *t*	1.35 (*m*), 1.20 (*m*)	21, 29	40.6, *t*	1.36 (*m*), 1.15 (*m*)	21, 29
21	27.9, *t*	1.45 (*m*), 1.35 (*m*)	20, 22, 29	27.9, *t*	1.44 (*m*), 1.38 (*m*)	20, 22, 29
22	41.7[e], *d*	2.05 (*m*)	21, 24, 30, 34	41.8, *d*	2.03 (*m*)	21, 24, 30, 34
23	150.6[d], *s*		21, 22, 24, 30, 34	150.6, *s*		21, 22, 24, 30, 34
24	18.7[a], *q*	1.64 (*s*)	22, 30	19.0[a], *q*	1.63 (*s*)	22, 30
25	109.4[b], *t*	4.67 (*br s*)[a]	1, 3	109.3[b], *t*	4.66 (*br s*)	1, 3
26	22.8, *q*	1.00 (*s*)	7	27.0, *q*	1.17 (*s*)	5, 7
27	24.9, *q*	1.03 (*s*)	11	24.3, *q*	1.13 (*s*)	11
28	21. 5, *q*	1.12 (*s*)	14	21.5, *q*	1.15 (*s*)	14
29	22.4, *q*	1.05 (*s*)	20	22.3, *q*	1.05 (*s*)	20
30	109.3[b], *t*	4.66 (*br s*)[a]	22, 24	109.2[b], *t*	4.66 (*br s*)	22, 24
31	20.3[f], *q*	1.00 (*d*, 5.7)	3, 4	20.1, *q*	1.01 (*d*, 7.0)	3, 4
32	17.4, *q*	0.79 (*d*, 6.6)	7, 8, 26	15.9, *q*	0.90 (*d*, 6.8)	5, 7, 8, 26
33	17.5, *q*	0.77 (*d*, 6.9)	29	17.5, *q*	0.78 (*d*, 6.2)	29
34	20.3[f], *q*	0.99 (*d*, 6.8)	21, 22	20.2, *q*	0.99 (*d*, 7.0)	21, 22

[a] Assignments in the same column may be reversed.
[b] Assignments in the same column may be reversed.
[c] Protons correlating with carbon resonance.
[d] Each couple of carbons gave two signals separated by less than 0.1 ppm in the ^{13}C NMR spectrum.
[e] Each couple of carbons gave two signals separated by less than 0.1 ppm in the ^{13}C NMR spectrum.
[f] Each couple of carbons gave two signals separated by less than 0.1 ppm in the ^{13}C NMR spectrum.

showed that: (i) the proton resonances of CH_3-26, CH_3-27 and CH_3-32 were shifted from δ 1.00, 1.03 and 0.79 to 1.17, 1.13 and 0.90, respectively, most probably due to inversion of stereochemistries at C-6, C-10 and C-7, respectively, (ii) the resonance for C-9 is shifted from δ 33.0 to 29.0, suggesting the existence of a γ-shielding interaction (Stothers, 1972), between H-9 axial and CH_3-32 which would be axially oriented at C-7. The ROESY experiment further supported these findings: the cross-peaks observed between (i) H-9 axial and CH_3-32, (ii) CH_3-26 and CH_3-32, and (iii) CH_3-27 and H-5a, allowed assignment of an axial orientation to CH_3-27, CH_3-32 and the side chain at C-6, and consequently an equatorial orientation to CH_3-26 and the C-10–C-11 bond linking the *trans*-THF to the THP ring.

Botryolins A and B are unique tetramethylated triterpenes having a regular arrangement of THF and THP rings. Triterpene polyethers are characteristic compounds from some marine red algae, such as *Chondria armata* (Ciavatta et al., 2001), and especially those of the genus *Laurencia* (Fernández et al., 2000). They have also been isolated, for example, from a mollusc (Spinella et al., 1997) and from a tree of the Rutaceae family (Harding et al., 1995). Together with the unusual carotenoids, braunixanthins (Okada et al., 1997), isolated from *B. braunii,* botryolins are the only triterpene polyethers identified so far in freshwater.

3. Experimental

3.1. General

CC: alumina from Merck (70–230 mesh; activity II). TLC purifications were performed on glass plates coated with silica gel 60 $PF_{254+266}$ (Merck) and visualised by UV light. Optical rotations were measured with a Jasco P-1010 digital polarimeter. IR spectra were recorded on a Perkin Elmer 1420. HRFAB mass spectra were obtained on a Jeol MS 700, in positive mode through inclusion of the products in a nitrobenzyl alcohol matrix and addition of NaI. EI MS were measured at 70 eV on a HP 5989 spectrometer. ^1H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded on a Bruker Avance 400 DPX, in $CDCl_3$.

3.2. Algal material and culture

The strain originating from the barrier lake of Kossou, Ivory Coast, in which the B and L races of *B. braunii* coexist (Metzger et al., 1990), was identified by Dr. A. Couté, Laboratoire de Cryptogamie, Museum National d'Histoire Naturelle, 75005 Paris, France. Registered as "Kossou 4″, the strain is conserved in the laboratory by periodic replications (every 4 months) on a modified CHU 13 medium (Metzger et al., 1985). The alga was grown under air-lift conditions (air enriched by 1% CO_2) and continuously illuminated as previously described (Metzger et al., 1985). The culture (10 l) was harvested when it entered the stationary phase of growth, and then freeze dried.

3.3. Extraction and isolation

The dry biomass of the Kossou strain (14 g) was extracted at room temperature twice with 500 ml heptane (1 h for each extraction). The extracts were combined, concentrated under reduce pressure (4.8 g) and dissolved in $CHCl_3$. Addition of an equivalent volume of methanol, caused a rubbery material to precipitate which was removed from the mixture by centrifugation. The "rubber"-free extract (3.6 g) was separated into three fractions via alumina CC by elution with: heptane (fr. I); toluene (fr. II); and $CHCl_3$/MeOH 2:1 v/v (fr. III). Fr. I contained botryococcenes (3.02 g). Fr. II (103 mg) was separated by preparative silica gel TLC; elution with heptane/Et_2O 93:7 v/v, to afford botryolins A (R_f 0.48; 23 mg) and B (R_f 0.43; 28 mg).

3.4. Botryolin A (**2**)

Clear oil; $[\alpha]_D^{25} = -6.0^\circ$ (*c* 2.6, $CHCl_3$). HRFAB–MS: obs. 539.4459 (calc. for $C_{34}H_{60}O_3 + Na$ 539.4440); ^1H and ^{13}C NMR spectra: see Table 1.

3.5. Botryolin B (**3**)

Clear oil; $[\alpha]_D^{25} = -0.6^\circ$ (*c* 3.0, $CHCl_3$). HRFAB–MS: obs. 539.4457 (calc. for $C_{34}H_{60}O_3 + Na$ 539.4440); ^1H and ^{13}C NMR spectra: see Table 1.

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