Phytochemistry, Vol. 25, No. 8, pp. 1869–1872, 1986.
Printed in Great Britain.

0031–9422/86 $3.00 + 0.00
Pergamon Journals Ltd.

AN *n*-ALKATRIENE AND SOME *n*-ALKADIENES FROM THE A RACE OF THE GREEN ALGA *BOTRYOCOCCUS BRAUNII*

P. METZGER*, J. TEMPLIER, C. LARGEAU and E. CASADEVALL

Laboratoire de Chimie Bioorganique et Organique Physique, UA CNRS 456, E. N. S. C. P. 11, rue P. et M. Curie, 75231 Paris Cedex 05, France

(*Revised received* 3 *December* 1985)

Key Word Index—*Botryococcus braunii*; Chlorophyceae; alga; non-isoprenoid very long chain hydrocarbons; structural determination.

Abstract—The various strains of the hydrocarbon-rich alga *Botryococcus braunii* (A race) examined up to now produce the *Z*-isomers of the following dienes: tricosa-1,14-diene, pentacosa-1,16-diene, heptacosa-1,18-diene, nonacosa-1,20-diene and hentriaconta-1,22-diene. A C_{29} triene was also detected in these strains, but its structure was not fully elucidated. In the present work, spectroscopic data and oxidative degradations, established the structure of the C_{29} trienic hydrocarbon. These techniques were also used to examine the dienic series of a new A strain, collected from nature and laboratory grown. In addition to the usual *Z*-dienes, *E*-isomers of tricosa-1,14-diene, pentacosa-1,16-diene, heptacosa-1,18-diene and nonacosa-1,20-diene, were characterized.

INTRODUCTION

The existence of two races was recently demonstrated in the hydrocarbon-rich green alga *Botryococcus braunii* [1]. They exhibit closely related morphological features, but sharply differ in the nature of their hydrocarbons. The A race produces odd, unbranched C_{23}–C_{31} hydrocarbons whilst the B race yields polyunsaturated, branched, C_{30}–C_{37} terpenoid hydrocarbons termed botryococcenes. In the B race, large variations in hydrocarbon composition were noticed according to geographical origin and culture conditions. Regarding the A race, all the studies reported up to now under various culture conditions, revealed a fairly constant composition of the hydrocarbon fraction [1–4]. It chiefly contains a series of odd C_{23}–C_{31} *n*-alkadienes and a C_{29} triene, the latter generally accounting for 1–10% of total hydrocarbons. The lack of important chemical variations noticed for A strains could be related to the low number of geographical areas where alkadiene- and triene-producing algae have been collected up to the present time. Indeed, all the collection A strains derive from the same sample collected from Maddingley Bricks Pits (U.K.) and the previously reported wild A strains originated from the Morvan region (France) or from Atlas (Morocco) [1].

All the dienic hydrocarbons (**1**) produced by the above strains were shown to contain terminal unsaturation and a *cis*-double bond, located in the 9,10-position relative to the terminal methyl group [5]. The C_{29} triene was also recently examined [6]. However the information obtained did not allow a positive identification and two possible structures **2** and **3** were proposed.

We recently succeeded in isolating a new A strain from a sample collected in Grosbois, Côte d'Or, France. The chromatogram of the total hydrocarbons from this strain grown in the laboratory, showed, in addition to the previously described dienes, a new series of odd carbon numbered hydrocarbons. In the present study we report the chemical structures of these new hydrocarbons from the Grosbois strain and that of the C_{29} triene produced by other collection strains. These determinations were carried out using oxidative degradation and spectroscopic examination.

RESULTS AND DISCUSSION

The composition of the hydrocarbon fractions was first examined by GC/MS (Table 1). The major hydrocarbons of the axenic strain from the culture collection of Austin were, as previously reported [1–4], odd C_{27}–C_{31} *n*-alkadienes and a C_{29} triene. In addition, minor C_{23} and C_{25} dienes, along with low amounts of other C_{29} and C_{31} trienes, were detected. In the Grosbois strain, besides the usual dienes comprising *cis*-unsaturation, new isomers of odd C_{23}–C_{29} dienes were discovered; they accounted for *ca* 57% of the hydrocarbon fraction.

Reversed-phase HPLC of the total hydrocarbons from the Austin strain provided three main eluates: C_{29} triene + C_{27} diene, C_{29} diene and C_{31} diene. Further separation of the first eluate on a silver nitrate–silica gel column afforded an enriched subfraction containing 76% of the major C_{29} triene. Its IR spectrum exhibited two bands at 910 and 990 cm^{-1} characteristic of a non-conjugated

$$\mathrm{Me(CH_2)_7CH{=\!=}CH(CH_2)_xCH{=\!=}CH_2} \qquad \mathbf{1}$$

x = odd from 11 to 19

$$\mathrm{Me(CH_2)_5CH{=\!=}CH{-\!-}CH{=\!=}CH(CH_2)_{17}{-\!-}CH{=\!=}CH_2} \qquad \mathbf{2}$$

$$\mathrm{Me(CH_2)_5CH{=\!=}CH(CH_2)_{17}{-\!-}CH{=\!=}CH{-\!-}CH{=\!=}CH_2} \qquad \mathbf{3}$$

---

* Author to whom correspondence should be addressed.

Table 1. Composition of hydrocarbon mixtures from A strains determined by GC/MS*

Hydrocarbons	Retention times/ hexane (sec)	Location and stereochemistry of the double bonds†	Relative %	
			Austin strain	Grosbois strain
$C_{23}H_{44}$	245	1,14 (*Z*)	trace	0.3
	250	1,14 (*E*)	—	0.6
$C_{25}H_{48}$	410	1,16 (*Z*)	0.4	2.3
	420	1,16 (*E*)	—	8.8
$C_{27}H_{52}$	640	1,18 (*Z*)	9.9	4.1
	650	1,18 (*E*)	—	18.0
$C_{29}H_{56}$	915	1,20 (*Z*)	43.8	25.1
	930	1,20 (*E*)	—	29.7
$C_{29}H_{54}$	980		1.3	trace
	990		1.6	trace
	1020	1,20 (*Z*), 22 (*Z*)	12.0	trace
$C_{31}H_{60}$	1240	1,22 (*Z*)	28.8	11.1
$C_{31}H_{58}$	1310		1.1	trace
	1380		1.1	trace

*The mass spectra (absence of $[M-15]^+$ ion), the ^{13}C NMR spectra (lack of tertiary-aliphatic and quaternary carbons) and the study of hydrogenation products, established that all the unidentified products (trienes from Austin strain and new dienes from Grosbois strain) are normal olefins.

†The *Z*-dienic series has previously been described [5].

terminal double bond; UV absorbance at 232 nm indicated the presence of two conjugated double bonds in the C_{29} triene. These spectroscopic observations therefore excluded structure **3**, but were consistent with **2**. Ozonolysis of the enriched subfraction was carried out in order to determine the location of the conjugated double bonds in the major C_{29} triene. The acids derived from oxidative cleavage of the resulting ozonides were analysed by GC/MS as their methyl esters. A C_{19} diester generated by 1,2 and 20,21 cleavages was principally detected; attempts to isolate a C_7 monoester resulting from 22,23 cleavage were unsuccessful. Nevertheless, these results, together with the spectroscopic observations, allowed the characterization of this hydrocarbon as a nonacosa-1,20,22-triene (**2**) in the Austin strain.

The stereochemistry of the conjugated double bonds was examined by NMR. The ^{1}H NMR spectrum of the C_{29} triene showed five signals in the olefinic proton region. By comparison with the spectra of the previously identified dienes, three were ascribed to the protons of the terminal double bond, at δ4.98, 5.04 and 5.88 ($^{2}J_{gem} = 2$ Hz, $^{3}J_{cis} = 10$ Hz, $^{3}J_{trans} = 17$ Hz, $^{3}J_{=\mathrm{CH,CH_2allyl}} = 7$ Hz, $^{4}J_{=\mathrm{CH_2,CH_2allyl}} = 1.5$ Hz), the two other signals at 5.50 and 6.31 were attributed to protons on carbons 20,23 and 21,22, respectively. The proton coupling constants ($^{3}J_{20,21} = {}^{3}J_{22,23} = 7.5$ Hz; $^{3}J_{20,19} = {}^{3}J_{23,24} = 7$ Hz; $^{4}J_{20,22} = {}^{4}J_{21,23} = 2.5$ Hz) were consistent with a *Z*,*Z* stereochemistry. This was confirmed by the ^{13}C chemical shifts of allylic and olefinic carbons (Table 2) [7, 8]. Thus the combination of spectroscopic data and oxidative degradation indicate that the main C_{29} triene produced by the Austin strain is the nonacosa-1,20*Z*,22*Z*-triene.

Freezing an hexane solution of the total hydrocarbons from the Grosbois strain led to the separation of the major unidentified dienes. A solid mixture composed of C_{29} (74%), C_{27} (21%) and C_{25} (5%) dienes was thus obtained. IR data established the existence of a terminal double bond (910 and 990 cm^{-1}) and of a *trans* internal double bond (970 cm^{-1}) for these dienes. The ^{13}C NMR chemical shifts of the allylic carbons (Table 2) confirmed a *trans* stereochemistry for the internal unsaturation at δ32.6, against 27.2 for the *cis* isomers [9, 10]. Ozonolysis of the diene mixture and subsequent treatments provided a C_9 monoester and three C_{15}, C_{17} and C_{19} diesters. The identification of these compounds established that the internal double bond of the *trans* series is located in the same position (9,10 relative to the terminal methyl), as the unsaturation of the *cis* isomers (**1**). Thus the new dienes isolated from the Grosbois strain are pentacosa-1,16*E*-diene, heptacosa-1,18*E*-diene and nonacosa-1,20*E*-diene. By analogy, the new C_{23} diene, whose isolation was impossible because of its low proportion in the hydrocarbon mixture, could be tricosa-1,14*E*-diene. Accordingly these new dienes differ from their previously identified counterparts only in the stereochemistry of the internal double bond.

In conclusion, the present results show the existence, for *B. braunii* A race, of a chemical variation as for the B race [1], i.e. hydrocarbons of similar nature but involving isomerism. Thus, the alkadiene series exhibits different stereochemistries of the internal double bond, in relation to the strain origin. These observations may be important in connection with studies about the factors (including the specificity of the involved enzymes) which control, along the biosynthetic pathway, the structure of these non-isoprenoid hydrocarbons. It has been established [11] that *cis*-dienes are produced via an elongation–decarboxylation mechanism with oleic acid as direct precursor (hence the stereochemistry of the internal double bond and its 9,10 location with respect to methyl). The terminal double bond of these dienes probably originates from a decarboxylation process probably via the activation by a β-hydroxy group [12]. The structure of the C_{29} triene strongly suggests that a similar biosynthetic mechanism is operating. In this case, the formation of the third double bond may originate either from the involvement of a dienic (*Z,Z*) fatty acid as direct precursor or from desaturation of the C_{29} dienic hydrocarbon chain. The structure of the *trans*-dienes from the Grosbois strain suggest that they also probably originate from an elongation–decarboxylation mechanism with elaidic acid as direct precursor (Scheme 1). However the concomitant formation of *cis* and *trans* dienes in this strain raises the question of the control of stereochemistry (nature of the precursor fatty acids, specificity of the elongation–decarboxylation system) in the biosynthesis of dienic hydrocarbons in *B. braunii*. From this point of view, the *E*/*Z* repartition in the hydrocarbon mixture from the Grosbois strain with regard to the hydrocarbon chain length, C_{23}: 2; C_{25}: 3.8; C_{27}: 4.4; C_{29}: 1.2 and C_{31}: 0, could be indicative of the enzymatic system capability to elongate and (or) decarboxylate long fatty acids > C_{30} involving an internal *trans* double bond.

EXPERIMENTAL

IR and UV spectra were recorded from CCl_4 and hexane solns, respectively. ^1H (250 MHz) and ^{13}C (125.76 MHz) NMR were obtained from $CDCl_3$ solns using TMS as int. ref.

Strain origins, culture conditions and hydrocarbon extraction. The collection strain was obtained from the University of Texas at Austin, U.S.A. (n° 572). The sampling of the wild algae was made in a barrier lake near Grosbois, Côte d'Or, France. The isolation techniques of the new strain, the culture conditions (batch air-lift 1 % CO_2) and the hydrocarbon extraction were as previously described [1].

Analyses and fractionation of hydrocarbons. The conditions used for GC/MS analyses were as reported in ref. [1]. Reversed-phase HPLC separation of the C_{29} triene from the hydrocarbon extract of the collection strain was performed on an instrument equipped with a 7000 psi inj. valve with a 20 μl loop. The detector was a differential refractometer, thermostated at 25°. The reversed-phase column was a Resolve 5μ spherical C_{18} Waters (two 150 × 3.9 mm). Hydrocarbon sample was injected in Me_2CO–THF (1:1) soln (20 μl, 10% in solvent). Mobile phase (dist. in glass, filtered through an AP 25 Millipore prefilter): Me_2CO–MeCN–THF (5:12:1), flow rate 120 ml/hr. R_ts (min) of the major components were C_{29} triene 11, C_{27} diene 11.5, C_{29} diene 17 and C_{31} diene 23. Successive injections of sample were carried out and the fractions collected. The first eluate (C_{29} triene + C_{27} diene) was then chromatographed on a silica gel–$AgNO_3$ (20%) column, using hexane as eluent with increasing amounts of Et_2O.

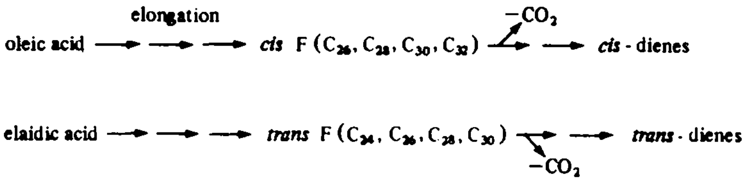
The Grosbois hydrocarbons were fractionated by freezing an hexane soln at −25° for 18 hr. GC analysis performed on the amorphous solid collected, showed three main peaks ascribed to the *E*-C_{25}, C_{27} and C_{29} dienes.

Oxidative cleavage. Ozonolysis at −20°, oxidative cleavage of the ozonides and esterification of the resulting acids with MeOH–HCl was conducted as described in ref. [13]. Me esters were analysed by GC/MS using EI. The apparatus was equipped with a fused silica column 25 m, WCOT SE 52. The column temp. was initiated at 120° for 3 min and then prog to 260° at 10°/min. MS *m*/*z* (rel. int.): nonanoic acid Me ester, 172 $[M]^+$ (2), 143 $[M - OMe]^+$ (10), 141 (11), 129 (11), 87 (34), 74 $[CH_2C(OH)Me]^+$

Table 2. ^{13}CNMR chemical shifts of *E*- and *Z*-alkadienes and C_{29} triene

Alkadienes	Me —	CH$_2$ —	CH$_2$ —	(CH$_2$)$_4$ —	CH$_2$ —	CH=CH —	CH$_2$ —	(CH$_2$)$_y$ —	CH$_2$ —	CH =	CH$_2$
Z isomers	14.1	22.7	31.9	29.0–29.8	27.2	129.9	27.2	29.0–29.8	33.8	139.2	114.0
E isomers	14.1	22.7	31.9	29.0–29.8	32.6	130.3	32.6	29.0–29.8	33.8	139.2	114.0

C_{29} triene	Me —	CH$_2$ —	CH$_2$ —	(CH$_2$)$_2$ —	CH$_2$ —	CH=	CH —	CH=	CH —	CH$_2$ —	(CH$_2$)$_{15}$ —	CH$_2$ —	CH=	CH$_2$
Z,Z	14.2	22.8	31.9	29.1–29.8	27.7	132.2	123.8	123.8	132.2	27.7	29.1–29.8	33.9	139.4	114.2



F = very long chain mono - unsaturated fatty acyl derivatives.

Scheme 1. Elongation–decarboxylation mechanisms for the biosynthesis of long chain alkadienes.

(100), 59 $[CO_2Me]^+$ (14), 57 (10), 55 (21), 43 (23). Pentadeca-1,15-dioic acid, diMe ester, 300 $[M]^+$ (1), 269 $[M-OMe]^+$ (21), 227 $[M-CH_2CO_2Me]^+$ (16), 112 (31), 98 (71), 84 (29), 74 $[CH_2C(OH)Me]^+$ (81), 69 (41), 59 $[CO_2Me]^+$ (47), 55 (100), 43 (38). Heptadeca-1,17-dioic acid, diMe ester, 297 $[M-OMe]^+$ (29), 255 $[M-CH_2CO_2Me]^+$ (16), 112 (29), 98 (100), 84 (33), 74 $[CH_2C(OH)OMe]^+$ (64), 69 (36), 59 $[CO_2Me]^+$ (40), 55 (100), 43 (45). Nonadeca-1,19-dioic acid, diMe ester, 325 $[M-OMe]^+$ (29), 283 $[M-CH_2CO_2Me]^+$ (19), 112 (31), 98 (69), 84 (43), 74 $[CH_2C(OH)OMe]^+$ (86), 69 (43), 59 $[CO_2Me]^+$ (52), 55 (100), 43 (55).

Acknowledgements—We are grateful to Dr. Coute, Museum National d'Histoire Naturelle, Paris, for the sampling of Grosbois lake. We thank Miss Michon and Mrs. Dupenhoat, ENS Paris and Mr. Davoud, Université P. et M. Curie, Paris, for recording the ^1H and ^{13}C NMR spectra and Dr. Sellier and Miss Mauroy, ENSC Paris, for GC/MS analyses.

REFERENCES

1. Metzger, P., Berkaloff, C., Casadevall, E. and Couté, A. (1985) *Phytochemistry* **24**, 2305.
2. Casadevall, E., Chirac, C., Largeau, C. and Metzger, P. (1985) *J. Phycol.* **21**, 380.
3. Casadevall, E., Dif, D., Largeau, C., Gudin, C., Chaumont, D. and Desanti, O. (1985) *Biotechnol. Bioengng.* **27**, 286.
4. Bailliez, C., Largeau, C. and Casadevall, E. (1985) *Appl. Microbiol. Biotechnol.* **23**, 99.
5. Knights, B. A., Brown, A. C. and Conway, E. (1970) *Phytochemistry* **9**, 1317.
6. Mc Coy, R. W. and Pauls, R. E. (1984) *J. Chromatogr. Sci.* **22**, 493.
7. Rossi, R., Carpita, A., Quirici, M. G. and Veracini, C. A. (1982) *Tetrahedron* **38**, 639.
8. Ando, T., Kusa, K., Uchiyama, M., Yoshida, S. and Takahashi, N. (1983) *Agric. Biol. Chem.* **47**, 2849.
9. Bus, J. and Frost, D. J. (1974) *Rec. Trav. Chim.* **93**, 213.
10. Pfeffer, P. E., Luddy, F. E. and Unruh, J. (1977) *J. Am. Oil Chem. Soc.* **54**, 380.
11. Templier, J., Largeau, C. and Casadevall, E. (1984) *Phytochemistry* **23**, 1017.
12. Mong-Sin, T. P. (1985) Thesis, P. and Curie, M., University, Paris.
13. Metzger, P., Casadevall, E., Pouet, M. J. and Pouet, Y. (1985) *Phytochemistry* **24**, 2995.