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A high molecular weight complex lipid, aliphatic polyaldehyde tetraterpenediol polyacetal from *Botryococcus braunii* (L race)

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Abstract

An aliphatic polyaldehyde tetraterpenediol polyacetal (APTP) has been isolated from the heptane extract of an Ivorian strain of the L chemical race of the green microalga, *Botryococcus braunii*. Size exclusion HPLC showed an unimodal distribution from M_r 5000 to 4000000, with a peak at 237000. The chemical structure of this soluble high M_r polymer was determined by spectroscopic methods and chemical degradation. The aliphatic polyaldehyde moiety is probably derived from the condensation–polymerization of a *n*-C_{32} diunsaturated α,ω-dialdehyde, involving an aldolization–dehydration mechanism. The acyclic tetraterpenediol is a 14,15-dihydroxy lycopa-18-ene, closely related to lycopadiene, the characteristic hydrocarbon produced by the L race of *B. braunii*. Acetalation of the polyaldehyde with the tetraterpenediol occurs for ca. seven aldehyde functions out of ten. Further extraction of the biomass with chloroform allowed isolation of an additional amount of polymer exhibiting by HPLC analysis a higher M_{peak} (400000), but with similar spectroscopic features. The polymers were isolated from the heptane and chloroform extracts in a yield of 13.2 and 2% of the dry biomass, respectively. Moreover, the algaenan, i.e. the non-hydrolyzable highly aliphatic insoluble biomacromolecular material, present in the outer walls, has been isolated in a yield of 12.3% of the biomass, using a new isolation process which avoids the formation of artifactual materials and allows for a more efficient hydrolysis of some sterically hindered oxygenated functions. FTIR analysis and 400°C pyrolysis of this algaenan, and comparisons with APTP clearly indicated that the molecular structure of the algaenan of the L race is based on an aliphatic polyaldehyde network. Moreover, the results suggested that the algaenan is likely to be formed from the hydrolysis of a highly condensed or reticulated form of APTP, which cannot be extracted with solvents. © 1998 Elsevier Science Ltd. All rights reserved.

Keywords: *Botryococcus braunii*; Chlorophyceae; Alga; L race; Aliphatic polyaldehyde; Tetraterpenediols; 14,15-dihydroxy lycopa-18-ene; Acetal; Absolute configuration; Algaenan

1. Introduction

The green microalga *Botryococcus braunii* biosynthesizes large amounts of various types of usual and specific lipids, the bulk of which is stored in its outer walls (Metzger, Casadevall, & Largeau, 1991). These outer walls are thought to be mainly formed of algaenan, an insoluble and chemically resistant aliphatic biopolymer (De Leeuw & Largeau, 1994). The molecular structure of the algaenan of the A race of *B. braunii*, which corresponds to the strains characterized by the production of *n*-alkadienes and trienes (Metzger et al., 1991), is a more condensed or reticulated form of the aliphatic polyaldehyde **1**. The latter, produced by algae of this race, is extractable with some solvents of low polarity, such as chloroform (Metzger, Pouet, Bischoff, & Casadevall, 1993; Gelin et al., 1994a). Feeding experiments with [1-^{14}C] and [10-^{14}C] oleic acid, indicated that this soluble polyaldehyde is biosynthesized from oleic acid, via a *n*-C_{32} diunsaturated α,ω-dialdehyde, by a condensation–dehydration mechanism (Metzger et al., 1993). Size-exclusion HPLC showed, moreover, an unimodal distribution in the three examined strains of the A race, from M_r 10000 to 4000000, with a M_{peak} varying between 123000 and 228000, depending on the strain origin.

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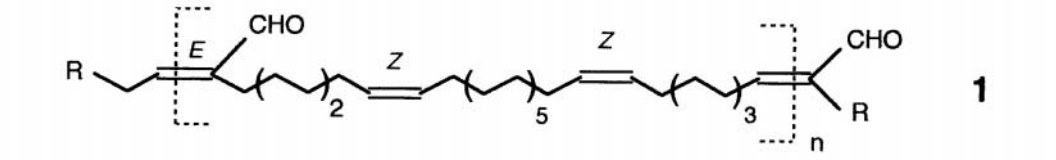

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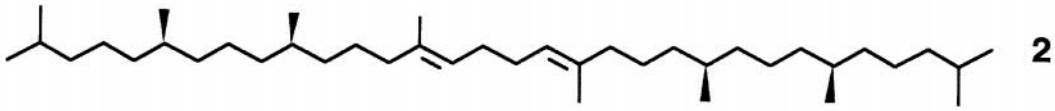

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To date, the precise molecular structures of the other algaenans isolated from numerous species of microalgae are not entirely established (De Leeuw & Largeau, 1994; Gelin et al., 1994a; Tegelaar, De Leeuw, Derenne, & Largeau, 1989; Derenne et al., 1992; Largeau & De Leeuw, 1995). Generally, algaenans are considered as deriving from the cross-linkage by ether bridges of some lipids exhibiting very long aliphatic chains (De Leeuw & Largeau, 1994). Some years ago, a resistant biopolymer was shown to occur in the L race of *B. braunii* (Derenne, Largeau, Casadevall, & Berkaloff 1989), chemically defined by the production of lycopadiene **2**, an acyclic tetraterpenoid hydrocarbon (Metzger et al., 1991; Metzger & Casadevall, 1987). Based on spectroscopic and pyrolytic investigations, this algaenan was thought to be composed of C_{40} isoprenoid units, cross-linked by ether bridges. A direct relationship between this chemically resistant material and the tetraterpenoid hydrocarbon synthesized by the L race, was thus suggested (Derenne et al., 1989; Derenne, Largeau, Casadevall, & Sellier, 1990).

Recently, examinations of the heptane and chloroform extracts obtained from the L race of *B. braunii*, allowed us to isolate a new type of soluble biopolymer of very high M_r: an aliphatic polyaldehyde tetraterpenediol polyacetal (APTP). The structure and the absolute configuration of this new polymer, presented here, have been established by means of spectroscopic techniques, chemical degradation and by spectroscopic comparisons with hemi-synthetic model compounds. Moreover, the recent design of a new process for isolating algaenans, avoiding the formation of artifactual melanoidin-like materials (Allard, Templier, & Largeau 1997), induced us to revisit the structure of the algaenan of the L race.

2. Results

2.1. Structure of aliphatic polyaldehyde teraterpenediol polyacetal (APTP)

2.1.1. Isolation and size exclusion HPLC

The dry biomass of a strain originating from a Yamoussoukro lake in Ivory Coast, was extracted with heptane at room temperature. After elimination of the solvent, the crude extract was dissolved in chloroform. Upon addition of an equivalent volume of methanol, a reddish rubbery material precipitated; it was repeatedly subjected to dissolution in chloroform and precipitation with methanol. Three repetitions of the procedure furnished a yellowish material which was finally kept in chloroform solution at −24°C for analyses. Unlike the aliphatic polyaldehyde extracted with chloroform from algae of the A race of *B. braunii* (Metzger et al., 1993), the present polymer could be redissolved in solvents of low polarity, such as methylene chloride, tetrahydrofuran and heptane, even after complete elimination of the solvent. The yield of purified rubbery material isolated from the heptane extract was 13.2% of the dry biomass when algae were in the stationary phase of growth and 6.8% with actively growing cultures. This material always appears as a substantial constituent of total biomass of the L race. Elemental analysis revealed the following composition (wt%): C, 82.9; H, 13.0 and O, 4.1 (Table 1), fairly constant whatever the culture age may be.

The M_r distribution of the polymer was determined by size-exclusion HPLC, using an Ultrastyragel column, with tetrahydrofuran as eluent; the M_r markers were polybutadiene standards. The chromatogram of the biopolymer extracted from the aged culture showed

Table 1
Elemental analysis of APTP and algaenans

	C %	H %	O %	Ash %	H/C[a]	O/C[a]
APTP	82.9	13.0	4.1	–	1.88	0.04
Algaenan L	79.3	12.1	6.8	1.8	1.83	0.06
Algaenan A	79.9	11.8	6.8	1.5	1.77	0.06

[a]Atomic ratio.

an unimodal distribution of M_r from 5×10^3 to 4×10^6, with a peak at 237000. A similar distribution was observed for a polymer extracted from an actively growing culture, but with a peak at M_r 68000.

An additional amount of polymer (2% of dry biomass) could be obtained by further extracting the heptane-extracted biomass with chloroform. This polymer showed by size-exclusion HPLC analysis a mass distribution shifted towards higher masses, up to 8×10^6 Da, and a M_{peak} at ca. 400000. FTIR and NMR investigations showed that it had similar spectroscopic features to those of the polymer extracted with heptane. ^1H and ^{13}C NMR spectra only differed in a pronounced line broadening of the signals of the chloroform extracted polymer, which could be related to a higher viscosity of the $CDCl_3$ solutions. The structural investigations presented below were performed on the polymer extracted with heptane.

2.1.2. Gross structural features

The FTIR spectrum (Fig. 1A) recorded from a thin film of polymer, obtained by evaporation of a chloroform solution on a KBr disc, showed an absorption for a CH aldehydic bond at 2706 cm^{-1}, associated with a band at 1690 cm^{-1} of carbonyl group influenced by an olefinic conjugation; absorptions for unsaturations occurred at 3000 and 1640 cm^{-1}. A vibration at 720 cm^{-1}, for $(CH_2)_n$, with $n \geq 4$, and a strong absorption at 1465 cm^{-1} indicated that the polymer contains very long polymethylenic chains. These IR characteristics were identical to those observed for aliphatic polyaldehyde **1** isolated from the A race (Metzger et al., 1993). The FTIR spectrum of the polymer isolated from the Yamoussoukro strain differed, however, by the presence of bands of medium

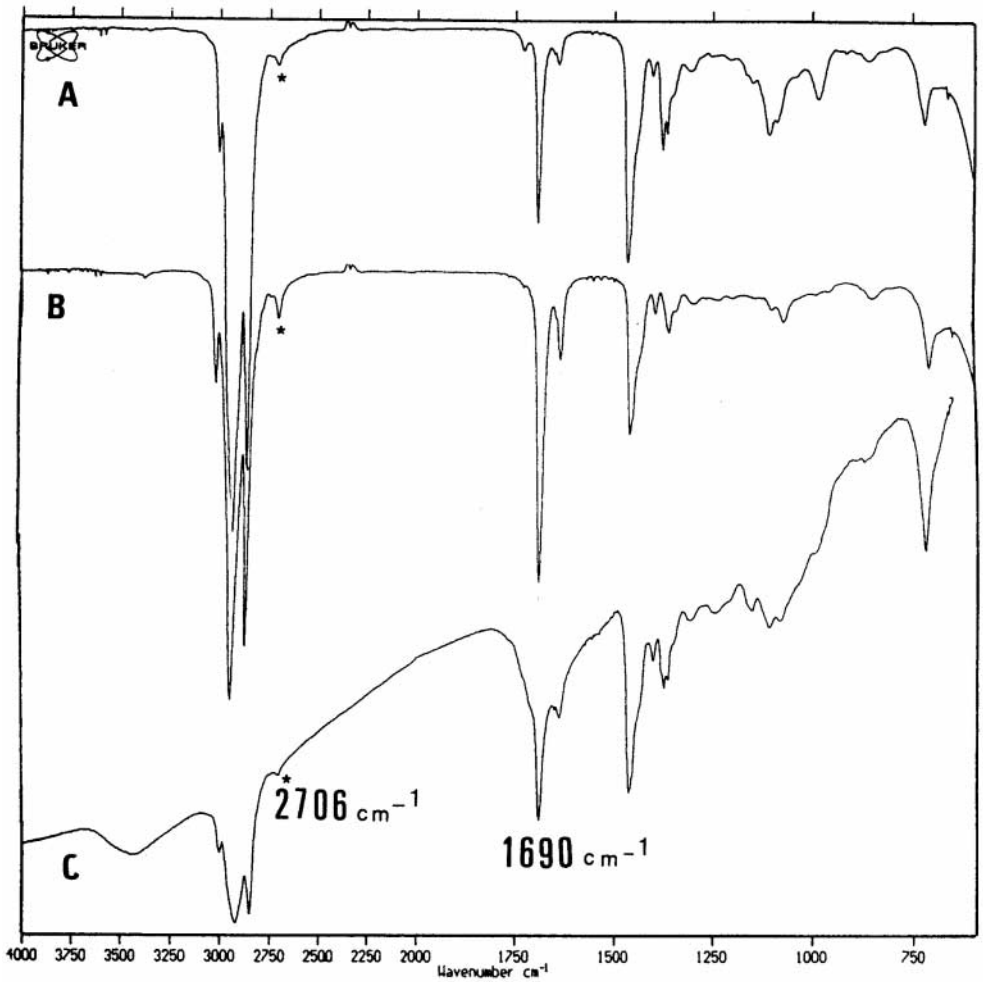


Fig. 1. FTIR spectra of APTP (A), hydrolyzed APTP (B) and algaenan L (C). A and B from films on KBr discs, C from dispersion in KBr.

Table 2
Selected ^{1}H and ^{13}C NMR data

	$\delta^{1}H$, multiplicity (J in Hz)	$\delta^{13}C$
Non-terpenoid skeleton		
Linear moiety far from the oxygenated functions		
vinylic CH	5.34, t (4.9)	130.1–129.7[a]
allylic CH_2	2.02, m	27.3, 27.2, 27.3
non-allylic CH_2	1.26, br	29.9–29.2[b]
Aldehyde moiety		
C-1	9.34, s	195.6
C-2		143.9
C-3	6.43, t (7.4)	155.7, 155.7, 155.6
C-4	2.34, dt (7.3, 7.2)	29.0
C-5	2.22, t (7.2)	24.1
Acetal moiety		
C-1′ (A)[c]	5.24, s	105.5
C-1′ (B)[c]	5.14, s	105.9
C-2′ (A)		136.3
C-2′ (B)		137.8
C-3′ (A)	5.55, t (7.2)	131.8
C-3′ (B)	5.63, t (7.2)	133.0
C-4′ (A and B)	1.90–2.00[d]	25.5, 25.5
Tetraterpenoid[e]		
C-1, C-32	0.83–0.87[d]	22.7
C-2, C-31	1.50, m	28.0
C-3, C-30	1.10, m	39.5
C-4, C-29	1.25, m	24.9
C-12	1.30, m	20.6
C-13	1.30, m	36.5
C-14 (A)		82.4
C-14 (B)		81.2
C-15 (A)	3.70, m	85.9
C-15 (B)	3.70, m	84.7
C-16 (A and B)	1.45, m; 1.60, m	28.8, 28.8
C-17 (A and B)	2.15, m	26.1, 26.0
C-18 (A and B)	5.12, t (6.3)	123.5, 123.4
C-19 (A and B)		136.4, 136.3
C-20	1.95, t (7.2)	40.1
C-33, C-40	0.81–0.87	22.8
C-36 (A)	1.20, s	22.6
C-36 (B)	1.23, s	21.8
C-37 (A and B)	1.59, s	16.0, 16.0

[a]130.1, 130.1, 130.0, 130.0, 129.9, 129.9, 129.8 and 129.7.
[b]29.9, 29.8, 29.5, 29.4, 29.4, 29.4, 29.3, 29.2, 29.2 and 29.1.
[c]A and B refer to the major and minor configurations respectively.
[d]Overlapping signals.
[e]^{1}H resonances of other protons were not determined due to the wide overlapping of signals in the range δ 1–1.50. Other ^{13}C resonances: CH_3: δ 19.8, 19.8; 19.7; CH_2: 38.0, 38.0, 37.8, 37.6, 37.6, 37.6, 37.5, 37.5, 37.5, 37.4, 37.4, 36.8, 36.5, 25.5, 25.5, 25.4, 24.9, 24.6, 24.6; CH: 33.0, 33.0, 32.9, 32.9, 32.8, 32.8.

intensity at 1375 and 1365 cm^{-1} indicative of methyl branchings, a C=O absorption of lower intensity and by the existence of bands at 1085 and 1105 cm^{-1} for C–O bonds.

The ^{1}H and ^{13}C NMR spectra (Table 2) confirmed the presence of a $–CH_2–CH=C(CHO)–CH_2–$ pattern in a highly predominant *E*-configuration, with signals for the CHO at δ_H 9.34 and δ_C 195.6, and a minor *Z* one, with signals at δ_H 10.10 and δ_C 191.2 (Metzger et al., 1993); *E*:*Z* ratio: 93:7, as determined from the ^{1}H integration. The ^{13}C NMR spectrum also showed numerous peaks of a high intensity around δ 29, ascribable to carbons of long methylenic chains and signals at δ ca. 129–130 for non-conjugated disubstituted double bonds of *Z* stereochemistry, as deduced from the resonance of their allylic carbons at δ 27.2 and 27.3. Moreover, all the ^{1}H and ^{13}C signals observed in the case of the aliphatic polyaldehyde **1** and ascribable to the olefinic and allylic carbons of the unsaturation conjugated with the CHO group, were easily identifiable for the *E*-configuration (Table 2). By comparison with those of **1**, the NMR spectra of the present polymer were, however, much more complicated. For instance, the ^{13}C and DEPT spectra revealed a total of 88 resonances, from which some corresponded undoubedtly to several carbons. The DEPT spectrum also suggested the presence in the polymer of an acyclic terpenoid moiety with, for example, signals of terminal methyls at δ_C 22.7 and 22.8 and in-chain methyls at δ_C 19.8, 19.8 and 19.7.

Inspection of the ^{1}H, ^{13}C and HETCOSY spectra suggested the presence of two different acetal-containing moieties A and B, in a 2:1 ratio, with signals at δ_C 105.5 and 105.9 correlating with ^{1}H singlets at δ_H 5.24 and 5.14, respectively. Four resonances for four other oxygen-bearing carbons, the aldehyde function excepted, were also noticed at δ_C 82.4 and 81.2 (quaternary carbons) and 85.9 and 84.7 (methine carbons). The two methine carbon signals correlated with a poorly resolved doublet in the ^{1}H domain at δ_H 3.70.

All these data suggested that the polymer isolated from the L race was based on an aliphatic polyaldehyde skeleton, of which seven aldehyde functions out of ten were acetalated with terpenediols, as deduced from the integration of the ^{1}H NMR spectrum.

2.1.3. Hydrolysis

Attempts to hydrolyse the acetal functions of the polymer dissolved in chloroform, using classical reagents, such as wet silica gel or silica impregnated with concentrated sulfuric acid, were unsuccessful, even after several days of reaction. This was finally achieved with the polymer dissolved in tetrahydrofuran using aqueous concentrated HCl as catalyst. After extraction of the reaction mixture with chloroform, the crude extract was concentrated; from this chloroform solution, the hydrolyzed polymer was isolated and purified by successive addition of methanol and dissolution in chloroform as described above for the native APTP. Determination of the structure of the hydrolyzed polymer and of the low M_r compounds present in the combined filtrates are described below.

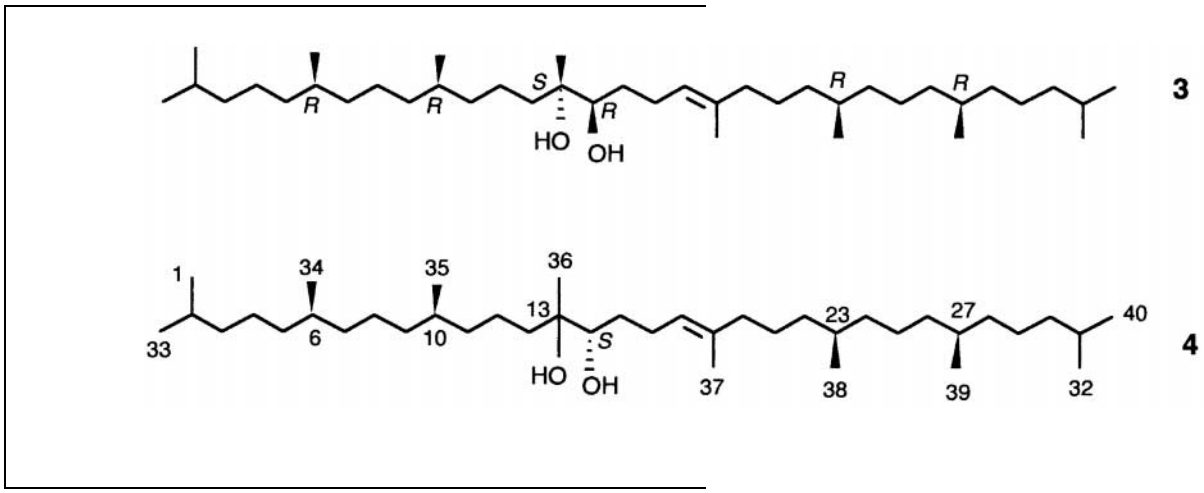
2.1.4. Hydrolyzed polymer

Size-exclusion HPLC analysis of the hydrolyzed polymer exhibited a mass distribution shifted towards lower M_r, from 2500 up to 1.8×10^6, with a M_{peak} at 110000. Moreover, the FTIR spectrum showed by comparison with that of the native APTP, an enhancement of the carbonyl absorption at 1690 cm^{-1}, along with a large decrease of the bands associated on the one hand with methyl groups at 1375 and 1365 cm^{-1} and on the other hand with C–O bonds at 1085 and 1105 cm^{-1} (Fig. 1B). The persistence of a band of medium intensity at 1370 cm^{-1} could be related to the H–C=O bending observed with aliphatic aldehydes (Lambert, Shurvell, Verbit, Cooks, & Stout, 1976). The hydrolysis of the acetal functions was confirmed by the ^1H and ^{13}C NMR spectra which only exhibited the same signals as those observed for the aliphatic polyaldehyde **1**.

Furthermore, ozonolysis of the hydrolyzed polymer, followed by the oxidative cleavage of the polyozonide, generated *n*-C_8, C_9 and C_{14} diacids identified by GC–MS of their dimethyl esters. Based on the formation of these three diacids in almost equal proportions, as previously observed in the ozonolysis of **1**, the position of the unsaturations in the aliphatic polyaldehyde backbone was suggested to be similar to that in **1**. In the latter case, these positions were confirmed by means of some feeding experiments with ^{14}C labelled tracers and subsequent analyses of the radio-labelled polymer (Metzger et al., 1993).

2.1.5. Tetraterpenediols

From the combined filtrates obtained from the purification of the hydrolyzed polymer, an oily product was obtained after silica gel CC and TLC purification, in a yield of 41% of the starting polymer. TLC analysis, CI(NH_3)MS, which revealed a $C_{40}H_{80}O_2$ molecular formula, IR, ^1H and ^{13}C NMR spectra suggested that this compound was 14,15-dihydroxy lycopa-18-ene, **3**, previously isolated as a free compound from the Yamoussoukro strain (Delahais & Metzger, 1997). However, the ^{13}C NMR spectrum showed, when compared with that of **3**, the splitting of three signals assigned to methine C-15, methyl C-36 and methylene C-13. This suggested that the isolated product comprised a mixture of isomers; this was confirmed by normal phase HPLC analysis which showed the presence of three partially overlapping signals, in a ratio of ca. 4:5:1. Coinjections revealed that the major compound coeluted with **3**; repeated injections allowed isolation of the second predominant compound, **4** (first eluted), as an oil ($[\alpha]_D = 0°$). It differed spectroscopically from **3** only by the ^{13}C NMR chemical shifts of two signals assigned to C-15 (78.28 instead of 78.26) and C-36 (23.60 instead of 23.56). Furthermore, the application of Mosher's ^1H method (Dale, Dull & Mosher, 1969; Othani, Kusumi, Kashman, & Kakisawa, 1991), using the *S*- and *R*-methoxy(trifluoromethyl)phenyl acetate (MTPA) derivatives of **4** (Fig. 2), allowed us to establish that the absolute configuration at C-15 was *S*, while it was found to be *R* in **3** (Metzger et al., 1993).



At this point of the work, two assumptions could be envisaged. Either three dihydroxy lycopaene isomers are involved in the acetalation of the aliphatic polyaldehyde, or, if we consider that only **3** occurs as a free compound in the alga, the other isomers are artificially formed during the hydrolysis treatment. In an attempt to solve this problem, model compounds were prepared by acetalation of methacrolein with pure dihydroxylycopaene **3**. Using *p*-toluene sulfonic acid as catalyst and molecular sieves to absorb the water formed (see Section 4), two acetals, **5** and **6**, were obtained in a ratio 7:93, respectively. NOE experiments established that isomers **5** and **6** differed by the orientation of the acetal proton H-1′ relatively to Me-

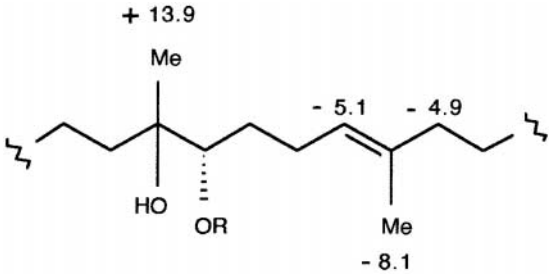
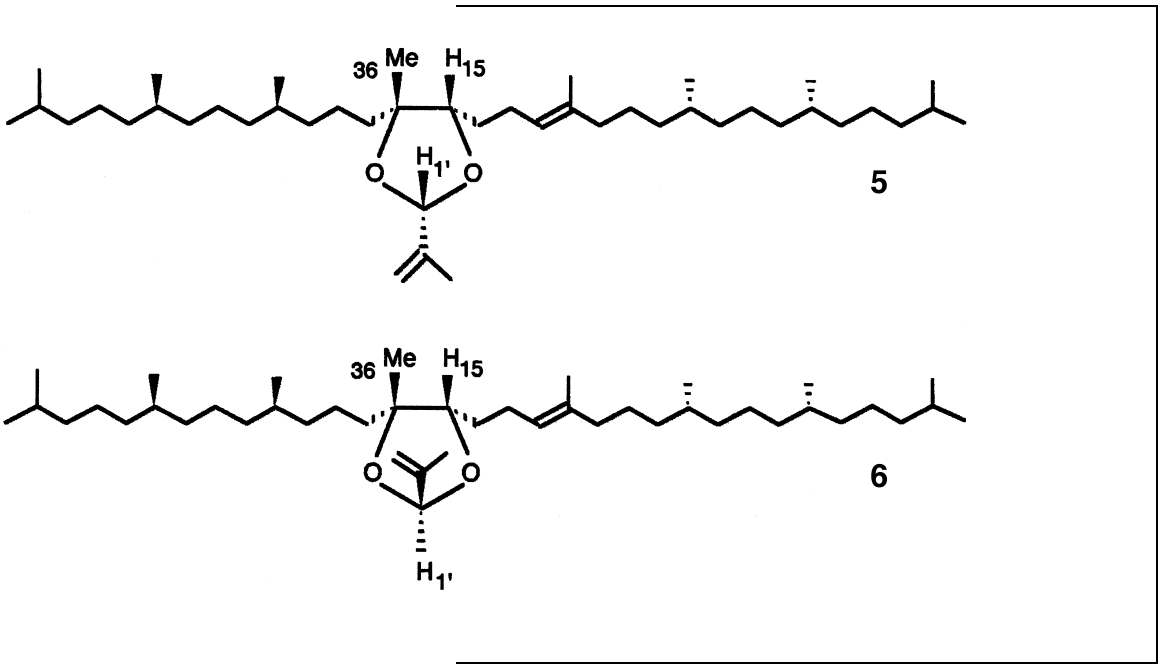


Fig. 2. $\Delta\delta_H$ values (Hz) from ^{1}H NMR data of MTPA esters of **4** (R = MTPA).

36 and H-15. Moreover, the absence of splitting of the signals ascribable to C-13, C-14, C-15 and C-36 in **6**, established that this acetal was not a mixture of isomers. By submitting this latter acetal to hydrolysis under the same conditions as those employed for APTP hydrolysis, and subsequently analyzing the crude product by HPLC, it appeared that **4** has been formed (16% of the whole). Thus, this result demonstrated that isomerization of **3** into **4** had occurred through the hydrolysis of methacrolein acetal **6**. With regard to the fact that acetal hydrolysis is known to occur with retention of configuration of vicinal diols, the present isomerization is, to our knowledge, unprecedented. This result, added to the non-occurrence of **4** as a free compound in the alga, strongly suggested that in the acetalation of the polyaldehyde network, only dihydroxy lycopaene **3** is involved.

figuration at C-15, it could be deduced that configuration is *S* at C-14, according to the general scheme of acid hydrolysis of epoxides. Moreover, since this diol obtained by hydrolysis of the epoxide and the natural dihydroxylycopaene **3** exhibits strictly identical ^{13}C NMR chemical shifts for all their carbons, it can be assumed that it has a similar configuration at C-14 (*S*) and C-15 (*R*). The observation in the ^{1}H and ^{13}C NMR spectra of the heptane extracted polymer of two sets of signals, A (major) and B (minor) (Table 2), could be interpreted as resulting from the existence of an isomerism related to the two possible configurations for the acetal carbon. This was confirmed by NOE experiments which clearly indicated that in B the acetal proton is on the same side of the acetal ring as Me-36 and H-15, while in A it is on the opposite side (Fig. 3). Moreover, the absence in the ^{13}C NMR spectrum of splitting of signals relative to C-13,14,15 and 36, both in A and B, argue for the formation of acetals from only one dihydroxy lycopaene. Therefore, it can be deduced that in A the acetal carbon is *S*, while in B it is *R*. Since the absolute configuration of the methine carbons 6, 10, 23, and 27 in **3** has already been determined as being *R* (Delahais & Metzger, 1997), the structure of the aliphatic polyaldehyde tetraterpenediol polyacetal was established as being **7** with absolute configurations 6*R*, 10*R*, 14*S*, 15*R*, 23*R*, 27*R*, 1′*S* for the predominant acetal group A and 6*R*, 10*R*, 14*S*,



2.1.6. Structure and absolute configuration

As described earlier (Delahais & Metzger, 1997), the dihydroxylycopaene obtained by acid hydrolysis of its naturally occurring parent epoxide exhibiting a *R*-con-

15*R*, 23*R*, 27*R*, 1′*R* for the minor one B. This structure does not take into account, however, a probable alternating arrangement, all along the normal chain, on the one hand of the aldehyde and acetal functions

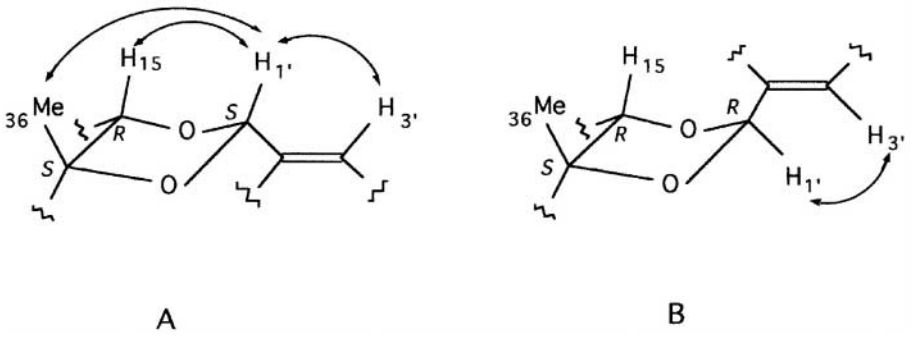


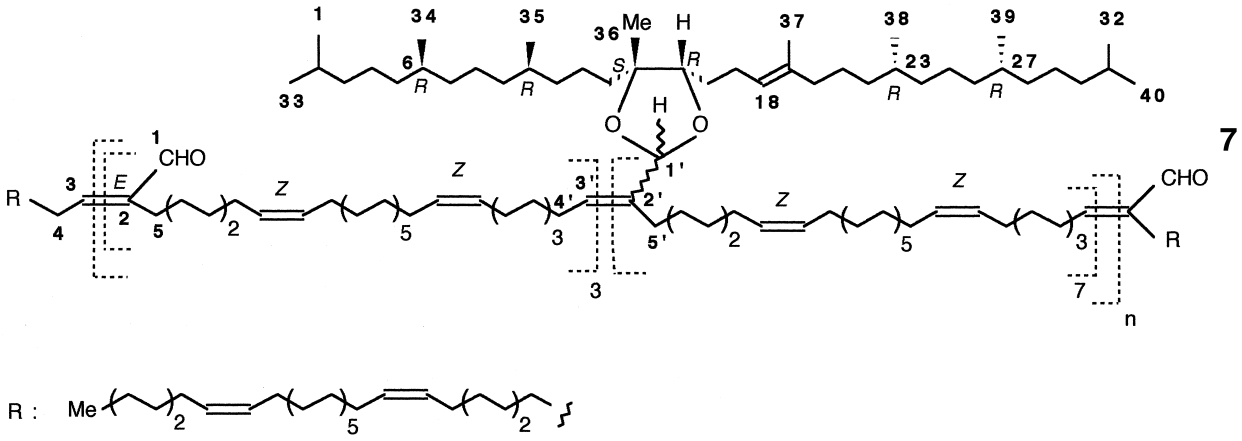
Fig. 3. Absolute configurations in acetal rings of APTP and NOEs.

and the other hand of the two acetal configurations. Moreover, as hypothesized in the case of **1** (Metzger et al., 1993), it is very likely that the polyaldehyde backbone is ended by the condensation with a monoaldehyde, such as a C_{32} aldehyde (group *R* in structure **7**).

2.2. Algaenan

2.2.1. Isolation

The residual biomass obtained after chloroform extraction was further extracted with chloroform–



The investigation of three other strains of the L race showed that APTP is a common polymer of the L chemical race of *B. braunii*. These strains originated from India (Kulavai lake, Metzger, Pouet, & Summons, 1997), Ivory Coast (another strain from Yamoussoukro lake) and Thailand (Khao Kho Hong reservoir, Metzger & Casadevall, 1987; Metzger, Allard, Casadevall, Berkaloff, & Couté, 1990); all exhibited lower amounts of APTP (heptane plus chloroform extractable polymers): 9, 6 and 7.5% of dry biomass, respectively. The only structural difference noticed from NMR investigations was noted for the Thailand strain: in this case, the *R*- and *S*-configurations of the acetal carbon were in almost equal proportions.

methanol in order to remove internal lipids. The subsequent chloroform–methanol extracted biomass (64% of the starting biomass) was then submitted to several successive acid hydrolyses with aqueous trifluoroacetic acid (TFA). Progressive increases in TFA concentration and in time duration were applied in order to avoid the formation of insoluble melanoidin-like polymers during algaenan isolation. These artifactual polymers were recently shown to be formed when a biomass containing polysaccharides and proteins is hydrolyzed with concentrated acids (Allard et al., 1997). The formation of such artifactual materials is frequently encountered when algal materials are treated with concentrated mineral acids such as phosphoric acid, to date currently used for algaenan isolation

(Berkaloff et al., 1983). The residue obtained through successive TFA hydrolyses (14.5% of starting biomass) was then submitted to a basic treatment, leading thus to the algaenan (12.3% of starting biomass). Furthermore, the combined chloroform and methanol filtrates which were recovered from the refluxes and washings performed on the algal residues after each acid hydrolysis, were concentrated under vacuum, giving an oily material (7.8% of the starting biomass), whose composition will be discussed below.

2.2.2. Chemical structure

The elemental analysis and the FTIR spectrum pointed to a very high aliphaticity for the algaenan (Table 1, Fig. 1C). For instance, the H:C ratio was similar to that of APTP and the FTIR spectrum showed bands of high intensity at 1465 cm^{-1} (CH_2 and Me asymmetric deformation vibration) and 720 cm^{-1} ($(CH_2)_n$ with $n \geq 4$). Moreover, a strong absorption at 1690 cm^{-1} associated with a small band at 2706 cm^{-1} indicated the presence of an aldehyde function conjugated with a carbon–carbon unsaturation. These IR argue for the existence of an aliphatic polyaldehyde network in the algaenan. However, the presence of bands at 1375 and 1365 cm^{-1}, indicated also the existence of some methyl branchings, but they were of lower intensity than in the case of APTP (Fig. 1A).

A 400°C 'off-line' pyrolysis of the algaenan was performed in order to obtain more structural information on this insoluble and chemically resistant material. The trapped pyrolysate, accounting for ca. 70% of the unheated algaenan, was separated by CC over alumina into heptane (74%), toluene (15%) and methanol (11%) fractions. The hydrocarbon fraction recovered from the heptane eluate was very complex as indicated by GC–MS analysis, the TIC trace showing numerous series of alkanes and alkenes from C_{13} up to C_{40}. Catalytic reduction of this fraction greatly simplified the GC trace (Fig. 4A). Then, it could be deduced that *n*-alkanes from C_{13} up to C_{33}, highly dominated (76% of the whole), with a maximum at C_{18}. *n*-Alkylcyclohexanes from C_{13} up to C_{23} (i.e. exhibiting normal chains from C_7 up to C_{17}, respectively) and terpenoids were the two other main series identified; they accounted for 9 and 15% of the reduced fraction, respectively. In the latter series, C_{14}, C_{15}, C_{16}, C_{18}, C_{19} (pristane), C_{20} (phytane), C_{21} and C_{40} (lycopane) were identified, the latter predominating. The toluene and methanol eluates were also examined by GC–mass spectrometry. However, they contained almost exclusively non-GC amenable compounds; only very minor amounts of trimethyl-6,10,14 pentadecan-2-one and of an unsaturated C_{40} ketone could be detected. Examination of these two eluates by IR, showed, however, that they were chiefly composed of unbranched compounds with strong absorption at 1465 cm^{-1} relative to that at 1375 cm^{-1}, a band at 720 cm^{-1} for polymethylenic chains and C=O absorptions in both spectra.

In a parallel experiment, APTP was pyrolyzed at 400°C, and the pyrolysate submitted to the same CC separation, as described above. GC–mass spectrometry of the reduced hydrocarbon fraction showed the presence of the same series of compounds. In this case, however, terpenoids highly dominated the chromatogram (Fig. 4B).

As already emphasized, the isolation of the algaenan involves successive hydrolyses so as to achieve a complete removal of saccharides. In addition, as shown by analysis of the oily material recovered from the refluxes and washings performed after each hydrolysis, substantial amounts of fatty acids and of a mixture of dihydroxy lycopaenes **3** and **4** were obtained. These released compounds accounted for 5.4 and 3.6% of the chloroform–methanol extracted biomass, respectively. The C_{16}–C_{32} fatty acid fraction was mostly composed of monounsaturated (82% of the total fraction) and saturated (16%) compounds with oleic (38.5% of the whole), $C_{28:1}$ (27%), palmitic (12%) and $C_{30:1}$ (7%) acids as major compounds.

All these spectroscopic, pyrolytic and analytical data suggested that the algaenan of the Yamoussoukro strain is similar to that of the A race of *B. braunii* (Gelin et al., 1994a), i.e. a highly condensed or reticulated form of the aliphatic polyaldehyde **1**. For instance, in the 400°C pyrolysis experiment, the same series of unbranched hydrocarbons and of cyclohexyl compounds were identified in both cases. These latter hydrocarbons were thought to originate from the cleavage of the C–C bond allylic to the olefin conjugated to the formyl group, followed by a cyclization, during the pyrolysis (Gelin et al., 1994a). The main difference observed between these two algaenans is the presence of terpenoids, as minor constituents, in the pyrolysate of the algaenan of the Yamoussoukro strain.

3. Discussion

The isolation of an aliphatic polyaldehyde tetraterpenediol polyacetal further substantiates *B. braunii* as a rich source of original lipids. This very high M_r soluble biopolymer of a novel type, isolated from the L race, is structurally related to the aliphatic polyaldehyde **1** previously isolated from the A race of *B. braunii* (Metzger et al., 1993). The similarity of the aliphatic polyaldehyde skeleton of these polymers, extractable by solvents of low polarity, suggests a common biosynthetic pathway. This pathway would involve a *n*-C_{32} diunsaturated α,ω-dialdehyde as a monomeric unit in the condensation–polymerization process leading to the formation of high M_r polymers.

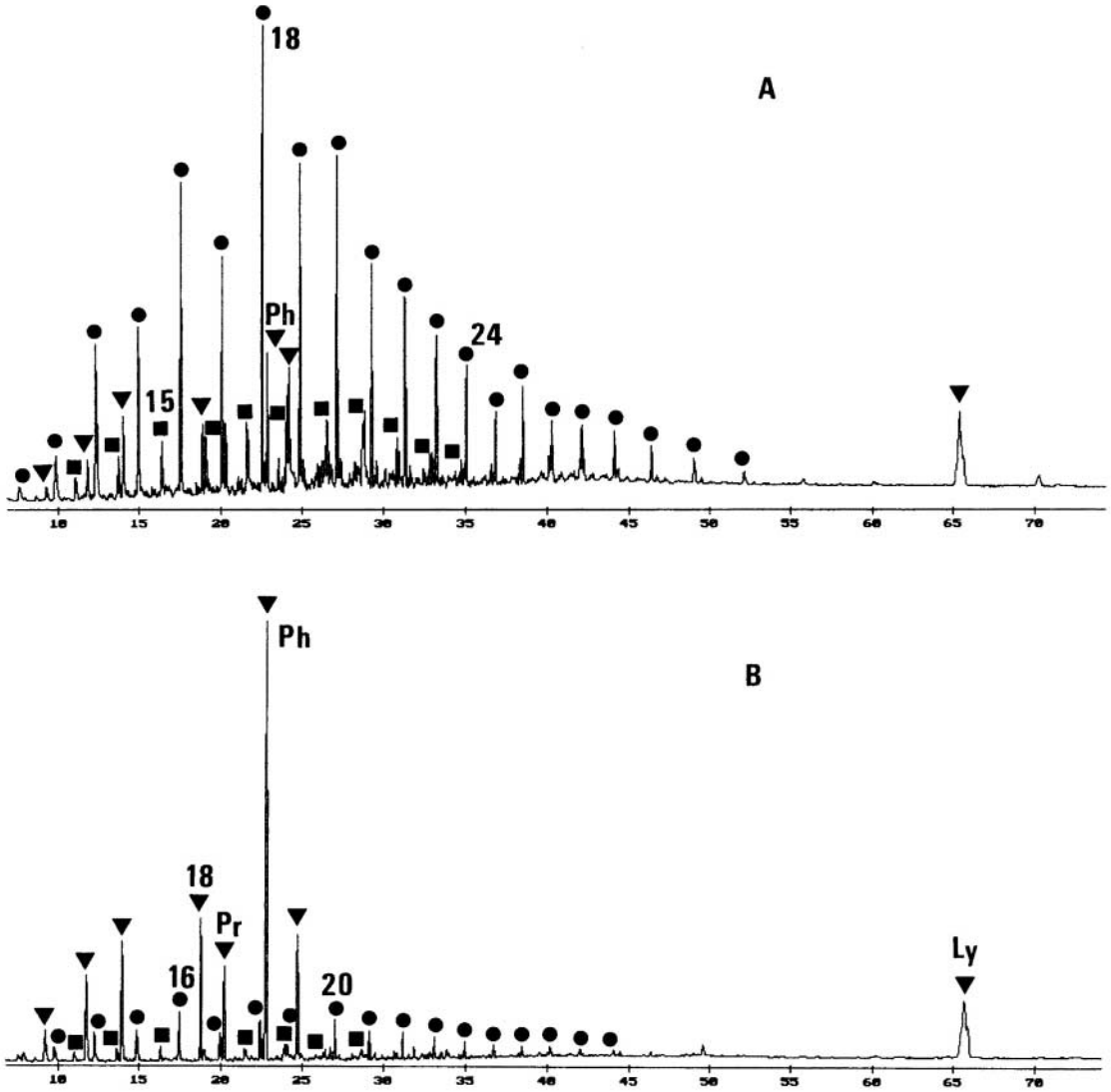


Fig. 4. Chromatograms of hydrogenated hydrocarbon fractions obtained from 400°C 'off-line' pyrolyses; A: algaenan L, B: APTP; ▼: terpanes, ●: *n*-alkanes, ■: *n*-alkylcyclohexanes, Ph: phytane, Pr: pristane, Ly: lycopane.

APTP produced by the L race is distinguishable, however, by the multi-incorporation of a tetraterpenediol, derived from lycopadiene, by acetalation of ca. seven aldehyde functions out of ten. The two possible configurations of the acetal carbon exist in APTP, in a ratio which depends on the strain origin. The proportion of the most sterically hindered acetal group B (Fig. 3) in the polymer, varies between 33 and 50% of the total acetal groups, which is very high when compared with the ratio observed in the synthesis of the methacrolein acetal models; in this latter case the most hindered compound is only formed in a low level of 7%. Furthermore, APTP is the first acetal-containing lipid isolated from *B. braunii*; an original ketal has been, however, recently described from the B race (Okada, Matsuda, Murakami, & Yamaguchi, 1996). This latter compound, named botryoxanthin, is a member of a new class of carotenoids; it results from the condensation of echinenone with a dihydroxy tetramethylsqualene.

By using the new isolation procedure recently described (Allard et al., 1997), a chemically resistant polymer has been obtained, from the L race, in a yield of 12.3% of the dry biomass. It must be noted that a much higher yield (33%) was previously observed, although the analyzed algae were in the same physiological state, through the usual isolation process (Derenne et al., 1989). Moreover, whereas the latter material was considered as essentially formed via the condensation of tetraterpenoid units derived from lycopadiene (Derenne et al., 1990), the IR and pyrolytic investigations performed on the present material demonstrate that tetraterpenoids are only minor constituents. In fact, the algaenan structure is predomi-

nantly based on an aliphatic polyaldehyde network, as clearly shown from IR data and GC–mass spectrometry analysis of the pyrolysate. The identification, among the pyrolysis products of a series of *n*-alkylcyclohexanes, occurring in substantial amount, and also identified in the pyrolysate of the algaenan of the A race, suggests a rather close similarity between the algaenans of the two races. Both are very likely to be of an aliphatic polyaldehyde type.

Our data, specially the recovery of dihydroxylycopaenes during the acid hydrolyses with TFA, strongly suggest that the algaenan of the L race derives from the hydrolysis of a highly condensed or reticulated, insoluble, form of APTP. The presence of some terpenoids in the polymer network as revealed by the GC–mass spectrometry analysis of the pyrolysate, would result from an incomplete hydrolysis of a reticulated form of APTP, some acetal groups being inaccessible to an aqueous catalyst in a highly aliphatic environment. Another explanation for the presence of tetraterpenoids which cannot be excluded, is the ether linkage of some lycopadiene derived units to the polyaldehyde network. Nevertheless, even if such a condensation exists, our results demonstrate that ether functions are not the major cross-linking groups in the algaenan of the L race, as previously proposed (Derenne et al., 1990; Gelin et al., 1994b). The polymeric nature of the algaenan results essentially from the formation of C=C bonds (conjugated to the formyl groups), during the condensation–polymerization of the *n*-C_{32} α,ω-dialdehyde, via an aldolization–dehydration pathway. The non-hydrolysis of the acetal functions during the previous isolation of a chemically resistant material from the same strain of the L race of *B. braunii*, could be related to unfavorable experimental conditions. Concentrated mineral acids would be unsuitable reagents, as suggested by the lack of reaction of concentrated sulfuric acid, observed here, with APTP.

4. Experimental

4.1. General

CC: silica gel (70–230 mesh), activity II alumina. TLC: silica gel 60 PF. Normal phase HPLC: silica gel 4 μm diam. (300 × 4.6 mm). IR: CCl_4; films on KBr discs; solids dispersed in KBr. NMR: $CDCl_3$, TMS as int. standard; ^1H: 250 MHz, ^{13}C: 62.5 MHz. $CHCl_3$ was stabilized with 0.6% EtOH and THF with 2,6-di-*t*-butyl-*p*-cresol. The strain investigated originated from a lake in Yamoussoukro, Ivory Coast (Metzger & Casadevall, 1987; Metzger et al., 1990); it was cultured under air-lift conditions (air enriched by 1% CO_2) and continuous illumination as previously described (Metzger, Berkaloff, Casadevall, & Couté, 1985).

4.2. Extraction and isolation of APTP 7

Three-week old cultures (10 l) were filtered through a 10 μm Monyl cloth; the algal concentrate was frozen in liquid N_2 and lyophilized. The dry biomass (11.9 g) was extracted at room temperature with 500 ml heptane, 2 × 1 h, and then with 500 ml $CHCl_3$, 18 h. The combined heptane extracts were concd under red. pres., giving a resin-like material (2.95 g). After dissolution in 60 ml $CHCl_3$, an equivalent vol. of MeOH was added, leading to the precipitation of an elastic material which was transferred into 60 ml $CHCl_3$ and redissolved by stirring. The precipitation–dissolution process was repeated twice and the final yellowish rubber (APTP) was kept as soln in $CHCl_3$ (60 ml), at −24°C for further analyses. The polymer yield, 1.57 g, was estimated by weighing an aliquot, dried under vacuum. The $CHCl_3$ extract, concd under red. pres. to a residual vol. of ca. 50 ml, was treated as the heptane extract; it furnished 0.24 g of polymer kept in $CHCl_3$ soln as described above. A 7 day-aged culture (3 l), in active growth phase, was also investigated; from 1.32 g of dry biomass, 90 mg of purified APTP were isolated from the heptane extract.

4.3. Size-exclusion HPLC

Analyses were performed as previously described (Metzger et al., 1993), with an instrument fitted with a differential refractometer, using an Ultrastyragel column (300 × 7.8 mm; linear functional range: M_r 500–8×10^6), and THF as mobile phase. Calibrations were performed with standards of polybutadienes from Scientific Polymer Products. Precision on retention vol. was estimated to be ±0.04 ml.

4.4. Hydrolysis

An aliquot of APTP (370 mg) in $CHCl_3$ soln (11.8 ml) was dild with 20 ml of THF and reacted under a N_2 atmosphere with 1 ml of conc. HCl for 18 h at room temperature and with stirring. Then, the reaction mixt. dild with 100 ml $CHCl_3$ was washed with H_2O until neutrality. The organic phase, dried (Na_2SO_4) was concd under red. pres. to a residual vol. of ca. 20 ml. After addition of an equiv. vol. of MeOH, a rubbery material was removed and purified by dissolution–precipitation, as described above, yielding the hydrolyzed polymer (53% of native APTP). The remaining $CHCl_3$–MeOH phases, free of polymer, were combined and concd under vacuum, yielding an oil. Purification by silica gel TLC, using heptane Et_2O MeOH (29:25:1), afforded a colorless oil exhibiting the

same R_f (0.47) as dihydroxy lycopaene **3**, previously isolated from this alga.

4.5. Dihydroxylycopaenes **3** and **4**

Analysis of this oil by normal-phase HPLC, using heptane–THF (25:1) at 1 ml min^{-1} showed the presence of three peaks, partially overlapping (R_t: 23, 25 and 26; respective ratio 4:5:1). The second eluted compound coeluted with a standard of dihydroxylycopaene **3** (Delahais & Metzger, 1997). Repeated inj. allowed isolation of 21 mg of the first eluting compound, **4**. Dihydroxy lycopaene **4**. $[\alpha]_D$: 0°C (*c* 1.08, heptane). CI(NH_3)MS (probe) *m/z* (rel. int.): 610 $[C_{40}H_{80}O_2 + NH_4]^+$ (100), 575 $[M + H - H_2O]^+$ (8). IR (CCl_4) ν_{max}: 3620, 3560, 2950, 2920, 2860, 1465, 1375, 1365 and 1090 cm^{-1}. ^1H NMR: δ 5.15 (H-18, t, $J = 7$ Hz), 3.41 (H-15, dd, $J = 10.1$, 2.0 Hz), 2.21 (H-17a, m), 2.10 (H-17b, m), 1.95 (H-20, t, $J = 7.2$ Hz), 1.62 (Me-37, s), 1.60–1.00 (overlapping CH and CH_2), 1.16 (Me-36, s), 0.88–0.83 (other methyls). ^{13}C NMR: δ 136.6 (C-19), 123.6 (C-18), 78.3 (C-15), 74.7 (C-14), 40.1 (C-20), 39.4 (C-3, C-30), 37.8, 37.5, 37.4, 37.3, 36.8, 36.5, 32.8, 32.8, 32.7, 31.3, 28.0 (C-2, C-31), 25.5 (C-21), 25.1 (C-17), 24.8 (C-4, C-29), 24.5, 23.6 (C-36), 22.7 (C-33, C-40), 22.6 (C-1, C-32), 20.8 (C-12), 19.8 (C-34, C-35, C-38, C-39), 16.0 (C-37).

4.6. Methacrolein acetals **5** and **6**

Acetals were prepared according to a previous published method (Roelofsen & Van Bekkum, 1972). A mixt. of dihydroxylycopaene **3** (0.2 mmol), methacrolein (0.8 mmol), cyclohexane (4 ml), *p*-toluene sulfonic acid (0.5 mmol) and molecular sieve powder type 5 Å (1 g) was stirred at room temp. for 2 days. Then, Et_3N (0.5 ml) was added to quench the reaction; the molecular sieves were removed by centrifugation and washed with pentane (2×10 ml). The combined supernatants were washed with 10% aq. NaOH (2×10 ml) and H_2O (3×10 ml). The organic phase was dried (K_2CO_3) and concd under red. pres. The crude product was submitted to silica gel (15 g) CC and eluted with heptane Et_2O (23:2, 200 ml). The concd eluate was then fractionated over silica gel TLC using heptane Et_2O (24:1) affording methacolein acetals **5** (R_f 0.50; 1.5 mg) and **6** (R_f 0.40; 21 mg). Methacrolein acetal **5**. CI(NH_3)MS (probe) *m/z* (rel. int.) 645 $[M + H]^+$ (3), 593 (5), 576 $[M - C_4H_6O_2 + NH_4]^+$ (100), 557 (17). IR (CCl_4) ν_{max}: 3070, 2940, 2910, 2850, 1465, 1370, 1360, 1090, 970, 910 cm^{-1}. ^1H NMR: δ 5.30 (H-1′, s), 5.15 (H-3′a, br s), 5.12 (H-18, t, $J = 7.0$ Hz), 4.99 (H-3′b, br s), 3.61 (H-15, dd, $J = 9.7$, 3.0 Hz), 2.21 (H-17a, m), 2.10 (H-17b, m), 1.95 (H-20, t, $J = 7.4$ Hz), 1.74 (Me-4′, s), 1.60 (Me-37, s), 1.60–1.00 (overlapping CH, CH_2 and Me-36), 0.88–0.83 (other methyls). Methacrolein acetal **6**. CI(NH_3)MS (probe) *m/z* (rel. int.) 645 $[M + H]^+$ (9), 593 (9), 576 $[M - C_4H_6O_2 + NH_4]^+$ (100), 557 (17). IR (CCl_4) ν_{max} identical to those observed for methacolein acetal **5**. ^1H NMR: δ 5.21 (H-1′, s), 5.20 (H-3′a, br s), 5.13 (H-18, t, $J = 7.0$ Hz), 5.05 (H-3′b, br s), 3.59 (H-15, dd, $J = 9.8$, 3.0 Hz), 2.21 (H-17a, m), 2.10 (H-17b, m), 1.95 (H-20, t, $J = 7.4$ Hz), 1.75 (Me-4′, s), 1.60 (Me-37, s), 1.60–1.00 (overlapping CH, CH2 and Me-36), 0.88–0.83 (other methyls). ^{13}C NMR: δ 142.4 (C-2′), 136.4 (C-19), 123.3 (C-18), 115.8 (C-3′), 103.7 (C-1′), 86.0 (C-15), 81.5 (C-14), 40.0 (C-20), 39.4 (C-3, C-30), 37.7, 37.5, 37.4, 37.4, 37.3, 37.3, 36.7, 36.1, 32.8, 32.8, 32.8, 32.7, 28.0, 25.4, 25.3, 24.8 (C-4, C-29), 24.5, 24.5, 22.7, 22.6, 22.4 (C-36), 20.7 (C-12), 19.8, 19.7, 15.9 (C-37), 15.9 (C-4′).

4.7. Preparation of MTPA esters of **4**

MTPA esters of dihydroxylycopaene **4** were obtained as previously described (Dale et al., 1969; Othani et al., 1991), using *R*(−) and *S*(+)-MTPA chlorides.

4.8. Hydrolyzed APTP and ozonolysis

FTIR (film) ν_{max}: 3000, 2920, 2850, 2706, 1690, 1465, 1400, 1370, 720 cm^{-1}. ^1H NMR: δ 9.35 (s), 6.43 (t, $J = 7.4$ Hz), 5.35 (t, $J = 6.5$ Hz), 5.33 (t, $J = 6.5$ Hz), 2.34 (dt, $J = 7.3$, 7.4 Hz), 2.22 (t, $J = 7.1$ Hz), 2.00 (m), 1.26 (br). Treatment by ozone of the hydrolyzed polymer, followed by oxidative degradation of the polyozonide by refluxing in H_2O_2–HCO_2H and then derivatization of the recovered diacids as dimethyl esters were as previously described (Metzger et al., 1993). Esters were determined by GC–MS (CP Sil 5CB capillary column, temp. prog. from 140 to 260°C at 4°C min^{-1}) and comparisons with authentic standards.

4.9. Algaenan isolation

The $CHCl_3$-extracted biomass was extracted for 18 h at room temp. with $CHCl_3$–MeOH (2:1, 500 ml). The dry residue (7.64 g) was refluxed in 150 ml aq. 2 N TFA for 2×3 h, 18 h and then in 4 N TFA for 15 h. Between each reflux, the residue was filtered and washed with 150 ml of H_2O. PhOH–H_2SO_4 assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) did not show the presence of sugars in the filtrate from the last washing. The acid hydrolyzed residue was then refluxed successively in 200 ml MeOH for 1 h, and in 200 ml $CHCl_3$ for 1 h (yield of acid hydrolyzed residue, 14.5% of starting biomass). Finally, a basic treatment was performed in 2-methoxyethanol (73 ml)–H_2O (10 ml)–KOH (1 g) at 110°C for 1 h. The algae-

nan was filtered, washed with H_2O until neutrality and refluxed successively in H_2O, MeOH and $CHCl_3$.

4.10. Analysis of lipids released during isolation of algaenan

The $CHCl_3$ and MeOH solns obtained after acid (TFA) hydrolyses were combined and concd under red. pres., affording 930 mg of lipidic material. This was refluxed in MeOH–HCl (generated by addition of a few drops of AcCl) for 1 h, concd under vacuum and separated by silica gel CC using heptane and increasing amount of Et_2O as eluent. The fr. eluted with heptane:Et_2O 19:1, essentially contained fatty acid Me esters; they were identified by GC–MS (CP Sil 5CB, temp. prog. from 180 to 300°C at 4°C min^{-1}). Dihydroxylycopaenes (**3** + **4**) were found to be the major compounds from the fraction eluted with Et_2O.

4.11. Algaenan and APTP pyrolyses

Pyrolyses at 400°C (1 h) under a 15 ml min^{-1} He flow and subsequent fractionation of the pyrolysates (trapped in $CHCl_3$ at −15°) by alumina CC were carried out as previously described (Largeau, Derenne, Casadevall, Kadouri, & Sellier, 1986). Heptane eluates (10 mg) were hydrogenated in the presence of Rh/C 5% in 10 ml heptane at 70°C, under 20 atm. of H_2, for 18 h. The catalyst was removed by centrifugation and the supernatants concd under red. pres. GC–MS analyses were performed using a CP Sil 5 CB capillary column, temp. prog. 100 to 300°C at 4°C min^{-1}.

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