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Bacterial Carotenoids XV *

On the Constitution of the Minor Carotenoids of *Rhodopseudomonas*

5. The Structures of P518 (2,2′-Diketo-spirilloxanthin), OH-R (OH-Spheroidenone), and OH-Y (OH-Spheroidene)

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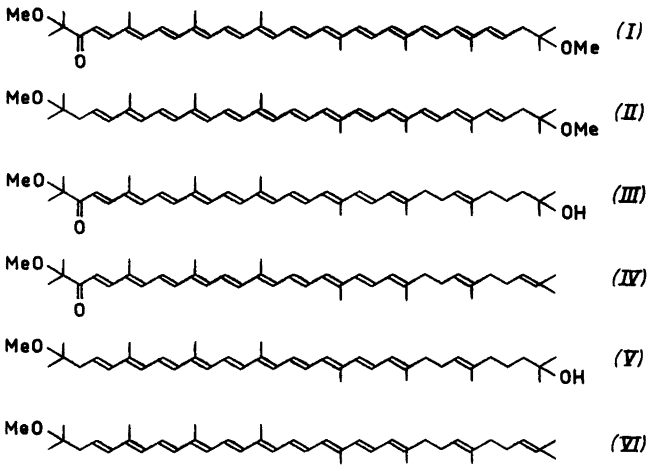
Nuclear magnetic resonance data and further chemical evidence have allowed a revision of the previously suggested structure (I) for the carotenoid P518, which is now shown to be 2,2′-diketo-spirilloxanthin (VII). The nuclear magnetic resonance spectra of the pigments OH-R and OH-Y confirm that these carotenoids are 1′,2′-dihydro-1′-hydroxy-spheroidenone (III) and 1′,2′-dihydro-1′-hydroxy-spheroidene (V), respectively. The dehydration of OH-R (III) to spheroidenone (IV) and of OH-Y (V) to spheroidene (VI) is reported.

In three preceding communications [1–3] in this series, structures have been suggested for P518,[1] OH-R [2] (now named OH-spheroidenone [3]), and OH-Y [4] (now named OH-spheroidene [3]).

The evidence previously available for the structure of P518 was consistent with this compound being 2-keto-spirilloxanthin (I). Spirilloxanthin itself had been assigned the structure (II) by Liaaen Jensen [5,6] and, independently on the basis of NMR data, by Barber, Davis, Jackman and Weedon.[7] The correctness of this structure was subsequently demonstrated by the total synthesis of Surmatis and Ofner.[8]

OH-Spheroidenone was formulated by Liaaen Jensen [2] as 1′,2′-dihydro-1′-hydroxy-spheroidenone (III). The structure of spheroidenone (Pigment R) had previously been established as (IV) by Davis, Jackman, Siddons and Weedon.[9] Similarly, OH-spheroidene was formulated as 1′,2′-dihydro-1′-hydroxy-spheroidene (V),[3] spheroidene itself having the structure (VI).[9]

* No. XIV of this series *Acta Chem. Scand.* **18** (1964) 343.



RESULTS AND DISCUSSION

The structure (I), previously suggested for P518,[1] was based on visible and infrared light absorption data, quantitative methoxyl determination, consideration of partition ratios, and a negative test for allylic methoxyl groups with P518, itself, and on the light absorption data, R_F-values and partition behaviour of the product obtained by lithium aluminium hydride reduction of P518. The structure (I) was, however, inconsistent with the stability of the reduction product towards $HCl-CHCl_3$ reagent. Insufficient material was available for elemental analysis.

In the present work a further quantity of P518 was isolated and its NMR spectrum determined. P518 was isolated from aerobic cultures of *Rhodopseudomonas spheroides* van Niel and *R. gelatinosa* (Molisch) van Niel as described elsewhere.[1,2] The NMR spectrum of P518 (Fig. 1) exhibits a broad band at

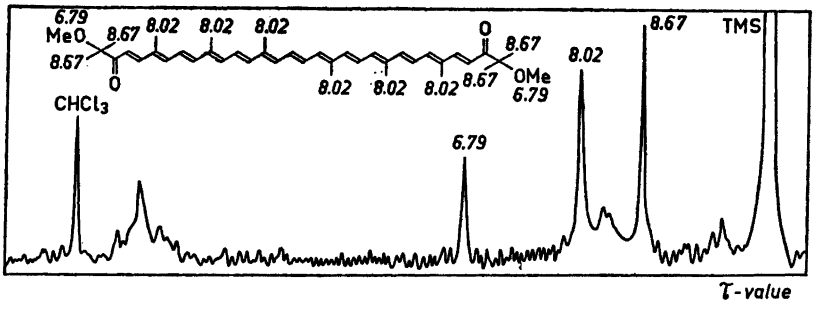
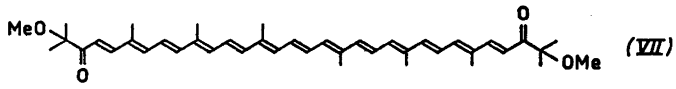


Fig. 1. NMR-spectrum of P518.

$\tau = 3.45$ (olefinic protons) and singlets at 6.79 (OMe), 8.02 (in-chain CH_3) and 8.67, this last absorption being characteristic of the methyl groups of the keto end-group of spheroidenone.[9] The ratio of the intensities of the singlets at 6.76, 8.02, and 8.67 is *ca.* 1:3:2. These data, together with the visible light absorption data for P518 and its reduction product can only be accommodated by structure (VII). Clearly, the absence of absorption at 8.83 and 7.70, characteristic of the terminal methyl groups and allylic methylene groups, respectively, of spirilloxanthin rules out structure (I).



(VII)

The P518 used in the previous and present investigation had been obtained from various batches of *R. spheroides* and *R. gelatinosa*. A re-examination of the experimental data does not favour the possibility that the two samples are different P518-pigments (*e.g.* I and VII). The mono- and diketo-compounds (I) and (VII) are not expected to differ much in the visible light absorption spectrum, since one keto-group in ω,ω'-conjugated diketones is known to be spectroscopically inefficient.[10] As seen from Fig. 2 the main absorption maxi-

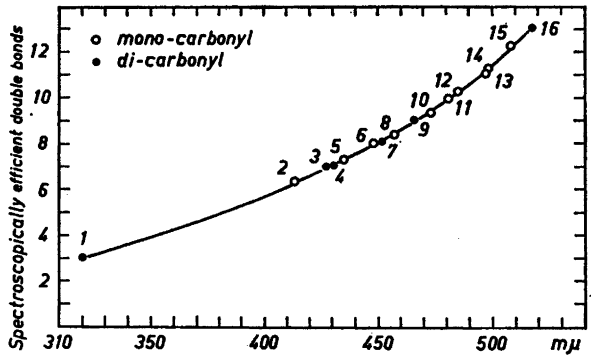


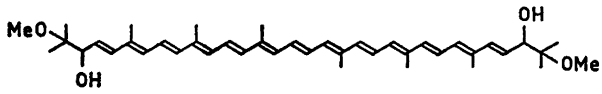
Fig. 2. The middle main absorption maximum in petroleum ether of various conjugated mono- and diketo-carotenoids as a function of spectroscopically efficient double bond. 1: 2,6-dimethyl-octa-2,4,6-triene; 2: β-apo-12′-carotenal (C_{25}); 3: crocetindialdehyde, azafrinon; 4: capsylaldehyde; 5: β-apo-10′-carotenal (C_{27}); 6: α-citraurin; 7: capsanthylal; 8: β-citraurin; 9: β-carotenone; 10: β-apo-6′-carotenal (C_{32}); 11: spheroidenone; 12: β-apo-4′-carotenal (C_{35}); 13: warmingone; 14: β-apo-2′-carotenal (C_{37}); 15: 3′4′-dehydro-18′-oxo-γ-carotene; 16: P518.

mum in petroleum ether of various conjugated carotenoid mono- and di-ketones as a function of the spectroscopically efficient double bond chain fit into a smooth curve.* It is, however, to be expected a possible separation of (I) and (VII) by circular paper chromatography.

* In this diagram no correction had been made for λ_{max} of conjugated aldehydes contra that of the corresponding ketones.

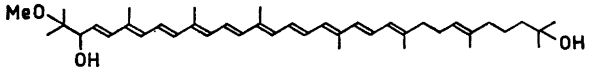
Accepting the structure (VII) for P518, the rather anomalous partition behaviour of P518 and the $LiAlH_4$-reduced compound can be explained by (1) the shielding effect of the methoxylated end-groups and (2) by possible hydrogen bonding in $LiAlH_4$-reduced P518.

The failure of $LiAlH_4$-reduced P518 to react with acidified chloroform [1] is compatible with the structure (VIII) for this compound.



(VIII)

Moreover, $LiAlH_4$-reduced OH-spheroidenone (IX) has now been shown to be less strongly adsorbed on deactivated alumina than $LiAlH_4$-reduced P518, which is consistent with the formulation of the latter as a diol (VIII).



(IX)

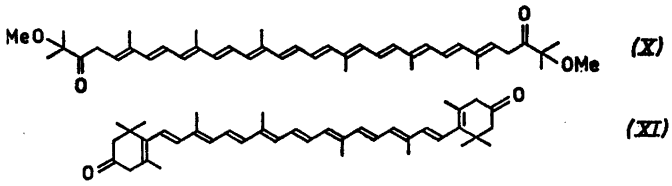
Additional proof of the structure (VII) of P518 has been obtained from a study of the reduction of this carotenoid with zinc-acetic acid-pyridine using the method of Kuhn and Winterstein.[11]

Several ω,ω'-conjugated diketones were shown by Kuhn and co-workers to be reduced to the corresponding ω,ω'-dihydropolyenes.[12-15] The latter compounds were readily oxidised, in high yields, to the original diketones by oxygen in the presence of various bases. This autoxidation of the dihydro-compounds was shown by Kuhn, Drumm, Hoffer and Møller [14] to involve the intermediate formation of a deeply coloured di-enolate ion, which subsequently yielded the ω,ω'-conjugated diketone and hydrogen peroxide on access to oxygen.

In the present investigation, mono-keto compounds, such as spheroidenone (IV), was found to be completely unreactive towards zinc in pyridine-acetic acid under conditions for which P518 was readily reduced. In the absence of air, the reduction product (X) gave a dark blue enol salt with alkali and this salt was converted quantitatively to P518 by air.

Even in the absence of alkali dihydro-P518 (X) readily reverted to P518. The dihydro-compound (X) could not be isolated in the pure state even in spectroscopic quantities. It therefore appears to be considerably more labile than dihydro-rhodoxanthin (XI).[15] The formation of the enolate anion from (XI) may be less favourable because steric hindrance prevents the double bonds in the rings from attaining co-planarity with the polyene chain,[16-18] thus reducing the resonance stabilisation of the enolate ion. The observed instability of dihydro-P518 (X) is furthermore in agreement with the statement of Kuhn *et al.*[14] that the oxygen affinity (enolization tendency) of such dihydro-compounds increases with increasing length of the polyene chain.

The biosynthetic evidence presented for the formation of P518 by Eimhjellen and Liaaen Jensen [3] is quite compatible with the diketo-structure for



P518 (VII). P518 is synthesized from spirilloxanthin (II) in *R. gelatinosa* on aeration of a previously anaerobically grown culture. The net result is the introduction of keto-group(s) in 2-position. Since spirilloxanthin (II) has two identical end-groups the introduction of two keto-groups is more plausible on enzymatic grounds. In stationary cultures grown with an excess supply of oxygen, the occurrence of (VII) rather than (I) is to be expected. The appar-

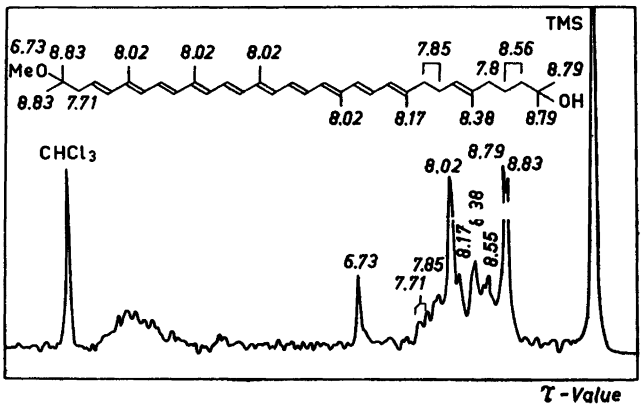


Fig. 3. NMR-spectrum of OH-spheroidene (OH-Y).

ent increase of P518 (VII) in resting cell suspensions of aerobically grown cells of *R. gelatinosa*, under anaerobic conditions in light, reported by Eimhjellen and Liaaen Jensen,[3] might possibly be caused by the formation of the mono-keto-compound (I), not identified as an individual carotenoid in our experiments.

OH-Spheroidene (OH-Y) and OH-spheroidenone (OH-R) were isolated from anaerobic and aerobic cultures respectively of *R. gelatinosa* as previously described [2,4] for NMR-investigation. The NMR-spectra are presented in Figs. 3 and 4. The spectrum of spirilloxanthin [7] is characterized by a singlet at 8.83 (saturated C-Me), a doublet at 7.70 (allylic—CH_2—) and a singlet at 6.76 (OMe). It is seen that the spectrum of OH-spheroidene exhibits the same bands with the expected intensities. Thus, OH-spheroidene has one end group which is the same or very similar to those of spirilloxanthin. The spectrum of OH-spheroidene contains absorption at 8.02 arising from four "in-chain" methyl groups and a band at 8.17 associated with one "end-of-chain" methyl group. Since the visible light absorption spectrum of the $LiAlH_4$-reduced compound

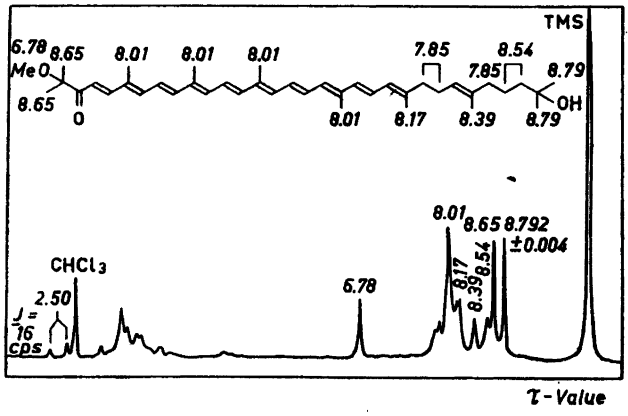
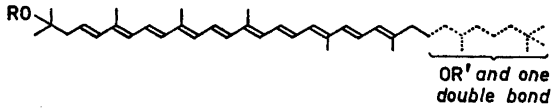


Fig. 4. NMR-spectrum of OH-spheroidenone (OH-R).

indicates the presence of a conjugated system of ten double bonds, the partial structure (XII; R = H, R′ = CH_3 or R = CH_3, R′ = H) may be written.



(XII)

The remaining double bond is readily located between the 5- and 6-positions since the spectrum shows the presence of one olefinic methyl absorption at 8.38. The lack of an absorption band at 8.31 proves the absence of a terminal isopropylidene group.[9] A prominent feature of the spectrum is the singlet at 8.79 which has the same intensity as that at 8.83. This must also arise from a grouping of the type -$C(CH_3)_2$-O- and accordingly OH-spheroidene has the structure (V) or the analogous structure with the positions of the methoxyl and hydroxyl group interchanged. That (V) is in fact the correct structure is shown by the conversion of OH-spheroidene (V) to spheroidene (VI) using phosphorus oxychloride in pyridine. Finally, light absorption data indicate an "all-*trans*" configuration of the conjugated system, and as the methyl group attached to the isolated double bond is at 8.38 rather than 8.31, it follows [9] that this double bond has the configuration indicated in (V). The remaining absorptions (7.85 and 8.56) are readily assigned on the basis of (V) (see Fig. 3).

The structure of OH-spheroidenone can also be derived from its NMR-spectrum (Fig. 4) since the region of this spectrum above $\tau = 5$ comprises of absorptions which on the one hand are characteristic of the keto end group of spheroidenone (IV) and P518 (VII) and on the other of the hydroxy end group of OH-spheroidene (V). Thus OH-spheroidenone has the structure (III) which accounts well for all the features of its NMR-spectrum (see Fig. 4). The stereochemical assignment (III) to the isolated double bond is based on the argument given above for the analogous bond in OH-spheroidene (V). One

further stereochemical assignment can be made on the basis of the NMR-spectrum. The doublet (J = 16 cps.) at 2.50 can be assigned to the olefinic proton β to the carbonyl group and the magnitude of the coupling constant proves that the double bond to which this proton is attached has the *trans*-configuration; *cf.* the argument used to establish the stereochemistry of the terminal carbon-carbon double bonds in bixin.[19] The possible ambiguity in the positions of the methoxyl and hydroxyl groups is removed by the observation that OH-spheroidenone (III) can be dehydrated to spheroidenone (IV) by $POCl_3$/pyridine.

EXPERIMENTAL

Materials and methods have been described in an earlier paper of this series.[2] Analytical grade reagents were used for all tests. All melting point determinations were carried out in evacuated capillary tubes and are uncorrected. Abridged thermometers were used.

P518

Isolation. Crystalline P518 (0.96 mg), m.p. 222°C, abs.max. 530, 561, and 603 mμ in CS_2, was isolated from *R. gelatinosa* as described elsewhere.[2] A chromatographically pure fraction containing 0.7 mg P518 was obtained from an aerobic culture of *R. spheroides* according to the previously described procedure.[1]

NMR-spectrum. The NMR-spectrum of 0.96 mg P518 in 0.15 ml $CDCl_3$ at 100 mc/sec is presented in Fig. 1. By cutting out and weighing the bands at 6.79, 8.02, and 8.67 the following intensity ratios were found: 6:19:14. Calc. for (VII): 6:18:12.

Zn-acetic acid reduction. The procedure of Kuhn and Winterstein[11] was adopted. To 0.40 mg P518 (spectrophotometrically determined) in 3 ml pyridine (previously dried over BaO) was added 0.1 ml of glacial acetic acid and 15 mg Zn-powder under nitrogen. The mixture was shaken at room temperature for 2 min whereupon a hypsochromic shift was observed. The carotenoids were transferred to petroleum ether in a separatory funnel, and the pet.ether extract was washed carefully with water. The reaction mixture exhibited abs.max at 440, 463, 490, 512, and 548 mμ (see Fig. 5); spectrophotometrically determined pigment recovery 74 %.

An aliquot (15 μg) of the orange-red reaction mixture in 3.5 ml ether was shaken with 2 drops of a 10 % KOH-methanol solution. The mixture immediately turned pink-blue, and the abs. spectrum in visible light had the characteristic appearance of that of P518, *cf.* Fig. 5. Paper-chromatographic examination of this reaction mixture on kieselguhr paper,[20] using 10 % acetone in pet.ether as developer revealed the presence of *cis* and *trans* P518 (*cf.*[1]) only.

Another aliquot (30 μg) of reduced P518 was transferred to dry pyridine (2 ml) and mixed with 1 ml of a saturated solution of KOH in butanol in a two-finger device at room temperature under vacuum (0.03 mm Hg). The red solution turned immediately blue. The blue colour remained stable until air was introduced, where-upon it turned blue-red. Paper-chromatographic examination of this reaction mixture demonstrated the presence of *cis* and *trans* P518 only.

Chromatographic separation of the reaction mixture from the Zn-acetic acid reduction, which judged from the abs.spectrum in visible light contained some unreacted P518, was best achieved on a 2 × 22 cm powdered sucrose column, packed hard with suction. Nitrogen pressure was employed during the chromatographic run. On development with 1 % acetone in pet.ether a cerise zone was eluted ahead of the 4 pink-bluish zones of the P518 stereoisomers. The cerise pigment constituted 43 % and the P518 stereoisomers 56 % of the recovered carotenoid. The abs. spectrum of the former compound had no fine-structure, and maxima at 494 and 530 mμ in pet. ether. On treatment with alkali in air a solution of this compound turned pink-blue, and showed the characteristic abs.spectrum of P518.

Further purification of the presumed dihydro-P518 was attempted by chromatography on kieselguhr-paper. However, the compound reverted very easily to P518 during this procedure. A small amount of a red-orange product (R_F = 0.54 using 10 % acetone in pet.ether as developer) was adsorbed between the *trans* and neo B isomers of P518.

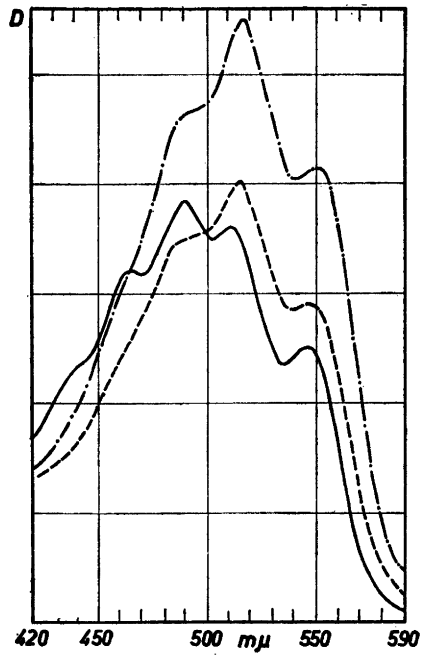


Fig 5. Zn-acetic acid reduction of P518 in pyridine. —·— Before treatment; ——— After reduction; — — — Reduced aliquot treated with alkali in the presence of air.

This pigment had abs.max. in acetone at 465, 495, and (515) mμ, and reverted to P518 on shaking with alkali in the presence of air, as seen by visual inspection as well as spectrophotometric determinations.

Other reduction experiments of P518 with Zn and acetic acid gave a similar result. Longer reaction periods resulted in the formation of yellow-coloured decomposition products. Isolation of the dihydro-compound by chromatography of the reaction mixture on columns of deactivated, neutral alumina was not possible, only recovered P518 and decomposition products being obtained.

An analogous reduction test to that described above was carried out with 1.19 mg of spheroidenone (isolated from an aerobic culture of *R. spheroides*[1]). No spectral shift was produced. A pigment recovery of 98 % was spectrophotometrically determined. Paper-chromatographic examination of the reaction mixture revealed the presence of *cis* and *trans* spheroidenone only.

OH-Spheroidenone

Isolation. Crystalline OH-spheroidenone (9.8 mg) m.p. 158.5—159.5°C, abs.max. 501 and 530 mμ in benzene, $E^{1\,\%}_{1\,cm} = 2070$ at 501 mμ, was isolated from an aerobic culture of *R. gelatinosa* as previously described.[2]

NMR-spectrum. The NMR-spectrum of 9.8 mg OH-spheroidenone in 0.5 ml $CDCl_3$ at 60 Mc/sec is presented in Fig. 3.

Conversion of OH-spheroidenone to spheroidenone. The dehydration method of Surmatis and Ofner [8] was adapted. To 0.38 mg of OH-spheroidenone in 5 ml dry pyridine was added 0.05 ml $POCl_3$. The reaction mixture was mechanically stirred for 30 min at 50°C under nitrogen. The carotenoids were transferred to ether in a separatory funnel, and the ether extract was washed with water; pigment recovery 74 %. The reaction mixture was submitted to column chromatography on neutral alumina, activity grade 2,[20] and found to contain 92 % spheroidenone (IV) and 8 % unreacted OH-spheroidenone. Spheroidenone (IV) was identified from its abs.spectrum in visible light, adsorptive pro-

perties on deactivated alumina (required eluant 4 % acetone in pet.ether compared to 10 % acetone in pet.ether necessary for elution of OH-spheroidenone) and co-chromatography tests of the *trans* and neo A isomer (*cf.* Ref.[1]) on kieselguhr paper with those of authentic spheroidenone (V) isolated from aerobically grown *R. spheroides* as described elsewhere.[1] The neo A and *trans* isomers exhibited $R_F = 0.74$ and 0.43, respectively, when 2 % acetone in pet.ether was used for develoment of the circular chromatogram.

OH-Spheroidene

Isolation. Crystalline spheroidene (9 mg), m.p. 157–157.5°C, abs. max. 440.5, 467.5, and 501.5 mμ in benzene, $E^{1\,\%}_{1\,cm} = 2785$ at 467.5 mμ was isolated from an anaerobically grown culture of *R. gelatinosa* as earlier described.[4]

NMR-spectrum. The NMR-spectrum of 5.3 mg OH-spheroidene in 0.5 ml $CDCl_3$ recorded at 60 Mc/sec is presented in Fig. 4.

Conversion of OH-spheroidene to spheroidene. The reaction was carried out as described above for OH-spheroidenone using 0.15 mg of OH-spheroidene. A pigment recovery of 73 % was spectrophotometrically determined. The reaction mixture contained 97 % spheroidene (VI) and 3 % of recovered OH-spheroidene. Spheroidene (VI) was identified from its abs.spectrum in visible light, adsorption properties on alumina activity grade 2^{21} (required eluant 3 % acetone in pet.ether contrary to 10 % acetone in pet.ether necessary for elution of OH-spheroidene) and co-chromatography tests on paper of the two main stereoisomers with those of authentic spheroidene (VI), isolated from anaerobically grown cells of *R. gelatinosa*. *Trans* spheroidene had $R_F = 0.52$ in the system 1 % acetone in pet.ether, and the main *cis* isomer $R_F = 0.72$ in the same system.

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Professor N. A. Sørensen has followed this work with inspiring enthusiasm and given helpful advice.

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