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THE STRUCTURES OF SIPHONEIN AND SIPHONAXANTHIN FROM *CODIUM FRAGILE**

T. R. RICKETTS

Cell Biology Unit, Department of Botany, The University, Nottingham

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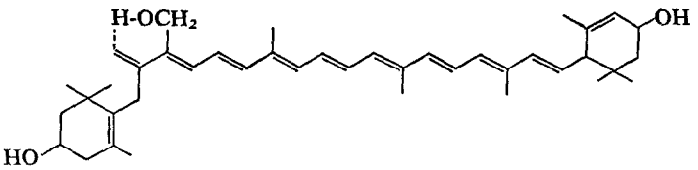
Abstract—Siphonaxanthin isolated from the siphonalean green alga *Codium fragile* has the structure 3,3',19-trihydroxy-7,8-dihydro-8-oxo-α-carotene. Siphonein isolated from the same source has been shown to be siphonaxanthin which is esterified with a variety of fatty acids.

INTRODUCTION

THE SIPHONALEAN green algae usually contain, in addition to chlorophylls *a* and *b*, the following carotenoids: α- and β-carotenes, lutein (and sometimes zeaxanthin), siphonein, violaxanthin, neoxanthin and siphonaxanthin. α-Carotene usually predominates over β-carotene.[1-4] Siphonaxanthin and siphonein (the ester of siphonaxanthin) account for a considerable proportion of the total xanthophylls.[1-4] Structure (I) has been proposed for siphonaxanthin,[5,6] based mainly upon chemical evidence and spectral properties in the visible region. It is suggested that siphonein is siphonaxanthin esterified to lauric acid through an hydroxymethyl group which replaces the usual methyl group at C9.[5,6]



SCHEME I. SIPHONAXANTHIN.

The present work was undertaken in order to provide a basis of comparison for the results of analyses on xanthophylls K1 and K2, which were isolated from some members of the Prasinophyceae.[7] Xanthophyll K1 showed the same spectral and chromatographic properties as authentic siphonein. Saponification converted it into a substance indistinguishable from siphonaxanthin with the tests employed. Some preliminary structural

* Cf. T. J. WALTON, G. BRITTON and T. W. GOODWIN, *Phytochem.* **9**, 2545 (1970).

[1] H. H. STRAIN, in *Photosynthesis in Plants* (edited by J. FRANCK and W. E. LOOMIS), p. 162, Iowa State College Press, Ames (1949).

[2] H. H. STRAIN, in *Manual of Phycology* (edited by G. M. SMITH), p. 243, Chronica Botanica Co., Waltham, Mass. (1951).

[3] H. H. STRAIN, *Chloroplast Pigments and Chromatographic Analysis*, p. 37, 32nd. Ann. Priestley Lect. Pennsylvania State University (1958).

[4] H. H. STRAIN, *Biol. Bull.* **129**, 366 (1965).

[5] H. KLEINIG and K. EGGER, *Phytochem.* **6**, 1681 (1967).

[6] H. KLEINIG, H. NITSCHE and K. EGGER, *Tetrahedron Letters* 5139 (1969).

[7] T. R. RICKETTS, *Phytochem.* **6**, 1375 (1967).

details are given in Ricketts.[7] The present paper reports mass spectral, i.r. and NMR data which supports the structure (I) for siphonaxanthin.

RESULTS AND DISCUSSION

Siphonaxanthin displayed the following absorption maxima: petrol (40–60°), (427), 450, 478 nm; ethanol, 445–451 nm; chloroform, 466 nm. After reduction and purification of the mixture the reduced product showed maxima at (375), 398, 421 and 448 nm in ethanol. Siphonein displayed the following absorption maxima: petrol (40–60°), (427), 450, 478 nm; ethanol, 452–465 nm; chloroform, 466 nm. Saponification of siphonein resulted in siphonaxanthin. Reduction of siphonein and examination of the purified product showed that this had an absorption spectrum in ethanol identical with that of reduced siphonaxanthin. Neither pigment showed any change in absorption maxima in ethanolic solution upon treatment with dilute HCl, indicating the absence of 5,6-epoxide groupings. Ethereal solutions of the pigments did not give a blue colour when treated with conc. HCl; many 5,8-epoxides give a positive reaction.

The single absorption maximum of siphonein and siphonaxanthin in ethanol compared with the triple maxima in petrol indicates that they possess at least one conjugated carbonyl grouping.

The i.r. spectrum of siphonaxanthin shows peaks at 1655 cm^{-1} and 1730 cm^{-1} which supports the presence of a conjugated carbonyl grouping (cf. fucoxanthin).[8] The absence of a signal in the NMR spectrum in the range 0·3–0·6 τ indicates that the carbonyl grouping is ketonic and not aldehydic. Reduction of the ketone group leads to a drop of approximately 30 nm in the main visible maximum; in carotenoids which possess a conjugated carbonyl group in the ring systems the shift of max. upon reduction is about 20 nm, whereas when the carbonyl group lies in the side chain (e.g. fucoxanthin,[7] capsanthin[9] and sintaxanthin)[10] there is a larger shift (e.g. fucoxanthin, 26 nm).[7] Thus, it appears probable that in siphonaxanthin a single ketone group is conjugated in the chain.

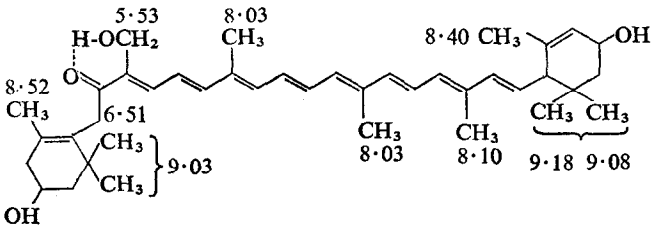
The lack of appropriate bands in the i.r. and NMR spectra excludes the presence of methoxy groupings in siphonaxanthin. The position of the absorption maxima of the reduced pigments indicate that the chromophore could consist of eight conjugated double bonds,[7] whilst those of the non-reduced pigments indicate that the ketone group is conjugated to the polyene chain in both siphonein and siphonaxanthin. Allene groupings and acetylenic linkages can be excluded because of the absence of bands in the i.r. spectrum near 1930 cm^{-1} and 2170 cm^{-1} respectively.

NMR Studies

The NMR spectrum of siphonaxanthin in $CDCl_3$ shows the signals expected for a 3′ hydroxy "α-end group" (cf. lutein)[8]: 9·08 (3H), 9·18 (3H), 8·40 (3H) in the ring system and 8·10 (3H), 8·03 ($\frac{1}{2}$ of 6H) in the conjugated chain (values in τ). The remaining signals at 8·03 ($\frac{1}{2}$ of 6H) and 9·03 τ (6H) can be attributed to a 3 hydroxy "β-end group" (cf. zeaxanthin).[8] The signal for the 5-methyl (8·52 τ, 3H) is about 0·2 τ higher than one would expect by analogy with zeaxanthin; this shift may be ascribed to the anisotropy of the carbonyl

[8] B. C. L. WEEDON, *Fortschr. Chem. Org. Naturstoffe* **27**, 81 (1969).
[9] A. L. CURL, *Agri. Food Chem.* **10**, 504 (1962).
[10] H. YOKOYAMA and M. J. WHITE, *J. Org. Chem.* **30**, 3994 (1965).

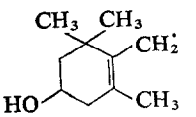
group. The two proton signal at 6·51 τ is in the region expected for the C7 methylene group in the environment shown in (II).[8,11] The broadened singlet at 5·53 τ can be ascribed to the —C$\underline{H}_2$O—H. group. Although no signal can be assigned to this —O$\underline{H}$ proton, on addition of D_2O to the solution the signal at 5·53 τ sharpens appreciably because of the removal of coupling to the —OH.



SCHEME II. NMR SIGNALS IN SIPHONAXANTHIN (IN τ).

Mass Spectra of Siphonaxanthin

A small molecular ion was detected at *m/e* 600 and successive losses of 3 molecules of water give significant peaks at *m/e* 582, 564 and 546 respectively. High resolution measurements of *m/e* 582 established its molecular composition as $C_{40}H_{54}O_3$ (measured 582·405, calculated 582·407); thus, the molecular formula for siphonaxanthin is $C_{40}H_{56}O_4$. In addition, significant peaks at *m/e* 429 and 411 can be ascribed to the expected loss of the fragment (III) from (M—H_2O) and (M—$2H_2O$) respectively by cleavage α to the carbonyl group.



SCHEME III.

The Structure of Siphonaxanthin

The molecular formula of siphonaxanthin is in agreement with the structure proposed (I & II). Since there can only be a maximum of nine double bonds in the polyene chain linking the cyclic end groups in normal bicyclic carotenoids (as indicated for siphonaxanthin in the NMR spectrum) and the extreme end of the molecule of siphonaxanthin from the ketone group is an "α-end group", then the chromophore of eight conjugated double bonds must lie in the 9,7′-positions. The conjugated ketone group must therefore lie in the 8-position. Fragmentation studies also provide good evidence for this.

Mass spectral data indicates that there are three hydroxyl groups, one of which is primary. The remaining oxygen atom indicated by the molecular formula must reflect the single conjugated ketone. The loss of fragment III probably indicates that there is a single hydroxyl group in the "β-end group". Data on the hydroxyl groups of siphonaxanthin[6] indicate that these are positioned as shown in (II). The NMR data also supports the positioning of the primary hydroxyl group at C19. Thus, the structure of siphonaxanthin is (II).

[11] ANON., *High Resolution NMR Catalogue, Spectrum No.* 545, Varian Associates (1963).

Siphonein

Siphonein isolated from *Caulerpa prolifera* has been assigned the structure siphonaxanthin monolaurate, the fatty acid being esterified to the primary hydroxyl group.[5] In *Codium fragile* GLC of the methyl esters released upon saponification of siphonein has shown that in this organism siphonaxanthin is esterified to a wide variety of fatty acids (Table 1). This may well account for the difficulty experienced in the isolation of siphonein in this alga.

TABLE 1. THE RETENTION VOLUMES AND RELATIVE ABUNDANCES OF THE METHYL ESTERS OF THE FATTY ACIDS DERIVED FROM THE SIPHONEIN OF *Codium fragile* (GLC)

Retention vol. relative to palmitate	Relative abundance %*	Tentative identity
0·47	0·8	C12
0·54	1·1	C13
0·63	26·4	?C13 unsat.
0·75	26·4	C14
0·85	0·5	?C15
0·90	1·2	?C15 unsat.
0·97	6·3	?C15 unsat.
1·00	6·6	C16
1·13	3·1	?C17
1·24	1·4	C18
1·30	7·4	?C18:1
1·41	0·5	?C18:2
1·52	7·1	C19
1·82	4·7	C20
2·13	6·3	C21

* Calculated from the areas of the peaks.

The lower absorption maxima of siphonaxanthin in the visible region as compared with siphonein has been attributed to hydrogen bonding in the former between the conjugated ketone group and the hydroxyl group which is involved in esterification in siphonein.[5] The positions of the hydroxyl and ketone bands found in the i.r. spectrum of siphonaxanthin are not inconsistent with this type of hydrogen bonding. The only major difference between the NMR spectra of siphonaxanthin and siphonein is that the signal at 5·53 τ in the former is not found in siphonein. A signal, of equivalent strength, appears at 4·90 τ and indicates the esterification of the primary hydroxyl group at C9.

EXPERIMENTAL

Materials

Siphonein and siphonaxanthin were extracted from *Codium fragile* (U.K. strain). About 3 Kg of frozen alga were very kindly provided by Dr. W. Mackie, The Astbury Department of Biophysics, The University, Leeds. In the final purification procedures, redistilled Analar solvents were used. For the final purification of the xanthophylls by chromatography the adsorbents (magnesium oxide and celite) were successively extracted with excesses of methanol, acetone and petroleum spirit (b.p. 40–60°). Without these precautions much non-pigmented material was found in the eluent.

Extraction of Pigments

Processing was batchwise, in about 160 g amounts. The total processed for the isolation of siphonaxanthin was about 2 Kg, whereas about 600 g was used in the isolation of siphonein. The batches of alga were ground

in dim light with sand and acetone in sequential volumes to remove the pigments. The aliquots of extract were centrifuged, combined and evaporated to dryness in the dark at low temperature. The pigment for siphonein extraction was processed directly, whereas that for siphonaxanthin isolation was saponified (in methanolic solution containing 10%, v/v of 60%, w/v aq. KOH at 40° for 10 min) before extraction and processing of the saponified pigments.

Chromatography

The pure pigments were separated by a combination of chromatography upon columns of icing sugar, developed with a gradient of acetone in petrol (b.p. 40–60°); and by chromatography upon MgO–Celite Hyflo-super-cel (1:1, w/w) developed with a gradient of *n*-propanol in petrol (b.p. 40–60°).

The purity of the siphonaxanthin was checked by TLC upon polyamide cellulose, developed with petrol (b.p. 100–120°)–MeOH–MeCOEt (8:1:1, by vol.) or with H_2O–MeOH–MeCOEt (1:5:5, by vol.);[12] and also upon silica gel G, developed either with hexane–Et_2O–HOAc (80:20:1, by vol.)[13] or with $CHCl_3$–MeOH–HOAc (85:25:1, by vol.) or with Et_2O.[14,15] After drying, the silica gel plates were sprayed with 10 per cent aq. H_2SO_4 and heated in an oven at 165° for 1 hr to demonstrate any non-pigment impurities. Siphonaxanthin gave R_fs of 0·0, 0·80 and 0·11 respectively in the three silica gel systems (monopalmitin gave an R_f of 0·51 in the last system). No impurities were detected using any of the methods. Purity was checked additionally by horizontal circular chromatography on Schleicher and Schüll kieselguhr paper, No. 287, developed with petrol (b.p. 60–80°) containing either 10% or 20%, v/v respectively of acetone.[16] Siphonaxanthin gave R_fs of 0·17 and 0·56 respectively.

The yield of purified siphonaxanthin was about 32 mg from 2 Kg of *Codium fragile*. After vacuum desiccation the extinction coefficient in ethanol was found to be $E^{1\%}_{1\,cm} = 1160$ at 447 nm; this value is probably low due to occluded solvent.

The purified siphonein fraction, which showed single spots on the two polyamide–cellulose TLC systems already described, was chromatographed upon silica gel G plates, developed with Et_2O,[15] or with 40%, v/v Et_2O in hexane (b.p. 67–70°),[13] or with $CHCl_3$–MeOH–H_2O, 65:25:4, by vol.[13] respectively. The R_fs obtained were 0·87, 0·0, and 0·99 respectively. No impurities were detected. The yield was about 2 mg from 600 g of *Codium fragile*.

GLC

The purified siphonein was saponified as described earlier (but for a 15 min period at 40°). The mixture was then diluted with water and the pigments extracted. The aqueous extract was acidified and the fatty acid component extracted with Et_2O. Methyl esters of the fatty acids were prepared by treatment of the dried extract with 3 ml BF_3 complex in a boiling water bath for 2 min. The methyl esters were then extracted with petrol (b.p. 40–60°) after dilution of the mixture with water. Aliquots of the methyl ester fraction were chromatographed on 2 m columns of 1,4 butanediol succinate on HMDS Chromosorb W., 80–100 mesh, 20:80, w/w, in a Perkin-Elmer F11 dual column gas chromatograph with flame ionization detector, using a temperature programme of 100–185° at 4°/min.

I.r. Spectroscopy

The siphonaxanthin was dissolved in a small amount of $CHCl_3$ and repeatedly evaporated to dryness under N_2 in order to remove any occluded solvent.

NMR Spectra

A 10 mg sample of siphonaxanthin, which had been treated as described above to remove occluded solvent, was dissolved in 1 ml $CDCl_3$ and evaporated to dryness under N_2. It was then dissolved in $CDCl_3$ for NMR spectra. The entire siphonein fraction was treated in a similar manner. Spectra were determined using a HA-100 100 MHz instrument (Varian Associates) and tetramethylsilane as an internal standard.

Mass Spectra

These were determined using an AEI MS902 mass spectrometer. The high resolution measurement was made relative to heptacosafluorotributylamine.

[12] K. EGGER and H. VOIGT, *Z. Pflanzenphysiol.* **53**, 64 (1965).
[13] B. W. NICHOLS, *Lab. Practice* 299, (1964).
[14] B. W. NICHOLS, In *New Biochemical Separations* (edited by A. T. JAMES and L. J. MORRIS), p. 321, Van Nostrand, London (1964).
[15] K. RANDERATH, *Thin Layer Chromatography*, p. 157, Academic Press, London (1966).
[16] A. JENSEN and S. LIAAEN-JENSEN, *Acta Chem. Scand.* **13**, 1863 (1959).

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