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Phytochemistry 65 (2004) 3159–3165
PHYTOCHEMISTRY
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C_{31}–C_{34} methylated squalenes from a Bolivian strain of *Botryococcus braunii*

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Received 22 January 2004; received in revised form 9 September 2004

Abstract

Three new triterpenes, synthesized by a Bolivian strain of the green microalga *Botryococcus braunii*, were isolated and their chemical structures determined by 1D and 2D NMR, and mass spectrometry. These compounds are tri-, di-, and mono-methylsqualenes, co-occurring with the previously identified tetramethylsqualene and some C_{30}–C_{32} botryococcenes. In this strain, methylated squalenes constitute up to 24% of the total hydrocarbons and 4.5% of the dry biomass. The results of a pulse-chase experiment with L-[Me–^{13}C] methionine provide evidence for the origin of these compounds via methylation of squalene at positions 3, 7, 18 and 22.

Keywords: *Botryococcus braunii*; Green alga; Methylated squalenes

1. Introduction

The green colonial microalga *Botryococcus braunii*, inhabiting freshwater ponds and lakes, is known as an especially rich source of lipids, including fatty acids, epoxides, alkyl phenols, ether lipids, and also hydrocarbons, sometimes in very high amounts (Metzger et al., 1991). Chemical analyses of wild samples and laboratory grown strains have lead to the recognition in this alga of three chemical races, according to the type of hydrocarbons produced: odd-carbon-numbered *n*-alkadienes and trienes synthesized by the A race (Metzger et al., 1985), C_nH_{2n-10} triterpenes (n = 30–37), called botryococcenes, produced by the B race (Metzger et al., 1985), and an acyclic tetraterpene, *trs trs*-lycopadiene, yielded by the L race (Metzger and Casadevall, 1987).

In addition to botryococcenes, 1′–3 linked triterpenes (Huang and Poulter, 1988), the B race also produces low amounts of squalene and sometimes a few compounds tentatively identified as methylated squalenes in a Martinique strain (Metzger and Casadevall, 1983), and in some Japanese isolates (Okada et al., 1995). During the course of a biosynthetic study, Huang and Poulter (1989a) induced the production of high proportions of tetramethylsqualene (**1**), by feeding the Berkeley strain with L-methionine, the methylating agent in the biosynthesis of botryococcenes (Metzger et al., 1987). From spectroscopic studies, Huang and Poulter (1989a) showed that this C_{34} squalene derivative exhibited non-isoprenoid methyl groups at positions 3, 7, 18 and 22. Later on, some mono- and di-epoxides likely derived from C_{32}–C_{34} methylsqualenes (Delahais and Metzger, 1997; Metzger, 1999), and unusual carotenoids with a tetramethylsqualene skeleton via ketal and ether linkages, were discovered in the B race of *B. braunii* (Okada et al., 1996, 1997, 1998). We report herein on the

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0031-9422/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2004.09.015

isolation and structural elucidation of three methylated squalenes: tri-, di-, and mono-methylsqualenes (**2**, **3** and **4**, respectively). The investigated strain originated from a freshwater lake of the Overjuyo valley in Bolivia (Metzger et al., 1988).

2. Results and discussion

2.1. Isolation of *1*, *2*, *3* and *4* and structural determination

Previous works on the localisation of botryococcenes in *B. braunii* colonies showed the existence of two pools for these hydrocarbons: (i) an extracellular one, located in the algal cell walls which are impregnated by oil, thus forming a dense matrix surrounding the cells, and extractable with apolar solvents such as heptane, and (ii) an intracellular pool, only extractable with polar solvents (Wolf et al., 1985; Metzger et al., 1987). Applying this sequential extraction to the dry biomass of the Bolivian strain, heptane and chloroform/methanol extracts were obtained (yield: 21% of dry weight for each extract). Column chromatography over alumina of these two extracts furnished by elution with heptane pure hydrocarbon fractions (yield in external and internal hydrocarbons: 13.2% and 4.5% of dry weight, respectively). GC and GC/MS analyses, including co-injections with authentic standards, showed that the external pool contained essentially C_{30}–C_{32} botryococcenes and low amounts of some other compounds (Fig. 1). In contrast, the GC trace of the internal pool exhibited high proportion of a tetramethyltriterpene (**1**, Fig. 1), accompanied by low amounts of botryococcenes and substantial quantities of squalene and C_{31}–C_{33} compounds (**2**–**4**, Fig. 1). Purification by HPLC of this latter fraction by successive injections on an analytical C_{18} column using acetonitrile as eluent, furnished four enriched subfractions of **4** (purity: 81%), **3** (purity:

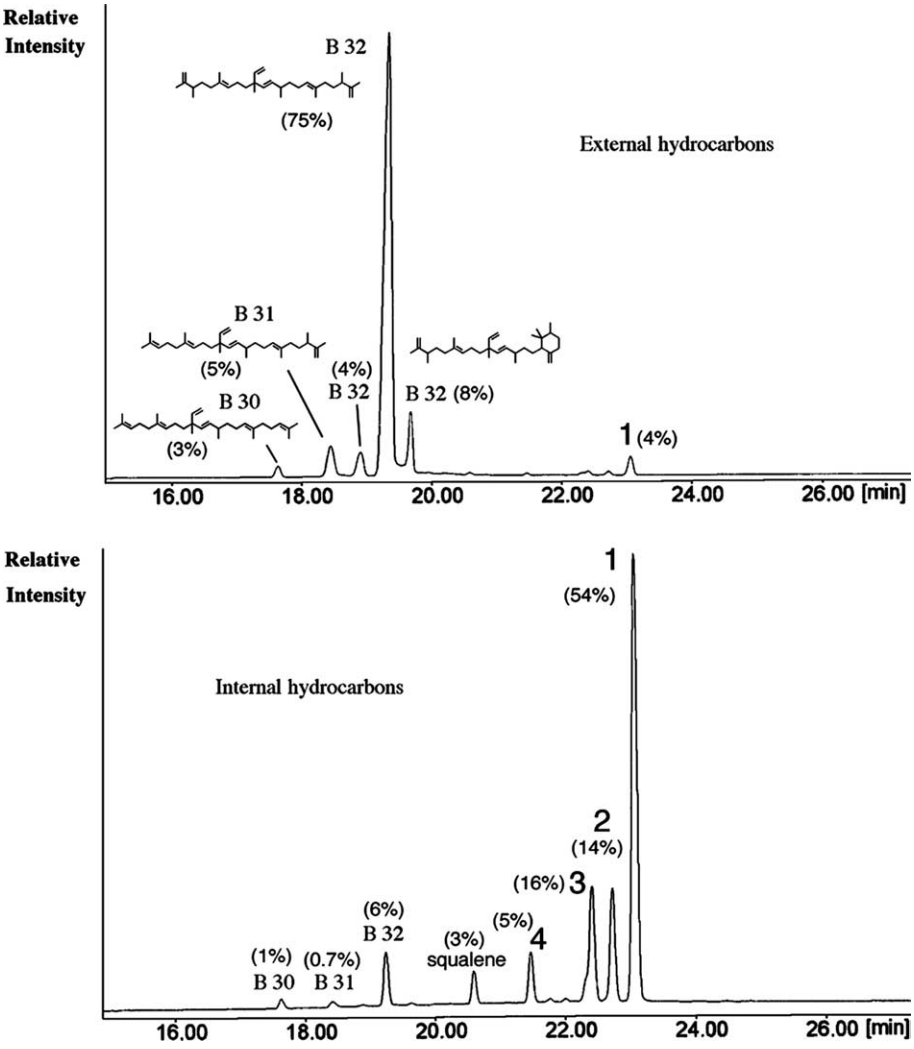

Fig. 1. GC analysis of the external and internal hydrocarbon pools isolated from *B. braunii* strain Overjuyo 2, grown 21 days under air-lift conditions (B: botryococcenes, followed by the number of carbon atoms); relative percentages in parentheses.

70%), **2** (purity: 72%) and **1** (purity: 85%). These enrichment levels were high enough to elucidate completely the structures of these compounds by ^{1}H and ^{13}C NMR. A rapid examination of the ^{1}H NMR spectra of **1–4** showed that they are not botryococcenes due to the absence of characteristic signals for the vinyl moiety and the quaternary methyl produced by the 1′–3 linkage.

Compound **1**, the major component of the $CHCl_3$/MeOH extract, had the molecular formula $C_{34}H_{58}$, based on HR–CI(CH_4)MS analysis (*m/z* 467.4613 $[M + H]^+$). ^{1}H and ^{13}C NMR data (Table 1) were found to be similar to those of the previously identified tetramethylsqualene (Huang and Poulter, 1989a). However, our HMBC experiment permitted us to invert the previous ^{13}C assignments for C-8 and C-9, which were erroneous. The detailed analyses of 2D NMR spectra, including a ^{1}H–^{1}H COSY spectrum, allowed to identify **1** as being 3,7,18,22-tetramethylsqualene (Fig. 2).

The molecular formula of compound **2** was determined to be $C_{33}H_{56}$ based on HR–CI(CH_4)MS analysis that showed a protonated molecular ion at *m/z* 453.4465 $[M + H]^+$, indicating the presence of six unsaturations. Compound **2** exhibited NMR spectral data (Table 1), rather similar to those of tetramethylsqualene **1**, but inspection of the ^{1}H and ^{13}C spectra indicated that it possesses three exomethylenes (instead of four in **1**)

Table 1
^{1}H and ^{13}C NMR data of methylated squalenes **1–4** recorded in $CDCl_3$

Assignment	Tetramethylsqualene (**1**)		Trimethylsqualene (**2**)		Dimethylsqualene (**3**)		Methylsqualene (**4**)	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	4.69 *m*	109.6	4.69 *m*	109.6	4.67 *m*	109.5	4.67 *m*	109.9
2		150.1		150.1		150.3		150.3
3	2.16 *m*	41.1	2.15 *m*	41.1	2.09 *m*	40.8	2.09 *m*	40.8
4	1.52 *m*, 1.43 *m*	33.5	1.50–1.35 *m*	33.5	1.46 *m*, 1.36 *m*	33.4	1.46 *m*, 1.35 *m*	33.4
5	1.89 *m*	31.7	1.89 *m*	31.7	1.89 *m*	37.6	1.89 *m*	37.6
6		155.0		155.0		135.2[b]		135.0[b]
7	2.06 *m*	39.7	2.07 *m*	39.7	5.10 *m*	124.2	5.11 *m*	124.5[c]
8	1.52 *m*, 1.34 *m*	34.0	1.52 *m*, 1.34 *m*	34.1	2.06 *m*	26.7	2.06 *m*	26.9
9	1.91 *m*	37.6	1.91 *m*	37.6[a]	1.98 *m*	39.9	1.98 *m*	39.9
10		135.5		135.5[b]		135.3[b]		135.2[b]
11	5.12 *m*	124.2	5.13 *m*	124.2[c]	5.14 *m*	124.4	5.14 *m*	124.4[c,f]
12	2.00 *m*	28.4	2.01 *m*	28.4	2.02 *m*	28.4	2.02 *m*	28.4
13	2.00 *m*	28.4	2.01 *m*	28.4	2.02 *m*	28.4	2.02 *m*	28.4
14	5.12 *m*	124.2	5.13 *m*	124.4	5.14 *m*	124.4	5.14 *m*	124.4[c,f]
15		135.5		135.3[b]		135.3[b]		135.1[b]
16	1.91 *m*	37.6	2.00 *m*	39.9	1.98 *m*	39.9	1.98 *m*	39.8[g]
17	1.52 *m*, 1.34 *m*	34.0	2.07 *m*	26.7	2.06 *m*	26.7	2.06 *m*	26.7[h]
18	2.06 *m*	39.7	5.10 *m*	124.1[c]	5.10 *m*	124.2	5.10 *m*	124.3[c]
19		155.0		135.2[b]		135.2[b]		135.3[b]
20	1.89 *m*	31.7	1.91 *m*	37.6[a]	1.89 *m*	37.6	2.06 *m*	39.8[g]
21	1.52 *m*, 1.43 *m*	33.5	1.50-1.35 *m*	33.4	1.46 *m*, 1.36 *m*	33.4	2.06 *m*	26.7[h]
22	2.16 *m*	41.1	2.11 *m*	40.8	2.09 *m*	40.8	5.10 *m*	124.5[c]
23		150.1		150.3		150.3		131.4
24	4.69 *m*	109.6	4.67 *m*	109.5	4.67 *m*	109.5	1.60 *s*	17.8
25	1.66 *br s*	19.0	1.66 *br s*	19.0	1.65 *br s*	19.0	1.65 *br s*	19.0
26	4.71 *m*	107.4	4.71 *m*	107.4	1.58 *br s*	16.1[e]	1.58 *br s*	16.1[i]
27	1.58 *s*	16.1	1.58 *br s*	16.1[d]	1.60 *br s*	16.1[e]	1.60 *s*	16.1[i]
28	1.58 s	16.1	1.60 *br s*	16.1[d]	1.60 *br s*	16.1[e]	1.60 *s*	16.1[i]
29	4.71 *m*	107.4	1.58 *br s*	16.1[d]	1.58 *br s*	16.1[e]	1.60 *s*	16.1[i]
30	1.66 *br s*	19.0	1.65 *br s*	19.0	1.65 *br s*	19.0	1.68 *br s*	25.8
31	1.02 *d* (7.0)	19.9	1.02 *d* (7.0)	19.9	1.00 *d* (6.9)	19.8	1.00 *d* (6.9)	19.8
32	1.00 *d* (6.8)	20.3	1.00 *d* (6.8)	20.3	1.00 *d* (6.9)	19.8		
33	1.00 *d* (6.8)	20.3	1.00 *d* (6.8)	19.8				
34	1.02 *d* (7.0)	19.9						

[a] 37.64 or 37.60.
[b] Signals may be interchangeable in a same column.
[c] Signals may be interchangeable in a same column.
[d] 16.13, 16.10 or 16.08.
[e] 16.12 or 16.09.
[f] 124.40 or 124.36.
[g] 39.84 or 39.82.
[h] 26.75 or 26.73.
[i] 16.14, 16.12, 16.09 or 16.08.

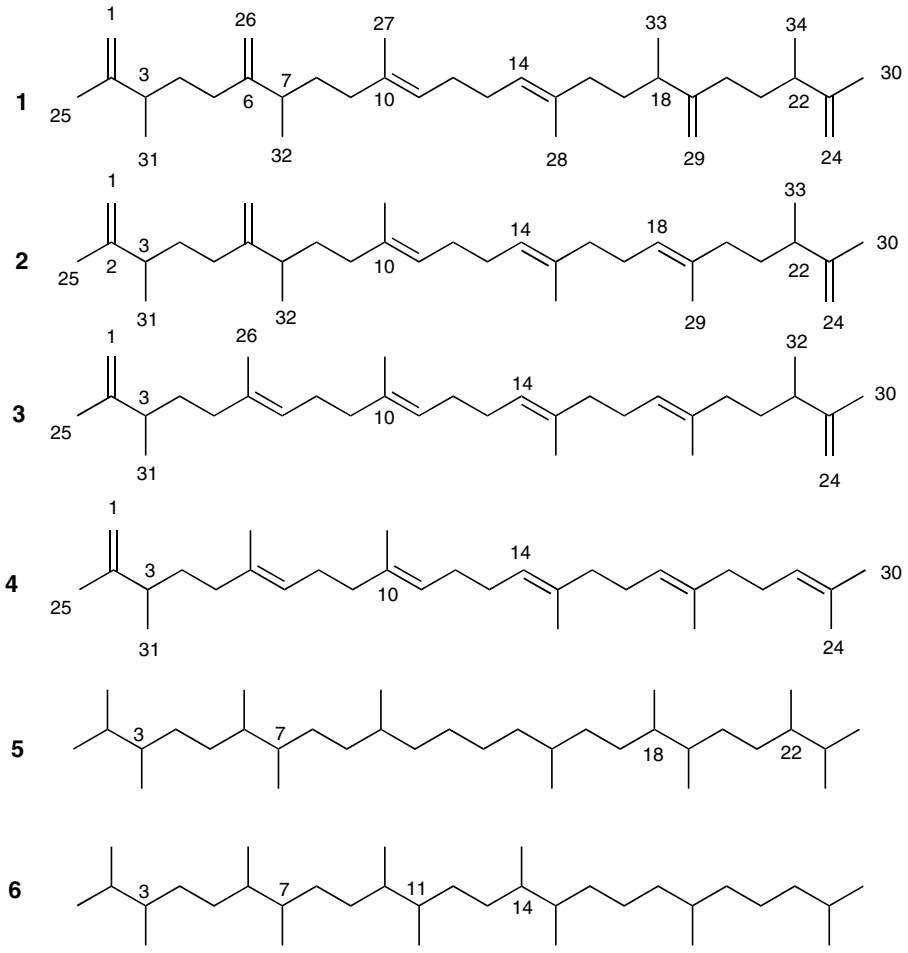

Fig. 2. Structures of methylated squalenes **1**–**4**, and tetramethylsqualanes **5** and **6**.

and three trisubstituted double bonds (instead of two in **1**). COSY crosspeaks were observed from the olefinic methylene protons at C-1 (δ_H 4.69) to the methyl protons at C-25 (δ_H 1.66) and to the allylic methine proton at C-3 (δ_H 2.15), and from the methylene protons at C-24 (δ_H 4.67) to the methyl protons at C-30 (δ_H 1.65) and to the allylic methine at C-22 (δ_H 2.11). In turn, the signals of the allylic methine protons at C-3 and C-22 correlated with those of methyl protons at C-31 and C-33 and with one of the diastereotopic protons at C-4 and C-21 (δ_H 1.35), respectively. This established that **2** derives from squalene by methylations at C-3 and C-22. Then, starting from the signals of the diastereotopic protons at C-4, HMBC correlations showed that a third non-terpenoid methyl group was present at position 7. Furthermore, all configurations of the trisubstituted double bonds C10–C11, C14–C15 and C18–C19 were established to be (*E*) on the basis of the chemical shifts of C-9 (δ_C 37.6), C-16 (δ_C 39.9) and C-20 (δ_C 37.6), respectively. These shifts would be reduced by about 5 ppm for (*Z*) geometry. Based on these data, the structure of **2** was unambiguously assigned to be 3,7,22-trimethylsqualene (Fig. 2).

Compound **3** which eluted before trimethylsqualene on the reversed-phase HPLC column, had the molecular formula $C_{32}H_{54}$, as determined by HR–CI(CH_4)MS analysis (*m*/*z* 439.4298 $[M + H]^+$), indicating the presence of six unsaturations. The ^{13}C NMR spectrum showed 16 peaks, and a DEPT 135 experiment indicated the presence of 4 CH_3, 6 CH_2, 3 CH and 3 C, including 1 CH_2, 2 CH and 3 C as olefinic carbons. These results strongly suggested the existence of a two-fold axis of symmetry in **3**. A long-range coupling observed between the vinylic methyl protons (δ 1.65) and the olefinic methylene carbon (δ 109.5) in the HMBC spectrum established the presence of an isopropylidene group at each terminus of the molecule, as in **1** and **2**. A non-terpenoid methyl group was shown to be at C-3 (and C-22) as an HMBC correlation was observed between the quaternary carbon of the exo methylene double bond (C-2 and C-23, δ_C 150.3) and the protons of a methyl group (Me-31 and Me-32, δ_H 1.00). Moreover the comparisons of the other ^1H and ^{13}C NMR data (Table 1), and COSY, HMQC and HMBC spectra with those of **1** and **2** clearly indicated that **3** is 3,22-dimethylsqualene (Fig. 2).

Compound **4**, which eluted between squalene and dimethylsqualene **3** on the reversed-phase HPLC column, had the molecular formula $C_{31}H_{52}$ based on HR–CI(CH_4)MS analysis (*m/z* 425.4142 $[M + H]^+$), indicating the presence of six double bonds. Inspection of the ^1H and ^{13}C NMR spectra (data in Table 1), indicated that compound **4** comprises a terminal isopropylidene group, and a non-terpenoid methyl group attached at C-3. Moreover, HMBC correlations between two methyl groups at δ_H 1.68/δ_C 25.8, and at δ_H 1.60/δ_C 17.8, respectively, indicated their geminal position on the olefinic carbon C-23. Detailed analysis of COSY, HMQC and HMBC spectra, and comparison of ^{13}C NMR data with those of squalene (Breitmaier and Voelter, 1987), gave evidence that the structure of **4** is 3-methylsqualene (Fig. 2). This compound had been tentatively identified in a previous work only on the basis of MS data (Metzger and Casadevall, 1983).

2.2. Biosynthetic relationship between methylated squalenes (*1–4*)

In *B. braunii* race B, botryococcenes and tetramethylsqualene (and squalene) are synthesized via the non-mevalonate pathway (Sato et al., 2003). These non-head-to-tail triterpenes result from 1′–3 (botryococcenes) and 1′–1 (squalene and tetramethylsqualene) linkages of two farnesyl moieties (Huang and Poulter, 1989b; Okada et al., 2004), likely via presqualene diphosphate as common precursor (Poulter, 1990). In order to investigate the relationship existing between methylated squalenes (**1–4**), a pulse-chase experiment was performed.

After feeding algae with L-[Me–^{13}C] methionine for two days, the algal colonies were transferred into a fresh culture medium and the growth allowed to continue for different periods of time. Internal hydrocarbons were isolated, purified and analysed by GC and GC–MS. The algal content in squalene and methylated squalenes, and their distribution are reported in Table 2. As can be seen from these data, a general trend is evident: the content in squalene and methylated squalenes increased from time 0 to day 6, but in higher proportion for tetramethylsqualene **1** than for its lower homologues. During the same period, the contribution of tetramethylsqualene increased from ca. 33% immediately after the pulse, up to 48% at day 6, while the percentages of all lower homologues decreased. Purification by reversed phase HPLC of internal hydrocarbons isolated at time 0, and days 2 and 6, yielded enriched fractions of **1** (purity: ca. 95%) and **4** (purity: ca. 90%). Attempts to obtain compounds **2** and **3** with satisfactory levels of purity were unsuccessful. ^{13}C NMR data of methylsqualene (**4**) and tetramethylsqualene (**1**), and comparison with data obtained for these compounds isolated from the culture in absence of L-[Me–^{13}C] methionine revealed that only resonances C-31 of **4**, and C-31,34, and C-32,33 of **1** were enhanced (Table 3). This demonstrated that methionine, likely via the *S*-adenosyl form, is the methylating agent. The labelling of C-31 in methylsqualene **4** was maximum at time 0, and then decreased. This large decrease of labelling, concomitant with a low increase in methylsqualene content of the alga, indicates that **4** is the precursor of its higher homologues, likely according to the sequence **4** → **3** → **2** → **1**. Such a pathway is corroborated by the enhancement of the intensity of methyl signal C-31,34 in tetramethylsqualene **1** between time 0 and day 2 (Table 3). The strong decrease of the intensity of signals C-31,34 and C-32,33 observed at day 6, indicates that tetramethylsqualene is not an end product, which is consistent with the isolation from *B. braunii* race B of several derivatives of **1**, like epoxides (Delahais and Metzger, 1997; Metzger, 1999) and carotenoids (Okada et al., 1996, 1997, 1998). The significantly higher enhancement of methyl signal C-32,33 at time 0, by comparison to that of C-31,34, could be related to differences in the proportions of precursors of **1** when L-[Me–^{13}C] methionine was added to the culture. These precursors underwent methylation at positions 7 and 18 (compounds **2** and **3**), and 3 and 22 (squalene and compound **1**).

The structural variation in this squalene-related family is certainly wider than illustrated by the present

Table 2
Variation in squalene and methylated squalene content and composition during the chase experiment[a]

Compounds	Time in fresh medium		
	t = 0	*t* = 2 days	*t* = 6 days
$C_{30}H_{50}$ (**Squalene**)	1.5 (13.9)	1.5 (10.3)	1.8 (8.8)
$C_{31}H_{52}$ (**4**)	1.8 (16.7)	1.9 (13.1)	2.5 (12.3)
$C_{32}H_{54}$ (**3**)	1.9 (17.6)	2.1 (14.5)	2.5 (12.3)
$C_{33}H_{56}$ (**2**)	1.1 (10.1)	1.2 (8.3)	1.8 (8.8)
$C_{34}H_{58}$ (**1**)	3.6 (33.3)	6.5 (44.8)	9.9 (48.5)
Other[b]	0.9 (8.4)	1.3 (9.0)	1.9 (9.3)
Total	10.8	14.5	20.4

[a] Expressed in mg/g dry biomass (relative percentages are shown in parentheses).
[b] Likely isomers of **2**, **3** and **4**.

Table 3
Enhancements[a] of ^{13}C NMR signals of non-terpenoid carbon atoms in methylsqualene (**4**) and tetramethylsqualene (**1**) during the chase experiment

Time in fresh medium	$C_{31}H_{52}$ (**4**)	$C_{34}H_{58}$ (**1**)	
	C-31	C-31, C-34	C-32, C-33
t = 0	16.3	6.8	16.5
t = 2 days	4.0	17.2	15.8
t = 6 days	0.4	0.8	1.1

[a] Determined by comparison with methylated squalenes **1** and **4** isolated from the alga cultivated in the absence of ^{13}C-labelled methionine.

compounds. Indeed, owing to: (i) the identification of epoxide derivatives of C_{32} and C_{33} methylated squalenes (Delahais and Metzger, 1997), exhibiting patterns for the non-terpenoid methyl groups different from those found in **3** and **2**, respectively, and (ii) the isolation of a diol deriving from a monocyclic trimethylsqualene (Metzger, P., Rager, M.N., Largeau, C., unpublished results), in the oils extracted from two different strains of *B. braunii*, the identification of some other structures can be expected. Moreover, the recent discovery of 3,7,11,14-tetramethylsqualane (**6**, Fig. 2), in some S.E. Asian oil shales and coal samples derived from fossil *Botryococcus* (Summons et al., 2002), in addition to botryococcanes (i.e. the fully hydrogenated derivatives of botryococcenes, specific biomarkers of *Botryococcus*) and 3,7,18,22-tetramethylsqualane (**5**, Fig. 2), speaks also for the production of some other tetramethylsqualenes by *B. braunii*. The richness of the present strain in these peculiar triterpenoids, essentially present in the alga as intracellular components, with a level above 4% of the total dry wt, is unequalled with regard to the numerous *Botryococcus* isolates investigated to date (Metzger et al., 1991). From a geochemical point of view, methylated squalanes, as botryococcanes, are important biomarkers of *Botryococcus* useful for establishing oil/source correlations.

3. Experimental

3.1. General experimental procedures

Column chromatography over alumina (Merck; 0.0630–0.2 mm, activity II); ^{1}H (400 MHz) and ^{13}C (100 MHz) were recorded in $CDCl_3$ solution on a Bruker Avance 400 DPX. The chemical shifts are given in ppm, and referenced to the residual $CHCl_3$ signal at δ_H 7.26 and $CDCl_3$ signal at δ_C 77.1. Reversed-phase HPLC analyses and quantitative separations were performed with a spherical 5 μm XTerra™ MS C_{18} column (4.6 × 250 mm) connected with a Waters 410 differential refractometer. GC/MS analyses were performed on a 6890N chromatograph from Agilent Technologies equipped with a fused silica DB-5MS column (30 m × 0.25 mm, film thickness 0.5 μm, temperature programmed from 220 to 300 °C at 4 °C/min), coupled to a 5973N mass spectrometer (spectra were recorded from electron impact at 70 eV). HR–CI mass spectra were obtained on a Jeol MS 700. The conditions for GC analyses were as above.

3.2. Algal strain and culture conditions

The strain originated from a lake in Overjuyo, Bolivia and was described in a previous paper (Metzger et al., 1988). The strain (Overjuyo strain 2) is conserved in the laboratory by periodic replications (every 4 months) on a modified CHU 13 medium (Largeau et al., 1980). The alga was grown at 25 °C, under batch air-lift conditions (air enriched by 1% CO_2) and continuous illumination (170 μE m^{-2} s^{-1}) as previously described (Metzger et al., 1985). After 21 days of growth, the culture (20 l) entered the stationary phase, it was harvested by filtration on 10 μm Nylon cloth, and the recovered biomass was then freeze-dried.

3.3. Extraction and isolation

The dry biomass of the Overjuyo strain (15.8 g) was extracted twice for 1 h with heptane (2 × 500 ml), at room temperature. The combined extracts were concentrated under reduced pressure and the oil (3.4 g) chromatographed on alumina CC; elution with heptane furnished the external hydrocarbon fraction (2.1 g). The heptane-extracted biomass was extracted with 250 ml $CHCl_3$ at room temperature overnight. Then, the $CHCl_3$ extracted biomass was extracted with 600 ml $CHCl_3$/MeOH 2:1 v/v 18 h, at room temperature. This lipid extract (3.4 g) was concentrated and chromatographed as above to furnish the internal hydrocarbon fraction (0.58 g). The external and internal hydrocarbon fractions were analysed by GC, GC–MS, and HPLC (elution with MeCN, at a flow rate of 2 ml/min). Methylated squalenes, **1**–**4**, were isolated by reversed-phase HPLC by repeated injection of internal hydrocarbon samples (20 μl, 10% in $CHCl_3$); retention times of squalene: 13.4 min, **4**: 16 min, **3**: 18.5 min, **2**: 20.8 min, **1**: 23.4 min. The GC retention indices relatively to squalene were as follows: **4** (1.052), **3** (1.107), **2** (1.126), **1** (1.146).

3.3.1. Tetramethylsqualene (*1*)

HR–CI(CH_4)MS (probe) *m/z*: 467.4613 $[M + H]^+$ (calcd. for $C_{34}H_{59}$ 467.4617); GC–EIMS 70 eV, *m/z* (rel. int.): 466 $[M]^+$ (0.8), 451 $[M - Me]^+$ (0.2), 217 (4), 205 (10), 189 (3), 177 (15), 163 (16), 149 (21), 135 (21), 123 (31), 121 (27), 109 (52), 107 (46), 95 (64), 93 (28), 81 (100), 79 (25), 69 (43), 67 (33), 55 (40). For ^{1}H and ^{13}C NMR spectral analysis, see Table 1.

3.3.2. Trimethylsqualene (*2*)

HR–CI(CH_4)MS (probe) *m/z*: 453.4465 $[M + H]^+$ (calcd. for $C_{33}H_{57}$ 453.4460); GC–EIMS 70 eV, *m/z* (rel. int.): 452 $[M]^+$ (0.8), 437 $[M - Me]^+$ (0.4), 217 (3), 205 (7), 189 (1), 177 (7), 163 (10), 149 (15), 135 (16), 123 (27), 121 (22), 109 (46), 107 (27), 95 (98), 93 (21), 81 (100), 79 (20), 69 (40), 67 (28), 55 (36). For ^{1}H and ^{13}C NMR spectral analysis, see Table 1.

3.3.3. Dimethylsqualene (*3*)

HR–CI(CH_4)MS (probe) *m/z*: 439.4298 $[M + H]^+$ (calcd. for $C_{32}H_{55}$ 439.4304); GC–EIMS 70 eV, *m/z* (rel. int.): 438 $[M]^+$ (0.8), 423 $[M - Me]^+$ (0.5), 217 (4),

203 (3), 189 (4), 175 (5), 163 (5), 149 (9), 135 (10), 123 (20), 121 (15), 109 (37), 107 (13), 95 (100), 93 (13), 81 (87), 79 (14), 69 (33), 67 (19), 55 (28). For ^{1}H and ^{13}C NMR spectral analysis, see Table 1.

3.3.4. Methylsqualene (**4**)

HR–CI(CH_4)MS (probe) *m/z*: 425.4142 $[M + H]^+$ (calcd. for $C_{31}H_{53}$ 425.4147); GC–EIMS 70 eV, *m/z* (rel. int.): 424 $[M]^+$ (1), 409 $[M - Me]^+$ (0.5), 217 (4), 203 (4), 189 (4), 175 (4), 163 (5), 149 (11), 135 (15), 123 (19), 121 (19), 109 (26), 107 (12), 95 (69), 93 (16), 81 (94), 79 (12), 69 (100), 67 (18), 55 (21). For ^{1}H and ^{13}C NMR spectral analysis, see Table 1.

3.4. Pulse-chase experiment

A culture was prepared by inoculating 100 ml of a maintenance culture into a 3 l Erlenmeyer flask containing 2.2 l of Chu-13 medium. The culture was shaken by magnetic stirring (150 rpm), aerated with sterile air enriched with 1% CO_2 and continuously illuminated. After 14 days of growth at room temperature, a 10 ml aqueous solution of 100 mg of L-[Me–^{13}C] methionine (isotopic purity: 99%), sterilised by filtration, was added, and the culture continued for two days. Then the colonies were separated from the medium under sterile conditions, by filtration on 10 μm Nylon cloth, rinsed with 3×200 ml sterile Chu-13 medium, re-suspended in 2 l fresh medium, and then equally distributed into three Erlenmeyer flasks. At appropriate time points after the rinse, the cultures were taken for analyses. The dry biomass was extracted as described above. Internal hydrocarbons were analysed by GC and separated by reversed-phase HPLC as above. The purity of each fraction was checked by GC–EIMS analysis. Three successive purifications were carried out to obtain C_{31} and C_{34} methylated squalenes from each run; purity: ca. 89% (**4**), and 95% (**1**).

Acknowledgements

We are grateful to Mrs. N. Morin, Ecole Normale Supérieure (Paris), for measurements of HR–(CI)MS.

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