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Six novel tetraterpenoid ethers, lycopanerols B-G, and some other constituents from the green microalga *Botryococcus braunii*

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Abstract

Six novel tetraterpenoid ethers, lycopanerols B-G, were isolated from lipidic extracts of the green microalga *Botryococcus braunii* (L race), along with a series of phytyl esters and α- and β-tocopherols. The structures of the compounds were determined by means of spectral analyses including 2D NMR techniques. A biogenetic relationship is proposed between lycopanerols and lycopadiene, the acyclic diunsaturated tetraterpenoid hydrocarbon synthesized by the alga.

Keywords: Botryococcus braunii; Chlorophyceae; Alga; Phytyl esters; Tocopherols; Lycopanerols; Tetraterpenoids; Ether lipids

1. Introduction

In the course of our analyses of the lipids extracted from the green microalga *Botryococcus braunii*, we recently isolated a series of terpenoid ether lipids of a new type which we termed lycopanerols. These compounds are synthesized from strains belonging to the L race of this alga, i.e. which are characterized by the production of lycopadiene **1**, an acyclic tetraterpenoid hydrocarbon (Metzger and Casadevall, 1987; Metzger et al., 1990). Lycopanerols A, **2**, the first-described members of this new family of natural products, comprise a *trans*-tetrahydrofuran (THF) containing lycopane connected by an ether bridge to a second lycopane, containing a tetrahydropyran ring (THP) in turn connected by another ether bridge to a very long *n*-alkenyl chain with a *cis* or a *trans* geometry for the mid-chain unsaturation (Metzger and Aumelas, 1997). We now report the isolation and the structural determination of six novel series of lycopanerols, **3–8**, characterized by the presence in their structures of at least one lycopane moiety, together with a series of phytyl esters and α- and β-tocopherols. Moreover, some strains of the A and B races of *B. braunii* have been also investigated for their content in phytyl esters and tocopherols and compared with the strains of the L race.

2. Results and discussion

The extraction of the Yamoussoukro strain with heptane yielded the external lipids which are stored in the outer walls of the alga. After removal of a rubbery polymer from this crude extract (Berthéas et al., 1999), the remaining lipids were fractionated and purified according to the procedures described in Section 3. In addition to the recently reported lycopanerols A, **2**, (Metzger and Aumelas, 1997), six novel series of tetraterpenoid ethers were isolated: lycopanerols B-G (**3–8**) and α- and β-tocopherols. Compound E was purified as an acetate derivative **6b**, and compounds D and G

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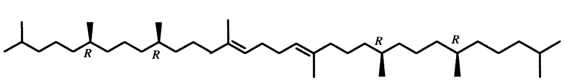
were analyzed as acetate derivatives **5b** and **8b**, respectively. From the internal lipids extracted with chloroform–methanol, a series of phytyl esters was also isolated comprising fatty acyl moieties with very long aliphatic chains.



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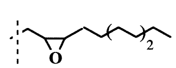

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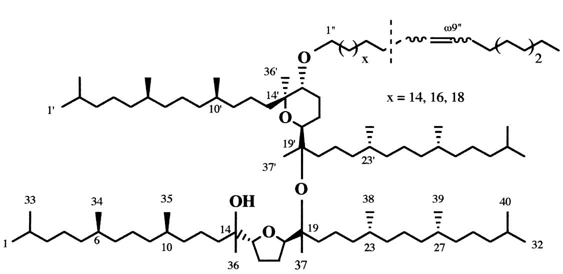

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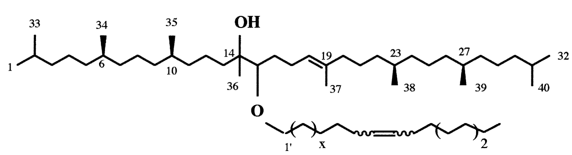
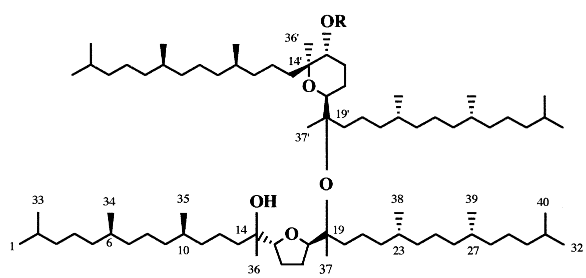


4

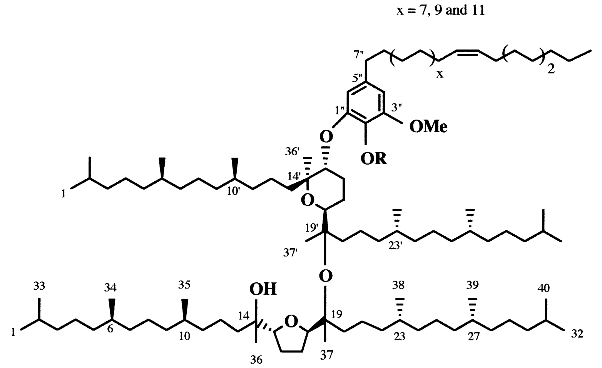
x = 14, 16, 18



5a R = H

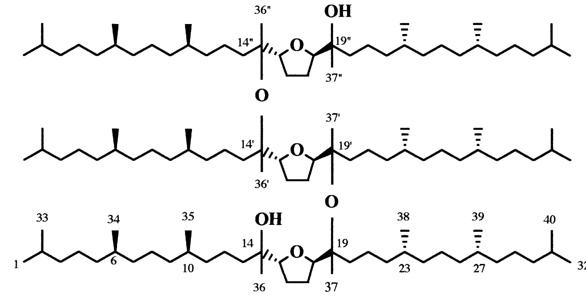
5b R = Ac

x = 7, 9 and 11



6a : R = H

6b : R = Ac



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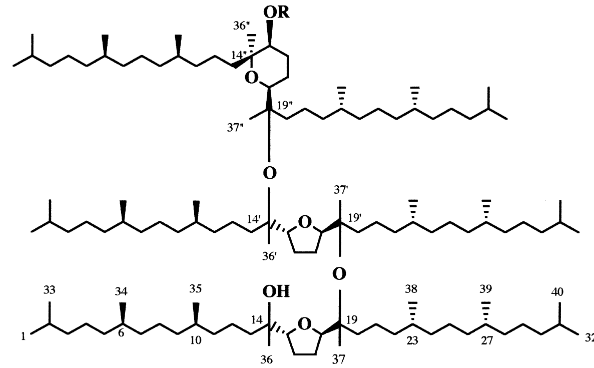


8a R = H

8b R = Ac

2.1. Phytyl esters

Phytyl esters were recovered from the internal lipids and they accounted for 0.7% of the dry biomass. The IR spectrum of the mixture showed a prominent band for C=O bond of esters at 1730 cm^{-1}. The ^{1}H and ^{13}C NMR spectra displayed signals for a trisubstituted carbon–carbon double bond at δH 5.34 (*m*) and δC 118.23 (*d*) and 147.57 (*s*) in the phytyl moiety, a (*Z*) disubstituted unsaturation at δH 5.35 (2H, *t*) and δC 129.93 (allylic carbons at δC 27.25) in the acyl moiety and an ester function at δC 173.94. Saponification of this lipid mixture generated phytol and a series of fatty acids, identified for their methyl esters by GC–MS. The very long chain monounsaturated acids dominated: *n*-$C_{30:1}$ (2%), $C_{28:1}$ (69%), $C_{26:1}$ (9%). Substantial amounts of oleic (11%), stearic (2%) and palmitic (7%) acids were also observed. The occurrence of phytyl esters in microalgae has been sparsely reported. To the best of our knowledge they were concerned with a marine dinoflagellatte (Withers and Nevenzel, 1977) and six chlorophytes (Cranwell et al., 1990). In these two cases, polyunsaturated fatty acids dominated, with chain lengths up to C_{22} and C_{20}, respectively. The marked predominance of very long chain fatty acids observed in the present study for phytyl esters again illustrates the richness of *B. braunii* in such fatty acids and in the three races of the alga (Metzger et al., 1990). However, no trace of phytyl esters was detected in one strain of the B race nor in five strains of the A race (see Section 3). The absence of phytyl esters in the A and B races of *B. braunii* precludes the possibility of artifacts with the Yamoussoukro strain, by esterification of phytol with free fatty acids during the isolation process.

2.2. Tocopherols

Tocopherols α and β detected in the lipids of the Ivorian strain were in the relative ratio of 77/23 and together accounted for 0.2% of the dry weight. The structures were confirmed by mass spectroscopy analyses and comparisons of the ^{13}C NMR data with those reported in the literature (Matsuo and Urano, 1976). Tocopherols, which prevent oxidation of various lipids, are considered as common compounds in a large number of microorganisms and especially in microalgae (Lewin, 1974; Fried et al., 1982; Ratledge and Wilkinson, 1988). During parallel analyses of other strains of *B. braunii* we identified α- and β-tocopherols in another strain of the L race (isolated from Kulavai lake in India, see Section 3), α-tocopherol in one strain of the B race (see Section 3) but no tocopherol in five isolates of the A race. The absence of vitamin E in the A race could be related to the synthesis of various non-isoprenoid alkenylphenols by these algae (Metzger and Largeau, 1999), sometimes in very high amounts, which can also play the role of antioxidant (Tyman, 1996).

2.3. Lycopanerols

The structural determination of lycopanerol A, (**2**), has been already described. However, the reported NMR data is essentially concerned with the hydrogenated derivatives (Metzger and Aumelas, 1997). The results of the unsaturated product are therefore included in Table 1 to allow comparisons with those

Table 1
NMR data of lycopanerols **2**[a] and **3**[a]

Position	δC **2**	δH (mult., *J*, Hz) **2**	δC **3**	δH (mult., *J*, Hz) **3**
13	38.07	1.40 (*m*)	38.07	1.40 (*m*)
14	72.94		72.95	
15	85.75	3.71 (*dd*, 9.4, 5.8)	85.75	3.71 (*dd*, 9.5, 5.7)
16	26.44	1.78 (*m*), 1.74 (*m*)	26.51	1.75 (*m*)
17	27.31	1.85 (*m*)	27.20	1.85 (*m*)
18	83.67	3.86 (*dd*, 6.6, 6.6)	83.66	3.88 (*dd*, 5.8, 5.8)
19	79.37		79.36	
20	38.68	1.35 (*m*), 1.54 (*m*)	38.68	1.35 (*m*), 1.55 (*m*)
36	24.60	1.18 (*s*)	24.60	1.18 (*s*)
37	19.60	1.13 (*s*)	19.58	1.13 (*s*)
13′	42.38	1.47 (*m*)	42.37	1.48 (*m*)
14′	79.97		79.96	
15′	84.98	3.10 (*dd*, 10.2, 2.6)	85.02	3.10 (*dd*, 10.0, 2.5)
16′	25.80	1.84 H eq(*m*), 1.46 H ax (*m*)	25.82	1.82 H eq.(*m*), 1.50 H ax.(*m*)
17′	28.25	1.64 (*m*)	28.25	1.62 (*m*)
18′	77.74	3.54 (*dd*, 9.7, 1.7)	77.73	3.54 (*dd*, 8.6, 2.2)
19′	80.83		80.84	
20′	41.93	1.40 (*m*), 1.56 (*m*)	42.92	1.38 (*m*), 1.53 (*m*)
36′	20.68	1.11 (*s*)	20.67	1.11 (*s*)
37′	21.26	1.05 (*s*)	21.25	1.04 (*s*)
1″	70.24	3.50 (*dt*, 8.8, 6.2)	70.57	3.52 (*dt*, 8.9, 6.7)
		3.23 (*dt*, 8.8, 6.6)		3.24 (*dt*, 8.9, 6.7)
2″	30.42	1.50 (*m*)	30.42	1.50 (*m*)
3″	26.44	1.30 (*m*)	26.43	1.30 (*m*)
4″ − ω12″	29.36-29.71	1.20–1.30 *br*	29.36-29.71	1.20–1.30 *br*
ω8″, ω11″	27.31, Δ *cis*	2.01 (*m*)	27.92	1.50 (*m*)
	32.72, Δ *trans*	1.97 (*m*)		
ω9″, ω10″	129.97, Δ *cis*	5.35 (*m*)	57.36, *cis*	2.90 (*t*, 4.1)
	130.44, Δ *trans*	5.38 (*m*)		
ω4″ − ω7″	29.36−29.71	1.20–1.30 *br*	29.36-29.71	1.20–1.30 *br*
ω3″	32.02	1.30 (*m*)	31.76	1.30 (*m*)
ω2″	22.79	1.30 (*m*)	22.76	1.30 (*m*)
ω1″	14.22	0.88 (*t*, 6.7)	14.21	0.88 (*t*, 6.7)

[a] Other resonances of the lycopane moieties, (i) CH_3: δC at 19.70–19.96, 22.64, 22.71, δH at 0.88 (overlapping signals); (ii) CH_2: δC at 20.82, 20.98, 21.45, 24.83–24.53, 37.36–37.94, 39.44, δH at 1.20–1.30 and (iii) CH: δC 28.00, 32.71–33.05 δH 1.40–1.50.

of other compounds of the lycopanerol family. The main structural information was obtained from 2D NMR experiments. Thus, in an HMBC experiment, the long-range connectivity observed between C-19 and H-18′ demonstrated the linkage of the THF and the THP units via the C-19-O-C-19′ ether bridge. Moreover, a ROESY experiment (Fig. 1) allowed to establish that the THF has a *trans* stereochemistry and that the large substituents at positions 14′, 15′ and 18′ on the THP ring are equatorially oriented.

HRFAB(NBA–LiCl) MS of compounds **3** showed the presence of two $[M + Li]^+$ ions at *m/z* 1668.7032 and 1640.6709, suggesting the presence of two homologous compounds, $C_{112}H_{220}O_6$ and $C_{110}H_{216}O_6$, respectively, differing one from the other by two methylene units. The adduct ions were in a ratio of ca. 86:14, respectively. The LRFAB(NBA–LiCl) mass spectrum showed a peak at *m/z* 1188 indicative of the ion $[C_{80}H_{157}O_4 + Li]^+$ and another one at *m/z* 1060 revealing the presence of the ion $[C_{72}H_{141}O_3 + Li]^+$. These mass data established on the one hand the linkage of two tetraterpenoids, likely via an ether bridge, and on the other hand the linkage of one tetraterpenoid with a C_{32} aliphatic chain (the lower homologue ion corresponding to the linkage of a C_{40} and a C_{30} was not clearly detected from the mass spectrum).

The IR spectrum was similar to the one of lycopanerols A, with bands for hydroxyl group (3580 cm^{-1}) and C–O bonds (1095 and 1070 cm^{-1}). The ^1H and ^{13}C NMR spectra of **3** were also very similar to those of lycopanerols A (**2**) and suggested that **3** was a mixture of two homologous epoxides (Table 1). Indeed, in the ^{13}C NMR spectrum of **3**, besides the nine signals in agreement with nine oxygen-bearing carbons as in **2** (four quaternary, four methine and one methylene), a new signal at δ 57.36 (methine) replaced the resonances of the olefinic carbons at δ 129.97 (Δ *cis*) and 130.44 (Δ *trans*) observed in **2**. Moreover, the ^{13}C chemical shift values of the carbons α to the epoxide group, 27.92, established that the epoxide is *cis* (Bascetta and Gunstone, 1985). Confirmation of the presence of an epoxide was given by the ^1H NMR spectrum showing

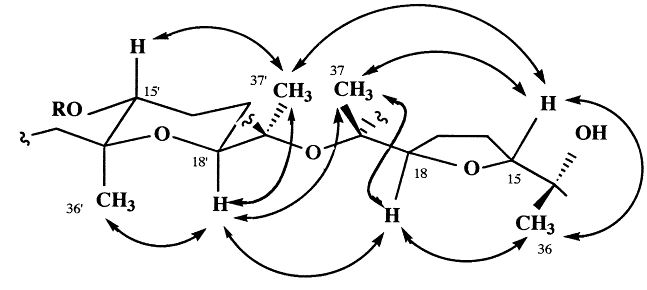


Fig. 1. Relative stereochemistry in **2** (R = *n*-alkenyl) and **5b** (R = Ac) and selected dipolar interactions.

a triplet for two protons at δ 2.90. Furthermore, the epoxidation of the parent compounds **2** (a mixture dominated by compounds with a *cis* double bond in the normal chain) with *m*-CPBA afforded essentially *cis* epoxides. The latter showed spectroscopic properties identical to those of the natural epoxides, thus establishing structure **3** for lycopanerols B. Not a single natural analogous compound with a *trans* epoxide function was isolated.

Lycopanerols C were found to be a mixture of six homologous compounds. The HRFAB(NBA–LiCl) MS yielded the following molecular formulae: $C_{72}H_{142}O_2$, $C_{70}H_{138}O_2$ and $C_{68}H_{134}O_2$, in a ratio of ca 48:35:17. The IR spectrum showed bands for hydroxyl group (3570 cm^{-1}, C–O bonds (1150, 1090 and 1060 cm^{-1}) and olefins (3000 cm^{-1}) in a *trans* configuration (970 cm^{-1}). Moreover, the EI-mass spectrum of the trimethylsilyl derivatives showed an ion at *m/z* 341 $[C_{18}H_{36}OSiMe_3]^+$, consistent with a hydroxyl at C-14 on a lycopane moiety. These data suggested the linkage of a tetraterpenoid with a C_{28}, C_{30} or C_{32} hydrocarbon chain, very likely via an ether bridge. The ^{13}C NMR spectrum (Table 2) showed three signals for three oxygen-bearing carbons, one quaternary (C-14), one methine (C-15) and one methylene (C-1′). It also displayed four signals for olefinic carbons, two ascribable to a three substituted double bond, δ at 124.06 (C-18, methine) and 135.85 (C-19, quaternary) and the other two to two mid-chain unsaturations, δ at 129.92 (Δ *cis*) and 130.38 (Δ *trans*). These configurations were confirmed by the chemical shift values of their respective allylic carbons: 27.23 (Δ *cis*) and 32.63 (Δ *trans*). The relative intensities of these peaks indicated a *cis*/*trans* ratio of ca 7:3. The ^1H NMR spectrum displayed three signals for three protons bound to two oxygen-bearing carbons: a triplet at δ 3.04 (H-15) and two doublets of triplets (at δ 3.62 and 3.49) assigned to the two diastereotopic protons H-1′ (Table 2 and HMQC experiment). A ^1H–^1H COSY experiment confirmed the existence of the pattern: -C(Me) = CH-CH$_2$-CH$_2$-CH(OR)-C(Me)OH-. Moreover, the ozonolysis of lycopanerols C, followed by the reductive cleavage of the ozonide, furnished *n*-nonanal and trimethyl-6,10,14-pentadecan-2-one, thus establishing that unsaturations were located at C-ω9′ and C-18. So, lycopanerols C were determined to be mono unsaturated lycopane, bearing a hydroxyl group at C-14, and linked via an ether bridge at C-15 to a C_{28}, C_{30} or C_{32} *n*-alkenyl chain comprising a *cis* or a *trans* unsaturation at ω9′, as shown in **4**.

Lycopanerol D was shown to have the molecular formula of $C_{80}H_{158}O_5$ by an HRFAB–MS analysis. The IR spectrum showed the presence of hydroxyls with bands at 3620 and 3560 cm^{-1}. The existence of a *trans*-THF containing lycopane was readily recognized by comparison of the ^1H [δ at 3.72 (H-15) and 3.88

Table 2
NMR data of lycopanerols **4**

Position	δC	δH (mult., J, Hz)
1,32	22.73	0.88
2,31	28.00	1.40–1.50
3, 30	39.42	1.20–1.30
4, 29	24.82	1.20–1.30
5, 28	37.33	1.20–1.30
6, 27	32.83	1.40–1.50
7, 26	37.48[a]	1.20–1.30
8, 25	24.51,24.53	1.20–1.30
9, 24	37.44[a]	1.20–1.30
10, 23	32.86, 32.73	1.40–1.50
11, 22	37.86, 37.39	1.20–1.30
12	20.84	1.20–1.30
13	36.75	1.30 (*m*)
14	75.01	
15	86.42	3.04 (*t*, 5.7)
16	31.02	1.50 (*m*)
17	25.35	2.30 (*m*)
18	124.06	5.12 (*t*, 5.5)
19	135.85	
20	40.04	1.95 (*t*, 7.4)
21	25.45	1.20–1.30
33, 40	22.64	0.88
34, 39	19.76	0.88
35, 38	19.76	0.88
36	23.90	1.14 (*s*)
37	16.02	1.60 (*s*)
1′	73.24	3.62 (*dt*, 8.7, 6.4)
		3.49 (*dt*, 8.7, 6.3)
2′	30.50	1.52 (*m*)
3′	26.31	1.30 (*m*)
4′ - ω12′	29.20-29.80	1.20–1.30 *br*
ω8′, ω11′	27.23 Δ *cis*	2.00 (*m*)
	32.63 Δ *trans*	2.05 (*m*)
ω9′, ω10′	129.92 Δ *cis*	5.35 (*t*, 4.9)
	130.38 Δ *trans*	5.38 (*t*, 7.2)
ω4′ − ω7′	29.20–29.80	1.20–1.30
ω3′	31.95	1.20–1.30
ω2′	22.71	1.20–1.30
ω1′	14.12	0.88

[a] Assignments may be reversed.

Table 3
NMR data of lycopanerol acetate **5b**[a]

Position	δC	δH (mult., J Hz)	HMBC[b]
13	38.00	1.45 (*m*)	15
14	72.94		13, 15, 36
15	85.79	3.72 (*dd*, 5.7, 9.5)	18, 36
16	26.52	1.80 (*m*)	15, 18
17	27.19	1.90 (*m*)	15, 18
18	83.70	3.88 (*dd*, 6.9, 6.9)	37
19	79.51		20, 37, 18′
20	38.59	1.30 (*m*)	18
36	24.59	1.18 (*s*)	15
37	19.70	1.14 (*s*)	18
13′	41.91	1.35–1.45 (*m*)	15′, 36′
14′	78.43		13′, 15′, 36′
15′	77.93	4.79 (*dd*, 11.3, 2.1)	36′, OCOC$\underline{H}_3$
16′	27.73	1.82 (H eq., *m*); 1.60 (H ax.,*m*)	18′
17′	26.46	1.75 (*m*)	18′
18′	77.31	3.56 (*br. d*, 8.3)	37′
19′	81.39		18′, 37′
20′	42.00	1.35–1.45 (*m*)	19′, 37′
36′	21.06	1.18 (*s*)	15′
37′	21.36	1.08 (*s*)	18′
O$\underline{C}$OCH$_3$	170.18		15′, OCOC$\underline{H}_3$
OCO$\underline{C}$H$_3$	21.46	2.01 (*s*)	

[a] Resonances of other protons and carbons from the lycopane moieties are similar to those of the corresponding atoms in **2** (Table 1).
[b] Protons correlating with carbon resonance.

(H-18)] and ^{13}C [72.94 (C-14), 85.79 (C-15), 83.70 (C18) and 79.51 (C-19)] NMR data, (Table 3), with those observed in **2** and **3**. In the CH-O region the ^{1}H NMR spectrum of the acetate derivative **5b** also displayed a doublet of doublets at δ 4.79 attributed to the proton bound to the acetate-bearing carbone, H-15′ in axial configuration ($J = 11.3$, 2.1 Hz), and a broad doublet at δ 3.56 ($J = 8.3$ Hz) assigned to H-18′. The COSY, HMQC and HMBC spectra allowed to assign all the signals relative to the THP ring. A ROESY experiment confirmed the *trans* stereochemistry of the THF ring and revealed, on the one hand, that Me-36′ and H-18′ were axially oriented and, on the other hand, that H-15/Me-36, H-18/Me-37 and H-18′/Me-37′ were *gauche* (Fig. 1). Thus, the structure of lycopanerol D was characterized as *trans*-THF containing lycopane connected at C-19, through an ether bridge, with C-19′ of a THP containing lycopane, as shown in **5a**.

Lycopanerols E, analyzed as acetate derivatives **6b**, appeared to be a mixture of three compounds, which displayed three adduct ions by HRFAB(NBA–LiCl)MS, compatible with three homologous compounds $C_{116}H_{218}O_8$, $C_{118}H_{222}O_8$ and $C_{120}H_{226}O_8$; they were in the relative ratio: 53:40:7, respectively. In the LRFAB-mass spectrum the presence of an ion at m/z 1188 $[C_{80}H_{157}O_4 + Li]^+$ suggested the existence in lycopanerols E of two ether-linked tetraterpenoid moieties. The IR spectrum showed a hydroxyl absorption at 3560 cm^{-1} and a carbonyl band at 1760 cm^{-1} for an acetoxy group substituting an aromatic ring (1500 cm^{-1}). The ^{1}H and ^{13}C NMR spectra (Table 4) showed resonances for the presence of a 1,2,3,5-tetrasubstituted benzene ring. Indeed, in the low field regions one singlet for two protons (H-4″ and H-6″) at δH 6.40, two peaks for two aromatic methine, at δC 104.56 and 106.72, C4″ and C6″, and four peaks for aromatic quaternary carbons at δC 152.13, 150.16, 141.36 and 127.58 were observed. These spectra also displayed signals for a methoxy group (δH at 3.81 and δC 56.05) and an acetate [δH 2.30 (3H, *s*) and δC 168.72 and 20.03]. The substitution of the benzene ring at C-5″ by a normal C_{27}, C_{29} or C_{31} alkenyl chain was supported on the one hand by prominent peaks around δH 1.29 and δC 29 ppm, CH_2 of polymethylenic chain, and on

Table 4
NMR data of lycopanerol acetates **6b**[a]

Position	δC	δH (mult., *J*, Hz)	HMBC[b]
13	37.95	1.40 (*m*), 1.50 (*m*)	36
14	72.97		15, 36
15	85.84	3.72 (*dd*, 5.7, 9.4)	16, 17, 18, 36
16	26.43	1.80 (*m*)	17
17	27.33	1.93 (*m*), 1.98 (*m*)	18
18	83.53	3.91 (*dd*, 7.4, 7.4)	17, 37
19	79.59		18, 37, 18′
20	38.07	1.35-1.55 (*m*)	18, 37
36	24.55	1.21 (*s*)	15
37	19.56	1.16 (*s*)	18
13′	41.79	1.63 (*m*)	36′
14′	79.45		15′, 16′, 36′
15′	82.44	4.13 (*br d*, 10.8)	16′,36′
16′	25.54	1.96 (H eq., *m*)	
		1.65 (H ax., *m*)	
17′	27.72	1.70 (*m*)	18′
18′	77.86	3.60 (*dd*, 5.9, 5.9)	37′
19′	81.28		18′, 37′
20′	41.97	1.45 (*m*), 1.52 (*m*)	18′, 37′
36′	20.52	1.24 (*s*)	
37′	20.76	1.12 (*s*)	18′
1″	150.16		15′, 6″
2″	127.58		4″, 6″, $OCOC\underline{H}_3$
3″	152.13		4″, $OC\underline{H}_3$
4″	104.56[c]	6.40 (*s*)	7″
5″	141.36		4″, 6″, 7″, 8″
6″	106.72[c]	6.40 (*s*)	7″
7″	36.74	2.56 (*t*, 7.5)	4″, 6″, 8″
8″	31.65	1.60 (*m*)	7″
9″-ω13″	29.40–29.90	1.20–1.30 *br*	
ω9″,ω10″	129.96	5.37 (*t*, 4.7)	ω8″, ω7″; ω11″, ω12″
ω8″, ω11″	27.30	2.02 (*m*)	
ω7″, ω12″	n. d.	1.34 (*m*)	
ω4″-ω6″	29.40–29.90	1.20–1.30 *br*	
ω3″	32.01	1.30 (*m*)	
ω2″	22.72	1.30 (*m*)	ω1″, ω3″
ω1″	14.20	c.a 0.8	ω2″, ω3″
OCH_3	56.05	3.81 (*s*)	
$O\underline{C}OCH_3$	168.72		$OCOC\underline{H}_3$
$OCO\underline{C}H_3$	20.03	2.30 (*s*)	

[a] Resonances of other protons and carbons from the lycopane moieties are similar to those of the corresponding atoms in **2** (Table 1).
[b] Protons correlating with carbon resonance.
[c] Assignments may be reversed.
n.d.: not determined due to the overlapping of signals.

the other hand by signals at δH 5.37 (2H), and δC 129.96, suggesting the presence of a disubstituted unsaturation, in *cis* configuration according to the δC value of allylic carbons at 27.30. The location of the unsaturation at position ω9″ was deduced from the ozonolysis of lycopanerols E, by GC–MS identification of *n*-nonanal. Furthermore, the HMBC experiment exhibited the following long range correlations between benzylic protons H-7″ and C-6″, methoxy protons and C-3″, protons of acetoxy group and C-2″, and benzylic protons and C-8″. The ^{1}H and ^{13}C NMR spectra also allowed to identify a *trans*-THF substructure (data for positions 14–19, 36 and 37 are very similar to those found in **2**, **3**, and **5**, Tables 1 and 3). Also detected in these spectra were signals for two oxygenated methine at δH 3.60 (H-18′*ax*, *dd*, *J* = 5.9, 5.9 Hz) and 4.13 (H-15′ *ax*, *br d*, *J* = 10.8 Hz)) and δC 77.86 and 82.44, respectively, and two oxygen-bearing quaternary carbons, at δC 81.28 and 79.45. COSY, HMQC and HMBC (Table 4) spectra pointed to the existence of a third substructure containing a THP ring. A ROESY experiment showed that Me-36′, H-15′ and H-18′ were axially oriented, thus establishing that the large substituents, at positions 14′, 15′ and 18′ were equatorial (Fig. 2). Furthermore, this last experiment established that H-15/Me-36, H-18/Me-37 and H-18′/Me-37′ were *gauche*, as already observed in **2** and **5**. Finally, the HMBC spectrum showing the key correlations H-18′/C-19 and H-15′/C-1″ established structure **6** for lycopanerols E as a *trans*-THF containing lycopane ether linked by C-19 by an ether bridge with C-19′ of a THP-containing lycopane, in turn linked at C-15′ via a phenoxy bond to a C_{27}, C_{29} or C_{31} *n*-alkenyl resorcinol derivative.

Lycopanerol F showed a $[M + Li]^+$ ion at *m*/*z* 1796.8430 in the HRFAB–MS mode, compatible with the molecular formula $C_{120}H_{236}O_7$ (calcd 1796.8271). Moreover, the LRFAB mass spectrum displayed two fragment ions at *m*/*z* 591 $[C_{40}H_{79}O_2]^+$ and 1204 $[C_{80}H_{157}O_5]^+$, suggesting that this compound would result from the coupling of three tetraterpenoid moieties, very likely via ether bridges. The IR spectrum

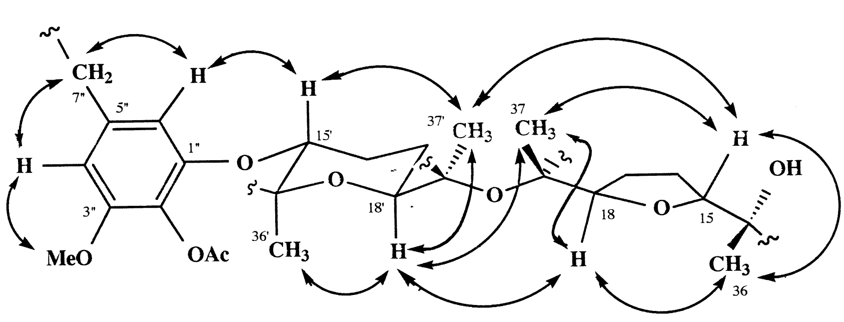


Fig. 2. Relative stereochemistry in **6b** and selected dipolar interactions.

showed the presence of C-O bonds (1065 cm^{-1} and hydroxyl groups (3560 cm^{-1}) borne by quaternary carbons because of the lack of reactivity of the alcohol functions with acetic anhydride in pyridine. ^{1}H NMR, ^{13}C NMR, HMBC (Table 5), COSY and HMQC spectra and comparisons with NMR data of **2** and **3** gave clear evidence for the presence of a *trans*-THF-containing lycopane moiety. Moreover, the ^{1}H NMR spectrum displayed only three signals for three protons borne by three oxygenated carbons (C-15, C-18 and C-18′) and the ^{13}C NMR spectrum showed six peaks for six oxygen-bearing carbons, namely three quaternary (C-14, C-19 and C-19′) and three methine (C-15, C-18 and C-18′), and also three signals for three methylene in THF rings. These data suggested that lycopanerol F has a two-fold axis of symmetry and thus comprises two *trans*-THF containing lycopanes, each one being linked by an ether bridge to a sandwiched third lycopane unit. Given the symmetry of the molecule, this third lycopane moiety can only contain a THF ring in its central part and not a THP one. A ROESY experiment confirmed the *trans* configuration of the THFs in the two "external" lycopanes: the absence of H-15/H-18 connectivity was consistent only with such a geometry (Fig. 3). Moreover, from this experiment it appeared that Me-36/H-15, Me-37/H-18 and Me-37′/H-18′ were *gauche*, but no clear evidence could be drawn for the configuration of the central THF. However, biogenetic considerations point for a *trans*-THF. Indeed, we have recently shown that the natural *anti*-diepoxy lycopane deriving from lycopadiene **1** and isolated from the same strain of *B. braunii*, which is a very likely precursor of lycopanerols, could be biomi-

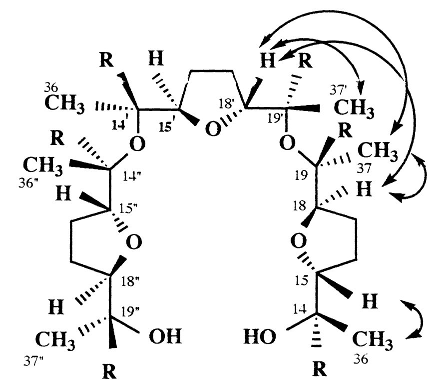


Fig. 3. Circular arrangement of **7** and selected dipolar interactions.

metically transformed into a *trans*-THF derivative, but not into a *cis* isomer (Metzger, 1999). A *cis*-THF-comprising lycopane could be only formed from the non-natural *syn*-diepoxide prepared by hemi-synthesis. Thus, we are inclined to consider that lycopanerol F has the structure shown in **7** and is a tri-*trans*-THF-containing lycopane. The alternate disposition of the three THF rings and the two non-cyclic ether functions, probably forming in lycopanerol F a circular arrangement (Fig. 3), is rather similar to that reported for some squalene derivatives such as teurilene and intricatetraol isolated from algae of the genus *Laurencia* (Suzuki et al., 1985, 1993) or glabrescol, a squalene-derived penta-THF diol from a *Rutacea* (Harding et al., 1995). In the present case, the arrangement would be stabilized by intramolecular hydrogen bonding between the hydroxyls at C-14 and C-19″.

The molecular formula of lycopanerol G was determined as $C_{120}H_{236}O_7$ by HRFAB-MS. The LRFAB mass spectrum displayed two fragment ions at *m/z* 598 $[C_{40}H_{79}O_2 + Li]^+$ and 1204 $[C_{80}H_{157}O_5 + Li]^+$, suggesting that this compound would result from the coupling of three tetraterpenoid moieties, as lycopanerol F, **7**. The IR spectrum showed absorptions for hydroxyls at 3610 and 3550 cm^{-1}. By treatment with acetic anhydride it retained, contrarily to **7**, one tertiary hydroxyl group. The comparison of ^{1}H and ^{13}C NMR data (Table 6) of the acetate derivative **8b** with those of **7** (Table 5), and examination of HMBC and ROESY spectra clearly indicated the presence of two *trans*-THF containing lycopane linked at C-19 and C-19′ by an ether bridge. Careful examination of the other NMR data indicated that the third tetraterpene moiety of **8b** could contain a THP ring. However, the data differed significantly from those of **5b**, especially the ^{13}C chemical shift values. The observation in the ROESY spectrum of dipolar interactions (Fig. 4), between Me-36″ and H-18″ (strong), Me-36″ and H-15″ and H-15″ and H-18″ (low) suggested that the

Table 5
NMR data of lycopanerol **7**[a]

Position	δC	δH (mult., *J*, Hz)	HMBC[b]
13, 20″	37.97	1.40 (*m*); 1.30 (*m*)	36
14, 19″	72.89		15, 36
15, 18″	85.72	3.71 (*dd*, 9.5, 5.7)	18, 36
16, 17″	26.46	1.79 (*m*)	15, 18
17, 16″	27.23	1.88 (*m*)	15, 18
18, 15″	83.38	3.89 (*dd*, 6.9, 6.9)	17, 37
19, 14″	79.24		37
20, 13″	38.80	1.52 (*m*); 1.57 (*m*)	18, 37
36, 37″	24.51	1.18 (*s*)	15
37, 36″	19.55	1.12 (*s*)	18
20′, 13′	42.54	1.37 (*m*); 1.55 (*m*)	37′
19′, 14′	80.46		18′, 37′
18′, 15′	76.06	3.57 (*br d*, 7.3)	37′
17′, 16′	28.00	1.73 (*m*)	
37′, 36′	21.12	1.08 (*s*)	18′

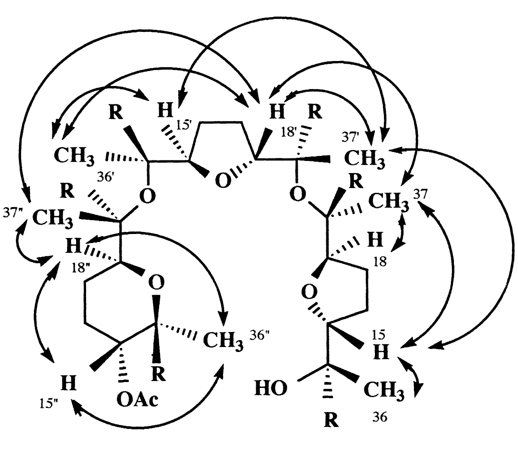
[a] Resonances of other protons and carbons from the lycopane moieties are similar to those of the corresponding atoms in **2** (Table 1).

[b] Protons correlating with the carbon resonance; only the correlations for one of the halves of the molecule are given.

Table 6
NMR data of lycopanerol acetate **8b**[a]

Position	δC	δH (mult., *J*, Hz)	HMBC[b]
13	38.03	1.40 (*m*)	15, 36
14	73.04		15, 36
15	85.78	3.71 (*dd*, 5.6, 9.5)	18, 36
16	26.52	1.80 (*m*)	15, 18
17	27.21	1.90 (*m*)	15, 18
18	83.27	3.89 (*dd*, 6.9, 6.9)	37
19	79.27		37, 18′
20	39.00	1.40 (*m*)	18, 37
36	24.55	1.18 (*s*)	15
37	19.59	1.13 (*s*)	18
13′	41.90	1.49 (*m*), 1.38 (*m*)	36′
14′	80.37		36′
15′	75.5[c]	3.65 (*m*)	36′
16′	28.09	1.61 (*m*)	
17′	28.09	1.52 (*m*)	
18′	75.9[c]	3.54 (*d*, 6.9)	37′
19′	80.41		18′, 37′
20′	43.63	1.37 (*m*)	
36′	21.08	1.06 (*s*)	
37′	21.43	1.10 (*s*)	
13″	40.94	1.42 (*m*), 1.30 (*m*)	15″, 36″
14″	75.03		15″, 18″, 36″
15″	74.79	4.43 (*dd*, 4.5, 11.1)	36″, $OCOC\underline{H}_3$
16″	25.02	1.82 (*m*), 1.52 (*m*)	15″
17″	24.95	1.53 (*m*)	16″, 18″
18″	71.55	3.43 (*br d*, 11.0)	37″
19″	79.42		18″, 37″
20″	37.94	1.60 (*m*), 1.40 (*m*)	18″
36″	15.60	1.18 (*s*)	15″
37″	19.44	1.03 (*s*)	18″
$O\underline{C}OCH_3$	170.50		15″, $OCOC\underline{H}_3$
$OCO\underline{C}H_3$	21.43	2.05 (*s*)	

[a] Resonances of other protons and carbons from the lycopane moieties are similar to those of the corresponding atoms in **2** (Table 1).
[b] Protons correlating with carbon resonance.
[c] Broad peak.



R = $C_{16}H_{33}$

Fig. 4. Circular arrangement of **8b** and selected dipolar interactions.

THP ring very likely adopted a rather distorted boat conformation. Moreover, the coupling constant values observed for H-15″, 4.5 and 11.1 Hz, which are of the same magnitude as those reported for protons of THP rings in boat from (Hashimoto et al., 1990), pointed also for such a conformation. Accordingly, the structure of lycopanerol G was assumed to be a *trans*-THF containing lycopane connected at C-19 via an ether bridge with C-19′ of a second *trans*-THF containing lycopane in turn ether-linked at C-14′ with C-19″ of a third lycopane containing a THP ring in a boat form, as shown in **8**.

Lycopanerols A–G are specific ether lipids of the L race of *B. braunii* and they differ markedly from ether lipids synthesized by algae classified in the A race. Indeed, in this latter case ether lipids derive from the coupling of (i) straight chain unsaturated hydrocarbons (odd carbon numbered), (ii) botryals C_{52}-C_{64}, which are triunsaturated aldehydes specific to these algae, and (or) (iii) *n*-alkenyl phenols (Metzger and Largeau, 1999). In the B race the only ether lipids described up to now, are two new carotenoids named braunixanthins which result from the ether linkage of echinenone, an alkyl phenol and a THF derivative of tetramethylsqualene (Okada et al., 1997). Many THF- and THP-containing natural compounds are thought to arise from polyepoxide precursors, both THF acetogenins (Cavé et al., 1997) and THF/THP derived squalenes (Sakemi et al., 1986; Hashimoto et al., 1990; Suzuki et al., 1993; Harding et al., 1995; Norte et al., 1997). In a previous paper (Metzger, 1999) we have shown that the natural *anti*-diepoxide deriving from lycopadiene **1** can be biomimetically converted, via acid catalysis, both into *trans*-THF- and THP-containing lycopanes. The existence of lycopanerols containing from one to three tetraterpenoid units highly suggests a biogenetic pathway through sequential condensations of anti-diepoxy lycopanes (or a monoepoxide in the case of lycopanerol **4**) rather than coupling by cascade mechanisms.

3. Experimental

3.1. General

CC: silica gel (70–230 mesh). TLC purifications were performed on glass plates coated with silica gel 60 $PF_{254\,+\,266}$ and visualised by UV light. Normal phase HPLC analyses and quantitative separations were performed with two spherical 3 μm silica columns (0.46 × 15 cm) connected in series, with differential refractometer detection. Acetylation and trimethylsilylation were carried out according to standard procedures (Kates, 1986). HRFAB mass spectra were obtained in positive mode through inclusion of the products in a nitrobenzyl alcohol matrix and addition of LiCl. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR: $CDCl_3$.

3.2. Algal materials and cultures

Strains of the L race originated from a lake in Yamoussoukro, Ivory Coast (Metzger et al., 1990) and from Kulavai lake in India (strain 1; Metzger et al., 1997). Strains of the A race came from the Austin Collection of Algae, Texas (UTEX no. 572), from lakes Jillamatong in Australia (Metzger et al., 1997), Overjuyo in Bolivia (strain no. 7; Metzger, 1994), Coat ar Herno in France (Metzger and Casadevall, 1991) and Chaumeçon in France (Metzger et al., 1985). The strain of the B race originated from a lake in La Manzo in Martinique (strain MLM1; Metzger et al., 1985). Algae were grown under air-lift conditions (air enriched by 1% CO_2) and continuous illumination, as previously described (Metzger et al., 1985). The cultures were harvested when they entered the stationary phase of growth, by filtration on 10 μm nylon cloth and then freeze dried.

3.3. Extraction and isolation

The dry biomass of the Yamoussoukro strain (22.1 g) was extracted at room temperature twice with 500 ml heptane (one hour for each extraction). The extracts were combined and concentrated under reduced pressure (6.5 g). Aliphatic polyaldehyde tetraterpenediol polyacetal was removed from the extract as described earlier (Berthéas et al., 1999). Then, the polymer-free extract (4.1 g), was separated into five fractions via silica gel CC by elution with: heptane (fr. I); heptane/Et_2O 19:1 v/v (fr. II); heptane/Et_2O 23:2 v/v (fr. III); heptane/Et_2O 17:3 v/v (fr. IV); and Et_2O (fr. V).

Fr. I contained pure lycopadiene **1** (15 mg). Fr. II (1.6 g) was separated by silica gel TLC; elution by heptane/Et_2O 22:3 v/v, afforded the monoepoxide of lycopadiene (R_f 0.57; 66 mg) (Delahais and Metzger, 1997), lycopanerols A, **2**, (R_f 0.48; 1.04 g) and C, **4**, (R_f 0.25; 25 mg). Fr. III (1.2g) purified by silica gel TLC by elution with heptane/Et_2O: 21:4 v/v afforded lycopanerols B, **3**, (R_f 0.65; 46 mg) and F, **7**, (R_f 0.5, 110 mg), *anti*-diepoxy lycopane (R_f 0.48, 22 mg; Metzger, 1999) and a wide band of R_f 0.4–0.25 corresponding to mixtures of compounds. The half part of the product from this last band was reacted with Ac_2O/Py, extracted as usually and purified by silica gel TLC, with heptane/Et_2O 17:3 v/v as eluent, to give the acetate derivative of lycopanerol E, **6b** (R_f 0.36; 105 mg). Separation of the other half part by normal phase HPLC (heptane/THF 100:1.5 at 1.5 ml/min) afforded α- and β-tocopherols, (R_{t^s} 14 and 20 min, 27 and 11 mg, respectively). Fr. IV (1.05 g), separated by silica gel TLC (elution heptane/Et_2O 7:3 v/v), afforded two major bands with R_f 0.53 and 0.46, respectively. The most eluted band was scraped off and further purified by normal phase HPLC (heptane/THF 200:7.5 at 1 ml/min). Repeated injections gave an enriched fraction in lycopanerol G, **8a**, (95%; 45 mg). A second purification of the band of R_f 0.46 by silica gel TLC with the same eluent furnished lycopanerol D, **5a**, (150 mg). Acetylation of lycopanerols **5a** and **8a** gave **5b** and **8b**, respectively.

Then, the heptane extracted biomass was extracted with $CHCl_3$ at room temperature overnight, in order to remove additional polyaldehyde. Finally, the residual biomass was extracted with $CHCl_3$/MeOH 2:1 v/v in 18 h, at room temperature. The recovered lipidic fraction (2.3 g) was separated by silica gel CC: elution by pure heptane, then by heptane/Et_2O 19:1, and finally by heptane/Et_2O 23:2. Purification of the second fraction by silica gel TLC (heptane/Et_2O 22:3) afforded phytyl esters (R_f 0.74; 158 mg).

3.4. Phytyl esters and hydrolysis

IR ν_{max} (CCl_4): 3000, 2920, 2850, 1730, 1465, 1370, 1360, 1150, 1110 and 720 cm^{-1}. ^{1}H NMR: δ 5.35 (2H, *t*, $J = 4.4$ Hz), 5.34 (1H, *t*, $J = 5.7$ Hz), 4.59 (2H, *d*, $J = 7.1$ Hz), 2.29 (2H, *t*, $J = 7.3$ Hz), 2.00 (6H, *m*), 1.69 (3H, *s*), 1.6 to 1.1: overlapping methinic and methylenic proton signals, 0.88 to 0.83 overlapping methyl signals. Phytyl esters were saponified in MeOH/toluene/H_2O 5:1:1, 1N KOH, under reflux one h. The reaction mixture was acidified with aqueous HCl 10%, extracted with Et_2O and then esterified by CH_2N_2. The analysis was performed by GC–MS; CPSil-5CB capillary column (25 m × 0.25 mm), temperature program: 150–300°C at 4° min^{-1}.

3.5. Tocopherols

They were identified by their mass spectra, EI(70eV)MS: α-tocopherol *m*/*z* (rel. int. %): 430 [M$^+$] (40), 205 (11), 165 (100); β-tocopherol *m*/*z* (rel. int. %): 416 [M$^+$] (79), 191 (33), 151 (100) and comparisons of the ^{13}C NMR data with those reported in the literature (Matsuo and Urano, 1976).

3.6. Lycopanerols A (**2**)

Clear oil; HRFAB–MS and IR data, see Metzger and Aumelas, 1997; ^{1}H and ^{13}C NMR spectra: see Table 1.

3.7. Lycopanerols B (**3**)

Clear oil; HRFAB–MS: obsd 1668.7032 (calcd for $C_{112}H_{220}O_6$ + Li 1668.7070) and 1640.6709 (calcd for

$C_{110}H_{216}O_6$ + Li 1640.6757); IR ν_{max} (CCl_4): 3580, 2960, 2920, 2840, 1460, 1370, 1150, 1095, 1070 and 720 cm^{-1}; ^1H and ^{13}C NMR spectra: see Table 1.

3.8. Epoxidation of lycopanerols A

A mixture of lycopanerols A (15 mg) dissolved in 5 ml of CH_2Cl_2, was treated with *m*-CPBA (5 mg) under stirring and at room temperature. After 2 h usual work-up gave an oily residue. It was chromatographed by silica gel TLC; elution with heptane/Et_2O 41:9 yielded 9 mg of **3** which showed spectroscopic properties identical to those observed for the natural compounds.

3.9. Lycopanerols C (4)

Clear oil; HRFAB–MS: obsd 1046.1211 (calcd for $C_{72}H_{142}O_2$+Li 1046.1169), 1018.0903 (calcd for $C_{70}H_{138}O_2$+Li 1018.0856) and 990.0574 (calcd for $C_{68}H_{134}O_2$+Li 990.0543); IR ν_{max} (CCl_4) 3570, 3000, 1460, 1375, 1365, 1150, 1090, 1060, 970 and 720 cm^{-1}; ^1H and ^{13}C NMR spectra: see Table 2.

3.10. Trimethylsilyl derivatives of lycopanerols C

EI(70 eV)MS, direct inlet, *m/z* (rel. int. %): 341 (54), 129 (39), 111 (57), 97 (82), 95 (44), 73 (59), 71 (69), 69 (74), 57 (100) and 55 (82).

3.11. Lycopanerol D (5)

Clear oil; $[\alpha]_D^{20} = -1.7°$ ($c = 7.5$, $CHCl_3$); HRFAB–MS: obsd 1206.2278 (calcd for $C_{80}H_{158}O_5$+Li 1206.2269); IR ν_{max} (CCl_4): 3620, 3560, 2940, 2910, 1460, 1370, 1140 and 1060 cm^{-1}; ^1H and ^{13}C NMR spectra of the acetate derivative **5b**: see Table 3.

3.12. Acetate of lycopanerol E (6b)

Clear oil; HRFAB–MS obsd 1746.6654 (calcd for $C_{116}H_{218}O_8$+Li 1746.6811), 1774.7029 (calcd for $C_{118}H_{222}O_8$+Li 1774.7125) and 1802.7377 (calcd for $C_{120}H_{226}O_8$+Li 1802.7438); IR ν_{max} (CCl_4): 3560, 2940, 2920, 2840, 1760, 1600, 1500, 1460, 1370, 1360, 1190, 1120 and 1070 cm^{-1}; ^1H and ^{13}C NMR spectra: see Table 4.

3.13. Lycopanerol F (7)

Clear oil; $[\alpha]_D^{20} = -2.4°$ ($c = 5$, $CHCl_3$); HRFAB–MS: obsd 1796.8430 (calcd for $C_{120}H_{236}O_7$ + Li 1796.8271); IR ν_{max} (CCl_4): 3560, 2940, 2920, 2860, 1460, 1370, 1360, 1065 cm^{-1}; ^1H and ^{13}C NMR spectra: see Table 5.

3.14. Lycopanerol G (8)

Clear oil; $[\alpha]_D^{20} = -7.4°$ ($c = 6.3$, $CHCl_3$); HRFAB-MS: obsd 1796.8136 (calcd for $C_{120}H_{236}O_7$+Li 1796.8271); IR ν_{max} (CCl_4): 3610, 3550, 2940, 2910, 2840, 1460, 1370, 1140, 1090 and 1070 cm^{-1}; ^1H and ^{13}C NMR spectra of the acetate derivative **8b**: see Table 6.

3.15. Ozonolysis

Lycopanerols **4** and **6b** (each 2 mg), in CH_2Cl_2 solution (2 ml), were treated by air enriched in ozone, at −15°C for 10 min. Then, excess ozone was eliminated by bubbling N_2 in the solution and the ozonides were reduced by addition of triphenyl phosphine (5 mg). After concentration of the reaction mixtures, the products were analyzed by GC–MS (CP Sil 5CB capillary column, temperature 5 min at 120° and then progress up to 300°C at 5°C min^{-1}). From **4**, *n*-nonanal and trimethyl-6,10,14-pentadecan-2-one were identified, while from **6b** only *n*-nonanal was detected. EI(70 eV)MS *m/z* (rel. int.): *n*-nonanal, 142 $[M]^+$ (1), 124 $[M–H_2O]^+$ (1.5), 98 (17), 82 (17), 70 (30), 57 (66), 43 (100); trimethyl-4,6,10-pentadecan-2-one, 268 $[M]^+$ (0.5), 250 $[M–H_2O]^+$ (3), 124 (12), 109 (23), 95 (28), 85 (23), 81 (23), 71 (43), 58 (75), 57 (45), 43 (100).

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