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HYDROCARBONS FROM THE GREEN FORM OF THE FRESHWATER ALGA *BOTRYOCOCCUS BRAUNII*

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Abstract—Using ozonolysis, followed by analysis of the products by gas chromatography and mass spectrometry, it has been shown that the three principal hydrocarbons of the green, exponential-growth stage of *Botryococcus braunii* are heptacosa-1,18-diene, nonacosa-1,20-diene and hentriaconta-1,22-diene.

INTRODUCTION

Botryococcus braunii (Kütz.) is a freshwater green colonial alga of widespread occurrence, which is known to occur in at least two physiologically distinct forms. The first of these is a green exponentially growing stage of limited abundance and the second is a brown resting stage which often arises as massive rust-coloured algal blooms on the surface of lakes.[1] From paleobotanical studies it has been suggested[2] that *B. braunii* may be the causal organism of the boghead coals (e.g. Torbanite), Coorongite, and also oil shales of the tertiary period[3] and a number of investigations of these theories have been undertaken (for brief reviews see Refs. 4 and 5). It has been shown in the brown resting stage that 70 per cent of the dry weight of *B. braunii* may be accounted for by two isomeric hydrocarbons, botryococcene and isobotryococcene, which occur in a 9:1 ratio.[5] In the green exponential form, however, we have found that only about 20 per cent of the dry weight of the alga could be accounted for as hydrocarbons[6,7] and also that less than 5 per cent of these hydrocarbons was botryococcene or its isomer. In fact three homologous series of hydrocarbons were demonstrated by GLC and the dominant "A" series with five members was found by mass spectrometry to have the general formula C_nH_{2n-2}. The next most-abundant series with four members was shown to have the formula C_nH_{2n-4}. These results were similar to those found for what was described as the "golden brown alga *B. braunii*",[3] when six compounds of the general formula C_nH_{2n-2} and one of the formula C_nH_{2n-4} were described.

It is the object of this paper to report the experiments which have enabled us to locate the position of the two double bonds of the "A" series hydrocarbons of the green stage of *B. braunii*.

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RESULTS

Hydrocarbons were isolated from the green form of *Botryococcus braunii* using acetone extraction followed by chromatography of the extract as previously described.[6] I.r. spectroscopy of these hydrocarbons indicated the presence of a vinyl group (1638, 990 and 908 cm^{-1}) and a *cis* disubstituted double bond (720 cm^{-1}). Gas–liquid chromatography (GLC) indicated that the mixture contained three components (> 90 per cent of the total fraction) and, by inspection of the data (see Table 1a), these were found to be members of the previously

TABLE 1a. GLC DATA (RETENTION INDICES, I) FOR THE OZONOLYSIS PRODUCTS FROM HYDROCARBONS OF *B. braunii*

Fraction	OV-17						SE-30					
	Peak 1	%	Peak 2	%	Peak 3	%	Peak 1	%	Peak 2	%	Peak 3	%
Hydrocarbon*	2705	12	2915	42	3115	46	2705	—	2905	—	3100	—
Aldehyde†	2355	8	2560	46	2760	46	2115	7	2330	46	2530	47
O-Methyloxime†	2525	9	2730	42	2930	49	2310	13	2520	42	2720	45
Methyl ester†	2550	19	2750	36	2955	45	2320	15	2525	40	2725	45

* 244°.
† OV-17, 223°; SE-30, 230°.

TABLE 1b. PARTIAL OZONOLYSIS PRODUCTS

Fraction		OV-17				SE-30			
		Mobile compound	Peak 1	Peak 2	Peak 3	Mobile compound	Peak 1	Peak 2	Peak 3
Aldehyde	*	—	2135	2340	2545	—	2030	2225	2420
	†	85	193	211	229	—	—	—	—
O-Methyloxime	*	—	2230	2435	2630	—	2130	2325	2520
	†	95	201	219	237	105	212	231	249

* 205°.
† Temperature of emergence 2°/min from 50°.

described[6] "A" series of hydrocarbons and to correspond to those compounds which had been shown by combined gas chromatography–mass spectrometry (GC–MS) to have the formulae $C_{27}H_{52}$ (peak 1), $C_{29}H_{56}$ (peak 2) and $C_{31}H_{60}$ (peak 3).

Ozonolysis of the hydrocarbon fraction was attempted using a Supelco microozonizer[8] and the method described by Beroza and Bierl.[9] Under the prescribed conditions, no reaction products could be detected using GLC, in spite of a positive reaction to ozone from the indicator solution. In addition it was found that triphenylphosphine and triphenylphosphine oxide could be detected in the GLC traces and it was thought that in this case these compounds might interfere with the analysis by GLC of possible products of ozonolysis. Ozonolysis was

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TABLE 3. MASS SPECTRAL DATA FOR PARTIAL OZONOLYSIS PRODUCTS FROM HYDROCARBONS OF *B. braunii*

Aldehyde fraction

		M	M-18	M-29	M-43	M-44	Eight most abundant ions							
Peak 1	Ion	266	248	237	223	222	55	41	43	57	69	83	29	67
	Abundance	11	9	7	10	9	1000	810	800	710	680	500	460	460
Peak 2	Ion	294	276	265	251	250	55	41	43	69	57	83	81	67
	Abundance	18	12	7	8	7	1000	760	610	560	530	430	370	340
Peak 3	Ion	322	304	293	279	278	55	41	43	69	57	83	29	67
	Abundance	14	8	4	6	5	1000	800	640	540	500	390	320	310

O-Methyloxime fraction

		M	M-15	M-31	M-41	M-43	Eight most abundant ions							
Peak 1	Ion	295	280	264	254	252	73	55	43	41	59	69	83	86
	Abundance	15	4	60	4	6	1000	390	360	340	280	240	200	150
Peak 2	Ion	323	308	292	282	280	73	55	41	43	86	69	57	29
	Abundance	34	7	103	8	7	1000	380	370	335	210	180	140	130
Peak 3	Ion	351	336	320	310	308	73	55	43	41	69	86	57	83
	Abundance	26	6	86	5	6	1000	350	300	290	170	160	140	115

Lower molecular weight compound detected by temperature programmed GLC

Aldehyde

	M	M-1	M-18	M-28	M-44	Eight most abundant ions							
Ion	142	141	124	114	98	57	41	43	29	44	56	55	27
Abundance	2	4	40	50	270	1000	1000	840	770	660	600	570	550
Literature value[11]	5	4	70	90	400	1000	700	680	360	560	630	500	260

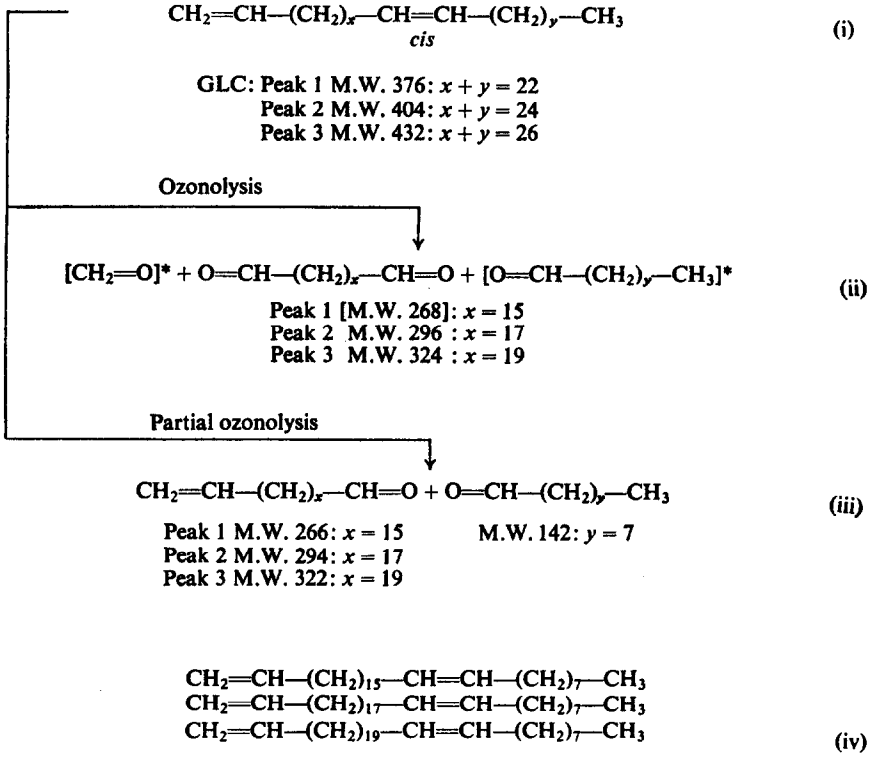
O-Methyloxime

	M	M-29	M-31	M-43	Eight most abundant ions							
Ion	171	142	140	128	73	43	41	86	29	28	27	55
Abundance	10	8	13	12	1000	330	320	200	190	180	140	140

containing one double bond. The molecular weights and GLC data, when compared with the dialdehyde series, were consistent with the double bond present in the monoaldehydes being the vinyl group. The low molecular weight aldehyde afforded a mass spectrum similar to that recorded by Gilpin and McLafferty for *n*-nonanal.[11] The data for the corresponding *O*-methyloxime were also in agreement with this compound being *n*-nonanal, the mass spectrum being similar to that obtained from the *O*-methyloxime of an authentic sample of *n*-decanal.

DISCUSSION

Previously it had been shown[6] that the principal series of hydrocarbons from the green exponentially growing stage of *Botryococcus braunii* had the empirical formula C_nH_{2n-2}. Further, for the three main members of this series, it was found that n was 27, 29 and 31 with the order of relative abundance being $C_{29}H_{56} \geqslant C_{31}H_{60} > C_{27}H_{52}$. Since the i.r. data showed the presence of a vinyl group and a *cis*-disubstituted double bond, it is possible to write a general formula (i) for these three hydrocarbons, as shown in Scheme 1. From the complete



* Not observed.

SCHEME 1.

ozonolysis experiment, three dialdehydes were produced in the same relative proportions as were found for the three parent hydrocarbons. The mass spectral data in Table 2 showed that the formulae for these three aldehydes may be expressed as indicated (ii) and that $X = 15$, 17 and 19 for GLC peaks (1), (2) and (3) respectively. Thus, it would be expected that $Y = 7$ for all three hydrocarbons, although the presumed aldehyde *n*-nonanal could not be detected in this reaction mixture using GLC. Partial ozonolysis was found to produce four monoaldehydes, together with small amounts of the dialdehydes and some unreacted hydrocarbon. Three of these monoaldehydes were closely related to the dialdehydes (ii) and had the structures (iii). The fourth aldehyde, a more mobile substance on GLC, had the correct molecular weight (142 as the aldehyde and 171 as the *O*-methyloxime) for *n*-nonanal and thereby confirmed that $Y = 7$. Thus the formulae for the three hydrocarbons represented by (i) are

therefore attempted by adapting the method of Munavalli and Ourisson[10] for use with the microozonizer. Using a flame ionization detector, tetracyanoethylene, which was incorporated into the reaction mixture to decompose ozonides, could not be detected under the conditions for GLC used in this work. Reaction with ozone was continued until hydrocarbons could no longer be detected by GLC. The aldehyde fraction so formed showed carbonyl absorption (1720 cm^{-1}) but no double bond absorption in the i.r. GLC (Table 1a) indicated three products derived from the three corresponding hydrocarbons. Using GS–MS, mass spectra (the most significant and the eight most abundant ions in these spectra are listed in Table 2) were obtained for peaks 2 and 3 and it was clear from inspection of these data that these two compounds were homologous, differing in mass by 28 units (i.e. C_2H_4). Fragmentations for the loss of water (M-18, M-36), ethylene (M-28) and ethylene plus water (M-46) from these compounds were observed, similar to those observed by Gilpin and McLafferty[11] for mass spectra of aldehydes. The ion arising by loss of hydrogen (M-1) formed via α-cleavage was not significant in the present work, and the corresponding ion at *m/e* 29 was only of medium intensity. The ions for M-43 and M-44 probably arise by β-fission processes.[11,12]

The aldehyde fraction was converted to the corresponding *O*-methyloxime derivatives and these were then analysed by GLC (see Table 1a) and GC-MS (see Table 2). The increase in molecular weight of 58 mass units over the corresponding aldehydes indicated the presence of two functional groups (ΔM.W. CH=O→CH=NOMe = + 29). Ions arising at M-31, M-46 and M-63 have been recorded previously in mass spectra of bis-*O*-methyloxime derivatives[13] and were thought to be derived by loss of fragments including methoxyl and methyl radicals, and methanol. The base peak of these spectra at *m/e* 73 and the ion observed at M-72 for each compound were probably formed by β-cleavage reactions. The ion at *m/e* 73 from *O*-methyloximes appears to be equivalent to the ion found by Goldsmith *et al.*[14] to occur at *m/e* 59 for the oxime derivatives of butyraldehyde and valeraldehyde. The same group also described an ion at *m/e* 72 from these compounds and an equivalent ion at *m/e* 86 was noted for the *O*-methyloximes in the present work.

Oxidation of the aldehyde fraction produced an acid fraction from which methyl esters were prepared. Analysis by GLC (see Table 1a) demonstrated the presence of three compounds in this fraction. GC–MS analysis indicated the presence of two carboxylic acid methyl ester groups and confirmed that the original fraction was composed of dialdehydes. The McLafferty rearrangements[12] produced the ion at *m/e* 74, in agreement with previous work on the mass spectra of methyl esters.[15] The spectra were similar to those reported in the literature for α,ω-dicarboxylic acid methyl esters.[4,16]

A second ozonolysis experiment using a shorter reaction time was carried out. GLC analysis indicated the presence of unreacted hydrocarbons together with three main aldehyde products. Retention data for these aldehydes are listed in Table 1b. In addition, a single, low molecular weight aldehyde was detected when using temperature programmed GLC. This compound was not detected in the previously described ozonolysis experiment.

Analysis by GC–MS (see Table 3) of the aldehyde and *O*-methyloxime fractions indicated that the three main components of the partial ozonolysis experiment were monoaldehydes

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TABLE 2. MASS SPECTRAL DATA FOR THE THREE MAIN COMPOUNDS PRODUCED UPON COMPLETE OZONOLYSIS OF HYDROCARBONS FROM *B. braunii*

Aldehyde fraction

		M	M-18	M-28	M-36	M-43	M-44	M-46	Eight most abundant ions							
Peak 2	Mass	296	278	268	260	253	252	250	55	41	43	57	69	95	67	81
	Abundance	8	13	4	3	11	11	12	1000	900	850	820	530	500	420	390
Peak 3	Mass	324	306	296	288	281	280	278	55	41	43	57	69	82	81	67
	Abundance	14	14	5	5	10	10	11	1000	860	800	800	550	550	500	460

O-Methyloxime fraction

		M	M-31	M-46	M-63	M-71	M-72	M-78	M-88	M-104	Eight most abundant ions							
Peak 1	Mass	326	295	280	263	255	254	248	238	222	43	73	55	41	57	69	71	83
	Abundance	45	72	11	40	25	150	11	12	12	1000	940	740	630	610	450	280	280
Peak 2	Mass	354	323	308	291	283	282	276	266	250	73	43	41	55	86	57	69	56
	Abundance	6	98	20	45	35	160	13	12	11	1000	750	500	500	320	260	260	190
Peak 3	Mass	382	351	336	319	311	310	304	294	278	73	43	55	41	57	86	69	83
	Abundance	5	94	22	48	35	150	13	11	11	1000	660	450	410	310	290	290	200

Methyl ester fraction

		M	M-31	M-64	M-73	M-92	M-105	M-106	M-123	M-146	Eight most abundant ions							
Peak 1	Mass	328	297	264	255	236	223	222	205	182	43	55	57	41	69	74	71	67
	Abundance	4	34	11	29	—	21	13	17	13	1000	880	780	770	580	450	400	240
Peak 2	Mass	356	325	292	283	264	251	250	233	210	55	43	41	57	69	74	98	83
	Abundance	4	73	20	58	13	35	18	15	20	1000	950	750	650	650	650	600	370
Peak 3	Mass	384	353	320	311	292	279	278	261	238	55	43	41	57	98	74	69	83
	Abundance	5	77	24	55	14	38	24	17	10	1000	900	700	670	660	630	620	370

as shown in (iv). This method of analysis does not rigorously exclude the possibility of a branched-chain structure, but it may be stated that no discontinuities could be detected in any of the mass spectra obtained. Since fragmentation is most likely to occur at highly branched carbon atoms,[17] irregularities in the relative abundances of fragmentations, due to C–C fission of long-chain molecules, would not be expected to occur in unbranched molecules. Further, the similarity between the mass spectra from the methyl esters and those recorded by Ryhage and Stenhagen[16] for α,ω-dicarboxylic acid methyl esters lends additional support for the view that these hydrocarbons from *B. braunii* are the unbranched, diunsaturated compounds heptacosa-1,18-diene, nonacosa-1,20-diene and hentriaconta-1,22-diene.

The presence of a terminal double bond (i.e. a vinyl group) in long-chain hydrocarbons of freshwater green algae has been previously reported for unnamed species of *Scenedesmus*[18] and *Chlorella*.[19] The *cis*-disubstituted double bond in each of the hydrocarbons of *B. braunii* is located at the same position with respect to the terminal methyl group of the carbon chain as the double bond of oleic acid. This is consistent with the current theories on hydrocarbon biosynthesis via decarboxylation of the corresponding fatty acid[20] and suggests that, in *B. braunii* at least, decarboxylation of an α,β-unsaturated fatty acid may occur to produce the vinyl group. Oleic acid has been demonstrated as a major component of the fatty acid fraction of the green stage of *B. braunii*[7] but no evidence can be advanced to support the occurrence of long-chain α,β-unsaturated fatty acids in this organism, although such compounds have been isolated from pollen.[21]

EXPERIMENTAL

Botryococcus braunii was obtained from the Cambridge culture collection (culture number 207/1B) and was originally isolated by Droop from Maddingley Brick Pits, England.

Culture conditions. The alga was cultured in modified Chu 13 medium under the high light intensity described previously.[6]

Hydrocarbons. Were isolated by acetone extraction of the dried (rotary evaporator) alga followed by chromatography on alumina.[6] In one isolation the hydrocarbon fraction consisted almost exclusively of the "A" series hydrocarbons and this material was taken for the principal ozonolysis experiment.

Ozonolysis. Ozone was generated from oxygen in a Supelco ozonizer and was bubbled into a solution of hydrocarbons (15 mg) and tetracyanoethylene (8 mg) in CH_2Cl_2 (2 ml) for 90 min. Reaction was carried out at room temp. and the disappearance of hydrocarbons and formation of aldehydes was followed by GLC. In a second experiment, a 30 min reaction time was used. In both cases the mixture obtained was used without further purification in all subsequent experiments.

O-methyloxime. Derivatives were prepared from the aldehyde fraction (5 mg) as previously described.[13]

Acids. The aldehyde fraction (5 mg) was oxidized in acetone solution using excess Jones reagent[22] for a period of 2 min. The acid fraction was isolated via partition into $NaHCO_3$ solution followed by acidification and ether extraction.

Methyl esters. Were prepared using CH_2N_2.

GLC. A Pye 104 model 14 instrument was used with 9 ft × ¼ in columns. Packing materials were 3% OV-17 coated on 100–120 mesh Gas Chrom Q and 5% SE-30 coated on 100–120 mesh Gas Chrom P. Columns were operated isothermally at 244° for hydrocarbons, 230° for dialdehydes, etc., and 205° for the shorter time ozonolysis experiment. Temperature programmed GLC analyses were carried out at 2°/min from 50° to 275°.

GC–MS. Mass spectra were obtained using an LKB 9000 gas chromatograph, fitted with a 10 ft × ¼ in. column, packed with 1% OV-17 coated on 100–120 mesh Gas Chrom Q. Operating conditions were molecular separator temp., 275°, ion source temp., 290°, and electron energy, 70 eV. The temp. of the column was programmed to produce GLC results comparable to those obtained using the Pye 104.

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Note added in proof—Since the acceptance of this paper two reports have appeared presenting new evidence and also proposing new mechanisms with respect to the fragmentations referred to as the McLafferty rearrangement: R. J. LIEDTKE, and C. DJEVASSI, *J. Am. Chem. Soc.* **91**, 6814 (1969); C. FENSELAU, J. L. YOUNG, S. MEYERSON, W. R. LANDIS, E. SELKE and L. C. LEITCH, *J. Am. Chem. Soc.* **91**, 6847 (1969).