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CHLOROPHYLLS AND CAROTENOIDS OF THE MARINE ALGA *EUTREPTIELLA GYMNASTICA*

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Key Word Index—*Eutreptiella gymnastica*; Euglenophyceae; chlorophylls; acetylenic carotenoids; neoxanthin; unidentified carotenoids.

Abstract—Monoalgal cultured *Eutreptiella gymnastica* contained chlorophyll *a* and *b*. The acetylenic carotenoids diatoxanthin and diadinoxanthin were among the main xanthophylls while their non-acetylenic analogues zeaxanthin and antheraxanthin were absent. The structurally most complex carotenoid was identical with neoxanthin. Three of the xanthophylls isolated could not be positively correlated with carotenoids previously reported from the Euglenophyceae. The ketocarotenoids astaxanthin, canthaxanthin and echinenone were absent.

INTRODUCTION

The number of autotrophic Euglenophyceae which has been analysed for chlorophylls and carotenoids is small. They all belong to the genera *Euglena* and *Trachelomonas* of the order Euglenales (systematics according to ref. [1]) and they are all freshwater representatives.

In earlier works some of the euglenophycean xanthophylls may have been confused with the common chlorophycean carotenoids antheraxanthin (**1**), lutein (**2**), violaxanthin (**3**) and zeaxanthin (**4**) [2–11]. The identity of the xanthophylls of *Euglena gracilis* with diadinoxanthin (**5**) and diatoxanthin (**6**) was revealed by Aitzetmüller *et al.* [12] and Johannes *et al.* [13], respectively. In both cases extensive use of modern physical methods (including IR, MS and ^{1}H NMR) ensured a reliable identification.

It is generally agreed that the structurally most complex carotenoid of the Euglenophyceae is identical with neoxanthin (**7**) [4, 5, 7–11, 14–17]. The chloroplasts of the Euglenophyceae thereby possess a biochemical similarity with the chloroplasts of the other two neoxanthin (**7**)-containing classes of the Chlorophyta, namely the Prasinophyceae and the Chlorophyceae. This similarity is opposed, however, by the presence of acetylenic xanthophylls in the Euglenophyceae and non-acetylenic xanthophylls in the two other classes. For surveys on carotenoid distribution within the Phycophyta, see refs. [18–20].

The present investigation extends our knowledge about euglenophycean pigments to *Eutreptiella gymnastica* (order Eutreptiales). This is also the first representative of the class from marine habitats which has been analysed for chlorophylls and carotenoids.

RESULTS AND DISCUSSION

The pigments were chromatographically pure and only exceptionally obtained in the crystalline state. Their identities were confirmed by co-chromatography [chlorophylls: paper (S&S 287) and TLC; carotenoids: TLC on two adsorbents] with authentic samples. In addition, the carotenoids were further characterized by UV/VIS, IR, ^{1}H NMR, MS, MS of their acetylated and silylated derivatives and by acid-catalysed epoxide-furanoid rearrangement. The qualitative and quantitative pigment composition of *E. gymnastica* is compiled in Table 1.

The carotenoid composition of *E. gymnastica* is, however, far more complex than revealed by Table 1. At least five more carotenoids were present in trace amounts, all of which were less polar than diadinoxanthin (**5**) on TLC (Si gel–$CaCO_3$, 1:1). The purification of these minor pigments is laborious and must rely on a sophisticated combination of different chromatographic systems. Their identification must be postponed until more biological material is available.

E. gymnastica contained chlorophyll *a* and *b* and thereby possessed the same chlorophyll complement as the other classes of the Chlorophyta [21]. No traces of more polar chlorophylls (degradative or native) could be observed on the chromatograms. Thus chlorophyll c_1 and c_2, which are regular components in the majority of classes within the Chromophyta [21, 22], were absent.

The carotene fraction consisted of β,ϵ-carotene (**8**) and β,β-carotene (**9**), both of which were identified by co-chromatography, UV/VIS and MS. The high III/II-value of 60% ensured that the β,ϵ-carotene (**8**) was not confused with *cis*-isomerized β,β-carotene (**9**). Within the Euglenophyceae, β,ϵ-carotene (**8**) has previously been detected in *Euglena gracilis* var. *bacillaris* [11] and the heterotrophic *Astasia ocellata* [23].

The xanthophylls of intermediate polarity possessed properties consistent with the acetylenic carotenoids diatoxanthin (**6**) and diadinoxanthin (**5**) while

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Table 1. Chlorophylls and carotenoids of *E. gymnastica*

Pigment	Amount (mg/g dry wt*)	(%)	Absorption maxima† (nm)			III/II (%)	R_f‡
Chlorophyll *a*	33	—	384 579	(414) 615	432 663	—	0.78
Chlorophyll *b*	21	—	(434) 646	457	597	—	0.75
β, ε-Carotene (**8**)	0.02	0.2	421	443	473	60	1.00
β, β-Carotene (**9**)	0.3	3	(427)	448	474	23	1.00
Unknown 1	2.2	20	(438)	458	487	50	0.97
Unknown 2	0.1	1	(440)	462	490	26	0.80
Diatoxanthin (**6**)	3.2	30	(434)	454	482	35	0.67
Unknown 3	2.3	21	(434)	453	480	10	0.64
Diadinoxanthin (**5**)	2.0	18	(428)	449	479	69	0.59
Neoxanthin (**7**)	0.7	6	413	438	466	87	0.25

*Lipid-extracted cells.

†Chl. *a*, chl. *b*, **5**, **6** and **7** in acetone; **8**, **9**, Unknown 1 (cryst.), Unknown 2 and Unknown 3 in petrol. Absorption maxima of Unknowns 1–3 were obtained from a separately cultured batch of algal cells.

‡TLC: Si gel–$CaCO_3$ (1:1), petrol–Me_2CO–C_6H_6–*iso*-PrOH (69.5 : 25 : 4 : 1.5).

their non-acetylenic analogues zeaxanthin (**4**) and antheraxanthin (**1**) were absent. In this respect, the present results are in accord with previous investigations with modern methods [12, 13]. Diatoxanthin (**6**) and diadinoxanthin (**5**) cannot be unequivocally distinguished from zeaxanthin (**4**) and antheraxanthin (**1**) by UV/VIS and IR [12, 24], but are more firmly retained on alkaline adsorbents [25]. The 7, 8-located triple bond of diatoxanthin (**6**) and diadinoxanthin (**5**) was revealed both by MS ($[M]^+$ and apparent absence of the $[M-106]^+$ fragment) and (notably) by ^1H NMR. Both xanthophylls possessed one end-group (d_1) with proton magnetic signals influenced by a triple bond in the 7, 8-position [diatoxanthin (**6**): δ 1.15, 1.20 and 1.92; diadinoxanthin (**5**): δ 1.15, 1.19 and 1.93] [24]. In addition, diatoxanthin (**6**) possessed one end-group (d_1) with proton magnetic properties as in zeaxanthin (**4**) (δ 1.08, 1.08 and 1.74) [24] while diadinoxanthin (**5**) possessed one end-group (f_1) with proton magnetic properties as in violaxanthin (**3**) (δ 0.98, 1.15 and 1.19) [26]. The epoxide group of diadinoxanthin (**5**) was further demonstrated by the proton signal at δ 1.93 of the in-chain methyl group at C-9 [26], mass spectral peaks at m/z 566 $[M-16]^+$, 502 $[M-80]^+$, 221 (263 for the diacetate) and 181 (223 for the diacetate) [24] and the positive epoxide test. The ^1H NMR spectra of both diatoxanthin (**6**) and diadinoxanthin (**5**) contained a signal at δ 2.00 equivalent to three protons of one in-chain methyl group. This is further support for a 7, 8 (7′, 8′)-location of the triple bond.

The chromatographically most polar xanthophyll possessed chemical and physical properties as expected for neoxanthin (**7**). Acetylation and silylation tests and subsequent MS of the reaction products revealed two primary/secondary hydroxyl groups and one tertiary hydroxyl group. The positive epoxide test further indicated that a fourth oxygen atom was present as an epoxide group. The epoxide nature of this xanthophyll was further supported by the mass spectral properties of the xanthophyll itself and its acetylated and silylated derivatives, as already described for diadinoxanthin (**5**). The allenic bond gave a characteristic IR band at 1940 cm^{-1} and its presence was further confirmed by the ^1H NMR signal at δ 1.80 (Me-9) [24]. The ^1H NMR spectrum possessed the expected methyl signals for end-group f_1 (δ 1.01, 1.16 and 1.21) [26] and end-group i_1 (δ 1.07, 1.34 and 1.34) [24]. However, the spectrum was slightly contaminated and of low intensity, and the expected signal at δ 1.92–1.93 (Me-9′) could not be distinguished in the broad signal at δ 1.96 (Me-13, 9′ and 13′).

Heteroxanthin (**10**) was not detected in the present investigation (cf. ref. [17]).

Members of the Euglenophyceae have been reported to contain ketocarotenoids such as echinenone (**11**) [3, 4, 8, 16, 27], canthaxanthin (**12**) [23, 27], astaxanthin (**13**) or its esters [5, 10, 16, 28] and other ketocarotenoids with less well-established structures. Orange and red ketocarotenoids have been claimed to be associated with the eyespot [4], a subcellular structure also present in *E. gymnastica* 1291. Conjugated ketocarotenoids with a polyene system as in echinenone (**11**) or canthaxanthin (**12**) do not possess fine structure in the UV/VIS spectrum even with petrol as a solvent [24]. Unknowns 1–3 are accordingly different from these and similar ketocarotenoids. Direct comparison on TLC (Si gel–$CaCO_3$, 1 : 1) demonstrated that Unknown 1 was less polar and distinctly more orange in colour than the brick-red echinenone (**11**). Unknowns 2 and 3 may, on the other hand, be easily misidentified on TLC (Si gel–$CaCO_3$, 1 : 1) with carotenoids repeatedly reported within the Euglenophyceae, namely canthaxanthin (**12**) [23, 27], β-cryptoxanthin (**14**) [3, 4, 6, 8, 10, 16, 27] and astaxanthin (**13**) [5, 10, 16, 28]. These pigments may nevertheless be distinguished by their UV/VIS spectral properties. Unknown 3 (unlike Unknowns 1 and 2) exhibited fine structure in the UV/VIS spectrum, which depended strongly on the solvent polarity. Further, alkali treatment quantitatively transformed this pigment to a more polar product with unchanged spectral properties. Unknown 3 is, therefore, most probably an ester with a keto function in conjugation with the polyene chain. An unidentified xanthophyll ester with similar UV/VIS spectral properties has been reported in isolated stigmata of *Euglena gracilis* 1224/5z ('strain z') [27]. However, examination of saponified and unsaponified extracts of whole cells of *E. gracilis* 1224/5z on TLC (Si gel–$CaCO_3$, 1:1) revealed that Unknown 3 (as well as Unknowns 1 and 2) was absent in this strain.

Further structural elucidation of Unknowns 1–3 has been undertaken in co-operation with Anne Fiksdahl and Synnøve Liaaen-Jensen, Norwegian Institute of Technology, University of Trondheim, and will be published separately.

EXPERIMENTAL

Biological material. E. gymnastica Throndsen was isolated by S. Saetrang from a serial dilution culture. The seawater sample for the dilution culture was taken at Nakkholmen, the inner part of the Oslo fjord, Norway, on 12 August 1964. The isolate is the type culture of the species and is maintained in the culture collection of the Section of Marine Botany, University of Oslo. A detailed fine structural investigation of the species as well as data on its distribution and ecology have been published [29, 30].

Culture conditions. Monoalgal culture of *E. gymnastica* was carried out in 300 conical flasks, each containing 300 ml of the enriched seawater medium IMR [31]. The salinity was adjusted to 25 parts per thousand with glass-distilled H_2O prior to the enrichment. The illumination was continuous with Philips fluorescent tubes (TL/32 and TL/33 from above, TL/55 from below). The light intensity was 45 $\mu E/m^2$ per sec as measured with a LI-188 integrating quantum photometer fitted with a 4π sensor (Lambda Instruments Corp.). The temp. was 15°. The algal material was harvested by continuous centrifugation (Kahlsico 903-1S) after 13 days. The dry-wt of the harvested cells was 2.82 g after extraction.

Extraction and chromatography. The harvested algal material was extracted immediately with Me_2CO, Me_2CO–MeOH (7:3) and MeOH until the residue was colourless.

The pigments were separated and purified by successive TLC on either one or both of the following adsorbents: Si gel –$CaCO_3$ (1:1) (TLC-I) and Si gel–$Ca(OH)_2$–MgO–$CaSO_4$ (10:4:3:1) (TLC-II). The developing solvents were different mixtures of petrol, C_6H_6, Me_2CO and *i*PrOH. The chlorophylls were purified on TLC-I while the carotenoids were purified on both TLC-I and -II.

One-dimensional circular paper chromatography was carried out on Schleicher & Schüll No. 287 (S&S 287) [32].

The identity of each isolated pigment was confirmed by co-chromatography with an authentic sample (chlorophylls: paper S&S 287 and TLC-I; carotenoids: TLC-I and -II). The authentic samples were obtained from the following sources: chlorophyll *a* and *b*: *Hordeum vulgare*; β, ϵ-carotene (**8**) and β, β-carotene (**9**): *Daucus carota*; diatoxanthin (**6**) and diadinoxanthin (**5**): *Thalassiosira decipiens* and neoxanthin (**7**): *H. vulgare*. In addition, the R_fs of the following carotenoids were established: antheraxanthin (**1**) (= zeaxanthin-5, 6-epoxide): *Lactuca sativa*; canthaxanthin (**12**): synthetic from Hoffmann–La Roche; β-cryptoxanthin (**14**): calyx of *Physalis alkekengi*; enchinenone (**11**): synthetic from Hoffmann–La Roche; lutein (**2**): *Medicago sativa*; violaxanthin (**3**): petals of yellow-flowered *Viola tricolor*; zeaxanthin (**4**): calyx of *P. alkekengi* and astaxanthin (**13**): *Haematococcus pluvialis*.

Physical and chemical methods. The amounts of chlorophyll *a* and *b* were calculated from the visible light spectrum of the total extract [33]. $E^{1\%}_{1\,cm} = 2500$ was used for the quantitative determination of chromatographically pure carotenoids. MS (AEI MS 902): 70 eV and 190–225°.

Alkaline hydrolyses was performed with KOH (5%) in MeOH at 20° for 12 hr. Acid-catalysed epoxide-furanoid rearrangement with HCl [34], acetylation [35] and silylation (SYLON BTZ, Supelco Inc.) [36] were carried out as described in the cited references.

Pigment data. The amounts (mg/g dry wt), TLC properties and UV/VIS spectra of the pigments are given in Table 1.

β, ϵ-Carotene (**8**). MS *m/z* (rel. int.): 536 $[M]^+$ (88), 480 $[M-56]^+$ (2), 444 $[M-92]^+$ (24) and 430 $[M-106]^+$ (2).

β, β-Carotene (**9**). MS *m/z* (rel. int.): 536 $[M]^+$ (70), 444 $[M-92]^+$ (11) and 430 $[M-106]^+$ (0.5).

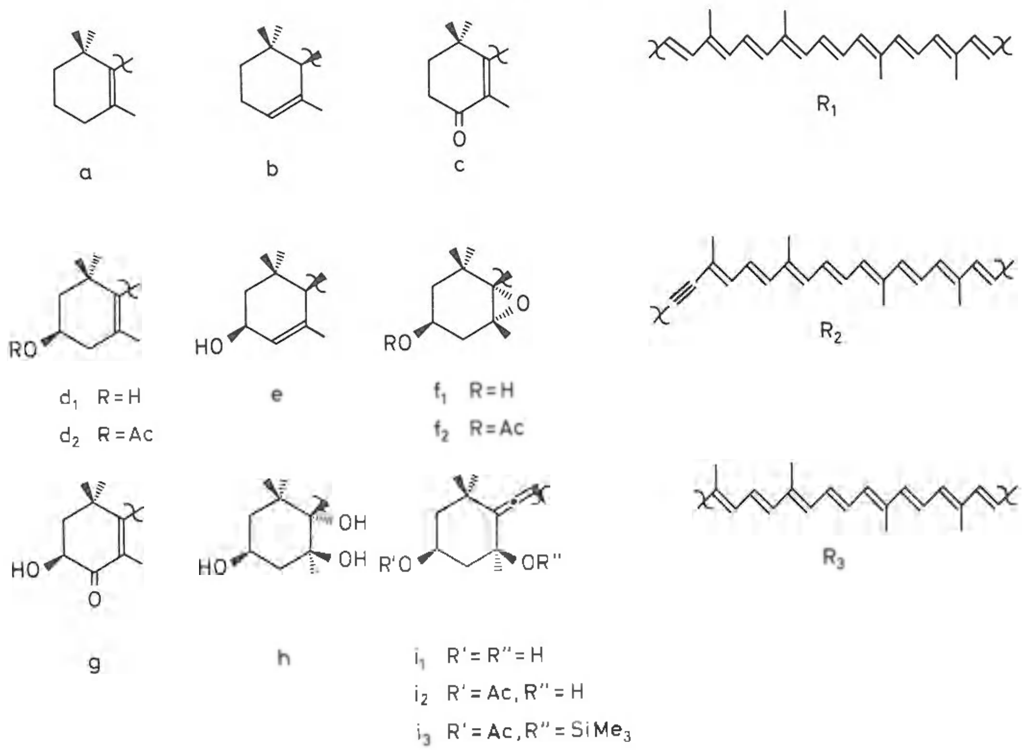
Diatoxanthin (**6**). IR ν_{max}^{KBr} cm^{-1}: 3370*s* (OH); 3040*w*, 2970*s*, 2930*s*, 2870*m*, 2840*m* (CH); 1565*w* (C=C); 1450*m* (ring-CH_2); 1370*m* (*gem.*–CH_3); 1180*w*, 1055*s* (C–O) and 965*s* (C=C): MS *m/z* (rel. int.): 566 $[M]^+$ (100), 564 $[M-2]^+$ (4), 551 $[M-15]^+$ (2), 548 $[M-18]^+$ (1) and 474 $[M-92]^+$ (4); ^1H NMR (100 MHz, $CDCl_3$, TMS as int. standard): δ 1.08 (Me–1′, 1′), 1.15 (Me–1), 1.20 (Me–1), 1.74 (Me–5′), 1.92 (Me–5), 1.97 (Me–13, 9′, 13′) and 2.00 (Me–9); contaminant signals: 1.56 and 2.17. The epoxide test was negative.

Diatoxanthin-3, 3′-*diacetate* (**15**). **6** was converted to **15** by a standard procedure. UV/VIS $\lambda_{max}^{Me_2CO}$ nm: (428), 449 and 476; III/II (%) = 19; MS *m/z* (rel. int.): 650 $[M]^+$ (44), 635 $[M-15]^+$ (0.6), 590 $[M-60]^+$ (10), 575 $[M-15-60]^+$ (4), 558 $[M-92]^+$ (2), 543 $[M-15-92]^+$ (0.6), 530 $[M-60-60]^+$ (3), 515 $[M-15-60-60]^+$ (5), 498 $[M-60-92]^+$ (4), 455 $[M-195]^+$ (1), 438 $[M-60-60-92]^+$ (3), 423 (7) and 395 (4).

Diadinoxanthin (**5**). MS *m/z* (rel. int.): 582 $[M]^+$ (100), 580 $[M-2]^+$ (21), 567 $[M-15]^+$ (4), 566 $[M-16]^+$ (2), 564 $[M-18]^+$ (2), 502 $[M-80]^+$ (12), 490 $[M-92]^+$ (8), 487 $[M-15-80]^+$ (3), 475 $[M-15-92]^+$ (1), 352 (22), 221 (homopyryllium) (74) and 181 (furyllium) (40). ^1H NMR (100 MHz, $CDCl_3$, TMS as int. standard): δ 0.98 (Me – 1); 1.15 (Me – 1, 1′); 1.19 (Me – 5, 1′); 1.93 (Me – 9, 5′); 1.96 (Me – 13, 13′) and 2.00 (Me – 9′); contaminant signals: 1.25, 1.57 and 2.17. The epoxide test was positive.

Diadinoxanthin-3, 3′-*diacetate* (**16**). **5** was converted to **16** by a standard procedure. UV/VIS $\lambda_{max}^{Me_2CO}$ nm: (424), 446 and 475; III/II (%) = 54; MS *m/z* (rel. int.): 666 $[M]^+$ (10), 664 $[M-2]^+$ (4), 651 $[M-15]^+$ (0.4), 606 $[M-60]^+$ (2), 591 $[M-15-60]^+$ (0.9), 586 $[M-80]^+$ (4), 574 $[M-92]^+$ (3), 559 $[M-15-92]^+$ (0.3), 550 (0.7), 546 $[M-60-60]^+$ (0.3), 531 $[M-15-60-60]^+$ (0.2), 526 $[M-60-80]^+$ (0.1), 520 (1), 514 $[M-60-92]^+$ (0.7), 511 $[M-155]^+$ (2), 263 (homopyryllium) (13) and 223 (furyllium (7).

Neoxanthin (**7**). IR ν_{max}^{KBr} cm^{-1}: 3400*s* (OH); 3060*w*, 2980*s*, 2940*s*, 2880*m* (CH); 1940*w* (C=C=C); 1720*w* (impurity); 1580*w* (C=C); 1465*m* (ring-CH_2); 1390*s*, 1380*s* (*gem.*–CH_3); 1190*m*, 1160*s* (*tert.* OH); 1080*m*, 1050*s*, 1020*m* (*sec.* OH) and



1 = $f_1 - R_1 - d_1$
2 = $d_1 - R_1 - e$
3 = $f_1 - R_1 - f_1$
4 = $d_1 - R_1 - d_1$
5 = $d_1 - R_2 - f_1$
6 = $d_1 - R_2 - d_1$
7 = $i_1 - R_3 - f_1$
8 = $a - R_1 - b$
9 = $a - R_1 - a$
10 = $d_1 - R_2 - h$
11 = $c - R_1 - a$
12 = $c - R_1 - c$
13 = $g - R_1 - g$
14 = $d_1 - R_1 - a$
15 = $d_2 - R_2 - d_2$
16 = $d_2 - R_2 - f_2$
17 = $i_2 - R_3 - f_2$
18 = $i_3 - R_3 - f_2$

Fig. 1. Carotenoid structures: the chirality of carotenoids ex Euglenophyceae has been established for diadinoxanthin (**5**) and heteroxanthin (**10**) only [37].

975*s* (C=C); MS *m*/*z* (rel. int.): 600 $[M]^+$ (14), 598 $[M-2]^+$ (1), 584 $[M-16]^+$ (4), 582 $[M-18]^+$ (20), 580 $[M-2-18]^+$ (3), 566$[M-16-18]^+$ (3), 564 $[M-18-18]^+$ (4), 520 $[M-80]^+$ (4), 508 $[M-92]^+$ (3), 502 $[M-18-80]^+$ (28), 484 $[M-18-18-80]^+$ (0.9), 352 (6), 221 (homopyrylium) (46) and 181 (furylium) (27); ^{1}H NMR (100 MHz, $CDCl_3$, TMS as int. standard): δ 1.01 (Me − 1'), 1.07 (Me − 1), 1.16 (Me − 1'), 1.21 (Me − 5'), 1.34 (Me − 1, 5), 1.80 (Me − 9) and 1.96 (Me − 13, 9', 13'); contaminant signals: 1.25, 1.58 and 2.17. The epoxide test was positive.

Neoxanthin-3, 3'-*diacetate* (**17**). **7** was converted to **17** by a standard procedure. UV/VIS $\lambda_{max}^{Me_2CO}$ nm: 418, 440 and 470; III/II (%) = 72; MS *m*/*z* (rel. int.): 684 $[M]^+$ (4), 682 $[M-2]^+$ (0.3), 666 $[M-18]^+$ (5), 651 $[M-15-18]^+$ (0.2), 624 $[M-60]^+$ (0.3), 606 $[M-18-60]^+$ (2), 604 $[M-80]^+$ (1), 592 $[M-92]^+$ (1), 586 $[M-18-80]^+$ (11), 550 (2), 526 $[M-18-60-80]^+$ (1), 263 (homopyrylium) (12) and 223 (furylium) (7).

Neoxanthin-5-*trimethylsilylether*-3, 3'-*diacetate* (**18**). **17** was converted to **18** by a standard procedure. UV/VIS $\lambda_{max}^{Me_2CO}$ nm: 419, 440 and 470; III/II (%) = 67; MS *m*/*z* (rel. int.): 756 $[M]^+$ (6), 754 $[M-2]^+$ (0.4), 696 $[M-60]^+$ (0.5), 681 $[M-15-60]^+$ (0.2), 676 $[M-80]^+$ (2), 666 $[M-90]^+$ (7), 664 $[M-92]^+$ (2), 606 $[M-60-90]^+$ (1), 586 $[M-80-90]^+$ (11), 550 (3), 263 (homopyrylium) (14) and 223 (furylium) (10).

Unknown 1. The sample was crystallized from Me_2CO–MeOH. UV/VIS λ_{max}^{petrol} nm: (438), 458 and 487; III/II (%) = 50; $\lambda_{max}^{Me_2CO}$ nm: (441), 461 and 491; III/II (%) = 47; TLC-I, petrol–Me_2CO (12:1): R_f 0.45 [echinenone (**11**), 0.38; β-cryptoxanthin (**14**), 0.06]. Unknown 1 was stable towards alkali. The epoxide test was negative.

Unknown 2. UV/VIS: λ_{max}^{petrol} nm: (440), 462 and 490; III/II (%) = 26; $\lambda_{max}^{Me_2CO}$ nm: (445), 467 and 495; III/II (%) = 14; TLC (system as in Table 1): R_f 0.80 [echinenone (**11**), 0.96; canthaxanthin (**12**), 0.82; β-cryptoxanthin (**14**), 0.82]. Unknown 2 was stable towards alkali. The epoxide test was negative.

Unknown 3. UV/VIS λ_{max}^{petrol} nm: (434), 453 and 480; III/II (%) = 10; $\lambda_{max}^{Me_2CO}$ nm: 452 and (464); $\lambda_{max}^{CHCl_3}$ nm: 471; TLC (system as in Table 1): R_f 0.64 [astaxanthin (**13**), 0.66]. Unknown 3 was unstable towards alkali and was quantitatively transformed to a more polar product (R_f 0.23);

UV/VIS λ_{max}^{petrol} nm: (433), 452 and 480; III/II (%) = 28; $\lambda_{max}^{Me_2CO}$ nm: 446 and (465); $\lambda_{max}^{CHCl_3}$ nm: 470. Unknown 3 gave a negative epoxide test.

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