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BOTRYOXANTHIN B AND α-BOTRYOXANTHIN A FROM THE GREEN MICROALGA *BOTRYOCOCCUS BRAUNII* KAWAGUCHI-1

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Key Word Index—*Botryococcus braunii*; Chlorophyceae; green alga; structural determination; carotenoid; tetramethylsqualene; botryoxanthin A.

Abstract—Two new carotenoids, botryoxanthin B and α-botryoxanthin A, were isolated from the Kawaguchi-1 strain of the green microalga *Botryococcus braunii* in addition to botryoxanthin A. They had 4-keto-β and ε-end groups instead of the β-end group present in botryoxanthin A. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Botryococcus braunii is a colonial green microalga which produces a large amount of hydrocarbons [1]. It was once believed that this alga had two interconvertible physiological states, green 'active' and orange 'resting', and that it produced different hydrocarbons at the two states [2]. However, it is known that this alga can be subclassified into at least three races, A, B and L, by the types of hydrocarbons present in the different races [3, 4]. The A race produces *n*-alkadienes and/or *n*-alkatrienes. The B and L races produce terpenoid hydrocarbons called botryococcenes and lycopadiene, respectively. The colony colour of the algal strains belonging to the B or L race changes from green in the linear phase of the growth to orange in the stationary phase because the secondary carotenoids (ketocarotenoids) in the colony matrix become more dominant than the primary carotenoids in the cells [5, 6]. Thus the algal colour can not be used as a marker to distinguish between the races of this alga.

Recently, botryoxanthin A (**1**), which is a member of a new class of carotenoids, was isolated from the colony matrix of the Berkeley strain belonging to the B race [7]. Botryoxanthin A is closely related to the hydrocarbons produced by the B race, because this carotenoid contains a tetramethylsqualene moiety which has been detected only in the B race [8, 9]. Therefore, it seemed timely to re-examine the relationship between colony colour and hydrocarbon production of this alga.

The Kawaguchi-1 strain isolated from a Japanese freshwater lake accumulates carotenoids in the colony matrix and looks moss green when cultured under the same conditions as other strains of the B race which exhibit green colour [9]. In the present paper, the structures of two new carotenoids, botryoxanthin B (**2**) and α-botryoxanthin A (**3**), isolated from the colony matrix of the Kawaguchi-1 strain are reported.

RESULTS AND DISCUSSION

The molecular formula of botryoxanthin B (**2**) was determined to be $C_{74}H_{110}O_3$ by the HRFAB mass spectrum (*m/z* 1046.8373 $[M]^+$ $\Delta -8.2$ mmu) and NMR data. This compound had similar UV-VIS absorption to *echinenone* [$\lambda_{max}^{n\text{-hexane}}$ nm (ε) 457 (162000), 294 (25000)]. The ^1H and ^{13}C NMR spectra resembled those of botryoxanthin A as a whole and indicated the presence of an *echinenone* type carotenoid moiety (Table 1). In the ^1H and ^{13}C NMR data of **2**, there were apparent differences from those of botryoxanthin A in the chemical shifts for three methyl groups [Me-16′, 17′ (δ_H 0.95), C-16′, 17′ (δ_C 27.58) and Me-18′ (δ_H 2.15), C-18′ (δ_C 14.27)], and a methylene group [H-3′, (δ_H 2.40), C-3′, (δ_C 34.55)]. The signal of a carbonyl carbon [C-4′ (δ_C 197.16)] was also present. The COSY 45 spectrum allowed the connection between the two methylenes of C-2′ and C-3′ [H-2′ (δ_H 1.50) and H-3′ (δ_H 2.40)]. HMBC correlations between Me-16′, 17′ and C-1′ (δ_C 35.56), Me-16′, 17′ and C-2′, Me-16′, 17′ and C-6′ (δ_C 159.37), H-3′ and C-4′, Me-18′ and C-4′, Me-18′ and C-5′ (δ_C 130.40), and Me-18′ and C-6′ established the presence of a β-end group with a carbonyl group. The other β-end group showed con-

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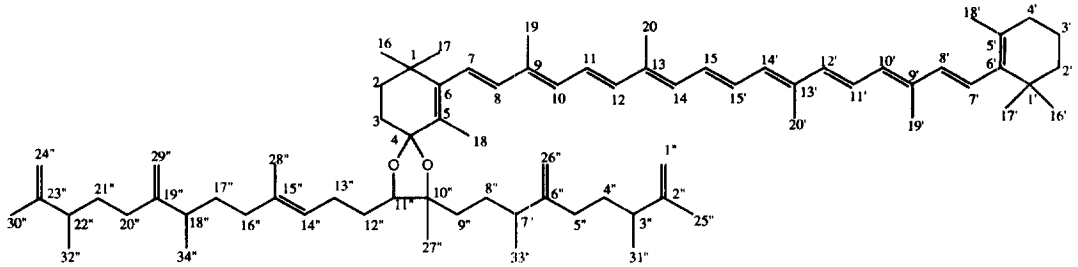

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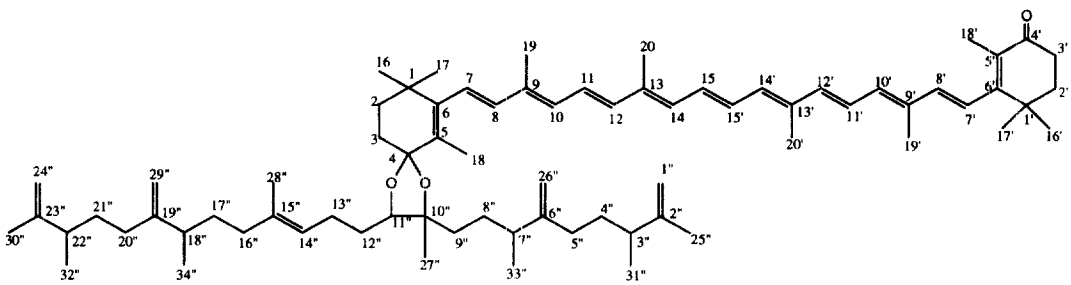

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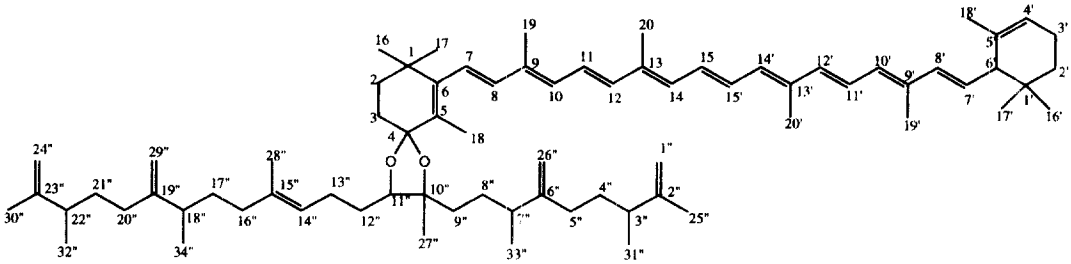

3

nectivity between two methylenes [H-2 (δ_H 1.78, 2.01), H-3 (δ_H 2.09, 2.24)] in the COSY 45 spectrum, and the remaining part of this β-end group was deduced by HMBC correlations. The quaternary carbon C-4 (δ_C 107.39) was assigned as an acetal from the chemical shift. The conjugated polyene chain was assigned by COSY 45, HSQC and HMBC. The coupling constants between H-7 and H-8, H-7′ and H-8′, H-11 and H-12, and H-11′ and H-12′ were 15 to 16 Hz. The PSNOESY spectrum showed correlations between H-7 and Me-19, H-8 and H-10, H-11 and Me-20, H-12 and H-14, H-14 and H-15′, H-7′ and Me-19′, H-8′ and H-10′, H-11′ and Me-20′, and H-12′ and H-14′. There was no *cis*-peak in the UV-VIS spectrum. Therefore the geometry of the conjugated polyene part of this compound was thought to be all-*trans*. The COSY 45, HSQC and HMBC data of the remaining part were almost identical to those of the tetramethylsqualene moiety in botryoxanthin A. Thus the whole structure of botryoxanthin B was determined as **2**.

The molecular formula of α-botryoxanthin A (**3**) was determined to be $C_{74}H_{112}O_2$ by the HRFAB mass spectrum [*m/z* 1032.8690 $[M]^+$ Δ 2.7 mmu] and NMR

Table 1. ^1H and ^{13}C NMR data of botryoxanthin B (**2**) in C_6D_6

Position	H (multi, *J* Hz)	C	HMBC (^1H)	Position	H (multi, *J* Hz)	C	HMBC (^1H)
1		34.98	16, 17	1″	4.82(*br s*) 4.84(*br s*)	110.23	25″
2	1.78(*m*) 2.01(*m*)	36.89	16, 17	2″		149.74	25″, 31″
3	2.09(*m*)	34.69		3″	2.19(*m*)	41.57	25″, 31″
4	2.24(*m*)	107.39	18	4″	1.48(*m*) 1.60(*m*)	33.76	31″
5		131.99	7, 18	5″	2.07(*m*)	32.56	7″, 26″
6		143.75	8, 16, 17, 18	6″		154.74	5″, 8″, 33″
7	6.23(*d*, 15.8)	126.54	8	7″	2.13(*m*)	40.98	8″, 33″
8	6.36(*d*, 15.8)	139.75	10, 19	8″	1.50(*m*) 2.01(*m*)	29.79	7″, 33″
9		135.79	7, 19	9″	1.21(*m*) 1.88(*m*)	33.35	27″
10	6.27(*d*, 11.2)	132.57	8, 12, 19	10″		81.77	11″, 27″
11	6.75(*dd*, 11.2, 15.0)	125.67		11″	4.06(*br d*, 10.0)	86.86	27″
12	6.47(*d*, 15.0)	138.25	20	12″	1.42(*m*) 1.79(*m*)	30.59	
13		137.19	20	13″	2.30(*m*) 2.46(*m*)	26.16	
14	6.32(*m*)	133.22	12, 15, 20, 15′	14″	5.32(*m*)	124.31	13″, 28″
15	6.68(*m*)	131.33		15″		136.15	13″, 16″, 28″
16	1.10(*s*)	29.16	2	16″	2.06(*m*)	37.98	14″, 28″
17	1.09(*s*)	27.40	2	17″	1.45(*m*) 1.67(*m*)	34.40	16″, 18″, 34″
18	2.15(*s*)	15.30		18″	2.12(*m*)	40.07	17″, 29″, 34″
19	1.88(*s*)	12.73	8, 10	19″		154.67	17″, 18″, 20″, 34″
20	1.86(*s*)	12.80	12, 14	20″	1.98(*m*)	32.01	22″, 29″
1′		35.56	2′, 3′, 16′, 17′	21″	1.48(*m*) 1.60(*m*)	33.76	32″
2′	1.50(*m*)	37.64	3′, 16′, 17′	22″	2.14(*m*)	41.38	24″, 30″, 32″
3′	2.40(*t*, 6.5)	34.55	2′	23″		149.71	22″, 30″, 32″
4′		197.16	2′, 3′, 18′	24″	4.79 (*br s*) 4.80 (*br s*)	110.15	30″
5′		130.40	7′, 18′	25″	1.63(*d*, 0.8)	18.88	1″, 3″
6′		159.37	2′, 8′, 16′, 17′, 18′	26″	4.93(*br s*) 4.96(*br s*)	108.08	7″
7′	6.14(*d*)	124.45		27″	1.25(*s*)	22.82	
8′	6.38(*d*, 16.0)	141.24	10′, 19′	28″	1.65(*s*)	16.22	14″, 16″
9′		134.82	7′, 8′, 19′	29″	4.88(*br s*) 4.89(*br s*)	108.02	18″
10′	6.28(*d*, 11.2)	134.76	8′, 12′, 19′	30″	1.60(*d*, 0.8)	18.88	22″, 24″
11′	6.69(*dd*, 11.2, 15.0)	124.84		31″	1.04(*d*, 7.3)	20.03	3″
12′	6.49(*d*, 15.0)	139.75	10′, 20′	32″	1.00(*d*, 6.5)	19.97	22″
13′		136.37	20′	33″	1.13(*d*, 6.7)	20.59	7″
14′	6.35(*m*)	134.28	12′, 20′	34″	1.06(*d*, 6.9)	20.41	18″
15′	6.66(*m*)	130.46					
16′	0.95(*s*)	27.58	2′				
17′	0.95(*s*)	27.58	2′				
18′	2.15(*s*)	14.27					
19′	1.82(*s*)	12.47	8′, 10′				
20′	1.87(*s*)	12.85	12′, 14′				

data. This compound had the similar UV-VIS spectrum to β,ε-carotene [$\lambda_{max}^{n\text{-hexane}}$ nm (ε) 473 (102 000), 445, (112 000), 421(sh), 268 (21 000)]. The ^1H and ^{13}C NMR spectra of this carotenoid resembled those of botryoxanthin A, but some differences were found (Table 2). Two singlets for two methyl groups [Me-16′ (δ_H 0.92), Me-17′ (δ_H 0.97)] appeared separately. The COSY 45 spectrum gave connectivities between the olefinic methine [H-4′ (δ_H 5.45), C-4′ (δ_C 121.27)] and C-3′ methylene [H-3′ (δ_H 2.02), C-3′ (δ_C 23.53)] which was coupled to the C-2′ methylene [H-2′ (δ_H 1.16 and 1.52), C-2′ (δ_C 32.01)]. The sp^3 methine [H-6′ (δ_H 2.25), C-6′

Table 2. ^1H and ^{13}C NMR data of α-botryoxanthin A (**3**) in C_6D_6

Position	H (mult, *J* Hz)	C	HMBC (^1H)	Position	H (mult, *J* Hz)	C	HMBC (^1H)
1		34.96	3, 16, 17	1″	4.81(*br s*) 4.84(*br s*)	110.23	3″, 25″
2	1.77(*m*) 2.01(*m*)	36.89	3, 16, 17	2″		149.71	3″, 4″, 31″
3	2.08(*m*) 2.24(*m*)	34.67		3″	2.19(*m*)	41.56	1″, 4″, 25″, 31″
4		107.41	2, 3, 18	4″	1.49(*m*) 1.60(*m*)	33.74	3″, 5″, 31″
5		131.93	7, 18	5″	2.07(*m*)	32.56	3″, 4″, 7″, 26″
6		143.76	8, 16, 17, 18	6″		154.73	5″, 7″, 8″, 33″
7	6.21(*d*, 15.9)	126.33	8	7″	2.13(*m*)	40.98	8″, 26″, 33″
8	6.35(*d*, 15.9)	139.81	10, 19	8″	1.49(*m*) 2.01(*m*)	29.79	7″, 9″, 33″
9		135.48	7, 8, 11, 19	9″	1.21(*m*) 1.87(*m*)	33.34	7″, 11″, 27″
10	6.27(*d*, 11.6)	132.67	8, 12, 19	10″		81.75	11″, 27″
11	6.74(*dd*, 11.6, 15.0)	125.48	10	11″	4.06(*dd*, 2.7, 10.0)	86.84	13″, 27″
12	6.47(*d*, 15.0)	138.42	10, 14, 20	12″	1.42(*m*) 1.78(*m*)	30.59	13″, 14″
13		136.55	11, 15, 20	13″	2.30(*m*) 2.45(*m*)	26.16	11″, 14″
14	6.31(*m*)	133.43	12, 15, 20, 15′	14″	5.32(*m*)	124.33	13″, 28″
15	6.66(*m*)	130.73	14	15″		136.13	13″, 16″, 17″, 28″
16	1.11(*s*)	29.16		16″	2.05(*m*)	37.98	14″, 17″, 18″, 28″
17	1.09(*s*)	27.40		17″	1.45(*m*) 1.65(*m*)	34.40	16″, 18″, 34″
18	2.15(*s*)	15.30	3	18″	2.12(*m*)	40.07	16″, 17″, 29″, 34″
19	1.88(*s*)	12.71	8, 10	19″		154.65	17″, 18″, 20″, 34″
20	1.86(*s*)	12.85	12, 14	20″	2.00(*m*)	32.01	18″, 21″, 22″, 29″
1′		32.76	2′, 3′, 16′, 17′	21″	1.49(*m*) 1.60(*m*)	33.78	20″, 22″, 32″
2′	1.16(*m*) 1.52(*m*)	32.01	4′, 16′, 17′	22″	2.15(*m*)	41.38	20″, 21″, 24″, 30″, 32″
3′	2.02(*m*)	23.53	2′, 4′	23″		149.71	21″, 22″, 30″, 32″
4′	5.45(*m*)	121.27	2′, 18′	24″	4.79(*br s*) 4.80(*br s*)	110.17	22″, 30″
5′		134.61	3′, 4′, 7′, 18′	25″	1.63(*d*)	18.88	1″, 3″
6′	2.25(*m*)	55.23	2′, 4′, 8′, 16′, 17′, 18′	26″	4.92(*br s*) 4.94(*br s*)	108.08	5″, 7″
7′	5.64(*dd*, 9.9, 15.6)	131.00	8′	27″	1.25(*s*)	22.80	11″
8′	6.26(*d*, 15.6)	136.94	10′, 19′	28″	1.65(*d*)	16.22	14″, 16″
9′		135.52	7′, 8′, 11′, 19′	29″	4.87(*br s*) 4.89(*br s*)	108.00	18″, 20″
10′	6.28(*d*, 11.6)	131.34	8′, 12′, 19′	30″	1.60(*d*)	18.89	22″, 24″
11′	6.74(*dd*, 11.6, 15.0)	125.31	10′	31″	1.03(*d*, 7.0)	20.03	3″, 4″
12′	6.47(*d*, 15.0)	137.87	10′, 14′, 20′	32″	0.99(*d*, 6.7)	20.00	21″, 22″
13′		136.72	11′, 15′, 20′	33″	1.13(*d*, 7.0)	20.59	7″, 8″
14′	6.31(*m*)	133.12	15, 12′, 15′, 20′	34″	1.05(*d*, 7.0)	20.41	17″, 18″
15′	6.67(*m*)	130.53	14′				
16′	0.92(*s*)	27.92	2′				
17′	0.97(*s*)	27.16	2′				
18′	1.71(*s*)	23.32	4′				
19′	1.84(*s*)	13.15	8′, 10′				
20′	1.86(*s*)	12.85	12′, 14′				

(δ_C 55.23)] was also coupled to the olefinic methine [H-7′ (δ_H 5.64), C-7′ (δ_C 131.00)] which is the end of the conjugated polyene in the carotenoid moiety. HMBC correlations between Me-16′, 17′ and C-1′ (δ_C 32.76), Me-16′, 17′ and C-2′, Me-16′, 17′ and C-6′, Me-18′ and C-4′, and Me-18′ and C-5′ (δ_C 134.61) established the ε-end group. The structure of the remaining part was deduced by comparison of the

HSQC, COSY 45 and HMBC data with those of botryoxanthin A. The coupling constants between H-7 and H-8, H-7′ and H-8′, H-11 and H-12, and H-11′ and H-12′ were 15 to 16 Hz. The PSNOESY spectrum showed correlations between H-7 and Me-19, H-8 and H-10, H-11 and Me-20, H-12 and H-14, H-14 and H-15′, H-7′ and Me-19′, H-8′ and H-10′, H-11′ and Me-20′, and H-12′ and H-14′. Therefore the structure of α-botryoxanthin A was determined as **3**.

The occurrence of botryoxanthins in two different strains, the Berkeley and the Kawaguchi-1, suggests a common distribution of these compounds in the B races of *B. braunii*. Therefore, the pigmentation of the colony matrix by botryoxanthins may be used as a marker at least to distinguish the B race from the other races because tetramethylsqualene has been detected only in the B race.

EXPERIMENTAL

General. NMR spectra including COSY 45, HSQC, HMBC and PSNOESY: C_6D_6 500 or 600 MHz referenced to the solvent peak. UV-VIS: *n*-hexane; FABMS: 70 eV using nitrobenzylalcohol as a matrix.

Algal sample. The Kawaguchi-1 strain was cultured under the conditions described elsewhere [9]. A 40-day-old culture of the alga was collected by filtration, lyophilized and stored below −20° until extraction.

Isolation of botryoxanthin B and α-botryoxanthin A. The lyophilized alga (10 g) was extracted with Me_2CO by sonication. The Me_2CO extract was subjected to silica gel CC. After elution of hydrocarbons with *n*-hexane, pigments remaining on the column were eluted with MeOH. The pigment fr. was subjected to ODS CC (YMC AM 170 × 37 mm i.d.) and fractionated into 4 frs with MeCN–CH_2Cl_2–MeOH (1:2:7–1:3:7–0:1:0). The second fr. was subjected to silica gel CC (Wakogel C-300 50 × 37 mm i.d.) with a mobile phase of *n*-hexane–Et_2O (40:1) to remove colourless contaminants. Then an orange fr. was sepd by normal-phase HPLC on a Develosil 60-3 column (250 × 10 mm i.d.) with a mobile phase of *n*-hexane–Me_2CO (93:7) followed by HPLC on a Cosmosil ODS AR column (250 × 10 mm i.d.) with MeCN–CH_2Cl_2 (3:1) to yield botryoxanthin B (**2**, 0.9 mg). The third fr. obtained by the first ODS CC was further sepd by HPLC on Develosil 60-3 (*n*-hexane–Et_2O 49:1) followed by HPLC on Cosmosil ODS AR (MeCN–Me_2CO 2:3) to yield botryoxanthin A (**1**, 3.3 mg) and α-botryoxanthin A (**3**, 1.1 mg).

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