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LOROXANTHIN, A UNIQUE XANTHOPHYLL FROM *SCENEDESMUS OBLIQUUS* AND *CHLORELLA VULGARIS**

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Abstract—A unique xanthophyll, which had been detected before in certain green algae, has now been isolated from *Scenedesmus obliquus* and *Chlorella vulgaris*. This pigment, here called loroxanthin, has also been isolated in its deuterated form from fully deuterated *Chlorella*. It forms a triacetate, and with methanol plus HCl, it yields monomethyl and dimethyl ethers. It can be oxidized to an aldehyde, loroxanthal. It has been characterized as a hydroxy lutein with the additional hydroxy group on a chain methyl group, probably that on C_9. The previously reported absence of this pigment in *C. pyrenoidosa* has been confirmed. It was present in two marine *Cladophora* species and in *Ulva rigida* but absent in *Spirogyra* sp. and in two marine siphonalean green algae. It is probably not identical with certain similar pigments reported in other vegetable sources and variously described as trollein and trollein-like.

NUMEROUS chromatographic investigations have shown that the principal xanthophylls of many green algae (Chlorophyceae) are identical with those of higher plants.[1–21] In addition to the usual xanthophylls, however, some, but not all, chlorophycean species contain a unique strongly adsorbed xanthophyll.[2,3,16,19–21] This xanthophyll has usually not been reported in *Chlorella vulgaris*,[8,11,18] in *C. pyrenoidosa*,[1,12,15,17] or in *Scenedesmus obliquus*.[12–14] There is, however, one report of the same or a similar pigment in *C. pyrenoidosa*.[20]

This chlorophycean xanthophyll has been partially characterized by its chromatographic sequence with the other chloroplast pigments.[2] In extensively developed, chromatographic

* Work performed under the auspices of the U.S. Atomic Energy Commission.

[1] H. H. STRAIN, in *Manual of Phycology* (edited by G. M. Smith), p 243, Chronica Botanica, Waltham, Mass. (1951).
[2] H. H. STRAIN, *Annual Priestley Lectures*, Penn. State Univ. **32** (1958).
[3] H. H. STRAIN, in *Biochemistry of Chloroplasts* (edited by T. W. GOODWIN), p. 387, Academic Press, New York (1966).
[4] Z. SESTAK, *J. Chromatog.* **1**, 293 (1958).
[5] Z. SESTAK, *Preslia* **35**, 123 (1963).
[6] Z. SESTAK, *Chromatog. Rev.* **7**, 65 (1965).
[7] Z. SESTAK and M. BASLEROVA, in *Studies on Microalgae and Photosynthetic Bacteria* (edited by Japanese Society of Plant Physiologists), p. 423, University of Tokyo Press, Tokyo (1963).
[8] S. W. JEFFREY, *Biochem. J.* **80**, 336 (1961).
[9] S. W. JEFFREY, *Biochim. Biophys. Acta* **162**, 271 (1968).
[10] T. W. GOODWIN, *Experientia* **10**, 213 (1954).
[11] M. B. ALLEN, T. W. GOODWIN and S. PHAGPOLNGARM, *J. Gen. Microbiol.* **23**, 93 (1960).
[12] J. M. ANDERSON, *U.S. Atomic Energy Comm.* UCRL-8870, I (1959).
[13] J. M. ANDERSON, *J. Chromatog.* **4**, 93 (1960).
[14] U. BLASS, J. M. ANDERSON and M. CALVIN, *Plant Physiol.* **34**, 329 (1959).
[15] J. SHERMA and G. ZWEIG, *J. Chromatog.* **31**, 589 (1967).
[16] J. S. BUNT, *Nature* **203**, 1261 (1964).
[17] A. HAGER and T. MEYER-BERTENRATH, *Planta* **69**, 198 (1966); **76**, 149 (1967).
[18] B. M. GILCHRIST, *Comp. Biochem. Physiol.* **24**, 123 (1968).
[19] R. MCLEAN, *Physiol. Plantarum* **20**, 41 (1967).
[20] P. J. HERRING, *Comp. Biochem. Physiol.* **24**, 187 (1968).
[21] N. I. KRINSKY and R. P. LEVINE, *Plant Physiol.* **39**, 680 (1964).

columns of powdered sugar and with light petroleum plus 0·5 per cent *n*-propanol as the wash liquid, the pigments of green algae, examined in the vegetative or rapidly growing condition, separated in the following sequence: neoxanthin (most strongly adsorbed), the additional xanthophyll (if present), violaxanthin, chlorophyll *b*, lutein plus traces of zeaxanthin, chlorophyll *a*, traces of cryptoxanthin, and carotenes.

Isolated by chromatography, the new xanthophyll was found to be spectroscopically similar to lutein and does not yield a blue color when the ethereal solution is treated with concentrated hydrochloric acid.[2] With respect to adsorbability and spectral properties, it is different from various, so-called "secondary" carotenoid pigments that are commonly found in green algae deprived of water and essential nutrients.[2,22,23]

The xanthophyll has now been isolated in larger quantities from two green algae, *Scenedesmus obliquus* (Scenedesmaceae) and *C. vulgaris* (Oöcystaceae), both of the order Chlorococcales. The deuterated pigment has also been prepared from fully-deuterated *C. vulgaris*.[24]

From various physical and chemical properties, the xanthophyll is now identified as a hydroxy lutein. As discussed below, it is probably not identical with similar, strongly adsorbed xanthophylls variously reported as trollein and trollein-like carotenoids observed in other green plants and in orange extracts after treatment with hydrochloric acid. This xanthophyll is now called loroxanthin.

CULTURE OF ALGAE

The algae were grown in flat, tilting vessels with about 2·5 l. of the culture medium and with continuous illumination from "daylight" fluorescent lamps as previously described.[25,26] The cultures, which produced 5 to 6 g of dry cells per l. after 5·5–6 weeks, were centrifuged, and the sedimented cells were either extracted immediately or preserved in a low-temperature refrigerator until they could be used.

EXTRACTION AND ISOLATION OF THE CAROTENOID PIGMENTS

For extraction of the pigments, the cells from two lots of the cultures were placed in nearly 2 l. of vigorously boiling water. After 2 min, the green suspensions were cooled with a bath of ice and water, and the cells were collected by centrifugation. The water was decanted and the pigments extracted with methanol (900 ml) plus diethyl ether (100 ml). After 20 min, the suspensions were centrifuged, and the residue re-extracted with methanol (700 ml) plus ether (300 ml). This mixture was centrifuged, and all the supernatants were combined and treated with KOH (100 g) in methanol (300 ml). After about 0·5 hr, the yellow pigments were transferred to ether by the addition of this solvent and aqueous salt solution. The ether extracts were evaporated to dryness in a rotary evaporator.

For their separation by TLC, the mixture of carotenoid pigments was dissolved in diethyl ether and chromatographed on an alumina sheet (Eastman Chromagram, 6062). Washed with ether:light petroleum:*n*-propanol (5:4:1), the pigments separated in the sequence neoxanthin, loroxanthin, violaxanthin, lutein and carotene.

For their separation by chromatography with powdered sugar, the carotenoids from two

[22] G. DERSCH, *Flora* **149**, 566 (1960).
[23] F. C. CZYGAN, *Arch. Mikrobiol.* **61**, 81 (1968).
[24] H. H. STRAIN, M. R. THOMAS, H. L. CRESPI and J. J. KATZ, *Biochim. Biophys. Acta* **52**, 517 (1961).
[25] W. CHORNEY, N. J. SCULLY, H. L. CRESPI and J. J. KATZ, *Biochim. Biophys. Acta* **37**, 280 (1960).
[26] H. L. CRESPI, S. M. CONRAD, R. A. UPHAUS and J. J. KATZ, *Ann. N. Y. Acad. Sci.* **84**, 648 (1960).

lots of the cultures were dissolved in ether (100 ml) which was then diluted with light petroleum (200 ml) and *n*-propanol (1·5 ml). Aliquot portions of this solution were added to six to ten columns (8 by 30 cm) packed with dry, powdered sugar. The columns were then washed with light petroleum plus 20 per cent diethyl ether and 0·5 per cent *n*-propanol to elute the carotenes and lutein. The topmost lemon-yellow zone contained neoxanthin; the second yellow zone contained loroxanthin; and the third, pale-yellow zone included small quantities of violaxanthin contaminated by lutein. Each pigment was eluted from the separate zones with ether plus ethanol, and the respective elutriate was evaporated to dryness. The residual pigment was dissolved in ether, crystallized by the addition of light petroleum or *iso*-octane, washed with light petroleum, and dried in a vacuum. In typical experiments, 55 mg of loroxanthin and 40 mg of neoxanthin were obtained from *Chlorella vulgaris* and 30 mg of loroxanthin and 29 mg of neoxanthin plus 55 mg of lutein from *Scenedesmus*.

For their preparation with magnesia, the carotenoids from two lots of the algae were dissolved in ether (75 ml) which was then diluted with light petroleum (67·5 ml) and propanol (7·5 ml). Aliquot portions were adsorbed in three columns (5 by 30 cm) of activated magnesia (1 part Seasorb 43 plus 1 part Celite 545, untreated) and washed with light petroleum plus 10 per cent *n*-propanol to elute carotene and lutein plus violaxanthin. The columns then contained an upper, orange-yellow zone with loroxanthin and a lower, lemon-yellow zone with neoxanthin. The *C. vulgaris* yielded 60 and 53 mg of eluted and crystallized loroxanthin and neoxanthin, respectively. The *Scenedesmus* provided 31 and 12 mg, respectively, plus 57 mg of lutein.

Loroxanthin in about the same yields and of the same adsorbability was obtained from freshly harvested cells and from frozen and stored cells. The chromatographic behavior of the loroxanthin preparations from the saponified extracts and from the unsaponified extracts was also the same; hence it did not occur as an ester.

PURIFICATION OF LOROXANTHIN

The loroxanthin isolated by chromatography and by crystallization, was contaminated with various colorless substances. Consequently, it was recrystallized from various solvents ranging from methanol plus water to ether, tetrahydrofuran or chloroform plus light petroleum or *iso*-octane. These recrystallized preparations did not exhibit reflective surfaces under the microscope.

PROPERTIES OF LOROXANTHIN

In agreement with earlier reports,[2] loroxanthin did not give the blue Et_2O–HCl test, and the spectral absorption curve was identical with that of lutein, λ_{max} in ethanol, 446, 474 nm. The spectral absorption properties of loroxanthin were not altered significantly by acetic acid in ethanol, and HCl in ethanol shifted the maxima only slightly toward shorter wavelengths, as in *trans* → *cis* isomerization and in the absence of rearrangeable 5,6-epoxide groups. Isomerization of loroxanthin with I_2 in light shifted the absorption maxima to slightly shorter wavelengths, namely, 440 and 467 nm, as did traces of HCl, indicating an all-*trans* arrangement of the double bonds.

The i.r. absorption spectrum of loroxanthin, determined with a thin layer of the pigment deposited on KBr plates (Beckman IR-12), exhibited λ_{max} (cm^{-1}) 3380 (assoc. —OH); 3040; 2965, 2925, 2865 (—CH_2, —CH_3); 1570 (conj. C=C); 1450 (—CH_2—); 1385, 1365 (—CH_3); 1040, 1024 (sec. —OH or allylic primary —OH); 967 (*trans* CHR=CHR); 831 (CHR=CR_2).

There were no maxima indicative of C=C=C, —CHO, C=O, —OCH_3, ester, tert. —OH or furanoid groups.

The NMR spectra were determined with about 5–15 mg of pigment in 0·3–0·4 ml C_5D_5N (Varian HA 100). Based upon hexamethylsiloxane (HMS) as internal standard, these spectra exhibited methyl peaks at τ(8·15+8·17), 8·25, 8·30, 8·45, 8·97, 9·03 and 9·20. Relative intensities were (2 or 3):1:1:1:2:1:1, respectively. With $CDCl_3$ as solvent, the peaks were at τ8·11, 8·16, 8·32, 8·44, 8·98, 9·07 and 9·22 with relative intensities 2:1:1:1:2:1:1.

The molecular weight of loroxanthin, determined with a high resolution mass spectrometer (AEI MS 902) equipped with a mass spectrometer data acquisition and analysis system (Picker Nuclear, White Plains, New York), was 584·4233, empirical formula $C_{40}H_{56}O_3$. The fragmentation pattern is shown by the bar graph in Fig. 1.

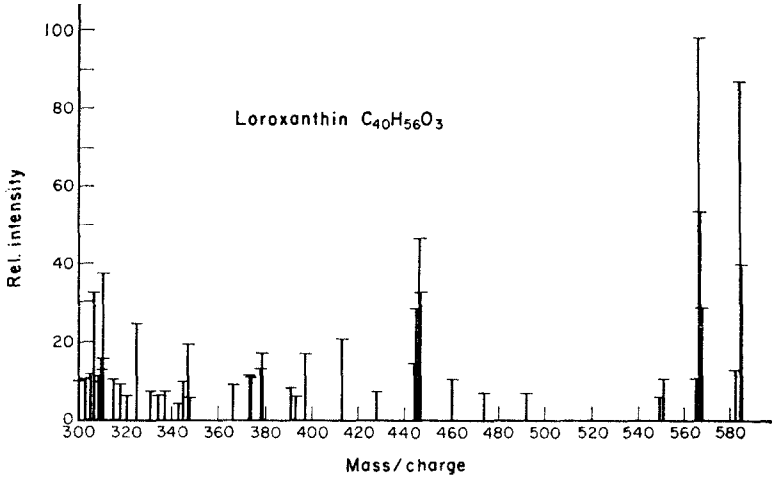


FIG. 1. MASS SPECTRUM OF LOROXANTHIN.

DEUTERATED LOROXANTHIN

Deuterated loroxanthin was isolated from fully deuterated *Chlorella vulgaris*[24] by chromatography on activated magnesia (Seasorb 43) as described above for the common loroxanthin. A typical experiment yielded 37 mg of deuterated loroxanthin, 44 mg of deuterated neoxanthin and 132 mg of deuterated lutein. The deuterated loroxanthin exhibited a molecular mass of 637·7557, empirical formula $C_{40}D_{53}(OH)_3$.

Fully deuterated loroxanthin was prepared from the deuterated, crystallized loroxanthin, which was allowed to stand in about ten times its weight of CH_3OD for an hour before the methanol was evaporated in a vacuum system. This re-exchange was repeated once. The resultant fully deuterated loroxanthin exhibited a molecular mass of 640·7749, empirical formula $C_{40}D_{53}(OD)_3$.

Ordinary loroxanthin exchanged with CH_3OD as described for deuterated loroxanthin exhibited a mass of 587, empirical formula $C_{40}H_{53}(OD)_3$.

LOROXANTHIN TRIACETATE

A few milligrams of loroxanthin were allowed to react with an excess of acetic anhydride (1 ml) in pyridine (10 ml) for about 8 hr. The product was transferred to light petroleum and

chromatographed in a column of powdered sugar. The product was primarily one, weakly-polar substance that was less sorbed than several monohydroxy carotenoids as lutein-3-acetate, lutein-3′-acetate, neoxanthin di-acetate and cryptoxanthin, in thin layers of powdered sugar washed with light petroleum (20–40°) plus 0·5 per cent *n*-propanol. The absorption curve in the visible was identical with that of loroxanthin, and characteristic absorption maxima in the i.r. were: 2923, 1740, 1450, 1368, 1245, 1025 and 970 cm^{-1}. The molecular mass was 710·4550, molecular formula $C_{40}H_{53}(O_2CCH_3)_3$. The fragmentation pattern is indicated in Fig. 2.

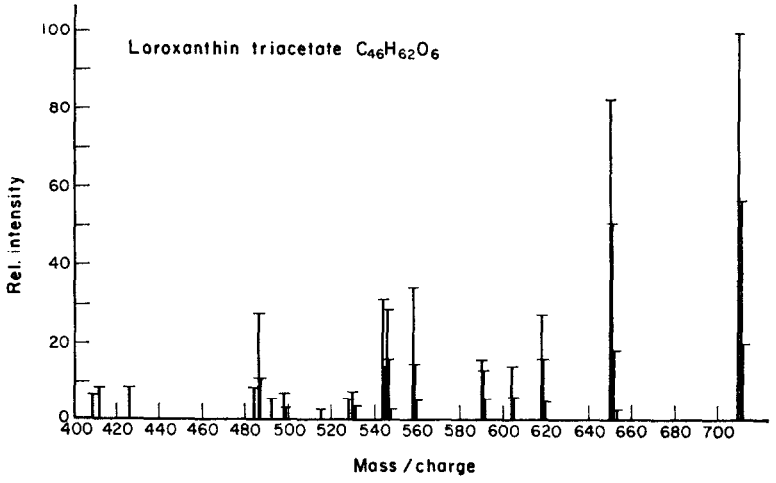


FIG. 2. MASS SPECTRUM OF LOROXANTHIN TRIACETATE.

LOROXANTHIN MONOMETHYL ETHER AND DIMETHYL ETHERS

Loroxanthin, dissolved in methanol (100 ml) plus conc. HCl (1 ml) for 17 min yielded three ethers (separated in columns of powdered sugar washed with light petroleum plus 0·4% *n*-propanol). One product was less sorbed than the residual loroxanthin; the other two were weakly sorbed and barely separable from each other. The spectral curves of all these products were like those of loroxanthin, but shifted to slightly shorter wavelengths (like partially *cis*-isomerized loroxanthin), and the i.r. exhibited a pronounced maximum at 1085 cm^{-1} (indicative of $—OCH_3$). The molecular mass of the most-adsorbed product was 598·4400, molecular formula $C_{40}H_{53}(OH)_2OCH_3$. The molecular mass of the two weakly-sorbed methoxy derivatives was 612·4544, molecular formula $C_{40}H_{53}(OH)(OCH_3)_2$.

LOROXANTHAL

An aldehyde of loroxanthin was obtained by treating the xanthophyll with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone, dissolved in dry benzene, at room temperature. In control experiments, however, this reagent destroyed lutein. Consequently, the loroxanthal was also prepared with a milder reagent, namely, *p*-chloroanil, in dry benzene, in the presence of iodine under sodium light.[27] This aldehyde was easily precipitated from ether solution by light petroleum, but it was difficult to obtain in the crystalline state.

[27] S. L. JENSEN, *Acta Chem. Scand.* **19**, 1166 (1965).

The loroxanthal exhibited one principal absorption maximum in the visible region, 480 nm in ethanol, 475 nm with a shoulder at about 500 nm in light petroleum. In the i.r., there was strong absorption at 1175 cm^{-1} and absorption at 2760 cm^{-1} (aldehydic C—H). The NMR showed strong methyl peaks at τ8·16, 8·27, 8·46, 8·96, 9·04 and 9·22 and aldehydic H at $\tau = 0{\cdot}38$ (in C_5D_5N, HMS).

Mass spectrometry indicated a molecular weight of 582·4072, formula $C_{40}H_{52}(OH)_2O$. A fragment equivalent to M—120 (probably toluic aldehyde) was observed. There was no fragment corresponding to loss of benzaldehyde.

MOLECULAR STRUCTURE OF LOROXANTHIN

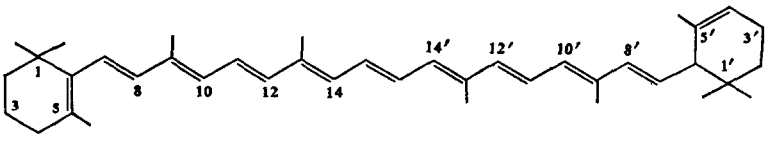
The mass spectra of loroxanthin and its triacetate and the exchange reactions with CH_3OD establish this pigment as a $C_{40}H_{56}O_3$ xanthophyll with three hydroxyl groups. The absorption spectrum in the visible region, which is not influenced by hydroxyl groups,[2] and that in the i.r. indicate a conjugated system of double bonds like that in α-carotene (I) and lutein (II). Close agreement of the NMR with that of the lutein indicates α-cyclogeranylidene and β-cyclogeranylidene rings plus at least three methyl groups in the chain. The presence of the α-ring is shown by unequivalent NMR resonances of the two geminal —CH_3 groups at 1′ (τ9·03 and 9·20) and of one —CH_3 on an unsaturated carbon atom, out of conjugation, at 5′ as in α-carotene and lutein. Based on experiments with lutein, the α-ring is also indicated by MS fragmentation leading to M − 56 peaks as expected for the retro-Diels-Alder fragmentation[28–32] of I, III, VI, VII, X, XI as well as from the absence of M − 56 fragmentation required for II, IV, V, VIII, IX, XII, XIII and XIV. Fragmentation effects reported herein will be considered in another report.

Comparative absorption spectra[33] and the NMR properties of loroxanthin support the presence of the β-cyclogeranylidene ring (i.e. with three methyl groups). As in the β-ring of lutein, α- and β-carotene, zeaxanthin, etc., two geminal —CH_3 groups are equivalent at τ8·97, and a single methyl is at τ8·30 (position 5).

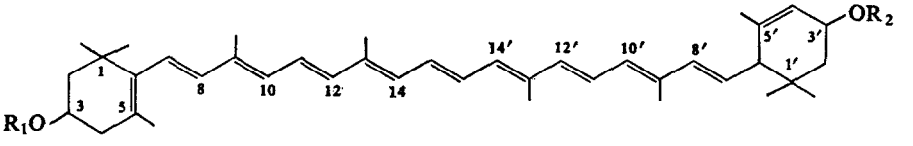
Two of the hydroxyl groups in loroxanthin are allylic; diethers are formed in methanolic HCl,[34] and intensity of the i.r. peak at 1023 cm^{-1} is greater than that at 1040 cm^{-1} (nonallyic, sec. —OH).[34–36] One hydroxyl group is primary, indicated for $\geqslant C{\cdot}CH_2OH$ by τ5·54 (*ca.* 2H)[34,37,38] and by the shift of this resonance in the acetate to τ5·05.[37,38] None of the alcohol groups is tertiary, inferred from the absence of absorption at 1150 cm^{-1} in the i.r. and from the ready formation of the triacetate. By elimination, there must be two secondary alcohol groups.

One alcohol group appears to be located at position 3 in the β-ring indicated by close agreement between the NMR spectra of lutein and loroxanthin. The two geminal —CH_3

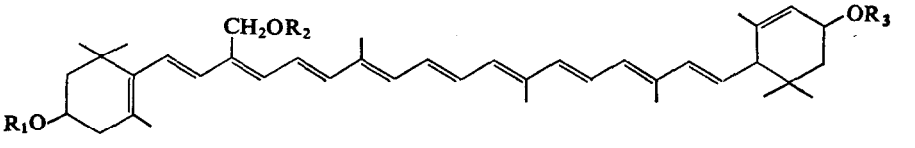
[28] U. SCHWIETER, H. R. BOLLIGER, L. H. CHOPARD-DIT-JEAN, G. ENGLERT, M. KOFLER, A. KÖNIG, C. v. PLANTA, R. RÜEGG, W. VETTER and O. ISLER, *Chimia, Switz.* **19**, 294 (1965).
[29] S. L. JENSEN, *Pure Appl. Chem.* **14**, 227 (1967).
[30] S. L. JENSEN, *Acta Chem. Scand.* **21**, 1972 (1967).
[31] B. C. L. WEEDON, *Chem. Britain* **3**, 424 (1967).
[32] H. BUDZIKIEWICZ, C. DJERASSI and D. H. WILLIAMS, *Mass Spectrometry of Organic Compounds*, Holden-Day, San Francisco (1967).
[33] A. HAGER and T. MEYER-BERTENRATH, *Ber. Deut. Botan. Ges.* **80**, 426 (1967).
[34] S. L. JENSEN and S. HERTZBERG, *Acta Chem. Scand.* **20**, 1703 (1966).
[35] C. BODEA, E. NICOARĂ, V. TĂMAS and H. MANTSCH, *Annalen* **666**, 189 (1963).
[36] A. J. AASEN and S. L. JENSEN, *Acta Chem. Scand.* **21**, 970 (1967).
[37] M. C. MARKHAM and S. L. JENSEN, *Phytochem.* **7**, 839 (1968).
[38] L. CHOLNOKY, J. SZABOLCS and E. S. WAIGHT, *Tetrahedron Letters* 1931 (1968).



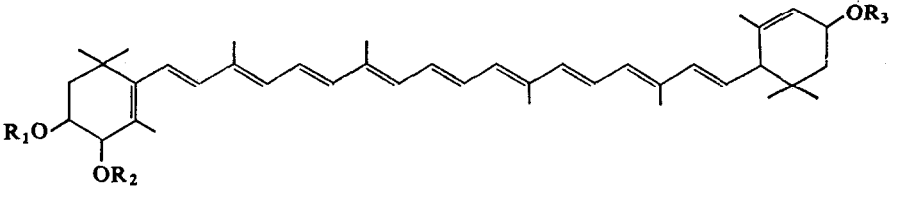
(I) α-Carotene



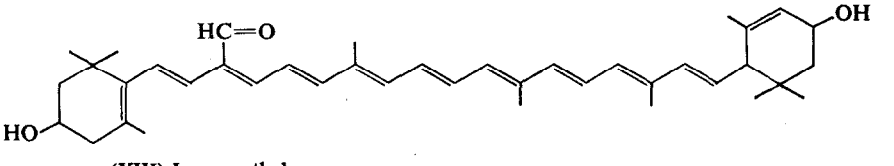
(II) $R_1 = R_2 = H$, Lutein
(III) $R_1 = H$, $R_2 = CH_3$, Lutein-3′-*monomethyl ether*
(IV) $R_1 = R_2 = COCH_3$, Lutein diacetate



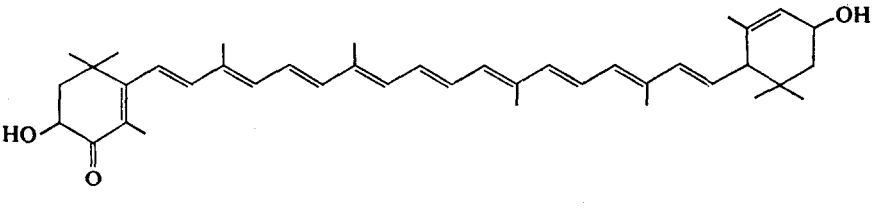
(V) $R_1 = R_2 = R_3 = H$, Loroxanthin (L)
(VI) $R_1 = R_2 = H$, $R_3 = CH_3$ L monomethyl ether
(VII) $R_1 = H$, $R_2 = R_3 = CH_3$, L dimethyl ether
(VIII) $R_1 = R_2 = R_3 = COCH_3$, L triacetate



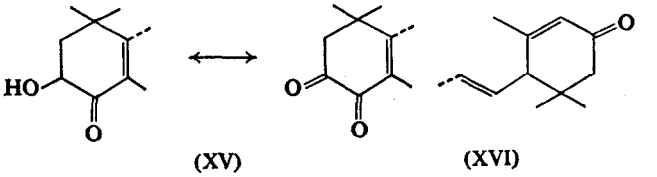
(IX) $R_1 = R_2 = R_3 = H$
(X) $R_1 = R_2 = H$, $R_3 = CH_3$
(XI) $R_1 = H$, $R_2 = R_3 = CH_3$
(XII) $R_1 = R_2 = R_3 = COCH_3$



(XIII) Loroxanthal



(XIV)



(XV) (XVI)

groups are equivalent at τ8·97, as is common for 3-hydroxy β-rings, but might not be equivalent with —OH in the 2 position. In natural pigments the hydroxy group is usually at 3. (At 4 the hydroxy group would be allylic.)

Another hydroxyl group must occur at the 3′ position in the α-ring. There it accounts for one secondary, allylic alcohol; it allows the M − 56 fragmentation of the diether (VII); and it accounts for the NMR. (No other location in the ring would be appropriate; at 2′ it would not be allylic and would not allow M − 56 fragmentation.[32,39] At 4′ it would be enolic, and at 6′ it would be tertiary.) The M − 56 fragmentation of the monomethyl ether also indicates that this substance is formed from an α-ring with a 3′ hydroxy group (VI, or possibly X).

The mass fragmentation pattern of loroxanthin triacetate VIII also indicates the occurrence of monohydroxy cyclogeranylidene rings. A peak at mass 528·3232 corresponds to $C_{35}H_{44}O_4 = C_{46}H_{62}O_6 - C_{11}H_{18}O_2$. This loss corresponds to the acetate of an hydroxy cyclogeranylidene group. There was no peak at mass 470 corresponding to $C_{46}H_{62}O_6 - C_{13}H_{20}O_4$ as would be required if one cyclogeranylidene group contained two hydroxy groups as the acetates.

The mass fragmentation pattern of loroxanthin dimethyl ether established the presence both of α- and β-cyclogeranylidene rings. A peak at mass 474·3505 corresponds to $C_{33}H_{46}O_2 = C_{42}H_{60}O_3 - C_9H_{14}O$. This loss indicates the presence of an hydroxy β-ring which would not have been methylated in loroxanthin treated with methanol plus HCl. A peak at mass 459·3227 corresponds to $C_{32}H_{43}O_2 = C_{42}H_{60}O_3 - C_{10}H_{17}O$. This loss indicates the presence of a methylated hydroxy α-ring, the methylation having taken place with the allylic hydroxyl when loroxanthin was treated with methanol plus HCl. From all these facts, one hydroxy group occurs at the 3 position in the β-ring; a second occurs at the 3′ position in the α-ring.

From its chemical properties and the NMR the third alcohol group must occur on an "in-chain" methyl. It must be allylic and oxidizable to an aldehyde (loroxanthal); hence, it cannot be located in the rings, for example, at 4 in IX, or on the geminal methyls. At the ring locations, it might be oxidized to a ketone or diketone (XV, XVI), but not to the observed aldehyde. On the geminal methyls it would not be allylic. The NMR also indicates that these geminal methyls and also the methyls at 5 and 5′ with τ8·30 and 8·45 are unsubstituted. The only other available locations for an allylic alcohol oxidizable to a conjugated aldehyde are the "in-chain" methyls.

Location of the third hydroxyl group on an "in-chain" methyl also follows from the mass spectrometry of loroxanthin itself. Fragments usually observed from carotenoids are M − 92 (toluene) and M − 106 (*m*-xylene), due to "coiling" and splitting.[28–32,40] With loroxanthin, the M − toluene peak is prominent, but the M − xylene peak is significantly reduced in intensity, and a prominent peak appears at M − 122, believed to be M − *m*-methylbenzyl alcohol. No peak for M − benzyl alcohol appears at M − 108. The peak for M − *m*-methylbenzyl alcohol is obtained with the monomethyl ether but not with the two dimethyl ethers, which exhibit a pronounced M − 136 peak instead, probably M − *m*-methylbenzyl ether. Moreover, the triacetate, VIII, lacks the M − *m*-methylbenzyl alcohol peak and exhibits one at M − 164, probably M − *m*-methylbenzyl acetate. At high resolution, the M − *m*-methylbenzyl alcohol and M − *m*-methylbenzyl acetate represent loss of $C_8H_{10}O$ and $C_{10}H_{12}O_2$, respectively, thus confirming the identity of the free and acetylated methylbenzyl alcohol. Loss of toluene also occurs, but no fragments corresponded to loroxanthin (V) less

[39] M. MOUSSERON-CANET, J.-C. MANI and D. LERNER, *Bull. Soc. Chim. Fr.* 3043 (1966).
[40] C. R. ENZELL, G. W. FRANCIS and S. L. JENSEN, *Acta Chem. Scand.* **22**, 1054 (1968).

benzyl alcohol, to 3′-methoxy loroxanthin (VI) less benzyl alcohol, to dimethoxy loroxanthin (VII) less benzyl ether or to loroxanthin triacetate (VIII) less benzyl acetate.

Loss of toluene by a "coiling" mechanism[28–32,40] may not involve the methyl groups at positions 9 and 9′. Inclusion of the 9 and 9′ methyls in toluene fragments requires splitting between carbons 6 and 7, a location next to the ring that is presumed to be sterically "protected" against "coiling" close to the ring. The toluene methyl must, therefore, come predominately from the 13 and 13′ methyls. Formulawise, steric hindrance of splitting at C-6 to C-7 reduces the opportunities for toluene formation relative to xylene formation.[40] With the methyl groups at 9 and 9′ prevented from forming toluene, the methyls at 13 and 13′ must be the source of the methyl in the toluene. Splitting of the chain at two locations will yield xylene with the methyls at 9 and 13, similarly for those at 9′ and 13′, but splitting at one position only will yield xylene with the methyls at 13 and 13′. Consequently, the hydroxy group on the chain methyl of loroxanthin must occur at the 9 or 9′ location, not at the 13 or 13′ location.

Location of the third hydroxyl on the methyl at the 9 or 9′ location also follows from the mass fragmentation of the triacetate. A peak at mass 404·2710 corresponds to $C_{28}H_{36}O_2 = C_{46}H_{62}O_6 - C_{18}H_{26}O_4$. If the acetate is not lost separately, the total lost fragment represents a diacetate with one acetate on the methyl at C-9 or C-9′.

Location of the third hydroxyl on the methyl at the 9 position follows from the mass fragmentation of the loroxanthin dimethyl ether. A fragment of mass 376·2780 corresponds to $C_{27}H_{36}O = C_{42}H_{60}O_3 - C_{15}H_{24}O_2$. From the hydrogen content, the lost portion must represent the β-ring (with free hydroxyl at 3) and a portion of the chain with methyl ether on the methyl attached to C-9. A similar fragment but with higher hydrogen content, must represent the opposite or α-end of the chain but without the methyl ether on the methyl attached to C-9′. This fragment with mass 373·2554 corresponds to $C_{27}H_{33}O = C_{42}H_{60}O_3 - C_{15}H_{27}O_2$. The higher hydrogen-content of this last fragment indicates that there was no methyl ether on the methyl at C-9′.

Comparison of the methyl region of the NMR of lutein and loroxanthin has revealed no shift of the methyl groups at positions, 5′, 1 and 1′. Relative to lutein, however, the methyl at 5 of loroxanthin is shifted slightly downfield, and one "in-chain" methyl is shifted slightly upfield. Similar effects have been observed with the acetylenic triple bond;[41–43] hence, the primary hydroxy group of loroxanthin is assigned to the methyl at the 9 position (V).

MOLECULAR STRUCTURE OF LOROXANTHIN METHYL ETHERS

Establishment of the structure of loroxanthin and the M − 56 fragmentation of the monomethyl ether prove that this ether must be the 3′ derivative (VI). Similarly the dimethyl ethers must be isomers of VII.

MOLECULAR STRUCTURE OF LOROXANTHAL

The molecular weight of loroxanthal, 582, indicates that this pigment is an aldehyde formed by oxidation (dehydrogenation) of the primary alcohol to a carbonyl (XIII). No resonance occurs in the τ5·54 region (primary —CH_2OH) and the presence of an aldehyde H is indicated by τ0·38. There is no shift of the 1 and 5 methyls in the NMR due to formation

[41] A. K. MALLAMS, E. S. WAIGHT, B. C. L. WEEDON, D. J. CHAPMAN, F. T. HAXO, T. W. GOODWIN and D. M. THOMAS, *Chem. Commun.* 301 (1967).
[42] M. S. BARBER, J. B. DAVIS, L. M. JACKMAN and B. C. L. WEEDON, *J. Chem. Soc.* 2870 (1960).
[43] K. AITZETMÜLLER, W. A. SVEC, J. J. KATZ and H. H. STRAIN, *Chem. Commun.* 32 (1968).

of the carbonyl group (as expected for XIV). The shift of the absorption maxima from 447 and 475 nm to 480 nm also corresponds to the shift observed with C=O located in the chain (as in fucoxanthin,[44] siphonaxanthin[45,46] and peridinin[45] or in a side-chain.[47,48])

Fragmentation of the loroxanthal did not yield M − benzaldehyde but it did provide a unit of M − 120 corresponding to M − C_8H_8O, probably *m*-tolualdehyde an oxidation product of xylene. Formation of this fragment is analogous to the loss of *m*-methylbenzyl alcohol by loroxanthin (V). No corresponding fragment was observed with keto carotenoids as canthaxanthin, astacin or fucoxanthin.

PIGMENTS OF VARIOUS GREEN ALGAE

With thin layers of nonreactive silica gel (Eastman Chromagram sheets) and with columns of powdered sugar, loroxanthin was found in small quantities in a marine *Ulva, U. rigida*, and in abundant quantities in two marine species of *Cladophora, C. trichotoma* and *C. ovoidea*, all from Mission Point, Carmel, California. Loroxanthin was not found in *Chlorella pyrenoidosa*, grown from cultures in which it had not been found before.[15] It was not detected in a freshwater *Spirogyra* (from Maple Lake, Cook County, Illinois) nor in two species of marine *Codium* (*C. fragile* and *Setchellii* from Carmel, California).

As noted above, strongly-sorbed xanthophylls somewhat similar to loroxanthin with respect to adsorbability have already been reported in various algae. One of these from *Chlorella pyrenoidosa* exhibited absorption maxima at 439 and 467 nm.[20] Another from *Dunaliella tertiolecta* (family, Polyblepharidaceae: order Volvocales) exhibited absorption maxima at 422 and 429 nm.[16] Both were, therefore, different from loroxanthin. Similar strongly adsorbed xanthophylls were also obtained from *Chlamydomonas Rheinhardii*,[49] *Spongiochloris typica*,[19] the euglenophycean *Euglena gracilis*,[49,50] various algae with scaly flagella,[51,52] and from *Pseudomonas echinoides*.[53]

Many of these strongly adsorbed pigments have been described as trollein or trollein-like xanthophylls.[49–53] The former was obtained by treatment of trollixanthin (from orange juice) with hydrochloric acid.[54,55] Although trollein exhibits some properties like those of loroxanthin, the method of preparation, the empirical composition, the absence of ether formation in the presence of methanolic hydrochloric acid, and the formation of a diacetate[50,54,55] instead of a triacetate do not support the identity of trollein and loroxanthin.

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[44] A. JENSEN, *Acta Chem. Scand.* **15**, 1605 (1961).
[45] A. L. CURL, *J. Food Sci.* **30**, 426 (1965).
[46] H. KLEINIG and K. EGGER, *Phytochem.* **6**, 1681 (1967).
[47] A. J. AASEN and S. L. JENSEN, *Acta Chem. Scand.* **19**, 1843 (1965).
[48] A. J. AASEN and S. L. JENSEN, *Acta Chem. Scand.* **21**, 2185 (1967).
[49] N. I. KRINSKY, *Anal. Biochem.* **6**, 293 (1963).
[50] B. P. SCHIMMER and N. I. KRINSKY, *Biochemistry* **5**, 1814 (1966).
[51] T. R. RICKETTS, *Phytochem.* **6**, 669 (1967).
[52] T. R. RICKETTS, *Phytochem.* **6**, 1375 (1967).
[53] F. C. CZYGAN and W. HEUMANN, *Arch. Mikrobiol.* **57**, 123 (1967).
[54] A. L. CURL and G. F. BAILEY, *J. Agr. Food Chem.* **2**, 685 (1954); **4**, 156 (1956).
[55] A. L. CURL and G. F. BAILEY, *Food Res.* **20**, 371 (1955).