Phytochemistry, Vol. 20, No. 11, pp. 2561–2564, 1981.
Printed in Great Britain.

0031-9422/81/112561-04 $02.00/0

OPTICAL PURITY OF (3*S*,3′*S*)-ASTAXANTHIN FROM *HAEMATOCOCCUS PLUVIALIS**

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(*Received* 14 *November* 1980)

Key Word Index—*Haematococcus pluvialis*; Chlorophyceae; carotenoids; esterified (3*S*,3′*S*)-astaxanthin; optical purity.

Abstract—*Haematococcus pluvialis* cultivated in a N-deficient medium produced astaxanthin (1% of total carotenoids), the monoester (76%) and diester (7%) of astaxanthin, β,β-carotene (1%), an adonirubin ester (3%), (3*R*,3′*R*,6′*R*)-lutein (7%), violaxanthin (2%) and neoxanthin (1%). The CD values of the mono- and diesters of astaxanthin, the HPLC properties of astaxanthin monoester further esterified with (−)-camphanic acid and the optical purity of astaxanthin [determined by HPLC analysis of the diester of (−)-camphanic acid] produced by saponification of the natural mono- and diesters of astaxanthin in the absence of oxygen showed that this green alga synthesizes pure (3*S*,3′*S*)-astaxanthin esters.

INTRODUCTION

The assignment of (3*S*,3′*S*)-chirality to astaxanthin monoester (**2a**) from *Haematococcus pluvialis* was based on conformational analysis of the diastereomeric tetrols (**4**) obtained by complex metal hydride reduction and CD correlation with (3*R*,3′*R*)-zeaxanthin (**5**) [1]. Astaxanthin monoester (**2a**) from *H. pluvialis* has been used as a reference compound for assignment of (3*S*,3′*S*)-chirality to astaxanthin (**1a**) from other sources by CD correlations [1, 2]. Recently an analytical method for the quantitative determination of the three stereoisomers (**1a**, **1b**, **1c**) in samples of astaxanthin has been developed [3]. The method is based on HPLC separation of diastereomeric esters of (−)-camphanic acid, and has been used to demonstrate the natural occurrence of **1b** (3*R*,3′*S*,*meso*)-, **1c** (3*R*,3′*R*)- and **1a** (3*S*,3′*S*)-astaxanthin in lobster eggs [4], and the nearly racemic nature of astaxanthin (**1a** + **1b** + **1c**) in shrimps (*Pandalus borealis*) [5].

In view of these findings and the availability of a pure optical standard, **1a** [6], it was of interest to examine the optical purity of astaxanthin (**1**) and its mono- (**2**) and diesters (**3**) from *H. pluvialis*.

RESULTS AND DISCUSSION

The carotenoids were isolated from cells of *H. pluvialis* that had been cultured in a N-deficient medium in order to promote the biosynthesis of astaxanthin [7].

Astaxanthin (**1**, 1% of total carotenoid) was identified from its vis. and mass spectra and by co-chromatography with authentic **1**. Its monoester (**2**, 76%) was the major carotenoid, and this was identified by its vis. spectrum, R_f and conversion to astacene (**6**) upon alkali treatment in the presence of oxygen [8]. Astacene (**6**) was identified by its mass spectrum and by co-chromatography with authentic **6**. Astaxanthin diester (**3**, 7%) was identified by the same criteria as the monoester **2**.

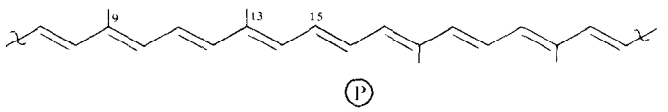
The other carotenoids isolated were β,β-carotene (**7**, 3%), lutein (**8**, 7%), violaxanthin (**9**, 2%) and neoxanthin (**10**, 1%). Of these **7**, **8** and **10** could not be separated from authentic standards and had the expected vis. and mass spectra [9]. ^{1}H NMR and CD spectra characterized lutein as (3*R*,3′*R*,6′*R*) [10]. No chiroptical properties were recorded for the epoxides **9** and **10**. Neoxanthin (**10**) was rearranged with acid to neochrome. Violaxanthin (**9**) had the expected vis. and mass spectral properties [9] and was rearranged with acid to a heptaene product compatible with auroxanthin [9]. Finally, a minor carotenoid (3%) that was slightly more strongly adsorbed than the astaxanthin diester (**3**) was identified as an adonirubin (**11**) ester after saponification in the absence of oxygen to adonirubin (**11**), which was identified from its vis. and mass spectra and by co-chromatography with authentic **11**. The configuration for adonirubin (**11**) was not examined, but it is probably 3*S* as for the astaxanthin esters (**2** and **3**).

The carotenoid composition for *H. pluvialis* is in fair agreement with that previously reported [7, 11].

The fatty acids esterified with astaxanthin (**1**) in the monoester (**2**), and therefore probably also in the diester (**3**), were as reported elsewhere [12] 16:0, 18:0, 18:1 (major), 19:0 and 20:0.

The CD data for astaxanthin monoester (**2**) and astaxanthin diester (**3**) obtained here in comparison with data for optically pure (3*S*,3′*S*)-astaxanthin (**1a**) [6] suggested predominant *S*-chirality. However, since 9- or 13-mono-*cis*-isomers produced during the isolation procedure have Cotton effect opposite to that of all-*trans*

* Part 3 in the series "Natural Occurrence of Enantiomeric and *meso*-Astaxanthin". For Part 2, see Müller, R. K., Bernhard, K., Mayer, H., Rüttimann, A. and Vecchi, M. (1980) *Helv. Chim. Acta* **63**, 1654.



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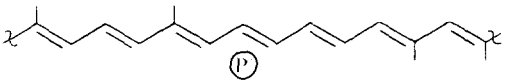

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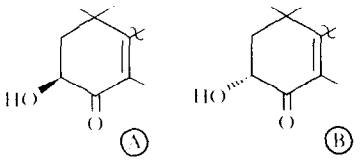
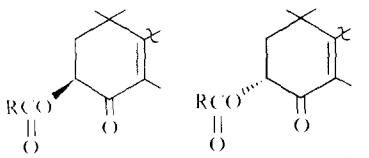


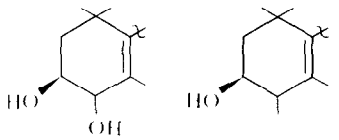
(A) (B)

Astaxanthin (**1**)
1a (3*S*, 3′*S*) A—P—A
1b (3*R*, 3′*S*, *meso*) B—P—A
1c (3*R*, 3′*R*) B—P—A



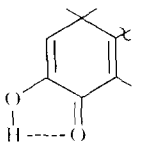
(A) (B)

Natural astaxanthin monoester (**2**)
2a (3*S*, 3′*S*) A—P—A′
2b (3*S*, 3′*R*) A—P—B′
2b (3*R*, 3′*S*) B—P—A′
2c (3*R*, 3′*R*) B—P—B′



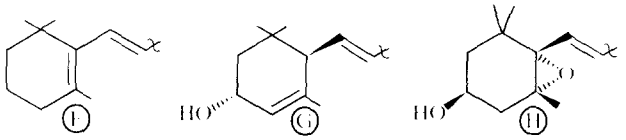
(C) (D)

Natural astaxanthin diester (**3**)
3a (3*S*, 3′*S*) A′—P′—A′
3b (3*S*, 3′*R*, *meso*) A′—P—B
3c (3*R*, 3′*R*) B′—P—B′



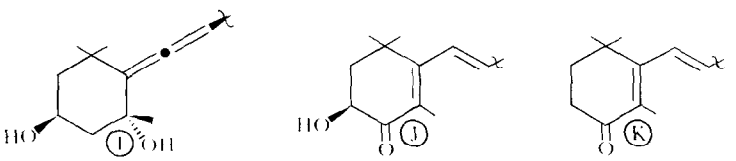
(E)

Tetrols (**4**) C—P—C
(3*R*, 3′*R*)-Zeaxanthin (**5**) D—P—D
Astacene (**6**) E—P—E



(F) (G) (H)

β,*β*-Carotene (**7**) F—P′—F
Lutein (**8**) D—P′—G
Violaxanthin (**9**) H—P′—H
Neoxanthin (**10**) I—P′—H
Adonirubin (**11**) J—P′—K



(I) (J) (K)

[6, 13], the optical purity is not readily determined from CD criteria alone.

Astaxanthin monoester (**2**) was reacted with (−)-camphanic acid chloride and the resulting ester of (−)-camphanic acid submitted to HPLC analysis. The result suggested, together with the CD data above, that the natural astaxanthin monoester (**2a**) had an optical purity of at least 90%. In contrast to natural astaxanthin monoester (**2**) from *Pandalus borealis*, whose diastereomeric esters with (−)-camphanic acid produced 4 peaks upon HPLC, ascribed to the (−)-camphanic acid ester of *cis* (**2a** + **2b**), *trans* (**2a** + **2b**), *cis* (**2b'** + **2c**) and *trans* (**2b'** + **2c**) [5], two badly resolved peaks with relative integral *ca* 9:1 were observed in the present case.

It has recently been reported [14] that diesters of astaxanthin in the strict absence of oxygen may be saponified to free astaxanthin at conditions where racemization at C-3(3') occurs only to a very minor extent. Natural astaxanthin monoester (**2**) was converted to astaxanthin (**1**) by this method and **1** thus produced by esterification with (−)-camphanic acid chloride and HPLC analysis of the diastereomeric esters found to consist of 99% (3*S*,3'*S*)-astaxanthin (**1a**, 87% all-*trans* + 12% *cis*) and 1% (3*R*,3'*S*)-astaxanthin (**1b**, all-*trans*). The latter minor component is likely to have been produced during the analysis [14]. Astaxanthin natural diester (**3**) was converted to astaxanthin (**1**) by the same procedure and found to consist of (3*S*,3'*S*)-astaxanthin (**1a**, 86% all-*trans* + 14% *cis*) besides traces of the *meso* form (**1b**).

It is concluded that astaxanthin (**1**) synthesized by the green alga *H. pluvialis* is the pure (3*S*,3'*S*)-isomer (**1a**). Astaxanthin produced by the yeast *Pfaffia rhodozyma* is known to be the pure enantiomer (3*R*,3'*R*) (**1c**) [14, 15], whereas astaxanthin from various marine animals represents mixtures of the three optical isomers [4, 5].

EXPERIMENTAL

Biological material. Haematococcus pluvialis Flot. em. Wille (clone NIVA CHL 9) was cultivated in 10% Z8 with addition of vitamin B_{12} [16]. Illumination from fluorescent lamps of *ca* 300 lx was applied with continuous light, temp. 20°. The algae were harvested when growth was limited by N-deficiency by filtration and frozen; yield 24 g wet wt.

Isolation of the carotenoids. Cells were kept in Me_2CO–H_2O (2:8) overnight and the green extract (containing no carotenoids) removed. The residue was extracted with Me_2CO–MeOH (7:3). Solvents were removed in a rotary evaporator with addition of C_6H_6, and the crude carotenoids chromatographed on TLC [Si gel (1 mm), Me_2CO–hexane, 3:7]. Rechromatography of coloured fractions was carried out with 10–50% Me_2CO in hexane. R_f values refer to Me_2CO–hexane (3:7) and spectra were recorded in Me_2CO. Individual carotenoids are described in order of increasing adsorbance.

β,β-Carotene (**7**), 0.4 mg, R_f 0.95, λ_{max} nm: (425), 450 and 475, %III/II [17] = 20; MS *m/z*: 536 (M), M-92, M-106, M-158; inseparable from authentic **7**.

Astaxanthin diester (**3**), 0.8 mg, R_f 0.93, CD (EPA; Me_2O–isopentane–EtOH, 5:5:2) nm ($\Delta\varepsilon$): 245 (−14.7), 271 (9.6), 314 (−12.2) cf. optically pure **1a** [6] (CH_2Cl_2): 249 (−14.4), 280 (12.5), 323 (−23.1). The diester **3** had λ_{max} nm 475 and on treatment with 5% KOH in MeOH for 1 hr in the presence of air gave astacene (**6**), R_f 0.32; λ_{max} nm 477; MS *m/z*: 592 (M), M − 16, M − 92, M − 106; inseparable from authentic **6**.

Adonirubin (**11**) *ester*, 0.3 mg, R_f 0.88, λ_{max} nm 475. Saponification in the absence of O_2 as for **2** and **3** provided adonirubin (**11**), λ_{max} nm 475; MS *m/z*: 580 (M), M − 16, M − 92, M − 106; inseparable from authentic **11**.

Astaxanthin monoester (**2**), 9.2 mg, R_f 0.69, λ_{max} nm 475; CD (EPA) nm ($\Delta\varepsilon$): 240 (−14.7), 271 (9.6), 314 (−12.2). Alkali treatment of **2** as for **3** provided astacene (**6**); R_f 0.32; λ_{max} nm 476; MS *m/z*: 592 (M), M − 16, M − 92, M − 106; inseparable from authentic **6**.

Violaxanthin (**9**), 0.2 mg, R_f 0.54, λ_{max} nm 417, 440 and 470, %III/II = 76; MS *m/z*: 600 (M), M − 16, M − 18, M − 18 − 18, M − 80, M − 92, M − 106, 221, 181. Treatment of **9** with 0.03 M HCl/$CHCl_3$ gave a product with λ_{max} nm: 378, 400 and 425 nm, %III/II = 98.

(3R,3'R,6'R)-*Lutein* (**8**), 0.4 mg, R_f 0.42, λ_{max} nm: (420), 445 and 473, %III/II = 47; MS *m/z*: 568 (M), M − 18, M − 18 − 18, M − 92, M − 106; ^{1}H NMR ($CDCl_3$): δ 0.85 (*s*, 3 H, Me-1'), 1.00 (3 H, Me-1'), 1.07 (*s*, 6 H, Me-1), 1.63 (*s*, 3 H, Me-6'), 1.74 (*s*, 3 H, Me-6), 1.91 (*s*, 3 H, Me-9'), 1.97 (*s*, 9 H, Me-9,13,13'), 5.6 (*s*, 1 H, H-4'), 6.1 (*s*, 2 H, 7,8), 7.2–6.2 (*m*, olefinic H); CD (EPA) nm ($\Delta\varepsilon$): 230–280 (+), 290 (−).

Astaxanthin (**1**), not readily separated from **8**, <0.1 mg, R_f 0.40, λ_{max} nm 475; MS *m/z*: 596 (M), M − 16, M − 16 − 16, M − 92, M − 106; inseparable from authentic **1**.

Neoxanthin (**10**), 0.2 mg, R_f 0.22, λ_{max} nm: 415, 438 and 466, %III/II = 57; MS *m/z*: 600 (M), M − 18, M − 18 − 18, M − 80, M − 92, M − 80 − 18, 221, 181; inseparable from authentic **10**. Treatment with 0.03 M HCl gave a product with λ_{max} nm: 400, 423 and 450, %III/II = 89.

Saponification of natural astaxanthin monoester (**2**) was carried out in CH_2Cl_2–MeOH with excess NaOH in the absence of O_2 at room temp. [14]. **2** (0.75 mg) in CH_2Cl_2 (3 ml) to which was added NaOH (1.5 mg) dissolved in MeOH (3 ml) was allowed to react for 45 min under N_2. To remove excess alkali the reaction mixture was drained through H_2O (5 ml) containing 10% NH_4Cl and the carotenoids collected in CH_2Cl_2 (3 ml) in the absence of O_2, before the common isolation procedure. According to TLC no astacene (**6**) was formed. The absence of O_2 from the apparatus was checked in a blank experiment with pyrogallol, which remained colourless.

Preparation of esters of (−)-camphanic acid was carried out by a published procedure [4, 5] with (−)-camphanic acid chloride in pyridine.

HPLC analysis was performed as described elsewhere [5, 18]. Improved separations were achieved using a 25-cm long column and a pressure of 46.6 kg/cm^2.

Acknowledgements—B.R. was supported by a research grant from Fa. Hoffmann-La Roche, Basel, to S.L.-J. A sample of synthetic adonirubin was provided by the same firm.

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