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Carotenoids of *Chrysochromulina polylepis* (Prymnesiophyceae)*

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Key Word Index—*Chrysochromulina polylepis*; Prymnesiophyceae, carotenoid composition; 19′-hexanoyloxyfucoxanthin; ichthyotoxic phytoplankton.

Abstract—The carotenoid composition is reported of the ichthyotoxic phytoplankton *Chrysochromulina polylepis* (Prymnesiophyceae), grown in pure cultures. Carotenoid yields correspond to *ca* 1 mg/20 l culture. The carotenoid composition consisted of (3*S*,5*R*,6*S*,3′*S*,5′*R*,6′*S*)-19′-hexanoyloxyfucoxanthin (81% of total carotenoid), fucoxanthin (10%), diadinoxanthin (9%), 19-hexanoyloxyparacentrone 3-acetate (1%), tentatively diatoxanthin (trace) and β,β-carotene (trace). The identifications included 500 MHz ^{1}H NMR and mass spectral evidence. The high proportion of 19′-hexanoyloxyfucoxanthin, readily detected by HPLC, serves to characterize this microalga.

Introduction

A bloom of the prymnesiophyte *Chrysochromulina polylepis* Manton and Parke 1962 [1] developed in Kattegat and Skagerrak, off the coasts of Denmark and Sweden in May 1988 and spread northwards towards Norwegian waters. There is a concentrated effort in Scandinavian countries not only to describe and characterize the bloom, but also to understand its initiation and the mechanisms by which this species was so successful in maintaining such high concentrations (up to 80×10^6 cells l^{-1}). The bloom caused severe fish killing and affected other marine life [2–4].

This paper describes the carotenoids of *C. polylepis*. Chlorophyll composition and *in vivo* absorption and fluorescence spectra were reported separately [5]. Together these data serve to characterize the spectral absorption and photosynthetic activity of this species.

Results and Discussion

Chrysochromulina polylepis, reisolated by Paasche from the natural bloom, was grown in pure culture as four batches in f/2 medium.

Yields of carotenoids were *ca* 1 mg carotenoid/20 l culture. Chromatographic separation was carried out by rp-HPLC (batch 1, Table 1) or TLC combined with normal phase semi-preparative HPLC (batches 2–4, Table 1). The individual carotenoids, Scheme 1, are treated in order of increasing polarity.

TABLE 1. CAROTENOIDS OF *CHRYSOCHROMULINA POLYLEPIS*

	Carotenoids in % of total carotenoid			
Carotenoid	Batch 1*	Batch 2	Batch 3	Batch 4
β,β-Carotene (**1**)	3		trace	
Diatoxanthin (**2**)	1		—	
Diadinoxanthin (**3**)	12		3 } 9	
Diadinochrome (**3b**)	—		6 }	
19-Hexanoyloxyparacentrone 3-acetate (**4**)	2		1	
Fucoxanthin (**5**)	14		10	
19′-Hexanoyloxyfucoxathin (**6**)	63 } 67		75 } 81	
9′-*cis*-19′-Hexanoyloxyfucoxanthin (**6a**)	4 }		6 }	
Total carotenoid in mg	<0.33	<0.61 (0.18)†	<0.85 (0.36)†	<0.87 (0.56)†
Carotenoids in % of dry wt‡	—	—	—	<0.35 (0.22)†
Culture volume (l)	8	29.5	15	15

*rp-HPLC estimate, batch 1 was grown at 73 and batches 2, 3 and 4 at 120 μmol quanta m^{-2}, s^{-1}.
†After TLC.
‡After acetone extraction.

*Part 44 in the series "Algal Carotenoids". For Part 43 see *Phytochemistry* (1989) **28**, 2797.

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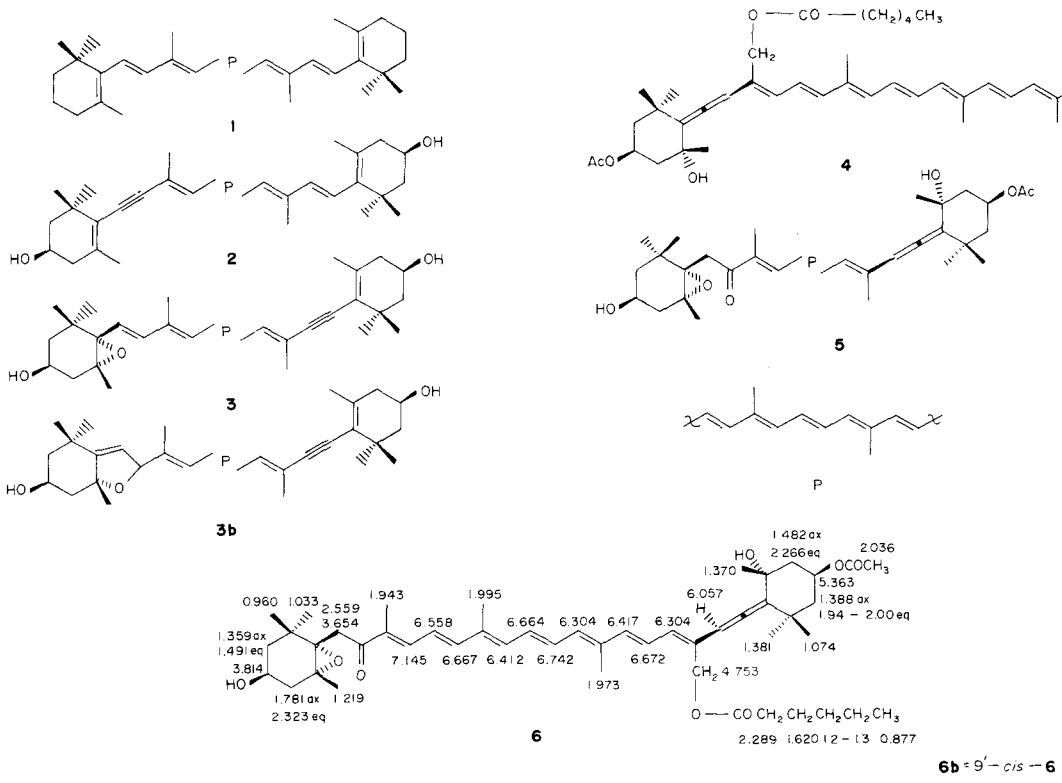

SCHEME 1.

β,β-Carotene (**1**, ≤3% of total carotenoid), characterized from batch 1 only by rp-HPLC and VIS absorption spectrum, represented a remarkably small carotene: xanthophyll ratio.

The acetylenic diol diatoxanthin (**2**) was detected in batch 1 (1% of total carotenoid) and characterized by rp-HPLC and VIS absorption spectrum only.

The epoxidic counterpart diadinoxanthin (**3**, 3–12%) was isolated as such and as the furanoid rearranged diadinochrome (**3b**, ≤6%) after longer work-up procedures. The identification of **3** was based on VIS absorption spectrum, MS, co-chromatography by HPLC with authentic **3** and rearrangement to **3b** in the presence of acid. Compound **3b** was identified by VIS absorption spectrum, MS and co-chromatography with an authentic sample.

The apo-carotenoid 19-hexanoyloxyparacentrone 3-acetate (**4**, 1% of total) was characterized by HPLC, VIS absorption spectrum and MS.

Fucoxanthin (**5**, 10% of total), separated from its 19′-hexanoyloxy derivative (**6**) by semi-preparative HPLC, was identified from VIS absorption spectrum, MS and co-chromatography with an authentic sample.

(3*S*,5*R*,6*S*,3′*S*,5′*R*,6′*S*)-19′-Hexanoyloxyfucoxanthin (**6**, 81% of total) was the major carotenoid. The HPLC-purified all-*trans* isomer was identified from its VIS absorption spectrum, exhibiting better spectral fine structure per cent III/II [6] than **5** [7], MS, ^{1}H NMR (500 MHz) with complete assignments, FT-IR, including the allene absorption and CD. The present high-field ^{1}H NMR data are superior to the earlier 100 MHz data [7, 8]. Assignments made by comparison with the fully interpreted 400 MHz ^{1}H NMR spectrum of 19′-butanoyloxyfucoxanthin [9] are included in Scheme 1. A minor component isolated by normal and rp-HPLC was suspected to represent 19′-butanoyloxyfucoxanthin [10], but proved to be the 9′-mono-*cis* isomer **6b** in direct HPLC (normal phase) comparison with the iodine

catalysed quasi-equilibrium mixtures prepared here of **6** and of 19′-butanoyloxyfucoxanthin *ex Pelagococcus subviridis.* As for the 19′-butanoyloxy derivative [10] the 9′-*cis* isomer was assumed to be the major isomer in the quasi-equilibrium mixture besides all-*trans.* The VIS absorption spectrum and MS for **6b** isolated here confirmed this conclusion.

The present results are compatible with the early TLC data reported by Riley and Wilson [11] for the Plymouth isolate. Moreover, our data demonstrating the presence of acetylenic carotenoids and fucoxanthin derivatives clearly place *C. polylepis* among the second pigment type of the Prymnesiophyceae which synthesizes 19′-acyloxyfucoxanthins in addition to **5** [12, 13]. The minor apocarotenoid **4** was first encountered in the prymnesiophyte *Emiliana* (*Coccolithus*) *huxleyi* [7]. The carotenoid distribution pattern and in particular the high proportion of **6**, readily detected by HPLC but not by TLC [7], serves to characterize this microalga.

Experimental

Biological material. The isolate of *C. polylepis* was obtained from Prof. E. Paasche, Department of Marine Botany, University of Oslo. This strain originates from the bloom that occurred in southern Norwegian waters in May–June 1988. The culture was maintained in f/2 medium [14], at 15°C and 34‰ salinity and at a scalar irradiance of either 73 (batch 1) or 120 (batches 2–4) μmol quanta m^{-2} s^{-1}. Culture volumes for the batches analysed are given in Table 1. The cultures were harvested in the late exponential growth phase. Cell densities (cells l^{-1}) were about 58.10^6 (batch 1), 120.10^6 (batch 2), 200.10^6 (batch 3) and 240.10^6 (batch 4).

Methods. If not otherwise specified, instruments and general physical and chemical methods were as specified elsewhere [15]. General precautions for work with carotenoids were taken.

rp-HPLC was carried out on a Hitachi HPLC instrument using a C-18 column, Brownlee 25 cm×4.6 mm, 5 μm particle size. Pigments were eluted in a low-pressure gradient system consisting of a linear gradient from 100% A to 100% B in 10 min and maintaining B for another 15 min. Solvent A consisted of 80:20 methanol:water that contained an ion-pairing agent [16]. Solvent B consisted of 60:40 methanol:ethyl acetate. Pigments were monitored by absorption at 440 nm, and quantified by calibration of the column with authentic pigments. Absorption spectra of the eluted pigments were recorded in the eluent on a Hitachi spectrophotometer Model U-2000 filled with a flow-through cell.

Normal phase HPLC was carried out on a Perkin Elmer Liquid Chromatograph Series 2 connected with a Pye Unicam PU 4021 Multichannel detector and a Merck Hitachi D-2000 Chromato-Integrator using a Spherisorb S-5CN column 25 cm×4.6 mm, particle size 5 μm, isocratic elution (*n*-hexane:*i*-propyl acetate:acetone:methanol in ratio 76:17:7:0.1), flow rate 1.5 ml min^{-1} and detector set point 445 nm. Semi-preparative separations of **5**, **6** and **6b** were effected with the same system and the individual components collected after 20 injections with 12–13-min intervals.

VIS absorption spectra refer generally to acetone using $E^{1\%}_{1cm}$=1650 (fucoxanthin), $E^{1\%}_{1cm}$=1500 (19′-hexanoyloxyfucoxanthin) and $E^{1\%}_{1cm}$=2500 (other carotenoids). Spectral fine-structure is defined as % III/II [6]. FT-IR spectra were recorded on a Nicolet 20 S×C FT-IR spectrometer.

Isolation of the carotenoids. Batch 1: cells were collected on Whatman GF/C filters under 50 mb of differential pressure. Pigments were extracted overnight at 4°C in the dark with 90% acetone. Extracts were cleared by filtration through Whatman GF/C filters and injected onto rp-HPLC column without further treatment; R_T (min) **5** (9.9), all-*trans* **6** (10.3), **6b** (11.0), **3** (11.4), **2** (11.9), **4** (12.1).

Batches 2–4: cells were collected by centrifugation and stored at −20°C prior to solvent extraction with acetone:methanol (7:3). The maximal total carotenoid content was estimated spectrophotometrically, see Table 1. Preparative TLC was carried out on kieselgel 60 DC Alufolien (Merck 5553) using hexane:acetone:*i*-propanol (68.5:40:1.5) as developer. The total extracts from each batch were separated into two fractions which were pooled, fraction 1 containing **1**, **2**, **3**, **3b** and **4** and fraction 2 containing **5**, **6** and **6b**. Fraction 1 was rechromatographed on alkaline plates [17] using hexane:acetone:*i*-propanol:benzene (57:33:1.3:17) as developer. Fraction 2 was submitted to semipreparative normal phase HPLC as described above.

R_F-values refer to TLC (SiO_2, 40% acetone in hexane), R_T-values if not otherwise specified to normal phase HPLC specified above.

β,β-*Carotene* (**1**). Available *ca* 1 μg; VIS λ_{max} nm (428), 454 and 479 (rp-HPLC eluent); (422), 447 and 472 (ether); rp-R_T see above.

Diatoxanthin (**2**). Available *ca* 3 μg, VIS λ_{max} nm 453 and 480 (rp-HPLC eluent); rp-R_T see above.

Diadinoxanthin (**3**). Available *ca* 70 μg, R_F=0.25, R_T=9.94 min; inseparable from authentic **3** by TLC and HPLC; VIS λ_{max} nm 424, 445 and 473, % III/II=36; MS (70 eV, 230°C) *m/z* (%): 582 $[M]^+$ (88); 580 $[M-2]^+$ (34); 566 $[M-16]^+$ (15); 564 $[M-18]^+$ (8); 562 $[M-2-18]^+$ (6); 548 $[M-16-18]^+$ (4); 502 $[M-80]^+$ (5); 490 $[M-92]^+$ (7); 291 $[M]^{2+}$ (2); 221 [homopyryllium]$^+$ (100); 181 [furyllium]$^+$ (43).

9′-*cis*-Diadinoxanthin had R_F=0.30; VIS λ_{max} nm (422), 441 and 467; % III/II=33.

Furanoid arrangement of **3** (10 μg) in ether in the UV/VIS cuvette upon addition of two to three drops 0.03 N HCl in $CHCl_3$ occurred during 2 min and resulted in a 19 nm hypsochromic shift of λ_{max}. The product was indistinguishable (TLC, HPLC, VIS) from **3b**.

Diadinochrome (**3b**). Available *ca* 42 μg, R_F=0.32, R_T=7.82 min, inseparable from authentic **3b** by TLC and HPLC; VIS λ_{max} nm 408, 429 and 456, % III/II=54; MS (70 eV, 210°C) *m/z* (%) 582 $[M]^+$ (100); 580 $[M-2]^+$ (27); 567 $[M-15]^+$ (5); 566 $[M-16]^+$ (5); 564 $[M-18]^+$ (4); 551 $[M-15-16]^+$ (4); 549 $[M-15-18]^+$ (4); 502 $[M-80]^+$ (10); 490 $[M-92]^+$ (9); 487 $[M-80-15]^+$ (4); 475 $[M-92-15]^+$ (4); 440 $[M-142]^+$ (3); 436 $[M-146]^+$ (6); 430 $[M-152]^+$ (5); 412 $[M-170]^+$ (7); 402 $[M-180]^+$ (7); 383 (64); 352 (23), 291 $[M]^{2+}$ (3); 221 (86); 181 (47); 91 (93).

19-*Hexanoyloxyparacentrone* 3-*acetate* (**4**). Available 5 μg, R_F=0.33, R_T=13.52 min; VIS λ_{max} nm 447 and 446, % III/II=8, MS (70 eV, 210°C) *m/z* (%) 618 $[M]^+$ (32); 600 $[M-18]^+$ (100); 576 $[M-42\ (C_2H_2O)]^+$ (79); 560 $[M-58]^+$ (23); 558 $[M-42-18]^+$ (13); 540 $[M-60-18]^+$ (24); 502 $[M-116]^+$ (72).

Fucoxanthin (**5**). Available 68 μg, R_F=0.24, R_T=20.77 min, inseparable from authentic **5** by TLC and HPLC; VIS λ_{max} nm (420), 447 and 469, % III/II=3; MS (70 eV, 230°C) *m/z* (%) 658 $[M]^+$ (48); 640 $[M-18]^+$ (44); 624 $[M-16-18]^+$ (12); 622 $[M-18-18]^+$ (13); 580 $[M-18-60]^+$ (22); 562 $[M-18-80]^+$ (5); 221 (76); 211 (37); 197 (100).

(3S,5R,6S,3′S,5′R,6′S)-19′-*Hexanoyloxyfucoxanthin* (**6**). Available *ca* 0.4 mg, R_F=0.24, R_T=22.41 min, inseparable from authentic **6** [7] by HPLC; VIS λ_{max} nm (423), 444 and 471, % III/II=43; MS (70 eV, 230°C) *m/z* (%) 772 $[M]^+$ (2); 754 $[M-18]^+$ (13); 752 $[M-2-18]^+$ (3); 738 $[M-16-18]^+$ (5); 736 $[M-18-18]^+$ (6); 694 $[M-18-60]^+$ (4); 676 $[M-18-18-60]^+$ (3); 662 $[M-18-92]^+$ (2), 658 $[M-114]^+$ (3); 656 $[M-116]^+$ (3); 640 $[M-18-114]^+$ (6); 638 $[M-18-116]^+$ (7); 622 $[M-16-18-116]^+$ (3); 620 $[M-18-18-116]^+$ (3); 602 $[M-18-60-92]^+$ (2); 600 $[M-172]^+$ (2); 580 $[M-190]^+$ (2); 578 $[M-192]^+$ (3); 576 $[M-18-60-116]^+$ (2); 560 $[M-18-18-60-116]^+$ (3); 536 $[M-236]^+$ (3); 474 $[M-298]^+$ (4); 386 (4); 326 (35); 311 (27); 271 (10); 247 (12); 221 (42); 195 (100); ^{1}H NMR (500 MHz, $CDCl_3$, 0.4 mg, 472 scans) δ (relative to $CHCl_3$ 7.26 ppm) 0.88 (*t*, *J*=6.9 Hz, 3H, CH_3 hexanoyl), 0.96 (*s*, 3H, Me-17), 1.03 (*s*, 3H, Me-16), 1.07 (*s*, 3H, Me-17), 1.22 (*s*, 3H, Me-18), 1.2–1.3 (*m*, *ca* 4H, γ,δ-CH_2 in hexanoyl), 1.36 (*t*, *J*=12.8 Hz, H-2 ax), 1.37 (*s*, 3H, Me-18′), 1.38 (*s*, 3H, Me-16′), 1.39 (*t*, *J*=12.0 Hz, H-2′ ax), 1.48 (*t*, *J*=12 Hz, H-4′ ax), 1.49 (*t*, *J*=11.9 Hz, H-2 eq), 1.62 m (2H?, β-CH_2 in hexanoyl), 1.78 (*dd*, 1H, H-4 ax), 1.94 (*s*, 3H, Me-19) 1.97 (*s*, 3H, Me-20′), 1.94–2.00 (H-2′ eq), 2.00 (*s*, 3H, Me-20), 2.04 (*s*, 3H, acetate Me), 2.26 (*m*, 1H, H-4′ eq), 2.29 (*t*, *J*=7.4 Hz, 2H, α-CH_2 in hexanoyl), 2.32 (*d*, 1H, H-4 eq), 2.56 (*d*, *J*=18.3 Hz, 1H, H-7), 3.65 (*d*, *J*=18.3 Hz, 1H, H-7), 3.81 (*m*, 1H, H-3), 4.75 (*d*, *J*=11.7 Hz, H-19′), 4.80 (*d*, *J*=11.8 Hz, H-19′), 5.36 (*m*, H-3′), 6.06 (*s*, 1H, H-8′), 6.30 (*d*, *J*=11.6, 1H, H-10′ or H-14′), 6.41 (*d*, *J*=10 Hz, 1H, H-14), 6.42 (*d*, *J*=15.1 Hz, 1H, H-12′), 6.59 (*dd*, J_1=15.1 Hz, J_2=10.8 Hz, 1H, H-11), 6.66 (*dd*, J_1=14.4 Hz, J_2=11.2 Hz, H-15), 6.67 (*d*, *J*=14.9 Hz, 1H, H-12), 6.67 (*dd*, J_1=15.2 Hz, J_2=11.2 Hz, 1H, H-11′), 6.74 (*dd*, J_1=14.4 Hz, J_2=11.4 Hz, H-15′, 7.15 (*dd*, *J*=11.0 Hz, *J* allylic=1.3 Hz, H-10). Assignments (see Scheme 1) were made by comparison with the fully interpreted 400 MHz ^{1}H NMR spectrum of all-*trans* and 9′-*cis* 19′-butanoyloxyfucoxanthin [10]; FT-IR (0.4 mg in KBr, 32 scans, ν_{max}; 3380s (OH); 2959s, 2922s and 2854s (CH); 1930w (C=C=C); 1773s (C=O, ester); 1656m (konj. C=O); 1608m, 1577w (C=C); 1533w; 1457m (CH_2); 1384m and 1365 (CH_3); 1262 and 1249m (ester); 1231w; 1202w; 1166m (tert. OH, –C–CO–C); 1031s (sec. OH); CD [nm (Δε), EPA=diethyl ether:*iso*-pentane:ethanol in ratio 5:5:2]; 221 (0); 234 (+0.70); 252 (0); 273 (–0.48); 279 (–0.40); 297 (0).

9′-cis-19′-*Hexanoyloxyfucoxanthin* (**6b**). R_F=0.24, R_T=28.24 min, inseparable by HPLC from the major *cis*-isomer prepared by iodine catalysed stereomutation of **6**, and differed by HPLC from all-*trans* and 9′-*cis* 19′-butanoyloxyfucoxanthin prepared by similar stereomutation. The iodine catalysed isomerizations were carried out with the samples (0.2 mg) in benzene (5 ml) and traces of iodine in hexane for 2 h in diffuse daylight and monitored by HPLC. Compound **6b** had VIS λ_{max} nm (419), 440, 465, % III/II=31. MS (70 eV, 230°C), *m/z* (%); 772 $[M]^+$ (0.3); 754 $[M-18]^+$ (1); 752 $[M-2-18]^+$ (1); 736 $[M-18-18]^+$ (2); 694 $[M-18-60]^+$ (1); 676 $[M-18-18-60]^+$ (1); 662 $[M-18-92]^+$ (2); 638 $[M-18-116]^+$ (2); 622 $[M-16-18-116]^+$ (1); 620 $[M-18-18-116]^+$ (1); 195 (100).

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