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OCCURRENCE OF A RESISTANT BIOPOLYMER IN THE *L* RACE OF *BOTRYOCOCCUS BRAUNII*

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Abstract—A resistant biopolymer, PRB *L*, occurs in the outer walls of the *L* race of *Botryococcus braunii* It accounts for a larger fraction of the total biomass than PRB *A* and *B* do in the *A* and *B* races The PRB *L* structure is mainly based on long (up to C_{30}) saturated, isoprenoid hydrocarbon chains probably linked by ether bridges Most of these isoprenoid chains exhibit regular head-to-tail linkages Therefore, PRB *L* does not belong to the same family as PRB *A* and *B*, the structures of which are based on long, normal hydrocarbon chains The only hydrocarbon produced by the *L* race algae is a lycopadiene so a relationship between hydrocarbon and PRB structure can be considered in this *L* race as has already been demonstrated for the *A* race.

INTRODUCTION

Biopolymers exhibiting a high resistance to drastic chemical treatments occur in the outer walls of some microalgae, including lichen phycobionts, [1–17] and fungi [2, 18–21] This type of material was also observed in higher plant cuticles [22] In previous studies [13, 23–25], we noticed that a high content of resistant material (9–12% of the dry wt of the total biomass) occurs in the *A* and *B* races of the hydrocarbon-rich microalga *Botryococcus braunii* * These quantities are substantially higher than those reported for other algae, e.g. 0 3% in *Prototheca wickerhamii* [28], 0 6% in *Chlorella fusca* [3] and 1 to 4% in *Pediastrum duplex* [29] The resistant biopolymers of these two races of *B braunii*, respectively termed PRB *A* and PRB *B*, have the same structure based upon long $(CH_2)_n$ chains, *n* up to 31, with ether bridges and ester functions sterically protected within the tridimensional network built up by the $(CH_2)_n$ chains. PRB *A* and *B* were shown to play a major role in the fossilization of *B. braunii* and in the formation of kerogens (Torbanites) with a high oil potential [24].

Recently [30], two strains of *B braunii* collected from the Ivory Coast and Thailand were shown to produce exclusively one hydrocarbon identified as the lycopa-14(E), 18(E)diene **1** The above strains exhibit the typical morphology and colony organization of *B braunii* but their average cellular size is smaller: 8 to 9 μm by 5 μm compared to 13 μm by 7 to 9 μm in the *A* and *B* race algae. These strains correspond to a third race which has been named *L*.

The aim of the present work was to test the occurrence of a resistant biopolymer in this third race, to determine its structure and to compare it to those of PRB *A* and PRB *B*

RESULTS AND DISCUSSION

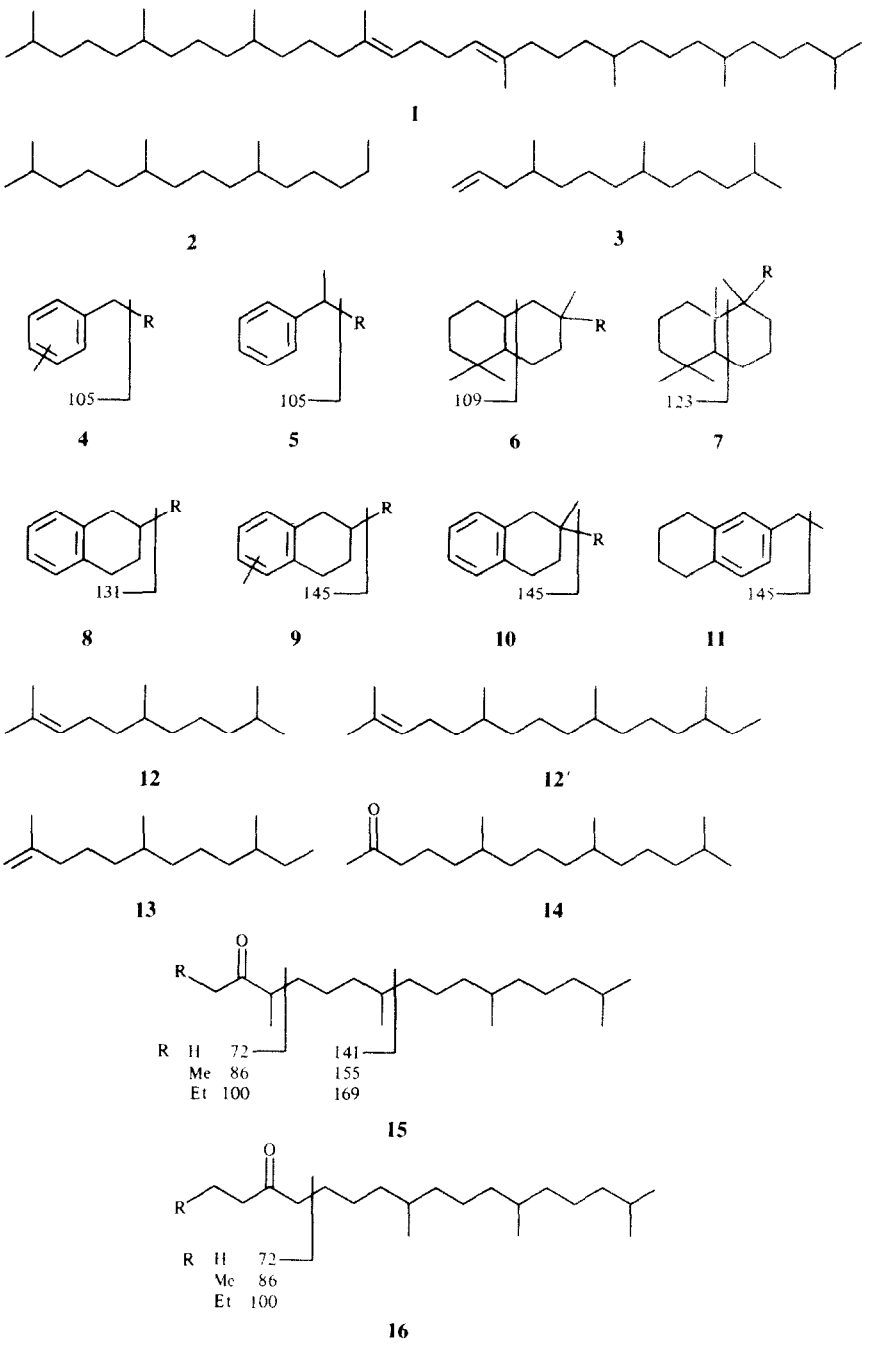
Occurrence, abundance and localization of resistant biopolymer

The algal biomass obtained at the end of batch cultures was submitted to the successive treatments already used for isolating PRB *A* and PRB *B* from the *A* and *B* race algae Two strains (Yamoussoukro from Ivory Coast and Songkla Nakarin from Thailand) of *L* race were treated in this way and, in both cases, a large amount of organic resistant residue was obtained This resistant biopolymer accounts for a still larger fraction of the total biomass (24 to 26% of the initial biomass dry wt) when compared to PRB *A* and PRB *B* (*ca* 10%) and was termed PRB *L*.

In addition, two cultures of the Ivory Coast strain but of different physiological stages were analysed. In the 'young' culture, the algae were harvested at the end of the exponential part of the growth curve (13 days of culture) and, in the 'old' one, during the prolonged stationary stage (42 days). PRB *L* was isolated from each sample and, whereas it accounts for 17% of the dry biomass of the 'young' algae, it represents 33% of the 'old' ones. A similar evolution has been noted for another green microalga, *Pediastrum duplex*, where the resistant polymer accounts for 1 to 4% of the biomass depending on the stage of the culture [29].

Ultrastructural observations using transmission electron microscopy showed that PRB *L* is located in the outer walls as are also PRB *A* and PRB *B*. After PRB *L*

*The *A* and *B* races of *B braunii* show a similar general morphology but are characterized by the different nature of their hydrocarbons [26, 27], the *A* race produces odd carbon numbered, C_{23} to C_{31}, dienic and trienic, unbranched hydrocarbons, the *B* race producing isoprenoid C_{30} to C_{37} hydrocarbons termed botryococcenes



isolation, these walls still retain their typical shape whereas all the other cell constituents have been entirely destroyed by the drastic chemical treatments (Fig 1)

Chemical structure of PRB L

Spectroscopic studies The FTIR spectrum of PRB *L* exhibits the same general features as those of PRB *A* and *B**, suggesting the occurrence of (i) long $(CH_2)_n$ alkyl chains (absorption at 720 cm^{-1}) (ii) oxygenated functions (carbonyl and/or carboxyl, ether and hydroxyl) and (iii) a low level of *cis*-unsaturations However, some quantitative differences are noticed between PRB *A* and *B* on the one hand and PRB *L* on the other hand, especially regarding the relative abundance of methyl and methylene groups In the PRB *L* spectrum, the band at 2930 cm^{-1} (CH_2 stretching vibration) exhibits a shoulder at 2960 cm^{-1} (Me stretching vibration) which was not detected in PRB *A* and *B* spectra the former band has a higher width at half height in the case of PRB *L* (*ca* 100 cm^{-1} instead of 45 cm^{-1}) In addition, the intensity ratio (2 8 instead of *ca* 4 for PRB *A* and *B*) of the bands at 1450 cm^{-1} (CH_2 and Me asymmetric deformation vibration) and 1375 cm^{-1} (Me symmetric deformation vibra-

*See ref [13] for a complete discussion of PRB *A* and *B* FTIR spectra

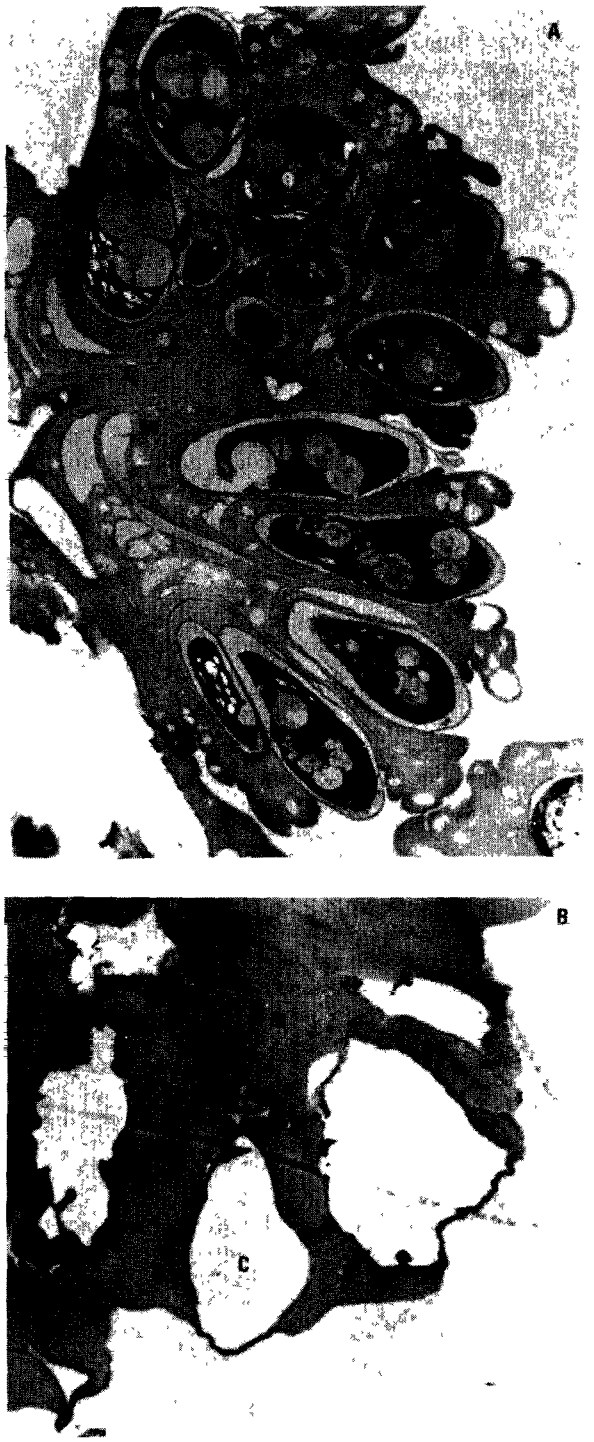


Fig 1A Untreated colony of *Botryococcus braunii*, *L* race The thick outer walls form the matrix (M) of the colony Part of each cell is occupied by a chloroplast crowded with starch (S) Some lipidic inclusions (I) are observed in numerous cells (× 7200). Fig 1B After the chemical treatments required for isolating PRB *L*, all colony constituents, apart from the outer walls have entirely disappeared The original locations of the cells, now emptied, however, are still well recognizable (C) The localization of PRB *L* in the outer walls allows for the conservation of the characteristic shape of the matrix (M) of the colony (× 7200)

tion) also indicates a more important contribution of Me groups relative to CH_2 in PRB *L* than in PRB *A* and *B*.

The high resolution ^{13}C NMR (high-power dipolar decoupling, cross-polarization, magic angle spinning) spectrum of PRB *L* at 25.1 MHz is also similar to those of PRB *A* and *B*. This spectrum is dominated by a peak at 30 ppm (CH_2 groups) exhibiting two shoulders at δ15 and 40. However, in agreement with FTIR observations, the shoulder at δ15 (Me groups) is relatively more important in PRB *L* The second shoulder (branched carbons) is also slightly more pronounced in the PRB *L* spectrum The other peaks of the PRB *L* spectrum have a low intensity and correspond to (i) unsaturated carbons (*ca* 130 ppm) which are assigned to aliphatic double bonds according to the FTIR spectrum which does not show any aromatic band (ii) ester functions (170 ppm) (iii) ether and/or hydroxyl functions (between 60 and 90 ppm) From a variable contact time experiment, we could estimate as 9% the level of unsaturated carbons. This percentage is similar to those encountered for PRB *A* and *B*.

The above findings suggested close structural relationships between PRB *L* and the resistant biopolymers isolated from the other two races of *B. braunii* but with some differences regarding the amount of methyl groups. To specify this level, we used a single-pulse sequence with high-power dipolar decoupling, the frequency of the carbons being 75 MHz. This sequence allows a better detection of the mobile carbons and especially those belonging to rotating methyl groups [31–34]. The aliphatic regions of the spectra of PRB *B* and PRB *L* recorded with this sequence exhibit important differences: numerous peaks on both sides of the main peak at 30 ppm in PRB *L* spectrum clearly show that its structure is substantially more branched and more methylated than the one of PRB *A* and *B* (Fig 2)

Pyrolysis. The above differences were confirmed in a parallel study carried out using Curie point-pyrolysis (770°) coupled with gas chromatography (Py-GC) and with gas chromatography-mass spectrometry (Py-GC-MS) [35] The pyrochromatogram of PRB *A* is domi-

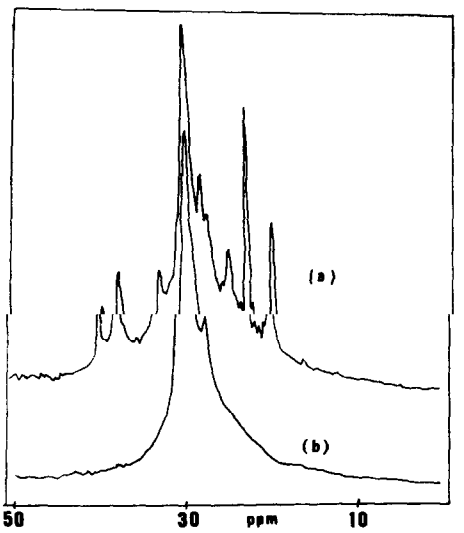


Fig 2 Solid state ^{13}C NMR spectra at 75 MHz of PRB *L* (a) and PRB *B* (b) with single-pulse sequence after 1200 scans

nated by a regular series of doublets (assigned to *n*-alkanes and *n*-1-alkenes) corresponding to the cracking of long $(CH_2)_n$ chains In sharp contrast, no homologous series of *n*-hydrocarbons was detected from PRB *L* under Curie point-pyrolysis conditions. In fact, identification of the main components of the pyrolysate by Py-GC-MS indicated that structure of PRB *L* could be mainly based on long isoprenoid chains instead of normal ones

However, from Py-GC-MS we could only get information about the major weakly polar components of the pyrolysates To detect less abundant products and identify polar compounds, we carried out pyrolyses under a helium flow with subsequent fractionation of the trapped products and GC-MS analysis Such a complete analysis of pyrolysate was previously performed on PRB *A* and *B* [24, 25] so it permitted a precise comparison of PRB *L* with both PRB *A* and *B* The material was first heated at 300° The weight loss at this temperature is higher than that in the case of PRB *A* and *B* (Table 1), however it is mainly due to highly volatile products, the trapped medium volatility compounds only accounting for a low percentage of the initial organic matter. The residue of this first pyrolysis, after being thoroughly extracted, was heated at 400° This temperature was shown, in the case of PRB *A* and *B*, to achieve an efficient cracking while minimizing secondary reactions This second pyrolysis also results in the case of PRB *L* in a large weight loss corresponding almost entirely to trapped products (Table 1)

The elemental composition and the FTIR spectrum of the final insoluble residue demonstrate that important structural modifications take place during 400° pyrolysis. The H/C atomic ratio (0.37 instead of 1 70 in the 300° residue and 1 86 in the unheated material [35]) and the low intensity of the CH_2 and Me FTIR bands confirm the loss of long hydrocarbon chains observed by Py-GC-MS In addition, the FTIR spectrum exhibits an important band at 1600 cm^{-1} corresponding to the aromatization of the residue The chromatogram of the products trapped during the 400° pyrolysis is very complex Accordingly the pyrolysate was first separated into three fractions by column chromatography. hydrocarbons (63%), ketones (21 5%) and polar compounds (15 5%) This distribution is similar to those observed with PRB *A* and *B*

Analysis of the hydrocarbon fraction obtained by pyrolysis

The total hydrocarbon fraction is complex so it was further separated into three subfractions on TLC using $AgNO_3$–silica gel

Subfraction I The highest R_f subfraction I is exclusively composed of saturated hydrocarbons Its GC trace shows two main series of peaks According to GC-MS, series *a* which dominates the short chain region, is assigned to regular isoprenoid alkanes from C_{13} to C_{21}, with head-to-tail linkages; it culminates at C_{18} (compound **2**) Series *b*, corresponding to *n*-alkanes from C_{12} to C_{30}, becomes more intense than the previous one after C_{19} No odd/even predominance could be noted in this series, whose maximum is also at C_{18} The intensity of this maximum is *ca* 20% of the C_{18} compound of series *a*. Two minor series *c* and *d* are observed, from C_{17} to C_{28} Their mass spectra are respectively characterized by a base peak at *m/z* 68 and 82 probably due to a saturated ring (C_5 and C_6) substituted by an alkyl chain This alkyl chain is probably branched as shown by the lack of a regular decrease of the other fragments but a precise structure (isoprenoid or not) could not be established because of the very low abundance of these series. Traces of mono-methylcyclohexanes, substituted by a C_8 to C_{18} alkyl chain have been characterized by the base peak of their mass spectra *m/z* 97 The relative position of the two substituents was not determined

Subfraction II This subfraction was not completely separated from the other two subfractions Its chromatogram is complex and does not clearly exhibit different homologous series By GC-MS, the main products have been identified as C_9 to C_{24} isoprenoid alkenes For a given carbon number, several isomers of these alkenes occur They differ from each other, on the one hand by the location of the double bond (which could not always be specified on the exclusive basis of the mass spectrometry data) and, on the other hand by the position of the methyl groups, that is by the linkage between C_5 units (head-to-tail, head-to-head or tail-to-tail) According to its mass spectrum, the major compound of this subfraction is a regular head-to-tail alkene, 4,8,12-trimethyl-1-tridecene **3**

Two series of alkylbenzenes from C_{14} to C_{29} were characterized in this subfraction The base peak of their mass spectra is respectively, *m/z* 105 or 106 and 119 or 120 These peaks result from the preferential cleavage of the alkyl chain As shown for compounds **4** and **5**, the base peak at *m/z* 105, 106 may be assigned to two structures differing by the position of the methyl group (on the ring or in the alkyl chain α to the ring). For the base peak at *m/z* 119 and 120, two methyl groups are involved In the two series the intensity of the fragments of higher masses than the base peak does not decrease regularly indicating that the alkyl chain is branched

Some bicyclic compounds from C_{14} to C_{27} were also detected in this subfraction They correspond to decalin,

Table 1 Pyrolysis of PRB *A* and PRB *L* (percentages are calculated with respect to initial organic matter)

	Weight loss (%)		Trapped products (%)		Highly volatile products (%)	
T	PRB *A**	PRB *L*	PRB *A**	PRB *L*	PRB *A**	PRB *L*
300°	11	33 5†	4 5	3 5†	6 5	30
400°	85	58 5‡	57	58 3‡	28	0 2

*Similar results were obtained with PRB *A* and *B* [24, 25]
†Average of eight experiments
‡Average of four experiments

tetralin and naphthalene units substituted by an alkyl chain Decalins are characterized by their important molecular ion C_nH_{2n-2} and a base peak at *m/z* 109 or 123 depending on the number of methyl groups on the A ring as shown for compounds **6** and **7** These structures probably originate from the cyclization of regular isoprenoid compounds with head-to-tail linkages On this basis, the relative positions of the methyl groups and of the alkyl chain which can be considered to establish the possible structures (e g. **6** and **7**) are limited. The mass spectra of tetralins exhibit an important molecular ion C_nH_{2n-8} and a base peak at *m/z* 131, 145 or 159 (see compounds **8** to **11**). Some of these tetralins may result from a partial aromatization of decalins, as for example **9** from **6** The complete aromatization of these structures will lead to substituted naphthalenes This type of product is detected by an important molecular ion C_nH_{2n-12} and a base peak at *m/z* 141 (no Me), 155 (one Me either on the ring or in the chain α to the ring) or 169 (two Me) corresponding to the preferential cleavage of the alkyl chain, β to the aromatic unit, this cleavage is similar to the one observed with alkylbenzenes, which leads to base peaks at *m/z* 91, 105 or 119 depending on the number of methyl groups.

Subfraction III The complex chromatogram of this lowest R_f subfraction is dominated by three peaks A, B and C of relative intensities 0 6, 0.55 and 1. In addition, a regular series is observed from C_{14} to C_{28}, maximum at C_{18}, the relative intensity of this maximum is 0 2 with respect to C.

A, B and C correspond to C_{14}, C_{15} and C_{16} regular head-to-tail isoprenoid alkenes, respectively With a base peak at *m/z* 70 and important fragment ions at *m/z* 83 (rel int 0.60) and 111 (0 48), the mass spectrum of A can be assigned to 2,6,10-trimethyl-2-undecene **12**. A C_{20} compound, **12**, of similar mass spectrometry features was also detected but its contribution to the chromatogram is minor.

The mass spectrum of B can be described as follows. *m/z* (rel. int), 210 [M^+] (0 7), 111 (17), 97 (17), 83 (31), 70 (70), 56 (100) and assigned to 2,6,10-trimethyl-1-dodecene **13** Less abundant compounds between C_{11} and C_{21} are also characterized by a base peak at *m/z* 56 due to the same McLafferty rearrangement and should therefore have a similar structure.

C also dominated the intermediate subfraction and was identified as 4,8,12-trimethyl-1-tridecene **3** Its presence in both subfractions II and III reflects the overlapping of the TLC bands due to the complex composition of the hydrocarbon fraction generated on PRB *L* 400° pyrolysis

The homologous series observed between C_{14} and C_{28} in subfraction III is also due to branched alkenes just as the other compounds contributing to the chromatogram Most of them are probably isoprenoid with different positions of the double bond and nature of the linkage between C_5 units (head-to-head, head-to-tail or tail-to-tail)

Analysis of carbonyl fraction

The main compound of the carbonyl fraction is a regular head-to-tail C_{18} isoprenoid ketone **14**, 6,10,14-trimethyl-2-pentadecanone The corresponding homologous series of methyl ketones can be observed from C_{11} to C_{19} but the only other important contribution is due to the C_{13} methylketone (which differs from **14** by a C_5 unit). Three C_{20} to C_{22} ketones are important in the chromatogram. Their mass spectra are respectively characterized by (i) a $[M]^+$ at *m/z* 296 (C_{20}), 310 (C_{21}) and 324 (C_{22}) (ii) a base peak at *m/z* 72, 86 and 100 (iii) an important fragment at *m/z* 141, 155 and 169. The base peaks are due to a McLafferty rearrangement but two structures are possible according to the mass spectrometry data as shown on compounds **15** and **16**. The carbonyl fraction appears therefore to be essentially made up of isoprenoid ketones

Comparison with PRB A and PRB B

A similar study carried with PRB *A* and *B* showed that these two biopolymers lead to the same products on 400° pyrolysis: *n*-alkanes, *n*-1-alkenes and *n*-ketones were released with chain lengths up to C_{31}. Alkylcyclohexanes and alkylbenzenes were also identified but, in both cases, the alkyl chain is normal In fact, no branched compound is detected in the pyrolysates of PRB *A* and *B* On the contrary, a quite low contribution of *n*-alkanes was observed in the saturated hydrocarbon subfraction (I) isolated from the 400° pyrolysate of PRB *L* with a relative intensity of the *n*-alkane series of only 40% with respect to the regular isoprenoid alkane series *a* Furthermore, no *n*-compound was identified either in the other two hydrocarbon subfractions or in the carbonyl fraction. In these subfractions the major constituents are isoprenoid As the contribution to the total hydrocarbon chromatogram of subfractions I and III is similar while subfraction II is less abundant, it appears clearly that *n*-alkanes are minor compounds (less than 20% of the total hydrocarbons).

The above observations therefore indicate that the structure of PRB *L* is mainly based on long isoprenoid hydrocarbon chains while normal chains only afford a low contribution The predominant isoprenoid hydrocarbons are characterized by regular head-to-tail linkages

An important question in the biosynthesis of these PRB is the occurrence of a possible relationship between the biopolymers and the hydrocarbons produced by the alga. For the *A* race, the hydrocarbons are long (up to C_{31}) unbranched alkadienes and alkatrienes and the structure of PRB *A* is based on long (up to C_{31}), normal hydrocarbon chains Therefore a relationship between the hydrocarbons and PRB *A* was suggested in this case by the structural observations This was confirmed by feeding experiments indicating the involvement of a common precursor (oleic acid) and of several common initial steps (elongation to very long chain fatty acyl derivatives) in the pathways leading to hydrocarbons and to PRB in the *A* race [36, 37] On the contrary, the hydrocarbons produced by the *B* race (botryococcenes) are isoprenoid while PRB *B* also exhibits a structure based on long normal hydrocarbon chains. Therefore there should exist no relationship between hydrocarbons and PRB in the *B* race. This was also confirmed by feeding experiments. botryococcenes originate from the terpenic pathway (labelling during feeding experiments with mevalonic acid) while PRB *B* derive from oleic acid [36, 37].

The third race described in this paper exclusively produces a C_{40} isoprenoid hydrocarbon (lycopadiene) and we have shown that most of the compounds released on 400° pyrolysis of PRB *L* are isoprenoid. Thus, a relationship between the hydrocarbon and the resistant biopolymer structures may exist, as in the case of the

algae of the *A* race. Indeed, it is of interest to note that all the regular head-to-tail compounds (hydrocarbons and ketones) dominating the 400° pyrolysate of PRB *L* have a chain length of less than C_{22}. When the chain was longer than this, the nature of the linkages was difficult to establish and the hydrocarbons are probably not regular. The lycopadiene **1** produced by the alga is a symmetrical C_{40} hydrocarbon resulting from the condensation of two phytyl (regular head-to-tail) units with a central tail-to-tail linkage. Therefore, if the PRB *L* structure is chiefly based on the cross-linking of units derived from lycopadiene **1** the regular isoprenoids generated by pyrolysis should admit, as observed here, less than 22 carbon atoms. We can also examine the structure of the released ketones. They probably result from the cleavage of ether bridges which were cross-linking the long hydrocarbon chains which build up the tridimensional network. If we consider the lycopadiene structure, it is likely that the cross-linkages would involve the carbons initially bearing the double bonds, C_{14} and C_{15} (or C_{18} and C_{19} because of the symmetry). During pyrolysis, this should lead to ketone **14** (cross-linkage on C_{14}) or to ketones **15** (cross-linkage on C_{15}) with a chain length up to C_{22}. The carbonyl fraction is actually dominated by these compounds and we therefore suggest that structure **15** is more likely than **16** for the C_{20} to C_{22} ketones. The above statement confirms the relationship between the lycopadiene 1 and PRB *L*.

As Torbanites are formed by selective preservation of PRB *A* and *B* during fossilization of the algal biomass, kerogens resulting from the selective preservation of PRB *L* should also exist. These kerogens should exhibit a similar morphology as Torbanites (fossil colonial algae) but their structure should be mainly isoprenoid.

EXPERIMENTAL

The description of the samples collected from Ivory Coast and Thailand, the isolation of unialgal strains, the growth conditions of laboratory cultures were as previously reported [30]. Ultrastructural observations, isolation of PRB *L*, spectroscopic studies (FTIR and solid-state ^{13}C NMR) pyrolysis under a He flow, fractionation and identification by GC-MS of pyrolysis products were carried out as previously described [13, 23–25, 30].

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