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Algal Carotenoids

VI.* The Carotenoids of *Trentepohlia iolithus*. Isolation of β,β-Carotene-2-ol, β,ε-Carotene-2-ol and β,β-Carotene-2,2′-diol

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The carotenoids of the green alga *Trentepohlia iolithus* have been reinvestigated and are shown to be β,β-carotene (*1*, 29 %), β,ε-carotene (*2*, 21 %), β,β-carotene-2-ol (*3*, 26 %), β,ε-carotene-2-ol (*4*, 20 %), and β,β-carotene-2,2′-diol (*5*, 1.2 %). The hydroxylated carotenoids occur as fatty acid esters. The structures of *3*, *4*, and *5* followed from their spectral properties (electronic, IR, NMR, and mass spectra) and co-chromatography tests with authentic mono- and dihydroxy derivatives of β,β-carotene (*6*, *7*, *8*, and *9*). Evaluation of CD data has led to the tentative absolute configuration 2*R* for *3*, 2*R*,6′*R* for *4*, and 2*R*,2′*R* for *5*.

This is the first demonstration of naturally occurring cyclic carotenoids hydroxylated in the 2-position.

The pigments of the green alga *Trentepohlia iolithus* (L.) Wallroth were first studied in 1892 by Zopf[1] who concluded that the pigments were closely related to the pigments obtained from carrots. Tischer[2] isolated β,β-carotene (*1*) and β,ε-carotene (*2*) in the crystalline state and identified zeaxanthin (β,β-carotene-3,3′-diol, *8*) and lutein (β,ε-carotene-3,3′-diol) from their electronic spectra alone. A carotenoid providing an intense blue colour when treated with concentrated hydrochloric acid was also encountered.

We have reinvestigated the carotenoid composition of *T. iolithus* using modern methods and now report that the pigments previously described as zeaxanthin (*8*) and lutein[2] are the hitherto unknown β,β-carotene-2-ol (*3*) and β,ε-carotene-2-ol (*4*), respectively. The compound giving a blue colour reaction with hydrochloric acid is β,β-carotene-2,2′-diol (*5*). The new carotenoid nomenclature, recommended by IUPAC,[3] is used here.

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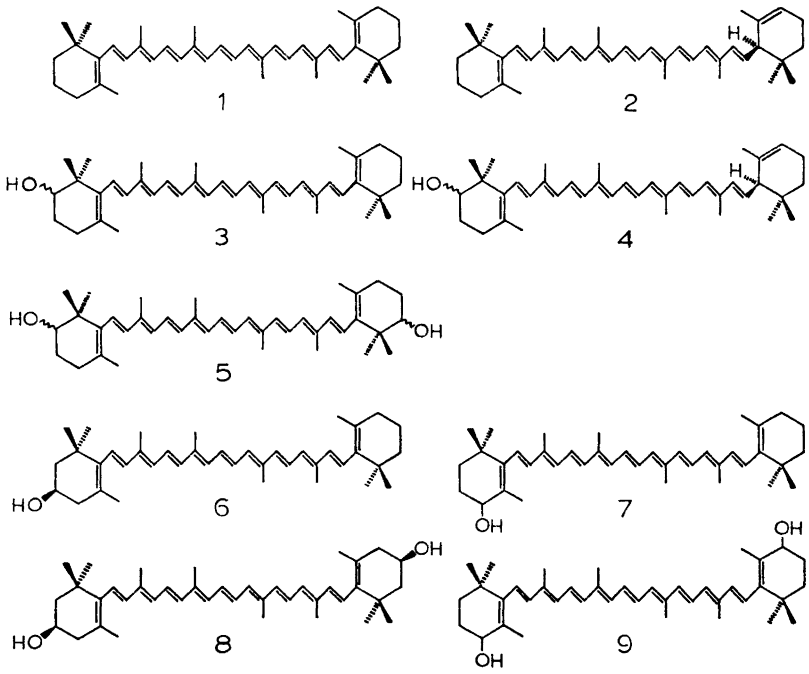


RESULTS AND DISCUSSION

Isolation

The biological material was harvested directly into acetone to minimize loss of pigment. The algae studied contained *ca.* 5.5 % carotenoids of the dried, extracted biological material. This organism is thus one of the richest carotenoid sources reported, *cf.* Ref. 4.

The best isolation procedure involved extraction with acetone, partition into petroleum ether from acetone-water, followed by chromatographic separation into carotene, monoester and diester fractions. The mixed carotenes were crystallized and further separated by chromatography on alumina. The monoester fraction was saponified and the mono-hydroxy compounds purified by chromatography on alumina, crystallized, separated into individual mono-ols by chromatography on magnesia columns and finally crystallized. The diester fraction was saponified, and the di-ol purified by chromatography on alumina and crystallized.

Prior to saponification the crude extract contained carotenes (50 %), monoesters (46 %), diesters (3 %) and others (1 %) estimated spectrophotometrically after separation by TLC on silica.

Individual carotenoids

Carotenes. β,β-Carotene (*1*, m.p. 177°C), constituting *ca.* 58 % of the carotene fraction, was identified from its spectral properties (electronic, IR, NMR,

and mass spectrum) and by co-chromatography with synthetic β,β-carotene (*1*).

β,ε-Carotene (*2*, m.p. 169–171°C) constituted *ca.* 42 % of the carotenes and was identified by the same spectroscopic methods and by co-chromatography with authentic β,ε-carotene (*2*) from carrots. *T. iolithus* is thus one of the best sources of β,ε-carotene (*2*) reported.

Monoesters. The mass spectrum of the mixed mono-esters showed molecular ions at *m/e* 816, 790, 788, 786, and 762. Less intense peaks at *m/e* 930, 902, 874, 846, and 734 could also be associated with molecular ions. After saponification the molecular ion of the mixed mono-ols occurred at *m/e* 552. The mono-ols were thus esterified with several fatty acids, mainly C_{14}, C_{16}, C_{16} mono-unsaturated, C_{16} di-unsaturated, and C_{18} mono-unsaturated fatty acids. Smaller amounts of C_{12}, C_{20}, C_{22}, C_{24}, and C_{26} fatty acids were also present. The fatty acids were not further investigated.

Mono-ols. β,β-Carotene-2-ol (*3*) comprised 57 % of the monohydroxy fraction and was obtained as bluish prisms; m.p. 177–178°C after recrystallization from chloroform-methanol. The electronic spectrum in acetone showed maxima at (430), 452.5 [E(1 %, 1 cm) = 2290] and 479 nm (% III/II [5] = 24) revealing a chromophoric system identical with that of β,β-carotene (*1*). Partition between petroleum ether and 95 % methanol [6] showed the pigment to be completely epiphasic. The mass spectrum, discussed below, exhibited the molecular ion at *m/e* 552.4334 (calc. for $C_{40}H_{56}O$ 552.4331). The formation of a mono-acetate (*m/e* 594 (M), M – 60, M – 92, and M – 106) defined the oxygen function as a primary or secondary alcohol. Co-chromatography on kieselguhr paper with authentic samples of cryptoxanthin (*6*, β,β-carotene-3-ol) and isocryptoxanthin (*7*,β,β-carotene-4-ol) showed the present mono-ol to be less polar.

The NMR spectrum (Fig. 1) exhibited a double doublet (J_1 = 7 Hz, J_2 = 4.5 Hz) at τ 6.45 which can be associated with a methine proton adjacent to the hydroxy group and coupled vicinally to two magnetically non-equivalent methylene protons of a cyclohexene ring. This is only possible if the hydroxy group is located either at C-2 or C-4. The latter position is ruled out by the co-chromatography test with and NMR data for isocryptoxanthin (*7*) and the hydroxy function is consequently located at C-2. Comparison of the NMR spectrum of this compound with those of isozeaxanthin (*9*, β,β-carotene-4,4′-diol) and zeaxanthin (*8*, β,β-carotene-3,3′-diol) shows that the methine protons of the carbon atom carrying the hydroxy group resonate at considerably lower field in the two latter compounds, τ 5.98 (broad triplet) and τ 6.06 (broad multiplet), respectively. The C-5,5′ methyl groups of isozeaxanthin (*9*) give rise to a singlet at τ 8.18. The corresponding signal of the compound *3* under investigation occurs at τ 8.28, *i.e.* about the same position as for zeaxanthin (*8*, τ 8.26).

A priori the hydroxy group in β,β-carotene-2-ol (*3*) would be expected to have considerable influence on the shift position of the neighbouring *gem.* dimethyl groups both due to the proximity of the hydroxy function and to the asymmetry of C-2. The magnetic non-equivalence of the two *gem.* methyl groups (τ 8.97, 8.91) is rather smaller than expected. The shift difference observed is larger than for zeaxanthin (*8*, τ 8.98, 8.98) and for

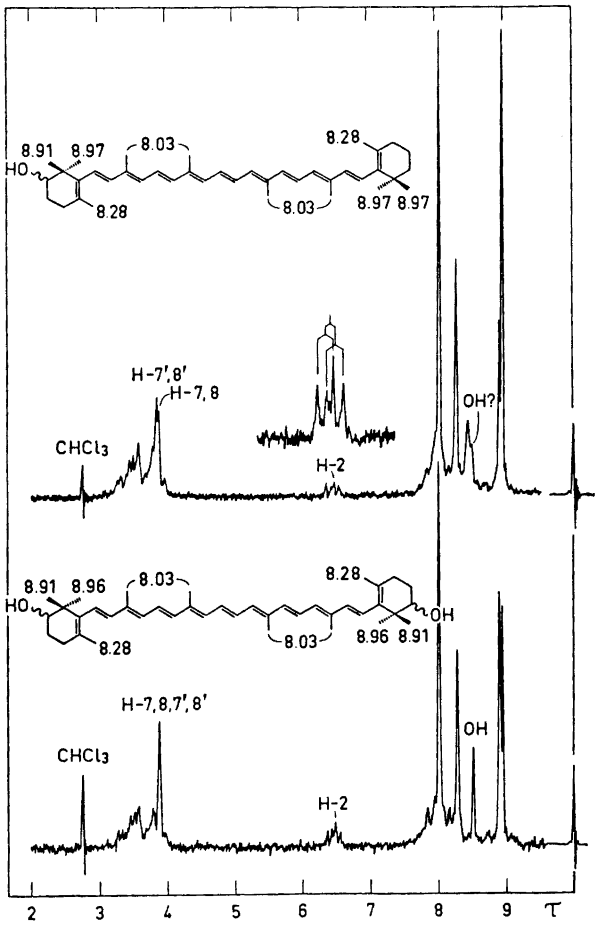


Fig. 1. NMR spectra of β,β-carotene-2-ol (*3*) and β,β-carotene-2,2′-diol (*5*) in $CDCl_3$ solution.

isozeaxanthin (*9*, τ 8.98, 8.96), but smaller than in ε-rings (β,ε-carotene (*2*), τ 9.16, 9.09).

Addition of $Eu(dpm)_3$[7,8] to the NMR sample caused strong down-field shifts of a number of signals and the effect is noticeable as far as C-13′ along the polyene chain. One set of signals which may be associated with the unsubstituted end of the molecule is not influenced by the shift reagent. The relative shifts of the methyl signals are given in Table 1 together with the relative shifts for the methyl signals[8] of zeaxanthin (*8*) and isozeaxanthin (*9*). These data again support the position of the hydroxy function as being at C-2. Smaller relative shift of the C-18 methyl group compared with those of *8* and *9* reflect the larger distance in *3* from the complexing site to the C-18

Table 1. Observed relative shifts for the methyl signals of some hydroxylated carotenoids upon addition of $Eu(dpm)_3$ to the NMR samples.

Carotenoid	Methyl groups										Experimental conditions	
	16 [a]	17	18	19	20	20′	19′	18′	17′	16′	Sample volume ml	Molar ratio $Eu(dpm)_3$: Substrate
β,β-Carotene-2-ol (*4*)	100	67.5	29.4	13.6	3.53	0.81	0.81	0.27	0.27	0.27	0.3	1:1
β,ε-Carotene-2-ol (*3*)	100	67.6	29.3	13.6	3.72	0.53	0.53	0.53	0.53	0.53	0.3	1:1
Zeaxanthin (*8*) [b]	100	92	64	26	8	8	26	64	92	100	0.8	2:1
Isozeaxanthin (*9*) [b]	100	87	231	39	9	9	39	231	87	100	0.3	1:1

[a] Arbitrarily chosen reference.
[b] From Ref. 8.

methyl group. It is pointed out that the smaller relative shift of the C-17 methyl group of the present mono-ol (*3*) only reveals that the C-16 and C-17 methyl grops are in different positions relative to the Eu atom. This is confirmed by inspection of models, provided the 2-hydroxy group is in axial position in the complex. For the free alcohols (*3*, *4*, and *5*), however, the hydroxy group appears to be equatorial since the coupling constants observed for the C-2(2′) methine proton ($J_1 = 7$ Hz, $J_2 = 4.5$ Hz) are compatible [9] with axial-axial and axial-equatorial coupling, respectively.

β,ε-Carotene-2-ol (*4*) comprised 43 % of the monohydroxy fraction and was obtained as purple prisms of m.p. 188 – 188.5°C after recrystallization from chloroform-methanol. The electronic spectrum, recorded in acetone solution, exhibited maxima at (425), 446.5 [E(1 %, 1 cm) = 2330] and 475 nm (% III/II = 68) revealing a chromophoric system identical with that of β,ε-carotene (*2*). The pigment was completely epiphasic on partition between petroleum ether and 95 % methanol. The mass spectrum, discussed below, showed the molecular ion at *m/e* 552.4334 (calc. for $C_{40}H_{56}O$ 552.4331) and *retro* Diels-Alder fragmentation (M – 56) [10] confirmed the presence of an ε-ring. Formation of a monoacetate (*m/e* 594 (M), M – 60, M – 92, M – 106) demonstrated the presence of a primary or secondary hydroxy group.

The NMR spectrum (Fig. 2) again showed a double doublet ($J_1 = 7$ Hz, $J_2 = 4.5$ Hz) at τ 6.45 and two singlets at τ 8.96 and 8.92 which may be associated with the same arrangement around the hydroxy function as for the mono-ol (*3*) described above. Two singlets at τ 9.09 and τ 9.17 and a broad singlet at τ 8.40 established the presence of an unsubstituted ε-ring.[15] Addition of $Eu(dpm)_3$ to the NMR sample again caused strong down-field shifts of several signals (Table 1, Fig. 2). The signals associated with the ε-ring remained virtually unshifted, thus demonstrating the location of the hydroxy function on the β-ring. The magnitude of the relative shifts are very close to those obtained for β,β-carotene-2-ol (*3*) and the two compounds must therefore

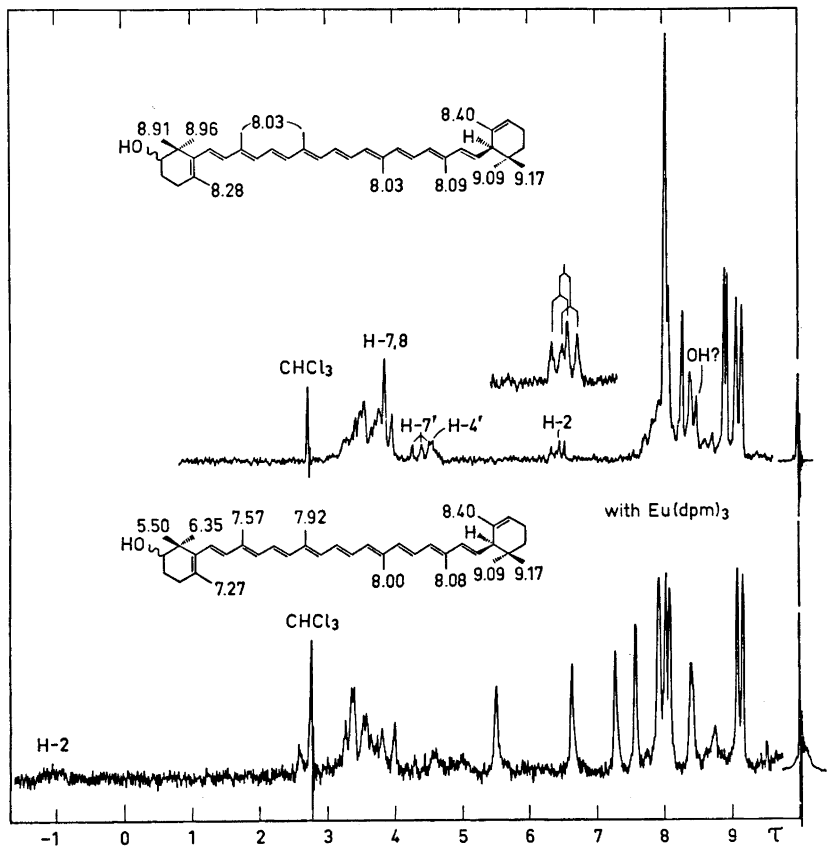


Fig. 2. NMR spectra of β,ε-carotene-2-ol (*4*) alone and with 0.5 relative moles of $Eu(dpm)_3$ in $CDCl_3$ solution.

have the same structural arrangement around the complexing function. The data discussed demonstrate that the compound in question is β,ε-carotene-2-ol (*4*).

β,ε-Carotene-2-ol (4) and β,β-carotene-2-ol (*3*) could be readily separated by coloumn chromatography and by TLC on magnesia, but not by any other TLC system tested. Cryptoxanthin (*6*) and isocryptoxanthin (*7*) were considerably more strongly adsorbed.

Diesters. The diesters comprised *ca.* 3 % of the total carotenoids and produced a single zone on alumina paper. The mass spectrum of the mixed diesters purified by TLC, had molecular ions at *m/e* 1096, 1070, 1068, 1066, 1042, 1040, 1038, 1036, 1016, 1014, 1012, and 988, all confirmed by losses of 92 and 106 mass units. After saponification the molecular ion occurred at *m/e* 568. This leads to the interpretation that the fatty acid composition of the diesters involves the same fatty acids which predominate in the mono-

esters discussed above, namely, C_{14}, C_{16}, C_{16} mono-unsaturated, C_{16} diunsaturated, and C_{18} mono-unsaturated acids. All possible combinations except $C_{16}+C_{16}$ were observed. The fatty acids were not further investigated.

Di-ol. β,β-Carotene-2,2′-diol (*5*) constituted the main compound (40 %) of the dihydroxy fraction and was obtained as purple prisms of m.p. 167°C after recrystallization from chloroform-methanol. The electronic spectrum (acetone) showed maxima at (425), 452 [E(1 %, 1 cm) = 2060] and 479.5 nm; % III/II = 34. The electronic spectrum corresponded to that of zeaxanthin (*8*), thus revealing the same chromophoric system. The partition ratio [6] between petroleum ether and 85 % methanol was 52:48, *i.e.* more epiphasic than *8* and *9*. The mass spectrum exhibited the molecular ion at *m/e* 568.4278 (calc. for $C_{40}H_{56}O_2$ 568.4280). Co-chromatography with zeaxanthin (*8*, β,β-carotene-3,3′-diol) and isozeaxanthin (*9*, β,β-carotene-4,4′-diol) on kieselguhr paper showed the new diol to be less polar.

The NMR spectrum (Fig. 1) showed the same signals as attributed to the 2-hydroxy-β-end group for the two mono-ols (*3* and *4*), and the spectrum indicated a symmetrical structure. A two-proton singlet at τ 8.52 disappeared on deuterium exchange and is consequently caused by the two hydroxy protons. Less prominent signals in similar position were also encountered for the two mono-ols but were not checked by deuterium exchange. The similarity of the NMR spectrum of this diol (*5*) with those of the two mono-ols (*3* and *4*) is striking. Also considering non-identity with zeaxanthin (*8*) and isozeaxanthin (*9*) it is concluded that this diol is β,β-carotene-2,2′-diol (*5*).

Some minor components present in the diol fraction were not further examined and may represent artefacts.

Mass and infrared spectra

The mass spectra of these 2-hydroxy carotenoids did not provide much structural information apart from the molecular composition. Beside the molecular ions, which in all three cases were strong, loss of water (M − 18) from the molecular ion (two consecutive losses in the case of the dihydroxy compound) were observed. Losses of 92, 106 (weak), and 158 mass units from the molecular ion, as observed for most carotenoids,[10,11] were also encountered. Loss of 153 mass units from the molecular ion may be associated with cleavage of the 7- (7′-) double bond(s) with hydrogen transfer to the minor fragment.[11] As already mentioned the β,ε-compound (*4*) also shows an M − 56 fragment ascribed to a *retro* Diels-Alder fragmentation of the ε-end group.[10]

All three compounds show losses of 78, 79, and 80 mass units from the molecular ion. High precision mass measurements confirm that these are losses of C_6H_6, C_6H_7, and C_6H_8 fragments from the molecular ions, respectively. Loss of 79 mass units from the molecular ion has previously been encountered for a number of carotenoids.[11] A rationale to the origin of this species is presented elsewhere.[12]

The IR spectra of the three hydroxy carotenoids were all virtually identical (Fig. 3) and did not give much structural information. The OH stretching frequency occurred at 3600 − 3300 cm^{-1} and the OH deformation or C − O stretching frequency at 1035 − 1040 cm^{-1}.

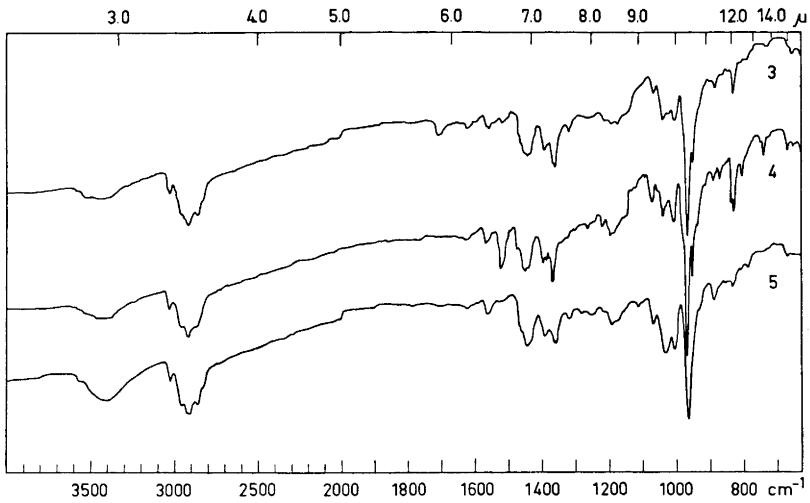


Fig. 3. IR spectra (KBr) of β,β-carotene-2-ol (*3*), β,ε-carotene-2-ol (*4*), and β,β-carotene-2,2′-diol (*5*).

Acid treatment

When treated with hydrochloric acid under the same conditions as in the colour test for epoxides and furanoxides [13] the diol *5* immediately gave a strong blue colour in the acid phase. The mono-ols *3* and *4* gave a very weak blue colour at the interphase after prolonged treatment. This fact demonstrates that this test is not specific for epoxides and furanoxides, and furthermore suggests that the compound of Tischer [2] giving a blue colour with hydrochloric acid must have been β,β-carotene-2,2′-diol (*5*).

Treatment of β,ε-carotene-2-ol (*4*) with 0.01 N HCl in chloroform-methanol (identical conditions as for the formation of lutein allylic methyl ether [14]) caused a rapid development of a green colour and resulted in rather severe decomposition of pigment (26 % recovery). Work-up of the reaction mixture with water afforded two new products more polar than the starting compound. Both products had identical electronic spectra with maxima at (380), 398.5, 422, and 449 nm (% III/II = 94) in acetone solution revealing an aliphatic octaene chromophore.[15] The mass spectra of the two products were virtually identical and showed the molecular ions at *m/e* 568, demonstrating a formal addition of one oxygen atom to the mono-ol (*4*, *m/e* 552). A number of characteristic fragment ions (M − 80, *m/e* 181 and 221) normally associated with epoxidic and furanoid carotenoids [16] were also present. We therefore assume that acid treatment gives rise to the furanoxides by rearrangement of the molecule through an unknown mechanism and upon introduction of an extra oxygen atom. The two products are considered as diastereomers at C-5 or C-8. It is known that this type of furanoid diastereomers may exhibit significant difference in polarity.[17]

Work on the chemistry of the new 2-hydroxy carotenoids will be pursued.

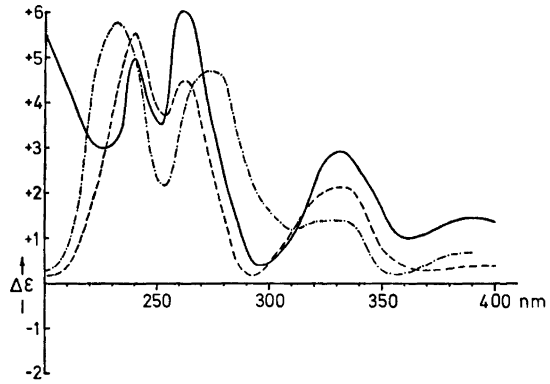


Fig. 4. Observed CD spectra of β,ε-carotene-2-ol (*4*, ———), β,ε-carotene (*2*, - - - - -) and the sum (—.—.—) of the CD spectra of β,ε-carotene (*2*) and β,β-carotene-2-ol (*3*) in EPA solution.

CD spectra and absolute configuration

One important structural feature remains to be discussed, *i.e.* the absolute configuration. CD curves of *2*, *3*, *4*, and *5* have been recorded (Figs. 4 and 5). The CD spectrum of β,ε-carotene (*2*, Fig. 4) from *T. iolithus* agrees well with that of β,ε-carotene reported by Buchecker and Eugster,[18] confirming the 6′*R*-configuration of β,ε-carotene (*2*) also from our source.

The CD curve of β,ε-carotene-2-ol (*4*) is reproduced in Fig. 4. Bartlett *et al.*[19] have advanced the hypothesis that two chiral centra in opposite ends of the carotenoid molecule do not influence each other and that the ORD curve of such dichiral carotenoids is approximately the sum of the curves of two relevant monochiral carotenoids. One would thus expect the CD curve of β,ε-carotene-2-ol (*4*) to be similar to the sum of the CD curves of β,β-carotene-2,2′-diol (*3*) and β,ε-carotene (*2*). Fig. 4 also includes this plot. Although the curves are not identical, the general trend is the same and it seems therefore justified to assume that the absolute configuration of *4* at C-2 and C-6′ is the same as for β,β-carotene-2-ol (*3*) and β,ε-carotene (*2*, 6′*R*), respectively.

β,β-Carotene-2-ol (*3*) and β,β-carotene-2,2′-diol (*5*) exhibit identical CD spectra apart from $\Delta\varepsilon$-values, thus revealing the same chirality at C-2(2′). The CD spectra of these compounds are, apart from $\Delta\varepsilon$-values, very close to the opposite of that [20] of natural zeaxanthin (*8*), see Fig. 5.

Mills' rule,[21] elaborated by Eliel,[22a] states that in cyclohexene systems with an asymmetric carbon in 4-position, the nature of the substituent at this carbon atom is irrelevant since the optical rotation is caused by a preferred (asymmetric) conformation of the cyclohexene ring.

It is seen (structures *3* and *6*) that both the 2-hydroxy-β-end group and the 3-hydroxy-β-end group may be regarded as 4-substituted cyclohexene systems. Assuming that the hydroxy groups in both cases prefer the equatorial

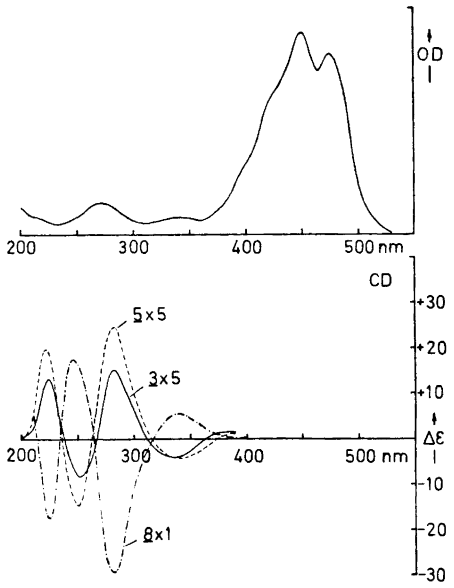
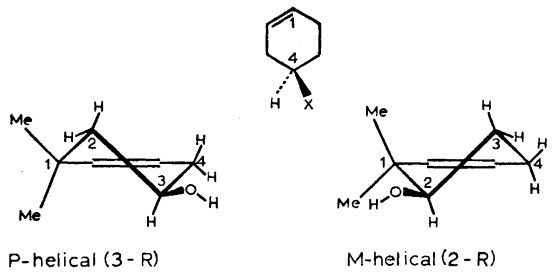


Fig. 5. Electronic and CD spectra of β,β-carotene-2-ol (*3*, ———) and CD spectra of β,β-carotene-2,2′-diol (*5*, - - - - -) and zeaxanthin (*8*, —.—.—.—) in EPA solution.

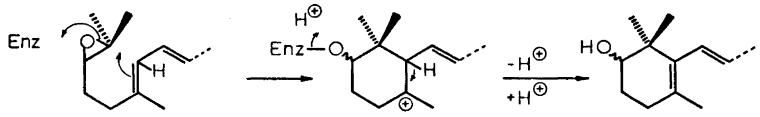
position (supported by the NMR evidence discussed above), the absolute configurations 2*R* and 3*R* of *5* (or *3* or *4*) and *8*, respectively, will result in opposite half-chair conformations as shown, M-helical and P-helical respectively,[22b] expected to cause opposite optical activity.



Zeaxanthin (*8*) has been shown to have 3*R* configuration[23] and provided Mills' rule can be extended to cover these cases, the CD data indicate that the 2-hydroxylated carotenoids have the 2*R* configuration. However, an unambiguous establishment of the absolute configuration of these 2-hydroxycarotenoids must await more rigorous correlation or X-ray analysis.

Biogenetic considerations

The biosynthetic route leading to these new 2-hydroxy-carotenoids is an open question. However, the structural relationship of these compounds with 3-hydroxylated cyclic triterpenes (*e.g.* lanosterol and cycloartenol) is pointed out. The latter two are formed biosynthetically from squalene epoxide.[24,25] Carotenes with 1,2-epoxy functions are naturally occurring [26] and one may consider a similar mechanism for the biosynthetic formation of 2-hydroxylated cyclic carotenoids as for 3-hydroxylated triterpenes:



The fact that the 2-hydroxy function was encountered only in the β-end group and that these compounds are optically active supports that the hydroxylation process is enzymatically controlled.

CONCLUSION

That carotenoids of this type have so far escaped detection may be due to restricted occurrence or inadequate analytical tools. Of their spectroscopic properties only the NMR data are sufficiently distinct from those of other mono- and di-hydroxylated cyclic carotenoids to allow conclusive identification.

For identification in the micro scale the lower polarity (R_F values and partition ratios) relative to other hydroxylated bicyclic carotenoids and the reaction with acid are of diagnostic importance.

EXPERIMENTAL

Materials and methods. For extraction purposes technical grade acetone was employed. Column chromatography was carried out on Merck neutral alumina or Baker magnesium oxide. Thick layer and thin layer chromatography was carried out on Merck Kieselgel P, Aluminium oxide PF_{254} or Baker magnesium oxide. Paper chromatography was carried out on Schleicher & Schüll Nos. 287 (kieselguhr containing) and 288 (alumina containing) circular papers.

For column chromatography distilled technical grade petroleum ether (b.p. 40 – 50°C), technical grade ether (passed through active alumina prior to use) and Merck analytical grade benzene were employed.

Electronic spectra were recorded on a Coleman Hitachi 124 spectrophotometer, IR spectra on a Perkin-Elmer 257 spectrometer and NMR spectra on a Varian A-60A instrument in deuteriochloroform solution with TMS as internal standard. Mass spectra were recorded on an AEI MS902 instrument at 70 eV with an ion source temperature of 160 – 180°C using the direct inlet system. High precision mass measurements were done with perfluorokerosene as reference. CD curves were recorded on a Roussel-Jouane Dicrographe.

Melting points were determined on an Electrothermal melting point apparatus in sealed evacuated tubes and are uncorrected.

Biological material. T. iolithus was collected at Støren near Trondheim in September 1971. The algae were scrubbed off the stones with a steel brush and collected directly into acetone.

Isolation. Extraction of the biological material was carried out with acetone at room temperature under a blanket of nitrogen; yield 4.57 g total carotenoids. After combustion of the extracted residue, the biological material was estimated to 84 g (*i.e.* 5.45 % carotenoids of the extracted residue).

Two thirds of the acetone extract was concentrated and the carotenoids (3.1 g) were transferred into petroleum ether by dilution with aqueous NaCl solution. The petroleum ether extracts were filtered to remove insoluble material (non-carotenoid), dried and concentrated. Chromatography on four columns (Alumina grade II) gave carotenes (1260 mg, eluent petroleum ether), monoesters (1340 mg, eluent 5 – 10 % ether in petroleum ether), diester (88 mg, eluent 10 – 30 % ether in petroleum ether) and others (66 mg, eluent 30 – 100 % ether in petroleum ether).

Carotenes

The mixed carotenes (1260 mg) were crystallized from acetone-methanol, yield 695 mg. The mixed crystalline carotenes consisted of *β,β*-carotene (*1*, 58 %) and *β,ε*-carotene (*2*, 42 %), estimated spectrophotometrically after separation by TLC on alumina. The mixed crystalline carotenes (250 mg aliquot) were separated on slightly deactivated alumina (1 % H_2O).

β,β-Carotene (*1*) was eluted with 100 % ether and crystallized from chloroform methanol, m.p. 177°C; $R_F = 0.53$ (alumina paper, petroleum ether) and inseparable from synthetic *β,β*-carotene; λ_{max} (acetone) (425), 451.5 and 476.5 nm, % III/II = 14; ν_{max} (KBr) 3050 – 2800 (CH), 1625, 1560 (C=C), 1445, 1390, 1365 (CH_3), 1010, 970 (*trans* –CH=CH–), and 830 (>C=CH–) cm^{-1}; τ ($CDCl_3$) 3.1 – 4.0 (14 H, olefinic), 8.03 s (12 H, in-chain CH_3), 8.28 s (6 H, end-of-chain CH_3), 8.47 (CH_2), 8.97 s (12 H, *gem.* CH_3); *m/e* 536 (M), 457 (M – 79), 444 (M – 92), 430 (M – 106), 399 (M – 137), and 378 (M – 158).

β,ε-Carotene (*2*) was eluted with 7 – 10 % ether in petroleum ether and crystallized from chloroform-methanol, m.p. 169 – 171°C; $R_F = 0.58$ (alumina paper, petroleum ether) and inseparable from *β,ε*-carotene from carrots; λ_{max} (petroleum ether) 420, 443 [*E*(1 %, 1 cm) = 2650] and 472 nm, % III/II = 71; ν_{max} (KBr) 3050 – 2800 (CH), 1560 (C=C), 1445, 1390, 1365 (CH_3), 1010, 968 (*trans* –CH=CH–) and 827 (>C=CH–) cm^{-1}; τ ($CDCl_3$) 3.1 – 4.1 (13 H, olefinic), 4.40 (1 H, H-7′), 4.58 m (1 H, H-4′), 8.03 s and 8.08 s (9 + 3 H, in-chain CH_3), 8.29 s (3 H, CH_3 at C-5), 8.44 (CH_3 at C-5′), 8.50 (CH_2), 8.97 s (6 H, *gem.* CH_3 *β*-ring), 9.09 s and 9.18 s (3 + 3 H, *gem.* CH_3 *ε*-ring); *m/e* 536 (M), 480 (M – 56), 457 (M – 79), 444 (M – 92), 430 (M – 106), 399 (M – 137), 388 (M – 92 – 56) and 378 (M – 158); CD (EPA) λ (nm) 240 pk (+5.50), 254 tr (+3.70), 263 pk (+4.50), 293 tr (+0.15), 340 pk (+2.10), 373 tr (+0.30). Δε values are given in parentheses.

Monoesters and mono-ols

Mixed mono-esters purified by TLC on silica had $R_F = 0.74$ (alumina paper, petroleum ether); λ_{max} (acetone) (425), 447.5 and 474.5 nm, % III/II = 38; *m/e* 930 (M_1), 902 (M_2), 874 (M_3), 846 (M_4), 816 (M_5), 790 (M_6), 788 (M_7), 786 (M_8), 762 (M_9), 734 (M_{10}), 724 ($M_5 - 92$), 710 ($M_5 - 106$), 698 ($M_6 - 92$), 696 ($M_7 - 92$), 694 ($M_8 - 92$), 684 ($M_6 - 106$), 682 ($M_7 - 106$), 680 ($M_8 - 106$), 670 ($M_9 - 92$), 658 ($M_5 - 158$), 656 ($M_9 - 106$), 642 ($M_{10} - 92$), 632 ($M_6 - 158$), 630 ($M_7 - 158$), 628 ($M_8 - 158$, $M_{10} - 106$), 535 (M – RCOO), 534 (M – RCOOH), 443 (M – 92 – RCOO), 442 (M – 92 – RCOOH), 429 (M – 106 – RCOO) and 428 (M – 106 – RCOOH).

Mono-ols. An aliquot of the monoester fraction (670 mg) was saponified with 1 l of 5 % KOH in methanol-ether (1:1) for 12 h; pigment recovery 100 %. The saponified pigments were chromatographed on alumina activity grade II and the mixed mono-ols (535 mg) were eluted with 10 – 20 % ether in benzene. The mono-ol fraction was concentrated and crystallized twice from ether-petroleum ether and ether-methanol;

yield 418 mg crystalline mono-ols free of lipid contaminant according to the NMR spectrum.

An aliquot of the mixed crystalline mono-ols (265 mg) was chromatographed on magnesia columns developed with benzene. The column material was extruded, the zones cut out and eluted individually with ether.

β,β-Carotene-2-ol (*3*) was crystallized from acetone-petroleum ether and dried *in vacuo*; yield 58.8 mg, m.p. 179 – 180°C. The crystals contained some acetone according to the NMR spectrum. Recrystallization from chloroform-methanol afforded bluish prisms of m.p. 177 – 178°C; $R_F = 0.34$ on MgO plates (20 % acetone in petroleum ether); λ_{max} (acetone) (430), 452.5 [$E(1\ \%, 1\ cm) = 2290$] and 479 nm, % III/II = 24; λ_{max} (methanol) 271, 338, (425), 448 and 474.5 nm, % III/II = 25; ν_{max} (KBr) 3600 – 3300 (OH), 3050 – 2800 (CH), 1440, 1390, 1360 (CH_3), 1070, 1040 (OH), 1005, 967 (*trans* –CH=CH–), 950 and 830 (>C=CH–) cm^{-1}; τ ($CDCl_3$) 3.0 – 4.1 (14 H, olefinic), 6.45 dd ($J_1 = 7$, $J_2 = 4.5$ Hz, 1 H, H-2), 8.03 s (12 H, in-chain CH_3), 8.28 s (6 H, end-of-chain CH_3), 8.43 (CH_2), 8.50 (OH?), 8.91 s and 8.97 s (3 + 9 H, *gem*. CH_3); *m/e* 552.4334 (calc. for $C_{40}H_{56}O$ 552.4331), 534 (M – 18), 474 (M – 78), 473 (M – 79), 472 (M – 80), 460 (M – 92), 446 (M – 106), 442 (M – 92 – 18), 415 (M – 137), 399 (M – 153), 394 (M – 158); CD (EPA) λ (nm) 225 pk (+2.65), 251 tr (–1.60), 281 pk (+3.05) and 334 tr (–0.80). $\Delta\varepsilon$ values are given in parentheses.

In co-chromatography tests of *3* with cryptoxanthin (*6*, *ex Arthrospira* sp., $R_F = 0.40$) and isocryptoxanthin (*7*, synthetic Hoffmann-La Roche, $R_F = 0.38$) on kieselguhr paper (2 % acetone in petroleum ether) *3* had $R_F = 0.56$.

β,ε-Carotene-2-ol (*4*) was crystallized from acetone-petroleum ether; yield 40.2 mg, m.p. 186 – 187°C. Recrystallization from chloroform-methanol afforded purple prisms of m.p. 188 – 188.5°C; $R_F = 0.52$ on MgO plates (20 % acetone in petroleum ether); λ_{max} (acetone) (425), 446.5 [$E(1\ \%, 1\ cm) = 2330$] and 475 nm, % III/II = 71; λ_{max} (methanol) 266, 330, 421, 443 and 471.5 nm, % III/II = 66; ν_{max} (KBr) 3600 – 3300 (OH), 3050 – 2800 (CH), 1560, 1515 (C=C), 1440, 1390, 1363 (CH_3), 1070, 1037 (OH), 1005, 964 (*trans* –CH=CH–), 949 and 825 (>C=CH–) cm^{-1}; τ ($CDCl_3$) 3.0 – 4.0 (13 H, olefinic), 4.40 (1 H, H-7′), 4.56 m (1 H, H-4′), 6.45 dd (1 H, $J_1 = 7$, $J_2 = 4.5$ Hz, H-2), 8.03 s and 8.09 s (9 + 3 H, in-chain CH_3), 8.28 s (3 H, CH_3 at C-5), 8.40 s (3 H, CH_3 at C-5′), 8.50 (OH?), 8.91 s and 8.96 s (3 + 3 H), *gem*. CH_3 β-end group), 9.09 s and 9.17 s (3 + 3 H, *gem*. CH_3 ε-end group); *m/e* 552.4334 (calc. for $C_{40}H_{56}O$ 552.4331), 534 (M – 18), 496 (M – 56), 474 (M – 78), 473 (M – 79), 472 (M – 80), 460 (M – 92), 446 (M – 106), 442 (M – 92 – 18), 399 (M – 153) and 394 (M – 158); CD (EPA) λ (nm) 200 pk (+5.50), 226 tr (+3.00), 240 pk (+4.95), 252 tr (+3.50), 262 pk (+6.00), 296 tr (+0.40), 331 pk (+2.90), 362 tr +1.00) and 391 pk (+1.45). $\Delta\varepsilon$ values are given in parentheses.

Diesters and diol

Diesters purified by TLC on silica had $R_F = 0.68$ (alumina paper, 1 % acetone in petroleum ether); λ_{max} (acetone) (425), 448.5 and 475 nm, % III/II = 26; *m/e* 1096 (M_1), 1070 (M_2), 1068 (M_3), 1066 (M_4), 1042 (M_5), 1040 (M_6), 1038 (M_7), 1036 (M_8), 1016 (M_9), 1014 (M_{10}), 1012 (M_{11}), 1004 ($M_1 - 92$), 990 ($M_1 - 106$), 988(M_{12}), 978 ($M_2 - 92$), 976 ($M_3 - 92$), 974 ($M_4 - 92$), 964 ($M_2 - 106$), 962 ($M_3 - 106$), 960 ($M_4 - 106$), 950 ($M_5 - 92$), 948 ($M_6 - 92$), 946 ($M_7 - 92$), 944 ($M_8 - 92$), 936 ($M_5 - 106$), 934 ($M_6 - 106$), 932 ($M_7 - 106$), 930 ($M_8 - 106$), 924 ($M_9 - 92$), 922 ($M_{10} - 92$), 920 ($M_{11} - 92$), 910 ($M_9 - 106$), 908 ($M_{10} - 106$), 906 ($M_{11} - 106$).

The diester fraction (88 mg) was saponified with 250 ml of 5 % KOH in methanol-ether (1:1) for 20 h and the diol was purified by chromatography on alumina grade III.

β,β-Carotene-2,2′-diol (**5**, 30 mg) was eluted with 20 – 30 % ether in benzene. Crystallization from chloroform-methanol gave 13.4 mg crystalline diol free of lipid contaminant. Recrystallization from chloroform-methanol gave purple prisms of m.p. 167°C; $R_F = 0.67$ kieselguhr paper (10 % acetone in petroleum ether); λ_{max} (acetone) (425), 452 [$E(1\ \%, 1\ cm) = 2060$] and 479.5 nm, % III/II = 34; λ_{max} (methanol) 271, (425), 447.5 and 474.5 nm, % III/II = 34; ν_{max} (KBr) 3600 – 3200 (OH), 3050 – 2800 (CH), 1570 (C=C), 1440, 1390, 1360 (CH_3), 1070, 1030 (OH), 963 (*trans* –CH=CH–), 885 and 830 (>C=CH–) cm^{-1}; τ ($CDCl_3$) 3.0 – 4.1 (14 H, olefinic), 6.45 dd (2 H, $J_1 = 7$, $J_2 = 4.5$ Hz, H-2,2′), 8.03 s (12 H, in-chain CH_3), 8.28 s (6 H, end-of-chain CH_3), 8.52 (2 H, OH), 8.91

and 8.96 s (6+6 H, *gem.* CH_3); *m/e* 568.4278 (calc. for $C_{40}H_{56}O_2$ 568.4280), 550 (M−18), 532 (M−18−18), 490 (M−78), 489 (M−79), 488 (M−80), 476 (M−92), 462 (M−106), 458 (M−92−18), 415 (M−153) and 410 (M−158). CD (EPA) λ (nm) 223 pk (+3.95), 250 tr (−2.95), 281 pk (+4.93) and 345 tr (−0.80). $\Delta\varepsilon$ values are given in parentheses.

In co-chromatography tests of *5* with zeaxanthin (*8*, *ex Flexithrix* sp., $R_F = 0.55$) and isozeaxanthin (*9*, synthetic Hoffmann-La Roche, $R_F = 0.53$) on kieselguhr paper (10 % acetone in petroleum ether) *5* had $R_F = 0.67$.

Acid treatment

β,ε-Carotene-2-ol (*4*, 0.74 mg) was treated with 0.01 N HCl in chloroform-methanol (3:2, 2 ml) for 15 min. Water (10 ml) was added and the pigments extracted with chloroform. The pigment recovery was 26 %. The individual components were separated by TLC on silica. Two main products ($R_F = 0.47$ and 0.22 on kieselguhr paper, 2 % acetone in petroleum ether) were observed. Both products had identical spectral properties: λ_{max} (acetone) (380), 398.5, 422 and 449 nm, % III/II = 94; *m/e* 568 (M), 550 (M−18), 488 (M−80), 221 and 181.

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